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Mukesh Verma *Editor*

Cancer Epigenetics

Risk Assessment, Diagnosis,
Treatment, and Prognosis

 Humana Press

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Cancer Epigenetics

Risk Assessment, Diagnosis, Treatment, and Prognosis

Edited by

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 **Humana Press**

Editor

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Preface

Cancer is a genetic and epigenetic disease. Both genetic and epigenetic changes occur simultaneously in an organism. Genetic alterations include mutations and single-nucleotide changes, deletions, insertions, changes in copy number, and translocations. Epigenetic alterations (methylation, histone and selected nonhistone protein alterations, noncoding RNA alterations, imprinting, and chromatin remodeling) broadly include non-genetic alterations that are capable of being transmitted from one cell generation to another. The epigenetic regulation plays an important role in normal development and maintenance of tissue-specific genes' expression in humans and the disturbance of these patterns lead to changes involved in tumor formation. Global epigenetic changes and genes' promoter-specific methylation patterns have been observed in many cancer types and play an essential role in carcinogenesis. Epigenetic changes have been observed in early stages of tumor development and together with the genetic alterations have been defined as abnormalities, necessary for cancer initiation and progression. Changes in histone modification patterns and microRNA expression also evolved as being important players in the carcinogenic process. Different cancer types express distinct methylation patterns but also share common epigenetic changes that are very important in early detection, progression, and prognosis as well as the design of new therapeutic tools against cancer cells. The technology available to detect these epigenetic changes is evolving rapidly and provides more understanding of these processes in normal and cancerous cells. Several of these technologies are discussed in this book. Recent studies identified several factors that may play a significant role in the initiation of the epigenetic changes in cancer. Some of the genetic and environmental factors that have been shown to be involved in these processes are also discussed.

The epigenome is dynamic and very susceptible to environmental changes. Toxicoepigonomics studies are conducted to explain the epigenetic effects of environmental exposures. Epigenetic programming occurs during development and reflects altered gene expression in disease states. This programming differs from genetic polymorphisms or mutations because genetic changes are reflected in all cells, whereas epigenetic changes are cell and tissue specific. Studies that involve the measurement of epigenetic changes that occur at the genome-wide level and their association with disease are called epigenome-wide association studies (EWAS). Epigenetic alterations respond quickly to environmental changes, and technologies are available to measure these changes. During the last 5 years, excellent progress has been made in the field of altered epigenomic profiling in response to toxins and environmental pollutants. Epigenetic marks are tissue specific; genomic marks are not. This fact has implications in tissue-specific toxicity, pharmacokinetics, and pharmacotoxicity. Genetic marks are static and can be measured at any point; epigenetic marks must be measured at different time points. Epigenetic marks contribute to the phenotypic characteristics of cells, tissues, and persons. The latest in cancer toxicoepigonomics is covered in the book.

All these epigenetic alterations can be used in clinical practice as biomarkers of early cancerous lesions or markers of progression and prognosis. Furthermore, epigenetic

inhibitors have potential in cancer treatment. FDA-approved epigenetic inhibitors and nutrients with chemoprevention potential are discussed.

The book is divided into four parts.

Part I. Background: Epigenetic mechanism.

Part II. Cancer specific type epigenetic changes.

Part III. Methods and technologies used for detecting epigenetic changes.

Part IV. Factors that influence epigenetic changes in cancer.

Different chapters covered in these parts provide the most up-to-date knowledge of epigenetics and its implication in cancer prevention by risk assessment and screening and cancer control by treatment.

Rockville, MD, USA

Mukesh Verma

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Part I

Background: Epigenetic Mechanism

Chapter 1

Cancer Epigenetics: An Introduction

Rajnee Kanwal, Karishma Gupta, and Sanjay Gupta

Abstract

Epigenetic and genetic alterations contribute to cancer initiation and progression. Epigenetics refers to the study of heritable changes in gene expression without alterations in DNA sequences. Epigenetic changes are reversible and include key processes of DNA methylation, chromatin modifications, nucleosome positioning, and alterations in noncoding RNA profiles. Disruptions in epigenetic processes can lead to altered gene function and cellular neoplastic transformation. Epigenetic modifications precede genetic changes and usually occur at an early stage in neoplastic development. Recent technological advances offer a better understanding of the underlying epigenetic alterations during carcinogenesis and provide insight into the discovery of putative epigenetic biomarkers for detection, prognosis, risk assessment, and disease monitoring. In this chapter we provide information on various epigenetic mechanisms and their role in carcinogenesis, in particular, epigenetic modifications causing genetic changes and the potential clinical impact of epigenetic research in the future.

Key words DNA methylation, Histone modification, microRNA, Epigenetics, Cancer

Abbreviations

5adC	5-Aza-2-deoxycytidine
8-OHdG	8-Hydroxy-2'-deoxyguanosine
APC	Adenomatosis polyposis coli
BRCA1	Breast cancer1
CDH1	Cadherin-1
ChIP	Chromatin immunoprecipitation
DAPK1	Death-associated protein kinase
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
DPP6	Dipeptidyl-peptidase 6
EZH2	Enhancer of zeste homolog 2
FHIT	Fragile histidine triad protein
GADD45	Growth arrest and DNA-damage-inducible protein GADD45 gamma
GSTP1	Glutathione S-transferase pi 1
HATs	Histone acetyltransferases
HDAC	Histone deacetylase

HDMs	Histone demethylases
HIC1	Hypermethylated in cancer-1
HMTases	Histone methyltransferases
HOX	Homeobox
IGF	Insulin-like growth factor
JARID1C	Jumonji/ARID domain-containing protein 1C
JMDJ3	Histone H3 lysine-27 demethylase
KLF4	Kruppel-like factor 4
LINE	Long interspread transposable element
LOI	Loss of imprinting
<i>MAGE</i>	<i>Melanoma-associated gene</i>
MBD	Methyl-CpG-binding domain
MeCP	Methyl-cytosine-binding protein
methyl H3K4	Histone H3 lysine 4 methylation
methyl H3K9	Histone H3 lysine 9 methylation
MGMT	O6-methylguanine–DNA methyltransferase
miR	microRNA
MLH1	Mismatch repair gene 1
MYOD1	Myogenic differentiation 1
OCT4	Octamer-binding transcription factor 4
PTEN	Phosphatase and tensin homolog
PTENP1	Phosphatase and tensin homolog pseudogene 1
RAR	Retinoic acid receptor
RARB2	Retinoic acid receptor b2
RASSF1	Ras association (RalGDS/AF-6) domain family member 1
Rb	Retinoblastoma
RNA	Ribonucleic acid
SETD2	SET domain containing 2
SINE	Short interspread transposable elements
SIRT1	Silent information regulator type1
SOX2	SRY (sex-determining region Y)-box 2
TMPRSS2	Transmembrane protease serine 2
TMS1	Target of methylation-induced silencing1
TRD	Transcription repression domain
TSA	Trichostatin A
UBE2C	Ubiquitin-conjugating enzyme
UTX	Ubiquitously transcribed tetratricopeptide repeat X chromosome
WRN	Werner syndrome RecQ helicase like
WWOX	WW domain-containing oxidoreductase

1 Introduction

Epigenetics is defined as the study of heritable changes in gene expression without alteration in DNA sequences. Epigenetic gene patterns play an important role in various biological processes including embryonic development, genetic imprinting, and X-chromosome inactivation [1]. Disruptions in these processes lead to a wide variety of pathologies including metabolic and

autoimmune diseases, neurological disorders, and cancer. The key processes responsible for epigenetic regulation are DNA methylation, chromatin modification (covalent alteration in core histones), nucleosome positioning (physical changes), and posttranslational gene regulation by noncoding RNAs. Deregulation of these processes causes aberrant gene function and altered gene expression that play critical role in cancer initiation, development, and subsequent progression [2, 3].

2 Epigenetic Regulation in Cancer

2.1 DNA Methylation

DNA methylation is a covalent modification of the cytosine ring at the 5' position of a CpG dinucleotide by addition of a methyl group added to the 5th carbon of the ring using S-adenosyl methionine as a methyl donor. Alterations in DNA methylation are recognized as the first epigenetic marker associated with cancer altering normal gene function. These alterations include hypermethylation, hypomethylation, and loss of imprinting (LOI) [4].

DNA hypermethylation, principally referred to as gain of methylation at specific sites, occurs mainly in promoter CpG islands. These modifications are catalyzed by enzyme DNA methyltransferase (DNMT). There are three main DNMTs, viz. DNMT1, DNMT3a, and DNMT3b. DNMT1 maintains the existing methylation patterns following DNA replication, and DNMT3a and DNMT3b are *de novo* enzymes that target unmethylated CpGs to initiate methylation, and are highly expressed during embryogenesis and minimally expressed in adult tissues. Another family member, DNMT-3L, lacks intrinsic methyltransferase activity and interacts with DNMT3a and DNMT3b to facilitate methylation of retrotransposons [5]. In normal cells, DNA methylation occurs predominantly in repetitive genomic regions, including satellite DNA and parasitic elements such as long interspread transposable element (LINE) and short interspread transposable elements (SINE) maintaining genomic integrity [6]. Loss of DNA methylation in genome-wide regions, referred to as “DNA hypomethylation” is associated with genomic instability and cancer progression. Furthermore, LOI or the loss of parental allele-specific monoallelic expression of genes due to aberrant DNA hypomethylation leads to an increased risk of cancer [7]. In tumor cells, specifically, global hypomethylation is accompanied by hypermethylation of localized promoter associated CpG islands that usually remain unmethylated in normal cells. This unique pattern of individual gene methylation is the characteristic commonly observed in various tumor suppressor genes in most types of human cancers and serve as a surrogate for point mutations or deletions that cause transcriptional silencing of tumor-suppressor genes [2, 8].

In addition DNA methylation can inhibit gene expression directly by inhibiting the binding of specific transcription factors and

indirectly by recruitment of methyl CpG-binding domain (MBD) proteins [9]. The associated MBD family members in turn recruit histone-modifying enzymes and chromatin-remodeling complexes to methylated states [10]. To date, six methyl-CpG-binding proteins, including methylcytosine-binding protein 2 (MECP2), MBD1, MBD2, MBD3, MBD4, and Kaiso, have been identified in mammals [11, 12]. MECP2 binds methylated DNA in vitro and in vivo. It contains an MBD at its amino terminus and a transcription repression domain (TRD) in the middle. Furthermore, it has been shown that nucleosome remodeling complex (NuRD) can methylate DNA by interacting with DNA methylation-binding protein MBD2, which directs the NuRD complex to methylate DNA [11]. A list of DNA methylation genes disrupted in various human cancers is shown in Table 1.

2.2 Histone Modification

Another epigenetic regulator is histone modification influencing chromatin structure, which plays important role in gene regulation and carcinogenesis. Chromatin is a highly ordered structure that packages eukaryotic DNA into a higher order chromatin fiber wrapped around an octamer of histone proteins. These octamers consist of double subunits of H2A, H2B, H3, and H4 core histone proteins. Histones can undergo multiple posttranslational modifications, which occur mainly in the terminal tails [13]. The enzymes that add and remove such modifications include: histone acetyltransferase (HATs) and histone deacetylase (HDACs and sirtuins), histone methyltransferase (HMTs) and histone demethylases (HDMs), kinases and phosphatases, ubiquitin ligases and deubiquitinases, and sumoligases and proteases [13, 14]. Genome-wide studies have shown that various combinations of modifications in specific genomic regions can lead to opening and closing of chromatin structure, responsible for activation or repression of gene expression [15]. For example, lysine monomethylation of H4K20 and H2BK5; trimethylation of H3K4me3, H3K36me3, and H3K79me3; and acetylation of H3K9 and H3K14 result in gene activation, whereas di- or trimethylation of H3K9 and trimethylation of H3K27 lead to gene repression. Descriptions and functions of posttranslational histone modification are beyond the scope of this chapter; however, a list of histone-modifying genes altered during cancer is provided in Table 2.

2.3 Noncoding RNAs

Noncoding RNAs are modulators of chromatin regulation and gene expression. The noncoding RNAs (ncRNA) are categorized into small (under 200 nucleotides) and large (>200 nucleotides) ncRNAs [16]. These RNAs are increasingly recognized to be vital for normal development and may be altered during cancer. The small ncRNA include small nucleolar RNA (snoRNA), PIWI-interacting RNA (piRNA), small interfering RNA (siRNA), and microRNA (miRNA) [17]. Many of these families show a high

Table 1
Alteration in DNA methylation genes in various human cancers

Alterations		Cancer type	Function
<i>DNA methyltransferase</i>			
DNMT 1	Upregulation, mutation	Colorectal cancer, ovarian cancer	Maintenance of methylation, repression of transcription
DNMT3a	Upregulation	Colorectal cancer, breast cancer, ovarian cancer, esophageal squamous cell carcinoma	De novo methylation during embryogenesis, imprint establishment, repression
DNMT3b	Upregulation	Breast cancer, hepatocellular carcinoma, colorectal cancer	De novo methylation during embryogenesis, repeat methylation repression
DNMT3L	Upregulation, mutation	Breast cancer, gastric cancer, cervical cancer	Interacts with DNMT3a & b and facilitate methylation
<i>Methyl-CpG-binding proteins</i>			
MeCP2	Upregulation, mutation	Prostate cancer, Rett syndrome	Transcription repression
MBD1	Upregulation, mutation	Prostate cancer, colon cancer, lung cancer	Transcription repression
MBD2	Upregulation, mutation	Prostate cancer, colon cancer, lung cancer	Transcription repression
MBD3	Upregulation, mutation	Colon cancer, lung cancer	Transcription repression
MBD4	Upregulation, mutation	Colon cancer, gastric cancer, endometrial cancer	DNA repair, glycosylase domain, repair of deaminated 5-methylC
Kaiso	Upregulation	Colon, intestinal, lung cancer	Transcription repression
<i>DNA demethylase</i>			
TET	Downregulation	Prostate cancer, breast cancer, lung cancer, pancreatic cancer	Convert 5mC to 5hmC (5 hydroxymethylcytosine)
TET1, TET2, TET3	Mutationally inactivated	Myeloid cancer, glioma, enchondroma, thyroid carcinoma	

DNMT DNA methyltransferase, *MBD* methyl-CpG-binding domain, *TET* ten eleven translocation

degree of sequence conservation across species and are involved in transcriptional and posttranscriptional gene silencing through specific base pairing with their targets [18]. Of these noncoding RNAs, miRNAs are most widely studied as they are vital to normal cell physiology and their mis-expression has been linked to several diseases, including cancer [19]. Recently, cancer development and

Table 2
Alteration in histone modification genes in various human cancers

Alteration		Cancer type
<i>Histone deacetylases</i>		
HDAC1	Upregulation/downregulation	Colorectal cancer, cervical dysplasias, endometrial stromal sarcomas, gastric carcinomas, colon cancer
HDAC2	Upregulation/mutation	Multiple gastric carcinomas, colon cancer
HDAC3	Upregulation	Colon cancer
HDAC4	Upregulation/downregulation/mutation	Prostate, breast, and colon cancer
HDAC5	Under-expression	Colon cancer, AML
HDAC6	Upregulation	Breast, AML
HDAC7	Upregulation	Colon cancer
HDAC8	Upregulation	Colon cancer
SIRT1	Upregulation/downregulation	Colon cancer
SIRT2	Downregulation, deletion	Glioma
SIRT3	Upregulation	Breast cancer
SIRT4	Downregulation	AML
SIRT7	Upregulation	Breast, thyroid carcinoma
<i>Histone acetyl transferases</i>		
p300	Mutation, translocation, deletions	Colorectal, breast, ovarian, hepatocellular, and oral carcinomas,
CBP	Mutation, translocation, deletions	Colon, breast, ovarian, and AML
pCAF	Mutation	Colon
MOZ	Translocation	Hematologic malignancy
MORF	Translocation	Hematologic, uterine leiomyomata
Tip60	Downregulation, translocation	Colorectal, prostate cancer
GCN5	Downregulation, mutation	Prostate, breast, ovarian cancer
PCAF	Rare mutation	Colon cancer
HBO1	Upregulation	Testis, breast, ovary, and bladder cancer
SRC1	Upregulation	Breast cancer, prostate cancer
ACTR	Upregulation	Breast cancer
ATF2	Upregulation	Breast cancer, skin cancer
ELP3	Upregulation	Breast cancer
<i>Histone methyltransferase</i>		
MLL1	Translocation, amplification	Hematologic malignancies
MLL2	Amplification	Glioma, pancreatic cancer
MLL3	Mutation, deletion	Hematologic malignancies, colon cancer

(continued)

Table 2
(continued)

	Alteration	Cancer type
MLL4	Amplification	Solid tumor
SUV39H1-2	Mutation overexpression	Ovarian, cancer
G9a	Gene repression	Colon, gastric, breast cancer
RIZ1/ PRDM2	Under-expression, mutations	Colorectal, gastric cancer
EVII	Chromosomal rearrangement	Myeloid leukemia
EZH2	Amplification, upregulation	Prostate, breast, ovarian cancer
SUZ12	Upregulation	Prostate, breast, ovarian cancer
BMI1	Upregulation	Prostate, breast, ovarian cancer
NSD1	Translocation, upregulation	AML
NSD2	Translocation	Multiple myeloma
NSD2	Translocation	Multiple myeloma
NSD3	Translocation, amplification	AML, breast cancer
SYMD2	Upregulation	Breast, colon cancer
DOT1	Upregulation	AML
SETDB1	Gene amplification transcriptional repression	Lung cancer, melanoma
CARM1	Transcriptional activation	Prostate cancer, breast cancer
Suv4-20h	Abrogation enhances telomere elongation	Breast cancer, prostate cancer
<i>Histone demethylases</i>		
LSD1/BHC110	Downregulation	Breast cancer, gastric cancer, lung cancer
JHDM1a, FBXL10,11	Upregulation	Glioblastoma, T cell lymphoma, prostate cancer
JHDM2A/ JMJD1A/TSGA)	Upregulation	Prostate cancer, breast cancer
JMJD2A/ JHDM3A	Upregulation	Squamous cell carcinoma, lung cancer
JARID1A-D	Upregulation/inactivation mutation	Leukemia, prostate, breast, renal carcinoma
UTX JMJD3	Downregulation, inactivation, mutation	Lung cancer, liver cancer, squamous cell carcinoma, renal cell carcinoma

CBP CREB-binding protein, *Dot1* disruptor of telomeric silencing, *EZH2* enhancer of zest homolog2, *Gcn5* general control nonderepressible, *HBO1* histone acetyltransferase binding to ORC1, *HDAC* histone deacetylase, *JARID* Jumonji, AT-rich interactive domain, *JHDM* JmjC domain-containing histone demethylase 1, *JMJD* Jumonji domain containing 2, *LSD1* lysine-specific demethylase 1, *MLL* myeloid/lymphoid or mixed-lineage leukemia-associated protein, *Morf* MOZ-related factors, *MOZ* monocytic leukemia zinc finger protein, *NSD1* nuclear receptor-binding SET-domain protein 1, *p300* E1A-binding protein p300, *PCAF* p300/CBP-associated factor, *PRMT* protein arginine methyltransferase 1, *RIZ1* retinoblastoma protein-interacting zinc finger 1, *SIRT* Sir2 histone deacetylase gene family, *SMYD2* split SET/MYND domain-containing histone H3 lysine 36-specific methyltransferase, *SUV39H* suppressor of variation 3-9 homolog, *TIP60* human HIV-1 Tat interactive protein 60, *UTX* ubiquitously transcribed tetratricopeptide repeat, X chromosome

miRNA profiles are being used to classify human cancers [20]. Approximately, 1,000 miRNA genes in the human genome have been computationally predicted to target multiple protein-coding transcripts [21]. It has been postulated that miRNAs regulate the translation rate of more than 60 % of protein-coding genes, and participate in the regulation of cellular processes [22]. Like mRNAs, miRNAs are mainly transcribed by RNA polymerase-II although miRNA synthesis is known to occur by RNA polymerase-III in those miRNAs that reside near tRNA, Alu, and mammalian-wide interspersed sequences [23, 24]. The first identified miRNAs, the products of the *Caenorhabditis elegans* genes *lin-4* and *let-7*, have important roles in controlling developmental timing and probably act by regulating mRNA translation. When *lin-4* or *let-7* is inactivated, specific epithelial cells undergo additional cell divisions instead of normal differentiation [25]. A list of miRNAs deregulated in various cancers is presented in Table 3.

2.4 Nucleosome Positioning

A growing body of evidence has revealed that nucleosome positioning changes correlate with alterations in gene expression and cancer [26]. Several studies have focused on nucleosome positioning around genes especially at transcription start site or in transcribed regions [27]. For example, nucleosome depletion has been observed in the promoter regions of a hypomethylated tumor suppressor MLH1 in normal cells, whereas in cancer cells the inactive hypermethylated MLH1 promoter is associated with nucleosome occupancy in a mitotically heritable fashion [28]. Prominently, DNA demethylation of the MLH1 gene causes nucleosome eviction and transcriptional activation, and this provide evidence that DNA methylation-mediated epigenetic silencing of tumor-suppressor genes may involve the insertion of nucleosomes into vacant positions [28, 29].

2.5 Chromosomal Looping

Chromosomal looping affects gene expression within the 3D context of nuclear architecture [29]. The knowledge of epigenetic silencing of imprinted genes requires long-range interactions between regulatory elements suggests that chromosome looping is a potential novel epigenetic regulatory mechanism [30]. Increasing evidence suggests that chromosome looping is important in cancer. For example, prostate cancer cells exhibit the formation of chromosomal loops between distal AR-binding sites and the proximal promoters of a few target genes including PSA, TMPRSS2, and UBE2C [31]. Further studies demonstrate that AR is capable of facilitating the formation of gene fusions, viz. TMPRSS2-ETS, in prostate cancer cells through inducing chromosomal looping that brings tumor translocation partners into close proximity [32, 33].

Table 3
microRNA alterations in various human cancers

microRNAs	Alterations	Cancer type	Target gene(s)
miR-127	Upregulation	Bladder cancer	Bcl-6
miR-124	Upregulation	Colon cancer	CDK6
miR-223	Upregulation	Acute myeloid leukemia	NFI-A, MEF2C
miR-34b/34c	Upregulation	Colon cancer	p53 network, CDK6, E2F3
miR-17, miR-92	Upregulation	Lung cancer	c-MYC
miR-372,miR-373	Upregulation	Testicular germ cell tumor and breast cancer	RAS, p53,CD44
miR-21	Upregulation	Glioblastoma, breast, lung, prostate, colon, and cervical cancer	PDCD4, PTEN, TPM1, REC, TIMP3, BCL2
miR-155	Upregulation	Burkitt's lymphoma, breast, colon, and lung cancer	RHOA
miR-146	Upregulation	Breast, pancreatic and prostate cancer	NF-κB
miR-92b	Upregulation	Brain primary tumor	PRMT5
miR-520	Upregulation	Breast cancer	CD44
miR-10b	Upregulation	Metastatic breast cancer	HOXD10
miR-9	Upregulation	Breast cancer	CDH1
miR-127, miR-199a	Upregulation	Cervical cancer	BCL6, E2F1
miR-421	Upregulation	Gastric cancer	CBX7, RBMXL1
miR-1228,miR-195, miR30b, miR-32, miR345	Upregulation	Malignant mesothelioma	CDKN2A, NF2, and JUN
miR-190, miR-196	Upregulation	Pancreatic cancer	HGF
miR-125	Upregulation	Breast cancer	AKT, ERBB2-4, FGF, FGFR, IGF, MAPKs, MMP11, SP1, TNF, VEGF
mir-29	Upregulation	Lung cancer	DNMT3a&b
mir-1	Upregulation	Hepatocarcinoma	FoxP1
mir-9-3	Upregulation	Colorectal, melanoma, head, and neck cancer	E-cadherin
mir-34a	Upregulation	Hematological, prostate, breast cancer	CD44, Notch1
mir-181c	Upregulation	Gastric, colorectal cancer	Notch4, K-Ras
miR-200c, miR-141, miR-429	Upregulation	Colorectal, breast, lung cancer	ZEB1/ZEB2

(continued)

Table 3
(continued)

microRNAs	Alterations	Cancer type	Target gene(s)
miR-126	Downregulation	Breast and lung cancers	CRK1, PIK3R2, SPRED1, VCAM1
miR-146a, miR-146b	Downregulation	Prostate cancer and papillary thyroid carcinomas	ROCK1, IRAK1, TRAF6
miR-340, miR-421, miR-658	Downregulation	Lymph node metastasis and gastric cancer	MYC, RB, PTEN
let-7a-3	Downregulation	Lung and ovarian cancer	RAS, IGF-II
miR-221, miR-222	Downregulation	Hepatocellular carcinoma	CDKN1C/P57 and CDKN1B/P27
miR-9	Downregulation	Ovarian and lung cancer	NF-κB
miR-218, miR-145	Downregulation	Breast, lung, and prostate cancer	PXN
miR-25, miR-32, miR-142	Downregulation	Lung cancer and solid tumor	ITGAα1
miR-124, miR-183	Downregulation	Lung cancer	ITGBβ1
miR-143	Downregulation	Cervical cancer	ERK5
miR-372, miR-373	Downregulation	Testicular germ cell cancer	LATS2
miR-181	Downregulation	Lung cancer	VGFR
miR-370	Downregulation	Cholangiocarcinoma	MAP3K8
miR-342	Downregulation	Breast and colon cancer	ER, PR, and HER2
miR-145	Downregulation	Colon and breast cancer	ER
miR-124, miR-183	Downregulation	Lung cancer	ITGB1 β
mir-101	Downregulation	Prostate, breast, lung cancer	EZH2
mir-143	Downregulation	Colorectal cancer	DNMT3a
mir-9-1	Downregulation	Breast, ovarian, pancreas cancer	FGF family
mir-137	Downregulation	Glioblastoma, oral, colorectal cancer	CDK6, E2F6, LSD
mir-129-2	Downregulation	Gastric, endometrial, colorectal cancer	SOX4
mir-145	Downregulation	Prostate cancer	OCT/SOX2/KLF
mir-148	Downregulation	Colorectal, melanoma, head, and neck cancer	TGIF2
mir-199a	Downregulation	Hepatocarcinoma, testicular ovarian cancer	IKKB

(continued)

Table 3
(continued)

microRNAs	Alterations	Cancer type	Target gene(s)
miR-203	Downregulation	Hematological, liver cancer	ABL1
miR-205	Downregulation	Bladder, breast, prostate cancer	ZEB1/ZEB2
miR-335	Downregulation	Breast cancer	SOX4/TNC
miR-342	Downregulation	Ovarian, breast cancer	PDGFRA

BCL2 B-cell lymphoma 2 protein, *CBX7* chromobox7, *CD44* cluster differentiation 44, *CDH1* cadherin-1, *CDK6* cyclin D kinase 6, *CDNK2A* cyclin-dependent kinase inhibitor 2A, *CRK1* Cdc2-related kinase1, *ER* estrogen receptor, *ERBB2-4* or (*HER4*) human epidermal growth factor receptor 4, *ERK5* extracellular signal-regulated kinase 5, *FGFR* fibroblast growth factor receptor, *FOXO1* Forkhead box protein O1, *HGF* hepatocyte growth factor, *HOXD10* homeobox D10, *IGF-II* insulin-like growth factor 2, *IRAK1* interleukin-1 receptor-associated kinase-1, *KLF* Kruppel-like factors, *LATS2* large tumor suppressor, homolog 2, *MAPKs* mitogen-activated protein kinase, *MEF2C* myocyte-enhancer factor 2C, *MMP11* matrix metalloproteinase 11, *NF2* neurofibromatosis, type 2, *NFIA* nuclear factor 1 A-type, *NF-κB* nuclear factor-κB, *OCT4* octamer-binding transcription factor 4, *P53* tumor protein 53, *PDcD4* programmed cell death 4, *PDGFRA* alpha-type platelet-derived growth factor receptor, *PIK3R2* phosphatidylinositol 3-kinase regulatory subunit beta, *PR* progesterone receptor, *PRNT5* protein arginine N-methyltransferase 5, *PTEN* phosphatase and tensin homologue, *PXN* paxillin, *ITGB1* integrin beta-1, *RAS* rat sarcoma, *Rb* retinoblastoma, *RBMX L1* RNA-binding motif protein X-linked, *RECK* reversion-inducing cysteine-rich protein Kazal Motif, *ROCK1* Rho-associated, coiled-coil containing protein kinase 1, *ROHA* ras homolog gene family member A, *SOX4* SRY (sex-determining region Y)-box 4, *SPRED1* sprouty-related, EVH1 domain containing 1, *TNFα* tumor necrosis factor-alpha, *TPM1* tropomyosin 1, *TRAF6* TNF receptor-associated factor 6, *VCAM* vascular cell adhesion molecule, *VEGF* vascular endothelial growth factor, *ZEB1* zinc finger E-box-binding homeobox 1

3 Cancer Epigenomics

Aberrant epigenomics contributes to neoplastic development by its involvement in the initiation, promotion, invasion, metastasis, and chemotherapy resistance [32, 34]. Reports suggest that more than 300 genes and gene products are epigenetically altered in human cancers through various epigenetic regulated mechanisms [35]. This advancement has been possible through the advent of high-throughput technology to map the human genome at single-nucleotide resolution [36, 37]. Similar to cancer genomics, cancer epigenomics has advanced due to large-scale epigenomics design for characterization of epigenetic alterations at the global and specific gene expression level [38, 39].

The first link between DNA methylation and cancer was established in 1983, demonstrating that genomes of cancer cells are hypomethylated relative to normal counterparts [40, 41]. Cancer cells frequently demonstrate genome-wide hypomethylation and site-specific CpG island hypermethylation in the gene promoter regions [41–43]. DNA hypermethylation at specific genes typically affects promoter CpG islands and inactivates transcription. The transcriptional inactivation caused by promoter hypermethylation affects genes involved in the major cellular pathways including DNA repair [*hMLH1* (mismatch repair gene 1), *MGMT* (O6-methylguanine–DNA methyltransferase),

WRN (Werner syndrome, RecQ helicase like), *BRCA1* (breast cancer 1), cell cycle control (*p16^{INK4a}*, *p15^{INK4b}*, *RB*), Ras signaling [*RASSF1A* [Ras association (RalGDS/AF-6) domain family member 1], *NORE1A*], apoptosis [*TMS1* (target of methylation-induced silencing 1), *DAPK1* (death-associated protein kinase), *WIF-1*, *SFRP1*], metastasis [cadherin 1 (*CDH1*), *CDH13*, *PCDH10*], detoxification [*GSTP1* (glutathione S-transferase pi 1)], hormone response (*ESR1*, *ESR2*), vitamin response [*RARB2* (retinoic acid receptor b2), *CRBP1*], and p53 network [*p14^{ARF}*, *p73* (also known as *TP73*), *HIC-1*] among others [44–49]. This provides tumor cells with higher growth advantage and increases genetic instability and malignant phenotype [45].

DNA hypomethylation in tumor cells is primarily caused by loss of methylation from repetitive regions of the genome causing genomic instability and changes in gene imprinting [45, 50, 51]. In cancer, hypomethylation is often associated with gain of function of oncogenes such as transcription factor c-Myc, which acts as an oncogene and is widely reported to be hypomethylated gene in cancer [52, 53]. Furthermore, hypomethylation at specific promoters can activate aberrant expression of oncogenes and induce loss of imprinting (LOI). The most common LOI event due to hypomethylation occurs in insulin-like growth factor 2 (*IGF2*), and is reported in wide range of cancers including breast, liver, lungs, and colon. Similarly, *S100P* in pancreatic cancer, *SNCG* in breast and ovarian cancers, and melanoma-associated gene (*MAGE*) in dipeptidyl peptidase 6 (*DPP6*) in melanoma are well-cited examples of hypomethylated genes in cancer [54].

In addition to changes in DNA methylation, histone modification patterns are also altered in human tumors. Studies have demonstrated that histone modification patterns are predictive for gene expression. For example, actively transcribed genes are characterized by high levels of H3K4me3, H3K27ac, H2BK5ac, and H4K20me1 in the promoter and H3K79me1 and H4K20me1 along the gene body [35, 55–57]. Loss of acetylation is mediated by HDACs that have been found to be over-expressed or mutated in different tumor types. Aberrant expression of both HMTs and HDMs is observed in various cancer types [15, 58, 59]. A recent study has described inactivating mutations in the histone methyltransferase *SETD2* and in the histone demethylase *UTX* and *JARID1C* in renal carcinomas [60, 61]. H3 acetylation and H3K9 dimethylation can discriminate between cancerous and nonmalignant prostate tissue and H3K4 trimethylation can predict occurrence of prostate-specific antigen serum level elevation after prostatectomy for cancer [57, 62]. *EZH2* (enhancer of zeste homolog 2) expression is an independent prognostic marker that is correlated with the aggressiveness of cutaneous melanoma, prostate, breast, and endometrial cancers [63, 64].

Altered epigenetic patterns in cancer cells are also associated with the deregulation of long noncoding RNAs and repositioning of chromatin-modifying complexes [38, 65]. For example, increased

expression of the ncRNA HOTAIR was found in primary and metastatic breast tumors. Over-expression of HOTAIR, which is normally expressed antisense to the HOXC locus during development and targets the PCR2 complex to the HOXD locus, leads to different PCR2 occupancy at chromatin sites, altered H3K27 methylation patterns, and increased cancer invasiveness in breast cancer cells [38, 65]. Another ncRNA involved in targeting of PCR complexes to tumor-suppressor genes is antisense noncoding RNA in the INK4 locus (*ANRIL*) [18]. *ANRIL* is transcribed from the antisense strand at the INK4b-ARF-INK4a locus, which is an important regulator of cell cycle progression, apoptosis, and cellular senescence. By recruiting PCR1 and PCR2 complexes to form heterochromatin surrounding the INK4b-ARF-INK4a locus, *ANRIL* mediates silencing of these tumor-suppressor genes [47, 66].

The new role of ncRNA has been unraveled which act as decoys for miRNA when they contain specific miRNA-binding sites [5, 47, 67]. For example, the competing endogenous RNA is the transcribed *PTENP1* pseudogene which contains numerous miRNA response elements also present in tumor suppressor *PTEN*. *PTENP1* has been shown to regulate cellular levels of *PTEN* by detracting miRNA from *PTEN* mRNA and is selectively lost in most human cancers indicating its tumor suppressive function [68, 69]. Similarly, miRNA expression patterns also confirm the malignant state. Altered expressions of various miRNAs have been observed in some tumor types [70]. The first association between miRNA and cancer development was described in chronic lymphocytic leukemia with chromosome 13q14 deletion [71, 72]. This deletion deregulates miRNA-15 and miRNA-16. Most of the targets of these two miRNAs are involved in cell growth and cell cycle [72]. The *let-7* is one of the most widely studied miRNA families in cancer. Alterations of *let-7* function have been described in several human cancer types, including carcinomas of the head and neck region, lung, colon, rectum and ovary. It acts mainly as a tumor-suppressor miRNA [25]. miRNA-145 is a well-known tumor-suppressor miRNA downregulated in many human cancers owing to aberrant DNA methylation of its promoter and/or p53 mutations. This miRNA is a pluripotency repressor which regulates silencing of *OCT*, *SOX2*, and *KLF4* in human embryonic stem cells; these genes are required for cell self-renewal and pluripotency maintenance [73–75]. Interestingly, it is becoming apparent that the expression of epigenetic regulatory enzymes such as DNMT, HATs, and HMTs can be controlled by miRNAs. In particular, the miRNA-29 family can directly regulate the expression of DNMTs such that downregulation of this family of miRNAs in small-cell lung cancer results in increased expression of DNMT3A and 3B causing a global genomic hypermethylation and specific methylation-induced silencing of tumor-suppressor genes such as *FHIT* and *WWOX* [76–78].

4 Cancer Epigenetics and Its Link to Genetics

It is evident that discrete genetic alterations in neoplastic cells alone cannot explain the process of multistage carcinogenesis. Neoplastic cells undergo number of transformation during complex phase of tumor development and progression reflected in their phenotype. This process is facilitated by altered epigenome and deregulated epigenetic mechanisms initiating genetic instability resulting in the acquisition of genetic mutation in tumor suppressor genes and activating genetic mutations in oncogenes. Moreover, epigenetic changes in tumors are generally of a clonal nature which occurs in early generation of cancer cells [79]. It is well established that 5-methylcytosine (m5C) residues are “hot spots” for mutations, which can destabilize gene structure and function. One-third of germ-line point mutations leading to human genetic diseases occur at CpGs and most of these mutations are C→T transitions [23]. This is because m5C is highly mutable by deamination, resulting in transitional mutations (i.e., C→T) at CpGs. In view of the symmetry of these CpG motifs, the methylcytosine on the opposite strand may also be affected, leading to (G→A) changes. As a consequence, CpGs are hot spots for mutations, in a variety of genes [80, 81]. G→A transitions are found in 44.8 % cases of leukemia and myelodysplasia, and in 60 % of colon cancer cases. C→T and tandem CC-TT mutations are found in basal cell and squamous cell carcinomas [82]. Methylation increases the rate of hydrolytic deamination and also increases the reactivity of neighboring guanines to electrophiles [82, 83]. The oxidation of m5C may contribute to the high frequency of C→T transitions at CpG sequences. Oxygen radicals can react with m5C to oxidize the 5, 6-double bonds and the intermediate product, m5C glycol, and then deaminates to form thymine glycol [83]. Oxidative stress can contribute to tumor development not only through genetic mechanisms but also through epigenetic mechanisms. As noted earlier, the presence of hydroxyl radicals can cause a wide range of DNA lesions including base modifications, deletions, strand breakage, and chromosomal rearrangements. Such DNA lesions have been shown to interfere with the ability of DNA to function as a substrate for the DNMTs, resulting in global hypomethylation [84]. The presence of 8-OHdG in CpG dinucleotide sequences has been shown to strongly inhibit methylation of adjacent cytosine residues [82, 85]. In addition, 8-OHdG may not be recognized by proof-reading enzymes and thus may persist as a mutation resulting in G→T transversions [86, 87]. These studies suggest that oxidative DNA damage can affect patterns of DNA methylation leading to aberrant gene expression and possibly contributing to the development of malignancy.

5 Clinical Applications

5.1 Epigenetic Biomarkers

In recent years, epigenetic markers have shown promise in establishing the diagnosis and prognosis of various human cancers [48, 88]. The methylated DNA sequences represent potential biomarkers for diagnosis, staging, prognosis, and monitoring of response to therapy. DNA methylation markers hold a number of advantages over other biomarkers, specifically their stability, their ability to be amplified at relatively low costs, and their restriction to limited regions of DNA methylation [89, 90]. Epigenetic markers can be detected in resected tumors and in body fluids. For example, the occurrence of hypermethylated *CDH13*, *MYOD1*, *MGMT*, *p16^{INK4b}*, and *RASSF1A* genes varies significantly among cancer types; their presence can be detected in body fluids as well as in plasma DNA [91, 92]. Hypermethylated cancer genes can also be detected in urine sediments and may prove useful in detecting bladder cancer. A more sensitive and specific screening test for prostate cancer is being evaluated for use in testing urine and plasma DNA. This diagnostic test targets a single methylated gene *GSTPI*. In addition, combined hypermethylation assays for small number of genes such as *RASSF1A*, *RARB2*, *APC*, and *GSTPI* have been used to help discriminate between benign and cancerous changes in the prostate [39, 93, 94]. The field of DNA methylation-based markers for prognosis and diagnosis is still emerging and its widespread use in clinical practice needs to be implemented.

Repetitive DNA elements such as short and long interspersed nuclear elements (SINEs and LINEs) and other repetitive sequences are often hypomethylated in human cancers. However, the utility of global hypomethylation as a prognostic marker in clinical practice remains to be determined [95].

Histone modification patterns also provide prognostic and diagnostic information in cancer. Repressive chromatin structures characterized by particular histone modifications such as H3K9, H3K27, and H4K20 methylation may precipitate DNA methylation [26]. Generalized changes in chromatin structure and histone modification also occur; for example, increased H3K4 dimethylation and H3K18 acetylation activation are reported to be associated with poor prognosis [96]. The major limitation is that it is unknown to what extent these changes correlate with alterations in gene activity in cancer cells. For instance, in addition to the H3K27 methylase EZH2, the corresponding demethylase JMJD3 has been reported to be over-expressed in metastatic prostate cancers [97]. Likewise, over-expression of the histone deacetylase HDAC1 is regularly observed in prostate cancers harboring the major TMPRSS2-ERG fusion [11]. Further assessment and validation in larger patient cohorts is needed before implementation in clinical practice.

Recently, miRNAs have also been proposed as potential epigenetic biomarkers in the diagnosis and prognosis of cancer. Some miRNAs, such as miR-199a, miR-200a, miR-146, miR-214, miR-221, and miR-222 have been found to be upregulated, whereas miR-100 is downregulated in human cancers [98, 99]. The miRNA let-71 was recently designated as a tumor suppressor and miR-429, miR-200a, and miR-200b were found to be clustered on a single primary transcript regulated by the epithelial-to-mesenchymal transition [100]. Studies have shown that two other miRNAs, miR-21 and miR-181a, can be used to identify the presence or absence of malignant phenotypes. A group of 27 miRNAs have been shown to be significantly associated with chemotherapy response and have been proposed as possible prognostic and diagnostic biomarkers, similar to DNA methylation biomarkers [101, 102].

5.2 Epigenetic Therapy and Prevention

The epigenome of a cancer cell is characterized by site-specific DNA hypermethylation, a global pattern of DNA hypomethylation, alterations in miRNA profile, and histone modifications [103–105]. Conceptually, epigenetic changes are reversible and consequently are rational targets for therapeutic approaches. To date, four epigenetic agents have been approved by the US Food and Drug Administration (USFDA) which include two main classes, namely DNA methyltransferase (DNMT) inhibitors and histone deacetylase (HDAC) inhibitors. DNMT inhibitors: 5-azacytidine (Vidaza) and 5-aza-2'-deoxycytidine (Decitabine), which are both analogs of cytosine, have demonstrated clinical activity at low doses against myelodysplastic syndrome and leukemia [104]. However, these nucleoside analogs show poor activity against solid tumors and are associated with severe toxic side effects. These drugs target the epigenome in a non-cell-specific way and therefore modify the methylation patterns in tumor cells as well as in normal cells. Several new DNMT anti-sense (MG98) and small molecule RG108 inhibitors are currently under development, which are more specific and less toxic [106].

Another class of agents is the HDAC inhibitors, which offer more promising targets for epigenetic anticancer therapy. The two HDAC inhibitors approved by USFDA are vorinostat (suberoyl-anilide hydroxamic acid, SAHA) and romidepsin (F-228) for the treatment of rare cutaneous T cell lymphoma [107, 108]. Interest in histone modifications has grown over the last few years with the discovery and characterization of a large number of histone-modifying molecules and protein complexes. Clinical trials show that HDAC inhibitors are well tolerated; can inhibit HDAC activity in peripheral mononuclear cells and tumors, and, more importantly, have clinical activity with objective tumor regression [108]. It is postulated that histone acetylation is associated with activation of gene transcription. Other HDAC inhibitors including butyrate, trichostatin A (TSA), oxamflatin and MS-275 induce expression of

several cell cycle regulatory molecules that inhibit cell-cycle progression, acting to block cyclin-dependent kinase activity and, as a consequence, causing cell-cycle arrest [109, 110]. Furthermore, inactivation of tumor suppressor genes by aberrant DNA methylation of the promoter region is complemented by another epigenetic event that alters the structure of chromatin—the hypoacetylation of lysines in histones, brought about by histone deacetylases. Because cross talk can occur between DNA methylation and histone deacetylation, a combination of these two epigenetic modifications represent an interesting approach for therapeutic intervention [109]. Inhibitors of these two pathways in combination have been shown to produce a synergistic reactivation of tumor-suppressor genes and an enhanced anticancer effect against tumor cells [111].

The use of miRNAs as potential therapeutic targets has been examined in several studies that have shown that specific miRNA deregulation (both over-expression and downregulation) in cancer cells is associated with pathogenic effect [112, 113]. Studies have demonstrated that reducing the expression levels of miR-10b, miR-21, the miR-146 family, miR-155, miR-373, and miR-520c in solid tumors by locked nucleic acid anti-miRNAs or antagomirs, or reexpressing miR-126, miR-148a, miR-206, miR-335, and miR-200 family by mimic miRNAs could be initially tested and validated in preclinical settings and then, if successful, could be considered for Phase I clinical trials alone or in combinations with existing regimens [113]. Additionally, manipulation of miRNA levels may also be a means of altering DNA methylation and therefore miRNA therapy in combination with DNMT inhibitors may be a better combination [114].

Accumulating research has established that environment, diet, and lifestyle factors contribute to cancer development by inducing both epigenetic and genetic changes that, in combination with genetic makeup, result in the disruption of key cellular processes leading to neoplasia [115]. The best studied example is the relationship between dietary methionine and DNA methylation. As an essential amino acid, methionine plays a central role in epigenetic regulation by serving as a methyl donor for methylation reactions [116, 117]. In the process of cytosine methylation, DNMT enzyme converts SAM to S-adenosylhomocysteine (SAH); therefore, an optimal supply of SAM or removal of SAH is essential for normal establishment of genome-wide DNA methylation patterns. CpG methylation patterns are largely erased in the early embryos and then reestablished in a tissue-specific manner. Therefore, early embryonic development may represent a sensitive stage, and dietary and environmental factors that affect DNA methylation reaction and the activity of DNMTs may result in permanent fixation of aberrant methylation patterns [118]. Another potential mechanism by which environmental and dietary exposures affect the epigenome may involve transposable elements [34].

Transposons are groups of mobile genetic elements that, when activated, may cause genetic mutations and transcriptional noise. They are shown to be heavily methylated and transcriptionally silent in somatic cells. Although it is well documented that the activation of transposable element-derived promoters may be a consequence of perturbed DNA methylation, transposable elements have been shown to be activated by different kinds of cellular stress [116]. Therefore, stress induced by environmental and dietary agents may activate transposable elements, leading to altered establishment and maintenance of epigenetic states [117]. Different classes of HDACs may also be altered by environmental and dietary agents. Interestingly, certain dietary chemopreventive agents such as butyrate, apigenin, diallyl disulfide, and sulforaphane have demonstrated HDAC inhibitory activity [119]. A recent study highlights the role of resveratrol in demonstrating that it is the most potent inhibitor of SIRT1, a member of the sirtuin family of NAD-dependent deacetylases, improves health and extends life span [120]. Certain dietary agents such as green tea polyphenols and phenethyl isothiocyanate have shown dual actions as DNMT as well as HDAC inhibitors in cancer cells. The dual action of these agents on both DNA and chromatin was more effective than 5'-aza-2'-deoxycytidine, or TSA, suggesting that they may be better epigenetic modifiers for cancer prevention, achieved through dietary intervention [121].

6 Conclusions and Future Directions

The importance of epigenetics in cancer has been recognized and the field has emerged rapidly over the years. Advancement in technology and the use of new high-throughput methods has made possible to study epigenetic process at the global level than a single gene. Techniques like next-generation sequencing allow studies of DNA methylation status of human cells at nucleotide resolution. In addition, high-density microarrays for miRNA profiling and ChIP-Chip and ChIP-Seq have ability to detect precisely the location of different covalent histone modifications at the global level. These epigenomics approaches have revolutionized cancer research and have expanded the understanding of tumorigenesis to provide information about epigenetic biomarkers for detection, prognosis, and therapeutic assessment. Additionally, understanding of the link between epigenetics deregulation and cancer will help in designing better treatment strategies. Moreover, the intrinsic reversibility of epigenetic modifications represents an exciting new opportunity for the development of novel strategies for cancer prevention and therapeutic intervention.

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Chapter 2

Community Resources and Technologies Developed Through the NIH Roadmap Epigenomics Program

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Abstract

This chapter describes resources and technologies generated by the NIH Roadmap Epigenomics Program that may be useful to epigenomics researchers investigating a variety of diseases including cancer. Highlights include reference epigenome maps for a wide variety of human cells and tissues, the development of new technologies for epigenetic assays and imaging, the identification of novel epigenetic modifications, and an improved understanding of the role of epigenetic processes in a diversity of human diseases. We also discuss future needs in this area including exploration of epigenomic variation between individuals, single-cell epigenomics, environmental epigenomics, exploration of the use of surrogate tissues, and improved technologies for epigenome manipulation.

Key words Bisulfite, Cell-type, Chemo-epigenetic, ChIP-seq, Chromatin, Immunoprecipitation, CRISPR, Crotonylation, Deacetylase, Hi-C, Ectoderm, ENCODE, Endoderm, Epigenetic, Epigenome, Genome-wide, HDAC, Histone, hmC, IHEC, mC, MeDIP-Seq, Methylation, MethylC-seq, Methylome, MRE-Seq, mRNA, Nucleosomes, Nucleus, Opto-epigenetic, Pluripotent, RNA-seq, Roadmap, RRBS, Transcription

1 Introduction: Overview and Goals of Program

Epigenomics is the study of functional, and sometimes heritable, changes in the regulation of gene activity and expression that do not change the underlying DNA sequence [1–3]. Although each cell type in the human body (with some exceptions) contains the same genetic information, the interplay between transcription factor programs and epigenetic regulation enables pluripotent stem cells to give rise to the diversity of differentiated cell types (e.g., skin cells, liver cells, neurons) [4, 5]. Furthermore epigenetic processes, from gametogenesis through embryonic and neonatal stages, and continuing throughout adolescence, adulthood, and

senescence, can influence both normal development and a variety of disease processes [6–9].

Even before completion of the first human genome sequence in 2000, a grassroots scientific effort began encouraging a large-scale, organized project in Epigenomics [10]. Meetings were held by a number of NIH institutes (e.g., NCI, NIDA, NIEHS) and by other groups (e.g., American Association of Cancer Researchers) that provided recommendations on how best to help the scientific community accelerate discovery in epigenomics [11, 12].

In 2007, Epigenomics was selected as a trans-NIH Roadmap (now Common Fund) project. A 2006 portfolio analysis conducted by the NIH revealed that between 1998 and 2006, increasing numbers of funded studies in the area of epigenetics were supported by the NIH Institutes and Centers. Moreover, there was a similar escalation in the number of publications associating altered gene expression profiles and epigenetic processes with adverse disease outcomes. Additionally in 2007, a scientific meeting was hosted by NIH to garner specific recommendations for a large-scale, transformative project in this area. Recommendations from this meeting and from prior scientific meetings were used to craft the four major initiatives of the NIH Roadmap Epigenomics Program:

1. To generate reference epigenome profiles for “normal” human cells and tissues, including new ways to generate, visualize, and analyze this data
2. To discover novel epigenetic marks
3. To develop revolutionary technologies with the potential to significantly change epigenetics research (with sub-initiatives in general epigenetic technologies, epigenetic imaging, and epigenomic manipulation)
4. To transform our understanding of the epigenomic basis of disease

These goals were translated into several distinct initiatives that are illustrated in Fig. 1. These projects included the development of reference epigenomes; the identification of novel epigenetic marks; the development of new technologies for epigenomic assays, imaging, and manipulation; investigations into epigenomic processes in a variety of diseases; and the development of monoclonal antibody tools for epigenomic research. To date a total of 78 grants have been funded by this program, including 61 R01s, 7 R21s, 5 RC1 “challenge” grants, and 5 U01s (<http://commonfund.nih.gov/epigenomics/fundedresearch>). The following sections describe the technologies and community resources generated from each of these initiatives.

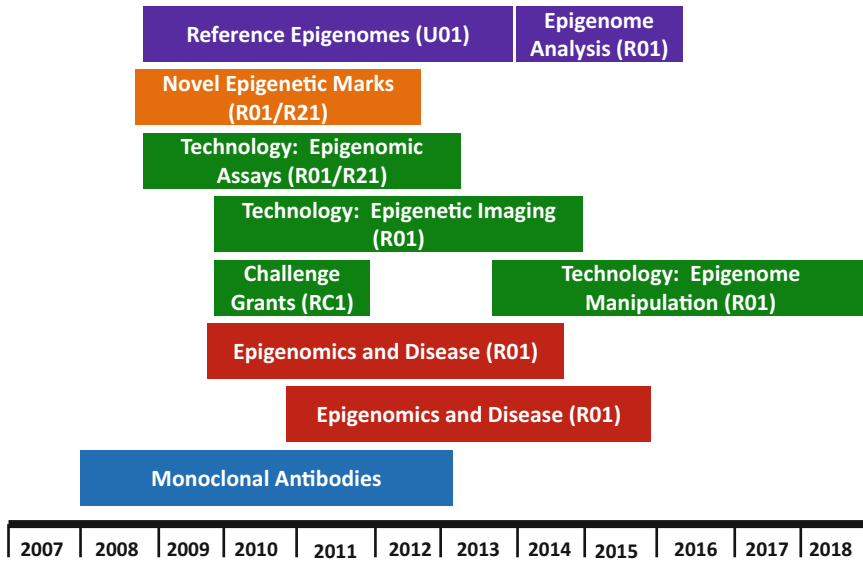


Fig. 1 Roadmap Epigenomics Program Projects and Timeline. This figure shows the major components of the program and the periods of Roadmap Epigenomics Program funding. Reference Epigenome activities are shown in *purple*, novel marks in *orange*, technology initiatives in *green*, disease studies in *maroon*, and antibodies in *blue*

2 Community Resources and Technologies Developed Through the Epigenomics Program

2.1 Comprehensive Reference Epigenome Maps

As indicated above, a key scientific recommendation was to generate comprehensive reference epigenome maps for normal human cells and tissues. These epigenomic maps would provide a public resource for the scientific community to use in investigator-initiated investigations of epigenetic regulation in biological processes and human disease. Specifically, these maps were intended to:

- Provide a normal baseline for investigators exploring environmental or disease epigenomics
- Reveal how epigenomes change during differentiation and development
- Discover how epigenomic modifications “interact” or co-occur with one another
- Reveal functional genomic elements
- Inform regenerative medicine studies by investigating the epigenomes of stem and induced pluripotent stem (iPS) cells
- Enable integration of epigenomic information with genetic information to better understand genome/epigenome interactions
- Provide a foundation for the future development of biomarkers and therapeutics.

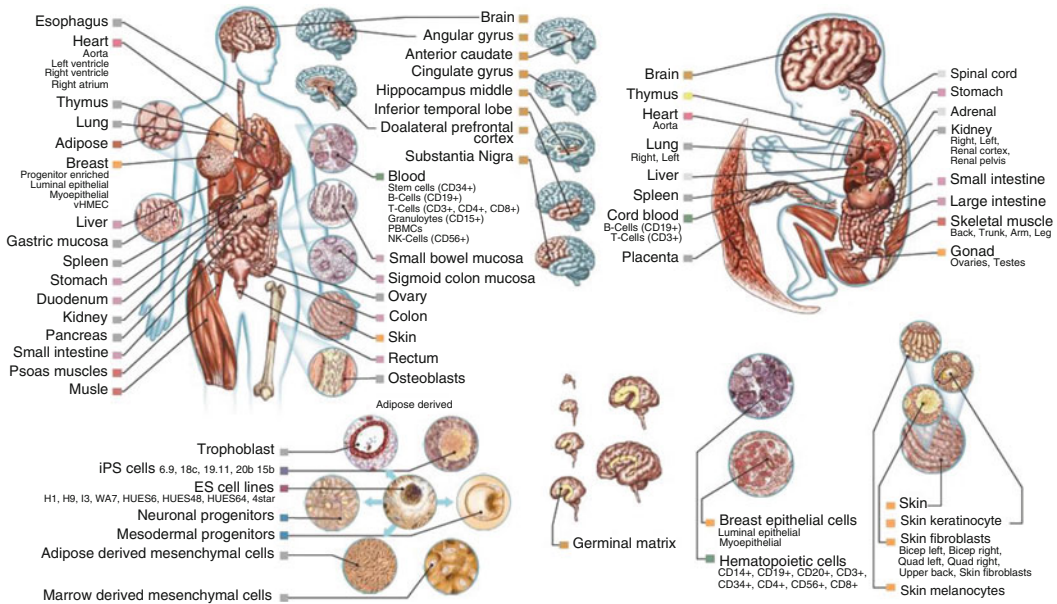


Fig. 2 Reference epigenomes from a diversity of human cells and tissues. The *top left* shows some of the characterized adult human cells and tissues including hematopoietic cells, gastrointestinal (GI) tract, adipose, muscle, heart, and several brain regions. The *top right* shows some of the mapped fetal cells and tissue types including muscle, lung, heart, GI tract, and placenta. The *bottom right* shows some of the fetal samples in more detail (brain, hematopoietic, and skin). The *bottom left* shows some of the cell lines characterized including human embryonic stem cells (hESCs), cell types differentiated into neural progenitor cells, trophoblast, etc., and induced pluripotent stem cells (iPSCs). Illustration by Rae Senarighi, University of Washington

The Roadmap Epigenome Mapping Consortium (REMC) was established to generate reference epigenome maps for normal human cells and tissues. The REMC consists of four mapping centers and one data coordination/analysis center to process the data through standardized pipelines [13]. The original goal of the REMC was to generate 25 reference epigenome maps by the end of the project. However due to rapid improvements in technology and cost reductions, the REMC has generated a total of 92 reference epigenome maps for a wide range of human cells and tissues from adults and fetuses as well as from embryonic stem (ES) cells and their derivatives (*see* Fig. 2). Normal cell or tissue types were prioritized for epigenome mapping based on their availability, the amounts of tissue available for the multiple necessary assays, to achieve a balance between purified cell types and heterogeneous tissues, and their anticipated relevance to human disease. Some of the comprehensive reference epigenomes generated were from embryonic stem (ES) cells, ES-derived cells, iPS cells, hematopoietic cell types, fetal tissues (adrenal, brain, spinal cord, heart, gastrointestinal, kidney, lung, muscle, placenta, thymus), and adult tissues (fat, breast, brain, gastrointestinal, muscle, skin, connective tissue).

The advent of next-generation sequencing coupled with development of improved or novel technologies (e.g., ChIP-seq, RNA-seq, MethylC-seq) and the requisite expansion of informatic capabilities provided an unprecedented opportunity to generate a catalog of reference epigenome maps for many human cells and tissues [8]. Of course, given the large number of known chromatin modifications and the likely existence of undiscovered chromatin modifications, it is not possible to generate “complete” epigenome maps. Thus the REMC has determined that a reference epigenome should include assays for DNA methylation, a core set of six posttranslational histone modifications (PTMs, H3K27me3, H3K36me3, H3K4me1, H3K4me3, H3K27ac, and H3K9me3), and mRNA expression analysis. Some samples were also assayed for chromatin accessibility using the DNase I hypersensitivity assay.

The reference epigenomes generated are categorized into four Classes (1–4) defined by the associated epigenomic data sets (*see* Table 1 for details). For example, Class 1 epigenomes have (1) DNA methylation maps generated by whole-genome bisulfite sequencing, (2) maps of six core histone PTMs plus up to 20 auxiliary histone PTMs, (3) chromatin accessibility data, and (4) transcript analysis by RNA-seq. As of January 1, 2014, the REMC has generated 92 Class 1–4 reference epigenomes. Additional useful data sets that do not contain all of the assays required for Class 1–4 epigenomes have also been generated by the REMC (<http://www.roadmapepigenomics.org/>). 23 of the 92 reference epigenomes are expected to be genotyped by whole-genome sequencing to help researchers probe the interactions between the genome and epigenome.

The REMC has undertaken the most comprehensive and coordinated effort to generate reference epigenome maps to date. In addition to the generation of this diverse panel of reference epigenomes, consortium investigators have delivered a number of critically important contributions to the field, a few of which are highlighted below. The consortium has developed and implemented a set of experimental and data quality standards/guidelines for these assay types (<http://www.roadmapepigenomics.org/protocols>). The Roadmap Epigenomics Program has also worked with other large projects performing epigenomic assays to develop congruent metadata standards and ontologies to maximize the utility and interoperability of data across these large projects (*see* Subheading 3.1). Early in the mapping efforts, consortium investigators developed the assay that produced the first complete mammalian methylomes [14]. Other major accomplishments include (1) identification of distinct epigenetic mechanisms regulating early and late stages of differentiation in embryonic stem cells [15]; (2) identification of epigenomic processes that transpire as ES cells give rise to the three embryonic germ layers, ectoderm, mesoderm, and endoderm [16]; (3) demonstration that chromatin

Table 1
Reference epigenome classes and progress

Assay	Class 1	Class 2	Class 3	Class 4
<i>Histone PTM ChIP-seq</i>				
H3K27me3	Required	Required	Required	Required
H3K36me3	Required	Required	Required	Required
H3K4me1	Required	Required	Required	Required
H3K4me3	Required	Required	Required	Required
H3K27ac or H3K9ac	Required	Required	Required	Required
H3K9me3	Required	Required	Required	Required
Up to 24 auxiliary histone modifications	Required	Not req.	Not req.	Not req.
<i>DNA methylation</i>				
WGBS/MethylC-seq	Required	Required	Not req.	Not req.
RRBS	Not req.	Not req.	Any assay	Any assay
MeDIP-seq/MRE-seq	Not req.	Not req.	Any assay	Any assay
<i>Chromatin accessibility</i>				
DNase I hypersensitivity	Required	If possible	Required	Not req.
<i>mRNA expression</i>				
mRNA-seq	Required	Required	Not req.	Not req.
Array based	Not req.	Not req.	Any assay	Any assay
<i>Number completed</i>	7	51	14	20

The Reference Epigenomes generated by the REMC have been classified according to the number and comprehensiveness of the assays performed. This table also indicates which assays are required or not required for a particular class of epigenome. Originally H3K9ac was a core histone PTM but it was replaced by the more informative H3K27ac. “Any assay” means that any assay of a given type (e.g., DNA methylation) was sufficient for a particular epigenome class. The most challenging assay, DNase I hypersensitivity, was encouraged if possible for Class 2 epigenomes. A total of 92 comprehensive reference epigenomes have been generated as of January 1, 2014. In addition, there are many available data sets for other tissues in which it was not possible to perform a complete set of assays (<http://www.roadmapepigenomics.org/>). Abbreviations include whole-genome bisulfite sequencing (WGBS), reduced representation bisulfite sequencing (RRBS), methylated DNA immunoprecipitation (MeDIP-seq), and methylation-sensitive restriction enzyme-sequencing (MRE-seq)

states change in response to developmental or environmental cues [17]; and (4) the discovery that, in addition to coding for amino acids, approximately 15 % of human DNA codons can specify transcription factor binding sites [18]. Consortium investigators have also developed remarkable insights into disease susceptibility by discovering that a large percentage of disease-associated variants from genome-wide association studies (GWAS) occur in DNA regulatory regions defined by DNase I hypersensitive sites [19]. Many of these regulatory DNA elements are enriched for gene variants

that have been associated with intrauterine exposure-relevant phenotypes and are frequently active during fetal development.

2.2 Reference Epigenome Data Access, Analysis, and Visualization

The data generated by the REMC constitute a rich resource that is broadly available to the scientific community. Although a brief overview is provided here, a more complete listing of different options for accessing, viewing and analyzing epigenomic data generated from these efforts is published elsewhere [20, 21]. These data can be classified into two major categories: data with associated human DNA sequence information and data without associated human DNA sequence information. In general, read density maps and data that do not include human DNA sequence information are submitted to the Gene Expression Omnibus (GEO), supported by the National Center for Biotechnology Information (NCBI), after data cleaning and can be obtained and downloaded for specific analyses (<http://www.ncbi.nlm.nih.gov/geo/roadmap/epigenomics/>). Data containing de-identified human DNA sequence information are, in many cases, also publically available through NCBI's Sequence Read Archive (SRA). In other cases, the precise language in the patient consent form and/or the Institutional Review Board of one of the epigenome mapping centers necessitated that these data be deposited into the controlled-access database of Genotypes and Phenotypes (dbGaP). Scientists may request access to use these data by completing standard dbGaP forms (<http://www.ncbi.nlm.nih.gov/gap>).

The complex nature of epigenomic data sets has stimulated the development of new integrative tools as well as multifaceted browsers to facilitate epigenome visualization. The Genboree Workbench, hosted by the Human Epigenome Atlas, allows investigators to analyze their own data with REMC-generated data using and take advantage of numerous comparative and integrative analysis tools that have been created through the REMC (<http://www.genboree.org/epigenomeatlas/index.rhtml>). Consortium data may be visualized directly on the UCSC Genome Browser using the Track Hub feature or can be browsed by cell type, mark, or a specific text query at several websites (e.g., <http://www.roadmapepigenomics.org/data>, <http://www.genboree.org/epigenomeatlas/index.rhtml>). REMC data can also be viewed in conjunction with ENCODE data (<http://encode-roadmap.org/>). Researchers interested in novel approaches for visualizing epigenomic data, including the ability to view long-range genomic interactions, should explore the Human Epigenome Browser (<http://epigenomegateway.wustl.edu/info/>).

These epigenomic data sets provide baselines for comparisons with diseased cells or tissues and have also shown great promise in elucidating the functional significance of non-coding genetic variant hits from GWAS experiments [19]. They also provide baselines

for allowing comparisons with diseased cells or tissues. Researchers may soon routinely use epigenome data sets to learn how chromatin state-defined cis-regulatory elements impact transcript distribution and abundance [8, 21]. Furthermore, with epigenome maps in hand, it may soon be possible to understand how environmental context (e.g., influences of drugs of abuse, stress, toxins, diet chemotherapy protocols, and other environmental exposures) alter the epigenome [22–24].

2.3 Discovery of Novel Epigenetic Modifications

At the time the Roadmap Epigenomics Program began, a number of histone modifications and just one DNA modification (mC) had been identified in eukaryotes. It was clear that our catalog of chromatin modifications was incomplete. Thus, an initiative was developed to discover additional epigenetic modifications in order to move toward the completion of this catalog. By 2013, researchers funded through this initiative generated numerous publications in this area and have identified and/or further characterized multiple new histone posttranslational modifications (PTMs) in yeast, *Drosophila*, and mammalian cells [25–27].

In one study alone, Dr. Yingming Zhao and his colleagues identified an additional 67 novel histone PTMs in mammalian cells including lysine crotonylation [26]. In addition, some projects have identified or implicated proteins in the specific deposition or removal of these marks on chromatin. These researchers have also discovered specific roles for the new histone PTMs, or combinations of PTMs, in the control of key biological functions such as cell cycle regulation, development, and DNA repair [28–31]. These studies move the field closer to establishing a more complete catalog of DNA and histone modifications. However, much work remains to be done to better characterize the biological functions of these DNA and histone modifications as well as the enzymes that establish, remove, or bind to these modifications.

2.4 Improved Assays for Monitoring the Epigenome

Prior to the start of the NIH Roadmap Epigenomics Program, there were several useful assays to monitor the epigenome including chromatin immunoprecipitation followed by microarray or sequencing (ChIP-chip, ChIP-seq) which detects chromatin features, reduced representation bisulfite sequencing (RRBS) which enables characterization of DNA methylation status at single base resolution at a subset of genomic regions, and the DNase I hypersensitive assay which detects the level of chromatin accessibility [8]. Although these assays were state of the art at the time, some were not sufficiently facile or comprehensive. Also there were limitations to the types of biological questions one could ask using these approaches. To address this scientific gap, an initiative on Technology Development in Epigenetics was initiated in 2008. In parallel the REMC expended great effort to improve current assay technology and develop new technologies [14, 32–34].

Four of the new or improved assays that were developed by these components of the Roadmap Epigenomics Program are described as follows. Prior to November 2009, methods for analyzing DNA methylation at single base resolution were limited and the largest existing methylome data set that existed was for the model plant *Arabidopsis thaliana* [35]. As a part of the REMC, researchers in the Ecker lab optimized their MethylC-Seq assay and applied it to two different human cell types, generating the first genome-wide single base resolution maps for any human cell type [14]. Since then, the use of MethylC-Seq and related assays for interrogating DNA methylation state has become widespread.

Most epigenomic assays measure a molecular phenotype from the chromatin of a population of cells but there is great interest in understanding how chromatin differs between individual cells. Researchers in the Soloway lab have recently published a paper that describes their bifurcated nanofluidic device for real-time detection and sorting of individual molecules of methylated DNA [36]. The DNA molecules were identified using fluorescently labeled methyl binding domain protein 1 (MDB1) which bound specifically to double-stranded, methylated DNA. They were also able to recover the sorted DNA by quantitative PCR for possible post-sorting applications. This device holds great promise for color-multiplexed epigenetic analyses and will allow recovery of genetic material for downstream studies.

Although the ability to monitor chromatin states across the genome is robust, a major obstacle has been the inability to interrogate the chromatin features that co-occur at a single genomic locus. Researchers in the Tackett and Taverna labs have developed chromatin affinity purification with mass spectrometry (ChAP-MS) that involves isolation of a single genomic locus which can then be subjected to mass spectrometry analysis to identify the proteins and associated PTMs at that locus [37]. As our ability to isolate genomic loci improves, this method may enable researchers to identify the complement of proteins that are physically associated with a given genomic locus in addition to pinpointing the precise histone modifications that are present. This technology will facilitate a deeper understanding of how these loci change during development or upon environmental exposure and also reveal in greater detail the cross-communication that occurs between epigenetic and transcriptional regulatory pathways.

Chromatin assays are frequently used to investigate a single point in time, obscuring dynamic biological processes. To address this limitation, researchers in the Henikoff lab developed a metabolic labeling strategy that enables affinity purification of tagged nucleosomes containing newly synthesized histones. This technique allows the measurement of histone turnover rates across the genome providing the ability to explore temporal chromatin changes [38].

2.5 Technologies for Improved Epigenetic Imaging

Many available epigenomic assays only probe a single time point; however it is often desirable to understand the dynamics of a biological system. Furthermore, each mammalian cell type is believed to have a distinct epigenomic profile, which may be altered in a disease state. As researchers understand more about how epigenomes are altered in specific disease states or by environmental perturbations, and develop “epigenetic therapeutics” to treat disease, our limited ability to visualize epigenetic modifying enzymes and modifications in vivo remains a significant scientific obstacle. Clinical diagnosis of diseases with a significant epigenetic component could be greatly enhanced if we improved our ability to monitor epigenetic modifying enzymes, binding proteins, or epigenetic states in specific tissues or cell types. While tissues such as blood or skin are readily available for this type of analysis, in the case of diseases impacting the brain, heart, bone, and other vital organs it may be difficult or impossible to obtain the appropriate tissue or cell type for epigenetic analysis.

Since the current technologies to determine the in vivo epigenetic state of tissues is extremely limited, a Roadmap Epigenomics Program initiative was developed to enable in vivo imaging or analysis of epigenetic changes at multiple levels ranging from a single cell to an entire organism. Several funded projects proposed a wide array of approaches including RNA aptamers, fluorescence complementation, soft X-ray tomography (SXT), magnetic resonance imaging (MRI), and positron emission tomography (PET). Two of these projects are described in more detail below.

Researchers in the Lomvardas and Larabell laboratories have been developing an in vivo fluorescence complementation approach to visualize epigenetic interactions between proteins when they physically interact. This approach will be linked to fluorescence microscopy-compatible SXT to analyze chromatin territories at high resolution in vivo. SXT analysis of olfactory nuclei indicates that downregulation of the lamin b receptor leads to the unusual nuclear architecture of the olfactory neuron. Downregulation of lamin b causes significant changes in epigenetic gene regulation in certain gene families [39]. Optimization of this technology could enable researchers to better correlate in vivo chromatin structure with chromatin features such as epigenomic marks, transcription factor binding, or chromatin-associated long noncoding RNAs.

In independent efforts, researchers in the Hooker and the Gelovani laboratories have been developing and optimizing PET radiotracers for in vivo imaging of Class I and Class III histone deacetylases (HDACs), respectively [40–42]. If robust PET ligands for these important epigenetic regulatory proteins are developed, they will be invaluable for monitoring in vivo changes in HDAC levels and/or activity over time and would be useful for monitoring difficult to access tissues such as the brain. If these ligands function

successfully in humans, they may be important for clinical diagnosis of disease as well as for monitoring the therapeutic efficacy of HDAC inhibitor treatment regimens.

2.6 Epigenomics in Health and Disease

The Reference Epigenome Maps developed by the Roadmap Epigenomics Program illustrate the critical role the epigenome plays in regulating cell type identity and function. One of the overarching goals of the Roadmap Epigenomics Program is to understand how alterations in the normal patterns of epigenetic regulation could contribute to the onset or progression of disease. The Epigenomics of Human Health and Disease initiative was developed to support epigenomic investigations of diseased, exposed, or otherwise compromised human tissues, in an effort to identify epigenetic changes that may underlie disease states.

At the time that this initiative was developed, the concept that epigenetic misregulation, rather than strictly genetic mutation, might underlie disease was not new. In 1983, Feinberg and Vogelstein published their landmark study showing that some genes became hypomethylated in human tumors relative to adjacent normal tissues, providing the first evidence that diseased cells could have different epigenetic profiles than their normal counterparts [43]. This initial finding sparked a long-lasting interest in cancer epigenetics. A 2006 portfolio analysis showed that the vast majority of NIH funding in disease epigenetics was in the area of cancer. However, it was not clear whether epigenetic misregulation is associated with other diseases and whether or not this misregulation plays a causal role. To address this knowledge gap, a Roadmap Epigenomics Program Disease initiative was implemented in 2008, which resulted in the co-funding (by the Common Fund and the relevant NIH Institutes and Centers) of 22 new grants, focused on a wide range of conditions. A second related initiative was implemented in 2010, which resulted in the funding of 11 additional grants by specific NIH Institutes and Centers. The breadth of disease research represented across these two initiatives is illustrated in Fig. 3. Below we describe the work of several investigators that used epigenomic approaches to identify key regulatory mechanisms that are altered in human disease.

The Young group used ChIP assays to define genomic regions containing large clusters of transcriptional enhancers (super-enhancers) highly enriched for transcription factors, the mediator complex, and chromatin modifications such as histone H3K27 acetylation. These super-enhancers drive transcription of key regulatory genes, such as master transcription factors, that are important in establishing cell type-specific gene expression programs during development and differentiation [44]. A subsequent study showed that a significant number of genetic variants associated with a diverse set of diseases are enriched in super-enhancers in disease-relevant cell types. This suggests that disruption of these

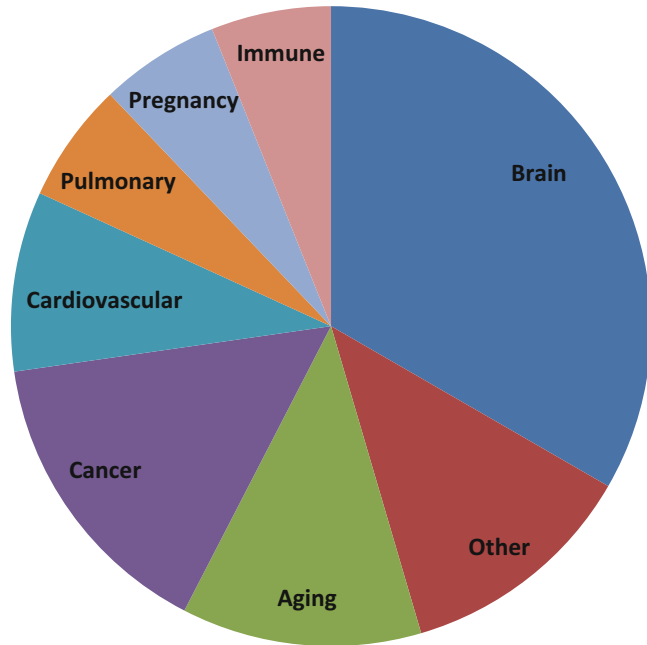


Fig. 3 The spectrum of epigenomics and disease investigations. This figure illustrates the diversity of tissues and diseases being investigated through the 33 disease projects funded as a part of the program. The major topics include brain disorders, aging, cancers, cardiovascular, pulmonary, immune research, and pregnancy

genomic elements may be a factor in the pathogenesis of a wide range of diseases [45].

The Huang group showed that in normal cells, estrogen mediates long-range epigenetic silencing through the transient formation of chromosomal loops flanked by estrogen receptor alpha (ESR1)-bound genomic regions [46]. However, in breast cancer, these loops stabilize and are associated with repressive epigenetic marks. In subsequent studies, Dr. Huang's group showed that these genomic regions, known as DEREs (distant estrogen response elements), were frequently amplified in breast carcinogenesis, and that gene expression changes associated with this phenomenon likely contribute to some breast cancers, as well as with the development of resistance to endocrine therapy.

One major issue that researchers contend with is sample origin: for example the disease affects the brain, but only peripheral blood samples are available. The Mill group took advantage of matched postmortem blood and brain samples from non-diseased individuals to compare DNA methylation patterns across several brain regions and blood [47]. As expected, they identified regions of tissue-specific methylation that differed between the brain and blood samples. These regions were mostly intragenic CpG islands

and low CpG density promoters, and were generally associated with genes that were differentially expressed across brain regions. However they also found that although the epigenomic profiles of blood and brain differed, a subset of the epigenetic variation observed between individuals within a tissue (such as the type of variation expected to contribute to phenotypic differences) was conserved across brain and blood. This suggests that although analysis of blood samples is unlikely to capture all of the disease-associated epigenetic changes found in the diseased tissue, it might have some utility as a surrogate tissue for certain human disease investigations. Furthermore, chromatin state information from specific blood cell types or other readily accessible cells or tissues may also be useful for developing biomarkers for predicting disease state or quantitating environmental exposures.

2.7 Epigenetic Antibodies

Another important resource gap at the start of the Epigenomics Program was the limited availability of high quality antibodies for probing epigenetic proteins or marks. Most of the available antibodies were polyclonal with only a finite supply of each batch. This limited the utility of these antibodies to the scientific community and made experimental validation between laboratories more challenging. Furthermore, antibodies varied dramatically in both their specificity and efficacy in ChIP experiments [48]. A minor initiative in the Roadmap Epigenomics Program aimed to develop a number of high-quality monoclonal antibodies against selected proteins and marks, and to make these antibodies available to the scientific community at low cost. As of 2013, 19 antibodies were generated and have been made available including: 5hmC, 5fC, Dicer, DNMT3L, Histone H3.3, Histone H3-pThr11, JMJD2A, NSD1, Histone H2A.Z/V, H3K56ac, Histone H2A Z-acetyl-Lys4/7/11, and Histone H3-acetyl-Lys56. These antibodies are available through NeuroMab at <http://neuromab.ucdavis.edu/catalog.cfm>.

2.8 Overall Roadmap Epigenomics Program Impact

There are many challenges in evaluating the impact of a scientific program since there is not one clear quantitative measurement that should be used. Depending on the nature of the project one could consider metrics such as web hits, data downloads, patent applications, or investigational new drug applications (INDs). Other less quantitative but salient metrics might include the number and types of spinoff projects, the extent to which the scientific community adopts protocols or reagents produced, the extent to which new protocols or assays lead to decreased costs or increased amounts of useful information, or how the program influenced the development of national and international efforts in this area. Often the effects of investment in a scientific program are not fully understood or appreciated for many years and sometimes not until well after the program ends.

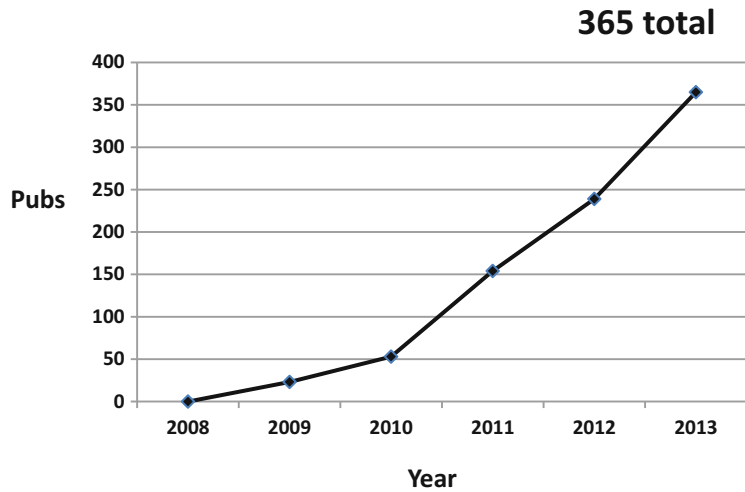


Fig. 4 Roadmap Epigenomics Program Publications Over Time. The Y-axis shows the total number of publications as of January 1, 2014 attributed to the program as a result of funding support, while the X-axis shows the year

Ultimately, scientific progress requires communication through publications. Publications can yield several useful impact metrics including (1) the absolute quantity of publications produced, (2) the quality of the publications produced, and/or (3) the number of times these publications have been cited. As shown in Fig. 4, there has been an increase in the absolute number of publications since the beginning of the Roadmap Epigenomics Program. As of January 1, 2014 there have been 365 publications attributed to the funded components of this program (<http://commonfund.nih.gov/publications?pid=5>). A significant number of Roadmap Epigenomics Program projects have ongoing funding (some began as recently as September 2013) so we expect that this number will continue to rise. Even some of the projects that have been completed 2 years ago are still publishing data obtained during Roadmap Epigenomics Program support.

With respect to publication quality, many of the results produced by projects within the program have been published in journals with high-impact factors. For example, as of January 1, 2014, there have been 23 publications in *Cell*, 12 in *Nature*, and 3 in *Science*. This is an indicator that many of the publications produced by the program are of potentially high impact.

Finally, several papers produced by the program have been highly cited suggesting they have made an important impact on the scientific community. One paper has been cited a total of 970 times as of January 1, 2014 [14]. A total of 26 papers have been cited between 100 and 401 times. Fifty-five publications have been cited between 30 and 100 times. Additionally, some of these publications have only been publically available for a short period of time and we anticipate that citations for these newer papers will increase over time.

3 Future Epigenomics Needs and Directions

3.1 *Expanding Epigenomic Data Sets*

The REMC will produce approximately 100 comprehensive epigenome maps by its conclusion. Although this is a remarkable achievement for the 5 years of this initiative, more epigenome maps are needed for uncharacterized cell types and tissues, diseased tissues, for cells and tissues with specific environmental exposures (e.g., environmental toxins, drugs of abuse), and for longitudinally-collected cells and tissues. These maps will be necessary for scientists to fully understand the role of the epigenome in human diseases. Building upon the NIH Roadmap Epigenomics Program, the International Human Epigenome Consortium (IHEC; <http://epigenomesportal.ca/ihec/>), an international consortium of funding agencies (Canada, European Union, Germany, Japan, South Korea, and the USA) aims to generate as many as 1000 epigenome maps. Hopefully, this will be a sufficient number of epigenomes to provide both a broad and comprehensive understanding of epigenomic regulation and epigenomic differences within and across disease states.

A sister project cataloguing the Encyclopedia Of DNA Elements (ENCODE; <http://www.genome.gov/encode/>) seeks to identify and catalog all of the functional elements in the human genome, along with a smaller effort to annotate the mouse genome. Although the goal of this NHGRI-funded project is not to generate epigenome maps, many of the assays used within this project are epigenome mapping assays. Roadmap Epigenomics Program and ENCODE data can be viewed together through the following web link (<http://www.encode-roadmap.org/>). The Roadmap Epigenomics Program has also worked with ENCODE and IHEC to develop congruent metadata standards and ontologies to maximize the utility and interoperability of data across these large projects. REMC, IHEC, and ENCODE data can be accessed via a common portal (<http://edcc-dev2.udes.genap.ca/edcc/ihec/index.html>). As these projects move forward, it will be important to continue to coordinate efforts such that the data from ENCODE, IHEC, the Roadmap Epigenomics Program and other entities will be as interoperable as possible, and ensure that there will be long-term support for data archiving since these rich data resources are certain to have useful synergies with one another as novel biological questions are explored.

3.2 *Epigenomic Analysis*

Although there has been a great deal of data generated by the Roadmap Epigenomics Program thus far, a deeper analysis effort is needed in order to mine this rich resource and achieve maximum utility for the scientific community. The key strength of these data is that they enable investigators to carry out analyses of already available reference epigenomic data and allow researchers to apply

this information to specific research questions. We anticipate that secondary analysis by the scientific community, especially through integration with other novel data sets, will maximize the utility of the data generated and provide models for diverse ways in which these data can be exploited to make important discoveries about biological processes and diseases. For example, analysis of epigenomic data with GWAS data was an extremely valuable use of epigenomics program data which was not foreseen when the program began [19]. To encourage further analysis projects in which REMC data is analyzed in concert with public data sets (e.g., ENCODE, TCGA, IHEC, Brainspan, dbGAP) or with investigator-generated data sets, a new Roadmap Epigenomics Program initiative was released that hopes to support around ten 2-year projects starting in 2014 that will hopefully lead to similar novel discoveries (<http://grants.nih.gov/grants/guide/rfa-files/rfa-rm-14-001.html>).

3.3 Integration with Genome Conformation Assays

Development of new genome conformation assays (e.g., Hi-C, ChIA-PET) enables researchers to probe three dimensional genome interactions in particular cell types [49, 50]. As these assays improve, it will be of great interest to correlate this data with existing epigenomic modification and transcription factor binding data to determine if/how they relate to one another. Additionally, emerging in vivo microscopy techniques may have the potential to reveal how these molecular phenotypes correspond to the three-dimensional structure of chromatin and to probe their spatial dynamics and functions within the nucleus [39].

3.4 The Utility of Surrogate Tissues for Disease Investigations

Studies investigating disease epigenomics typically focus on the diseased tissue or cell type. However, for diseases involving some organs (e.g., brain, bone), obtaining human tissue specimens can be difficult or impossible. This challenge can be overcome by obtaining post-mortem tissue or using in vivo epigenetic imaging strategies, although this imaging technology is still in its infancy. An alternative strategy could be to use readily accessible or “surrogate” tissues (e.g., blood cell types, olfactory neurons, skin) that may in part reflect epigenomic changes that have taken place in the primary diseased tissue. Although some work has been published in this area, the extent to which use of surrogate tissues is appropriate for disease investigations remains unclear [47]. To explore this issue, NIH convened a workshop in August 2012 to obtain recommendations on the use of surrogate human cells in disease epigenomics investigations. Fifteen scientists with expertise in this area converged on three major scientific recommendations. The first recommendation was to develop and explore human samples to see whether or not disease-associated epigenetic patterns from a primary tissue could also be found in readily accessible surrogate cell types and would provide information that was as useful as that provided by the primary tissue involved in the disease.

One confounding issue with human epigenetic studies is that that one cannot readily control for genotype and environmental exposures. Therefore a second recommendation was to develop and explore model organism systems in which genotype and environmental exposure can be well controlled to see how epigenetic patterns associated with a disease state compare between primary and surrogate tissues. The third recommendation was to perform a systematic study of normal human epigenetic variability in different tissues to provide information about the normal range of variability. This information will help researchers design informative epigenome-wide disease association studies. The latter recommendation may be addressed in respect to genotype part by the Genotype-Tissue Expression Program (GTEx, *see below*). However, the investigation of how the epigenome changes in different tissues over human lifespan also needs to be investigated since age may have a significant influence on the epigenome [51, 52].

3.5 Human Epigenomic Variation

A major goal of the REMC was to explore epigenomic differences between cell and tissue types. Although some pilot work was performed investigating epigenomic differences between individuals in a single cell or tissue type, this program was not designed or powered to investigate this issue rigorously. However the Common Fund-supported GTEx Program (<http://www.ncbi.nlm.nih.gov/gtex/GTEX2/gtex.cgi>) is characterizing human gene expression in multiple post-mortem and surgical tissues and correlating this information with human genotype. If this project were to expand to include additional molecular phenotypes (e.g., DNA methylation, histone modifications) this would enable for the first time a robust exploration of the relationship between genomic and epigenomic variation in specific human tissues.

3.6 Epigenomic Variation Within a Cell Type: The Promise of Single-Cell Epigenomics

Two related major challenges are to understand how the epigenome impacts gene expression in individual cells and how cell-to-cell variation influences function at the tissue, organ, and systems level. To date, the study of epigenetic modifications typically involves analyzing complex tissue samples or mixed cell populations. It is clear that distinct epigenomic signatures are associated with specific cell or tissue types and that these epigenomic signatures likely play a role in determining cellular phenotypes [16, 17]. In progenitor cell populations, the epigenome has a clear connection to fate determination and cell-type specification [53]. Even cell lines cultured in vitro are heterogeneous to varying degrees and most existing data is the result of ensemble averaging; very little is known about how the epigenome differs across individual cells within a particular cell type [54]. Technological limitations have hampered our ability to evaluate epigenetic states at the resolution of a single cell. Available evidence suggests that in certain circumstances the epigenome is modifiable. Therefore microenvironmental differences in

and around individual cells could lead to epigenomic changes in certain cells that would impact their individual cellular functions. An intriguing example of this can be found in the field of learning and memory where recent results suggest that neural activity induces learning-specific changes at the level of the epigenome [55].

The ability to perform epigenomic analysis at single-cell resolution would help us to better understand these mechanisms. Researchers in the field are improving methods that may permit the analysis of DNA methylation states in single cells [56]. Evaluating histone modifications and other chromatin marks at specific loci in single cells will likely prove to be more challenging, but the technology is evolving [57]. The Common Fund Single Cell Analysis program may help develop or improve technologies to address some of the obstacles in this area (<https://commonfund.nih.gov/Singlecell>). As new methodologies are developed and reproducibility improves, more studies will incorporate single-cell approaches and a clearer picture will emerge regarding epigenomic variability within a defined cell population and, more importantly, the functional implications of any observed differences.

3.7 Environmental Epigenomics

No Roadmap Epigenomics Program initiative explicitly addressed the effects of environmental exposures on the epigenome. Yet it has become clear that well-controlled investigations in this area are critical, since it is likely that environmental exposures (e.g., environmental toxins, substances of abuse, psychosocial stress, diet, exercise) impact our epigenomes. However, the precise mechanisms by which an environmental exposure can lead to a chromatin change are still emerging [58–60]. Furthermore, it is not well understood how long these epigenetic alterations perdure in somatic cells or how these modifications are subsequently maintained or removed [61]. Also, as is the case for many epigenetic modifications, limited work has been done to rigorously test the functional effects of modifications associated with environmental exposures. Explicit investigation into these issues for a diverse set of environmental exposures would greatly enhance our understanding of how the environment influences the epigenome and whether or not different environmental stressors impact common epigenomic processes or perhaps impact a common set of genomic loci.

3.8 Functional Epigenomics Tools and Technologies

Over the past decade there have been dramatic improvements in our ability to scrutinize chromatin. Despite these improvements in our ability to monitor epigenomes, our ability to manipulate the epigenome has remained relatively crude and largely relies on pharmacological or genetic manipulation of epigenetic regulatory proteins. Pharmacological modulators of epigenetic enzymes and processes are of great value; however these modulators typically have pleiotropic effects and rarely impact a specific tissue, cell type, or locus. Genetic manipulation of epigenetic enzymes can achieve

results similar to those obtained by pharmacological manipulation, although gene targeting approaches can sometimes limit these effects to selected tissues or cell types. In addition, whether or not tissue or cell-specific epigenomic manipulation is achieved, the technologies currently available do not have robust temporal control and typically yield changes that impact the entire genome of a cell rather than focused effects on one or a few key loci. The potential pleiotropic effects resulting from genome-wide epigenome manipulation obscure our understanding of how epigenomic changes at specific loci may influence phenotype and are an obstacle to the development of future epigenomic therapeutics.

To address this scientific barrier, a Roadmap Epigenomics Program initiative was developed to stimulate innovative research to develop novel tools and technologies to enable one or more of the following: (1) tissue- or cell-specific manipulation of epigenetic modifications or their effector molecules, (2) temporal manipulation of the epigenome, and (3) locus-specific manipulation of the epigenome. As a part of this initiative, ten projects were funded that use some of the following general strategies: (1) use of TALE, Zn finger, CRISPR, or lncRNA strategies to target epigenetic modifying enzymes to specific loci for manipulation of the epigenome in a locus-specific manner; (2) development of opto-epigenetic or chemo-epigenetic switches to enable temporal manipulation of the epigenome using light or chemical ligands; and (3) the integration of the above techniques with genetic resources to enable cell-type-specific manipulation of the epigenome. We look forward to seeing the fruits of this research in coming years.

4 Summary

We hoped to convey how some of the resources and technologies generated by the NIH Roadmap Epigenomics Program may be exploited for studying various human diseases including cancer research projects. Some of the key resources and technologies generated by the program include reference epigenome maps for a wide variety of human cells and tissues, the development of new technologies for epigenetic assays and imaging, the identification of novel epigenetic modifications, and an improved understanding of the role of epigenetic processes in a diversity of human diseases. Despite the many accomplishments of the NIH Roadmap Epigenomics Program, much future work is needed in this research area including exploration of epigenomic variation both in different individuals and within cells of the “same” type, environmental epigenomics, exploration of the use of surrogate tissues, and improved technologies for epigenome manipulation. Ultimately our collective efforts to understand the epigenome will provide the foundational knowledge necessary to elucidate the role of

epigenomic mechanisms in a wide variety of human diseases and may lead to the development of new preventative measures, diagnostics, and therapeutics for these diseases.

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Epigenome-Wide Association Studies (EWAS): Past, Present, and Future

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Abstract

Just as genome-wide association studies (GWAS) grew from the field of genetic epidemiology, so too do epigenome-wide association studies (EWAS) derive from the burgeoning field of epigenetic epidemiology, with both aiming to understand the molecular basis for disease risk. While genetic risk of disease is currently unmodifiable, there is hope that epigenetic risk may be reversible and or modifiable. This review will take a look back at the origins of this field and revisit the past early efforts to conduct EWAS using the 27k Illumina methylation beadarrays, to the present where most investigators are using the 450k Illumina beadarrays and finally to the future where next generation sequencing based methods beckon. There have been numerous diseases, exposures and lifestyle factors investigated with EWAS, with several significant associations now identified. However, much like the GWAS studies, EWAS are likely to require large international consortium-based approaches to reach the numbers of subjects, and statistical and scientific rigor, required for robust findings.

Key words EWAS, Methylation, Risk, Biomarker, Cancer, Peripheral blood, Exposures

1 Origins and Definitions

Genetic epidemiology aims to use the natural variation in the genome, namely single-nucleotide polymorphisms (SNPs) and also copy number variants (CNVs) to look for associations with cancer risk, prognosis and in pharmacogenetic studies to explain drug response or resistance. It has been used to investigate why some patients have tumours that metastasize or have a particular pathology and has been used to look at environmental interactions. Over the last decade, starting with the Human Genome Project (HGP) and HAPMAP catalogue of human variation the development of experimental study design and statistical analysis to perform genome-wide association studies (GWAS) within large international consortia has begun to bear significant fruit with numerous diseases and phenotypes [1]. Using the recent iCOGs consortium as an example approximately 70 novel genetic associations for

breast [2], ovarian [3], and prostate cancers [4] were identified in a large international effort. The current challenges in genetic epidemiology are now in translation of this new information into risk prediction and or stratified medicine-based approaches that can be used in the clinic.

New research over the last few years has aimed to look further into the genome at the natural variation in the epigenome, in particular at DNA methylation, which is the addition of a methyl group to cytosines as a mechanism of gene regulation. For all of the natural epigenetic variation in the human population you can ask all of the same questions as in genetic epidemiology studies. The term *epigenetic epidemiology* was first mentioned in the literature as a future research topic by Issa in a review of colon cancer epigenetics in 2000, in which was described the need to understand the interaction between dietary folate and colon cancer risk [5]. The concept was further developed and defined in a review by Jablonka and Lamb in 2002 in which the authors argue that inheritance of epigenetic information may provide a mechanism of inherited traits including risk of disease [6]. More recently numerous reviews have defined the area and various aspects of epigenetic epidemiology [7–13].

The abbreviation EWAS is now widely used to mean *epigenome-wide association study*, initiated by the review by Rakyan and colleagues [14] and typically used to define the analysis of DNA methylation. However, we must acknowledge that the term “epigenome” should also encompass a wide array of histone modifications that also influence gene function as part of the epigenome and have been largely ignored to date in EWAS due to technological constraints and appropriate sample availability. Furthermore, the abbreviation EWAS has also been used as an acronym for “environment-wide association studies” [15, 16], “exposome-wide association studies” [17], and even “early-walking aids” [18] and potentially other definitions. However, in the majority of studies published to date, and in this review, the term EWAS is used to define studies of DNA methylation to identify the common normal variation in the DNA methylome using genome-wide technologies most frequently using peripheral blood DNA.

The motivation for pursuing genome-wide approaches came from studies of DNA methylation of candidate genes measured in peripheral blood DNA. One of the earliest of these was the association between loss of imprinting (LOI) of the insulin growth factor II (*IGF2*) gene and colorectal cancer risk in cross-sectional studies [19–22]. However, *IGF2* hypomethylation in peripheral blood was not related to colorectal or breast cancer risk in a prospective cohort study using pre-diagnostic samples, indicating that this may rather be a biomarker of disease, but not of risk [23]. Furthermore, epimutations have been identified in the *MLH1* and *MSH2* genes associated with familial colon cancer, Lynch syndrome patients [24, 25].

However, in both of these cases the DNA methylation phenotype (epimutation) was driven by rare genetic polymorphisms *in cis* [26, 27]. This argues against the hypothesis that epimutations themselves may be heritable across generations as a mechanism to explain a proportion of the missing heritability of these diseases. Subsequently, candidate gene studies conducted by us [28] and others [29–32] have reported associations between candidate gene methylation and cancer risk. Specifically, using the *ATM* gene example we have investigated *common epigenetic variation* focussing on regions of the genome that are more variable, such as gene-body or intragenic sequences [28]. Much like GWAS, it is in the common variation in the population, rather than in rare epimutations, that EWAS will be most powerful for identifying robust biomarkers.

2 EWAS Reviews of Methods

The goal of this chapter is not to reiterate many of the numerous reviews of methods for EWAS, study design, statistical and bioinformatic approaches nor the various plagues that blight them [8, 14, 33–36]. There have been many excellent reviews on novel pre-processing methods and study design issues [9, 37–45]. This chapter rather focuses on the initial studies that have been performed, predominantly on white blood cell (WBC) DNA and what has been discovered thus far that have been robustly *replicated and or validated*. It is not intended to be an exhaustive review of all published studies. Studies have been identified in the literature using the search terms “EWAS,” “epigenome-wide association,” and “epigenetic epidemiology,” supplemented with some known studies that did not contain these keywords. However, several important methodological issues that have been consistently raised as concerns, caveats, and/or complications in EWAS as compared to GWAS need to be discussed.

The first concern is often on the use of WBC DNA where methylation variation may reflect differences in the proportions of different blood cell types that could obscure or confound associations. While it may be preferable to identify a more appropriate cell type [46], the fact remains that most large cohort studies have only collected blood samples that are thus the only DNA source available for identifying incident disease cases prior to disease onset. However, at this stage current evidence suggests that cell composition explains only a minor proportion of variation in blood DNA methylation [43]. Data generated on the 27k Illumina array [47, 48] and 450k array [49] using fractionated blood samples can be used to infer the cell population in WBC DNA [50] and to determine if identified biomarkers vary across cell type, most successfully used in the recent investigation of the autoimmune disease rheumatoid arthritis [51]. Apart from this disease, which

plausibly could be explained by the hematological nature of the disease, cell type composition has not explained a large proportion of significant associations in most EWAS analyses of non-hematological diseases published thus far. However, it remains important to consider cell type as a potential confounding factor.

There is a strong case to be made for longitudinal studies not only for risk studies (including DNA samples prior to disease onset), but also in intervention studies using pre- and posttreatment with specific exposures to investigate environmental impacts on the epigenome [40]. However, only few studies have investigated temporal variation of WBC DNA methylation in serial samples from the same individuals reporting associations with BMI or age [52–54]. Only methylation traits that are stable over time can be well characterised using a single blood sample. It is, therefore, important to establish the temporal variability of methylation markers in WBC DNA in order to select informative markers, i.e., those that are variable in the population, yet stable over time when only one blood sample is available. Failing this, it would be vital to use multiple serial blood samples from the same individuals to report robust associations with a particular phenotype.

The extent to which WBC DNA methylation is genetically determined and/or represents acquired changes through life is not known. A genetic component is supported by evidence in families [52] and twins [55, 56], in numerous studies showing allele-specific methylation [57, 58], and lastly with the examples of genetically driven epimutations in *MSH2* and *MLH1* [26, 59]. However, strong conclusions about a meiotically heritable mechanism for an epigenetic trait remain elusive. Age is associated with methylation and is a key risk factor for cancer and other disease; thus it is important to evaluate its potential confounder effects [60]. However, without controlled longitudinal studies it will be almost impossible to rule out any accumulated exposures throughout life from the effect of aging alone [54].

Lastly, unlike GWAS studies in which independent replication of genotyping has become less necessary (although still required for poor-performing SNPs), largely because the genotype calling is categorical, DNA methylation is a continuous quantitative variable and the various technical and statistical processing in generating this data strongly argues for the need for validation of the reported methylation values using independent quantitative methods such as Epityper or Pyrosequencing. It is not surprising that the many associations that have been reported and repeatedly validated in subsequent studies were often independently validated by alternative methods, such as the methylation at F2RL3 associated with tobacco smoking which was validated by Epityper in the original study and in subsequent reports [61–63].

3 Past: 27k Studies

The Illumina 27k Illumina array covers on average 2 CpG sites in the promoter regions of approximately 14,000 genes and represents less than 0.1 % of the 28 million CpG sites in the human genome. Unlike GWAS studies that can use tag SNPs that can conceivably cover the majority of the haplotype blocks in the genome, the 27k array falls far short from being representative of “epigenome-wide.” Nevertheless some studies have used these arrays to identify blood-based DNA methylation signatures of risk in ovarian cancer [64], bladder cancer [65], head and neck squamous cell carcinoma (HNSCC) [66], and breast cancer [67]. Although these epigenome-wide cancer studies report intriguing associations, independent replication is required before any associations can be considered robust, since they were small studies (~200 cases/200 controls), and in some cases used blood samples collected after diagnosis or treatment. Furthermore, none of these early EWAS using the 27k used independent validation to verify the associated probes, and only time will tell if they can be further replicated.

In the first of these studies Teschendorff and colleagues used a mixture of at-diagnosis ($n=113$) and posttreatment blood samples from ovarian cancer patients ($n=122$) compared to unmatched healthy controls ($n=148$). This study design was not ideal given that the healthy controls were not matched to the cases and as such were significantly biased on several subject characteristics including age, OC use, and pregnancy as well as the cases being confounded either by the presence of disease or prior treatment with chemotherapeutic cytotoxic drugs. Nevertheless, using a supervised classification system the investigators identify a signature using the top 100 probes that can accurately predict case–control status using the at-diagnosis set that also predicted ovarian cancer in the post-treatment samples. However, from this first report one interesting observation was a bias in the differences between cases and controls towards non-CpG island probes (which were significantly under-represented in this array design), arguing strongly for the use of the latterly designed 450k array which does cover non-CpG islands with a higher density of probes.

Several studies from Kelsey and colleagues have reported signatures for both bladder and HNSCC from case–control studies using the 27k array [65, 66]. In the first of these they report on $n=223$ cases blood DNA collected approximately 1 year after diagnosis and treatment, compared to $n=237$ healthy unmatched (and untreated) controls that were also biased by smoking status and gender. This study reports a signature of the top nine probes that differentiate cases from controls with a predictive value of $AUC=0.7$ to predict the presence of bladder cancer [65]. In a smaller study of 92 cases of HNSCC compared to 92 healthy

unmatched controls (biased on smoking, alcohol, and HPV sero-positivity), using a similar supervised analysis they report a 6-CpG signature that predicts the presence of cancer in the patients (AUC=0.73) [66]. A reanalysis of these data using a 50-probe signature associated with different blood cell populations more accurately predicted the case-control status of the subjects, suggesting that much of this association is driven by blood cell changes that may have occurred as a result of the presence of disease or prior treatment in the patients [48]. Like the previous ovarian cancer study, these studies have not used independent methods to validate these small probe-set signatures, and have thus far not been replicated by independent studies.

In a more recent study Xu and colleagues used the 27k Illumina DNA methylation array to perform an EWAS on prospective incident breast cancer cases ($n=298$) and unmatched controls ($n=612$) from the Sister Study [67]. Results suggest 250 probe associations of methylation detected in WBC DNA with breast cancer risk (FDR $p<0.05$). However, without independent replication or methodological validation, conclusions from this study need to be tempered with caution. As described in the accompanying editorial [68], the strongly zero-inflated p-value distribution might either reflect yet-undiscovered potential confounding factors, or failure to capture and correct for the correlation between probes. An interesting observation in this study was that the majority of associated probes showed a loss of methylation reminiscent of the global hypomethylation observed in total methylation in cancer patients compared to controls [69]. If validated, this association and those reported for other tumour types may yet prove useful as risk biomarkers if independent of other known risk factors.

4 Present: 450k Studies

The Illumina 450k array is the most widely used platform in the last two years for studies reporting EWAS. The array still only covers less than 2 % of the CpG sites in the genome, but does attempt to cover all known genes with a high density of probes in the promoters (including CpG islands and surrounding sequences), but also covers with a lower density across the gene bodies, 3'UTRs, and other intergenic sequences.

There have been two EWAS using the 450k array to investigate *diabetes*, in specific subpopulations of WBC including CD14+ monocytes for type1 diabetes in which several markers were further analyzed by pyrosequencing but were shown not to validate the reported association individually [70]. An important advance in this study, however, was the use of multiple study designs including discordant monozygotic twin pairs and singleton case-control group in which many of the associated markers were replicated [70].

CD4+ T cells were analyzed in an EWAS for type 2 diabetes, in which one gene *ABCG1* was associated with the phenotypic correlate insulin levels and a model for insulin resistance [71]. Further validation in independent cohorts for both of these findings is therefore warranted.

The first 450k-based EWAS to gain from adjusting for the blood cell proportions was performed by Liu and colleagues investigating $n=354$ *rheumatoid arthritis* cases compared to $n=337$ healthy matched controls reporting 4 CpG sites in the MHC cluster that epigenetically mediates a genetic association with the disease [51]. In addition to peripheral blood, validation specifically in monocytes suggested the possibility of a myeloid mechanism driving the peripheral blood association. While no studies have replicated this methylation phenotype yet, several GWAS reports have identified SNPs in the MHC locus associated with rheumatoid arthritis validating this genetic association with the disease (www.genome.gov/gwastudies). This supports the view that combining both genetic (GWAS) and epigenetic (EWAS) approaches in parallel may prove a fruitful approach for understanding mechanisms of disease risk [72].

The most robustly validated findings thus far with EWAS have been the associations between DNA methylation in blood and *smoking status* [61, 62, 73–83]. There may be many reasons why smoking associated methylation changes have been so strongly validated; firstly, that unlike other environmental exposures smoking is much easier to categorize subjects into presence or absence of the exposure and most cohort studies will have collected this variable; and secondly the methylation difference between smokers and nonsmokers for most probes is >5 % which is a large enough change to be detected on multiple platforms. The genes that have shown the most robust associations across multiple studies (at least 3) are in *AHRR*, 2q37.1, 6p21.33, *F2RL3*, *GPR15*, *GFIL*, *CYP1A1*, *MYO1G*, and *CNTNAP2*. These have been replicated in peripheral blood DNA, but also in lung tissue [62], cord blood of smoking mothers [79], and different genetic populations [82]. A number of interesting observations have been made regarding this association with smoking. Firstly, the methylation alterations are detectable in blood DNA even in former smokers that have quit up to 10–20 years prior to giving blood [62, 73, 83]. We have used this knowledge to create a predictive model of former smoking status that might be more quantitative and useful as an epidemiological variable (AUC = 0.80) to study risk of disease associated with former smoking [73]. Mechanistically, given that the majority of white blood cell types have life-spans of ~30 days, this suggests that the exposure must also be affecting the hematopoietic stem and progenitor cells which perpetuate the epigenetic alterations in the daughter differentiated cells. If exposures such as smoking can increase an individual's risk of cancer, even in former smokers, then

it is conceivable that those exposures throughout life must also affect the adult stem and progenitor cells and potentially the cell of origin for the initial carcinogenic events. This provides an interesting model for other exposures that also induce long term increased risk of disease. The second interesting observation is that the most consistently altered gene across all studies is the aryl hydrocarbon receptor repressor (*AHRR*), and the majority of changes occur in an intragenic CpG island showing a loss of methylation >20 %. The *AHRR* gene is involved in the regulation of the aryl hydrocarbon receptor (AHR) pathway which metabolizes the carcinogenic dioxins and dioxin-like compounds and therefore is a highly plausible candidate. However, while we have shown that hypomethylation of this intragenic locus is associated with over-expression of the gene in lung tissue, whether this results in a further repression of the pathway is not yet clear [62].

There have now been numerous studies that have reported an association between WBC DNA methylation and *increasing age*. Initially using the 27k array for EWAS two studies reported methylation signatures of aging in developmental genes defined by those that are bivalently marked or targeted by the PRC2 polycomb group complex [60, 84]. This association with age was also reported in a twin study using the 27k array, which showed further evidence for a genetic component of epigenetic variation [57] and was replicated in an interactome analysis of several 27k array datasets [85]. More recently using the 450k array Florath and colleagues showed that the majority of probes (78/94) that associate with age in a cross-sectional study design were also associated with age in a longitudinal study design (+8 years) [54]. This is important in ruling out confounding factors not accounted for in the cross-sectional study design in unrelated individuals. These investigators also developed a 17-probe set signature to estimate an individual's age with average difference between predicted and observed ages of ± 2.8 years [54]. Using a similar approach, Hannum and colleagues have developed a 71-probe set signature derived from the 450k array to predict age in multiple tissues with an accuracy of ± 3.9 years [86]. Importantly, this study showed that in tumour tissue DNA, the apparent age of the cells and ageing rate are far higher than their matched normal tissue counterparts providing direct evidence for an epigenetic mechanism for increased cancer risk with increased age. Intriguingly, males appeared to age faster than females, with no apparent hypothesis for a mechanism at this stage [86]. The overlap between the 71-probe signature and another dataset comparing newborns and centenarians [87] had 70/71 overlapping, while at least 39/78 probes defined by Florath and colleagues were also overlapping with these datasets. Across all of these studies several genes appear most consistently associated with age, including *ELOVL2*, *CCDC102B*, *OTUD7A*, and *FHL2*. Most importantly, some of

these associations have also been replicated using alternative technologies for *ELOVL2* and *FHL2* [88]. Therefore, apart from clearly displaying the need to adjust for age in any other EWAS analyses, these studies have shown some important findings regarding the consequences of increasing age in human biology and provided a novel molecular epigenetic measure of cellular age [89].

5 Future: Bis-Seq and Consortium-Based Approaches

Thus far, there have been no sequencing based EWAS reported; however, it is an obvious evolution for future EWAS. While the 450k array is an advance on the previous 27k version, it is clear that targeting only 2 % of the CpG sites is only scratching the surface of the epigenome. Several reports have shown that the most variable sites of the genome are in intragenic CpG sites (gene-body) which are only sparsely covered by the 450k array. We have previously hypothesised that the most variable region of the genome is in the first few kb of a gene, which can only be targeted using sequencing based methods [90]. Given that NGS based methods will soon be used for clinical genetics, if any of the epigenetic markers we identify could become clinically useful, then using this same technology would be feasible. Therefore, the future challenges will be finding an appropriate method at a reasonable cost to allow such studies with large enough study sizes to have sufficient power for EWAS [91]. This new approach will undoubtedly lead to further debate about appropriate study design, statistical methods and bioinformatic processing of this data with new biases and confounding factors to take into account. Already, it is clear that the international consortium based approaches will be needed to combine datasets, whether for 450k arrays or Bis-Seq data, to approach the level of replication and robustness that have been achieved with GWAS consortia, and several EWAS consortia have recently been initiated to do just that.

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Chapter 4

Epigenetic Biomarkers in Liver Cancer

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Abstract

Liver cancer (hepatocellular carcinoma or HCC) is a major cancer worldwide. Research in this field is needed to identify biomarkers that can be used for early detection of the disease as well as new approaches to its treatment. Epigenetic biomarkers provide an opportunity to understand liver cancer etiology and evaluate novel epigenetic inhibitors for treatment. Traditionally, liver cirrhosis, proteomic biomarkers, and the presence of hepatitis viruses have been used for the detection and diagnosis of liver cancer. Promising results from microRNA (miRNA) profiling and hypermethylation of selected genes have raised hopes of identifying new biomarkers. Some of these epigenetic biomarkers may be useful in risk assessment and for screening populations to identify who is likely to develop cancer. Challenges and opportunities in the field are discussed in this chapter.

Key words Biogenesis, Biomarkers, Epigenetics, Epidemiology, Hepatitis, Liver cancer, miRNA, Treatment

Abbreviations

AFP	Alpha-fetoprotein
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
miRNAs	microRNAs
pri-miRNA	primary-microRNA
RISC	RNA-induced silencing complex

1 Introduction

Biomarkers are used for cancer diagnosis, prognosis, and surveillance in different populations [1–22]. Liver cancer or hepatocellular carcinoma (HCC) is one of the most common cancers worldwide, with an annual incidence of close to one million cases. This cancer affects more than 500,000 people worldwide [23]. The prevalence of HCC is higher in Asian countries due to infection with the hepatitis B virus (HBV) and hepatitis C virus (HCV) [24].

Several risk factors, such as alcohol, infection (HBV, HCV), dietary aflatoxin, and environmental pollutants, have been proposed as contributing to the development HCC [25–28]. The incidence of HCC in the USA has almost doubled during last two decades [29]. The prognosis for the disease is poor if it is not detected early. HCC is generally cirrhotic, and liver cirrhosis is considered the precursor of HCC [30, 31]. HCV-infected patients with liver cirrhosis are at very high risk of developing HCC. Among environmental factors, aflatoxin B1 is a major factor. Aflatoxin B1 is produced by the fungus *Aspergillus flavus*, which is a common food contamination in developing countries. If aflatoxin B1 is not properly metabolized by microsomes in the liver, it may generate G-T transversions that contribute to the development of HCC.

2 Need to Identify Biomarkers for Diagnosis and Prognosis

Epigenetics is a developing science with potential for use in cancer diagnosis, prognosis, and treatment [3, 9, 12–16, 18–21]. A list of publications on epigenetic biomarkers in liver cancer is shown in Table 1. While analyzing the literature, terms should be selected carefully. For example, the term “liver cancer epigenetics” generated 106 publications, whereas “liver cancer epigenetic biomarker” generated 117 publications. The topic of methylation was covered more in the literature than were microRNA (miRNA) or histones. The field of epigenetics is new, but 65 publications included epigenetic inhibitors for the treatment of liver cancer.

All stages of cancer, including initiation, progression, and metastasis, involve genetic and epigenetic regulation in most HCC tumor types [14, 15, 32]. Epigenetic regulation is required for normal development, and any abnormality in the process may lead to the development of cancer [14, 25, 33, 34]. Epigenetics consists of altered gene expression without a change in the nucleotide sequences. Four major components of epigenetics machinery are promoter methylation, histone modification, altered miRNA expression, and chromatin reorganization and packaging [14, 34]. Assays, both biochemical and imaging, have been developed to measure epigenetic abnormalities in normal and cancer tissues and cells [35]. A role in this process also has been proposed for nonhistone proteins and other complexes, such as the polycomb group of repressor proteins [36]. Chen et al. demonstrated that polycomb-group proteins help to maintain the stemness of hepatic cells and have a role in liver regeneration [36].

The infection of liver cells by hepatitis viruses (HBV, HCV, and hepatitis D [or delta] virus [HDV]) has been demonstrated in liver carcinogenesis [37, 38]. HDV infects HBV-infected cells, whereas HBV or HCV can infect liver cells independently. The severity of liver cirrhosis is greater in dual infection compared

Table 1
Liver cancer biomarker publications^a

PubMed terms	Number of publications
Biomarker	627,095
Cancer	2,870,669
Cancer and biomarker	226,581
Liver cancer	184,970
Liver cancer biomarker	17,248
Epigenetics	7,860
Epigenetics biomarker	650
Liver cancer epigenetics	106
Liver cancer epigenetic biomarkers	117
Liver cancer and methylation	1,872
Liver cancer and histones	514
Liver cancer and miRNA	798
Liver cancer and chromatin	1,317
Liver cancer epidemiology	21,998
Liver cancer treatment	92,649
Liver cancer treatment and epigenetic inhibitors	65

^aPublications were searched from January 1990 to November 2013

to single-infection cases [37]. HDV infection induced DNA methyltransferase (DNM3b) activity and hypermethylation of gene-encoding transcription factor E2F1. The HBV genome integrates into the host genome, and HBV gene X plays a significant role in integration, maintenance of integration, and replication of HBV. The HBx gene product binds with the C-terminal of p53 and inactivates it, resulting in abnormal apoptosis [25]. Epigenetic approaches have been applied to HCC diagnosis, prognosis, and follow-up of survival and treatment [32]. Epigenetic changes can be reversed by chemicals and drugs and thus have potential in cancer treatment [39].

3 Epigenetic Biomarkers

Both methylation of specific genes and histone deacetylation have been reported to alter the expression of genes involved in HCC carcinogenesis [32, 40]. The Tristetraprolin (*TTP*) gene promoter has one specific CpG site that reduced expression of c-myc upon

hypermethylation as tested in liver cell line and clinical samples [41]. This epigenetic regulation also resulted in resistance to the antiproliferative effect of transforming growth factor beta (TGF- β). Further analysis of the repressor transcriptional complex indicated participation of MECP2, c-Ski-2, and DNMT3a. In another study, HBV-infected samples showed inactivation of E-cadherin (*CDH1*) gene expression and deacetylation of histones [40]. Hypermethylation of genes encoding *SOX-1* and secreted frizzled-related proteins (SFRPs) was observed in HCC patient samples analyzed by methylation-specific polymerase chain reaction (MSPCR) [42]. Loss of *CDH1* previously has been shown to be associated with enhanced metastasis. Furthermore, miR-373, an activator of *CDH1*, was downregulated in these patients. This study indicated that multiple epigenetic mechanisms were involved in HCC carcinogenesis and that methylation, deacetylation, and miRNA expression could be used as biomarkers to follow HCC development. Dysregulation of epigenetically regulatory protein EZH2 has also been reported [23]. Table 2 lists selected biomarkers for HCC diagnosis and prognosis.

In an independent study, the proportion of different methylated regions in (a) paired normal, (b) normal, and (c) tumor + normal liver tissues was determined. Based on data that were adapted from Xueguang Sun et al. (source: a Zymo Research American Association for Cancer Research (AACR) Poster entitled “Epigenetic Biomarker Discovery, Validation for Diagnosis, and Therapeutic Intervention for Hepatocellular Carcinoma, 2011”), the percentage of methylated regions in normal, infected, and cancer tissue samples were determined. The results are shown in Table 3 and Fig. 1. The regions analyzed for methylation quantitation were LTR promoter, miRNA, small interspersed nuclear elements (SINE), and long interspersed nuclear elements (LINE). Note that SINE and LINE sequences of the genome contain repeat sequences that, with development, undergo hypomethylation. Table 3 and Fig. 1 demonstrate differential methylation in different regions of tissue samples.

The role of miRNAs in liver metabolism and hepatocellular adenoma and HCC mediated through peroxisome proliferator-activated receptors (PPAR) was suggested by Peyrou et al. [43]. This nuclear receptor is involved in lipid metabolism, glucose metabolism, liver regeneration, and activation of inflammatory response in the infected liver. Investigators also have proposed microRNAs as therapeutic targets [44].

5-Methylcytosine (5-mC) is regularly evaluated in cancer patient samples. 5-mC is converted to hydroxyl-5-mC (5-hmC) enzymatically and has been reported in different cancers. Chen et al. evaluated HCC patient samples by trapping/capillary hydrophilic interaction liquid chromatography (cHILIC)/in source fragmentation tandem mass spectrometry and reported the presence of 5-hmC in HCC patients. Its levels could be detected at the early stages of cancer development [45].

Table 2
Liver cancer biomarkers and their characteristics

Biomarker name	Implications/comments
Altered methionine metabolism and global DNA methylation	Useful for disease progression [33]
ALF	Oversecretion of ALF in HCC [60]
AFP, AFP-L3, DCP, GP73	Useful for monitoring treatment response [61]
Autoantibodies to tumor-associated antigens	Useful for monitoring disease progression [62]
CYFRA 21-1, TPA, TPS, and CEA	Serum levels elevated in HCC [63]
E-cadherin	Upregulated in HBV-infected liver cells [40]
EZH2-mediated H3K27me3	Upregulated in HCC, useful for diagnosis [64]
Macrophage migration inhibitory factor (MIF) and other inflammatory biomarkers	Upregulated in HCC [65]
MAGE-4 gene m-RNA and TGF	High serum levels in HCC [66]
MAGE-3	Hypermethylated in HCC [67]
miRNA profiling	Differential expression of a group of miRNAs, useful for diagnosis and prognosis [43, 68]
P16 and c-myc	Hypermethylation in HCC [39]
P16	Hypermethylation in HCC [69]
Proteomic profiling	Differentially expressed proteins in samples from HCC patients [56, 70, 71]
RASSF1a	Hypermethylation in HCC [36]
SSCA antigen	Useful for diagnosis [67]
TP53, CTNNB1, AXIN1, CDKN2A	Mutated in HCC [23]

Animal models that demonstrate the activity of epigenetic inhibitors in suppressing HCC have been successfully developed [39]. Epigenetic regulation affects genomic instability, which may lead to the development of cancer [33]. The Illumina HumanMethylation 450 BeadChip has been used successfully to follow genome-wide methylation profiling in cases of HCC and controls [32]. This analysis included CpG islands, shores, and shelves.

4 Other Biomarkers (Genomic, Proteomic, Transcriptomic, and Metabolomic)

Different imaging techniques are used in diagnosing HCC. The most common are abdominal ultrasound magnetic resonance imaging (MRI) and contrast-enhanced computed tomography (CT) scan in combination with histology [46, 47]. Among biochemical biomarkers, serum alpha-fetoprotein levels have been

Table 3
Hypermethylation regions in various tissues

	miRNA	LTR	LINE	SINE	Percentage
A. Hypermethylation regions in paired normal tissue					
Strong hypermethylation	30	20.0	16.7	10.0	53.3
Hypermethylation	19	73.7	21.1	0.0	5.3
Insignificant	12897	1.1	16.0	14.7	68.2
Hypomethylation	109	8.3	60.6	4.6	26.6
Strong hypomethylation	1883	0.2	39.2	6.8	53.8
B. Hypermethylation regions in normal tissue					
Strong hypermethylation	120	11.7	30.0	14.2	44.2
Hypermethylation	54	59.3	27.8	9.3	3.7
Insignificant	11293	1.1	22.5	14.4	62.0
Hypomethylation	112	1.8	82.1	4.5	11.6
Strong hypomethylation	1788	0.1	46.2	10.1	43.6
C. Hypermethylation regions in tumor and normal tissue					
Strong hypermethylation	9	55.6	11.1	0.0	33.3
Hypermethylation	8	87.5	12.5	0.0	0.0
Insignificant	15330	1.0	22.8	13.1	63.0
Hypomethylation	53	15.1	67.9	1.9	15.1
Strong hypomethylation	362	0.3	44.8	1.9	48.3

used for HCC diagnosis [38, 48, 49]. Long et al. reported increased glutamine synthase levels in HCC [50]. Among genetic alterations found in HCC are aneuploidy, genomic instability, deletions, mutations, gene amplifications, and genomic rearrangements [25]. The main chromosomes in which genomic instability was observed were 1p, 4q, 6q, 8p, 13q, 16p, 16q, and 17p. These regions are likely to have known and not-yet-known tumor-suppressor genes. Ozen et al. reported mutations in *CTNNB1*, *TP53*, *AXIN1*, and *CDKN2*, but the frequency of these mutations is low in HCC patients [23]. These markers should be combined with other markers to improve the sensitivity of detection. Dysregulation of signal transduction pathways such as transforming growth factor beta, Wnt, Notch, and Hedgehog have also been proposed for use in diagnosing HCC [51]. Kohles et al. used immunogenic cell death biomarkers such as high-mobility group box-1 (HMGB1), soluble levels of receptor for advanced glycation endproducts (sRAGE),

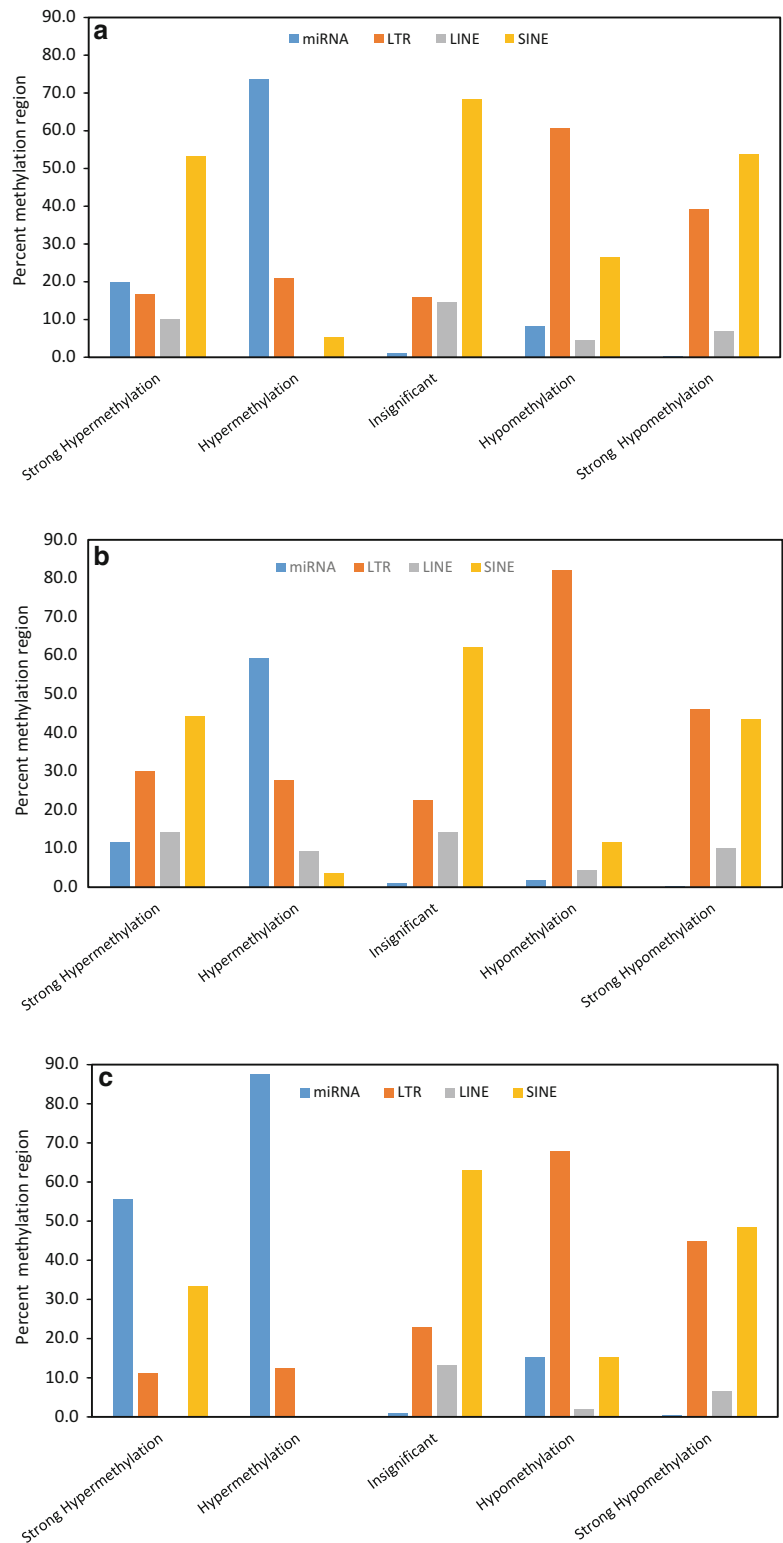


Fig. 1 Proportion of different methylated regions in (a) paired normal, (b) normal, and (c) tumor + normal liver tissues. The data have been adapted from Xueguang Sun et al. (source: a Zymo Research AACR Poster entitled “Epigenetic Biomarker Discovery, Validation for Diagnosis, and Therapeutic Intervention for Hepatocellular Carcinoma”)

and DNase to follow up treatment [52]. Metabolomic profiling may also be useful in HCC diagnosis [53]. Proteomic biomarkers were useful in distinguishing HCC patients from patients with liver cirrhosis [54]. In one study, HCC patients undergoing surgery were followed for survival and recurrence of HCC, and the AFP-L3 biomarker was found to be useful for following survival [55]. Proteomic biomarkers may also be useful in preventing the development of HCC in HBV-infected patients [56]. HBV and HCV co-infection in the same patient has not generally been observed.

5 Potential of miRNA Biomarkers in Liver Cancer Diagnosis and Prognosis

microRNAs in HCC can be used as biomarkers for diagnosis and follow-up of treatment [8, 44]. Technology exists that can determine miRNA profiles at the genome level. A recent case-control epidemiology study conducted by Shen et al. in normal subjects and HCC patients evaluated genome-wide expression of circulating miRNAs associated with HCC. The results indicated that miR-483-5p was significantly elevated in the serum of HCC patients [29]. Validation of these results was accomplished in another set of samples. HCV status was known in samples used for this study. The sensitivity and specificity of miR-483-5p were 75.5 % and 89.8 %, respectively. Other investigators also found miRNAs to be useful as biomarkers for HCC diagnosis [44]. Anwar et al. identified methylation of miRNA genes in selected cell lines, and potential miRNAs were then used to analyze human tissue samples [44]. Results indicated at least three miRNA genes that were hypermethylated in HCC patients only. Chen et al. demonstrated that methylation-mediated repression of miR-129-2 resulted in overexpression of oncogenic *SOX-4* in 75 tumor samples [57]. All HCC subjects in this study were HBV positive.

6 Future Directions and Conclusion

Epigenetic biomarkers and epigenetic inhibitors (demethylating agents and histone-deacetylating agents) have shown promise for HCC use. Four epigenetic inhibitors have been approved by the US Food and Drug Administration. For early HCC detection, the approach of combining two types of biomarkers was proposed in a study in which messenger RNA (mRNA) expression and DNA methylation were followed in patient samples. The investigators observed that aberrant mRNA and DNA methylation levels of *ABCB6* were more useful indicators than a single marker alone [58]. The combination of genetic and epigenetic biomarkers for HCC diagnosis and prognosis was proposed by Ozen et al. [23]. HCC is quite resistant to conventional chemo- and radiotherapy.

Interferon has been effective in the treatment of HCC, especially in HCV-infected patients [59]. Epidemiology studies should be conducted to identify additional risk factors for HCC and to develop new risk-prediction models. Epigenetic information in these areas should be very beneficial in developing new therapeutics for HCC as well.

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Part II

Cancer Specific Type Epigenetic Changes

Chapter 5

Cancer Type-Specific Epigenetic Changes: Gastric Cancer

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Abstract

Gastric cancer (GC) remains a major cause of mortality despite declining rate in the world. Epigenetic alterations contribute significantly to the development and progression of gastric tumors. Epigenetic refers to the number of modifications of the chromatin structure that affect gene expression without altering the primary sequence of DNA, and these changes lead to transcriptional activation or silencing of the gene. Over the years, the study of epigenetic processes has increased, and novel therapeutic approaches have emerged. This chapter summarizes the main epigenomic mechanisms described recently involved in gastric carcinogenesis, focusing on the roles that aberrant DNA methylation, histone modifications (histone acetylation and methylation), and miRNAs (oncogenic and tumor suppressor function of miRNA) play in the onset and progression of gastric tumors. Clinical implications of these epigenetic alterations in GC are also discussed.

Key words Gastric cancer, DNA methylation, Histone modification, miRNA

1 Background

Gastric cancer (GC) is the fourth most frequent cancer and is the second leading cause of cancer-related death worldwide [1]. They are classified into two main types of gastric adenocarcinoma: intestinal and diffuse [2]. The intestinal type mostly progresses through the successive multistep from chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and finally to gastric tumor [3]. In contrast, the pathogenesis of diffuse type is poorly understood, although a subset of diffuse type GC appears to develop independently of preneoplastic lesions [4, 5]. Differences in the clinico-pathological characteristics between these two histological types indicate that development occurs through distinct molecular pathways [6–10]. Each histological type is a consequence of a gradual accumulation of various genetic and epigenetic alterations, leading to gain of function in oncogenes and loss of function in tumor-suppressor genes [11].

Epigenetic events, including mainly DNA methylation of CpG islands, posttranslational modifications of histones, non-coding RNAs, and nucleosome positioning, affect gene expression without altering the primary DNA sequence, and these changes lead to transcriptional activation or silencing of the gene [11].

In recent years, altered epigenetic control of gene expression had emerged with a central role in many different diseases, as GC [12–14]. A greater understanding of epigenetics and the therapeutic potential of intervention into these processes is necessary to help GC treatment.

In this chapter, we provide an overview of main recently described epigenomic mechanisms involved in gastric carcinogenesis, focus on the roles that aberrant DNA methylation, histone modifications and microRNAs play in the onset and progression of gastric tumors, and the development of compounds that target enzymes that regulate the epigenome.

2 DNA Methylation and Histone Modification Genes in Gastric Cancer

DNA methylation and histone posttranslational modifications have been implicated in the regulation of higher order chromatin structure, the maintenance of genome integrity, and stable patterns of gene expression [14].

The methylation status is controlled by DNA methyltransferases (DNMT1, DNMT3A, and DNMT3B) [15] and many studies have shown that overexpression of DNA methyltransferases is closely related to gastric tumorigenesis [16–24] (Table 1).

The cross talk between DNA methylation and histone modifications is established by different nuclear factors, such as methyl-CpG-binding proteins (MBPs) [25, 26]. In cancer, the roles of MBPs are related to their functions as transcriptional repressors or chromatin remodelers [26–28]. However, a few studies have reported MBPs in GC (Table 1). Mutations in *MBD4* have been found in gastric tumors in association with microsatellite instability [31, 32]. *MBD4* encodes a protein that interacts with the mismatch repair protein hMLH1. Therefore, it has been postulated that mutations in *MBD4* may result in mismatch repair deficiency [34]. Recently, we showed reduced mRNA expression of *MBD2* and *MBD3* in gastric tumor compared with paired adjacent nonneoplastic specimens, gastritis, and paired non-gastritis tissues using relative quantification real-time PCR (qRT-PCR). These reduced mRNA expression may have a specific role in the gastric carcinogenesis process and could be used as biomarkers for the detection or monitoring of cancer development and progression [30].

Among all the posttranslational modifications on histone tails, histone acetylation and methylation are the main modifications that have been clinically associated with pathological epigenetic

Table 1
Methylation machinery in GC

Gene	Function	Alteration	References
<i>DNMT1</i>	Maintenance of methylation Transcription repression	Upregulation	Kanai et al. [16] Fang et al. [17] Ding et al. [18] Yang et al. [19] Mutze et al. [20]
<i>DNMT3A</i>	De novo methylation during embryogenesis Imprint establishment Repression	Upregulation	Ding et al. [18] Fan et al. [21] Yang et al. [19]
<i>DNMT3B</i>	De novo methylation during embryogenesis Repeat methylation Repression	Upregulation	Ding et al. [18] Su et al. [22] Hu et al. [23] Yang et al. [19]
<i>MBD2</i>	Transcription repression DNA demetilase	Downregulation	Kanai et al. [29] Fang et al. [17] Pontes et al. [30]
<i>MBD3</i>	Transcription repression, but requires MBD2 to recruit it to methylated DNA	Downregulation	Pontes et al. [30]
<i>MBD4</i>	Transcription repression DNA repair	Downregulation Mutation	Pinto et al. [31] D'Errico et al. [32]
<i>Kaiso</i>	Transcription repression	Upregulation	Ogden et al. [33]

DNMT DNA methyltransferase, *MBD* Methyl-CpG-binding domain

disruption in GC [13, 14]. A growing number of studies have analyzed the histone acetyltransferase (HATs) and histone deacetylases (HDACs), histone methyltransferases (HMTs), and histone demethylases (HDMs) in tumor cells, whereas few genes involved in histone acetylation or methylation activity have been described for GC (Table 2).

Inactivation of HAT activity through gene mutation has been described in different tumors. Somatic mutations in E1A-binding protein p300 (*EP300*), a transcriptional coactivator protein and functions as a HAT, have been identified in GC. Using reverse transcription-polymerase chain reaction-single-strand conformation polymorphism (RT-PCR-SSCP) analysis, Koshiishi et al. [35] found that loss of heterozygosity (LOH) was significantly correlated with advanced stage and lymph node metastasis in intestinal-type GC, suggesting the *EP300* gene behaves as a tumor suppressor gene in the intestinal type of gastric adenocarcinoma. Recently, Kim et al. [36] reported the frameshift mutations of *EP300* and its expressional loss may be a feature GC with high microsatellite instability.

Table 2
Histone modification genes altered in GC and clinical implications

Gene	Alteration	Clinical implications	References
<i>Histone acetyltransferases</i>			
<i>EP300</i>	Mutation	Advanced stage Intestinal type Lymph node metastasis	Koshiishi et al. [35] Kim et al. [36]
<i>KAT5</i>	Downregulation	Depth of tumor Lymph node metastasis patient age	Sakuraba et al. [37]
<i>PCAF</i>	Downregulation	Depth of tumor TNM stage Tumor size	Ying et al. [38]
<i>KAT7</i>	Upregulation	–	Iizuka et al. [39]
<i>Histone deacetylases</i>			
<i>HDAC1</i>	Upregulation	Advanced stage Histological subtype Poor prognostic Overall survival	Choi et al. [40] Mutze et al. [41] Sudo et al. [42] Gao et al. [43]
<i>HDAC2</i>	Upregulation	Advanced GC Shorter survival	Song et al. [44] Weichert et al. [45] Mutze et al. [41]
<i>SIRT1</i>	Upregulation Downregulation	Depth of tumor Intestinal subtype Lymph node metastasis Overall survival TNM stage	Cha et al. [46] Feng et al. [47] Kang et al. [48] Yang et al. [49]
<i>Histone methyltransferase</i>			
<i>RIZ1</i>	Mutation Promoter hypermethylation		Sakurada et al. [50] Tokomaru et al. [51] Oshimo et al. [52] Pan et al. [53] Oue et al. [54]
<i>BMI1</i>	Upregulation	Advanced stage	Liu et al. [55]
		Depth of tumor Histological subtype Lymph node metastasis Poor prognosis Tumor size Sex	Xiao and Deng [56] Lu et al. [57] Zhang et al. [58] Li et al. [59] Lu et al. [60]

(continued)

Table 2
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Gene	Alteration	Clinical implications	References
<i>EZH2</i>	Upregulation Polymorphism	Poor survival Depth of invasion Distant metastasis Histological subtype Lymph node metastasis Overall survival Tumor size Sex Vessel invasion	Matsukawa et al. [61] Cai et al. [62] Choi et al. [63] Zhou et al. [64] Gao et al. [43] Lee et al. [65] He et al. [66]
<i>Histone demethylases</i>			
<i>JMJD2B</i>	Upregulation	Depth of tumor Differentiation status Distant metastasis Lymph node metastasis TNM stage Tumor size	Li et al. [67] Kim et al. [68] Zhao et al. [69]
<i>RBP2</i>	Upregulation	–	Zeng et al. [70]

BM11 polycomb ring finger oncogene, *EP300* E1A-binding protein p300, *EZH2* Enhancer of zest homolog2, *HDAC1* histone deacetylase 1, *HDAC2* histone deacetylase 2, *JMJD2B* Jumonji domain containing 2 B, *KAT5* K (lysine) acetyltransferase, *KAT7* K (lysine) acetyltransferase 7, *PCAF* P300/CBP-associated factor, *RBP2* retinoblastoma-binding protein 2, *RIZ1* Retinoblastoma protein-interacting zinc finger 1, *SIRT1* sirtuin 1, *SUZ12* polycomb-repressive complex 2 subunit

In general, HDACs have been upregulated in gastric tumors. However, studies indicate that sirtin 1 (*SIRT1*) is an HDAC that also downregulated in GC. These contradictory findings may be attributable to the diverse functions of SIRT1, which can interact with both tumor suppressors and tumor promoters. SIRT1 expression was associated with the cytoplasmic expression of β -catenin in gastric adenocarcinoma, a key regulator of Wnt signaling. These results suggested that SIRT1 may be an important regulator of β -catenin in tumorigenesis, which could mediate the role of β -catenin by deacetylation. Moreover, SIRT1 expression were significantly associated with younger age (<60 years old), lower histologic grade, intestinal type, lower depth tumor, lower TNM stage, absence of lymph node metastasis, and better patient survival [48].

Enhancer of zest homolog2 (*EZH2*), an HMT that plays a role in trimethylation of H3K27 and leads to silencing of important genes in carcinogenesis, is overexpressed in several types of cancer, including GC. Cai et al. [62] reported that *EZH2* plays an important role in the multi-step process of intestinal-type GC. In addition, Zhou et al. [64] found that five *EZH2* gene variants (rs734004, rs734005 rs2072407, rs6464926, rs12670401) were significantly associated with GC susceptibility.

Among the HDTs, retinoblastoma-binding protein 2 (RBP2) is a newly identified member of the JARID family, and RBP2 specifically targets tri- and dimethylated H3K4 for demethylation in cancer [71, 72]. Zeng et al. [70] reported that *RBP2* is overexpressed in GC and suggested that HDT inhibition by targeting RBP2 may be an anticancer strategy.

3 DNA Methylation and Their Clinical Significance in Gastric Cancer

Epigenetic silencing of tumor-related genes by hypermethylation is increasingly recognized as having an important role in the screening early diagnosis and prognosis of cancer. DNA hypermethylation, which refers to the gain of methylation at a locus originally unmethylated, usually results in stable transcriptional silencing, which functions in regulating gene expression [73].

GC is one of the tumors with a high and early frequency of aberrant methylation and that could be useful for detection on gastric tumor [13]. Thus, DNA methylation could be useful biomarker in GC risk evaluation, early diagnosis, predicting patient prognosis and evaluating the sensitivity to chemotherapeutic drugs. Table 3 summarizes the hypermethylation genes and clinical outcomes reported in GC.

Shigematsu et al. [111] found hypermethylation of region around cg06436185 CpG site (*PRKAG2* gene) predicted the presence of lymph node metastases at sensitivity of 43 % and specificity of 85 % using genome-wide methylation analysis and validation the isolated candidate biomarkers. In addition, the authors suggested the hypermethylation of the region was associated with poor survival rate among GC patients with lymph node metastases. This information is important because imaging modalities used in clinical practice are almost powerless to detect micrometastasis present in lymph node [112].

Several studies have shown that the methylation status has influenced in prognostic of GC patients with specific tumor location. The hypermethylation status of Raf kinase inhibitory protein (*RKIP*), a member of a novel class of molecules which implicated in cancer progression and suppress the metastatic spread of tumors, has been report in gastric cardia adenocarcinoma and associated with TNM stage, histological differentiation, depth of invasion, lymph node metastasis, distant metastasis or recurrence, and upper gastrointestinal cancers family history [109].

Reports have indicated that infection by *Helicobacter pylori* or Epstein–Barr virus (EBV), pathogens with a substantial role in development of GC, is associated with elevated levels of aberrant DNA methylation in GC. *H. pylori* alters DNA methylation of E-cadherin (encoded by *CDH1* gene), as well as likely promoting the methylation of others genes *APC*, *p16* (*INK4a*), *bMLH1*,

Table 3
Genes commonly methylated in GC and clinical-pathological features related

Gene	Role	Assay	Clinicopathological features	References
<i>APC</i>	Tissue cell invasion and metastasis Signal transduction	MSP	Age-specific marker EBV Improved patient's outcome	Ksiaa et al. [74] Gedder et al. [75] Borges et al. [76]
<i>BNIP3</i>	Apoptosis	Q-MSP MSP	Lower response to chemotherapy Poor prognosis	Murai et al. [77] Hiraki et al. [78] Sugita et al. [79]
<i>CDH1</i>	Tissue invasion and metastasis	MSP PS	Depth of tumor invasion Diffuse type <i>H.pylori</i> infection Lymph node metastasis Male gender Poor prognosis Tumor size	Leal et al. [80] Kang et al. [81] Borges et al. [82] Tahara et al. [83] Al-Moundhri et al. [84] Yu et al. [85] Lee et al. [86]
<i>CHFR</i>	Cell cycle regulation	MSP Q-MSP	Lymph node metastasis	Oki et al. [87] Hiraki et al. [88] Hu et al. [89]
<i>DAPK</i>	Apoptosis	MSP Q-MSP	Lymph node metastasis	Zou et al. [90] Hu et al. [89] Tahara et al. [83] Sugita et al. [79] Ben Ayed-Guerfali et al. [91] Yao et al. [92]
<i>FLNC</i>	Cell morphology	MSP	Lymph node metastasis Poor prognosis	Kim et al. [93] Shi et al. [94]
<i>LOX</i>	Tissue cell invasion and adhesion	MSP methylation-specific DNA microarray	Depth of tumor invasion Lymph node metastases TNM stage Poor survival	Maekita et al. [95] Shin et al. [96] Tamura et al. [97]
<i>MGMT</i>	DNA repair	MSP	Lymph node metastasis Cancer-related death	Ksiaa et al. [74] Zou et al. [90] Hiraki et al. [88] Balassiano et al. [98] Shi et al. [94]

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Table 3
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Gene	Role	Assay	Clinicopathological features	References
<i>MLH1</i>	DNA repair	MSP	Differentiation Poor prognosis	Poplawski et al. [99] Hiraki et al. [88] Kim et al. [100] Shin et al. [101]
<i>P14ARF</i>	Cell cycle regulation Apoptosis Cell differentiation		EBV	Balassiano et al. [125] Geddert et al. [75]
<i>P16</i>	Cell cycle regulation	MSP	Cardiac location EBV Intestinal type <i>H. pylori</i> infection Lymph node metastasis Poor survival Tumor differentiation	Ksiaa et al. [74] Dong et al. [102] Zou et al. [90] Shin et al. [96] Hu et al. [89] Geddert et al. [75] Balassiano et al. [98] Al-Moundhri et al. [84] Shin et al. [101] Borges et al. [76]
<i>RAR-beta</i>	DNA binding Activation transcription	MSP	Improved patient's outcome Lymph node metastasis	Ksiaa et al. [74] Yao et al. [92]
<i>RASSF1A/</i> <i>RASSF2</i>	DNA repair Cell cycle regulation	MSP Q-MSP	Depth of tumor invasion Differentiation Lymph node metastasis TNM stage Poor prognosis	Zou et al. [90] Guo et al. [103] Shin et al. [101] Yao et al. [92]
<i>RUNX3</i>	Signal transduction	MSP Q-MSP	Depth of tumor invasion Distant metastasis <i>H. pylori</i> infection Lymph node metastasis tumor stage	Sakakura et al. [104] Lee et al. [105] Zou et al. [90] Hiraki et al. [88] Tamura et al. [98] Hu et al. [89] Fan et al. [106] Al-Moundhri et al. [84] Lu et al. [107]
<i>SHP1</i>	Signal transduction	MSP	EBV	Ksiaa et al. [74]
<i>TIMP3</i>	Tissue cell invasion and adhesion	MSP Q-MSP	Antrum localization Differentiation	Yao et al. [92] Guan et al. [108]

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Table 3
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Gene	Role	Assay	Clinicopathological features	References
<i>GSTP1</i>	Apoptosis	MSP	Diffuse type	Ksiaa et al. [74]
<i>PAX6</i>	Transcriptional regulation	MSP Q-MSP	Lymph node metastasis Poor prognosis Tumor stage	Yao et al. [92]
<i>CALCA</i>	Calcium regulation and acts to regulate phosphorus metabolism	MSP Q-MSP	Lymph node metastasis	Yao et al. [92]
<i>RKIP</i>	Ras pathway	MSP	Depth of tumor invasion Differentiation Distant metastasis Lymph node metastasis Tumor location	Guo et al. [109]
<i>TFF2</i>	Repair gene	MSP	<i>H. pylori</i> infection	Perteson et al. [110]

MSP methylation-specific polymerase chain reaction, PS pyrosequencing, Q-MSP quantitative methylation-specific polymerase chain reaction

MGMT, *TFF2*, and *RUNX3* [110, 112]. Moreover, EBV infection has been associated with hypermethylation of promoter region in *APC*, *p16*, *MINT1*, *MLH1*, *TP73*, *HOXA10*, *CXXC4*, *TIMP2*, and *PLXND* [113].

Recent studies have demonstrated that detecting circulating methylated DNA in serum or gastric washes is a potent and practical approach for GC patients. Watanabe et al. [114] suggest that methylation status in promoter of *MINT25* gene alone and in a combination of *MINT25*, *ADAM23*, and *GDNF* from gastric washes can be appropriated alternative to DNA from biopsied tissue for determination of methylation status in GC and to screen for this deadly disease. Lim et al. [115] found that promoter methylation of *p16* gene was high in preoperative serum and had a tendency towards decreased frequency postoperative from GC patients, suggesting that postoperative disappearance of *p16* promoter methylation could be an available prognostic factor for node positive GC. Ling et al. [116] observed that circulating methylated *XAF1* DNAs may be a useful biomarker for diagnosis of GC, predicting patients prognosis and monitoring tumor recurrence after surgery treatment.

The second important DNA methylation alteration in cancer is DNA hypomethylation. DNA hypomethylation refers to the loss of DNA methylation and affects chromosomal stability and increases aneuploidy [117].

The level of long interspersed transposable elements (LINEs) methylation has emerged as a promising prognostic or predictive biomarker in GC [118]. Bae et al. [119] observed that LINE-1 methylation decreased during the transition from intestinal metaplasia to gastric adenoma and no further decrease occurred during the transition from gastric adenoma to GC. The authors also found that LINE-1 hypomethylation was strongly associated with poor prognosis in GC. Other study reported that LINE-1 hypomethylation is correlated with shorter overall survival and suggested that hypomethylation in LINE-1 is potential prognostic biomarker in GC [120].

4 miRNA Roles and Their Clinical Significance in Gastric Cancer

Improved understanding of microRNA (miRNA) in GC could lead to novel prevention strategies, early detection and improved therapeutics. A large number of miRNAs and their targets have been identified are upregulated or downregulated in GC (Table 4). The oncogenic or tumor suppressor function of miRNA (oncomiRNA or tumor-suppressor miRNA, respectively) depends on the outcome of the target mRNA. Increased activity of oncomiRNA leads to cell proliferation and inhibition of apoptosis. On the other hand, decreased activity of tumor-suppressor miRNA (tsmiRNA) leads increased to an oncogene translation others miRNA and tumor formation [186].

In GC, studies have reported consistently the oncogenic activity of miR-21 by suppressing the expression of *PTEN* or *PDCD4* [125, 127]. Furthermore, miR-21 upregulation has been associated with lymph node metastasis in GC patients [128]. MiR-106b was also consistently reported as an upregulated miRNA in GC and associated with lymph node metastasis [129, 136]. Recently, Shiotani et al. [187] suggested that the combination miR-106b with miR-21 may provide a novel and stable marker of increased risk for early gastric cancer after *H. pylori* eradication.

Other studies also have investigated the role of miRNAs in *H. pylori* infection. Xiao et al. [176] have shown that after *H. pylori* infection there is a strong induction of miR-155 which inhibits the production of the potent pro-inflammatory cytokine IL8. In addition, *H. pylori* infection also induced miR-146a. Interestingly, similar to miR-155, miR-146 also modulates the inflammatory response induced by *H. pylori* by reducing the levels of IL8, MIP-3 α , and GRO- α [177], suggesting that these miRNAs play an essential role in the control of the inflammatory response to *H. pylori* and possibly in the limitation of tissue damage observed in patients with gastritis and GC.

Table 4
Deregulated miRNA in GC

miRNA role	Targets	Clinicopathological features	Specimen	References
<i>OncomiRNA</i>				
miR-21	<i>PTEN</i> <i>PDCD4</i> <i>RECK</i> <i>SERPINI1</i>	Differentiation Lymph node metastasis <i>H. pylori</i> infection TNM stage Tumor size	Cell line Blood gastric juice Plasma Tissue Serum	Zhang et al. [121] Belair et al. [122] Motoyama et al., [123] Zhang et al. [124] Zheng et al. [125] Yamanaka et al. [126] Cao et al. [127] Xu et al. [128] Wang et al. [129] Song et al. [130] Cui et al. [131] Komatsu et al. [132]
miR106b	<i>P21</i>		Cell line Tissue Plasma	Kim et al. [133] Tchernitsa et al. [134] Tsujiura et al. [137] Kim et al. [136] Wang et al. [129] Cai et al. [137]
miR-106a	<i>TIMP2</i>	Depth of tumor invasion	Tissue Gastric juice Plasma	Xiao et al. [138] Tsujiura et al. [135] Cui et al. [131] Zhu et al. [139]
miR-196a	<i>P27</i>	pN stage pT stage Tumor size Survival	Cell line Tissue	Tsai et al. [140] Sun et al. [141]
miR-200b	<i>ZEB1</i> <i>ZEB1</i> <i>ZEB2</i> <i>SUZ12</i>	Diffuse type Poor overall survival <i>H. pylori</i> infection	Cell line Tissue	Belair et al. [122] Kurashige et al. [142] Song et al. [143] Cui et al. [138]
miR-27a	<i>PHB</i> <i>ZBTB10</i> <i>HOXA10</i>	<i>H. pylori</i> infection	Cell line Tissue	Liu et al. [144] Belair et al. [122] Sun et al. [145]
miR-20a	<i>EGR2</i>	Overall survival Relapse-free survival	Plasma Tissue	Cai et al. [137] Wang et al. [147] Wang et al. [129] Li et al. [148]
miR-222	<i>PTEN</i> <i>RECK</i>	Shorter metastasis-free survival	Cell line Tissue	Belair et al. [122] Chun et al. [149] Li et al. [150] Kim et al. [136]

(continued)

Table 4
(continued)

miRNA role	Targets	Clinicopathological features	Specimen	References
miR-221	<i>PTEN</i>	Shorter metastasis-free survival <i>H. pylori</i> infection	Tissue Plasma	Cai et al. [137] Chun et al. [149]
<i>tsmiRNA</i>				
miR-148a	<i>ROCK1</i> <i>MMP7</i>	Clinical stage Lymph node metastasis Poor clinical outcome	Cell line Tissue Serum	Kim et al. [136] Zheng et al. [151] Sakamoto et al. [152]
miR-182	<i>CREB1</i>	Intestinal type	Cell line Tissue	Li et al. [153] Kong et al. [154]
miR-9	<i>cyclin D1</i> <i>Ets1</i> <i>CDX2</i> <i>GRB2</i> <i>NF-kappaB1</i>		Cell line Tissue	Luo et al. [155] Wan et al. [156] Rotkrua et al. [157] Zheng et al. [158]
miR-146a	<i>LICAM</i> <i>CARDIO</i> <i>COPS8</i> <i>NASF2</i> <i>SAMD4</i>	Tumor size differentiation pN stage	Cell line Tissue Serum	Kogo et al. [159] Hou et al. [160] Hou et al. [161] Zhou et al. [162] Crone et al. [163] Xiao et al. [164] Yao et al. [165] Kim et al. [136]
miR-200c		Lymph node metastasis Poor overall survival	Blood Tissue	Song et al. [143] Valladares et al. [166]
let-7a	<i>RAB40C</i> <i>CDKN1</i> <i>SPHK2</i> <i>FN1</i>	Differentiation Lymph node metastasis	Cell line Tissue plasma	Zhu et al. [167] Yang et al. [168] Zhu et al. [169] Tsujiura et al. [135]
miR-223	<i>EPB4IL3</i> <i>STMN1</i>	Poor metastasis-free survival	Cell line Tissue	Li et al. [170] Kang et al. [171]
miR-449	<i>GMNN</i> <i>MET</i> <i>CCNE2</i> <i>SIRT1</i>		Cell line Tissue	Bou Kheir et al. [172]
miR-429	<i>MYC</i>	<i>H.pylori</i> infection Lymph node metastasis	Cell line Tissues	Sun et al. [173] Liu et al. [174]
miR-433	<i>RAB34</i> <i>KRAS</i>	pTNM stage Overall survival	Cell line Tissue	Luo et al. [155] Ueda et al. [175] Guo et al. [176]

(continued)

Table 4
(continued)

miRNA role	Targets	Clinicopathological features	Specimen	References
<i>Controversial</i>				
miR-155	<i>SMAD2</i>	Depth of tumor invasion Lymph node metastasis <i>H. pylori</i> infection	Cell line Tissue	Belair et al. [122] Xiao et al. [177] Liu et al. [178] Li et al. [179] Kim et al. [180]
miR107	<i>FOXO1</i> <i>DICER1</i> <i>CDK6</i>	Depth of tumor invasion differentiation Lymph node metastasis Tumor size Tumor stage Overall survival	Cell line Tissue	Feng et al. [181] Inoue et al. [182] Li et al. [183]
miR-126	<i>SOX2</i> <i>CRK</i> <i>CRKL</i>	Depth of tumor invasion Lymph node metastasis TNM stage Tumor size	Cell line Tissue	Feng et al. [184] Otsubo et al. [185] Wang et al. [186]

Despite this has been well established that miR-155 is upregulated in *H. pylori* infection and lead to inflammatory response, this miRNA role in GC is controversial. Kim et al. [180] reported that the miR-155 is upregulated in advance GC than in paired non-cancerous samples, and their high expression was closely related to depth of tumor invasion and lymph node metastasis. However, miR-155 also may function as a tumor suppressor by targeting SMAD2 in GC cells. Li et al. [179] found that miR-155 was significantly downregulated in GC cell lines compared with an immortalized gastric epithelial cell line. Therefore, the same miRNA may act as both oncomiRNA or tsmiRNA.

Other miRNA have been reported with these functional differences in GC, as miR-107 and miR-126. This dual role of miRNA might be explained because a single miRNA is capable of targeting of multiple genes.

The single-nucleotide polymorphisms (SNPs) in miRNA have been associated with remarkable modification of the target gene expression and so modify the GC susceptibility. However, the role of genetic variants in miRNAs on GC susceptibility and their prognostic significance remain largely unknown.

Several studies have reported that SNPs in miRNA-27a to affect the corresponding miRNA production and GC susceptibility.

Table 5
miRNA related to the risk of in GC

miRNA	SNP	Country	Population	Genotyping methods	Number of cases/controls	Reference
<i>miR-27a</i>	rs895819	<i>China</i>	Asian	<i>PCR-RFLP</i>	304/304	Sun et al. [145]
		<i>China</i>	Asian	<i>massARRAY</i>	295/413	Zhou et al. [189]
	rs11671784	<i>China</i>	Asian	<i>real-time PCR</i>	892/978	Yang et al. [146]
<i>miR-146a</i>	rs2910164	<i>China</i>	Asian	<i>PCR-RFLP</i>	304/304	Zeng et al. [190]
		<i>Japan</i>	Asian	<i>PCR-CTPP</i>	583/1637	Hishida et al. [191]
		<i>China</i>	Asian	<i>real-time PCR</i>	1686/1895	Zhou et al. [162]
		<i>Korean</i>	Asian	<i>PCR-RFLP</i>	461/447	Ahn et al. [192]
<i>miR-196a</i>	rs11614913	<i>Japan</i>	Asian	<i>PCR-RFLP</i>	552/697	Okubo et al. [193]
		<i>China</i>	Asian	<i>PCR-RFLP</i>	213/213	Peng et al. [194]
		<i>Korean</i>	Asian	<i>PCR-RFLP</i>	461/447	Ahn et al. [192]
		<i>Greece</i>	Greek	<i>PCR-RFLP</i>	163/480	Dikeakos et al. [195]
<i>miR-499</i>	rs3746444	<i>Japan</i>	Asian	<i>PCR-RFLP</i>	697/552	Okubo et al. [193]
		<i>Korean</i>	Asian	<i>PCR-RFLP</i>	461/447	Ahn et al. [192]
<i>miR-149</i>	rs2292832	<i>China</i>	Asian	<i>PCR-RFLP</i>	274/269	Zhang et al. [196]
		<i>Korean</i>	Asian	<i>PCR-RFLP</i>	461/447	Ahn et al. [192]
		<i>Greece</i>	Greek	<i>PCR-RFLP</i>	163/480	Dikeakos et al. [195]

PCR-CTPP polymerase chain reaction with confronting two-pair primers, *PCR-RFLP* polymerase chain reaction-restriction fragment length polymorphism, *real-time PCR* real-time polymerase chain reaction

Sun et al. [145] found AG polymorphism within *miR-27a* (rs895819) was associated with a significantly increased risk of GC, conferred a higher level of *miR-27a*, which accompanied significantly reduced *ZBTB10* mRNA. Recently, Yang et al. [146] found that the G/A polymorphism in *miR-27a* (rs11671784) decreased mature *miR-27a* expression, increased level of its target *HOXA10* and reduced GC risk, suggesting that this variant may promote loss of function of the oncogenic *miR-27a*. These results could be useful to assess individual susceptibility of GC and will improve our understanding of the potential contribution of miRNA SNPs to cancer pathogenesis. Table 5 summarizes several SNPs in miRNA and their implications described in GC.

SNPs in miRNA target genes also could affect the binding of miRNA with the target and contribute to the susceptibility of human cancers. In GC, was reported that SNP rs4143815 in *B7-H1* gene, a target of *miR-570*, can affect *B7-H1* protein expression by interfering with *miR-570* suppressive function. Moreover, this SNP was significantly related to the risk of gastric adenocarcinoma and to the clinicopathological features of GC including tumor size, differentiation grade, depth of tumor infiltration, lymph node metastasis, and TNM stage [197].

5 Future Perspectives

The silencing of cancer-related genes by DNA methylation and chromatin modification are reversible and may represent a viable epigenetic therapeutic target. In the last decade, drugs that modify chromatin or DNA methylation status have been used alone or in combination in order to affect therapeutic outcomes. Although a growing number of studies regarding epigenomics in GC the complete understanding and the called “epigenetic therapy” has not yet been used in treatment of GC patients. Currently, surgery remains the primary curative treatment for gastric tumors and adjuvant and neoadjuvant therapies are accepted [198].

In the last years, a phase I trial of first-line vorinostat, an orally bioavailable histone deacetylase inhibitor, in combination with capecitabine plus cisplatin (XP) was performed to assess recommend phase II trial dose in patients with advanced GC. This phase I trial evaluated the 3-weekly vorinostat-XP regimen as first-line chemotherapy in unresectable or metastatic gastric cancer. The change in H3 acetylation after treatment with vorinostat correlated significantly with the vorinostat dose (300 mg/day vs. 400 mg/day) and the baseline level of H3 acetylation before treatment. A phase II trial based on the results of the present study is now ongoing. However, further clinical trials are warranted to evaluate biomarkers as well as the safety and efficacy of this regimen [199].

In summary, aberrant DNA methylation and histone modification play a crucial role in gastric carcinogenesis. Thus, the recognition of the methylation machinery, genes with aberrant methylation status, and histone methylation levels in gastric carcinogenesis exemplified in this review allow us to contemplate the possibility of dealing with the aforementioned oncological issue in a new way that may have a significant impact on the therapy and management of GC.

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Beyond the Island: Epigenetic Biomarkers of Colorectal and Prostate Cancer

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Abstract

Epigenetic dysregulation is a common feature across all cancer types. Epigenetic mechanisms, from DNA methylation to histone modifications, allow for a vast number of cellular phenotypes to be created from the same genetic material. Just as certain genetic changes play a key role in tumor initiation and progression, epigenetic changes may also set the course of tumor development and be required for malignant transformation. The most frequently studied epigenetic changes investigated thus far are global genomic DNA hypomethylation along with specific hypermethylation, predominantly at promoter CpG islands of tumor suppressor genes. In addition to DNA methylation changes at CpG islands, there is an abundance of other epigenetic alterations occurring within cancer cells including DNA methylation alterations outside of CpG islands, non-CpG methylation, changes in cytosine oxidative species (hydroxymethylcytosine, formylcytosine, carboxylcytosine) levels, and histone modifications. This chapter examines epigenetic alterations beyond the island, and summarizes recent findings in DNA-based epigenetic regulation of the two most commonly diagnosed cancers in the Western world: colorectal cancer and prostate cancer.

Key words Epigenetics, Colorectal cancer, Prostate cancer, DNA methylation, CpG island shores, 5-Hydroxymethylcytosine, Non-CpG methylation, Histones, Polycomb repressive complex 2

1 Colorectal Cancer Overview

Colorectal cancer (CRC) is the third most commonly diagnosed cancer overall in the USA and the second leading cause of cancer-associated deaths in both men and women, with a 5-year survival rate of 64 % [1]. Worldwide, there are an estimated 1,233,700 new cases yearly and 608,700 deaths from CRC with similar incidence and mortality rates reported in developed countries as worldwide [2]. The most common genetic aberration in CRC is mutation to *adenomatous polyposis coli* (*APC*). This causes dysregulated Wnt signaling which leads to uncontrolled cell growth and proliferation. *APC* mutation is often followed by mutations to *KRAS*, *TP53*, *PIK3CA*, and *PTEN* [3, 4]. Some of the first studies of cancer-associated DNA methylation changes were performed in colorectal tumors, including the finding that tumors have lower

global methylation levels than normal DNA [5]. In addition to global decreases in methylation, a subset of CRC tumors was shown to harbor widespread gene-specific promoter hypermethylation, which became known as the CpG island methylator phenotype (CIMP) [6]. A gene often methylated in CRC tumors is *MutL homolog 1 (MLH1)*, a DNA mismatch repair gene required for correcting DNA mismatches [7]. Promoter DNA hypermethylation of *MLH1* leads to loss of expression resulting in mismatch repair deficiency and microsatellite instability (MSI), which occurs in about 15 % of CRC. While the majority of MSI cases are due to *MLH1* methylation, MSI can also occur due to *MLH1* mutation or deficiency in other mismatch repair proteins (MSH2, MSH6, PMS2). CRC patients with MSI tumors have better survival than those with microsatellite stable (MSS) tumors and also require different chemotherapeutic treatment [8, 9]. While 5-fluorouracil-based treatments are used to treat MSS CRC, it confers no survival benefit to patients with MSI CRC [10, 11]. Though MSI and CIMP tumors overlap, overall prognosis for CIMP CRC is unclear as some reports show worse prognosis for patients with CIMP, such as when located distally and in individuals with BRAF mutation but no MSI [12, 13]. A better understanding of the epigenetic events driving different CRC subtypes may aid in patient diagnosis, prognosis, and treatment.

2 Prostate Cancer Overview

Prostate cancer (PCa) is the most frequently diagnosed cancer and the second leading cause of cancer deaths in American men [1]. Worldwide there are an estimated 903,500 new PCa cases diagnosed yearly with 258,400 estimated deaths. Incidence and mortality due to PCa are higher in developed countries than worldwide, due to differing risk factors and screening practices [2]. Though one in six men is expected to be diagnosed with PCa in the USA, there is a very high 5-year survival rate of 99 % [1]. While PCa and CRC differ in incidence and mortality rates, both diseases are marked by genetic and epigenetic events. Loss of the *NKX3.1* homeobox gene, involved in normal differentiation of prostatic epithelium, is an initiating event in many prostate tumors [14]. Loss of the tumor-suppressor *PTEN*, leading to aberrant PI3K signaling, and the *TMPRSS2:ERG* gene fusion, which activates the oncogene *ERG*, are frequent events in PCa and are associated with more aggressive disease [15, 16]. Other common genetic aberrations in PCa include *ABL*, *BRAF*, *EGFR*, *HRAS*, *KIT*, *KRAS*, *NRAS*, and *PIK3CA* [17]. The current biomarker in clinical usage for PCa diagnosis is prostate-specific antigen (PSA), measured in serum. PSA, however, lacks specificity and sensitivity and also fails to distinguish indolent from aggressive disease [18, 19].

Another current biomarker for PCa is the non-coding RNA PCA3, though it also lacks the ability to distinguish aggressive disease [20]. Prostate-specific membrane antigen (PSMA) is a promising protein biomarker, which is more highly expressed in metastatic and androgen-independent PCa and is currently being explored as a potential therapeutic target for treatment [21, 22]. Determining genetic and epigenetic biomarkers for accurate diagnosis and, importantly, prognosis of aggressive PCa is a major current goal of researchers in the field.

3 Global DNA Hypomethylation

Global DNA methylation loss in cancer is a well-established and early event which can be utilized as a marker of CRC and PCa. CpG dinucleotides are rare across much of the genome, occurring at a lower frequency than other dinucleotide combinations. Most of these CpGs are highly methylated in normal cells, except for small regions of dense CpGs, known as CpG islands, which are largely unmethylated [23]. A global decrease in DNA methylation levels in cancer was first reported 30 years ago when comparing colon tumor DNA with normal cells and this hypomethylation has since been recognized as a hallmark of all cancers [5]. Methylation at repetitive DNA sequences maintains chromosomal stability such as at centromeric regions and also suppresses transposable elements for genomic stability [24, 25]. Global DNA hypomethylation may contribute to increased genomic instability, as well as overexpression of oncogenes [26, 27]. Genomic imprinting, in which DNA methylation occurs on a specific parent-of-origin allele, may also be lost during cancer-associated DNA methylation loss. This is particularly well illustrated in CRC at the *IGF2* locus [28]. Hypomethylation of repetitive regions, such as LINE-1 sequences, may also promote expression of genes at antisense promoters situated next to LINE-1 repeats [29].

In CRC, hypomethylation covers nearly half of the genome [26]. PCa is also marked by significant losses in DNA methylation [30]. Hypomethylation at gene-specific promoter CpG islands is relatively infrequent in cancer. In a comparison of tumor versus normal adjacent colon tissue, 26 % of methylation loss was at CpG islands and only 3 % was at gene promoters [26]. Two examples from this study, *B3GNTL1* and *TACSTD2*, incur hypomethylation at their enhancers, leading to increased expression, in CRC versus normal. In PCa, the oncogenes *Wnt5a*, *CRIP1*, and *SI00P* have shown hypomethylation [31]. Hypomethylation may also reveal alternative transcription start sites (TSS). For example, in the PCa cell line LNCaP, *KLK4* incurs promoter CpG island hypermethylation with concurrent hypomethylation at a CpG island in exon 2 which results in transcriptional activation of a new TSS at exon 2 [32].

Further investigation of these hypomethylated genes may serve to increase our knowledge of their oncogenic potential in CRC and PCa, and uncover information regarding relevant regulatory regions such as alternative start sites.

4 CpG Island Hypermethylation and the CpG Island Methylator Phenotype

A CpG island is defined as a DNA region 200 base pairs in length with a GC content greater than 50 % and an observed/expected CpG ratio greater than 0.6 [33]. These CpG islands are found in more than half of the promoters of genes but may also be found within gene bodies and, less frequently, in intergenic regions. Most CpG islands are unmethylated in normal somatic cells and expression of CpG island-containing genes is controlled by transcription factors. Specific regions may be repressed by methylation under normal conditions such as imprinted regions and the inactive X chromosome [34, 35].

All cancer types incur DNA methylation changes of some kind, including aberrant CpG island methylation. In some CRCs, this occurs at a large number of CpG islands across the genome leading to the designation of CIMP. Several different panels for CIMP have been created to categorize these tumors and their associated clinicopathological status. A unified panel for the classification of CIMP in CRC is not yet defined, though several genes and associated features are concordant [36–39]. Tumors with high levels of CIMP (CIMP-high/CIMP-H/CIMP1) are usually associated with the MSI phenotype, *BRAF* mutation, and a higher number of methylated genes while lower CIMP level (CIMP-low/CIMP-L/CIMP2) is associated with *KRAS* mutation and a fewer total number of methylated genes. Tumors without evidence of CIMP (CIMP-negative/CIMP0) are associated with *TP53* mutation [36]. One CIMP panel consists of *CACNA1G*, *IGF2*, *NEUROG1*, *RUNX3*, and *SOCS1* along with *MLH1* DNA hypermethylation [37]. A further development defines all CIMP tumors (CIMP-H and CIMP-L) as incurring methylation in three or more genes among a panel consisting of *B3GAT2*, *FOXL2*, *KCNK13*, *RAB31*, and *SLIT1* with further methylation at *FAM78A*, *FSTL1*, *KCNK1*, *MYOCD*, and *SLC6A4* defining CIMP-H [38]. Another CIMP panel consists of the classical markers *MLH1*, *MINT1*, *MINT2*, *MINT12*, *MINT17*, and *MINT31*. CIMP1 CRCs, as defined by this panel, have a much higher frequency of mutations, with an average of 425 non-synonymous mutations per tumor, with only 73 mutations per case in CIMP2 or CIMP0 CRCs [39]. Among the genes frequently mutated in these CIMP1 CRCs, there was enrichment for mutations in genes that encode for chromatin regulatory proteins, including *CHD7* and *CHD8*. These results were also confirmed in a dataset from

The Cancer Genome Atlas [39]. The CIMP phenotype was later modified, with different cancer-specific gene panels, to apply to other cancers including glioblastoma (gCIMP), prostate, bladder, breast, and endometrial cancers, to name but a few [40–44]. In PCa, the methylator phenotype was associated with clinicopathological features of poor prognosis including higher Gleason score and high PSA [41].

5 CpG Island Shores

Much focus has been given to DNA hypermethylation at promoter CpG islands of Tumor suppressor genes as potential biomarkers for CRC, PCa, and other cancers. Beyond CpG islands lie regions which may incur methylation to modulate transcription of target genes: CpG island shores. CpG island shores are regions located 500–2,000 base pairs away from CpG islands. These CpG island shores flank some islands, upstream and/or downstream, and have a lower CpG content than islands. CpG island shores are a transition zone between high-CpG-density islands and low-CpG-density “open sea” of the genome. Despite the distance from TSSs and lower CpG content, CpG island shores are more often differentially methylated between different normal tissues of the same individual, and between normal colon and colon tumor DNA from the same individual [45].

As CpG island shores are a recently defined DNA element, the role of differential methylation at these regions in CRC, PCa, and tumorigenesis overall is still being elucidated. Still, some interesting observations have emerged thus far. CpG island shore methylation differs not only between cancer and normal, but also between cell lines. In a comparison of two CRC cell lines, HT-29 and HCT 116, 12 % of CpG sites located within CpG island shores were differentially methylated between the two cell lines [46]. Another study found distinct methylation patterns in colorectal adenomas depending on site: proximal (right-sided) versus distal (left-sided). Of 168 significantly differentially methylated CpGs between adenomas from the right versus left colon, 35 % were in CpG island shores compared to 24.4 % in CpG islands [47]. The genes associated with the significant CpG island shores were enriched for homeobox (HOX) genes, and those involved in development, DNA binding, and transcription. Among these, 14 *HOX* genes, represented by 30 CpGs, were differentially methylated. 20/30 of these CpGs were in CpG island shores, and 28/30 were hypermethylated in left-sided adenomas compared to right. Hypermethylation was also found in right colon adenomas at the CpG island shores of *PRAC* and *PRAC2*, which are PCa susceptibility genes predominantly expressed in the prostate and distal colon.

Differential methylation at CpG island shores between tumor and normal has been shown in a variety of cancers. Comparing PCa and adjacent normal tissue, *SPON2*, encoding the extracellular matrix protein spondin 2, was the second most significantly upregulated gene [48]. It was found to have hypomethylation at five CpG sites in its CpG island shore, thus showing the inverse association between CpG island shore methylation and transcription. In a comparison of malignant peripheral nerve sheath tumors to benign neurofibromas and normal Schwann cells, there was highly significant enrichment for hypermethylation at shore regions in tumors [49]. Integration of shore DNA methylation data with gene expression patterns was able to discriminate between benign and malignant disease phenotypes. In some cases, the association between shore methylation and gene expression is not as clear. For example, in glioma tumors and glioma cell lines, *NCX2* showed varied expression levels across samples despite identical methylation patterns at CpG island shores both upstream and downstream of the *NCX2* TSS [50].

Methylation at the CpG island shore, rather than island, can control expression of certain genes, such as at *CAV-1* in breast cancer. CpG island shore methylation at *CAV-1* in breast tumor samples is more highly correlated with decreased gene expression than at other regions, including its CpG island [51]. In another study of CpG island shores and breast cancer, the CpG island shore region in the promoter of *ESR1* was investigated. It had a wide range of methylation values, from 1.8 to 100 % methylated, compared to mild-to-moderate methylation (5–50 %) at two other regions in the CpG island of *ESR1* [52]. Interestingly, larger tumor size was the strongest predictor of methylation at this region, compared to other clinicopathological features. Like these breast cancer samples, CpG island shore methylation can differ greatly between tumor samples, as well as between different tissues and cell lines.

The role of CpG island shores is still being elucidated, and these regions may have different mechanisms depending on the genomic context in which they occur. Our group has studied DNA methylation at the CpG island shore of *MLH1* in peripheral blood cells of CRC patients and healthy controls and found hypomethylation in this region to be significantly associated with a single nucleotide polymorphism (SNP), rs1800734, located over 1 kb downstream in the *MLH1* promoter [53]. SNP-associated hypomethylation at the *MLH1* CpG island shore was found in both CRC cases and controls. This same association was found in normal colonic mucosa of cancer patients, but any association between SNP genotype and methylation was lost in tumor DNA (unpublished data). This same SNP is also associated with hypermethylation at the *MLH1* promoter in MSI CRC [54, 55]. Enhancer elements also may be located within CpG island shores, for example in the gene *GLT-1*. Shore methylation at this enhancer differs

between cell types of the brain depending on required expression levels [56]. Another possible role for CpG island shores is their ability to control transcription of alternative transcripts if a CpG island shore is hypomethylated while its CpG island is hypermethylated [45]. CpG island shores may act in concert with a host of other DNA regulatory regions and proteins, and be further modulated by DNA sequence, to fine-tune gene expression in the appropriate time- and space-dependent context. Further study of these dynamic regions will help elucidate their role in tumorigenesis and methylation changes at these CpG island shores can potentially be used as biomarkers.

6 5-Hydroxymethylcytosine

5-Hydroxymethylcytosine (5hmC), regarded as the sixth base of the genome, was discovered in 2009 by two separate groups [57, 58]. It is produced through the oxidation of 5-methylcytosine by Ten-Eleven Translocation family proteins (TET1, TET2, TET3) [59]. During DNA replication, the maintenance DNA methyltransferase DNMT1 will duplicate the methylation pattern from mother to daughter strand. DNMT1 recognizes hemi-methylated DNA but has greatly reduced activity for hemi-hydroxymethylated DNA, and thus any 5hmC will be unmethylated after replication in this passive demethylation process [60]. It was later found that 5hmC could be further oxidized to 5-formylcytosine (5fC) and then 5-carboxylcytosine (5caC) in an active process to demethylate cytosine without cell division and DNA replication [61]. This discovery implies that DNA cytosine methylation is not a final step in gene silencing, but can be further regulated and fine-tuned, and may be an important epigenetic biomarker. 5hmC is generally absent from non-gene regions and is instead found at gene promoters and intragenic regions, which are enriched for genes linked to angiogenesis and hypoxia [62, 63]. In a normal healthy cell, most CpG islands remain unmethylated; this state may be maintained by active demethylation by TET proteins [64]. 5hmC is also enriched at regions marked with enhancer-associated histone modifications H3K4me1 and H3K27ac [65].

Unlike the relatively constant 5mC levels which are at 4.5 % of all cytosines, it was found that 5hmC levels differ greatly across tissues [66]. 5hmC levels are highest in the central nervous system (0.65 % of total cytosine in cerebral cortex, for example) and lower in other tissues (0.05 % in liver) [67]. These levels are even further reduced across many solid tumors, including CRC and PCa. Using the cellular architecture of the colonic crypt, researchers investigated the relationship between 5hmC content and level of cellular differentiation. Cells at the bottom of colonic crypts represent regenerative tissue stem/progenitor cells while at the top of the

crypt are terminally differentiated cells. Differentiated cells had much higher 5hmC staining than basal cells of the crypts [68]. Using dot blots with an anti-5hmC antibody, 72.7 % (16/22) of CRC samples had significantly reduced 5hmC levels compared to normal colon, as well as normal liver, brain, kidney, lung, and skeletal muscle. 50 % of tumors from this study had decreased TET1 expression, 73 % of which also had reduced 5hmC. Additionally, this 5hmC loss was only associated with a modest decrease in 5-methylcytosine. This suggests that TET1 loss is only one mechanism of 5hmC reduction in CRC and decreased 5hmC is not solely due to a loss of methylated DNA by global hypomethylation. 5hmC levels were not associated with clinicopathological features such as grade and stage. Even low-grade tumors had reduced 5hmC levels, indicating that its loss may be an early tumorigenic event. Increased levels of 5hmC in more differentiated cell types were confirmed in normal prostate. In the NIH/3T3 mouse fibroblast cell line, stable expression of *BRAF* harboring the V600E mutation allows cells to undergo cellular transformation [69]. These transformed cells had significant downregulation of 5hmC levels, along with reduced Tet1, Tet2, and Tet3, though Tet1 knockdown itself was not sufficient for cellular transformation. Very few studies have looked at specific gene regions incurring or losing 5hmC in their promoter regions in CRC or PCa, and this is a current area of active research. Overall, decreased 5hmC levels are found in CRC and PCa, and further study may reveal specific areas of 5hmC change that may be utilized as biomarkers of cancer.

7 Non-CpG Methylation

Non-CpG methylation, which includes methylation of C at CpA, CpT, and CpC dinucleotides, while common in plants, is a rare occurrence in animals. In humans, non-CpG methylation is present in pluripotent cell types, varying in level between different pluripotent cell lines, but decreases when cells differentiate [70]. It is nearly absent in various somatic cell types, though has higher levels in human brain tissue compared to other tissues [71]. In normal rectal mucosa, CpA is methylated in 0.07 % of all CpA dinucleotides, 0.02 % at CpT, and 0.02 % at CpC [70]. Non-CpG methylation has also been detected in PCa tumors and the LNCaP cell line [72]. It was found that the DNA methyltransferases Dnmt3a, Dnmt3b, and Dnmt3L are required for non-CpG methylation, specifically CpA, in mouse embryonic stem cells (ESCs) [73]. possible, another study found that Dnmt3b was not required for non-CpG methylation in mouse germinal vesicle oocytes [74]. It is possible that different DNMTs are required in different cell types for non-CpG methylation.

The utility of non-CpG methylation as a marker of CRC or PCa is currently less characterized than CpG methylation, as it is almost undetectable in normal rectal mucosa as well as CRC tumor DNA [26, 70]. Using a microarray to target the 5' UTR of 271 genes implicated in cancer, only a few genes were found to have non-CpG methylation in the MCF7 breast cancer cell line and in bone marrow samples from children with acute lymphatic leukemia (ALL). In MCF7 cells, *MSH2*, *EREG*, and *HSPA2* were found to harbor non-CpG methylation, and in the ALL samples, non-CpG methylation was detected in *HIST1H2AG*, *PGF*, *CPEB4*, and *TJP2*. Using quantitative PCR for validation, non-CpG methylation was confirmed only for *TJP2* [75]. Interestingly, both non-CpG methylation and 5hmC have their highest levels in ESCs, pluripotent cells, and the brain, with very low levels in other somatic tissues, including colon and prostate cells, indicating a role for these epigenetic marks in development. No studies have yet been published comparing non-CpG methylation in normal versus tumor DNA of either CRC or PCa in patient samples. Thus, even though non-CpG methylation may play a role in early development, its regulatory role in carcinogenesis of CRC and PCa is currently unknown.

8 Proteins Involved in Epigenetic Maintenance and Regulation

The proteins which establish, interpret, change, and remove epigenetic marks all work in concert to maintain epigenetic regulation within cells. These are broadly classified as writers, readers, and erasers. Epigenetic writers establish the epigenetic marks, which are recruited to appropriate loci by sequence context, chromatin marks already present, or other proteins. Readers recognize these marks and aid in carrying out the appropriate response required for cellular function. Erasers remove the marks when required, while remodelers can alter nucleosome positioning, and insulators form the boundaries between domains [76].

Writers of histone modifications include histone methyltransferases (HMTs) and histone acetyltransferases (HATs) which are counteracted by histone demethylases (KDMs) and histone deacetylases (HDACs), of which there are many to catalyze reactions at specific histone residues. CCCTC-binding factor (CTCF) and CTCFL/BORIS are insulator proteins which bind to enhancer blocking elements to prevent enhancers from interacting with promoters. CTCF can also provide a barrier between active and repressive regions of the chromatin [77]. Any alterations in the expression and/or function of these epigenetic regulators may drive tumorigenesis.

Writers of DNA methylation include DNA methyltransferase 1 (DNMT1), DNMT3A, and DNMT3B. Readers of DNA methylation

include methyl-CpG-binding domain (MBD)-containing proteins MBD1, MBD2, MBD4, and MeCP2, as well as zinc fingers Kaiso, ZBTB4, and ZBTB38. MBDs and Kaiso may also aid in transcriptional repression at gene promoters [76]. DNA methylation erasers include TET1, TET2, and TET3 [59].

9 Polycomb Repressive Complex 1 and 2

Control of gene expression is in part regulated by the degree of accessibility with which the DNA is packaged and compacted. DNA is packaged into the nucleus by forming nucleosomes which consist of an octamer of histone proteins. The DNA wraps around each octamer consisting of two copies each of histone 2A (H2A), histone 2B (H2B), histone 3 (H3), and histone 4 (H4) [78]. The histones may be post-translationally modified, usually on their protruding amino-terminal tails, by acetylation, methylation, phosphorylation, ubiquitylation, glycosylation, sumoylation, ADP-ribosylation, and carbonylation [79, 80]. Just as the presence or absence of DNA methylation can affect gene transcription, these histone modifications can also alter the repressive or active state of DNA.

Polycomb group (PcG) proteins can facilitate this DNA packaging by catalyzing histone modifications and recruiting other complexes. PRCs are recruited to target DNA regions in part by genome sequence [81]. Polycomb repressive complex 2 (PRC2) consists of key players EZH2, SUZ12, and EED, and catalyzes the repressive histone H3 lysine 27 trimethylation (H3K27me3) mark. PRC2 can also recruit other polycomb complexes, HDACs and DNMTs, which act in concert to further repress transcription and compact the chromatin at specific loci. H3K27me3 is a docking site for PRC1. PRC1 monoubiquitylates H2AK119 by the core unit RING1B, which blocks RNA polymerase II elongation. The repressive effects of PRC2 and PRC1 are counteracted by the Trithorax group complex which lays down H3K4 methylation by the writer MLL, and also contains the eraser KDM6A/UTX to remove H3K27me3 where appropriate [76]. Both activating H3K4me3 and repressive H3K27me3 in ESCs mark transcription factors that regulate differentiation and development; genes with both active and repressive marks are poised for transcription, known as a bivalent state [82].

10 DNA Methyltransferases

DNMTs catalyze the addition of a methyl group to cytosine. DNMT3A and DNMT3B are essential *de novo* methylators, which act during embryonic development. DNMT3L, while unable to methylate DNA itself, aids DNMT3A and DNMT3B in germ cells

by increasing their catalytic activity [83, 84]. DNMTs can be recruited to the DNA by proximal sequence elements as well as certain histone modifications, including H3K27me3 [85, 86]. DNMT1 is a maintenance methyltransferase which binds to hemimethylated DNA and copies the DNA methylation patterns from parent to daughter strand post-synthesis [87]. Alteration of these proteins can affect epigenetic regulation and have shown evidence of dysregulation in cancer. DNMT1 mutations were found in 7 % of CRCs while other studies in larger cohorts have not found mutations in DNMT3A in CRC or PCa [88, 89]. Elevated Dnmt3b1 expression in the *Apc*^{Min/+} CRC mouse model leads to increased colorectal carcinogenesis and methylation of tumor suppressor genes [90]. HCT 116 CRC cells have less tumor-initiating ability when injected into immune-deficient mice when *DNMT1* is knocked down, indicating its importance in tumorigenesis [91]. Indeed, levels of *DNMT1* mRNA increase from the normal rectal epithelium of ulcerative colitis (UC) patients without neoplasia to normal epithelium of UC patients with neoplasia, and are highest in colorectal neoplasia, indicating that increasing *DNMT1* may predict the risk of CRC in UC patients [92]. PCa is also marked by higher levels of DNMTs. DNMT1, DNMT3a, and DNMT3b protein levels were higher in cell cultures derived from PCa compared to benign prostatic hyperplasia samples, and increased level was also associated with higher Gleason score [93]. DNMT levels were also higher in a more aggressive cell line PC3-LN4 than in the PC3 cell line from which it was derived. Thus, altered levels of both DNA methylation and the proteins which catalyze the methylation reaction are both markers of CRC and PCa.

DNMTs work to initiate and maintain DNA methylation and work together with histone modifications to exert transcriptional control and can interact directly with SUV39H1 and EZH2, HMTs which lay down H3K9me3 and H3K27me3 marks, respectively [86, 94]. Polycomb group targets, marked by H3K27me3 in ESCs, are much more likely to be hypermethylated in cancer versus non-PcG targets [86]. It has been reported that 49 % of genes methylated in CRC are PcG targets in ESCs [95]. Another study showed that 29 % of regions prone to methylation in CRC versus normal DNA are located in gene promoters, and 95 % of these are located in CpG islands enriched for PcGs in ESCs [26]. Even outside of gene promoters, methylation-prone regions in CRC are located in CpG islands and overlap with polycomb marks. These methylation-prone regions do not overlap with repetitive elements such as Alu repeats, SINEs or LINEs. Methylated regions in PCa are also PcG targets, and many of these methylated regions in cancer are also methylated in tumors with higher Gleason score and positive ERG expression [96].

However, some studies have found the two marks, DNA methylation and H3K27me3, to be mutually exclusive in cancer.

Many genes that are bound by PcG in the normal prostate cell line PrEC lose PcG binding but incur hypermethylation at those same regions in PC3 cells [97]. Chromatin immunoprecipitation (ChIP) for H3K27me3 followed by bisulfite sequencing in the CRC cell line HCT 116 showed regions of methylated DNA in genic and intergenic regions outside of CpG islands, indicating that both marks are present in these regions. However, in promoter CpG islands, H3K27me3 and DNA methylation are mutually exclusive [98]. Further study of this phenomenon is required to fully understand the interplay between DNA methylation and histone modifications in tumorigenesis.

11 Histone Modifications

Similar to aberrant DNA methylation, dysregulation of histone modifications occurs in CRC and PCa. The most commonly studied of these histone marks are methylation and acetylation. The modifications themselves may be biomarkers of cancer, but altered levels of the histone writers and erasers are also associated with cancer. The first histone modifications found to be dysregulated in cancer were the loss of global H4K16Ac and H4K20me3 [99]. H4K16Ac regulates higher order chromatin structure, and its loss leads to a more relaxed chromatin state, which may lead to genomic instability [100]. The HDAC sirtuin 1 (SIRT1), which removes H4K16ac, has shown increased expression in PCa, CRC, and CRC cell lines [101, 102]. H4K20me3 is also associated with repressed chromatin state and heterochromatic domains [103]. Levels of this mark are lower in circulating nucleosomes from sera of CRC patients compared to healthy controls [104, 105]. H4K20me3 is also lower in castration-resistant PCa compared to normal prostate and non-castration-resistant tumors [106].

As previously stated, H3K27me3 is marked by PRC2 and is associated with repressive chromatin. EZH2 is overexpressed in PCa and associated with metastasis, invasion, and progression [107, 108]. Interestingly, the counteracting HDM which removes the H3K27me3 mark, JMJD3, has also shown upregulation in metastatic PCa [109, 110]. EZH2 is also more highly expressed in CRC tumors than normal epithelial cells, and is even higher in patients with metastases [111]. Knockdown of EZH2 in CRC cell lines decreases cell proliferation and invasion while pharmacological inhibition of EZH2 with DZNep increases apoptosis [111–113]. DZNep was also able to reduce cellular invasion in PCa cell lines. Treatment of PCa cell lines LNCaP and DU145 with DZNep before injection into immunocompromised mice led to smaller tumor size than untreated cells [114].

A specific gene targeted by EZH2 for H3K27me3 is vitamin D receptor (*VDR*), which has lower expression in more aggressive,

metastatic CRCs when marked by H3K27me3 [111]. *Wnt5a* also has higher H3K27me3 levels, along with lower expression, in the more metastatic CRC cell line SW620 compared to non-metastatic SW480 cells [115]. *DCC* is another gene marked by H3K27me3 in CRC [116]. *Wnt5a* and *DCC* also incur promoter CpG island DNA methylation in CRC [116, 117]. *TIMP3*, a tumor suppressor often downregulated in PCa, is associated with H3K27me3 at its promoter in DU145 PCa cells [118]. With increased EZH2 expression, there are many more genes which incur H3K27me3 and subsequent downregulation across the genome in both CRC and PCa. Overall, increased EZH2 and H3K27me3 are biomarkers for more aggressive and metastatic disease.

Both H3K4me2 and H3K4me3 are marks associated with transcriptional activation, found near transcriptional start sites in genes which are highly expressed. High H3K4me2 level is a predictor of PCa recurrence and H3K4me2 and H3K4me3 levels were significantly higher in hormone-refractory PCa compared to hormone-dependent PCa [119, 120]. H3K4 methylation is performed by SET1 and MLL family HMTs, including SMYD3, while methylation is removed by LSD1 and JARID1 family KDMs. SMYD3 is highly expressed in CRC [121]. The counteracting protein LSD1 also has elevated expression in CRC [122]. Interestingly, LSD1 knockout in the HCT 116 cell line did not change global H3K4me2/3 levels, but it significantly altered expression level of 72 genes and reduced cell proliferation [123]. LSD1 is also higher in PCa, with increased expression levels associated with recurrence [124]. Inhibition of LSD1 by the selective inhibitor Namoline decreased cell viability in PCa LNCaP cells, significantly decreased androgen-induced expression of genes, and even decreased tumor growth in mouse xenografts [125]. Though there are somewhat contradictory findings in regards to levels of H3K4me2/3 and its removal by LSD1 in CRC and PCa, increased expression of LSD1 may be a marker of cancer and its inhibition is promising as a target for future studies.

Another repressive mark is H3K9me3, found on heterochromatin. In a study of 219 CRCs, 25 % showed elevated mRNA expression of SUV39H1, the HMT which catalyzes H3K9me3 [126]. Knockdown of G9a, another HMT responsible for this mark, inhibited cell growth in the PCa cell line PC3 leading to cellular senescence [127]. Yet H3K9me3 has also been found to be reduced in PCa compared to normal prostate tissue [120]. H3K9 may also be acetylated, where it is then considered a marker of active chromatin. Decreased H3K9ac is associated with tumor progression, poor prognosis, and higher grade and stage in PCa [119, 128]. H3K9ac levels are also lower in circulating nucleosomes in sera of CRC patients compared to controls [104, 105]. Overall, increased repressive H3K9me3 and decreased activating H3K9ac are markers of CRC and PCa.

H3K12ac and H3K18ac are markers for active transcription. H4K12ac loss is predictive for PCa recurrence and H3K18ac loss is correlated with poor prognosis and increased risk of recurrence in low-grade PCa [129]. H4K12ac and H3K18ac levels are higher in moderately to well differentiated CRC tumors, but decreased in poorly differentiated tumors. Higher HDAC2 expression, which removes these marks, is associated with progression of adenoma to carcinoma in CRC [130].

H3K36me3 is an intragenic marker of active genes [131]. Recently it was shown that the H3K36me3 mark is required in vivo for the recruitment of proteins during DNA mismatch repair (MMR) [132]. Methylation of *MLH1* or mutation to *MLH1* or other MMR genes including *MSH2* and *MSH6* can contribute to MSI. It was found that H3K36me3 recruits the dimer MutS α (consisting of MSH2 and MSH6) onto chromatin for DNA repair. Cells lacking SETD2, the writer for the H3K36me3 mark, exhibited MSI and had a higher mutation frequency. This finding may explain how individuals with CRC can exhibit the MSI phenotype without *MLH1* methylation or mutation found in other known MMR genes. This finding adds another layer of complexity to the role of histone modifications in tumorigenesis and how they can interact in other cellular processes.

12 Conclusions

In summary, there are a whole host of epigenetic marks which can initiate tumorigenesis as well as modulate and fine-tune gene expression and maintain genomic stability. These modifications, from DNA methylation to histone modifications, are written, read, and erased by an even greater number of regulatory proteins. Thus far, the majority of studies in cancer epigenetics have been aimed at CpG islands in promoters of genes, yet emerging research shows an abundance of epigenetic changes occurring in all cancer types beyond these islands. What makes epigenetics such an attractive subject for cancer research is that these changes are dynamic, undergoing modulation based not only on genetic background, but also environmental factors, including diet, physical activity, environmental toxins, smoking, and alcohol use. Further study of these environmental factors affecting DNA methylation, DNA hydroxymethylation, non-CpG methylation, and histone modifications, will lead to new understanding of the carcinogenic process as well as potential methods for cancer prevention. Additionally, the flexible nature of epigenetics allows for treatment options targeting epigenetic marks and/or the proteins responsible for these changes. Just as certain genetic changes play a key role in tumor initiation and progression, such as *APC* mutation in CRC, epigenetic changes may also be “driver events” setting the course of tumor development. Distinguishing epigenetic drivers versus passengers

and elucidating the epigenetic landscapes of CRC and PCa will reveal targets for chemotherapeutic treatments and provide important biomarkers for diagnosis and prognosis in cancer patients.

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Prostate Cancer Epigenome

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Abstract

Prostate cancer is a major health burden within the ever-increasingly aging US population. The molecular mechanisms involved in prostate cancer are diverse and heterogeneous. In this context, epigenetic changes, both global and gene specific, are now an emerging alternate mechanism in disease initiation and progression. The three major risk factors in prostate cancer: age, geographic ancestry, and environment are all influenced by epigenetics and additional significant insight is required to gain an understanding of the underlying mechanisms. The androgen receptor and its downstream effector pathways, central to prostate cancer initiation and progression, are subject to a multitude of epigenetic alterations. In this review we focus on the global perspective of epigenetics and the use of recent next-generation sequencing platforms to interrogate epigenetic changes in the prostate cancer genome.

Key words Androgen receptor, Epigenome, Methylation, Prostate cancer

1 Prostate Cancer

Prostate cancer (PCa) is a complex, multifactorial disease that continues to be a significant factor in morbidity and mortality in the USA. Age, geographic ancestry, family history of (PCa), and more recently environmental factors are the only established risk factors. Many putative risk factors [1–3] have also been implicated, but their role in PCa etiology remains unclear (reviewed in ref. 4). Familial PCa is a genetically heterogeneous disease suggesting that many gene loci rather than a specific major susceptibility gene are responsible for its predisposition ([5] and reviewed in ref. 6). The highest incidence rates for PCa in the world are among African-American men [7]. Mortality from PCa among African-American men is also more than double that of men of European ancestry.

2 Known Mechanisms in Prostate Cancer

Over the past several years significant efforts have been invested in understanding the molecular basis of prostate cancer. A number of mouse models (*Pten*^{-/-} [8], *Myc*^{hi/lo} [9], *Nkx3-1*^{-/-} [10], and *Id4*^{-/-} [11] and reviewed in ref. [12]) have been developed that recapitulate several aspects of human disease initiation and progression. Most if not all of the studies have pointed towards the central role of androgen receptor (AR) in prostate cancer [13, 14]. AR, a member of the nuclear receptor superfamily, is required for normal prostate function and is involved in cyto-differentiation of the prostate epithelial cells [15]. In a majority of prostate cancer cases, AR function contributes to the survival and proliferation of cancer cells in primary disease and in most cases the presence of AR continues to be indispensable after progression to hormone-independent disease. However, the prostate epithelial specific AR knockout mouse [16, 17] also develops prostate cancer, suggesting that AR function is not absolutely required for disease development. Also unlike other cancers (most notably breast and colorectal) where a familial mutation in a single gene leads to a higher risk of developing the respective cancers, no such major risk allele has been identified for prostate cancer to date. The heterogeneity of prostate cancer [18] suggests that there could be multiple initiating events leading to inactivation of tumor suppressors and/or activation of tumor promoters/oncogenes that could at some point of disease progression cross-talk with AR [18]. These early or late events may promote the transition of androgen receptor from a tumor suppressor to an oncogene.

3 Epigenetics

The definition of epigenetics has evolved over a period of time since the term was introduced by Waddington in 1942 as “causal interactions between genes and their products, which bring the phenotype into being” [19]. In broadest terms, epigenetics is a bridge between genotype and phenotype—a phenomenon that changes the final outcome of a genetic locus without changing the underlying DNA sequence [20]. Chromatin modifications, both covalent and non-covalent, are currently considered as hallmarks of changes in an epigenetic landscape [21]. Both DNA and histones, two basic components of chromatin, undergo these modifications to influence chromatin structure and gene expression. Depending upon the type and location, these modifications alter multiple cellular processes such as gene regulation [22], DNA replication [23, 24], cellular differentiation [25], and stem cell development [26].

3.1 Epigenetics and Cancer

Multiple genetic changes must occur to promote the cancer phenotype [27]. According to the classical mechanism, genomic instability (deletions or translocations) due to inactivating mutations in tumor suppressor genes or gain of function mutations in oncogenes resulting in uncontrolled growth is considered as a tumor initiating event. More recently, loss of function (expression) through epigenetic changes due to altered pattern of DNA methylation and histone modification is gaining significant attention as an additional pathway involved in cancer [28–30]. Thus combined genetic and epigenetic changes are now considered as cancer initiating events. As discussed above, aging, environment, and ancestry are the three major risk factors in prostate cancer. All three of these risk factors are intricately linked to epigenetic alterations in genomes that can lead to global changes in gene expression patterns which are generally pro-tumor. Several epigenetic alterations, such as global hypomethylation and CpG island hypermethylation, are progressively accumulated during aging and directly contribute to cell transformation [31].

Recent advances suggest that epigenetic mechanisms mediate the effect of environmental factors on the genome by disrupting cellular processes which contribute to higher cancer risk [32]. More recently, the concept of generational threshold in prostate cancer incidence is also gaining attention. Prostate cancer risk is usually low in recently migrated populations but within three generations, the risk of prostate cancer among these migrant populations is almost similar to or higher than the native host country population [33]. This generational threshold appears in part due to epigenetic alterations (parental/offspring adopting a more westernized lifestyle) and not due to genetic mutations in the F0/F1 generations. These epigenetic changes could predispose the inherited genome of subsequent generations to mutagenic/genotoxic alterations leading to the development of sporadic prostate cancer [33].

Differences in epigenetic changes are also observed that associate with the high risk of prostate cancer seen in African American men [34]. Specifically, increased methylation is observed for *NKX2-5* and *TIMP3*, genes known to be involved in prostate carcinogenesis, in the normal prostate of African American men when compared to that of Caucasians [35].

3.2 Histone Modifications Versus DNA Methylation and a Link Between These Two Events in Relation to Prostate Cancer

Histones are no longer considered as simple “DNA packaging” molecules as they are involved in the dynamic regulation of a large set of genes through reversible posttranslational modifications that occur on their N-terminal tails, which are rich in positively charged amino acids [36]. The histone posttranslational modifications include acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation, deimination, and proline isomerization [37, 38]. Among these modifications, acetylation and

methylation are well studied in prostate cancer. Evidence indicates that the accumulation of acetylation and methylation at specific histone lysine or arginine residues plays a prominent role in gene regulation [38, 39]. In general, histone acetylation is associated with gene activation whereas histone de-acetylation is correlated with gene silencing [40, 41]. Histone methylation appears to play a more complex role in gene regulation. Methylation of H3K4 and to a lesser extent H3K48 and H3K79 are associated with gene activation whereas methylation at H3K9 and H3K27 are associated with gene silencing [42]. Some promoters simultaneously carry both gene-activating H3K4me3 and -inactivating H3K27me3 marks and are known as bivalent promoters [43]. Such bivalent marks could be required for the maintenance of embryonic stem cell gene expression patterns [43]. Whether such modifications are also present in cancer stem cells is proposed but remains to be investigated [44]. In prostate cancer increasing evidence suggests that large numbers of histone modifications are associated with changes in histone-modifying enzymes such as histone methyltransferases (HMTs) and histone acetyltransferases (HATs) [29]. Histone acetylation and deacetylation catalyzed by a group of enzymes collectively termed as histone acetyltransferases (HAT) and histone de-acetylases (HDAC) respectively, have emerged as the central mechanism in regulating various events leading to prostate cancer [45]. Multiple proteins with HAT and HDAC (HDAC1-11) activities are known that also target nonhistone proteins [46]. As would be expected, certain HDACs are increased in prostate cancer (HDAC1, HDAC2, and HDAC3) (reviewed in ref. 47). Paradoxically, the expression of HAT p300 is also increased in prostate cancer. In prostate cancer, HATs such as p300/CBP may target nonhistone proteins such as AR. Acetylation of AR is a gain-of-function modification that promotes prostate cancer cell growth [48].

DNA methylation includes the addition of a methyl group to cytosine residues of CpG dinucleotides [49]. DNA methylation in mammals is established by two DNA methyltransferase enzymes. Current evidence suggests that members of the DNMT3 family (DNMT3A and DNMT3B) are involved in de novo methylation while DNMT1 is involved in the maintenance of methylation. De novo DNA methylation of certain gene promoter associated CpG dinucleotides occurs rarely in normal prostate epithelial cells but its frequency increases in prostate cancer. This phenomenon is in part thought to be supported by an increase in the expression of DNMT1 in prostate cancer cells when compared to their normal counterparts [50]. Thus DNA methylation in the promoter region of tumor-suppressor genes is likely an important epigenetic mechanism involved prostate cancer initiation and progression [51].

Histone modifications and DNA methylation can occur independently of each other; however initial histone modifications can

lead to altered DNA methylation. Significant cross talk exists between the histone modifying and DNA methylating machinery [52, 53]. An example includes the interactions between EZH2, the SET domain methyltransferase, part of the polycomb repressive complex 2 (PRC2), and DNA methyltransferases [54, 55]. For instance, it has been observed that *MYT1*, a gene which is regulated by EZH2, is also regulated by DNMTs [55]. Almost 47 % of the genes regulated by DNMT3B are also bound by the PRC1/PRC2 complexes in colon cancer [56]. However, there are other studies that show that EZH2 occupancy at a given locus is not associated with DNA methylation at that same locus. For example, a recent study showed that *CDH1* and *KLF2*, both known to be regulated by EZH2 in prostate cancer, have no alterations of DNA methylation status on their promoters upon EZH2 overexpression. This observation suggests that DNA methylation has no role in *CDH1* and *KLF2* repression, mediated through EZH2 [57].

Histone deacetylases and the DNA-binding proteins associated with them could also be attracted to regions of methylated DNA during chromatin modification. Methylated DNA-binding proteins, such as MBD2, also interact with the nucleosomal remodeling complex (NuRD) and direct the complex to methylate DNA [58]. Chromatin-immunoprecipitation (ChIP) coupled with meta-analysis also showed a link between polycomb group protein (PcG)-mediated tri-methylation on H3K27 and de novo DNA methylation in cancer [59]. Genome-wide analysis combined with methylation-predicted models revealed that CpG islands which are methylation prone have a strong association with embryonic targets of PRC2 and a subset of PRC2 targets were also more likely to be hypermethylated in cancer (reviewed in ref. 60).

Changes in HMTs and HATs can result in changes in histone modification at both the global and gene-specific levels. Global histone modifications (acetylation and methylation) are also predictive of clinical outcome in prostate cancer. Histone acetylation and di-methylation of five residues in histones H3 and H4 (acetylation: H3K9, H3K18, and H4K12 and di-methylated H4R3 and H3K4) identified two prostate cancer disease subtypes with distinct risks of tumor recurrence in patients with low-grade prostate cancer. These histone modification patterns were predictors of outcome independently of tumor stage and preoperative prostate-specific antigen levels, and were also associated with invasion [61]. In another study, H3K4me1, H3K9me2, H3K9me3, H3Ac, and H4Ac were significantly reduced in prostate cancer as compared to nonmalignant prostate tissue [62].

3.3 Gene-Specific Epigenetic Changes in Prostate Cancer

In addition to recently discovered global epigenetic changes in prostate cancer, many gene-specific epigenetic modifications are also observed in prostate cancer which are summarized in Table 1 [57, 63–70].

Table 1
List of genes that are epigenetically silenced in prostate cancer

Target	Function	Epigenetic modification
<i>DAB 2 interacting protein (DAB2IP)</i>	Inhibition of NF-κB/Ras pathway	Histone methylation
Adrenoceptor beta 2 (<i>ADRB2</i>)	β-adrenergic signaling	Histone methylation
E-cadherin (<i>CDH1</i>)	Cell-cell adhesion, tumor suppressor	Histone methylation
Prostate secretory protein of 94 amino acids (<i>PSP94</i>)	Inhibits MMP secretion	Histone methylation
Slit homolog 2 (<i>SLIT2</i>)	Chemo-repellent protein	Histone methylation
TIMP metalloproteinase inhibitor 2/3 (<i>TIMP2/3</i>)	ECM degradation	Histone methylation
Raf-1 kinase inhibitor protein (<i>RKIP</i>)	Inhibition of Raf and NF-κB pathways/ metastasis suppressor	Histone methylation
Kruppel-like factor 2 (<i>KLF 2</i>)	Tumor suppressor	Histone modification
Prostate cancer-associated transcript 1 (<i>PCAT-1</i>)	Transcriptional repressor lincRNA	Histone methylation
Glutathione S-transferase Pi (<i>GSTPI</i>)	DNA repair, intra cellular detoxification	DNA methylation
Adenomatous polyposis coli (<i>APC</i>), retinoic acid receptor beta (<i>RARβ</i>), <i>RAAF1</i> , inhibitor of DNA binding (<i>Id4</i>),	Tumor-suppressor genes	DNA methylation
Androgen receptor (<i>AR</i>), estrogen receptor 1 (<i>ESR1</i>), estrogen receptor 2 (<i>ESR2</i>)	Hormone receptor genes	DNA methylation
<i>CDH13</i> , <i>CD44</i>	Cell adhesion genes	DNA methylation
Cyclin D2 (<i>CCND2</i>), <i>CDKN1B</i> , Stratifin (<i>SFN</i>)	Cell-cycle control genes	DNA methylation
Growth arrest and DNA-damage-inducible protein GADD45 alpha (<i>GADD45α</i>), PYD And CARD Domain (<i>PTCARD</i>), <i>RPRM</i> , <i>GLIPR1</i>	Apoptotic genes	DNA methylation
<i>O</i> -6-methylguanine DNA-methyltransferase (<i>MGMT</i>)	Remove alkyl adducts from <i>O</i> ⁶ -guanine	DNA methylation

3.3.1 Epigenetic Inactivation of Id4 in Prostate Cancer

The inhibitor of differentiation-4 helix-loop-helix protein (ID4) is a member of the inhibitor of differentiation gene family (along with Id1, Id2, and Id3) and acts as a dominant negative transcriptional regulator of basic helix-loop-helix (bHLH) family of transcription factors. Our recent studies have shown that ID4 is highly expressed in the normal prostate but is epigenetically silenced in prostate cancer due to promoter hyper-methylation [70, 71]. Prostates from Id4-/- mouse models display frequent PIN like lesions that supports its role as a tumor suppressor [11].

4 Epigenetic Regulation of AR Dependent Pathways

The AR itself as well as its downstream pathways are regulated by diverse epigenetic mechanisms in prostate cancer. As might be expected, these epigenetic modifications in the AR pathway are generally defined as pro-tumorigenic. Studies have shown that histone lysine methylation status contributes significantly to AR signaling which correlates with an increased expression of several histone demethylase proteins (LSD1, JMJD2C, GASC1, and KDMC4) in prostate cancer [72–74]. Most of the histone demethylase proteins physically interact with AR to facilitate its recruitment to androgen response element (ARE) sites and subsequent activation of the downstream target genes [73]. AREs are particularly enriched for H3K4me1 or H3K4me2, generally associated with transcriptional enhancers [75].

5 Role of Histone Methyl-Transferase in Prostate Cancer

The expression of EZH2, a key component of the PRC2 complex, which specifically tri-methylates H3K27, is increased in prostate cancer [64, 76]. The consequence of high EZH2 expression together with the observations that show increased mutations in KDM6A [72, 73] which can de-methylate H3K27me3 suggests the inactivation of genes primarily associated with prostate differentiation. A surprising new role of EZH2 has also emerged in prostate cancer. Recent studies have shown that EZH2 could also act as a co-activator of AR and requires the intact methyl-transferase activity EZH2 [77].

6 Data Sources for Detection of Epigenetically Altered Genomic Loci in Prostate Cancer

Until recently, epigenetic analyses for DNA methylation and histone tail modification were carried out on *individual* genetic loci by assays that utilized traditional DNA sequencing or polymerase

chain reaction (PCR) of genomic DNA that had been modified by bisulfite treatment or enriched by chromatin immunoprecipitation (ChIP) with antibodies against specific histone tail post translational modifications. However with the advent of next-generation sequencing (NGS) and high-density arrays, and global genomic profiles of both DNA methylation and histone tail modifications, generated using techniques such as methylation-sensitive restriction enzyme sequencing (MRE-SEQ), methylated DNA immunoprecipitation sequencing (MEDIP-SEQ), reduced representation bisulfite sequencing (RRBS) [78], array hybridization, and ChIP-SEQ, are now widely available for public interrogation [79–82].

Multiple cultured cell types and tissues have been investigated and the quantitative data have been published and deposited in publicly accessible databases found on the World Wide Web where all of the data can be accessed by individuals for visualization and/or download for their own use. Experiments using cell culture models and human tissues, representing both prostate cancer epithelia as well as healthy prostate epithelia, have been profiled. Here, we present examples of two data sources that are useful for prostate cancer researchers interested in epigenetic alterations. These examples are not meant to be exhaustive and will likely have been superseded or augmented with additional data by the time the reader is performing their specific research. One example is the UCSC Genome Browser (<http://genome.ucsc.edu>) site that contains reference sequence and working draft assemblies for a large collection of genomes. In addition, this site contains data and visualization tracks for independent research results including those interrogating the underlying chromatin structure of complete genomes. One must be familiar with the basic workings of the UCSC genome browser and track visualization methods in order to take advantage of these data sets. We refer readers to the “Guide to the UCSC Genome Browser” (<http://www.nature.com/scitable/ebooks/guide-to-the-ucsc-genome-browser-16569863>) for an easily accessible introduction to its usage.

One specific model system for examining comparative DNA methylation profiles in prostate cancer can be found on the Human Feb. 2009 (GRCh37/hg19) Assembly on the UCSC Genome Browser. This assembly contains a super track under the “Regulation” section of tracks named “ENC DNA Methyl” that contains DNA methylation data that was produced using several experimental methods by the ENCODE production groups (<http://encodeproject.org>) [79]. A track named “HAIB Methyl RRBS” from within this supertrack contains Reduced Representation Bisulfite Seq data. Replicate subtracks for a representative sample of the prostate epithelial cell line, PrEC (Lonza-CC-2555), and for LNCaP (ATCC CRL-1740) are contained within this track. The user can choose to display on the UCSC Browser only the subtracks for these cell lines in addition to the RefSeq Gene Track and

CpG island track for additional genomic annotations to aid in result interpretation. Representative images for our favorite prostate cancer-related genes, *ID4* and *ZIC2*, are represented in Fig. 1. The UCSC Genome Browser allows one to view the entire gene of interest to identify regions of differentially methylated CpG dinucleotides and their overlapping coordinates relative to the various parts of the gene of interest and any neighboring CpG islands (Fig. 1a, c). Alternatively the user can zoom in to the level of nucleotide visualization and see the actual genomic DNA sequence surrounding the individual CpG that is being interrogated (Fig. 1b, d). In this figure, individual CpGs are represented by tick marks on the tracks labeled on the left, where green represents unmethylated CpGs or 0 % methylation, yellow represents 50 % methylation and red indicates 100 % methylation. One can easily distinguish between areas that are unmethylated in PrEC and methylated in LNCaP, indicating hypermethylation has occurred in cancer in the 5' region of *ID4* and in the 3' region of *ZIC2*.

Another example using a modified version of the UCSC Genome Browser, known as the UCSC Cancer Genomics Browser, can be found at <https://genome-cancer.ucsc.edu>. This site contains data for copy number variation, mutation, gene expression, DNA methylation and other genomic interrogations of multiple organ specific cancer tissues from “The Cancer Genome Atlas” (TCGA) (<http://cancergenome.nih.gov>) as well as from other sources. Again we refer the reader to the User Guide under the Help section of this site (<https://genome-cancer.ucsc.edu/proj/site/help/>) for detailed instructions on viewing specific data from within the Cancer Genomics Browser. The prostate cancer data from TCGA includes data from currently over 300 prostate adenocarcinoma (PRAD) tissue samples that have been interrogated for multiple genomic assays including global DNA methylation by the HumanMethylation450 BeadChip (www.illumina.com), gene expression by RNA-Seq, and other genome-wide cancer assays. The TCGA prostate samples also include results from adjacent normal epithelia as well as epithelia from non-diseased prostate tissue. The user can load both DNA methylation and gene expression data vertically contiguous on the browser to allow for additional functional genomic interpretation and hypothesis generation about the correlated consequences of specific DNA methylation patterns and associated gene expression levels. This allows the user to compare the groups of samples (normal versus cancer) against each other from within the single assay as well as between different assays. For example, Fig. 2a shows the results of DNA methylation in the top panel and exon-level gene expression by RNA-SEQ in the lower panel for *ID4* in PRAD. The results on the Cancer Genomics Browser are depicted in heat-map format with the genomic loci representing the column(s) of the heat map and the samples represented in rows. Samples can be labeled further with clinical



Fig. 1 UCSC Genome Browser-generated images displaying differentially methylated CpG dinucleotides relative to the various parts of the gene of interest and any neighboring CpG islands (**a** and **c**). Alternatively the user can zoom in to the level of nucleotide visualization and see the actual genomic DNA sequence surrounding the individual CpG that is being interrogated (**b** and **d**). In this figure, individual CpGs are represented by *tick marks* on the tracks labeled on the *left*, where *green* represents unmethylated CpGs or 0 % methylation, *yellow* represents 50 % methylation, and *red* indicates 100 % methylation

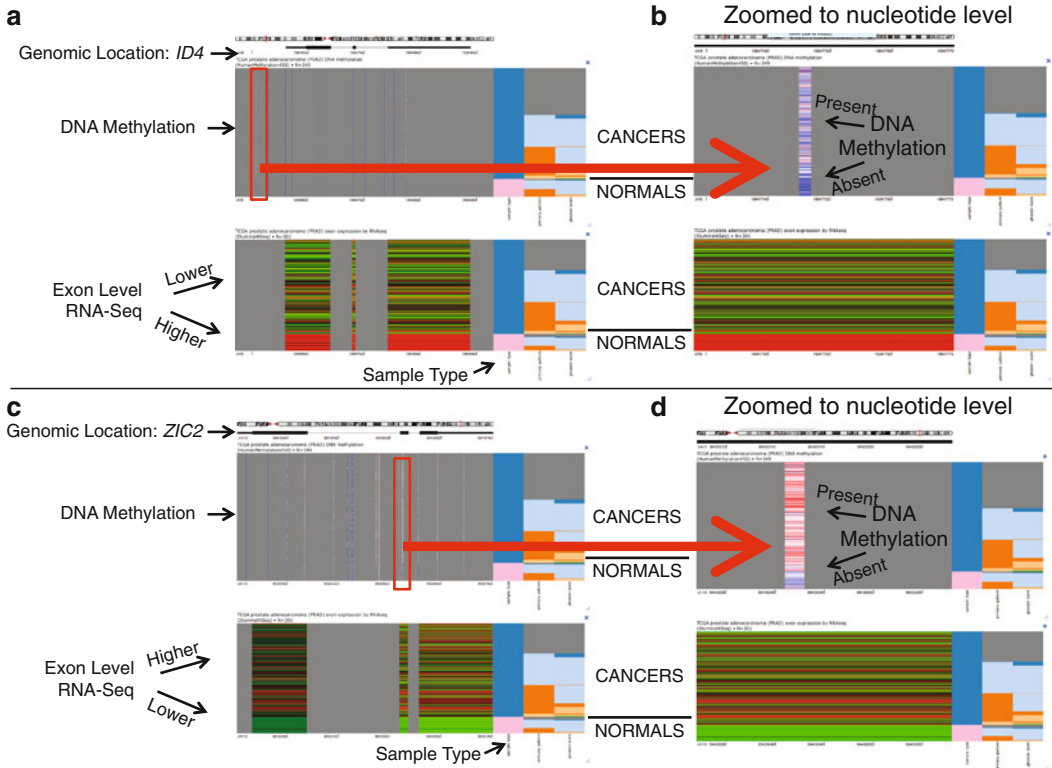


Fig. 2 The Cancer Genomics Browser displays epigenetic data from cancer and normal tissues. Panels **a** and **c** show the results for prostate adenocarcinoma (PRAD) DNA methylation in the *top* heatmap (red and blue) and sample normalized exon-level gene expression by RNA-SEQ in the *lower* heatmap (red and green) for *ID4* and *ZIC2*, respectively. Panels **b** and **d** are zoomed to nucleotide level to clearly demarcate the difference in methylation at specific CpG dinucleotides between normal and cancer samples

annotations such as normal versus cancer, Gleason score, and primary pattern. In each experimental panel (e.g., DNA methylation or RNA-SEQ) normal and cancer samples are sorted from bottom to top, respectively, and are represented by pink and blue coloring in the sample type annotation column found on the right of each individual heat map. Additional sample annotation columns, selected by the user, can also be viewed next to sample type. Samples are sorted by the annotation column that is displayed immediately to the right of the heatmap. The user has the option to sort by any of the annotation columns that are displayed by moving it to the first position adjacent to the heatmap.

It is readily observable from the normalized exon level gene expression heat map that the normal tissue expresses higher levels of *ID4* (red rows) than the cancers (green, black, and red rows). One can zoom in (Fig. 2b) to the individual levels to reveal the locations of methylated CpG dinucleotide locations in cancer (red) and the corresponding unmethylated locations in normal (blue).

These data suggest that methylation of these CpG locations near to and internal to *ID4* are correlated with downregulation of the tumor-suppressor *ID4* in prostate cancer, consistent with our previously reported data [70, 71]. These results are also consistent with the methylation patterns found in PrEC and LNCaP data described above. When the same data are depicted for *ZIC2*, similar methylation patterns are seen in normal and tumor tissues as were found in PrEC and LNCaP cell lines, i.e., hypermethylation in cancer (Fig. 2c). However, the gene expression data reveal a different pattern from that of *ID4*. Even though regions of *ZIC2* are hypermethylated in cancer, there is an extreme increase in *ZIC2* gene expression in cancer tissue samples (Fig. 2d), consistent with previously published reports [83]. Where *ID4* is methylated and downregulated in PRAD, *ZIC2* is methylated but is significantly *up-regulated* in PRAD. In the case of *ZIC2*, these data allow one the opportunity to formulate an alternative hypothesis as to why DNA methylation does not result in the downregulation of gene expression but instead results in the up-regulation of gene expression. As can be seen from the positional information, the increased DNA methylation is not in the region of the promoter or transcription start site (TSS) of *ZIC2*, as is seen in *ID4*. It is instead found downstream in the 3' region of *ZIC2*, conceivably indicating that the binding of a repressor of *ZIC2*, such as GATA3, is excluded from binding by methylated DNA in that region of *ZIC2* and can no longer down-regulate *ZIC2* transcription in cancer. These examples of comparative DNA methylation and expression analyses in prostate cancer illustrate the confirmatory and discovery powers of functional epigenomics in the genes of *ID4* and *ZIC2*, respectively. Finally, if so desired the user-processed dataset can be downloaded in tabular quantitative format from the Cancer Browser for generation of custom figures from the user's own statistical and graphical software.

7 Concluding Remarks

Epigenetic alterations have now emerged as major contributors to prostate cancer disease initiation and progression. The detailed investigation of the molecular mechanisms involved in regulating these epigenetic changes will remain the major focus of current and future research. With the advent of newer genome-wide technologies, it is now possible to investigate the global epigenome and understand the role that risk factors (e.g., age, geographic ancestry, and environment) and molecular mechanisms (e.g., AR and other tumor suppressors/oncogene regulation, cellular heterogeneity, the transition to castration-resistant prostate cancer, and individual responses to therapies) play in the clinical course of

PCa. Since epigenetic changes involve alteration in the expression of key enzymes and pathways without altering the DNA sequence (e.g., somatic mutations), strategies that target these proteins in order to reverse these epigenetic changes are attractive as future preventive and therapeutic interventions.

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Chapter 8

CpG Island Hypermethylation as a Biomarker for the Early Detection of Lung Cancer

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Abstract

Lung cancer is the most frequent cause of cancer-related deaths and causes over one million deaths worldwide each year. Despite significant strides in the diagnosis and treatment of lung cancer, the prognosis is extremely poor, with the overall 5-year survival rates still remaining around 15 %. This is largely due to occult metastatic dissemination, which appears in approximately two-thirds of patients at the time of detection. Thus, the development of efficient diagnostic methods to enable the early detection of cancer for these patients is clearly imperative.

One promising approach is the identification of lung cancer-specific biomarkers at an early stage. The de novo methylation of CpG islands within the promoters of tumor suppressor genes is one of the most frequently acquired epigenetic changes during the pathogenesis of lung cancer and usually associated with transcriptional downregulation of a gene. The analysis of DNA methylation patterns in sputum, bronchial fluid, plasma, or serum could become a powerful tool for the accurate and early diagnosis of lung cancer with unparalleled specificity and sensitivity.

Key words Lung cancer, Hypermethylation, Early detection, Biologic fluid, CpG island

Abbreviations

NSCLC	Non-small cell lung cancer
SCLC	Small cell lung cancer
CXR	Chest X-ray
TSGs	Tumor suppressor genes
MSP	Methylation-specific polymerase chain reaction
ER	Estrogen receptor
DAP	Death-associated protein
BAL	Bronchoalveolar lavage
MeDIP	Methylated DNA immunoprecipitation
EWAS	Epigenome-wide association study
NGS	Next-generation sequencing

DMRs	Differentially methylated regions
ROC	Receiver operating characteristic
PCA	Principal component analysis (PCA)
LDA	Linear discriminant analysis

1 Lung Cancer

1.1 Introduction

Lung cancer is the most common cancer worldwide, with 1.3 million new cases being diagnosed each year. Lung cancer is classified into two major histological types, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC). Adenocarcinoma, squamous cell carcinoma, and large cell carcinoma are the major histological subtypes of NSCLC. In the past, squamous cell carcinoma was the predominant form of NSCLC, but during the last few decades, it has been replaced by adenocarcinoma in many countries. At present, nearly 40–50 % of lung cancers are adenocarcinoma, which mostly originates at the peripheral areas of the lung and is more common in women. Squamous cell carcinoma is more common in males and accounts for about 30–40 % of all lung cancers. Squamous cell carcinoma typically occurs centrally in the large bronchi, and is not easily visualized on X-ray. This type of cancer is characterized by having cells that are moderate to poor in differentiation. Large cell carcinoma makes up 15–20 % of all lung cancers and also has poorly differentiated cells; in addition, it tends to occur in the outer part of lung and to invade subsegmental bronchi or large airways. SCLC accounts for 20–25 % of all bronchogenic carcinomas and usually arises in the larger airways and follows a highly aggressive clinical course compared to NSCLC.

1.2 Diagnostic Tests

Historically, chest X-ray and sputum cytology were the only diagnostic tests available for the detection of lung cancer in its early stages. Sputum makes for a good biological specimen for screening because it is readily accessible and the technique is noninvasive. The efficacy of chest X-ray in combination with sputum cytology as screening tools of lung cancer has been evaluated in randomized controlled trials sponsored by the National Cancer Institute and conducted at the Memorial Sloan-Kettering Cancer Center, the Mayo Clinic, and the Johns Hopkins University in the 1970s and early 1980s. Over 30,000 male smokers aged 45 years or older participated in the trials and randomly assigned to chest X-ray and sputum cytology at baseline and repeated at interim periods over 3–5 years. However, these trials have not been proven effective in reducing disease-specific mortality between the study groups [1, 2].

The likelihood of detecting abnormal cells in sputum depends on number of sputum samples, the tumor location, histology, and pathologic stage. Sputum specimens are known to be most

effective in detecting centrally located tumors, but unfortunately the overall sensitivity of sputum cytology lingers around 50 % [3–6]. The low sensitivity results partially from the presence of cancerous cells in only a small fraction of sputum samples; a longstanding demand exists for molecular biomarkers that can improve the sensitivity and specificity of lung cancer detection. The advent of early detection of lung cancer through CT screening and autofluorescence bronchoscopy seems promising but has yet to be fully implemented.

1.3 Prognosis

In spite of significant breakthroughs in radiological tests (particularly CT and positron emission tomography scanning) and combined treatment modalities, the long-term survival from lung cancer has not improved significantly over the last 30 years: the overall 5-year survival rate has improved marginally from 12 % in 1977 to 16 % in 2007 [7]. The poor prognosis of lung cancer patients is largely a result of occult metastatic dissemination, which appears, at the time of diagnosis, in more than half of all patients.

The majority of patients whom achieve long-term survival are those who had curative surgical resection of early stage tumors without metastases. The lack of sensitive screening methods for the early detection of lung cancer is responsible for the high mortality rate. Surgical resection of early stage lung cancer is currently the only potential curative therapy; if stage I NSCLC is curatively treated with surgery, the overall 5-year survival rate for the patient remains up to 83 % [8]. Accordingly, it is essential to develop efficient diagnostic methods which can detect the cancer at the earliest stages of lung cancer in which curative surgical resection is feasible.

Screening and early detection of lung cancer brings up many challenges compared with other cancers such as cancers of the cervix, breast, stomach, and colon. Above all, preinvasive lesions that will eventually develop into a lung cancer in the entire bronchial epithelium are difficult to identify. To obtain tissue samples through invasive bronchoscopy for pathological or cytological diagnosis is difficult. Thus, paradigm for lung cancer screening needs to be different compared with other cancers.

2 DNA Methylation

The major epigenetic modification of mammalian DNA arises at the cytosine residues of CpG dinucleotides. The addition of a methyl group ($-\text{CH}_3$) at the fifth carbon position of cytosine by DNA methyltransferases results in the formation of methylcytosine. DNA methylation in normal cells has many diverse functions including development, chromatin structure, genomic imprinting, X-chromosome inactivation, aging, and carcinogenesis.

Aberrant methylation at CpG dinucleotides plays a major role in carcinogenesis by genetic and epigenetic mechanisms. 5-Methylcytosine is unstable and may convert to thymine. The C-to-T transition mutation that arises via spontaneous deamination of methylcytosine at CpG dinucleotides has been considered a source of genetic mutation in cancer. Global hypomethylation and regional hypermethylation play important roles for epigenetic gene regulation in carcinogenesis. Global hypomethylation is observed in some cancers and is known to be involved in carcinogenesis via microsatellite instability through activation of repetitive elements or retrotransposons, loss of imprinting, and transcriptional activation and overexpression of oncogenes.

Approximately 70 % of CpG dinucleotides in bulk genomic sequence are typically methylated, with the remaining CpGs being non-methylated. Most non-methylated CpGs occur in discrete clusters known as CpG islands. Approximately 60 % of human genes have CpG islands near their 5' ends and with an average length of 1.0 kb and are located in promoter regions of many genes; these islands exist at the 5' end of most house-keeping genes, which code for the many proteins essential for cell viability and seem to remain unmethylated in most cell types [9]. Regional hypermethylation of CpG islands at promoter regions of tumor suppressor genes (TSGs) is one frequent mechanism of gene inactivation in human neoplastic cells and is one of the most commonly acquired epigenetic changes occurring in the pathogenesis of lung cancer.

3 Tumor-Specific Methylation

The primary concern in methylation analysis of tumor suppressor genes for the detection of lung cancer at early stage is the false positives that can be caused by patient age and environmental factors, mainly smoking, which could affect methylation patterns even in subjects who never develop cancer.

3.1 *Environmental Factor-Related Methylation*

Not all methylation of CpG islands at promoter region of TSGs indicate that the methylation is tumor-specific, since CpG island hypermethylation of numerous TSGs occurs after the onset of neoplastic evolution but others are already hypermethylated in normal epithelial cells by environmental factors such as exposure to tobacco, and by aging. For example, Belinsky et al. [10] reported that hypermethylation of the *p16* promoter was present in lung cancer induced by inhalation of cigarette smoke in F344/N rats and in the bronchial epithelium and sputum of the smoker; hypermethylation of *p16* was also associated with smoking duration in primary NSCLC [11]. Altered methylation of *RARβ2*, *CDH13* (H-cadherin), *p16*, and *RASSF1A* genes was found in bronchial

epithelial cells from heavy smokers [12]. Altered methylation in genomic DNA from peripheral blood lymphocyte was compared in current smokers, former smokers, and never-smokers with the use of the Illumina HumanMethylation 27K BeadChip [13]. A locus, cg03636183, located in F2RL3 was found to be less methylated in smokers. Differentially methylated genes in small airway epithelium were also found to be more frequent in smokers than non-smokers [14].

3.2 Age-Related Methylation

Age-related methylation has also been detailed for several genes. Hypermethylation of ER, N33, MYOD, and thrombospondin-4 genes in normal colon mucosa increases progressively with age and occurs in all colon cancers, implying that aging per se of the normal epithelium is a risk factor for promoter hypermethylation of the *ER* gene [15–17]. The insulin-like growth factor 2 (*IGF2*) gene also shows, with age, extensive methylation of the originally unmethylated paternal allele in colon cancer [18]. In glioblastoma multiforme, hypermethylation of ER and N33 was detected more frequently in patients over the age of 40 [19]. In addition, a significant increase in promoter methylation of RAR β 2, RASSF1A, GSTP1, NKX2-5, and ESR1 genes was found to correlate with age in normal aging prostate [20].

Furthermore, epigenome-wide analysis showed changes in DNA methylation with age. Florath et al. [21] analyzed over 480,000 CpG sites using the Infinium HumanMethylation450 BeadChip in whole blood DNA of 965 participants aged between 50 and 75 years of a population-based cohort study and identified 65 novel CpG sites with a significant association between methylation with age; Bell et al. [22] also documented that epigenome-wide scans differentially identify methylated regions for age and age-related phenotypes in a healthy aging population. Consequently, differentiating age- or environmental factor-related methylation in patients who do not develop cancer from tumor-specific methylation in patients who develop cancer is essential to the early detection of lung cancer.

4 CpG Island Hypermethylation in Biological Fluids as a Surrogate Marker for Early Detection of Lung Cancer

4.1 Sputum

Cytologic examination of sputum that contains exfoliated cells shed from larger respiratory passages turned out to be neither additive to value in enhancing lung cancer detection nor reductive of lung cancer mortality. Nonetheless, cytological examination of sputum is still the routine clinical practice followed for suspected lung cancer cases. To improve the poor efficiency of cytologic examinations which miss half of the lung cancer cases, a number of groups have analyzed hypermethylation of CpG islands at the

promoter regions of TSGs in sputum using MSP, qMSP, or pyrosequencing (Table 1), proposing that methylation profiling of TSGs in multiple sputum samples may be beneficial to the detection of central tumors (squamous cell and small-cell carcinomas) arising from the larger bronchi.

Hypermethylation of CpG islands at the promoter regions of p16 and/or O⁶-methyl-guanine-DNA methyltransferase (MGMT) was first analyzed using MSP in sputum samples from 123 cancer-free subjects with a high risk of lung cancer and found in 100 % of patients with squamous cell lung carcinoma up to 3 years before clinical diagnosis [23], suggesting that detection of hypermethylation in sputum can be a promising approach for the early detection of lung cancer.

Since then, methylation profiling of different genes in sputum has been analyzed in comparison with sputum cytology, bronchoscopy, and spiral CT. Promoter methylation of p16, MGMT, death-associated protein (DAP) kinase, and Ras effector homologue (RASSF1A) genes was not detected in sputum from cancer-free never-smokers but in sputum from current and former smokers [24]. Some of current and former smokers showing aberrant methylation of RASSF1A gene in sputum developed lung cancer within 12–14 months of bronchoscopy [25]. Molecular abnormalities such as p16 hypermethylation and K-ras mutation in sputum of 50 patients with NSCLC were found at a frequency similar to abnormal cytology (42 %), but combined analysis of K-ras/p16 and cytology increased the diagnostic yield up to 60 % [27].

Aberrant methylation of RAR β 2, p16, and RASSF1A promoters was found in sputum of spiral CT-detected lung cancer patients [30], suggesting that methylation profiling in sputum samples can be a complementary approach to spiral CT for early detection of lung cancer. The number of hypermethylated genes in sputum was found to increase as the time to lung cancer diagnosis decreased [31]. Sputum was also found to be useful as a surrogate for tumor tissue to predict the methylation status of advanced lung cancer where bronchoscopic biopsy is not feasible [35]. In addition, the ability to predict the risk of lung cancer by analyzing methylation profiling of promising genes was replicated between different regions [39]. These observations suggest that methylation profiling of TSGs in sputum can be a universal biomarker for the early detection of lung cancer.

4.2 Bronchial Aspirates

The limitations of sputum cytology have led to the development of invasive procedures for lung cancer diagnosis, including bronchoscopic examination, and a number of groups have reported abnormal hypermethylation of many promoters including p16, RASSF1, RAR β 2, and APC genes in bronchial fluid (Table 2).

Hypermethylation of the p16 gene ensued in 19 of 50 NSCLC tumors obtained at the time of resection, and methylated p16

Table 1
Studies on CpG island hypermethylation in sputum

Study (year)	Cases	Controls	Methods	Genes	Ref.
Palmisano et al. (2000)	SCC (N=32)	Cancer-free subjects (N=91)	MSP	p16, MGMT	[23]
Belinsky et al. (2002)	All (N=52)	Smokers (N=89)	MSP	MGMT, DAPK, RASSF1A, p16	[24]
Honorio et al. (2003)	All (N=32)	Smokers (N=13)	MSP	RASSF1A	[25]
Wang et al. (2003)	NSCLC (N=77)		MSP	hMLH1, hMSH2	[26]
Destro et al. (2004)	NSCLC (N=50)	Smokers (N=100)	MSP	p16	[27]
Konno et al. (2004)	All (N=109)	Cancer-free subjects (N=101)	MSP	p16, APC, RARB	[28]
Belinsky et al. (2005)	All (N=56)	Smokers (N=121)	MSP	MGMT, p16, DAPK, RASSF1A, PAX5 α , PAX5 β , HCAD	[29]
Cirincione et al. (2006)	All (N=29)	Smokers (N=112)	MSP	p16, RARB2, RASSF1A	[30]
Belinsky et al. (2006)	All (N=98)	Smokers (N=92)	MSP	p16, MGMT, PAX5 a, PAX5 b, DAPK, GATA4, GATA5, RASSF1A, SFRP1, HLHP, BETA3, HCAD, LAMC2	[31]
Machida et al. (2006)	All (N=134)		MSP	ASC/TMS1	[32]
Georgiou et al. (2007)	All (N=150)	Smokers (N=48)	MSP	p16	[33]
Hsu et al. (2007)	All (N=82)	Cancer-free subjects (N=37)	MSP	p16, RARB	[34]
Belinsky et al. (2007)	All (N=72)		MSP	p16, MGMT, PAX5 a, PAX5 b, DAPK, GATA5, RASSF1A, HCAD	[35]
Shivapurkar et al. (2007)	NSCLC (N=13)	COPD (N=25)	qMSP	TCF21	[36]

(continued)

Table 1
(continued)

Study (year)	Cases	Controls	Methods	Genes	Ref.
Baryshnikova et al. (2008)		Smokers (<i>N</i> = 820)	MSP	p16, RASSF1A, NORE1A	[37]
Hwang et al. (2011)	All (<i>N</i> = 40)		Pyro-sequencing	HOXA9	[38]
Hubers et al. (2012)	All (<i>N</i> = 53)	COPD (<i>N</i> = 47)	qMSP	RASSF1A, APC, CYGB	[39]
Leng et al. (2012)	All (<i>N</i> = 104)	Smokers (<i>N</i> = 154)	Nested MSP	31 genes	[40]
Bruse et al. (2014)		Smokers (<i>N</i> = 1390)	MSP	11 genes	[41]

Abbreviations: *All* all types of lung cancer included, *NSCLC* non-small cell lung cancer, *SCC* squamous cell carcinoma, *COPD* chronic obstructive pulmonary disease, *qMSP* quantitative methylation-specific PCR

Table 2
Studies on CpG island hypermethylation in bronchial aspirates

Study (year)	Samples	Cases	Controls	Methods	Genes	Ref.
Ahrendt et al. (1999)	BAL	NSCLC (<i>N</i> = 50)		MSP	p16	[42]
Kersting et al. (2000)	BAL, BB	All (<i>N</i> = 51)	Smokers (<i>N</i> = 25)	MSP	p16	[43]
Soria et al. (2003)	BB		Smokers (<i>N</i> = 100)	MSP	p16, DAPK, GSTP1	[44]
Zöchbauer-Müller et al. (2003)	BAL, BB		Smokers (<i>N</i> = 107)	MSP	Retinoic acid receptor β2, H-cadherin, p16, RASSF1A	[12]
Kim et al. (2004)	BAL	NSCLC (<i>N</i> = 85)	Cancer-free subjects (<i>N</i> = 107)	MSP	p16, RARβ2, RASSF1A, FHIT, H-cadherin, FHIT	[45]
Grote et al. (2004)	Bronchial aspirates	All (<i>N</i> = 155)	Benign lung disease (<i>N</i> = 67)	qMSP	APC	[46]
Topaloglu et al. (2004)	BAL	All (<i>N</i> = 31)	Cancer-free subjects (<i>N</i> = 10)	qMSP	CDH1, APC, MGMT, RASSF1A, GSTP1, p16, RARβ2, ARF	[47]

(continued)

Table 2
(continued)

Study (year)	Samples	Cases	Controls	Methods	Genes	Ref.
Russo et al. (2005)	Bronchial aspirates	NSCLC (N=49)	Cancer-free subjects (N=27)	MSP	p16, MGMT, ECAD, DAPK, GSTP1, SMAD8	[48]
Schmiemann et al. (2005)	Bronchial aspirates	All (N=89)	Benign lung disease (N=102), others (N=56)	qMSP	APC, RASSF1A, p16	[49]
Grote et al. (2005)	Bronchial aspirates	All (N=75)	Benign lung disease (N=64)	qMSP	p16, RARB2, SEMA3B	[50]
Schmidt et al. (2010)	Bronchial aspirates	All (N=281)	Cancer-free subjects (N=242)	qMSP	SHOX2	[51]
van der Drift et al. (2012)	Bronchial washing	All (N=129)	Cancer-free subjects (N=28)	qMSP	RASSF1A	[52]
Dietrich et al. (2012)	Bronchial aspirates	All (N=125)	Cancer-free subjects (N=125)	qPCR	SHOX2	[53]
Nikolaides et al. (2012)	Bronchial washing	All (N=333)	Cancer-free subjects (N=322)	Pyro-sequencing	p16, TERT, WT1, RASSF1	[54]

Abbreviations: *All* all types of lung cancer included, *NSCLC* non-small cell lung cancer, *BAL* bronchoalveolar lavage, *BB* bronchial brush, *qMSP* quantitative methylation-specific PCR

alleles were first detected in the BAL fluid of 63 % (12 of 19) of the corresponding patients [42]. Aberrant methylation of the *p16* gene in sputum, bronchial lavage, and bronchial brush samples from 25 cancer-free chronic smokers with greater than 20 pack-years has also been described by Kersting et al. [43]. Hypermethylation of the *p16* promoter was found in 7 (28 %) of 25 chronic smokers: 16 % of sputum samples, 12 % of bronchial lavage samples, and in 8 % of bronchial brushings. Zöchbauer-Müller et al. [12] also analyzed the methylation statuses of RARβ2, H-cadherin, p16, and RASSF1A genes in exfoliated samples of upper aerodigestive tract epithelium from heavy smokers without evidence of lung cancer. Forty-eight percent of smokers exhibited aberrant methylation of at least one gene. Bronchial washing in patients with peripheral tumors also showed high value in detecting RASSF1A hypermethylation [52].

To discriminate between age- or smoking-related methylation and tumor-specific methylation, aberrant methylation of five genes (*p16*, *RAR β 2*, *RASSF1A*, *FHIT*, and *H-cadherin*) was investigated by MSP in the tumor tissues and bronchial lavage of 85 NSCLC patients and in bronchial lavage samples from 127 cancer-free individuals [45]. The prevalence of hypermethylation for *p16*, *RAR β* , *H-cadherin*, and *RASSF1A* was significantly different between cancer-free subjects and NSCLC patients, but not for the *FHIT* gene; hypermethylation of the *FHIT* gene may not be tumor-specific.

4.3 Circulating Tumor DNA

Although many studies have shown that DNA circulates freely in plasma or serum, particularly that of cancer patients, the source of this DNA remains to be elucidated. It is currently assumed that tumor DNA enters the circulation following lysis of the cancer cell, breakdown of circulating cancer cells, or destruction of tumor micrometastasis. The existence of DNA and RNA in the plasma of cancer patients has been recognized since the 1970s, but it was not until 1989 that the neoplastic characteristics of plasma DNA in cancer patients were recognized.

Recent efforts have identified DNA alterations in the plasma or serum from lung cancer patients as a tool that, when developed, holds great potential for the diagnosis of lung cancer. Aberrant methylation of TSGs in serum from lung cancer patients was first reported by Esteller et al. [55]. Since then, a number of groups have reported CpG island hypermethylation of TSGs in the serum or plasma from lung cancer patients (Table 3). Approximately 30–70 % of aberrant methylation that was detected in tissue samples from lung cancer patients was also found in matched plasma or serum samples. Aberrant methylation status of DCC, Kif1a, NISCH, and RAR β in plasma from lung cancer patients was also comparable with abnormalities of lung cancer detected on chest CT [61]. These observations suggest that abnormal methylation of TSGs in circulating cell-free DNA can be used as a surrogate biomarker for early detection of lung cancer.

5 CpG Island Hypermethylation in Premalignant Lesions of the Lung

Premalignant lesions for squamous cell carcinoma of lung include hyperplasia, metaplasia, dysplasia, and carcinoma in situ. Adenomatous dysplasia and atypical adenomatous hyperplasia (AAH) are premalignant lesions for adenocarcinoma. To discover molecular biomarkers for the early detection of lung cancer, it is imperative to analyze molecular alterations in premalignant lesions of lung cancer. However, a few studies have investigated CpG island hypermethylation of TSGs in premalignant lesions. Belinsky et al. [10] was the first to analyze the timing of *p16* methylation in

Table 3
Studies on CpG island hypermethylation in serum or plasma

Study (year)	Body fluid	Cases	Controls	Methods	Genes	Ref.
Esteller et al. (1999)	Serum	NSCLC (N=22)		MSP	DAPK, GSTP1, p16, and MGMT	[55]
Beazarto et al. (2002)	Plasma	NSCLC (N=35)	Cancer-free subjects (N=15)	F-MSP	P16	[56]
Usadel et al. (2003)	Serum, plasma	All (N=89)	Cancer-free subjects (N=50)	qMSP	APC	[57]
Russo et al. (2005)	Serum	NSCLC (N=49)	Cancer-free subjects (N=27)	MSP	P16, MGMT, ECAD, DAPK, GSTP1, SMAD8	[48]
Belinsky et al. (2005)	Serum, plasma	All (N=56)	Cancer-free subjects (N=195)	MSP	MGMT, p16, DAPK, RASSF1A, PAX5 α , PAX5 β , HCAD	[29]
Fujiwara et al. (2005)	Serum	All (N=91), other cancer (N=9)	Benign lung disease (N=100)	MSP	P16, RAR β , DAPK, MGMT, RASSF1	[58]
Belinsky et al. (2007)	Serum	All (N=72)		MSP	p16, MGMT, PAX5 a, PAX5 b, DAPK, GATA5, RASSF1A, HCAD	[35]
Umemura et al. (2008)	Serum	All (N=11)	Cancer-free subjects (N=87)	MSP	MGMT, p16, RASSF1A, DAPK, RAR β 2	[59]
Hoffman et al. (2009)	Serum	NSCLC (N=76)		qMSP	DAPK, MGMT, and GSTP1	[60]
Ostrow et al. (2010)	Plasma	All (N=93)		qPCR	DCC, Kif1a, NISCH, RAR β	[61]
Begum et al. (2011)	Serum	All (N=76)		qMSP	APC, AIM, <i>CyclinD2</i> , <i>CALCA</i> , <i>CDHL</i> , <i>DCC</i> , <i>p16</i> , <i>MGMT</i> , <i>RASSF1</i> , <i>MINT31</i> , <i>CyclinA1</i> , <i>ESR1</i> , <i>HIC1</i> , <i>PGP9.5</i> , <i>TIMP3</i>	[62]
Zhang et al. (2011)	Plasma	NSCLC (N=110)	Cancer-free subjects (N=50)	MSP	APC, CDH13, KLK10, DLEC1, RASSF1A, EFEMP1, SFRP1, RAR β and p16(INK4A), RUNX3, hMLH1, DAPK, BRCA1, p14(ARF), MGMT, NORE1A, FHIT, CMTM3, LSAMP, and OPCML	[63]
Kncip et al. (2011)	Plasma	All (N=188)	Cancer-free subjects (N=155)	qPCR	SHOX2	[64]

Abbreviations: *F-MSP* fluorescent methylation-specific PCR, *qMSP* quantitative methylation-specific PCR, *qPCR* quantitative real-time PCR

an animal model of lung carcinogenesis. Hypermethylation of the *p16* gene was frequently detected in precursor lesions to lung cancer in rats treated with tobacco-specific NNK, and the prevalence of hypermethylation increased with disease progression from basal cell hyperplasia (17 %) to squamous metaplasia (24 %) and then to carcinoma in situ (50 %).

Licchesi et al. [65] evaluated atypical adenomatous hyperplasia, adjacent normal lung tissues, and synchronous lung adenocarcinoma for promoter methylation of genes implicated in lung tumorigenesis. Hypermethylation of *MGMT*, *RAR β* , *RASSF1A*, and *hTERT* genes was detected in AAH as well as in adjacent normal lung, thereby suggesting that these genes may be targeted during the earliest stages of lung tumorigenesis. In the case of *p16*, promoter methylation was rarely detected in normal-adjacent lung and was never found in low-grade AAH, but was detected in high-grade AAH (30 %) and in adenocarcinomas (50 %).

6 Locus-Specific Detection of DNA Methylation

A wide spectrum of techniques exists that allow detection of DNA methylation. Based on the principle of 5-methylcytosine detection, they can be grouped into three categories (Table 4): (1) immunoprecipitation of 5-methylcytosine using anti-5-methylcytosine antibodies and enrichment of methylated DNA using methyl binding domain (MBD) moieties; (2) digestion of DNA by methylation-sensitive restriction endonuclease; and (3) chemical modification of DNA by sodium bisulfite.

Methylation-sensitive restriction digestion followed by PCR across the restriction site is a very sensitive technique that is still used in some applications today. This method is still applicable for some locus-specific studies that require linkage of DNA methylation information across multiple kilobases, either between CpGs or between a CpG and a genetic polymorphism. Restriction endonuclease-based analysis is limited by providing methylation data only at the restriction enzyme recognition sites or adjacent regions and is prone to false-positive results that may result from incomplete digestion other than DNA methylation.

Methylated DNA immunoprecipitation (MeDIP) [66] requires immunoprecipitation of DNA using an antibody that specifically recognizes 5-methylcytosine. It is straightforward, and data is relatively easier to analyze. MeDIP is specific for methylated cytosines and not confounded by the presence of hydroxymethylated cytosines. However, it shows low resolution and does not yield information on individual CpG dinucleotides. In addition, methylated CpG-rich sequences may give higher enrichments than methylated

Table 4
Overview of locus-specific methylation analysis

Enzyme digestion	Affinity enrichment	Bisulfite conversion
AIMS	MeDIP-PCR	Bisulfite sequencing
HpaII-PCR		COBRA
MS-AP-PCR		EpiTYPER
Southern blot		MethyLight
		MS-HRM
		MSP
		MS-SNuPE
		Pyrosequencing

Abbreviations: *AIMS* amplification of inter-methylated sites, *MS-AP-PCR* methylation-sensitive arbitrarily primed PCR, *MeDIP-PCR* methylated DNA immunoprecipitation-PCR, *MSP* methylation-specific PCR, *MS-SNuPE* methylation-sensitive single nucleotide primer extension

CpG-poor sequences and therefore needs adjustment for bias associated with CpG density.

Methylation analysis by bisulfite treatment may be hampered by DNA degradation by high temperature and low PH and by incomplete conversion of unmethylated cytosine that can result from high GC density regions, histone protection, and stable secondary structure elements.

Pyrosequencing methylation analysis (PMA) and quantitative methylation-specific PCR (qMSP) [67] are currently considered as the gold standard methods for locus-specific analysis of methylation, or for the validation of DNA methylation results derived from high-throughput platforms such as microarrays or next-generation sequencing. Both methods utilize a bisulfite-converted DNA template. PMA is essentially a primer extension method to analyze short- to medium-length DNA sequence, and the area of interest is amplified using methylation independent (non-CpG containing) primers. In the subsequent pyrosequencing reaction the methylation level of each CpG is calculated as the ratio of C/T incorporation at this position. The limitation of the method is that it can accurately detect DNA methylation levels of >5 %. PMA provides the advantage of quantitatively sequencing information over a longer stretch of DNA.

However, qMSP can accurately detect minute DNA methylation levels down to 0.1 %, but this method utilizes methylation-specific primers; therefore, it essentially interrogates only the CpGs in the primer region. Thus, the selection method depends on the particularities of the questions asked in each research project.

7 Genome-Wide Detection of DNA Methylation

The biggest bottleneck for epigenome-wide association study (EWAS) has been the lack of an appropriate technology for DNA methylation profiling on a genomic scale; in recent years, technologies have improved to enable assessment of locus-specific DNA methylation on genome-wide scale. A variety of both array- and next-generation sequencing (NGS)-based methods (Table 5) have been developed by coupling locus-specific technologies for methylation detection with high-throughput technologies. Each of two approaches has inherent strengths and weaknesses. Although sequencing-based assays become more affordable and are anticipated to be more widely used in EWAS, they remain costly. Consequently, array-based assays are alternative methods for determining methylation levels on genome level.

7.1 Array-Based Technologies

In parallel with recent advances in microarray technologies, high-density oligonucleotide-based whole-genome microarrays have emerged as a preferred platform for genomic analysis and have

Table 5
Overview of genome-wide methylation analysis

	Enzyme digestion	Affinity enrichment	Bisulfite conversion
Array-based analysis	CHARM DMH HELP MAD MCAM Methyl-Scope MMASS MSNP PMAD	MeDIP-Chip MIRA	BiMP GoldenGate Infinium
Sequencing-based analysis	HELP-seq LUMA MCA-seq Methyl-seq MSCC RLGS	MeDIP-seq MethylCap-seq	BC-seq BSPP DHPLC RRBS WGSBS

Abbreviations: *BC-seq* bisulfite conversion followed by capture and sequencing, *BiMP* bisulfite methylation profiling, *BS* bisulfite sequencing, *BSPP* bisulfite padlock probes, *CHARM* comprehensive high-throughput arrays for relative methylation, *COBRA* combined bisulfite restriction analysis, *DMH* differential methylation hybridization, *DHPLC* denaturing HPLC, *HELP* *HpaII* tiny fragment enrichment by ligation-mediated PCR, *LUMA* luminometric methylation assay, *MCA* methylated CpG island amplification, *MCAM* MCA with microarray hybridization, *MeDIP* mDIP and mCIP, methylated DNA immunoprecipitation, *MIRA* methylated CpG island recovery assay, *MMASS* microarray-based methylation assessment of single samples, *MSCC* methylation-sensitive cut counting, *NGS* next-generation sequencing, *RLGS* restriction landmark genome scanning, *RRBS* reduced representation bisulfite sequencing, *-seq* followed by sequencing, *WGSBS* whole-genome shotgun bisulfite sequencing

been used to provide unbiased whole-genome coverage of DNA methylation profiles. For instance, the comprehensive high-throughput *arrays* for relative methylation (CHARM) assay [68] are based on methylation-sensitive restriction enzymes and cover approximately 2.1 M probes genome-wide and do not make assumptions about where functionally important hypermethylation of TSGs occurs. In addition, CHARM assay uses a novel genome-weighted smoothing algorithm in order to correct for CpG density and fragment biases present in methyl-enrichment assay.

Methylated DNA immunoprecipitation and array-based hybridization (MeDIP-Chip) [69] method first immunocaptures methylated genomic fragments with an antibody directed against 5-methyl-cytosine. Input DNA and immunoprecipitated methylated DNA are labeled with Cy5 and Cy3, respectively, and are cohybridized on a 2-channel, high-density genomic microarray. Main limitation of MeDIP-Chip technique is that methylation profiles analyzed are not base pair-specific but reflect methylation levels on a resolution restricted by sonicated DNA fragments. It also requires large amount of genomic DNA.

The Illumina Infinium HumanMethylation450K BeadChip (Illumina Inc.) quantitatively measures 485,577 CpG sites at a single nucleotide resolution with 99 % of NCBI Reference Sequence (RefSeq) genes and 96 % coverage of CpG islands. The 450K array is designed to detect CpG methylation of bisulfite treated DNA and to achieve increased coverage of CpGs by including two different types of chemical assay on the same array: the Infinium I assay ($n=135,501$ probes) and Infinium II assay ($n=350,076$ probes). However, the assay is susceptible to certain polymorphisms not known to or considered at the time the array was designed.

Array-based genome-wide DNA methylation analysis is easy to perform and data is easily interpreted with well-characterized software programs. However, its resolution is low and it is not easy to distinguish one repetitive element from another.

7.2 Sequencing-Based Technologies

Initially, sequencing-based methylation analyses in a genomic region relied primarily on Sanger sequencing. However, it is cost inefficient, labor intensive, and too time consuming to sequence the whole genome. To overcome the limitations, high-throughput sequencing platforms are employed as an alternative to analyzing DNA methylation on a genome scale and are applied to DNA methylation analysis in conjunction with techniques such as enzyme digestion, affinity enrichment, and bisulfite modification.

For example, Down et al. [70] first described MeDIP and high-throughput sequencing (MeDIP-seq) assay. The technique uses an antibody that is specific to 5-methyl-cytosine to retrieve methylated fragments from sonicated DNA and then the enriched DNA undergoes high-resolution next-generation sequencing.

Although MeDIP-seq generates unbiased and full-genome methylation levels without the limitations associated with methylation-sensitive restriction enzymes, it is of far low resolution and produces inaccurate levels of methylation when alignment accuracy to repetitive regions is low.

Methylated DNA capture by affinity purification (MethylCap)-seq consists of the affinity purification of methylated DNA with the MBD (methyl-binding domain) of MeCP2 and subsequent deep sequencing of eluted DNA [71]. The assay has advantages that can detect regional methylation changes at a genome-wide scale with CpG resolution and cover genome independent of restriction site. However, sequencing and specific antibody to identify genomic regions are relatively expensive. Although affinity-based enrichment methods of methylated DNA sequences such as MeDIP-seq, MethylCap-seq, and MBD-seq are less quantitative and don't provide methylome status at single-base resolution, they are highly automatable and economical.

BC-seq [72], bisulfite conversion followed by capture and sequencing, is widely used to analyze the methylation state of a whole genome at single-base resolution. BS-seq approach provides the highest level of coverage and resolution and can sequence large fragments compared to MeDIP-seq. However, BS-seq is not able to distinguish between methylated and hydroxymethylated cytosine bases.

Reduced representation bisulfite sequencing (RRBS) [73] technique combines digestion of genomic DNA with specific restriction enzymes and bisulfite sequencing in order to enrich for the regions that have a high CpG content. In addition, this approach provides useful nucleotide-level quantitation of cytosine methylation. However, this method can introduce a bias toward CpG-rich regions of the genome that typically include unmethylated CpG islands in normal cells. RRBS method has limited coverage of the methylome in CpG poor regions and provides about 10 % genome coverage.

8 Epigenome-Wide Association Study (EWAS)

To discover CpGs that are involved in lung cancer pathogenesis, many groups have analyzed different methylation of CpGs between biologic materials from lung cancer patients and healthy individuals. However, most studies were restricted to few candidate genes and were performed with inadequate genome coverage. Recently, epigenetic studies of human cancer have shifted from candidate gene analyses toward epigenome-wide analysis with rapid advancements of technology. Analyses that were limited to specific CpGs can now be performed on a genome scale, and a number of groups have been reporting results of EWAS in lung cancer.

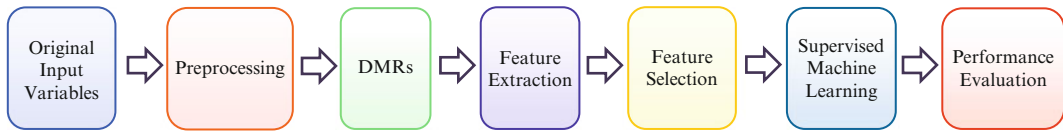


Fig. 1 Pipeline of data processing for epigenome-wide association study

For example, Lockwood et al. [74] performed a large-scale analysis of DNA methylation in NSCLC by using single-CpG resolution Illumina HumanMethylation27 bead array to identify subtype-specific molecular alterations relevant to the choice of target chemotherapeutic agents. The group found candidate genes involved in distinct pathogenetic pathways of adenocarcinoma and squamous cell carcinoma. Carvalho et al. [75] performed genome-wide methylation analysis of NSCLC using MethylCap-seq and identified 57 differentially methylated regions (DMRs) present in NSCLC tumor tissues. Heller et al. [76] searched methylated CpGs in tumors and normal tissues from 101 NSCLC patients by using MeDIP-chip and found that HOXA2 and HOXA10 methylation serve as prognostic indicator in squamous cell carcinoma. Sato et al. [77] performed EWAS using the Infinium HumanMethylation27 chip to identify genes involved in recurrence of adenocarcinoma and found that altered methylation of ADCY5, EVZ1, GFRA1, PDE9A, and TBX20 genes at precancerous stages determines the aggressiveness of adenocarcinoma.

The analysis of epigenomic data to discover CpGs for the early detection of lung cancer requires several steps (Fig. 1): data preprocessing, identification of differentially methylated CpGs or DMRs, dimensionality reduction, and classification of unknown samples using supervised machine learning.

9 Preprocessing

Epigenome-wide analysis covers an increasing number of CpG sites, but random noise and systematic variation arise from variations in experimental process. Therefore, preprocessing such as background correction and normalization is important to identify real biological variations by eliminating technical bias.

Several pipelines have been developed for the preprocessing, quality control, and methylation estimation for microarray and NGS data. Sun et al. [78] reported two LOESS (or LOWESS, locally weighted scatterplot smoothing) normalization protocol for differential methylation hybridization (DMH) microarray data. Analytic pipelines targeted to MeDIP-seq data have been reported by several groups.

Chavez et al. [79] showed a pipeline for normalization of MeDIP-seq data in comparison to available whole-genome

bisulfite sequencing data. Huang et al. [80] also reported MeQA pipeline that allows the easy and straightforward analysis of sequencing data on DNA methylation by providing the general control of the sequence reads and assessing the quality of the methylation analysis experiment. MEDIPS [81] was developed for genome-wide differential coverage analysis of sequencing data derived from MeDIP-seq. However, this method requires considerable efforts to prepare the data and run packages for sequencing and quality control packages, which potentially increase analysis time and introduce processing errors.

A number of research groups have been developing a complete preprocessing pipeline for the Infinium HumanMethylation450 (450K) BeadChip data. However, there currently is no consensus on how to process this type of data. The 450K array uses a combination of two distinct probe types, Infinium I and II, to cover more CpGs across human genome. This unique probe design produces technical differences between two probe types. Different softwares to reduce the bias between Infinium I and II probes and to correct batch effect, background and dye effect are being developed: Illumina Methylation Analyzer (IMA) [82], Subset Quantile Normalization (SQN) [83], Subset Quantile Within Array Normalization (SWAN) [84], ChAMP [85], and Beta-Mixture Quantile Normalization (BMIQ) [86].

10 Identification of Differentially Methylated Regions

Previous publications have focused on precisely estimating methylation levels at single-base resolution and statistical methods such as the Wilcoxon rank-sum test and Student's *t*-test have been used to identify CpGs differentially methylated among multiple samples. However, recently differentially methylated regions (DMRs), as regions of the genome at which multiple adjacent CpG sites show different methylation statuses among multiple samples, are regarded as possible functional regions involved in gene transcriptional regulation. Identification of DMRs is essential for determining local differences in the methylation profiles of diverse biological samples. With the progress of high-throughput technologies over recent years, there have been considerable efforts in identifying DMRs across large number of samples and developing DMR detection algorithm.

Zhang et al. [87] developed quantitative differentially methylated region (QDMR), a quantitative method to quantify methylation difference and identify DMRs from genome-wide methylation profiles by adapting Shannon entropy. QDMR works on the fraction or percentage methylation across multiple samples and is independent of specific methylation mapping techniques. In addition, QDMR can reflect the biological characteristics of methylation

difference such as the relationship between methylation difference and CpG density.

Hansen et al. [88] reported BSmooth algorithm, an alignment, quality control, and analysis pipeline for low coverage whole-genome bisulfite sequencing (WGBS) data. BSmooth identifies DMRs taking biological variability into account and is based on a statistic that summarizes consistent differences. Recently, Morris et al. [85] presented ChAMP package offering a new Probe Lasso method for the identification of DMRs.

11 Dimensionality Reduction

From a theoretical point of view, increasing the number of features in a model should lead to better performance. However, in practice the inclusion of more features leads to worse performance. “Curse of dimensionality” [89], also known as the Hughes effect, refers to the problems that arise when analyzing and organizing data in high-dimensional spaces that do not occur in low-dimensional settings. All problems become tougher as the dimensionality increases. High-dimensional data is difficult to work with since adding more features can increase the noise and there usually aren’t enough observations to get good estimates. In addition, query accuracy and efficiency degrade rapidly as the dimension increases. These problems result in overfitting and increase in running time and number of samples required. The curse of dimensionality can be overcome by reducing the dimensionality.

Dimensionality reduction in machine learning or statistics is the process of reducing the number of random variables under consideration. Dimensionality reduction improves by projecting high-dimensional data onto 2D or 3D, compresses data by efficient storage and retrieval, and removes noise by positive effect on query accuracy. The dimensionality reduction can be achieved by feature extraction and feature selection. Feature extraction creates new features by combining all original existing features (reduce dimensionality by combining correlated redundant features, rather than eliminating features) linearly or nonlinearly, and all transformed features are combinations of the original features. In contrast feature selection chooses only a subset of all the original features. In either case, the goal is to find a low-dimensional representation of the data that preserves most of the information or structure in the data.

12 Feature Extraction

The most common approaches for feature extraction, particularly for continuous data, use techniques to project the data from a high-dimensional space to a lower-dimensional space. Techniques that

are commonly used for feature extraction are principal component analysis (PCA) and Fisher's linear discriminant analysis (LDA). PCA is closely related to LDA in that they both look for linear combinations of variables which best explain the data. However, PCA does not take into account any difference in class, whereas LDA explicitly attempts to model the difference between the classes of data. PCA seeks a projection that preserves as much information in the data as possible, but LDA seeks a projection that best separates the data.

12.1 PCA

PCA is one of the most popular methods for feature extraction. PCA can project the data from the original space into a lower-dimensional space in an unsupervised manner. PCA creates a completely new set of values of linearly uncorrelated variables called principal components that are linear combinations of the original attributes and are orthogonal to each other. Orthogonal transformation is conducted to convert a set of observations of possibly correlated variables into principal components.

All principal components (PCs) start at the origin of the ordinate axes. First PC is the direction of greatest variability (covariance) in a data and contains the greatest amount of variation. Second is the next orthogonal (uncorrelated) direction of greatest variability. For second PC, all the variability along the first component is removed and the next direction of greatest variability is then found. The number of principal components is less than or equal to the number of original variables. Dimensionality reduction implies information loss, but PCA preserves as much information as possible, that is, it minimizes the error. Traditional PCA applies linear transformation and may not be effective for nonlinear data. However, PCA can also apply transformation to potentially high-dimensional space in a nonlinear way by means of the kernel trick. The resulting technique is capable of constructing nonlinear mappings that maximize the variance in the data.

12.2 Linear Discriminant Analysis (LDA)

LDA is a method used in statistics and machine learning to find a linear combination of features which characterizes or separates two or more classes. The objective of LDA is to perform dimensionality reduction while preserving as much of the class discriminatory information as possible. The resulting combination may be used as a linear classifier, or more commonly, for dimensionality reduction before later classification. LDA works when the measurements made on independent variables for each observation are continuous quantities. LDA assumes unimodal Gaussian likelihoods. If the densities are significantly non-Gaussian, LDA may not preserve any complex structure of the data needed for classification. LDA has a tendency to overfit training data.

13 Feature Selection

Feature selection, another way to reduce the dimensionality of the data, is a process of selecting an optimal subset of features for use in model building by identifying and removing as many redundant and irrelevant features as possible. Feature selection provides many potential benefits: it removes features that are unlikely to be useful, improves mining performance, enhances generalization by reducing overfitting, facilitates data visualization and data understanding, increases predictive accuracy, reduces training times of a final model, and defies the curse of dimensionality to improve prediction performance. There are three standard approaches to feature selection: filter, wrapper, and embedded.

13.1 Filter Methods

Filter methods are computationally simple and fast and most frequently used in the literature [90]. Features in filter approaches are selected before the data mining algorithm is run, using some approach that is independent to the classification algorithm and does not incorporate learning of the data mining task. Features are scored according to the evidence of predictive power and then are ranked. Methods such as t -test, F -test, and signal-noise ratio are used for scoring. Features with high score are selected and used by the classifier. The number of features selected, based on the cutoff point in score, is determined by cross-validation. Although genes are considered independently in filter method, redundant genes may be included. In addition, filter method is relatively robust against overfitting but may fail to select the most useful features.

13.2 Wrapper Methods

Wrapper methods use a classification algorithm to score feature subsets without incorporating knowledge about the specific structure of the classification and thereby to find the best subset of features [91]. Wrapper methods create all possible subsets from feature vectors and use a classification algorithm to induce classifiers from the features in each subset and consider the subset of features with which the classification algorithm performs the best. Wrapper methods can in principle find the most useful features. However, since the classifier needs to be trained for each feature subsets considered, wrapper methods are computationally intensive and therefore exhaustive searching is impossible. In addition, wrapper methods are prone to overfitting and have a higher risk of overfitting than filter methods

13.3 Embedded Methods

Feature selection in embedded methods occurs naturally as part of learning procedure and is usually specific to given learning machines [90]. The algorithm itself decides which attributes to use and which to ignore during the operation of the algorithm. Therefore, the learning part and the feature selection part cannot be separated

in embedded methods. Although feature selection is dependent on classifier, embedded method is not only less prone to overfitting but also computationally much more efficient than wrapper methods.

14 Classification

Classification of cancer based on selected features is performed using supervised machine learning algorithms. Classification is a two-step process involving the model building, the testing, and the usage of the classification model: given a set of data representing examples of a target concept, a classification model is constructed to explain the concept. The model is next used for classifying unknown cases. An algorithm that implements classification is known as a classifier. Classification techniques are divided into base classifiers and ensemble classifiers. Base classifiers include decision tree, k -nearest-neighbor, artificial neural networks, Naïve Bayes, and support vector machines, etc. Ensemble classifiers include AdaBoost, bagging, stacking, and random forest.

The choice of which specific machine learning algorithm we should use is critical for accurate classification of cancer. A classifier for routine use is most often selected based on the performance of a classifier, including prediction accuracy. Ensemble methods require increased storage and computation, and its comprehensibility is decreased with involvement of multiple classifiers in decision-making. For these reasons, ensemble methods are usually applied when interested in the best classification accuracy.

14.1 Artificial Neural Network

Artificial neural network (ANN) is a mathematical model based on biological networks which are typically organized in layers, which are made up of a number of interconnected nodes of artificial neurons [92]. The nodes receive input, and process them to obtain an output. An ANN has several advantages over other models. First, an ANN is a parametric model. Second, an ANN has a high tolerance to noisy data and can classify patterns on which it has not been trained. However, an ANN often overfits by considering the noise as part of the pattern for any given pattern in cases that training goes on too long.

14.2 Decision Tree

Decision trees are one of most popular learning methods commonly used for data mining. Decision trees use a tree-like graph or flow chart of decision for classification and its possible consequence is used to create a plan to reach a goal. Decision trees can be learned by progressively splitting the labeled training data into subsets based on a numerical or logical test. This process is repeated on each derived subset in a recursive manner until either further splitting is not possible or a singular classification is achieved.

Decision trees have many advantages: they are simple to understand and to interpret, they require little data preparation, they can handle many types of data including numeric, nominal, and categorical data, they generate robust classifiers, they are quick to learn, and they can be validated using statistical tests. In addition, decision trees are inexpensive to construct, extremely fast at classifying unknown records, easy to interpret for small-sized trees, and its accuracy is comparable to other classification techniques for many simple datasets.

However, decision trees do not generally perform as well as ANNs in more complex classification problems. Decision trees can also be unstable because small variations in the data might result in a completely different tree being generated. In addition, decision tree learners create biased trees if some classes dominate. It is therefore recommended to balance a dataset prior to fitting with decision trees in order to prevent the tree from creating a biased tree. Decision trees tend to overfit on data with few samples in high-dimensional space. Therefore, getting the right ratio of samples to number of features is important. It is also necessary to consider dimensionality reduction beforehand for a better chance of finding high discriminative features.

14.3 Support Vector Machine (SVM)

Support vector machine (SVM) is based on statistically learning theory and has shown promising results in many practical applications [93]: especially SVM works very well with high-dimensional data and avoids the curse of dimensionality problem. A linear SVM creates a hyperplane that separates the data into two classes with the maximum margin. In contrast, nonlinear SVM, which is called a nonlinear kernel [94], is a method for computing similarity in the transformed space using the original attribute set. A nonlinear kernel transforms the data from a linear feature space to a nonlinear feature space and improves the SVM performance by applying different kernels to different datasets. SVMs are well suited to nonlinear classification problems, as are k -nearest neighbor approaches.

14.4 Random Forest

A random forest [95] is a class of ensemble methods specifically designed for decision tree classifiers. Random forests consist of many decision trees and often used when training dataset and the number of input variables are very large. It combines tree predictors such that the response of each tree depends on a set of predictor values of a random vector chosen independently. The random vectors are generated from a fixed probability distribution for all trees in the forest.

Random forests are resistant to overfitting and generalized well to new data. In addition, random forests show high levels of predictive accuracy and maintain accuracy by imputing missing data when a large proportion of the data are missing. Random forests do not need for prior feature selection because it trains

rapidly even with thousands of potential predictors. It has shown excellent performance in settings where the number of features is much larger than the number of samples. However, random forests are prone to overfitting for some datasets and do not handle large number of irrelevant features.

14.5 *K*-Nearest Neighbor (*K*-NN)

The *K*-nearest neighbor (*K*-NN) algorithm is a nonparametric method for classification and is based on the idea that a sample with similar-valued features is likely to have the same level in feature space. *K*-NN classifies an unlabeled sample based on their similarity with examples in the training set and is analytically simple and nearly optimal in the large sample limit. However, it requires large storage and is highly susceptible to the curse of dimensionality.

The use of large values of *K* yields smoother decision regions and provides probabilistic information. However, too large values of *K* are detrimental: it destroys the locality of the estimation. In addition, it increases the computational burden. To avoid the effects of the curse of dimensionality, dimensional reduction is usually performed before applying the *K*-NN algorithm for high-dimensional data.

15 Evaluating the Performance of a Model

A common problem with machine learning is overfitting, meaning a bias toward the samples in the training set. An algorithm may perform well on training dataset, but not generalize to other samples. Therefore, once a model is identified based on training dataset, the performance of the model is evaluated to identify the best from amongst the different models.

Cross-validation is most commonly used for the evaluation of model performance, where the data is partitioned into training and testing data in several different ways. The testing dataset is used as the final unbiased estimate of the performance of a model, and the performance of a model averages over all of these folds.

15.1 *Cross-validation*

Cross-validation is a simple concept. A dataset is usually partitioned into training and test datasets by using *N*-fold or leave-one-out method. For *N*-fold cross-validation, a dataset is divided into *N* random sample subsets with equal size. Each subset is used as a test dataset and the rest *N*−1 subsets are used as a training dataset to learn a classifier. The performance of a model is evaluated using the test dataset. The procedure is run *N* times, which give *N* accuracies. The final estimated accuracy of learning is the average of the *N* accuracies. Fivefold and tenfold cross-validations are commonly used.

The leave-one-out (LOO) method is a special case of cross-validation and is used when the dataset is very small. Each fold of the cross-validation has only a single test example and all the rest of the data is used in training. If the original data has *M* examples, this is *M*-fold cross-validation.

15.2 Measure of Performance

The performance of a model can be measured using error rate, accuracy, precision, sensitivity, specificity, recall, confusion matrix, and ROC curve. The simplest performance measure of machine learning algorithms is accuracy, the proportion of correctly classified samples. The precision of a model is the ratio of the number of true positives to the total number of predicted positives. It is a measure of how accurate the positive predictions are, or how precise the model is in predicting. A recall of a model is just another name for the true positive rate. Recall (or true positive rate, or detection rate) is proportion of positive examples that are classified correctly.

A confusion matrix, also known as an error matrix, is appropriate when predicting a categorical target. Receiver Operating Characteristic (ROC) curves is an alternative to precision/recall curves. Area under ROC curve (AUC) indicates probability that classifier will rank a randomly chosen positive instance higher than a randomly chosen negative instance.

16 Concluding Remarks

A number of groups have studied CpG island hypermethylation in biologic fluids to discover candidate CpGs for the early detection of lung cancer. However, the majority of studies have been performed with samples from invasive lung cancer. Epigenetic or genetic changes in the bronchial tree are an initial step in field cancerization, which subsequently progress to premalignant foci such as hyperplasia and dysplasia. The progression of dysplasia to invasive carcinoma generally reflects ongoing selection of clonally advantageous genetic or epigenetic changes. Methylation profiling in invasive cancer may not totally reflect molecular changes associated with field cancerization and therefore, analysis of CpG island hypermethylation in premalignant lesions is required.

Bronchial aspirates are valuable in the evaluation of peripheral lung lesion. In contrast, sputum samples may be beneficial to the detection of central tumors (squamous cell and small-cell carcinomas) arising from the larger bronchi and be important sources of diagnosis in bronchoscopically nonvisible tumors. Therefore, a combination examination of prebronchoscopic sputum and bronchial aspirates is complementary to each other and is likely to enhance the total diagnostic yield.

The degree of hypermethylation of TSGs is usually measured quantitatively in most experiments. Candidate CpGs for the early detection of lung cancer are then selected by comparing the difference of methylation levels between cancer patients and healthy individuals. A serious problem in analyzing hypermethylation of CpG islands in bronchial aspirate from cancer patients is the technical contamination of normal bronchial epithelial cells in the aspirate.

This may result in reduction of true methylation levels in cancer patients and in misclassification of the cancer patient. Accordingly, analysis of CpG islands in bronchial biopsy samples may show higher accuracy in classifying lung cancer than in bronchial aspirates or sputum. The same problem also occurs in plasma and serum since there is normal DNA released from breakdown of normal lymphocyte. The adjustment for the biased methylation levels in individual CpGs is required for accurate classification of lung cancer in plasma or serum.

Although a large number of genes that are frequently methylated in NSCLCs have been identified, only single genes or small numbers of genes were investigated for methylation in a large number of studies. Most cancers arise from alteration of multiple genes and therefore profiling of DNA methylation across the genome is paramount in discovering epigenetic biomarkers for the early detection of lung cancer.

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Analysis of DNA Methylation in Pancreatic Cancer: An Update

Christian Pilarsky and Robert Grützmann

Abstract

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive tumor and the fourth common cause of cancer death in the Western world. The lack of effective therapeutic strategies is due to the late diagnosis of this disease. Methylation markers could improve early detection and help in the surveillance of PDAC after treatment. Analysis of hypermethylation in the tumor tissue might help to identify new therapeutic strategies and aid in the understanding of the pathophysiological changes occurring in pancreatic cancer. There are several methods for the detection of methylated events, but methylation-specific PCR (MSP-PCR) is the method of choice if a small number of genes will be tested in a larger set of patients samples. After isolation of the DNA by standard procedure, the DNA is then modified using sodium bisulfide.

Key words Pancreatic cancer, Methylation, Bisulfide, Plasma tissue, PCR

1 Introduction

Pancreatic cancer is still one of the most malignant and aggressive types of cancer in humans with a very dismal prognosis. 40,000 new cases are diagnosed in the USA each year making pancreatic cancer the fourth male and the fifth female leading cause of cancer-related death [1]. The most abundant form of exocrine pancreatic cancer is ductal adenocarcinoma (PDAC) [2]. In the last decades only small improvements could be made in the therapy of this disease, which is mainly due to the delayed appearance of symptoms causing a late diagnosis. Only approximately 85 % of the patients show an organ overlapping growth of the tumor when the disease is discovered and only the remaining patients have an opportunity of curative surgical treatment. Therefore, the actual 5-year survival rate after surgical resection is about 20 %, and for all patients about 5 % [1].

DNA hypermethylation of the coding areas of the human genome is a hallmark in cancer development and was identified in a series of landmark investigations in the late twentieth century [3, 4]. Since then DNA hypermethylation has been investigated in nearly

all cancers and it has been shown that DNA hypermethylation might be a useful tumor marker [3]. Moreover, based on data analysis, it has been assumed that hypermethylation of genes might be an early event in tumorigenesis [5]. Hypermethylation has been shown to occur already in pancreatic intraepithelial neoplasia (PanIN) lesions indicating that epigenetic changes might be interesting candidates for the development of early diagnosis marker [6, 7]. Methylation marker candidates might be identified in different ways, either direct by using small-scale analysis of the methylome [8, 9] or by bioinformatics analysis of gene expression data, linking the observed under expression of genes with data from other sources. Next-generation sequencing provides a new approach into the methylome for the identification of new marker and insight into the basic changes of tumor development [10–12]. Since sequencing of the complete human is still cost and time intensive, other large-scale methods for the detection of promoter methylation are now used to characterize the methylome of tumor cells. Illumina's Infinium HumanMethylation450 BeadChip is now the method of choice to investigate the methylation status for over 450,000 sites [13–15]. The availability of such tools for the large-scale characterization leads to the need of post-discovery validation of the identified methylated sites. This validation can be done by different techniques but MSP-PCR remains a quick and easy method for such purposes.

In pancreatic cancer several studies have investigated hypermethylation in tumor tissue and body fluids revealing more than 100 possible marker genes available for testing in different settings [6, 16–33]. However, a major impediment is the low number of primary samples analyzed. Therefore new studies are needed to investigate those markers in a large number of samples of PDAC, other forms of pancreatic cancers, and chronic pancreatitis to establish reliable methylation marker for early diagnosis, clinical monitoring, and prognosis.

2 Materials

2.1 Tissue

PDAC tissue can be used from different sources like fresh frozen or formalin fixed paraffin embedded. However due to the heterogeneity of PDAC it has outmost importance that each tissue sample should be evaluated by a trained pathologist.

Blood plasma can be obtained easily from patients during routine blood draws. It is critical that the plasma is free from white blood cells; therefore the plasma should be centrifuged twice before storage.

2.2 Isolation and Modification of DNA

QIAamp® DNA Mini Kit (Qiagen, Hilden, Germany)

EZ DNA Methylation-Gold Kit (Zymo Research, Orange, CA, USA). In recent years this technology has become the mainstay

for methylation analysis and the EZ DNA Methylation-Gold Kit has been the best performer.

2.3 PCR

For primer design several tools are available. However, we have made our best experience with MethPrimer (<http://www.urogene.org/methprimer/index1.html>). Another source is the usage of already describes primer combinations. It might be feasible to use the computational modified DNA i.e. from Methprimer to generate own primer with the use of other programs in which the parameter can be better controlled like Primer 3 (<http://bioinfo.ut.ee/primer3-0.4.0/primer3/input.htm>). To identify the ideal sequence for PCR it might also be worthwhile to analyze the primer and the target sequences with a methBLAST (<http://medgen.ugent.be/methBLAST/>) to identify sequence homologies. All primers should be tested on fully methylated DNA, which can be obtained from Millipore (Billerica, MA, USA).

For all the experiments routine labware is needed, but the source is not of importance as long as a Tier1 provider supplied it. The performance of enzymes, chemicals, plasticware, and equipment from such high-quality providers is nearly identical. Beware of your source of water; sloppy prepared water is the number one cause of contaminants of reactions and therefore the number one reason why experiments fail. Performing a large number of PCR experiments requires high standard of cleanliness to reduce the risk of cross contaminants to bare minimum.

3 Methods

3.1 DNA Isolation from Plasma

Add 200 μ l plasma to the microcentrifuge tube. If the sample volume is less than 200 μ l, add the appropriate volume of PBS (*see Note 1*).

Add 200 μ l Buffer AL to the sample. Mix thoroughly by pulse-vortexing for 15 s.

Incubate at 56 °C for 10 min.

Add 200 μ l ethanol (96–100 %) to the sample, and mix again by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid (*see Note 2*).

Carefully apply the mixture from **step 5** to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at $6,000\times g$ for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate (*see Note 3*).

Open the QIAamp Mini spin column and add 500 μ l Buffer AW1 without wetting the rim. Close the cap and centrifuge at $6,000\times g$ for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and discard the collection tube containing the filtrate.

Carefully open the QIAamp Mini spin column and add 500 μ l Buffer AW2 without wetting the rim. Close the cap and centrifuge at $20,000\times g$ for 3 min.

Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at $20,000\times g$ for 1 min.

Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 μ l Buffer AE or distilled water. Incubate at room temperature (15–25 °C) for 1 min, and then centrifuge at $6,000\times g$ for 1 min (*see Note 4*).

3.2 From Frozen or Formalin-Fixed Paraffin-Embedded Tissue

Excise the tissue sample or remove it from storage. Determine the amount of tissue. Do not use more than 25 mg (*see Note 5*).

If samples are large mechanically disrupt the tissue sample (*see Note 6*).

Add 20 μ l proteinase K (source of proteinase K or units), mix by vortexing, and incubate at 56 °C until the tissue is completely lysed. Vortex occasionally during incubation to disperse the sample, or place in a shaking water bath or on a rocking platform (*see Note 7*).

Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

Add 200 μ l Buffer AL to the sample, mix by pulse-vortexing for 15 s, and incubate at 70 °C for 10 min. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid.

It is essential that the sample and Buffer AL are mixed thoroughly to yield a homogeneous solution.

Add 200 μ l ethanol (96–100 %) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from inside the lid. Follow the protocol for plasma DNA isolation from **step 6**.

3.3 Bisulfite Modification

For the bisulfite modification samples containing 500 pg to 2 μ g of DNA can be used. For optimal results, the amount of input DNA should be from 200 to 500 ng.

The DNA you have prepared using a photometer to determine the absorbance at 260 nm (*see Note 4*).

Prepare the conversion Reagent. Add 900 μ l water, 300 μ l of M-Dilution buffer, and 50 μ l M-Dissolving buffer to a tube of CT conversion reagent.

Mix at room temperature with frequent vortexing or shaking for 10 min. Note: It is normal to see trace amounts of undissolved reagent in the CT conversion reagent (*see Note 8*).

Preparation of M-Wash buffer Add 24 ml of 100 % ethanol to the 6 ml M-Wash buffer concentrate (D5005) or 96 ml of 100 % ethanol to the 24 ml M-Wash buffer concentrate (D5006) before use.

Add 130 μl of the CT conversion reagent to 20 μl of your DNA sample in a PCR tube.

Place the sample tube in a thermal cycler and perform the following steps (*see Note 9*):

98 °C for 10 min

64 °C for 2.5 h

4 °C storage up to 20 h.

Add 600 μl of M-Binding buffer to a Zymo-Spin IC Column and place the column into a provided collection tube.

Load the sample (from **step 2**) into the Zymo-SpinTM IC Column containing the M-Binding buffer. Close the cap and mix by inverting the column several times.

Centrifuge at $20,000\times g$ for 30 s. Discard the flow-through.

Add 100 μl of M-Wash buffer to the column. Centrifuge at $20,000\times g$ for 30 s.

Add 200 μl of M-Desulfonation buffer to the column and let stand at room temperature (20–30 °C) for 15–20 min. After the incubation, centrifuge at $20,000\times g$ for 30 s.

Add 200 μl of M-Wash buffer to the column. Centrifuge at $20,000\times g$ for 30 s. Add another 200 μl of M-Wash buffer and centrifuge at $20,000\times g$ for an additional 30 s.

Place the column into a 1.5 ml microcentrifuge tube. Add 10 μl of M-Elution buffer directly to the column matrix. Centrifuge for 30 s at $20,000\times g$ to elute the DNA (*see Note 10*).

3.4 Results

We have isolated, modified, and amplified DNA from the various sources including formalin-fixed paraffin-embedded (FFPE) tissue from 15 years ago. We were also able to demonstrate changes in methylated genes between different types of pancreatic cancer [34]. It is however easier to use DNA isolated from frozen tissue, since the DNA quality is higher even after long time storage.

4 Notes

1. To avoid the lysis of white blood cells in the samples several precautions have to be made. The plasma should be drawn using a Vacutainer and can be stored up to 4 h before centrifugation. The centrifugation should be performed at +4 °C without brakes. Plasma should be centrifuged twice and aspiration of cells should be avoided.
2. It is possible to add QIAGEN Protease (or proteinase K) to samples that have already been dispensed into microcentrifuge tubes. In this case, it is important to ensure proper mixing after adding the enzyme.

If the sample volume is larger than 200 μl , increase the amount of QIAGEN Protease (or proteinase K), Buffer AL,

and ethanol proportionally. Do not add QIAGEN Protease or proteinase K directly to Buffer AL. Close each spin column in order to avoid aerosol formation during centrifugation.

3. Incubating the QIAamp Mini spin column loaded with Buffer AE or water for 5 min at room temperature before centrifugation generally increases DNA yield. A second elution step with a further 200 µl Buffer AE will increase yields by up to 15 %. Volumes of more than 200 µl should not be eluted into a 1.5 ml microcentrifuge tube because the spin column will come into contact with the eluate, leading to possible aerosol formation during centrifugation. Elution with volumes of less than 200 µl increases the final DNA concentration in the eluate significantly, but slightly reduces the overall DNA yield.
4. For long-term storage of DNA, eluting in Buffer AE and storing at -20°C is recommended, since DNA stored in water is subject to acid hydrolysis. UV spectroscopy is the main method to determine DNA concentrations. However during purification of genomic DNA contaminants such as RNA and small single stranded DNA are co purified. This will lead to a high divergence in the measured concentration of the DNA. If high-throughput techniques for methylation analysis are used the DNA concentration should be determined more carefully. It is not sufficient to ascertain the DNA concentration by UV spectroscopy. Instead a combination of gel electrophoresis and Picogreen (Invitrogen, Carlsbad, CA) should be used.
5. The QIAamp DNA Mini Kit can also be used to isolate DNA from fixed tissues. However, the length of DNA isolated from fixed tissues is usually <650 bp, depending on the type and age of the sample and the quality of the fixative used [35]. Use of fixatives such as alcohol and formalin are recommended. Fixatives that cause cross-linking, such as osmium tetroxide, are not recommended as it can be difficult to obtain amplifiable DNA from tissue fixed with these agents. Cut slices of the embedded tissue and collect them in a 1.5 ml microcentrifuge tube and proceed with **step 3**. It is not necessary to remove the paraffin in advance since it will melt during the incubation at 56°C .
6. Some tissues require undiluted Buffer ATL for complete lysis. In this case, grinding in liquid nitrogen is recommended. Samples cannot be homogenized directly in Buffer ATL, which contains detergent.
7. Proteinase K must be used. QIAGEN Protease has reduced activity in the presence of Buffer ATL. Lysis time varies depending on the type of tissue processed. Lysis is usually complete in 1–3 h. Lysis overnight is possible and does not influence the preparation. In order to ensure efficient lysis, a shaking water bath or a rocking platform should be used. If not available, vortexing 2–3 times per hour during incubation is recommended.
8. Each tube of CT Conversion Reagent is designed for ten separate DNA treatments. Storage: The CT Conversion

Reagent is light sensitive; so minimize its exposure to light. For best results, the CT Conversion Reagent should be used immediately following preparation. If not used immediately, the CT Conversion Reagent solution can be stored overnight at room temperature, 1 week at 4 °C, or up to 1 month at -20 °C. Stored CT Conversion Reagent solution must be warmed to 37 °C, and then vortexed prior to use. Do not use old Conversion Reagent.

9. For DNA volumes >20 µl, an adjustment needs to be made during the preparation of the CT Conversion Reagent. The amount of water is decreased 100 µl for each 10 µl increase in DNA sample volume. For example, for a 40 µl DNA sample, 700 µl of water is added to make the CT Conversion Reagent. The maximum DNA sample volume to be used for each conversion reaction is 50 µl. Do not adjust the volumes of either the M-Dissolving Buffer or M-Dilution Buffer. The capacity of the collection tube with the column inserted is 800 µl. Empty the collections tube whenever necessary to prevent contamination of the column contents by the flow- through. Alternatively, water or TE (pH ≥ 6.0) can be used for elution if required for your experiments.
10. The DNA is ready for immediate analysis or can be stored at or below -20 °C for later use. For long-term storage, store at or below -70 °C. We recommend using 1–4 µl of eluted DNA for each PCR; however, up to 10 µl can be used if necessary. The elution volume can be >10 µl depending on the requirements of your experiments, but small elution volumes will yield more concentrated DNA. Do not store the modified DNA for a longer time, since it tends to degrade. We have obtained our best results with modified DNA stored for less than a month at -20 °C.

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Epigenetics of Urothelial Carcinoma

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Abstract

Urothelial carcinoma is the most frequent type of bladder cancer. Improvements in diagnostics and therapy of this common tumor are urgently required and need to be based on a better understanding of its biology. Epigenetic aberrations are crucial to urothelial carcinoma development and progression. They affect DNA methylation, histone modifications, chromatin remodeling, long noncoding RNAs, and microRNAs. Compared to other cancers, DNA hypomethylation, especially at LINE-1 retrotransposons, and mutations in enzymes establishing or removing histone acetylation or methylation are particularly prominent. Accumulating evidence suggests that disturbances in DNA methylation, histone modifications and noncoding RNAs may contribute especially to altered differentiation and metastatic potential. With proper selection, histone-modifying enzymes may constitute good targets for therapy. For diagnostics, DNA methylation and miRNA biomarkers are well suited because of their relatively high stability. There are indeed excellent biomarker candidates for DNA-methylation-based diagnostics of urothelial carcinoma, whereas miRNAs are well investigated, but there are still many discrepancies between studies published to date.

Key words Biomarker, Diagnosis, Epigenetics, Methylation, Urothelial carcinoma

1 Introduction

Urothelial carcinoma (UC) is the most prevalent histological subtype of bladder cancer, a common cancer worldwide [1]. Urothelial carcinoma again comprises two different, but not entirely distinct, kinds of cancer, namely papillary and invasive UC which differ in terms of biology and clinical behavior [2, 3]. Low-grade papillary carcinoma carries a low risk of mortality, but tends to recur frequently. High-grade papillary carcinoma, in particular, may progress towards the more lethal invasive carcinoma. More commonly, invasive carcinoma develops from high-grade dysplastic lesions designated carcinoma in situ.

A major difference between papillary and invasive UC is the extent of genetic and chromosomal instability [2, 3]. Low-grade papillary tumors are usually near-diploid with a limited number of

mutations in oncogenes that drive hyperproliferation such as *FGFR3*, *PI3KCA*, and *HRAS*, complemented by inactivation of *CDKN2A* encoding the cell cycle inhibitor p16^{INK4A}. In contrast, invasive carcinomas have a high rate of point mutations in various oncogenes and tumor suppressors, and numerous numerical and structural chromosomal aberrations. The most common mutations inactivate *TP53* and activate the *hTERT* (telomerase) promoter, whereas *FGFR3* mutations are rarer. Chromosomal instability is pronounced and may be driven by mutations and deregulation of cell division and checkpoint proteins. *CDKN2A* homozygous deletions are found in invasive carcinomas alternative to the more severe loss of *RBI*. Loss of cell cycle control is exacerbated in many cases by amplification of *CCND1*/Cyclin D1 and *CCNE1*/Cyclin E1.

The heterogeneity of urothelial carcinoma requires an individualized approach to its treatment. Therapeutic options include transurethral resection for early-stage tumors, radical cystectomy, radiotherapy and chemotherapy for invasive cancers, and immunotherapy for prevention of recurrences. Unfortunately, these treatments cure only some patients with muscle-invasive urothelial cancer and modern targeted therapies have proven beneficial in only few cases [1]. Moreover, long-term monitoring of all patients is required and several consecutive interventions may become necessary, making the treatment of bladder cancer very expensive.

Given these clinical conundrums, improved therapies for urothelial carcinoma are urgently required. Moreover, biomarkers for prognosis, diagnostic monitoring, and prediction of response to chemotherapy would be very helpful in clinical practice. In this regard, although several biomarkers have been introduced for detection and monitoring of urothelial carcinoma that can conveniently be used on urine samples, none of them is considered fully satisfactory.

There is now considerable evidence that epigenetic disturbances contribute to the pathogenesis of urothelial carcinoma [4–8]. In addition to the many alterations in DNA methylation that have been catalogued over the last two decades, recent results highlight the importance of other epigenetic factors such as histone modifiers that are commonly mutated or deregulated in urothelial carcinoma, at an overall similar frequency to *TP53*. Moreover, a large body of recent literature addresses functions of noncoding RNAs in this cancer type. We will discuss these findings in the light of the following questions: How many common epigenetic alterations contribute to the pathogenesis of urothelial carcinoma? How could these alterations be exploited to develop more efficacious therapies for this cancer? Which options do epigenetic alterations offer for the development of robust biomarkers for detection, monitoring and prognosis to improve individualized therapy of patients with urothelial carcinoma?

2 DNA Hypermethylation

Until recently, DNA hypermethylation in urothelial carcinoma (UC) was mainly studied by investigating CpG-island promoters of candidate genes, typically using qualitative methylation-specific PCR (MS-PCR). However, this elegant and straightforward method is not well suited for partially and heterogeneously methylated sequences. In retrospect, this limitation, together with the notorious heterogeneity of UC, may explain why the results from different groups yielded quite different results. The studies from that period have been comprehensively reviewed [5, 6, 9]. Despite their limitations, collectively they demonstrated a strong tendency for DNA hypermethylation to increase with tumor stage and grade and indicated that suitable panels of DNA methylation markers might be useful for detection and monitoring of UC in urine. Indeed several studies in patients have been performed to explore the diagnostic potential of various methylation marker panels [10–16]. The application of methylation markers was aided by the development of novel techniques more robust or more quantitative than MS-PCR, such as quantitative MS-PCR and bisulfite pyrosequencing. Furthermore, hypermethylation of several candidate genes was found to correlate with their decreased expression and the function of these genes was explored. However, the overall impact of DNA hypermethylation on UC biology is difficult to estimate from the investigation of individual candidate genes. For instance, several genes involved in apoptosis regulation were found to be hypermethylated at significant frequencies [6], but it is still unclear to which extent these changes act as an impediment to apoptosis in UC.

The advent of techniques allowing more global and less biased screening for DNA methylation alterations such as the bead arrays or methylation-dependent immunoprecipitation (MeDIP) coupled to CpG-island array hybridization has provided the potential for obtaining a more comprehensive picture of both hyper- and hypomethylation changes, thereby improving the selection of potential biomarkers and deeper insights into UC biology.

In addition, screening of methylation changes across the genome allows to address the question of how changes in DNA methylation relate to genomic changes such as chromosomal gains and gene amplification [17, 18]. For instance, gene silencing in some human cancers tends to occur across larger chromosomal regions encompassing many genes. Although this phenomenon, termed long-range epigenetic silencing (LRES) appears to be associated more with a shift in histone modification patterns, DNA hypermethylation may also contribute [19]. In UC, it will be interesting to learn whether several regions exhibiting LRES described in one study [20] will also show up in further studies.

Table 1
Comprehensive studies of DNA methylation changes in urothelial carcinoma

Purpose	Approach	Result	Ref No.
Tumor detection in urine	Azacytidine treatment of cell lines	Biomarker panel for detection	[22]
Differences between UC subtypes	Bead array (early version)	Distinct profiles for subtypes; evidence for field effect	[23]
Distinction tumor vs. normal	Based on Wolff [23]	Biomarker panel for detection	[30]
Distinction between UC subtypes	Bead array	Biomarker for identification of high-grade cases	[31]
Tumor detection in urine	Bead array	Biomarker panel for detection; candidate for prognosis	[26]
Identification of progressive pTa tumors	CpG-island array + bead array	Biomarker candidates, distinct profiles for subtypes	[27]
Differences between UC subtypes	Bead array	Distinct profiles for subtypes	[18]
Biomarkers for recurrence of non-muscle invasive UC	Bead array	Biomarker candidates	[29]

Conversely, DNA hypomethylation events also appear to cluster in large genomic regions, particularly blocks of sequence that are associated with the nuclear membrane and less transcriptionally active in normal cells [21]. To which extent this observation pertains to UC with its pronounced hypomethylation (*see* below) cannot yet be definitely discerned.

The primary aim of the large-scale studies of DNA methylation in UC published to date has been the identification of suitable biomarkers for diagnostic and prognostic purposes. A number of key studies are listed in Table 1 [18, 22–30]. A comprehensive analysis of DNA methylation changes is also expected from the ongoing integrative analysis of genetic and epigenetic alterations of bladder cancers by the TCGA consortium (<http://cancergenome.nih.gov/>).

Costa et al. [22] identified hypermethylated genes initially as those becoming substantially upregulated upon treatment with the DNA methylation inhibitor 5-azacytidine in UC cell lines as well as being substantially downregulated in tumor vs. normal tissues. Genes containing CpG-islands were then analyzed by MS-PCR to select those actually showing hypermethylation. This approach yielded a three gene panel capable of distinguishing tumor tissues and detecting tumors from urine sediments by quantitative MS-PCR with exceptional sensitivity and specificity.

Wolff et al. [23] applied an early version of the Illumina methylation array to directly compare DNA methylation between non-invasive urothelial tumors, invasive tumors and urothelium from

cancer-carrying bladders. In line with expectations, there were more hypermethylation events in muscle-invasive cancers, but somewhat unexpectedly, a number of CpG sites were specifically hypomethylated (compared to urothelium and invasive cancers) in noninvasive tumors. A degree of hypermethylation was also observed in morphologically normal-appearing tissues. As these hypermethylation events are unlikely to result from occult expansion of tumor clones, these findings support a field effect in the bladder of UC patients with widespread epigenetic changes in the urothelium from which the actual tumors and their recurrences arise. This study was continued by Chihara et al. 2013 [30] who validated a panel of hypermethylation markers in tissues and urine samples by bisulfite pyrosequencing, achieving nearly ideal accuracy in distinguishing tumor and normal tissues.

Marsit et al. [31] likewise used a methylation bead array in order to detect hypermethylation events distinguishing between more or less aggressive forms of bladder cancer. Their screening yielded several loci associated with muscle-invasive and particularly high-grade cancers which they validated in an independent cohort using bisulfite sequencing. Intriguingly, the same group also identified a panel of methylation biomarkers in blood cells strongly associated with bladder cancer [32].

Array-based screening in a Danish bladder cancer cohort [26] yielded another panel of hypermethylated loci. Interestingly, these authors too observed changes in *HOX* and *KRT* (cytokeratin) genes. Several candidates were validated in an independent cohort, using methylation-specific high-resolution melting, yielding high specificity and sensitivity in urine-based diagnostics. Methylation at *TBX4* was suggested as a prognostic marker.

Kandimalla et al. [27] focussed on the progression of noninvasive papillary (pTa) tumors. For screening they employed a CpG-island array and performed validation with a custom-designed bead array. Interestingly, their set of candidates again comprised many *HOX* genes and genes encoding tissue-specific transcription factors; validated hypermethylated genes were *TBX2*, *TBX3*, *GATA2*, and *ZIC4*. A large fraction of pTa tumors containing oncogenic mutations in *FGFR3* displayed overall less hypermethylation than those with wild-type *FGFR3* and their methylated genes were quite distinct, a finding corroborated by Serizawa et al. [33]. This finding hints at the existence of “methylation subtypes” in UC.

Lauss et al. [18] addressed this issue across all UC stages using methylation analysis by bead arrays. Clustering by DNA methylation indeed yielded several “epitypes.” This analysis concurs in several respects with the others in that muscle-invasive tumors presented with the highest degree of hypermethylation, *HOX* genes were frequent targets of hypermethylation, a cluster of tumors with frequent gene hypomethylation was discernible, and *FGFR3* mutant tumors had overall less hypermethylation.

Additionally, this study provided insights into the mechanisms underlying these patterns. Thus, one epitype encompassing tumors of various stages and grades was characterized by strong overexpression of *EZH2* and frequent hypermethylation of developmental genes (including *HOX* genes) that are polycomb targets. These tumors therefore appear to be subject to an epigenetic pathogenicity mechanism observed in other cancer types [34] in which genes bound by polycomb PRC2 complexes and carrying the H3K27me3 modification are prone to DNA hypermethylation in cancer. This association has also been observed in UC cell lines [28]. It remains unknown what drives methylation changes in the other UC epitypes, in particular another cluster consisting of high-grade cases harboring a different pattern of frequent hypermethylation.

Employing novel bioinformatic methods allow to infer copy number changes from bead array methylation data. Lauss et al. [18] searched for correlations between DNA methylation and copy number changes. Positive correlations with DNA methylation changes were only observed for copy number gains, in particular for cytokeratin (*KRT*) genes. The prevalence of altered methylation of *KRT* genes in methylation screening studies may therefore be related to a high frequency of copy number changes at these genes.

Kim et al. [29] searched for hypermethylation biomarkers in non-muscle-invasive (i.e., pTa and pT1) tumors by first screening of a subset using bead arrays. Candidate genes were further selected by their diminished expression in tumor tissues and validated by bisulfite pyrosequencing in the full cohort. Three genes, including *HOXA9*, turned out each as independent predictors of disease recurrence.

While further studies are under way, several conclusions are evident from those published to date. First, even if one takes positive publication bias into account, DNA hypermethylation biomarkers appear to be excellently suited for the detection of bladder cancer in urine. All studies addressing this issue have reported sensitivities and specificities far exceeding those of current techniques such as urine cytology, cytogenetics, or other molecular biomarkers. Prospective studies should be begun with the aim of translating these exciting findings into clinical routine. The issue of prognostic markers is more complex and will require more exploratory studies. On the biological side, much of the data converges towards a common set of genes with altered methylation enriched in transcription factors, cytokeratin genes and polycomb targets. A tentative interpretation of these findings is that DNA methylation alterations in UC could be involved particularly in altered cell fate and differentiation.

This conclusion is quite different from that which one would have made on the basis of the older candidate gene studies, which preferred genes regulating cell proliferation and apoptosis.

Notably, few if any of these have reappeared in the recent, less biased investigations. On a note of caution, most recent studies have used the same technique, namely bead arrays. While these have developed over time to cover a larger part of the genome, some bias might have become introduced this way. Studies using screening strategies by a different technique (such as methylation capture followed by sequencing or reduced representation bisulfite sequencing) should be enlightening. Of course, the question whether pro-apoptotic genes or genes involved in cell differentiation are primarily hypermethylated in urothelial cancers is not academic. Reactivation of silenced pro-apoptotic genes by DNA methyltransferase inhibitors is expected to be therapeutically efficacious in a straightforward manner, but what reactivation of differentiation-associated genes might achieve is more difficult to predict.

3 DNA Hypomethylation

Bead arrays give a quantitative measure of methylation at individual CpG-sites and thereby allow investigating hypomethylation at individual genes, which had been tedious in the past. When interpreting such results it is important to consider that most CpG-islands are unmethylated in normal tissues. Therefore, CpG-sites hypomethylated in cancer, of necessity, must be located in the minority of CpG-islands methylated in normal tissues, in gene bodies or at intergenic sites, including CpG-island shores and enhancers. In addition, about half of human gene promoters have moderate or low CpG-content and tend to be partly and heterogeneously methylated. Therefore, while promoter CpG-island hypermethylation is often quite straightforwardly correlated with decreased gene expression or a switch to a different transcript of the same gene, this is not the rule for hypomethylation. Heterogeneous and cell-type-specific methylation in particular may create pitfalls in the interpretation of hypomethylation data [35]. As summarized above, large-scale investigations of methylation changes in urothelial carcinoma have revealed subsets of genes as well as epigenetic subtypes of the cancer with conspicuous patterns of gene hypomethylation that require further investigation for their functional impact. In particular, a thorough investigation of CpG-island shores and enhancers, which are often methylated in a tissue-specific manner, is still lacking in urothelial carcinoma and may require larger coverage of the genome than provided by array techniques.

One class of sequences strongly methylated in normal tissues are repeats, including CpG-rich satellite repeats such as SAT2 and SAT3, long-interspersed retroelements such as LINE-1, short-interspersed retroelements like ALUs and remnants of ancient

retroviruses such as HERV-K littering the human genome. Such sequences may become hypomethylated in cancer [36–38]. However, due to technical limitations in the analysis of repeat sequences by current techniques, mostly global data are available. A detailed comprehensive analysis of repeat sequence methylation will likely become feasible only with third-generation sequencing techniques delivering long reads with sufficient accuracy.

In urothelial carcinoma, hypomethylation of repeat sequences is highly prevalent and often pronounced. While hypomethylation also affects satellite repeats [39–41], the best investigated repeats are LINE-1, a class of LTR-less retroelements comprising approximately 18 % of the human genome. Full-length elements contain an internal promoter within their 5'-UTR that is relatively CpG rich. These promoters are pervasively methylated in normal somatic tissues ensuring repression of transcription and preventing adverse effects, especially retrotransposition [42, 43]. LINE-1 methylation decreases by up to 70 % in UC, with significant changes in almost all tumors [44, 45]. This hypomethylation is associated with increased transcription of full-length elements [46], of which some should be capable of actually retrotransposing. It is not yet known to which extent retrotranspositions occur in bladder cancer, as this requires dedicated bioinformatic analysis of whole-genome sequences [47] or specialized enrichment techniques [48].

Nevertheless, the data available so far predict that the impact of activated LINE-1 sequences on genomic instability may be substantial. In particular, the recent large-scale sequencing efforts have revealed a novel pattern of mutations named “kataegis” which appears to be caused by endogenous DNA cytidine deaminases, in particular APOBEC3B [49, 50]. These enzymes normally protect the genome from retroviruses and endogenous retroelements, especially LINE-1, but may be misdirected to the cell genome by LINE-1 elements attempting to reintegrate. Therefore, the unexpected high frequency of kataegis in UC might relate to the high activity of LINE-1 retrotransposons.

LINE-1 hypomethylation in UC may ultimately promote genomic instability and mutagenesis, but is also likely to exert local effects. In particular, hypomethylated LINE-1 promoters are bidirectional. While the sense promoter directs transcription of the retroelement itself, the antisense promoter leads to hybrid transcripts including exons from neighboring genes. One such transcript leads to overexpression of the MET receptor tyrosine kinase, which can promote cell proliferation and migration, in many bladder cancers [51]. Intriguingly, the hybrid transcript was also found across the urothelium of cancer-carrying bladders, providing another indication of the epigenetic field effect in urothelial carcinogenesis.

In cancer patients, LINE-1 hypomethylation can often be detected in blood cells and provides another potential biomarker. This change appears to be an indirect consequence of tumorigenesis,

as there is no correlation between the methylation in the tumor itself and the patients' blood cells [52]. Instead, hypomethylation in blood cells is influenced by multiple genetic and environmental factors. LINE-1 hypomethylation in blood can be relatively easily ascertained and has therefore been proposed as a biomarker for bladder cancer [53]. However, meta-analyses of studies in various cancers suggest that this phenomenon, while clearly valid, may be of limited clinical utility [54, 55].

4 Histone Modifiers

Another big surprise from the recent large-scale mutational analyses of urothelial carcinoma is a high prevalence of mutations in genes encoding histone modifiers. It had been known for several years that the histone methyltransferase EZH2, which catalyzes (tri)methylation at histone H3 lysine 27 (H3K27me₃), is frequently overexpressed in UC [56–59]. There were also hints that inactivating mutations in the histone demethylase UTX (*KDM6A*), which catalyzes the reverse reaction, occur not only in renal cell carcinoma, but also UC [60]. The first whole-genome mutational analysis [61] indeed revealed rather frequent mutations in UTX, but frequent mutations were also observed in MLL H3K4 methyltransferases (*KMT2A-C*), in the histone acetyltransferase coactivators CBP and p300 (*CREBBP*, *EP300*) and in the histone demethylase Jarid-1 (*KDM5A*). A follow-up paper by the same group [62] added another histone deacetylase coactivator, TRRAP, to the list and reported a few mutations in the UTX paralog UTY. Yet another factor in this context is NCOR1, a transcriptional co-repressor interacting with the histone deacetylase HDAC3 [63]. Mutations in histone deacetylases are rare in cancer, but several members of the family are deregulated in urothelial carcinoma [64]. In addition, frequent mutations target chromatin remodelers like ARID1A and CHD6. The reported changes in chromatin regulators are corroborated by the data from the TCGA consortium as summarized in Table 2.

In molecular terms, the effect of these mutations can be predicted straightforwardly. All factors are involved in setting up patterns of histone modifications at promoters and enhancers [65–67]. In particular, they control methylation at H3K4 (MLL proteins, UTX vs. JARID-1A) and histone acetylation (CBP, p300, TRRAP vs. HDACs) which are modifications associated with active genes, or control methylation at H3K27 (EZH2, UTX), a modification associated with inactive genes [65]. Thus, mutations or altered expression of these enzymes are expected to change the pattern of histone modifications at promoters and enhancers that are crucial for transcription.

Table 2
Mutations in histone modifier and remodeling genes in UC

Guo et al., 2013					TCGA						
Gene	Main gene function	Types of mutations					Types of mutations				
		Overall mutations	Missense mutations	Nonsense, Frameshift, Splice site	Synonymous	Samples tested	Overall mutations	Missense	Nonsense, Frameshift, Splice site	Synonymous	Samples tested
ARID1A	ATP-dependent DNA helicase activity	21	4	17		99	42	9	32	1	120
CABIN1	Interaction with HDAC1 and HDAC2	8	6	1	1	99	3	1		2	103
CHD2	ATP-dependent DNA helicase activity	4	4			99	7	6	1		105
CHD4	ATP-dependent DNA helicase activity	4	3	1		99	8	8			105
CHD5	ATP-dependent DNA helicase activity	7	5		2	99					105
CHD6	ATP-dependent DNA helicase activity	7	4	1	2	99	12	8	2	2	112
CREBBP	Histone acetyltransferase	18	4	13	1	99	30	10	17	3	116
EP300	Histone acetyltransferase	16	7	9 ^a		99	32	20	9 ^a	3	117

<i>EP400</i>	Member of complex with histone acetyltransferase activity	7	3	4	99	11	4	7	103
<i>JARID1A</i>	Lysine-specific demethylase	5	5		99	4	2	2	105
<i>JMJD1B</i>	Lysine-specific demethylase	3	2	1	99	5	4	1	105
<i>MLL</i>	Histone methyltransferase	11	5	5	99	23	14	8 ^a	112
<i>MLL2</i>	Histone-lysine N-methyltransferase	2	2		99	27	10	14	105
<i>MLL3</i>	Histone-lysine N-methyltransferase	9	2	6	99	34	21	12	109
<i>NCOR1</i>	Recruitment of histone deacetylases	7	5	2	99	14	9	5	111
<i>SMARCA4</i>	ATP-dependent DNA helicase activity	6	5	1	99	10	7	3	106
<i>UTX</i>	Histone demethylase	40	6	32	99	39	5	32 ^a	141
<i>UTY</i>	Histone demethylase	5	1	4	99	3	1	1	105

Mutations reported by Guo et al., 2013, and by the TCGA consortium (accessed through the COSMIC database as of 10.1.2014) are listed. Genes considered had >5 % mutations in at least one of these studies

^aIncludes in-frame deletion

More difficult questions are which genes will be affected by the altered function or level of these chromatin modifiers and which processes in the tumor cells are accordingly changed. Histone modifiers typically regulate large sets of genes. Factors with similar enzymatic activity, even the closely related CBP and p300 acetyltransferases, have overlapping, but not identical target gene sets and transcription factor interaction partners [68]. Their effects are moreover pleiotropic and the same co-activator may interact with an oncogenic transcription factor such as MYC as well as a tumor suppressor like p53 [69]. Similarly, H3K4 trimethylation is required not only for the transcription of genes involved in cell differentiation, but also for the transcription of genes driving, e.g., cell proliferation [65]. In addition, maintenance and resetting of histone modifications is not only required for transcription, but also for other processes, including DNA replication and DNA repair. These different and even divergent requirements may explain why mutations observed in histone modifier genes appear to inactivate often only one allele. Obviously, while many arguments can be made a priori, the biological effects of these mutations will have to be elucidated by thorough experimental investigations in suitable models.

Another important question that will have to be addressed in the near future is how the mutations and expression changes in the various histone modifiers are related to clinical parameters. Some data is already available for EZH2 overexpression, which appears to be associated with more aggressive and high-grade cancers [56, 59, 70]. Conversely, UTX mutations tend to be more frequent in lower stage and lower grade cancers. However, further studies on sufficiently large cohorts are required to answer these questions.

Last, not least, the high frequency of mutations and expression changes in histone-modifiers suggests novel approaches to urothelial carcinoma therapy. All the factors involved are enzymes that can in principle be targeted by small-molecule drugs. Such drugs are already available for histone deacetylases, albeit with limited success so far in urothelial carcinoma [64], and are currently under investigation for EZH2. The novel findings on mutations of histone modifiers hint at the possibility that drugs against these enzymes may be more efficacious in some tumors than in others. For instance, specific HDAC inhibitors might be particularly active in cases with HAT mutations. Again, a better understanding of the precise impact of the observed mutations will be required as a basis for following that line of development.

5 Long Noncoding RNAs

Another great surprise of the last decade was the discovery that the human genome encodes only about 20,000 protein coding genes, accounting for less than 2 % of the genome [71]. However, the

advent of sensitive high-throughput techniques, especially next-generation sequencing revealed that about 90 % of the genome is actively transcribed [72] and led to the identification of a plethora of novel transcripts. Many belong into the category of long non-coding RNA (lncRNA) [73, 74] which are distinguished from small noncoding RNAs (*see below*) by being longer than 200 nucleotides. Many share characteristics of mRNAs such as being transcribed by RNA polymerase II, becoming polyadenylated and spliced. These transcripts may exert regulatory functions in gene regulation, development and metabolism [75–77]. Only the tip of the iceberg regarding their functions and underlying mechanisms may have been discovered. However, these initial insights are exciting enough to envision the complexity of further hidden layers in regulation of biological processes and nuclear topography.

The first reported lncRNAs were not yet recognized and named as such. An early example was H19 originating from an imprinted twin locus with *IGF2* [78] which is involved in epigenetic regulation and disease. The XIST RNA has a central function in X-chromosome inactivation [79, 80]. There is not yet a consensus classification for the many new lncRNAs. Designations like natural antisense transcript (NAT), enhancer RNAs (eRNA) [81], or large intergenic noncoding RNA (lincRNA) all define RNAs longer than 200 nucleotides.

One classification utilizes their genomic location without specific reference to their function. Thus, lncRNAs residing in distinct transcription units apart from protein coding genes have been referred to as lincRNAs [82, 83]. Natural antisense transcripts are transcribed in antisense direction to other genes with varying degree of overlap (reviewed in ref. 84). Pseudogenes are products of gene duplications or retrotranspositions having lost their coding potential, but may influence gene expression by epigenetic or post-transcriptional mechanisms. For instance, there are many pseudogenes of *POU5F1* encoding the transcription factor OCT4 regulating embryonic stem cell pluripotency and suspected to contribute to a stem cell phenotype in cancer. However, it is often unclear whether reported OCT4 increases in cancer cells are due to true OCT4A transcripts or pseudogenes [85]. A thorough analysis has recently been published for normal urothelium and urothelial cancer [86] confirming earlier indications that *POU5F1* as such is not upregulated in urothelial carcinoma [87]. A fourth class is defined by their localization in introns of annotated genes (long intronic noncoding RNAs). Yet another heterogeneous group of transcripts includes transcripts nascent from enhancers (eRNA) or promoters with regulatory function on genes in their proximity.

While many lncRNAs function in *cis*, others exert effects in *trans*, such as the lncRNA HOTAIR from the *HOXC* gene cluster [88, 89]. Several lncRNAs control allele-specific expression, as does XIST during X-inactivation [90, 91] and a number of

lncRNAs are involved in genomic imprinting. Many imprinted loci contain reciprocally expressed lncRNAs, e.g., *IGF2/H19*, *CDKN1C/LIT1* (also known as *KCNQ1OT1*), and *DLK1/GTL2* (also known as *MEG3*). They may function as recruiting factors for polycomb group protein complexes to maintain imprinting or serve as microRNA precursor as shown for H19 [92]. Aberrant expression of such regulators leads to imprinting disorders like Beckwith-Wiedemann syndrome. Accordingly, aberrant expression of lncRNA from imprinted loci is often associated in cancers with aberrant expression of the linked protein coding genes regulating cell proliferation and differentiation. All three mentioned imprinted loci are in fact deregulated in bladder cancer (Greife et al., submitted) [93, 94].

Some lncRNAs have been proven to be key factors in regulation of development, pluripotency, and lineage-specific differentiation [88]. Two well-studied lncRNA genes, *HOTTIP* and *HOTAIR*, reside in *HOX* clusters on different chromosomes. They were discovered by profiling polyadenylated transcripts from adult primary fibroblasts of different anatomic sites using a tiling array [89]. Amelioration of the signal intensity quantification by a signal processing algorithm adapted from use in computer vision (Otsu's method) yielded a quantitative measure of transcript abundance. The authors identified 407 transcribed regions within the *HOX* cluster and classified them into known gene exons and introns, as well as newly identified intergenic lncRNAs. Of these, *HOTTIP* and *HOTAIR* have been shown to be involved in regulating *HOX* gene expression in *cis* or in *trans* by recruitment of histone-modifying protein complexes, leading to activating H3K4 methylation (via binding to WDR5/MLL), repressive H3K27 methylation (via binding to EZH2), and H3K4 demethylation (via binding to LSD1). Chromosome conformation capturing to determine long-range interactions between distant chromosomal regions revealed that lncRNAs from the *HOXA* locus play a role in formation of 3D chromosomal loops [95] and may thus regulate the access of *HOXA* genes to transcription factories. Homeotic *HOX* genes encode key transcription factors for body pattern formation during embryonic development and tissue homeostasis in the adult organism [96]. Accordingly, many lncRNAs are expressed in a tissue specific manner and at subtle levels.

Aberrant expression of lncRNAs has been observed in a number of diseases including cancer [97–99]. The number of cancer associated noncoding RNAs has burgeoned over the last years, due to the evolution of microarray platforms and more affordable large-scale sequencing techniques [74, 100]. Both oncogenic and tumor-suppressive functions have been reported for these lncRNAs. In most cases, knowledge about the underlying mechanisms is still accumulating. Generally, interactions between lncRNAs and proteins are a common theme.

Such interactions can be investigated by RNA immunoprecipitation [101] or UV cross linked immunoprecipitation (CLIP) that will also provide positional information about the interaction [102]. Both techniques employ pull-down of ribonucleoprotein complexes by means of specific antibodies. The bound RNA can subsequently be analyzed by PCR or high-throughput RNA-sequencing. Of note, these techniques leave the question open how lncRNAs can specifically recruit protein complexes to specific target genes.

Although the mechanisms underlying the interaction between proteins and lncRNAs or the recruitment of protein complexes are far from completely understood, secondary and tertiary structures are clearly important. A number of different methods have been developed to characterize such structures, including RNase footprinting, FragSeq, PARS, and SHAPE [84, 103, 104]. Information on structure, function, and annotation of lncRNAs is available in databases [74, 105].

Technical approaches to analyze RNA-DNA interactions typically use RNA as a bait. In chromatin RNA immunoprecipitation (ChIRP) a specific RNA is used for capturing lncRNA-chromatin-complexes thereby allowing to map its binding sites genome wide [106]. Similar approaches comprise chromatin oligo-affinity precipitation (ChOP) [107] and capture hybridization of RNA targets (CHART) [108]. Of note, ChIRP is distinct from chromatin RNA immunoprecipitation (ChRIP) [109] using immunoprecipitated chromatin (ChIP) to extract the chromatin-associated RNA after DNase I treatment resulting in information about association between the RNA and a specific chromatin mark.

Genome-wide studies have revealed that lncRNAs function as scaffolds or recruit chromatin-modifying protein complexes such as polycomb repressive complex 2 (PRC2), leading to changes in chromatin condensation and transcription activity [76, 89].

Reported cancer-associated lncRNAs include HOTAIR, KCNQ1OT1/ LIT1, XIST, and ANRIL (Table 3). HOTAIR was initially reported overexpressed in progressive breast cancers [110], and subsequently found associated with an aggressive phenotype and poor prognosis in many other cancers including tumors of the endometrium [111], lung [112], pancreas [113], liver [114], estrogen receptor-positive primary breast cancer [115], gastrointestinal stromal tumors [116], and nasopharyngeal carcinoma [117]. To our knowledge, its feasibility as a diagnostic marker in UC has not yet been comprehensively investigated. According to our own data, HOTAIR is indeed overexpressed in a subset of bladder cancers, but modulation of its expression does not generally lead to the expected changes in tumor cell phenotypes. Rather, our functional studies suggest that HOTAIR can have cell-type-dependent effects on cell proliferation and invasiveness (Heubach et al., submitted).

Table 3
Cancer-associated long noncoding RNAs

lncRNA	Chromosomal location	Mode of action	Reference
GAS5	1q25	Decoy	[112, 151]
Linc-UBC1	1q32	Chromatin remodeling	[111]
Linc-p21	6p21	Translational repressor Transcriptional co-repression	[136–138]
PANDA	6p21	Transcriptional co-repression	[147]
HOTTIP	7p15	Scaffold/chromatin remodeling	[225, 226]
PTENP1	9p13	Decoy/miRNA sponge	[163, 227]
ANRIL/ CDKN2B-AS	9p21	Chromatin remodeling	[123–126]
MALAT-1	11q13	Alternative splicing, scaffold function, regulation of transcription	[153, 154, 157, 158, 160, 161, 228, 229]
LIT1/KCNQ1OT1	11p15	Chromatin remodeling	[94, 109, 118, 159, 230]
H19	11p15	Chromatin remodeling and posttranscriptional modification	[74, 92, 93, 131 –135, 231]
HOTAIR	12q13	Chromatin remodeling	Heubach et al. (submitted), [88, 89, 110, 113–117, 232–234]
MEG3/ GLT2	14q32	Transcriptional co-activation	[139–146, 235, 236]
UCA1/CUDR	19p13	Regulation of transcription? Chromatin remodeling?	[166–170]
TUG1	22q12	Transcriptional repression/ chromatin remodeling	[76, 128, 129]
XIST	Xq13	Chromatin remodeling	[79, 80, 90, 91, 119–122, 237, 238]
Terra	Transcribed from telomeres	Protein inhibition	[239]

Aberrant imprinting and DNA methylation patterns leading to expression changes of KCNQ1OT1/LIT1 were observed in colorectal cancers [118] and bladder cancers [94] and are associated with downregulation of the cell cycle inhibitor CDKN1C/p57^{KIP2}. Changes in XIST expression have been reported, too [119, 120] and unmethylated XIST was discussed as a potential serum marker [121, 122].

ANRIL (also named *CDKN2B-AS*) is located at chromosome 9p21 adjacent to the *CDKN2* locus encoding the tumor suppressors p14^{ARF}, p15^{INK4B}, and p16^{INK4A}. This is the most often deleted region in UC, but neither the effect of these deletions on *ANRIL* nor the function of *ANRIL* in tumors without deletions are fully understood. *ANRIL* with its interaction partner CBX7 is thought to contribute to repression of the 9p21 tumor suppressors in stem cells and tumors, e.g., in prostate cancers [123]. Genome-wide association studies (GWAS) revealed that *ANRIL* polymorphisms are associated with risk for various diseases like coronary artery disease, periodontitis [124], squamous cell carcinoma of the lung [125], and lymphoblastic leukemia (ALL) [126], but not bladder cancer [127]. We discovered increased expression of *ANRIL* isoforms in bladder cancer tissues and cell lines without chromosome 9p21 deletions, but no stringent inverse correlation with the adjacent tumor-suppressor genes (Hoffmann et al., in preparation).

Like *ANRIL*, the lncRNA *TUG1* interacts with chromatin-modifying enzymes [128]. Overexpression of *TUG1* has been found in a cohort of 44 bladder cancers, especially in high grade and stage cases. Knockdown by siRNA resulted in decreased cell proliferation and induction of apoptosis [129].

A direct scaffold function is exerted by *NEAT1*, from the neighbor gene of *MALAT1/NEAT2* (see below) on chromosome 11q13. This RNA regulates nuclear compartments and topography by supporting the formation and maintenance of paraspeckles (nuclear bodies). It could thus be involved in many cellular processes like gene transcription in response to cellular stress or differentiation [130].

Many lncRNAs function in the regulation of transcription. The *H19* RNA is normally exclusively expressed from the maternal allele of its gene at 11p15.5. Loss of imprinting leading to overexpression is found in several human cancers, including UC [74]. *H19* may have both oncogenic and tumor suppressive functions through its various interaction partners, e.g., c-MYC and pRB1 and by serving as a precursor of miR-675, which is involved in the regulation of developmental genes. In UC, Ariel et al. [131] suggested *H19* as a prognostic tumor marker for early recurrence. *H19* overexpression increased proliferation [132] and migration [133]. It also has been exploited for an elegant targeted therapy approach by using a promoter vector expressing diphtheria toxin A-fragment under the control of a *H19* regulatory sequence for intravesical instillation in bladder cancer patients failing Bacillus Calmette-Guerin therapy [134]. For bladder cancers without *H19* overexpression an IGF2-P4 regulatory sequences was added [135]. Application to cell lines and animal models resulted in growth inhibition and clinical trials have been initiated to investigate the efficacy of intravesical instillation of the double promoter vector.

The lncRNAs LincRNA-p21 and MEG3 influence signaling pathways involved in carcinogenesis. LincRNA-p21 is regulated by p53 and in mice serves as a repressor during p53-dependent transcriptional responses after DNA damage [136, 137]. A post-transcriptional effect on JUNB and β -catenin function was discovered in HeLa cells [138].

The imprinted, maternally expressed gene 3 (*MEG3*) encodes the first lncRNA proposed to function as a tumor suppressor by inhibiting growth of cancer cells [139–143] acting through both p53-dependent and—independent pathways [144, 145]. Its last intron encodes miR-770. In gastric cancer, downregulated MEG3 expression was significantly correlated with tumor progression and prognosis [142]. In UC, MEG3 expression is diminished which may be associated with increased proliferation, inhibition of apoptosis and activation of autophagy [146].

In yet another mechanism, lncRNAs may function as decoys for transcription factors, e.g., PANDA which keeps NF-YA away from its proapoptotic target genes [147]. Similarly, lncRNAs may affect the localization of transcription factors. The cytoplasmic lncRNA NRON can prevent NFAT (nuclear factor of activated T-cells) from shuttling into the nucleus [148–150]. The GAS5 RNA binds to the DNA-binding domain of nuclear glucocorticoid receptors inhibiting receptor binding of the receptor to glucocorticoid response elements on genomic DNA [151]. GAS5 moreover inhibits the cell cycle via repression of CDK6. GAS5 is downregulated in a number of tumor types with consequences on proliferation and apoptosis. This is also the case in bladder cancer [112]. Thus, rescue of GAS5 expression might be useful to suppress cell proliferation.

MALAT1 (NEAT2) serves as an example for an lncRNA functioning in both transcriptional and posttranscriptional regulation. It may control alternative splicing of pre-mRNAs by modulating the activity and cellular distribution of splicing factors towards active transcription sites within nuclear speckles. An unusual processing mechanism targeting the 3'-end of MALAT1 generates nuclear MALAT1 lncRNA and a second small tRNA-like noncoding RNA localized to the cytoplasm, termed mascRNA, whose function is unknown [152]. Nuclear MALAT1 regulates the transcription of genes associated with metastasis or proliferation in lung cancer and HeLa cells, interacting with a huge number of proteins, and might also mediate chromatin modifications [153]. The longer MALAT1 RNA was first shown to be associated with metastatic potential and poor prognosis in non-small-cell lung cancer [154]. MALAT1 is expressed in normal human tissues, but is upregulated in many different tumor types [153]. RNAi-based knockdown of MALAT-1 was reported to decrease tumor cell motility and proliferation, although knockdown of predominantly nuclear-located RNAs is not very efficient. More efficient techniques

are based on zinc finger nucleases (ZFNs) [155] or LNA-ASOs (locked nucleic acid-antisense oligo) which trigger cleavage by RNase H [156].

Two moderate size studies in bladder cancer have reported overexpression of MALAT1 in bladder cancer, with higher levels in more aggressive cases [157, 158]. Functional analyses using siRNA suggested MALAT1 to be involved in the induction of EMT and increased migration capacity [157] or exerting pro-proliferation and anti-apoptotic effects [158]. Interestingly, no obvious phenotype was observed in Malat1 knockout mice even though MALAT1 is broadly conserved among mammals, except slight changes in gene expression profiles [159, 160]. Knockout studies in a transgenic mice model developing invasive bladder cancers with occasional lung metastasis might be informative [161].

In posttranscriptional regulation, lncRNAs may act as “sponges” for microRNAs. This is particularly likely for transcripts from pseudogenes. For instance, PTENP1 RNA functions as a microRNA decoy for microRNAs targeting PTEN and thereby helps restricting cell proliferation [162, 163].

Importantly, most publications on the best investigated cancer-associated lncRNAs emphasize their similar functions across different tumors. This is in so far surprising, as many lncRNAs are expressed and subtly controlled in a tissue-specific manner [98]. Tissue- and cancer-specific lncRNA functions obviously require more studies. Thus, it remains unknown whether observed differences in function for MALAT1 or HOTAIR are a consequence of different applied techniques, of tissue specificity, or of different isoforms.

So far, only quite few individual lncRNAs have been functionally investigated in UC [164]. Often, these lncRNAs were first identified and investigated in other tumor types and then analyzed in UC for the same properties. For the reasons discussed above, this approach may not be fully appropriate for transcripts with specific functions in the normal urothelium or UC. Moreover, because of the pronounced tissue-specific expression of many lncRNAs, urothelial-specific candidates may not only be functionally more relevant, but may also provide better biomarkers than ubiquitous species. For that reason, profiling studies for urothelial carcinoma are very desirable and should be more feasible now as a catalogue of lncRNAs becomes available.

To date, only a single study for UC using RNA-Seq is listed in Pubmed [165]. It aimed at distinguishing progressive from non progressive T1 tumors using archived specimen. Studies employing microarray based techniques are also few. A microarray expression analysis with bladder cancer samples compared to adjacent normal tissues on an Agilent platform identifying 85 differentially expressed lincRNAs with at least two-fold expression change [111]. The expression of lincRNA UBC1 (up-regulated in bladder

cancer 1) was verified in a larger cohort of 102 patients in which up-regulation was associated with lymph node metastasis and poor survival. Moderate UBC1 downregulation by siRNA knockdown attenuated bladder cancer cell proliferation, motility, invasion, colony formation ability, as well as tumorigenicity and metastatic potential in xenografts. RIP and ChIP assays indicated that some of the functional effects might be explained by regulation of gene expression via interaction with EZH2 and SUZ12.

Another presumably UC-specific lncRNA is UCA1 (urothelial carcinoma associated 1). It was discovered by comprehensive expressed sequence tag analysis, serial analysis of gene expression and microarray analysis [166]. The initial study focused on its biomarker potential. UCA1 expression was correlated with tumor stage, grade, and multifocality. In voided urine, 80 % of all tumors ($n=94$) and all high-grade invasive cancers ($n=20$) were detected with an UCA1 assay that performed better than NMP22 and cytology. Another study [167] indicated a role in embryonic development and confirmed overexpression in bladder cancers. Expression modulation affected proliferation, motility, and tumorigenic potential in xenografts. These effects may be partly mediated by UCA1 target genes, e.g. *EP300/CREB* and *AKT* [168]. UCA1 itself may be regulated by the ETS2 transcription factor [169]. The predominantly important UCA1 isoform seems to be UCA1a [170].

Similarly, the lncRNA ncRAN initially identified in neuroblastoma [171] was overexpressed especially in high-stage bladder cancers and its overexpression in a low-grade cell line-enhanced tumorigenic properties [172].

The list of lncRNA candidates for UC biomarkers is still very short. One interesting candidate needs mention. Rivas and colleagues [173] used the knowledge on lncRNAs originating from mitochondria to develop a FISH-based method (S-FISH). Their method allows to discriminate between normal and malignant cells obtained from urine by analysis of two different transcripts. It could be used as a new noninvasive diagnostic test for bladder cancer from patients' urine. The FISH technique as such is already in clinic use for bladder cancer diagnosis in form of the Urovysion assay [174].

In conclusion, an increasing number of studies on individual lncRNAs illustrates that these are key regulatory factors in tumorigenesis and cancer progression. This knowledge will be expanded once high-throughput sequencing data for specific tumor types becomes evaluated for tumor specific or patient specific transcripts for personalized medicine. Pioneer studies have proven that candidates might be useful as appropriate markers for detection or prognosis, even in a noninvasive fashion by analysis of urine. An FDA approved assay for urine samples (Progensca PCA3 urine test) is already on the market for prostate cancer [175]. By being often stable in serum and urine, lncRNA are well suited for the development of such new diagnostic assays, which might reduce the number of

necessary invasive diagnostic interventions such as cystoscopy. On the other hand expanding knowledge on the function of lncRNAs in shaping the phenotype of cancer cells might lead to targeted therapies. A proof of concept has already been given by the H19-promoter driven toxic plasmid (BC-819) discussed above. The advancement of new technologies for specific delivery of nucleotides might allow to correct the expression levels of selected lncRNAs impacting on the balance between proliferation and apoptosis in cancer cells or inducing their terminal differentiation. Trend-setting results have already been obtained for small noncoding RNAs, e.g., synthetic RNA molecules (antagomirs). As lncRNAs form extensive secondary and tertiary structures that are important for their functions, there are some extra challenges for therapeutic approaches targeting lncRNAs, but also opportunities. Chang and colleagues [176] speculate that targeting the structure of candidates could inhibit their interaction with binding partners thus abrogating the described tumor-promoting effects. These considerations highlight the importance of further studies on lncRNA structures.

6 Small Noncoding RNAs

Small noncoding RNAs constitute the second large group of non-coding RNAs, comprising tRNAs, piRNAs, tiRNAs, snoRNAs, and microRNAs. A rapidly increasing number of publications refer to microRNAs in UC, but there are no publications yet on piRNAs and snoRNAs in bladder cancer.

Mature miRNAs are single-stranded RNA molecules of about 20-23 nucleotides after they have been processed from their precursors in a coordinated series of processing steps [177]. Drosha, Exportin5 and Dicer perform and regulate miRNA processing during development, differentiation and in cancers. These proteins were reported to be upregulated in UC [178]. This finding was confirmed by Han et al. [179], especially for high-grade carcinoma, although others found a downregulation of Dicer [180]. Han et al. investigated additionally how these changes in the miRNA processing machinery could be used to inhibit proliferation of bladder cancer cells and to induce apoptosis by siRNA-mediated knockdown [179].

The mature miRNA is assembled in a ribonucleoprotein complex named RISC (RNA-induced silencing complex) that mediates target recognition and inhibitory effects in two ways: Perfect complementary binding of the miRNA seed region to target mRNA 3'UTRs leads to their cleavage. In cases of imperfect complementarity translational initiation is inhibited [177].

Commonly used methods for measuring expression of precursors or mature miRNA candidates are based on microarray platforms, sequencing or real time RT-PCR [177, 181–183].

Specific recommendation for procedures during RT-PCR analysis are described by Benes and Castoldi [184]. Strengths and pitfalls of the respective methods have been discussed by Farazi et al. [185] and specifically for use with FFPE materials [186]. Protocols for the quantitative analysis of miRNAs in epithelial cells and tissues and an overview about algorithms for target prediction have been collected by Bitzer et al. [187]. A comprehensive list of online resources including data mining tools and databases has been provided by Gana et al. [188].

Several techniques have been applied to determine changes in miRNA expression profiles for various cancer types [185] including UC, in which many of the differentially expressed miRNAs are downregulated [189]. These studies can essentially be categorized into three categories.

The first category consists of a huge number of studies analyzing the expression and especially the function of single miRNAs as tumor suppressors or oncogenes and their effects on central pathways in the respective cancer type. A summary of growth factor receptor signaling and the predicted interaction with deregulated miRNAs in UC has been provided by Fendler et al. [190]. A general overview on “oncomiRs” involved in tumor progression by metastasis is available [191]. Expression profiling of human metastatic cancers including UC revealed a number of up- and down-regulated candidates, including the well known “metastamiR” miR-10b from the *HOXD* gene cluster [192]. Particularly interesting findings in UC are a tumor suppressor function of miR-145 via apoptosis [193] and the implication of the miR-200 family in EMT and EGFR signaling [194].

The second category of publications deals with the epigenetic regulation of miRNAs themselves and how this could be targeted by epigenetic inhibitors. Coordinated repression of the miR-200 family and miR-205 in muscle-invasive bladder cancers and undifferentiated bladder cancer cell lines is caused by promoter hypermethylation and repressive chromatin marks [195]. Kunej et al. [196] have provided a literature survey on epigenetically regulated miRNAs in 22 different cancer types. Interestingly, they identified epigenetically regulated miRNAs associated only with one specific tumor type, namely in UC miRs-134, -183, -192, -429, -494, and -498. These miRNAs might provide targets for therapeutic approaches using epigenetic inhibitors such as 5-azacytidine or HDAC inhibitors [190].

The third category contains studies seeking to identify expression signatures of miRNAs from either tissues or body fluids associated with clinicopathological parameters that might serve as biomarkers for diagnosis, prognosis or response to chemotherapy. A recent review on such studies has been published [197]. It highlights miRs downregulated in low-grade, non-muscle-invasive UC of which some also target FGFR3 signaling, like miRs-145, -101,

-100, and -99a. Rather increased expression of miRs is reported for muscle-invasive bladder cancers, including miR-21 and -373 targeting p53 among others [178]. Li et al. [198] identified common changes among genitourinary cancers but also many UC-specific differentially expressed miRNAs as potential biomarkers. Other literature reviews on miRNAs in genitourinary cancers also emphasize the association of cancer-specific deregulated miRNAs with relevant signaling pathways. Importantly, these include the molecular pathways that are distinct between the different histological types of bladder cancers [199, 200]. These findings have recently been expanded and complemented target genes by [189]. MiRNA signatures distinguishing between normal and tumorous bladder tissues or even between tumor stages were identified by Dyrskjot et al. [201]. A further classification distinguished progressing and nonprogressing tumors and carcinoma in situ. A similar approach was used by Veerla et al. [202]. Another study analyzed miRNA signatures with their target genes, e.g., cytokeratin 7 (KRT7) [203]. Catto et al. [178] identified signatures distinguishing low-grade and high-grade bladder cancers. MiRs- 21, -99a, and -100 were found to be predictive for progression-free survival.

In various tumors, miRNAs have been implicated in chemoresistance, a major problem in bladder cancer treatment, too [204, 205]. Selected candidates potentially involved in chemoresistance of UC to treatment with cisplatin, doxorubicin or gemcitabine have only recently been discovered [206–209]. Such miRNAs may be candidates for biomarkers predicting therapeutic response or as targets to overcome therapy resistance. Potential therapeutic approaches using inhibitors, small molecules, sponges, or synthetic molecules such as antagomiRs, tested for miRNA targeting in other tumor types, were reviewed by [176, 190]. Uchino et al. used synthetic RNA molecules for transurethral injection in mouse models and achieved reduced tumor growth and metastasis [210]. An alternative method to inhibit selectively miRNAs has been named miRNA-mower [211].

An obvious problem in the development of miRNA based biomarkers is the fact that many of the published studies reveal differing sets of miRNAs with only limited overlap [212]. In addition, promising candidates often lack independent validation. Such problems have especially become apparent when circulating miRNAs are investigated in body fluids like serum or urine (general review [213–215]), also because it is incompletely understood whether they are passively or actively (exosomes) secreted [216]. Roos and Jakubowski published methods for detection of low-abundance biomarkers, including miRNAs, in biological fluids of patients with bladder cancer [217]. Further studies using mainly PCR-based methods identified different candidates [218–222]. For the many downregulated miRNAs detection of the associated

DNA hypermethylation as a biomarker is an interesting alternative [223]. A literature review on circulating miRNAs found in urine or serum of patients with different types of genitourinary cancers and their purpose has been recently published [224]. These include 13 miRNAs found in patients with bladder cancer.

7 Perspectives

Changes in DNA methylation, histone modifications, long non-coding RNAs, and microRNAs have been linked to urothelial carcinoma development and progression and many have been shown to contribute to essential aspects of the cancer phenotype. Compared to other cancers, DNA hypomethylation, especially at LINE-1 retrotransposons, and mutations in enzymes establishing or removing histone acetylation or methylation are particularly prominent. Accumulating evidence suggests that disturbances in DNA methylation, histone modifications and noncoding RNAs may contribute especially to altered differentiation and metastatic potential. There are excellent biomarker candidates for DNA-methylation-based detection of UC. Many studies suggest that miRNAs may be similarly well suited, but obviously several technical issues need to be resolved before miRNA-based diagnostics becomes practical. For both DNA methylation and miRNA biomarkers, their potential as prognostic biomarkers remains uncertain. The frequency of alterations in histone-modifying enzymes reported so far is remarkable. With proper selection, they may constitute prime targets for therapy. However, for that purpose the distribution of the mutations and expression changes across the subtypes of UC and their functional effects need to be better explored. Long noncoding RNAs will likely turn out to represent crucial modulators of the cancer phenotype. Again, their precise mode of action and the relation of their expression changes to clinical parameters will need to be better defined. Investigations on tissue-specific lncRNAs may be particularly valuable because of their presumable suitability as urothelial-specific biomarkers and therapeutic targets.

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Chapter 11

Epigenetics of Prostate Cancer

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Abstract

The introduction of novel technologies that can be applied to the investigation of the molecular underpinnings of human cancer has allowed for new insights into the mechanisms associated with tumor development and progression. They have also advanced the diagnosis, prognosis and treatment of cancer. These technologies include microarray and other analysis methods for the generation of large-scale gene expression data on both mRNA and miRNA, next-generation DNA sequencing technologies utilizing a number of platforms to perform whole genome, whole exome, or targeted DNA sequencing to determine somatic mutational differences and gene rearrangements, and a variety of proteomic analysis platforms including liquid chromatography/mass spectrometry (LC/MS) analysis to survey alterations in protein profiles in tumors. One other important advancement has been our current ability to survey the methylome of human tumors in a comprehensive fashion through the use of sequence-based and array-based methylation analysis (Bock et al., *Nat Biotechnol* 28:1106–1114, 2010; Harris et al., *Nat Biotechnol* 28:1097–1105, 2010). The focus of this chapter is to present and discuss the evidence for key genes involved in prostate tumor development, progression, or resistance to therapy that are regulated by methylation-induced silencing.

Key words Epigenetics, Methylation, -Methylome, MicroRNA, Prostate cancer

1 Introduction to DNA Methylation and Histone Acetylation in Prostate Cancer

DNA methyltransferases (DNMTs) are a family of enzymes that carry out post-replicative methylation of newly synthesized strands of DNA. These include DNMT1, DNMT3A and DNMT3B [3]. Studies have shown that DNMT1 and DNMT3B can cooperate to maintain the silencing of specific genes in cancer cells, thus contributing to the malignant phenotype [4, 5]. The initial findings suggesting methylation events that might contribute to prostate cancer development and progression surfaced in the early 1990s. These studies demonstrated that cytidine methylation of regulatory sequences near the glutathione-S-transferase gene (GSTP1) was associated with prostate carcinogenesis [6]. Subsequent studies have shown that global hyper- and hypo-methylation occurs in both primary and metastatic prostate cancers, and has been associated with increased tumor stage [7, 8]. The availability of technologies

to conduct genome-wide methylation studies have shown hypermethylation at a number of other genetic loci including RARB, APC, PYCARD, PTGS2, ABCB1, and RASSF1 in prostate cancer [9, 10]. In addition to cytidine methylation at the site of CpG islands epigenetic modification can have an influence on gene expression through chromatin acetylation, methylation, and other specific histone modifications [11]. The process of histone acetylation at lysine residues is regulated by histone acetyl transferase (HAT) and histone deacetylases (HDACs) [12, 13]. Androgen receptor (AR) activity is down regulated by HDACs in prostate cancer, and some HDACs are required for activation of AR target genes [14–18], and global patterns of histone methylation have been shown to predict prostate cancer recurrence [19, 20]. Methylation of histones also plays a role in prostate cancer progression having been linked to high levels of lysine-specific demethylase 1A (KDM1A) which correlates with increased risk of disease relapse [21, 22]. Demethylation of KDM4C and KDM1A has also been shown to regulate androgen receptor function [23].

Thus, epigenetic modifications have been shown to play an increasingly pivotal role in development and progression of prostate cancer. We here review some of the recent advances in the field and discuss their implications for improved diagnosis, prediction of disease progression and relapse, and novel therapies.

2 Gene Methylation in Prostate Cancer

There are a number of methodologies for discerning the methylation status of genes. Older approaches employed the use of methylation-sensitive restriction endonucleases, which cut recognition sites differently depending on whether 5-methyl CpG is present, or modification of DNA using sodium bisulfite, a procedure that promotes C deamination to U but spares 5-methyl cytosines [24]. Another method uses column- or bead-immobilized recombinant methylated-CpG-binding domain (MBD) proteins to enrich for methylated DNA fragments for subsequent detection by Southern blot, PCR or microarray hybridization [25]. A modification of this methodology designated COMPARE-MS combines the use of methylation-sensitive restriction enzymes with MBD-assisted capture and enrichment of methylated DNA followed by quantitative PCR for sensitive, specific, and rapid quantitation of hypermethylated CGI sequences [26]. More recent methods use the power of methylation bead-chip technology to survey up to 485,000 methylation sites per sample at single nucleotide resolution including miRNA promoter regions (Illumina Technologies). These new technologies have provided a method to perform large gene methylation surveys in prostate cancer.

There are a number of genetic loci that are silenced by methylation in prostate cancer (Table 1). One of the first to be identified was glutathione-transferase pi 1 (GSTP1), an enzyme responsible for detoxification by catalyzing the conjugation of hydrophobic and electrophilic compounds with reduced glutathione [6]. The data show that the majority of human prostate carcinomas fail to express GSTP1 and that this is primarily due to hypermethylation of promoter sequences in the GSTP1 gene. Benign prostatic hyperplastic (BPH) tissue fails to exhibit hypermethylation at this site, as does normal prostate epithelium. Hypermethylation of the GSTP1 gene promoter has also been observed in prostatic intraepithelial neoplasia (PIN) tissue, a possible premalignant precursor lesion of the prostate [27]. High-grade PIN lesions also exhibit a nearly complete loss of GSTP1 expression, mainly due to hypermethylation at the gene promoter [27, 28]. In addition to GSTP1 multiple other genes have been shown to exhibit hypermethylation at the promoter site, greatly reducing their expression. For example, APC, MGMT, and RASSF1A were methylated in at least 60 % of prostate cancers, while other genes including COX-2, DAPK1, CDH1, CDKN2A, RUNX3, and THBS1 were methylated at frequencies lower than 35 % [10]. Overall methylation levels have been found to be higher in prostate cancer compared to normal prostate, with the levels in BPH being statistically similar to those found in the normal tissue [7]. Recent advances in methylation profiling have provided the ability to survey gene methylation patterns across the entire genome to compare these patterns in tumor versus normal tissues. The analysis of the LNCaP prostate cancer cell line and normal prostate epithelial cells (PrEC) using Methyplex next-generation sequencing methods to survey global methylation differences demonstrated a 1.7-fold difference between them [29]. Of the 6,691 methylated promoters in prostate tissue 2,841 differentially methylated regions are cancer-specific. Further analysis demonstrated methylation in the promoter regions of several genes including RASSF1, KCTD, CHMP4A, APC, CDKN2A, SHC1, LAMC2, TSPAN1, CALML3, AOX1, TME246, and TINAGL1 in LNCaP cells. The gene SPON2 was methylated in both LNCaP and PrEC cells. Studies on a limited set of human prostate tumor samples showed similar results. One novel methylation target, WFDC2, was methylated in all six prostate cancer cell lines tested and in 17/22 (77 %) of cancer tissues, but not in benign prostate tissue. There also are DNA methylation patterns associated with ETS-positive and ETS-negative prostate cancer, specifically a decrease in repeat element methylation in the ETS-negative disease [29]. Other novel differentially methylated genes in prostate cancer cell lines identified by these investigators include MAGI2, MEIS2, NTN4, GPRC5B, TMEM 246, FGFR2, VAMP5, C14orf159,

Table 1

Genes exhibiting altered methylation patterns in prostate cancer and prostate cancer cell lines

Gene symbol	Gene name	Function
ABCB1	ATP-binding cassette, subfamily B	ATP-dependent transporter [9, 10]
AMT	Aminomethyltransferase	Glycine cleavage pathway [29]
AOX1	Aldehyde oxidase 1	Superoxide formation [29, 41]
APC	Adenomatous polyposis coli	Tumor suppressor [9, 10]
BNC1	Basonuclin 1	Transcription factor [31]
C14orf159	Chromosome 14 open reading frame 159	Unknown [29]
CALM3	Calmodulin 3 (phosphorylase kinase, delta)	Ion channel control [29]
CAV1	Caveolin 1,	Scaffolding protein [29]
CCDC181	Coiled-coil domain containing 181	Unknown [41]
CDH1	E-Cadherin	Cell-cell adhesion [10]
CDKN2A	Cyclin-dependent kinase inhibitor 2A	Cell cycle control [10]
CHMP4A	Charged multivesicular body protein 4A	Endosomal protein sorting/ transport [29]
CRIP1	Cysteine-rich protein 1	Zinc transport [43]
CXXC5	CXXC-type zinc finger protein	NF-kappa-B/MAPK pathways [31]
CYB5R2	Cytochrome B5 reductase 2	Drug metabolism/elongation of fatty acids [31]
DAPK1	Death-associated protein kinase 1	Apoptosis [10]
FGFR2	Fibroblast growth factor 2	FGF pathway signaling [29]
FLNC	Filamin C, gamma	Membrane-anchoring protein [43]
FZD1	Frizzled family receptor 1	Wnt pathway signaling [31]
GPRC5B	G protein-coupled receptor family C	Unknown [29]
GSTP1	Glutathione S-transferase Pi 1	Detoxification [6, 9, 10, 27, 28]
HAAO	3-Hydroxyanthranilate 3,4-dioxygenase	Quinolinic acid synthesis [43]
HAPLN3	Hyaluronan and proteoglycan link protein 3	Cell adhesion [41]
HIF3A	Hypoxia-inducible factor 3, alpha subunit	Transcription factor [43]
HS3ST2	Heparan sulfate (glucosamine) 3-O-sulfotransferase 2	Glucosamine sulfonation [43]
KCTD1	Potassium channel tetramerization domain containing 1	Transcriptional repressor [29]
KDM1A	Lysine (K)-specific demethylase 1A	Histone deacetylation [21, 22]

(continued)

Table 1
(continued)

Gene symbol	Gene name	Function
KDM4C	Lysine (K)-specific demethylase 4C	Histone demethylase [23]
LAMC2	Laminin, gamma 2	Cell adhesion [29]
LMX1B	LIM homeobox transcription factor 1, beta	Transcription factor [31]
MAGI2	Membrane-associated guanylate kinase	Interacts with atrophin 1 [29]
MEIS2	Meis homeobox 2	Transcription factor [29]
MGMT	O-6-methylguanine-DNA methyltransferase	DNA repair of alkylated guanine [10]
NTN4	Netrin 4	Unknown [29]
PITX2	Paired-like homeodomain 2	Transcription factor [39, 40]
PPARGC1A	Peroxisome proliferator-activated receptor gamma	Energy metabolism [30]
PPP1R3C	Protein phosphatase 1 regulatory subunit 3C	Regulates protein phosphatase activity [29]
PTGS2	Prostaglandin-endoperoxide synthase 2	Deoxygenase/peroxidase [9, 10]
PYCARD	PYD and CARD domain containing	Apoptosis [9, 10]
RARB	Retinoic acid receptor, beta	Transcriptional regulator [9]
RASSF1A	Ras association domain family member 1	Tumor suppressor [9, 10, 29]
RASGRF2	Ras protein-specific guanine nucleotide releasing factor	Calcium-regulated nucleotide exchange factor [43]
RPL39L	Ribosomal protein 39-like protein	Ribosomal protein [31]
RUNX3	Runt-related transcription factor 3	Transcription factor [10, 43]
S100A16	S100 calcium-binding protein A16	Calcium binding [29]
SHC1	SHC Src homology 2 domain containing transforming protein	Signaling adapter protein [29]
SPON2	Spondin 2, extracellular matrix protein	Cell adhesion [29]
SYN2	Synapsin II	Regulates neurotransmitter release [31]
TINAGL1	Tubulointerstitial nephritis Antigen-like 1	Adrenocortical zonation [29]
THBS1	Thrombospondin 1	Cell cell/cell matrix adhesion [10]
TMEM 246	Transmembrane protein 246	Unknown [29]
TSPAN1	Tetraspanin 1	Mediates signal transduction [29]
VAMP5	Vesicle-associated membrane protein 5	Vesicle and cell membrane fusion [29]
WFDC2	WAP four-disulfide core domain 2	Protease inhibitor [29]
ZNF783	Zinc finger family member 783	Transcriptional regulation [31]

PPP1R3C, S100A16, and AMT [29]. The hypermethylation of the promoter regions of AOX1 and SPON2 was confirmed in another study using the Illumina Human Methylation bead chip that tests for methylation in greater than 480,000 CpG sites [30]. AOX1 methylation was much greater in prostate cancer compared to normal tissue, and strongly correlated with Gleason score, with higher Gleason score tumors exhibiting lower AOX1 expression. These genes along with GSTP1 and PPARGC1A exhibit the greatest hypermethylation of CpG sites associated with prostate cancer compared to normal prostate tissue, and are associated with advanced Gleason score. At the same time relatively small changes in global methylation patterns were observed to be associated with prostate cancer progression suggesting these specific gene changes are relevant to the disease process. Other genome-wide methylation studies have identified additional genes as targets for silencing including BNC1, FZD1, SYN2, RPL39L, LMXB1, CXXC5, ZNF783, and CYB5R2, all of which contain promoter-associated sites that exhibit significant differences in methylation between HGPIN or prostate cancer, compared to normal prostate [31]. BNC1 is a gene that is involved in maintaining the proliferative potential of immature cells and has previously been shown to be hypermethylated in renal cell carcinoma and CLL [32–34], while FZD1 is involved in the Wnt signaling pathway. Altered expression of FZD has also been reported in colon cancer, breast cancer cells, and neuroblastoma [35–37] suggesting a role in tumor invasion. The CYB5R gene encodes an enzyme involved in the metabolism of drugs, including many chemotherapeutic agents [38] and thus may play a role in chemoresistance. Several hypermethylated genes have been suggested to have prognostic value in predicting biochemical failure (PSA recurrence). The PITX2 gene was shown to have prognostic value in two prostate cancer patient cohorts [39, 40]. A more recent analysis using the Infinium 27 K methylation assay on both a training and validation cohort of prostate cancer patients demonstrates the potential utility of the CCDC181 gene, or a combination of CCDC181 and AOX1/HAPLN3 genes to predict time to PSA recurrence independent of clinical or pathologic parameters [41]. In one methylation array analysis of metastatic prostate cancer of all 27,578 CpG sites assayed 1,361 were methylated across all samples corresponding to 1,133 different genes [42]. A combination of methylation array analysis and pyrosequencing has validated that HIF3A and HAAO genes as genes that are significantly hypermethylated in prostate cancer, and that the CRIP1, FLNC, RASGRF2, RUNX3, and HS3ST2 genes are significantly methylated in the recurrence compared to the non-recurrence patient group [43]. A compilation of the genes discussed in this chapter that are frequently methylated in association with human prostate cancer and supporting citations is shown in Table 1.

3 The Epigenetic Modifications in Prostate Cancer with/by miRNA

MicroRNAs are a group of conserved, single-strand small, noncoding RNA (18–25 nucleotides) that act posttranscriptionally to regulate protein expression [44]. MiRNAs are important contributors to gene regulation and their expression levels are often changed in many disease models. MiRNAs represent about 1 % of the genome but target somewhere in the range of 30 % of genes [45]. The activity of miRNA depends on its complementarity to the mRNA sequence it binds to. If they are perfectly complementary, it results in the degradation of the mRNA; where the sequence complementation is not perfect, this leads to altered translation of the mRNA, either increasing or decreasing it (<2-fold) [46]. Unlike other larger members of the RNA families, microRNAs are inherently resistant to RNase activity due to their small size and distinctive structure [47]. They are also very stable in FFPE tissues and can be found in both serum and plasma [47]. MiRNAs primarily silence their target genes by binding to the 3'-UTR (3'-untranslated region) of its target's mRNA but there are instances when they increase the translation of their targets by binding to the 5'-UTR of the mRNA target [48].

MiRNAs can act as tumor suppressors or tumor promoters (oncogenic miRNA) [49]. Oncogenic miRNAs are increased in cancer and promote tumorigenesis by suppressing either genes that inhibit proliferation or genes associated with differentiation or apoptosis [47]. In contrast, decreased availability of miRNAs that are tumor suppressors can promote the development of cancer by increasing the rate of cellular proliferation [47].

Epigenetic events are generally reversible, heritable changes that do not involve changes to the gene sequence or chromosomal structure but alter expression of gene products including noncoding RNAs [50]. Since they are reversible, epigenetic changes may be considered dynamic and therefore, it is reasonable that these processes are dysregulated in cancer [50]. There are two basic ways that miRNA can be involved in epigenetics. The first are epigenetic changes in the regulation of miRNAs. These changes can be broadly classified into two groups: miRNAs that are regulated by DNA methylation of their promoter regions and miRNAs that target DNA methyltransferases or chromatin enzymes. Secondly miRNA are involved in the “epigenome” by their ability to change the expression of other components of the epigenetic machinery and thereby the expression of tumor suppressors or oncogenes [48].

In prostate cancer Ambs et al. [51] found that the most highly upregulated miRNA was miR-32 followed by miR-182, miR-31, miR-26a, miR-200c, and miR-196a. In addition, Ambs et al. reported that miR-338 was the only miRNA found to be upregulated by androgen [51]. The miR-106b-25 cluster (miR25/miR93/miR106) is also overexpressed in prostate cancer. In contrast miR-502 h, miR-494, miR-490, and miR-1-133a are downregulated in prostate cancer [51].

4 MiRNAs Regulated by Methylation

Like other genetic products, miRNAs are regulated through methylation of their promoters. Examples include: miRNA-34a, miR-34b/c, miR-126, MiR-193b, MiR-145, miR-205, miR-21, miR-615, and miR-196b [52]. MiR-34a is a p53 target that plays an important role in metastasis. Its expression is controlled via methylation of the CpG island upstream of its transcript. The promoter site contains a binding site for p53 suggesting it also has a role in the regulation of this miRNA [48]. The expression of miR-34a is thought to be induced in part by DNA damage [53]. MiR-34a is downregulated in CD44+ prostate cancer stem cells [54]. Its downregulation may contribute to the role of PCa stem cells especially in metastasis [55–57]. Studies where forced expression of miR-34a occurs in CD44+ cells leads to growth inhibition, and decrease in metastatic behavior [56].

MiR-34b and -34c directly target DNA methyltransferase 1 (DNMT1), and several histone deacetylases (specifically, HDAC 1, 2, and 4) [45]. Other targets of miR-34b/c include MYC (myelocytomatosis oncogene), CDK4 cyclin-dependent kinase 4, CDK6 (cyclin-dependent kinase 6), CREB (cAMP response element-binding protein), and MET (proto-oncogene) (and are involved in the cell cycle arrest and metastasis [58–61]. High expression of miR-34b is associated with recurrence free and overall survival and has been shown to interfere with EMT (epithelial to mesenchymal transition) inhibiting mesenchymal markers including vimentin and Snail [62]. Decreasing the expression of miR-34b increases H3K4me3 (trimethylated histone H3 lysine 4) a sign of active chromatin [45]. These miRNA are also downregulated by methylation [62] and their expression levels are inversely correlated with the methylation levels of the CpG island adjacent to their genes [53]. P53 also plays a role in the regulation of miR-34b/c but alone cannot induce their expression when they have been silenced by hypermethylation.

MiR-126 is another miRNA frequently downregulated by methylation of its promoter region in prostate cancer [48]. Treatment with either 5-aza-2'-deoxycytidine (5-AZA) or 4-phenylbutyric acid (PBA) that act as demethylating compounds lead to increased expression of this miRNA [48]. MiR-126b has been shown to be downregulated in high grade (Gleason score >7) prostate cancer, but this change in express level was limited to the tumor stroma and did not occur in the actual tumor itself [63].

MiR-145 and -143 are also downregulated through hypermethylation of the promoter region. For miR-145, this downregulation occurs in 81 % of prostate cancers. Methylation of its promoter has been shown to prevent the binding to and regulation

of miR-145 by p53 [45, 64]. Unsurprisingly, treatment of cells with 5-AZA and trichostatin A (TCA, an HDAC inhibitor) in combination leads to increased expression of miR-145 [48]. Both miR-143 and -145 play a role in EMT [54]. As methylation of their promoter decreases, they decrease expression of mesenchymal markers like fibronectin and increase the epithelial marker E-cadherin [65]. Xu et al. [66] provided clear evidence that miR-143 regulates genes involved in cell proliferation, migration and sensitivity to chemotherapy through its regulation of the proteins cyclin D1, K-RAS, and ERK1/2 (aka MAPK1/3, mitogen-activated protein kinase 1/3). It is not surprising that the expression of these genes is downregulated in metastatic tissue and negatively correlated with bone metastasis, higher Gleason score and levels of free PSA in prostate cancer patients [54]. However, Friedman et al. found that deregulation of miR-143 and -145 was not seen in metastases to the lymph nodes when compared to primary lesion, so it may be that they are cell-type specific and they have a role in suppressing bone metastasis but not those to lymph nodes [67].

5 miRNAs Regulating Components of the Epigenome

MiRNAs are also involved in the regulation of epigenetic machinery in cells. MiR-101 expression is inhibited and leads to the elevated EZH2 (enhancer of zeste homolog 2), a histone methyl transferase component of PRC2 (polycomb repressor complex) in prostate cancer. EZH2 stops transcription by trimethylating histone H3 lysine 27 (H3K27me3) and is overexpressed in many cancers including prostate cancer and is linked to decreased survival [68]. Loss of miR-101 is associated with an aggressive phenotype that includes increased cell migration and invasion [69]. Over-expression of miR-101 can be overcome by overexpression of EZH2 making this a self-regulatory loop [70]. It also appears that in prostate cancer, physiological conditions and/or microenvironment influences miR-101 expression as AR (androgen receptor) and HIF-1alpha and -1beta are able to induce its expression [48].

Another miRNA that is regulated by members of the epigenetic machinery is miR-26a. A large study of matched normal and prostate cancer tumor tissues indicated that hypermethylation of miR-26a can lead to rearrangement of ERG (ETS-related gene) without TMPRSS2-ERG gene fusion [71]. MiR-26a expression inhibits EZH2 and EZH2 is stimulated by MYC, which also inhibits miR-26a [72, 73]. The interaction of MYC, EZH2, and miR-26a was first identified in Burkitt lymphoma cells [73].

6 Histone Deacetylases: HDAC-1, -2, and -4

There are also several miRNAs in prostate cancer that regulate and/or are regulated in part by histone deacetylases. For example, miR-449a directly targets HDAC1 in prostate cancer and this miRNA is often down regulated compared to normal tissues. MiR-449a functions to regulate cell growth and viability by inhibiting the expression of HDAC1 [74]. Restoration of miR-449a has been linked to cell cycle arrest, loss of clonogenicity and senescence [48]. MiR-449a has also been shown to bind directly to the 3'-UTR of the androgen receptor (AR) and may play a role this pathway in prostate cancer by decreasing cell proliferation [75].

The miR-200 family consists of five members: miR-200b, miR-200a, miR-429, miR200c, and miR-141. They are frequently altered in prostate cancer and they, along with miR-205, regulate EMT and migration/invasion by targeting ZEB1 (zinc-finger E-box binding homeobox 1), ZEB2 and Snail2 [54, 76]. It is interesting to note that NF-kappaB has been shown to upregulate ZEB1 and ZEB2, which in turn, suppress miR-200 family members. Thus there is a negative feedback loop between the miR-200 family and ZEB1/ZEB2 and EMT; that is ZEB1 (EMT activator) suppresses miR-200c, but miR-200c targets ZEB1 [53, 77]. It has also been shown that upregulation of PDGF-D (platelet-derived growth factor-D) also inhibits expression of the miR-200 family [77].

Other members of the miR-200 family are also epigenetically regulated [48, 78, 79]. MiR-205 is generally downregulated in prostate cancer and aids prostate cancer cells undergoing EMT and may facilitate metastatic spread [45]. It is considered a tumor suppressor that works by inhibiting protein kinase C (PKC). MiR-205 works with members of the miR-200 family (*see above*) and together they target ERBB3 (receptor tyrosine kinase erbB-3 gene), E2F1 (transcription factor), ZEB2 and PKC [80]. MiR-205 is also involved in the posttranslational regulation of EZH2 overexpression [80]. Restoration of miR-205 leads to a mesenchymal to epithelial transition and reduction of invasiveness and cell migration [80].

MiR-499a targets HDAC1 and is frequently downgraded in prostate cancer. Over-expression in prostate cancer leads to cell cycle arrest and downregulation of HDAC1 [45, 74]. This in turn ultimately leads to the stimulation of p27^{KIP1} (a cyclin-dependent kinase), another key growth regulator [45].

7 MiRNA and the Androgen Receptor Pathway

Given the importance of the androgen receptor pathway in prostate cancer, it is not surprising to find miRNA involved in this pathway as well. Ottman et al. reported that 43 miRNAs were significantly

altered in prostate cancer cell lines as the cells progressed from androgen deprivation therapy to CRPC (castration resistant prostate cancer) [81]. MiR-21 is oncogenic miRNA that is upregulated in prostate cancer and involved in the regulation of tumor growth, invasiveness, and metastasis [54, 70, 82]. Many genes have been identified as targets of miR-21. Overexpression of miR-21 inhibits PTEN (phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase) and PDCD4 (programmed cell death protein 4) leading to decreased apoptosis [83]. In the presence of androgen, AR binds to miPPR-21, a miR-21 promoter, leading to over-expression and castration resistance [84]. But in at least one study with patient samples, miR-21 knockdown did not correlate with a reduction in either proliferation or invasiveness in prostate cancer cells. In this study they were not able to document a significant difference in a cohort of 36 patients in the expression level of miR-21 in prostate cancer versus normal tissues. Clearly a complete understanding of the role(s) of this miRNA remains to be settled [85].

Significantly higher expression of miR-221 and -222 in CRPC leads to reduced dihydrotestosterone effects on PSA and increased androgen-independent growth [86]. Higher expression levels are also associated with more aggressive tumors [87]. There is however, contradictory data where suppression of androgen also leads to lower levels of miR-221/-222. There is some speculation that this may be due to a dosage effect [51]. MiR-221/-222 has been shown to bind to and inhibit the cyclin-dependent kinases p27^{KIP1} and p57^{KIP2} (cell cycle inhibitors) leading to tumor growth [45, 70, 88]. Overexpression of miR-221/-222 induces a G₁-to-S phase shift and greatly enhances colony formation in vitro [45, 88]. MiR-221 overexpression in LNCaP cells, induced neuroendocrine differentiation (NED) and the expression of NSE (neuron specific esterase), both of which are important in CRPC and associated with poor prognosis [89].

Stable overexpression of miR-616 and miR-125b are additional miRNAs associated with CRPC [90]. MiR-125b overexpression and is key to cell proliferation. Reduction in its expression or depletion with siRNA transfections is shown to induce a proliferation defect in PC-3 cells [54]. MiR-125b targets the pro-apoptotic BAK (Bcl-2 homologous antagonist killer), a member of the BCL-2 family, as well as EIF4EBP1 (eukaryotic translation initiation factor 4E-binding protein), one member of a translation repressor family [45]. MiR-616 targets and inhibits of TFPI-2 (tissue factor pathway inhibitor-2), inducing androgen independent prostate cancer growth. Re-expression of TFPI-2 in prostate cell lines restored androgen dependence and inhibits cell growth [91].

MiR-331-3p is another miRNA that has been linked to the androgen pathway. Initially, miR-331-3p was shown to target the mRNA of ERBB-2 (Her2) a tyrosine kinase receptor that is over expressed in prostate cancer. ERBB-2 is inhibited by miR-331-3p

in prostate cancer and subsequent inhibition of the PI3K/AKT pathway [90]. Epis et al. found that there are two specific miR-331-3p binding sites within the 3'-UTR of ERBB-2. MiR-331-3p also decreased AR signaling by decreasing prostate specific androgen binding [90]. In subsequent studies, Epis et al. found that miR-331-3p and miR-642 inhibit DOHH (deoxypusine hydroxylase) expression resulting in decreased cell growth by inhibiting the activity of eIF5a (eukaryotic translation initiation factor) [92].

MiR-15a and -16-1 are thought to work as tumor suppressors and are mediated through multiple oncogenes including BCL2 (basal cell lymphoma 2), MCL1 (induced myeloid leukemia cell differentiation protein), CCND1, and WNT3a [93]. All of these are upregulated in advanced prostate cancer and are associated with decreased survival and increased invasiveness [47]. The expression of miR-15a and -16-1 are downregulated in about 80 % of tumors compared with normal tissue and result in the inhibition of cell proliferation, invasion, and angiogenesis [54]. Thus their loss during cancer initiation is a significant event in cancer development. Re-expression of miR-15a/miR-16 results in growth arrest and apoptosis supporting their role as tumor suppressors in prostate cancer [94]. Interestingly, miR-15a/miR-16 have also been shown to be downregulated in stromal cells in prostate cancer suggesting that they would inhibit stromal support of prostate tumors if expressed. Specifically, downregulation of these miRNAs in cancer-associated fibroblasts increasing the expression of Fgf-2 (basic fibroblast factor 2) and its receptor Fgfr1, which in turn are associated with cell survival, migration, and proliferation. So this cluster of miRNA act on both the cancer cells directly as well as the supporting stromal tissue surrounding the tumor [95].

MiR-1-13a is a cluster of miRNA that includes miR-1, miR-133a, and miR-196a that regulate cell differentiation. MiR-1 is downregulated in prostate cancer, and is a homologue of miR-206. MiR-206, in breast cancer, is described as a suppressor of metastasis. This miRNA acts as a tumor suppressor and decreases expression of exportin-6 (XPO6) and phosphotyrosine kinase 9 (TWF1) [51]. Alshalalfa, and co-workers, recently reported that miR-1 is one of a small class of "master regulators of miRNA mediated regulation in prostate cancer." This ability to act as a master regulator was shown in part by miR-1's (and miR-16's) to disrupt protein complexes involving SMAD4 (mothers against decapentaplegic homolog 4) and HDAC-1 and to disrupt the NF-kappaB and RAS and Syndecan-pathways [96].

MiR-106b is part of the MiR-106-25 cluster that includes miR-25, miR-106b, and miR-93. This cluster has been shown to be involved in transformation of epithelial cells in prostate cancer into aggressive disease with androgen resistance and

neuroendocrine differentiation (NED). This NED phenotype is especially common in tumors with Gleason scores >8. MiR-106b assists in this process by downregulating the transcription repressor REST (RE-1 silencing transcription factor) [97, 98]. Upregulation of this cluster was also reported by Hudson et al. and associated with recurrence and when co-occurring with high miR-106b and caspase-7 (CASP7) was an independent marker for early recurrence [99]. MiR-106b directly targets p21/WAF decreasing the translation of its gene CDKN1A and also inhibits E2F1. Inhibition of E2F1 is thought to protect prostate cancer cells from apoptosis. Suppression of p21/WAF (cyclin-dependent kinase inhibitor-1 or CDK-interacting protein 1) expression mediates cell cycle arrest with exposure to chemotherapeutic agents [51].

8 Future Directions

There has been a plethora of evidence published, as delineated in this chapter, linking alterations in the human methylome for genes encoding both proteins and miRNAs with either the incidence or progression of human prostate cancer. However, to date the utility of this information for the diagnosis, prognosis and treatment of this disease has been very limited. The discovery of alterations in methylation patterns using the new technologies at hand promises to further expand the panel of genes that exhibit altered promoter methylation in prostate cancer. However, if we are to successfully bring these markers into the clinic for the improvement of patient care, then we must demonstrate sufficient specificity and sensitivity of these markers through rigorous correlative studies that incorporate well-annotated tissues and a sound statistical plan. Assays must then be developed and validated under the standards of the Clinical Laboratory Improvement Amendment (CLIA) in a CLIA-certified laboratory environment. Only then will we be able to more broadly apply these methylation markers and include them in the armamentarium of diagnostic, prognostic and predictive assays to improve the care and treatment of prostate cancer patients.

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Methylation Profile Landscape in Mesothelioma: Possible Implications in Early Detection, Disease Progression, and Therapeutic Options

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Abstract

Malignant Pleural Mesothelioma (MPM) is an aggressive malignancy of the pleura associated with asbestos exposure. Incidence of MPM is expected to increase over the course of next decade in both Europe and the developing countries. Although significant progress has been made in terms of etiology and pathogenesis of this disease, currently available therapeutic options have not significantly improved the survival outcome of patients on standard chemotherapeutic regimens. Integrity of the cellular DNA is often altered in many cancers. Understanding of the molecular mechanisms that regulate cellular DNA alterations to facilitate cancer initiation and development has potential to allow better design of cancer cell inhibitory strategies. In this context, there is a need to explore the gamut of “omics” strategies to provide a comprehensive epigenetics profile for MPM. This chapter discusses the functional genomics and epigenetic patterns observed by various investigators studying MPM patient populations on global fronts, and attempts to present a holistic approach in combating this insidious disease. Here we provide investigators in this field with novel insights and methodologies used in other types of cancers that might have profound impact in the early detection, prognosis and potential therapeutic strategies for MPM.

Key words Biomarker, Cancer, Histone inhibitor, Mesothelioma, Methylation

1 DNA Methylation and Cancer Development

The hallmarks of cancer are aberrant epigenetic alterations that include global changes in DNA methylation, histone modification patterns, and chromatin-modifying enzyme-expression profiles, which alter the gene expression profiles, and are tightly associated with cancer initiation and progression [1]. In addition to a large number of genetic alterations that are associated with many diseases; recent advances in the field of biomedical research have established that prominent epigenetic abnormalities play important roles in human diseases including cancers [2]. In fact, alteration of DNA methylation is one of the most commonly observed

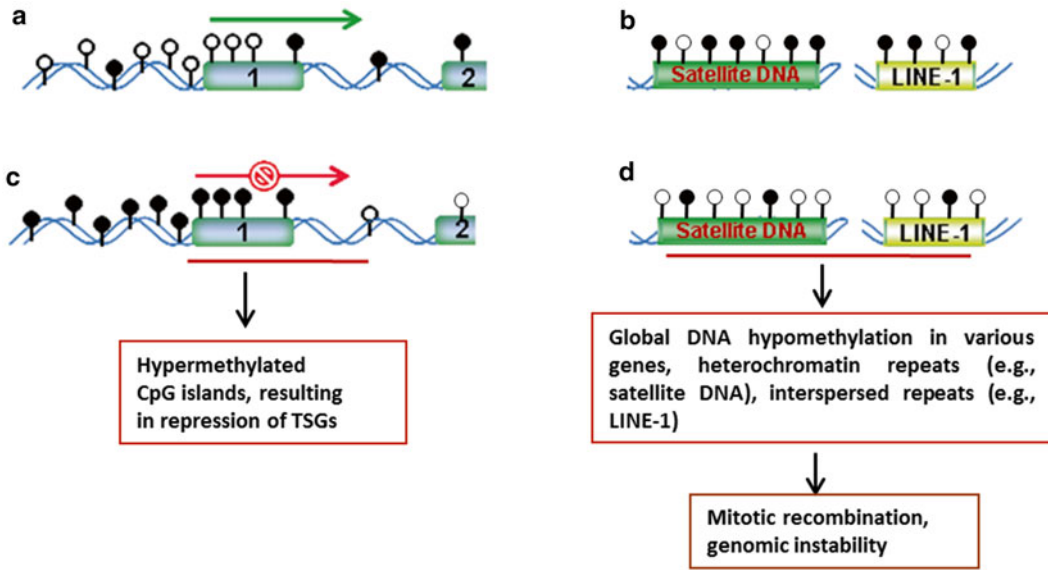


Fig. 1 A schematic comparison of DNA methylation between normal (**a, b**) and cancer cells (**c, d**). *White circles* unmethylated cytosine residues, *black circles* methylated cytosine residues, *1, 2* the exons of the predicted tumor suppressor gene (TSG), *⊗* blocked gene transcription

epigenetic events in tumorigenesis (Fig. 1). An aberrant DNA methylation profile has been recognized as a common thread in many if not all types of human neoplasia [3]. MPM has also been studied extensively for alterations in gene expression and aberrant epigenetic changes have been observed in a cohort of patient populations on a worldwide scale [4, 5].

2 DNA methylation in Mesothelioma

2.1 CpG Island Methylation in Mesothelioma

Malignant pleural mesothelioma (MPM) is a fatal neoplasm associated with a known history of asbestos exposure [6]. Asbestos fibers have been shown to be both cytotoxic and clastogenic in in vitro studies [7, 8]. The mitotic process interfered by asbestos can lead to abnormal chromosome segregation and deletion events [9]. The molecular mechanisms of carcinogenesis from mesothelial cells to mesothelioma are largely unknown. Although mutations and deletions of p53 and Rb occur frequently in many cancers these changes are rare in mesothelioma. Recent study indicates that increased asbestos exposure is significantly associated with an increased prevalence of gene promoter methylation of cell cycle control genes including Adenomatous Polyposis Coli (APC), *Cyclin D* (CCND)2, *Cyclin-Dependent Kinase Inhibitor* (CDKN)2A, CDKN2B, a human open reading frame sequence HPPBP1 and *Ras Association* (RalGDS/AF-6) Domain Family Member (RASSF)1 genes [10]. In addition, there is increasing evidence that DNA

methylation alteration plays important role in the development of malignant mesothelioma although it was viewed previously that methylation imbalance was not a consequence of neoplastic transformation of mesothelial cells [11]. Recent studies have revealed the spectrum of somatic alterations in mesothelioma that is similar to other human cancers and includes both genetic and epigenetic events that act in concert to drive tumorigenesis and promote progression to a malignant phenotype. Now, epigenetic changes are increasingly recognized as one of major features of malignant mesothelioma. A high-throughput, array-based analyses of mesothelioma DNA revealed that increased asbestos exposure was significantly associated with an increased prevalence of gene promoter methylation of cell cycle control genes [12]. In general, the cytosine within the islands containing the CpG dinucleotides in the promoter and other regulatory regions of genes are targeted for methylation, and the methylated genes are often silenced/repressed (Fig. 1). A number of published studies have shown hundreds of differentially methylated CpG loci in MPM compared with non-tumor pleura [13]. Differentially methylated CpG clusters in MPM cause the disruption of gene expression for normal biological processes that often result in transformation and tumorigenesis of mesothelial cells. For example, cell cycle control genes *WEE1* and *RASSF1* both had significantly higher methylation in MPM, suggesting for an uncontrolled proliferative potential, a hallmark of cancer. The *Janus activated kinase* (JAK)2 had significantly decreased methylation, and concomitant silencing of *Suppressor of Cytokine Signaling* (SOCS) genes may then result in increased, or constitutive *Signal Transducer And Activator of Transcription* (STAT) activation, which is a recognized inflammation-associated mechanism of tumor initiation and promotion [13]. *WNT-inhibitory factor* (WIF)-1 expression was downregulated in mesothelioma by approximately 72-fold when compared with the normal mesothelial tissue [14]. WIF-1 contains a Wnt inhibitory factor domain that interacts with Wnt proteins and inhibits Wnt signaling. WIF-1 functions as a tumor suppressor gene, and has been found to be epigenetically silenced in various cancers. Thus, epigenetic alterations are common in mesothelioma and discriminate the malignant phenotype from normal pleura.

2.2 Hypermethylated Promoters in Mesothelioma

As mentioned above, some of the differential CpG islands in mesothelioma cells become aberrantly methylated, and this aberrant methylation targeting promoter regions of specific genes has been observed in various cancer types, including mesothelioma. Table 1 shows a growing number of genes that have been recently discovered and added to a panel of genes methylated in mesothelioma. The genes affected by hypermethylation are involved in pathways of cell-cycle regulation, DNA repair, apoptosis, angiogenesis, invasion, detoxification, and adhesion. Although the promoter

Table 1
Selected genes affected by aberrant methylation in MPM

Gene	Gene function	Methylation frequency of the genes in MPM patients	References
BMP3b	BMP3 belongs to the transforming growth factor-beta (TGFB) superfamily. Bone morphogenic protein, also known as osteogenin, induces bone formation	53 % methylation in Japanese patients versus 8 % in American patients	[15]
IGFBP3	This gene is a member of the insulin-like growth factor binding protein family. The protein forms a ternary complex with insulin-like growth factor acid-labile subunit and either insulin-like growth factor I or II. In this form, it circulates in the plasma, prolonging the half-life of IGFs and altering their interaction with cell surface receptors	75 % methylation in Japanese patients versus 15 % in American patients	[16]
WIF-1	The protein functions to inhibit WNT proteins, which are extracellular signaling molecules that play a role in embryonic development. This gene functions as a tumor suppressor gene, and has been found to be epigenetically silenced in various cancers	Promoter hypermethylation with decreased gene expression in the tumor	[14, 17]
Secreted frizzled-related protein family	The Secreted frizzled-related protein (SFRP) family consists of five secreted glycoproteins in humans (SFRP1, SFRP2, SFRP3, SFRP4, SFRP5) that act as extracellular signaling ligands. SFRPs are able to bind Wnt proteins and Fz receptors in the extracellular compartment. The interaction between SFRPs and Wnt proteins prevents the latter from binding the Fz receptors	One or more members are methylated in 85 % of primary tumors. Promoter methylation occurs in >80 % of primary mesothelioma	[14, 18, 19]
ESR1	This gene encodes an estrogen receptor, a ligand-activated transcription factor composed of several domains important for hormone binding, DNA binding, and activation of transcription	Highly methylated in MPM	[20]
SLC6A20	Transport of small hydrophilic substances across cell membranes is mediated by substrate-specific transporter proteins which have been classified into several families of related genes. The protein encoded by this gene is a member of the subgroup of transporter with unidentified substrates within the Na ⁺ and Cl ⁻ coupled transporter family. This gene is expressed in kidney	Highly methylated in MPM	[20]

SYK	This gene encodes a member of the family of non-receptor type Tyr protein kinases. This protein is widely expressed in hematopoietic cells and is involved in coupling activated immunoreceptors to downstream signaling events that mediate diverse cellular responses, including proliferation, differentiation, and phagocytosis. It is thought to be a modulator of epithelial cell growth and a potential tumor suppressor in human breast carcinomas	Highly methylated in MPM	[20]
CDH1	This gene is a classical cadherin from the cadherin superfamily. The encoded protein is a calcium dependent cell-cell adhesion glycoprotein comprised of five extracellular cadherin repeats, a transmembrane region, and a highly conserved cytoplasmic tail. Loss of function is thought to contribute to progression in cancer by increasing proliferation, invasion, and/or metastasis	Methylated in 71.4 % of MPM patients	[21]
FHIT	This gene, a member of the histidine triad gene family, encodes a diadenosine 5',5'''-P1,P3-triphosphate hydrolase involved in purine metabolism. The gene encompasses the common fragile site FRA3B on chromosome 3, where carcinogen-induced damage can lead to translocations and aberrant transcripts of this gene. In fact, aberrant transcripts from this gene have been found in about half of all esophageal, stomach, and colon carcinomas	Methylated in 78.0 % of MPM patients	[21]
APC1A	This gene encodes a tumor suppressor protein that acts as an antagonist of the Wnt signaling pathway. Defects in this gene cause familial adenomatous polyposis, an autosomal dominant pre-malignant disease that usually progresses to malignancy	Methylated in 14.3 % of MPM patients	[21]
RASSF1A	This gene encodes a protein similar to the RAS effector proteins. Loss or altered expression of this gene has been associated with the pathogenesis of a variety of cancers, which suggests the tumor suppressor function of this gene. The inactivation of this gene was found to be correlated with the hypermethylation of its CpG island promoter region	Methylated in 19.5–22 % of MPM patients	[12, 21, 22]

(continued)

Table 1
(continued)

Gene	Gene function	Methylation frequency of the genes in MPM patients	References
DAPK	Death-associated protein kinase 1 is a positive mediator of gamma-interferon-induced programmed cell death. DAPK1 encodes a structurally unique 160-kD calmodulin dependent serine-threonine kinase that carries 8 ankyrin repeats and 2 putative P-loop consensus sites. It is a tumor suppressor candidate	Methylated in 20.0 % of MPM patients	[21]
APC1B	Promotes rapid degradation of CTNNB1 and may function as a tumor suppressor. May function in Wnt signaling	Methylated in 32.5 % of MPM patients	[21]
p14ARF	This ARF product functions as a stabilizer of the tumor suppressor protein p53 as it can interact with, and sequester, MDM1, a protein responsible for the degradation of p53. This gene is frequently mutated or deleted in a wide variety of tumors, and is known to be an important tumor suppressor gene	Methylated in 44.2 % of MPM patients	[21, 22]
p15INK4B	This gene lies adjacent to the tumor suppressor gene CDKN2A in a region that is frequently mutated and deleted in a wide variety of tumors. This gene encodes a cyclin-dependent kinase inhibitor, which forms a complex with CDK4 or CDK6, and prevents the activation of the CDK kinases, thus the encoded protein functions as a cell growth regulator that controls cell cycle G1 progression	Methylated in 19 % of MPM patients	[22]
RARB	This gene encodes retinoic acid receptor beta, a member of the thyroid-steroid hormone receptor superfamily of nuclear transcriptional regulators. It binds retinoic acid, the biologically active form of vitamin A which mediates cellular signaling in embryonic morphogenesis, cell growth and differentiation	Methylated in 55.5 % of MPM patients	[21]
TEMEM30B	Components of phospholipid-translocators	Methylated in 38 % of MPM patients	[23]

KAZALDI	This gene encodes a secreted member of the insulin growth factor binding protein (IGFBP) superfamily. Studies of the mouse ortholog suggest that this gene product may have a function in bone development and bone regeneration	Methylated in 48 % of MPM patients	[23]
MAPK13	MAP kinases act as an integration point for multiple biochemical signals, and are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation, and development. This kinase is closely related to p38 MAP kinase, both of which can be activated by proinflammatory cytokines and cellular stress	Methylated in 38 % of MPM patients	[23]
MGMT	Involved in the cellular defense against the biological effects of O6-methylguanine (O6-MeG) in DNA. Repairs alkylated guanine in DNA by stoichiometrically transferring the alkyl group at the O-6 position to a cysteine residue in the enzyme. This is a suicide reaction: the enzyme is irreversibly inactivated	Methylated in 13.7 % of MPM patients	[24]
THBD	The protein encoded by this intronless gene is an endothelial-specific type I membrane receptor that binds thrombin. This binding result in the activation of protein C, which degrades clotting factors Va and VIIIa and reduces the amount of thrombin generated. Mutations in this gene are a cause of thromboembolic disease, also known as inherited thrombophilia	Promoter hypermethylation with decreased gene expression in the tumor	[25]
TYMP	This gene encodes thymidine phosphorylase that converts thymidine into two smaller molecules, 2-deoxyribose 1-phosphate and thymine. This chemical reaction is an important step in the breakdown of thymidine, which helps regulate the level of nucleosides in cells. Thymidylate phosphorylase also catalyses the reaction that converts capecitabine to active 5-FU	Promoter hypermethylation with decreased gene expression in the tumor	[26]

hypermethylation of APC, *O*-6-Methylguanine-DNA Methyltransferase (MGMT), CDKN p16, and RASSF1A genes represents paradigmatic cancer-related epigenetic silencing events in a variety of cancers, while some of these genes are hypermethylated in specific cancers [27, 28]. Overall, it seems that there are two types of hypermethylated genes in tumors: cancer-type nonspecific and cancer-type specific. For example, research studies have demonstrated frequent epigenetic inactivation of RASSF1A and CDKN p16 in a wide variety of tumors. In fact, RASSF1A and p16 are the most frequently inactivated proteins ever identified in human cancers including mesothelioma [29]. On the other hand, genes such as *guanosine triphosphate* (GTP)-binding nuclear protein GSP1 is hypermethylated in more than 90 % of prostate cancers, but is largely unmethylated in acute myeloid leukemia [30]. Among the genes commonly hypermethylated in mesothelioma are *secreted frizzled-related protein* (SFRP) (80 %), *Fragile Histidine Triad* (FHIT) (78 %), and *cadherin* (CDH)1 (71.4 %). However, a number of hypermethylated loci have been detected in MPM. Four genes in particular, *transmembrane protein* (TMEM)30B, *Kazal-Type Serine Peptidase Inhibitor Domain* (KAZALD)1, *Solute Carrier Family 6* (Proline IMINO Transporter), *Member* (SLC6A)20, and *mitogen activated protein kinase* (MAPK)13 (also known as *Stress activated protein Kinase 4/p38δ*), are specifically methylated in MPM although aberrant methylation of TMEM30B was also identified in melanoma [31]. It is worthy to mention that genetic alterations including methylation of cancer-associated genes such as APC, indicate that the accumulation of multiple genetic and epigenetic alterations might be required before a cell is fully converted into a neoplastic phenotype.

3 Methylated Genes as Potential Biomarkers

Mesothelioma is a fatal and asbestos-associated disease with patient survival from presentation of <12 months [32]. Because most of the patients have an advanced stage at presentation, the early diagnosis of MPM is critical for both the clinical and pathological perspectives. It is not unusual for patients to undergo several medical investigations without definitive diagnosis early in their course of illness. Thus, there is intense interest in the discovery of markers that can be assessed in pleural effusions, histological specimens, and serum to facilitate early diagnosis of MPM [33]. Among the molecular markers, DNA methylation patterns have shown promise for cancer diagnosis, prognosis, and treatment strategies.

As described above, the hallmark of cancer epigenetics is aberrant DNA methylation consisting of global hypomethylation and regional hypermethylation of many tumor suppressor genes. Since global DNA hypomethylation has also been observed in many

cancers, including some benign tumors, it is believed to constitute an early event in transformation of normal cells [34]. Therefore, global hypomethylation along with a panel of hypermethylated cancer-type nonspecific genes such as CDKN p16, RASSF1A, and MGMT could present a signature of all types of cancer, and thus serve as potential common biomarkers for screening or surveillance for the early detection of cancer. The panels of methylated cancer-type specific genes can be used as potential biomarkers for early detection of specific cancers, in particular the most malignant cancers such as mesothelioma. In this context, one study investigating the methylation status of the 28 loci has revealed that three loci, *Estrogen Receptor* (ESR)1 (also known as ER α), SLC6A20 and *Spleen Tyrosine Kinase* (SYK) had a substantially elevated frequency and/or level of methylation in MPM compared to non-tumor lung tissues. Combination of SLC6A20, SYK, and APC yields a sensitivity of 92 % and specificity of 73 % based on evaluation of the current collection of tissues, indicating that these three loci may serve as candidate early detection markers for MPMs [20]. However, the ability to detect methylation of these three genes in pleural fluid or blood of the patients with MPM is required for further investigation.

Methylation of some genes may be associated with prognosis of patients with MPM. Fischer et al. [21] have reported that combining *retinoic acid receptor* (RAR)- β with either *death-associated protein kinase* (DAPK) or RASSF1A showed a significantly shorter overall survival of those patients who had both genes methylated compared to those with only one or neither epigenetic alteration, even though the methylation status of none of the single genes significantly influenced prognosis. Similarly, the combination of all three genes revealed a worse prognosis for patients with double or triple methylations compared to the group which had only one or no gene methylated. These results support the idea that the prognostic value of a combination of epigenetic alterations is superior to the impact of an individual gene alone on overall survival [21]. In addition, Destro et al. showed that methylation of tumor suppressor genes such as p15, p16, RASSF1A, and Nore1A (*a homolog of RASSF1A*) is associated with poor prognosis of MPM [22]. Elucidation of aberrant DNA methylation patterns anchored in large majority of human tumor samples will open a new avenue in diagnostic and prognostic applications for these cancer patients.

4 Targeting of Methylated Genes to Restore Gene Functions

Tumor-specific DNA methylation patterns result in silencing tumor suppression genes in cancer cells. These aberrant epigenetic modification patterns are established and maintained by DNA (Cytosine-5-)-Methyltransferases (DNMTs). Epigenetic cancer

therapy aims to restore normal epigenetic modification patterns through the inhibition of epigenetic modifier enzymes. Therefore, DNMTs serve as logical targets for oncology drug development. There are two strategies that can be utilized to revert the local hypermethylation in cancer: DNMT inhibitors and interference with DNMTs expression. There are two types of DNMT inhibitors: nucleoside analogues and non-nucleoside DNMT inhibitors. Two prominent examples are the cytosine analogs 5-azacytidine (Azacytidine, Vidaza) and 20-deoxy-5-azacytidine (Decitabine, Dacogen), which are potent inhibitors of DNMTs and have been approved for the treatment of myelodysplastic syndrome, a pre-leukemic bone marrow disorder [35].

Although DNMT inhibitors are currently being used as cancer therapies after decades of studies, progress made in MPM therapy has been rather little. One study showed that both DNMT1 silencing and Decitabine treatment substantially induced MPM cell death. However Decitabine, in addition of being an inhibitor of DNMT1 function, also mediates a p53-independent p21 upregulation, and is characterized by causing the arrest of MPM cells at the G2/M phase of the cell cycle. In contrast, no changes in expression of p53 and p21 proteins were observed in cells where DNMT1 expression was silenced [36]. These findings highlight the possibility that both DNMT inhibitors and DNMT silencing could be effective alternative approaches for mesothelioma treatment. Since nearly all MPM patients progress during or after first-line treatment, second-line chemotherapy is being increasingly used in clinical practice. Therefore, testing and development of new chemotherapy agents as well as new chemotherapeutic strategies are needed as second-line therapies for MPM [37].

Combination therapies are expected to enhance or extend the efficacy of the drugs, in part by countering the molecular effects of drugs that abrogate their efficacy and to reduce the side effects through applying lower dosages of one or both drugs [38]. Combination therapies employing DNMT inhibitors and other agents are under investigation. Interleukin-13 receptor $\alpha 2$ (IL-13R $\alpha 2$) is known as a cancer antigen and high expression of this receptor is found in various human cancers including malignant mesotheliomas. Immunotoxin or cytotoxin therapy for cancer targeting IL-13R $\alpha 2$ has already been investigated in preclinical and clinical trials. Recent study revealed that IL-13R $\alpha 2$ expression was augmented with epigenetic modulation by a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine. Not surprisingly then that a combination of 5-aza-dC and anti-IL-13R $\alpha 2$ antibody suppressed tumor growth mediated by IFN- γ and prolonged the survival of mice bearing mesothelioma xenografts. These observations indicate a promising role for IL-13R $\alpha 2$ as a target for antibody treatment in malignant mesothelioma, and, in combination with epigenetic modulators, suggest a novel strategy to enhance

therapeutic potency [39]. Future research on mesothelioma therapy should be directed toward targeted DNMT inhibition and toward exploring interactions between epigenetic pathways such as DNA methylation and histone deacetylation to maximize cancer specificity.

5 Future of Genetics and Epigenetics in Mesothelioma

Based on the comprehensive review of the current information with regards to potential biomarkers and therapeutic options available to mesothelioma patients, it is an emerging field that is likely to contribute significantly in early detection, better prognostication, and rapid discovery of novel combination therapeutic strategies. Although several clinical trials have been reported using Histone Deacetylase (HDAC) or DNMT inhibitors, but the most promising one appears to use valproate-doxorubicin combination [4, 5]. There is a growing body of evidence that suggests influence of metabolism on epigenetics and disease that need to be explored in greater depth to unravel chemotherapy unresponsiveness in recalcitrant cancers, due to underlying defects among various cellular pathways [40–42]. There is also a need to develop and integrate database repositories from various sources, initiate new techniques and new collaborations among surgeons, oncologists, basic and translational scientists to complement resources available to combat MPM.

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Part III

Methods and Technologies Used for Detecting Epigenetic Changes

Chapter 13

Techniques to Access Histone Modifications and Variants in Cancer

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Abstract

Recent years have witnessed an explosion of epigenetic research on the role of histone variants and modifications in cancer. To understand the global dynamics of chromatin structure and function, analysis of histone variants incorporated into the nucleosome and their covalent modifications, is required. The nucleosome is the fundamental structural unit of chromatin, contains an octamer of core histones H3, H4, H2A, and H2B. The differential alterations in diverse histone variants and their accompanying modifications patterns will provide a deeper insight into their biological role in structural and functional properties of chromatin. Here we provide a step-by-step protocol to investigate these aspects, the histone modifications and variants, their localization and dynamics within specific regions of chromatin under distinct condition and the recruitment/retention of epigenetic regulators at their target sites in chromatin to influence cell growth and differentiation.

Key words Chromatin, Histone, Variants, Post-translational modification, Cancer

1 Introduction

Eukaryotic genomic DNA is tightly packaged into chromatin by histones inside the nucleus. Covalent modifications of histones and their variants are central to the regulation of chromatin dynamics; therefore, many biological processes involving chromatin, such as replication, repair, transcription, and genome stability, are regulated by histones [1]. The perturbation of the enzymatic machinery involved in different types of histone modification like acetylation, ubiquitination, phosphorylation, and methylation can cause developmental defects and disease [1]. Further, the diversity of histone into multiple variants results in structural plasticity of chromatin via deposition in functionally distinct chromosomal domains. Thus highlighting the distinct regulatory mechanisms for gene expression under various physiological states [2]. However,

the mechanisms of this fundamental process are poorly understood so far.

The role of epigenetic phenomena in cancer biology is increasingly being recognized in the recent past. Global loss of acetylation of histone H4 at lysine 16 (H4K16Ac) and loss of trimethylation of histone H4 at lysine 20 (H4K20me3) were the first histone marks reported to be deregulated in cancer cells [3]. In prostate and ovarian tumors, decrease of H3K9ac has been linked with histological grading, tumor progression and poor prognosis [4–6]. Further, levels of H3K27me3 and H3K18ac have been found to have prognostic value in multiple cancers; patients with low level of H3K27me3 and H3K18ac have shorter overall survival time [7–12]. In recent times, histone variants of H2A family and their correlation with cancer have been of immense interest. H2A.Z has been reported to be over expressed in colorectal, breast, lung, and bladder cancer and the overexpression of H2A.Z in breast tumors correlates with increased probabilities of cancer metastasis and decreased patient survival [13–15]. Also, H2A.X deficiency has implications on genomic stability and thereby on cancer incidences in coherence with p53 deficiency. In addition, macroH2A, heteromorphous variant, has been associated with several tumor types, including melanoma, lung, testicular, bladder, colon, ovarian, breast, cervical, and endometrial cancers. Macro H2A is found to be able to suppress tumor progression of malignant melanoma through regulation of CDK8 [16]. Also, the over expression of H2A.1, homomorphous variant of H2A, correlates with sequential development of hepatocellular carcinoma with decrease of H2A.2, [17]. CENP-A, a variant of H3 has a critical role in shaping the centomere/kinetochore structure is upregulated in colorectal cancer, lung adenocarcinoma, invasive testicular germ cell tumors, breast cancer, and hepato-cellular carcinoma [18]. H3.3 one of the highly conserved variant differing from canonical H3.1 and H3.2 in five amino acids, has been reported to be over expressed in various human tumors. Recently, exome sequencing studies have revealed the mutations in H3.3 in pediatric Glio-Blastoma Multiforme (GBM) and Diffuse Intrinsic Pontine Gliomas (DIPGs) [19]. Though, the alterations in the histone variants still remains to be explored in understanding the mechanism and functional significance of histones in cancer biology. The Histone Infobase: Histome (www.histome.net) provides detailed information on histone variants and their post-translational modifications [20].

The following protocols will provide investigator with an excellent platform to begin their own research in the field of histone biology. The chapter deals with specific protocols to delineate the post-translational modifications and variants of histone; followed by protocols for understanding the functional significance of histones when incorporated into the genome.

2 Materials

2.1 Extraction of Histones

1. Dounce homogenizers and Mortar/pestle.
2. 10× Phosphate Buffer Saline (PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na₂HPO₄, and 0.24 g KH₂PO₄ in 100 ml milli-Q water.
3. Cell Lysis Buffer: 250 mM Sucrose, 50 mM Tris-HCl, pH 7.5, 25 mM KCl, 5 mM MgCl₂, 50 mM NaHSO₃, 45 mM Sodium butyrate, 10 mM β-mercaptoethanol, 0.2 % Triton-X100 + Inhibitors.
4. Inhibitors: 0.2 mM PMSF, 0.15 mM Spermine, 0.5 mM Spermidine, 2 mM EDTA, 10 mM Sodium fluoride, 1 mM Sodium orthovanadate, 10 mM β-glycerophosphate.
5. Histone Extraction Solution: 0.34 M Sucrose, 50 mM Tris-HCl pH 7.5, 25 mM KCl, 5 mM MgCl₂, 1 mM DTT, 0.1 % Triton-X100, 0.1 mM EDTA, 0.1 mM EGTA, 0.1 mM PMSF, 0.5 mM Spermidine.
6. 0.2 N H₂SO₄ (Sulfuric acid): Mix 218.57 μl of 18 M H₂SO₄ in 19.87 ml of milli-Q water.
7. Acidified Acetone: 50 mM HCl: 1.077 ml HCl in 250 ml of Acetone, store at 4 °C.
8. 100 % Acetone, store at 4 °C.

2.2 Resolution of Histones

2.2.1 Sodium Dodecylsulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

1. Gel running apparatus and Power pack.
2. Stock Acrylamide: 30 % Acrylamide, 0.8 % Bis-acrylamide. Dissolve 75 g of Acrylamide and 2.0 g of *N,N'*-methylene bis-acrylamide in 150 ml of milli-Q water. Make the volume to 250 ml, filter and store at 4 °C.
3. 1.5 M Tris-HCl, pH 8.8.
4. 1 M Tris-HCl, pH 6.8.
5. Ammonium persulfate solution (10 %w/v), freshly prepared.
6. *N,N,N',N'*-Tetramethylene diamine (TEMED).
7. 18 % Resolving gel (30 ml): 30 % Acrylamide (18 ml), milli-Q water (4.035 ml), 1.5 M Tris-HCl, pH 8.8 (7.5 ml), 10 % SDS (300 μl), TEMED (15 μl), 10 % APS (150 μl).
8. Stacking gel (10 ml): 30 % acrylamide (1.3 ml), milli-Q water (7.29 ml), 1.5 M Tris-HCl, pH 6.8 (1.25 ml), 10 % SDS (100 μl), TEMED (10 μl), 10 % APS (50 μl).
9. 2× Loading dye: 0.0627 M Tris-HCl pH 6.8, 1 % w/v SDS, 10 % Glycerol, 1 % β-mercaptoethanol, 0.0025 % Bromophenol Blue (BPB).

**2.2.2 Acetic Acid Urea
Triton Polyacrylamide Gel
Electrophoresis
(AUT-PAGE)**

1. Lamp, light source required for gel polymerization.
2. Acrylamide stock solution: 60 % (w/v), storage at 4 °C.
3. *N,N'*-methylene bis-acrylamide stock solution: 2.5 % (w/v) in milli-Q water.
4. Separating gel solution: 15 % w/v Acrylamide, 0.1 % w/v Bis-acrylamide, 1 % v/v acetic acid, 0.05 M ammonium hydroxide, 8 M urea, 0.56 % w/v Triton X-100, 0.5 % TEMED, 0.0004 % w/v flavin mononucleotide.
5. Stacking gel solution: 4 % w/v Acrylamide, 0.16 % w/v Bis-acrylamide, 1 % v/v acetic acid, 0.05 M ammonium hydroxide, 0.5 % v/v TEMED, and 0.0004 % w/v flavin mononucleotide.
6. Electrophoresis buffer: 1 M Acetic acid and 0.1 M Glycine.
7. Sample Buffer: Weigh DTT into a sample buffer preparation tube for a final concentration of 1 M. Add 0.9 ml of 8 M urea stock solution, 0.05 ml of phenolphthalein, and 0.05 ml of concentrated ammonium hydroxide to the tube to obtain the intensely pink sample buffer. Add 0.05 ml of sample buffer per sample tube with lyophilized protein (25 µg) to be analyzed. Acidify the sample by addition of 1/20 volume of glacial acetic acid. Add pinch of methylene blue, a running front dye to above sample.

**2.2.3 Two Dimensional
(2D)-SDS-AUT PAGE**

1. Equilibration buffer: 1 % w/v protamine sulfate, 5 % v/v 2-β mercaptoethanol (β-ME), 0.75 M potassium acetate pH 4.8, 20 % v/v glycerol, 1 % cysteamine HCl, and 6 M urea.
2. Sealing buffer: 1 % w/v Agarose, 0.75 M Potassium acetate pH 4, 20 % v/v Glycerol, and 0.001 % Pyronin Y.

**2.3 Western Blotting
of SDS and AUT-PAGE**

1. Protein molecular weight standard marker.
2. PVDF membrane, Whatman 3 mm filter paper, and Electro blotting apparatus.
3. SDS-Transfer buffer: 0.19 M Glycine, 25 mM Tris-HCl base, 0.01 % SDS, and 20 % methanol.
4. Tris Buffered Saline with Tween 20 (TBST): 20 mM Tris-HCl, 500 mM NaCl, 0.1 % (v/v) Tween 20, pH 7.4.
5. Blocking buffer: 5 % skimmed milk or 5 % BSA in TBST.
6. Destaining Solution: 50 % methanol, 40 % milli-Q water, and 10 % glacial acetic acid.
7. Fast green stain: 0.03 % in Destaining solution.
8. Histone H3 Serine 10 phosphorylation antibody (Abcam, AB5176).
9. Horseradish peroxidase (HRPO)-anti-IgG conjugate secondary antibody.

10. AUT-Equilibration Buffer 1: 0.05 M acetic acid, 0.5 % w/v SDS.
11. AUT-Equilibration Buffer 2: 0.0625 M Tris-HCl pH 6.8, 2.3 % w/v SDS, 1 % β -ME.
12. AUT-Transfer Buffer, pH 10: 25 mM CAPS, 20 % v/v methanol.

2.4 Immuno-fluorescence

1. Humidified chamber, Glass slides and Coverslips, Nail Polish.
2. Fixing solution: 4 % w/v paraformaldehyde in 1 \times PBS.
3. Washing solution 1: 1 \times PBS, pH 7.4.
4. Washing solution 2: 0.1 % NP-40 in 1 \times PBS.
5. Permeabilizing solution: 0.5 % Triton X-100 in 1 \times PBS.
6. Blocking buffer: 0.3 % BSA, 0.1 % NP-40 in 1 \times PBS.
7. Vecta-shield mounting solution.

2.5 Immuno-histochemistry

1. Paraffin-embedded tissue sections.
2. Glass slides: Coated with 0.01 % Poly-L-Lysine solution.
3. Slide rack, Oven (temperature range 30–100 °C), Humid chamber, Glass coupling jars, and Microwavable plastic coupling jars.
4. Reagents: 1 \times TBS (pH 7.4), Xylene, Ethanol, Methanol, and Hydrogen peroxide.
5. Antigen Retrieving Buffer: 10 mM Sodium citrate buffer, pH 6.2 in milli-Q water.
6. Universal Vectastain ABC kit (PK 6200).
7. Counter-staining solution (Hematoxylin solution).
8. Peroxidase activity removing solution: 30 % H₂O₂ in 1 \times TBS.
9. Blocking solution: 1:50 horse serum diluted in 1 \times TBS.
10. Antibody dilution buffer: 1 \times TBS.
11. DAB staining solution: 75 mg DAB in 300 ml of 1 \times TBS, mix well, filter through blotting paper, add 100 μ l of H₂O₂ just before staining.

2.6 Chromatin Immunoprecipitation

2.6.1 Isolation and Cross-Linking of Nuclei

1. Reagents: 37 % Formaldehyde and 2.5 M Glycine.
2. ChIP Buffer: 10 mM HEPES pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT + Inhibitors.
3. Inhibitors: 100 mM PMSF, 45 mM Sodium butyrate, 0.15 mM Spermine, 0.5 Spermidine, 10 mM Sodium Fluoride, 10 mM β -Glycerophosphate, 2 μ g/ml Aprotinin, 1 μ g/ml Leupeptin, 0.7 μ g/ml Pepstatin.

**2.6.2 Micrococcal
Nuclease (MNase)
Digestion of Chromatin
and Immunoprecipitation**

1. Protein A/G (50 % slurry).
2. Micrococcal Nuclease (MNase) enzyme.
3. ChIP 10× buffer: 150 mM Tris-HCl pH 7.4, 150 mM NaCl, 600 mM KCl.
4. ChIP solution: 0.34 M sucrose, 1× ChIP buffer, 2 mM CaCl₂ + Inhibitors.
5. 2×RIPA buffer: 100 mM Tris-HCl pH 8, 300 mM NaCl, 0.2 % SDS, 1 % sodium deoxycholate, 2 % Triton X-100 + Inhibitors.
6. Nuclear Lysis Buffer: 5 M Urea–2 M NaCl.

**2.6.3 DNA Extraction
from Immunoprecipitates**

1. Low salt buffer: 0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 150 mM NaCl.
2. High salt buffer: 0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.0, 500 mM NaCl.
3. LiCl buffer: 0.25 M LiCl, 1 % NP40, 1 % Sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl pH 8.0.
4. Elution buffer: 2 % SDS, 0.1 M NaHCO₃, and 10 mM DTT.
5. 4 M NaCl.
6. 3 M sodium acetate pH 5.2.
7. 100 µg/ml Proteinase K.
8. 100 µg/ml RNaseA.

**2.7 Fluorescence
Recovery After
Photobleaching**

1. Confocal microscope preferably with focus drift compensation and stage with accessories to maintain temperature and CO₂.
2. A slow migrating cell line like HeLa Koyoto expressing histone-GFP fusion protein.
3. Live cell chamber.
4. ImageJ Software.

**2.8 Histone Dimer
Pull Down**

1. Purified histone monomers (either native or with 6×His tag).
2. Superdex 200 Gel Filtration Column and 6–8 kDa cut-off dialysis tubings.
3. Ni-NTA beads.
4. HD Buffer A: 20 mM potassium phosphate, 10 mM HCl, 100 mM NaCl, 1 mM PMSF, 5 % glycerol.
5. HD Buffer B: 20 mM potassium phosphate, 100 mM NaCl, 1 mM PMSF, 5 % glycerol.
6. HD Buffer C: 20 mM potassium phosphate, 1.5 mM MgCl₂, 420 mM NaCl, 0.5 mM DTT, 25 % glycerol, 0.2 mM EDTA + Inhibitors.

7. HD Buffer D: 20 mM potassium phosphate, 1.5 mM MgCl_2 , 100 mM NaCl, 0.5 mM DTT, 25 % glycerol, 0.2 mM EDTA, 10 mM Imidazole + Inhibitors.
8. Laemmli Buffer: 60 mM Tris-HCl pH 6.8, 2 % SDS, 10 % glycerol, 5 % β -mercaptoethanol 0.0025 % Bromophenol Blue (BPB).

3 Methods

3.1 Extraction of Histones for Cell Lines and Tissues

3.1.1 From Cell Lines

1. Remove media from confluent culture plates. Wash the cells twice with chilled 1×PBS. Scrap cells into fresh 1×PBS and pool in the 1.5 ml centrifuge tube (*see Note 1*).
2. Centrifuge at $1,500\times g$ for 10 min at 4 °C. Measure the packed cell volume (pcv) and resuspend the cell pellet in freshly prepared ice cold cell lysis buffer in ~5 volumes of pcv.
3. Incubate for 10 min at 4 °C and confirm cell lysis using trypan blue exclusion (lysis should be >80 %) (*see Note 2*).
4. Centrifuge at $1,500\times g$ for 15 min at 4 °C to pellet nuclei and wash twice with cell lysis buffer.
5. The nuclei pellet obtained is processed for histone extraction.

3.1.2 From Tissues

1. Powder the frozen tissue (400 mg) in mortar and pestle with liquid nitrogen (*see Note 3*).
2. Homogenize the powdered tissue in Dounce homogenizer for 7–8 strokes with chilled 4 ml histone extraction solution. Incubate on ice for 10 min (*see Note 4*).
3. Centrifuge the homogenate at $1,500\times g$ at 4 °C for 15 min to pellet nuclei. Resuspend the pellet containing nuclei in 4 ml of chilled histone extraction solution.
4. Centrifuge the homogenate at $1,200\times g$ at 4 °C for 15 min.
5. The nuclei pellet obtained is processed for histone extraction.

3.1.3 Acid Extraction of Histones

1. Add of 0.2 M H_2SO_4 (6 vol.) to nuclear pellet (**step 5** in Subheadings 3.1.1 and 3.1.2), followed by intermittent vortexing at 4 °C for 90 min (*see Note 5*).
2. Centrifuge at $12,000\times g$ at 4 °C for 20 min. Transfer the supernatant containing histone protein into fresh tube and discard pellet containing DNA.
3. Add 5 volumes of acetone, mix gently, and leave overnight at –20 °C for histones precipitation.
4. Centrifuge at $12,000\times g$ at 4 °C for 20 min.
5. Wash precipitated histone pellet twice with 50 mM acidified acetone followed by washing with chilled acetone with in-between centrifugation as in **step 4** of Subheading 3.1.3.

6. Dry the histone pellet in refrigerated speed vacuum at 4 °C for 20 min.
7. Dissolve the histone pellet in 0.1 % β -mercaptoethanol in H₂O and store at –20 °C.

3.2 Resolution of Histones

3.2.1 SDS-PAGE

1. Wipe the glass plates with methanol, assemble the apparatus and pour resolving gel mixture, and allow the 18 % gel to polymerize for 45 min at room temperature (RT).
2. Pour the stacking gel mixture over the polymerized resolving gel and place the gel comb inside. Leave it for polymerization for 60 min.
3. Mix the histones with equal volume of 2 \times loading dye and incubate in boiling water bath for 15 min. Load the samples and allow the bands to stack on resolving gel at 15 mA and resolve at 30 mA (constant current).
4. Remove the gel from the assembly as confirmed either by run length of prestained protein marker on gel or when BPB dye reaches to the bottom of gel.
5. Gel can be stained either by coomassie (if concentration of histone is >5 μ g/lane) or silver staining (if concentration of histone is <5 μ g/lane) (Fig. 1a) (*see* **Notes 6** and **7**).

3.2.2 AUT-PAGE

1. The separating gel solution is allowed to polymerize in assembled glass plates in the presence of light, 1–2 h (*see* **Note 8**).
2. After polymerization of separating gel, stacking gel solution is poured with gel comb and allowed to polymerize for 45 min–1 h.
3. The polymerized wells are cleaned properly by electrophoresis buffer and the gel is pre-electrophoresed for 2 h at constant voltage of 200 V. The positive (+ve) electrode is connected to the upper reservoir and negative (–ve) electrode is attached to lower reservoir, as histones are positively charged and will move toward negatively charged electrode (cathode) (*see* **Note 9**).
4. The histone samples are prepared in sample buffer and are resolved at constant voltage of 200 V until front running methylene blue dye reaches to the bottom of gel.
5. The gel is stained as per “SDS-silver” staining method (Fig. 1b).

3.2.3 Resolution of Histones on Two Dimensional (2D)-SDS-AUT PAGE

Histones are resolved on 18 % SDS-PAGE gel for the first dimensional electrophoresis on the basis of molecular weight followed by 15 % AUT-PAGE for the second dimensional electrophoresis to resolve major and minor histone variants and post-translationally modified isoforms on the basis of charge, mass, and hydrophobicity.

1. Resolve total histones (20–25 μ g) on first dimensional SDS-PAGE, perform the coomassie staining, and cut out the core histones, H2A, H2B, H3, and H4 region (*see* **Note 10**).

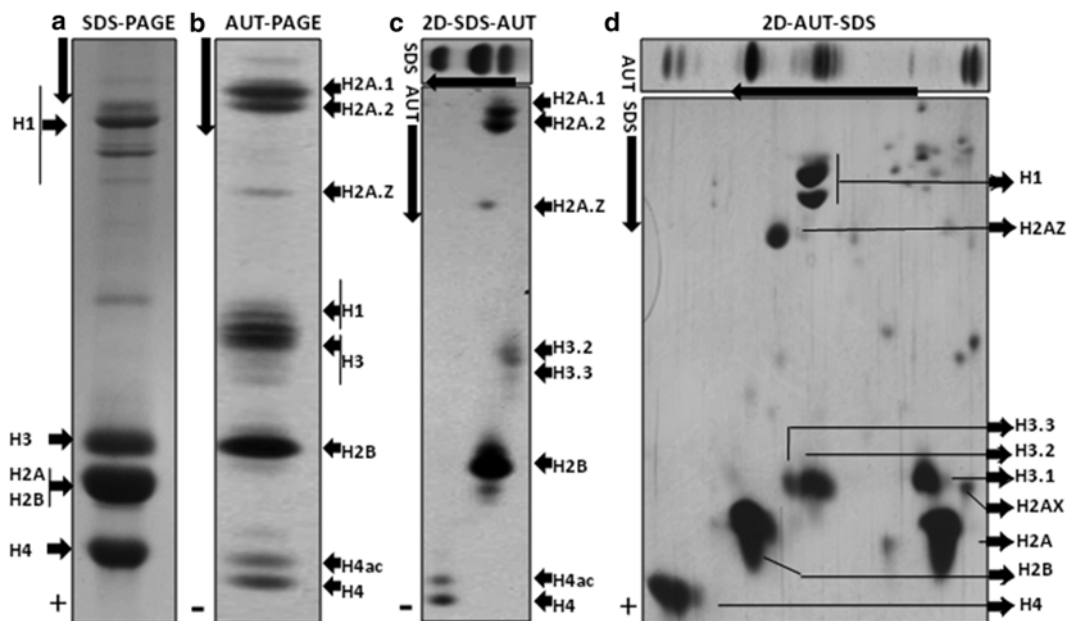


Fig. 1 Total histone profiling of acid extracted histones from rat liver. **(a)** Total histones (2 μ g) were resolved on 18 % SDS-PAGE followed by silver staining. Linker histones are marked as H1 region. **(b)** Total histones resolved on 15 % AUT-PAGE (5 μ g) and silver stained. **(c)** Total histones (20–25 μ g) were resolved on 18 % SDS-PAGE gel followed by coomassie staining. After destaining, the core histones were excised and electrophoresed on second dimensional 15 % AUT-PAGE, and **(d)** 20–25 μ g of histone was resolved on 15 % AUT-PAGE. After staining and destaining of gel, the AUT-lane was excised and electrophoresed on second dimension 18 % SDS-PAGE

2. Prepare the AUT-PAGE as mentioned above without wells in the stacking gel and pre-electrophorese for 2 h at constant voltage of 200 V.
3. Equilibrate the gel slice of SDS-PAGE containing core histones for 60 min in equilibration buffer.
4. Place equilibrated gel slice horizontally to the top of pre-electrophorese AUT-PAGE and seal the gel slice using sealing buffer.
5. Carry out electrophoresis at constant voltage of 200 V till the pyronin Y dye pass out the gel, followed by 2 h of over running the gel for better resolution of histones or standardize. Stain the gel either by “SDS-silver” or “coomassie” or “amido black-silver” staining method (Fig. 1c).

3.3 Western Blotting

3.3.1 SDS-Immunoblotting

1. On completion of electrophoresis, SDS-gel is removed and processed for Immunoblotting.
2. Activate PVDF membrane equal to the gel size in 100 % methanol for 30 s, and immediately wash with milli-Q water for 30 s.

3. Equilibrate gel, Whatmann paper, and PVDF membrane for 15 min at RT in SDS-transfer buffer. Assemble the transfer stack with membrane on anode side just above the gel, three paper of Whatmann on both the sides and place the cassette in transfer apparatus (*see* **Note 11**).
4. Transfer proteins at 4 °C, employing a constant current of 300 mA for 180 min with chilled SDS-transfer buffer to minimize the heat generated during electro transfer (*see* **Note 12**).
5. Remove membrane on completion, mark the orientation and prestained marker bands with lead pencil and check the transfer of protein on membrane by staining with fast green (0.03 % in destaining solution) and destain.

3.3.2 AUT- Immunoblotting

1. Transfer the AUT-gel in a staining tray and wash with AUT-equilibration buffer 1 (30 min × 2) and AUT-equilibration buffer 2 (15 min × 2).
2. Activate PVDF membrane equal to the gel size in 100 % methanol for 30 s, and immediately wash with milli-Q water for 30 s.
3. Pre-equilibrate the gel, whatmann and PVDF membrane in AUT-transfer buffer for 10 min.
4. Set up the transfer with AUT-transfer buffer as mentioned in **steps 2–4** of Subheading **3.3.1**.

3.3.3 Immunoprobng

1. Membrane with transferred protein is washed with TBST for ~5 min to destain.
2. Block the membrane with 5 % non-fat skimmed milk or 5 % BSA in TTBS for 1–2 h at RT or as per manufacturer's specification or standardization of antibody to be used.
3. Remove membrane from blocking solution and incubate with primary antibody (e.g., phospho-H3Serine10). Primary antibody is diluted in 5 % or 1 % BSA/milk (e.g., phospho-H3Serine10, 1:1,000 in 5 % BSA) followed by incubation at 4 °C overnight or as per manufacturer's specification of antibody to be used.
4. Post-incubation wash the membrane with TBST (5 min × 2 and 10 min × 2) at RT on shaker.
5. Incubate the membrane at RT for 1 h with secondary antibody (e.g., 1:8,000 dilutions of anti-rabbit for phospho-H3Serine10) horse radish peroxidase conjugated secondary antibodies.
6. Wash the membranes as in **step 4** of Subheading **3.3.2**.
7. Visualize the immunoreactive bands with enhanced chemiluminescence reagent (Millipore) or femto-west (Pierce) as per the manufacturer's instructions followed by autoradiography.
8. The representative data is shown as Fig. **2a** and **c** for immunoblotting with antibody against histone H3 Serine 10 phosphorylation.

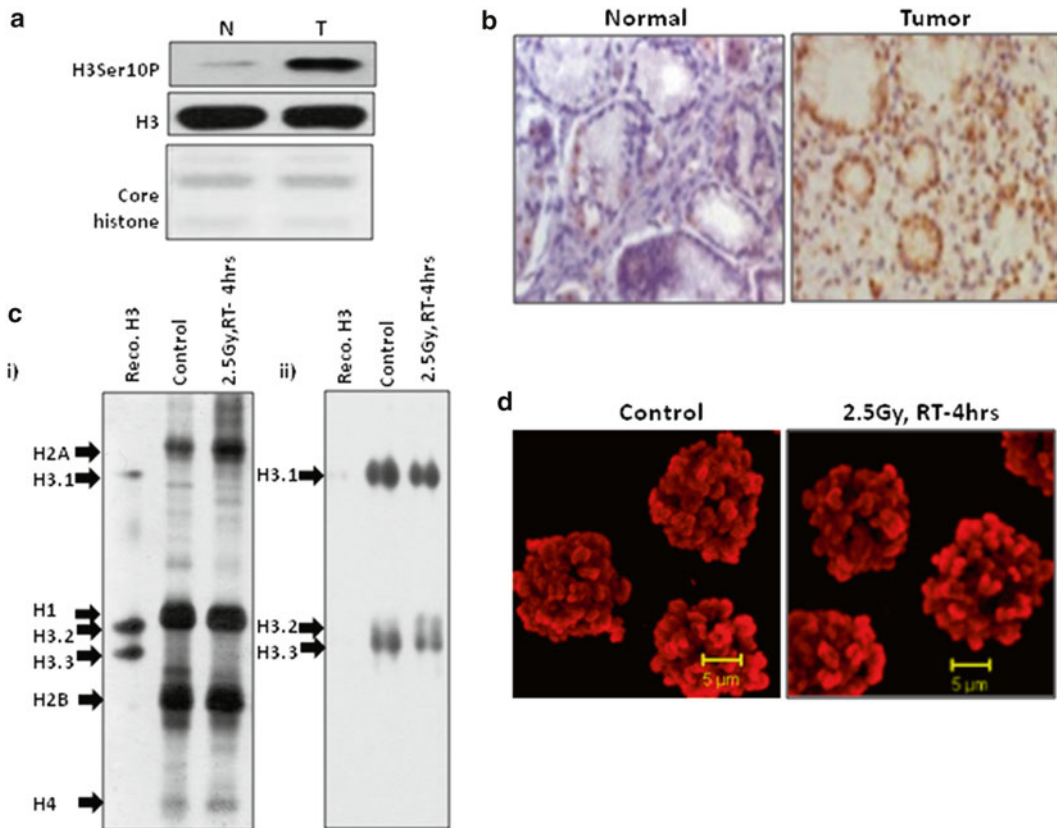


Fig. 2 Identification of histone post-translational modifications: (a) Immunoblot of H3 Serine10 phosphorylation after SDS-PAGE of histones from human normal and gastric tumor tissues. Tumor shows higher level of H3Ser10phospho compared to normal tissues. Anti-H3 is used as loading control. (b) Immunohistochemistry with anti-H3Ser10phospho on human normal and gastric tumor tissue. Nuclear staining reveals higher level of H3Ser10P in tumor than normal tissue. (c) (i) Silver stained and (ii) Immunoblot of anti-H3Ser10phospho after AUT-PAGE of histones isolated from G2/M arrested control and irradiated WRL68 cell line along with recombinant H3 variants H3.1, H3.2, and H3.3. Anti-H3Ser10P shows maximum signal intensity with H3.1 along with H3.2 and H3.3, but without a significant alteration in signal intensity in response to irradiation. (d) Immunofluorescence with anti-H3Ser10phospho in G2/M arrested control and irradiated WRL68 cells. RT-Recovery time after DNA damage

3.4 Immuno-fluorescence

Fluorescent microscopy is a powerful technique to study the localization and distribution of proteins and their post-translational modifications with in cells or sub-cellular compartments (Fig. 2d).

3.4.1 Culturing, Fixation, and Permeabilization of Cells on Coverslip

1. Place the cover slips (number as required) in a cell culture plate and seed cells for 30–40 % confluency. Start the following protocol once 70–80 % confluency is achieved.
2. Discard the media, give three washes of PBS, and transfer the coverslips to 30 mm cell culture dish or 6-well plate.

3. Incubate coverslips in freshly prepared fixing solution at RT for 20 min. Aspirate out the solution and wash with washing solution 1 (3 min×3) on shaker at 50 rpm (*see Note 13*).
4. After washing, incubate cells containing cover slip in permeabilizing solution at RT for 20 min. Post-incubation, wash with washing solution 2 (3 min×2) at RT on shaker at 50 rpm (*see Note 14*).

3.4.2 Immuno-staining and Mounting of Cells

1. Incubate the coverslips at RT for 1 h in blocking buffer (just overlay the cover slip with minimum buffer). Post-incubation, wash as in **step 3** of Subheading 3.4.1.
2. Overlay coverslip with 30–50 µl of primary antibody solution (e.g., phospho-H3Serine10 dilution 1:100) and incubate for 1–2 h in humid chamber at RT (*see Note 15*).
3. Wash cover slip alternatively in washing buffer 1 and 2 (3 min×4) on shaker at 50 rpm.
4. Prepare fluorescent tagged secondary antibody solution (e.g., 1:300 dilution for antibody against rabbit) in blocking buffer. Add 30–50 µl/cover slip and incubate for 2 h in humid chamber at RT in dark or in a black box (*see Note 15*).
5. Wash as per **step 3** of Subheading 3.4.2 and counter stain cells with DAPI for 30 min in humid chamber in dark (*see Note 16*).
6. Put a small drop of Vecta-shield mounting solution on glass slide and keep the cover slip in an inverted position carefully to avoid air bubbles. Seal the sides of cover slip with transparent nail polish.

3.5 Immuno-histochemistry

Immunohistochemistry (IHC) is a method for demonstrating the presence and location of proteins in tissue sections (Fig. 2c). Though, less sensitive quantitatively than immunoassays such as Western blotting or ELISA, it enables the observation of processes in the context of intact tissue.

3.5.1 Preparation of Tissue Mounted Glass Slides and Deparaffinization

1. Section the paraffin-embedded tissue block at 4 µm thickness on a microtome, transfer on poly-L-lysine coated glass slides and allow the slide to dry overnight at 30 °C.
2. Transfer the glass slides to slide rack and incubate it for 16 h at 60 °C.
3. Immerse the slide rack for 15 min in three coupling jars with 100 % xylene, step-wise (*see Note 17*).
4. Keep the slide rack on tissue paper for 15 s to remove extra xylene.
5. Immerse the slide rack for 10 min in three coupling jars with 100 % ethanol one by one.

6. Transfer the slide rack in to a plastic box with slow running tap water for 10 min. Remove extra water as in **step 4** of Subheading 3.5.1 (*see Note 18*).

3.5.2 Peroxidase Activity Removal and Antigen Retrieval

1. Transfer the slide rack into glass coupling jars containing freshly prepared peroxidase activity removal solution and incubate for 30 min at RT in dark.
2. Remove extra peroxidase activity removal solution as mentioned earlier in **step 4** of Subheading 3.5.1.
3. Incubate the slide rack in milli-Q water containing boxes for 5 min each, one by one.
4. Transfer the slide rack in to a plastic box with slow running tap water for 10 min. Remove extra water onto tissue paper.
5. For Antigen Retrieval, preheat the double volume of antigen retrieving buffer required to immerse the slide rack in microwave at full power for 1 min (*see Note 19*).
6. Immerse the slide rack into antigen retrieving buffer and heat in microwave at full power for 3×5 min each. The decreased volume of buffer should be supplemented with Milli-Q water. Allow the slides containing buffer to cool down to RT for ~30 min.
7. Immerse the slide rack for 5 min in two boxes with 1× TBS pH 7.4, one by one. Remove extra TBS from slides by tapping on the tissue paper or by wiping carefully around the tissue section.

3.5.3 Staining and Mounting of Tissue Sections

1. Draw a circle around the tissue section using pap-pen and layer 20–50 µl of blocking solution on tissue sections. Incubate the slides in humid chamber at RT for 30 min. Rinse extra blocking solution as mentioned earlier.
2. Incubate tissue sections with 20–50 µl of primary antibody solution (e.g., 1:200 dilution against phospho-H3Serine10) in humid chamber at 37 °C for 1 h (*see Note 20*).
3. Immerse the slide rack for 10 min each in four different boxes containing 1× TBS, one by one. Rinse extra solution from slides by tapping on the tissue paper.
4. Layer 20–50 µl of secondary antibody solution (1:100 dilution, provided with vectastain kit) in antibody dilution buffer on tissue sections. Incubate the slides in humid chamber at RT for 30 min.
5. Wash secondary antibody solution as in **step 3** of Subheading 3.5.3.
6. Layer 20–50 µl of pre-mix 2 % reagent A and B of vectastain kit in antibody dilution buffer on tissue sections. Incubate the slides in humid chamber at RT for 30 min.

7. Wash vectastain solution as in **step 3** of Subheading 3.5.3 and rinse extra solution as mentioned earlier.
8. Incubate slides in DAB staining solution till light brown color develops on the sections (*see Note 21*).
9. Immediately, transfer the slide rack in a box with slow running tap water for 10 min.
10. Stain the slides with counter-staining hematoxylin solution for 1 min followed by washing of the stain for 2 min under slow running tap water.
11. Incubate the slide rack in 1× TBST for 30 s followed by washing as in **step 10**.
12. Incubate the slide rack four times in different 100 % ethanol containing boxes for 10 min each, one by one. Transfer the slide rack in hot air-oven at 60 °C for 5 min to evaporate ethanol.
13. Incubate the slide rack for 10 min each in four coupling jars with 100 % xylene, one by one. Take out one slide at a time and rinse extra xylene from slides by tapping the slide on the tissue paper.
14. Mount the slide with a drop of DPX mounting solution on the coverslip and place the slide on it carefully to avoid air bubbles on the tissue section. Dry the slides at RT and process for analysis and imaging.

3.6 Chromatin Immunoprecipitation

The following protocol describes how Chromatin Immunoprecipitation (ChIP) can be used to identify binding partners of histones or the DNA sequence to which a particular protein binds (Fig. 3a). Native ChIP may be used for stable associations whereas cross-linking ChIP (X-ChIP) can be used for lesser stable complexes.

3.6.1 Isolation and Cross-Linking of Nuclei (for X-ChIP)

1. Wash and harvest 1×10^6 cells in 2 ml 1×PBS.
2. Centrifuge at $3,000 \times g$ for 10 min at 4 °C to pellet cells and wash with 200 µl of ChIP buffer A.
3. Resuspend the cell pellet in 200 µl ChIP buffer A supplemented with 0.1 % (v/v) Nonidet-P-40 to lyse the cells.
4. Incubate on ice for 10 min with intermittent vortexing for 10 s to assist in lysis.
5. Centrifuge at $5,000 \times g$, 1 min, 4 °C to pellet nuclei. Wash with ChIP buffer A supplemented with 0.1 % (v/v) Nonidet-P-40 and centrifuge as mentioned above to remove remaining cell debris. Take out 2 µl of this suspension and mix with 198 µl of 5 M Urea-2 M NaCl for estimation of nuclei at A260 nm.
6. Resuspend the nuclei in 1×PBS, add drop wise 37 % formaldehyde solution such that the final concentration becomes 1 %, and incubate with rocking at RT for 10 min (*see Note 22*).

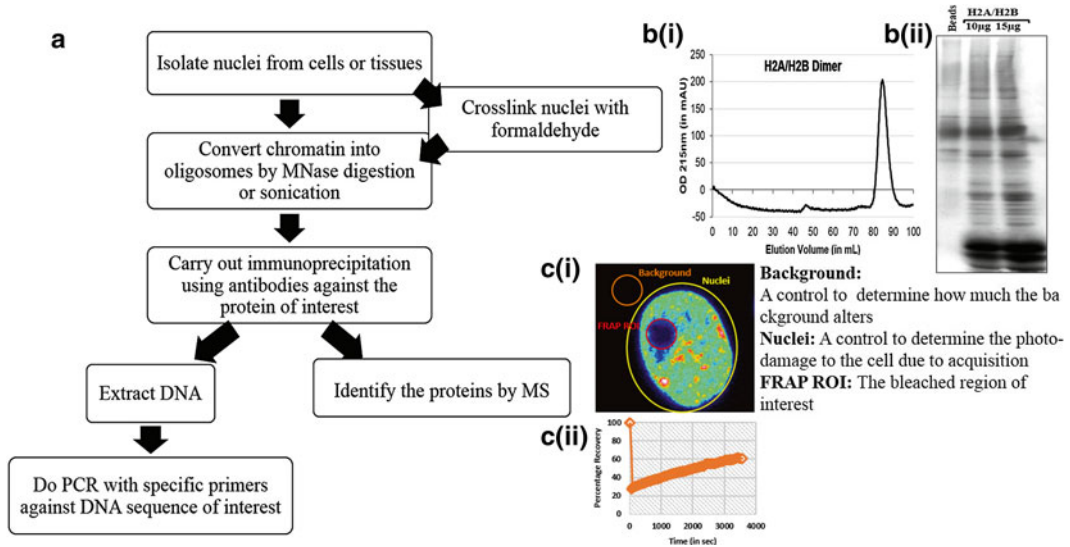


Fig. 3 In vitro pull down and chromatin dynamics. **(a)** Flowchart showing the sequential steps to be followed during ChIP. **(b)** (i) Elution profile of H2A/H2B dimer loaded onto Superdex 200 16/60 gel filtration column. (ii) H2A/H2B dimer pull down with two different concentrations of dimer resolved onto 4–20 % SDS PAGE. **(c)** (i) The measurements to be carried out to analyse the FRAP data. (ii) Plot showing percentage recovery with time for histone H2A

7. Quench the reaction by addition of 2.5 M glycine at a working concentration of 0.125 mM and incubate by rocking at RT for further 10 min.
8. Pellet nuclei by centrifugation at $5,000\times g$ for 10 min at RT. Wash the nuclei thrice with $1\times$ PBS and pellet down by centrifugation as mentioned above.

3.6.2 MNase Digestion of Chromatin and Immunoprecipitation

1. Resuspend the nuclei in 500 μ l of ChIP solution A.
2. Centrifuge at $5,000\times g$ for 10 min, 4 $^{\circ}$ C and resuspend the nuclear pellet in ChIP solution A at the concentration of 1 mg/ml.
3. Add 50 Units of MNase/mg of DNA and incubate at 37 $^{\circ}$ C for varying length of time (*see Note 23*).
4. Stop the reaction by adding $2\times$ RIPA buffer.
5. Centrifuge at $13,000\times g$ for 20 min at 4 $^{\circ}$ C to pellet down high molecular weight DNA and transfer the supernatant, which contains oligosomes, to a fresh tube.
6. Store 10 % of the supernatant for loading onto gel as input along with the immunoprecipitate, and mix 20 μ l of the supernatant with 1 % SDS (make final concentration 0.1 %) and load onto a 1.8 % agarose gel for confirmation of MNase digestion.

7. Preclear the lysate (supernatant **step 5**) by adding 20 μl /ml of protein A/G bead cocktail: 50 % slurry, 100 μg of salmon sperm DNA/ml, 500 μg of BSA/ml by rocking at 4 °C for 1–2 h.
8. To the lysate add antibody and incubate overnight at 4 °C on a rocker. Add 20 μl of bead cocktail per ml of lysate and incubate for more 2–4 h rocking.
9. Harvest beads by centrifugation at $3,000\times g$ at 4 °C for 5 min. Wash beads four times with 1 \times RIPA buffer. Immunoprecipitate can be directly loaded on the gel and stained for visualizing the proteins or DNA can be recovered.

3.6.3 DNA Extraction from Immunoprecipitates

1. Wash the beads twice each with low salt buffer, high salt buffer, and LiCl buffer.
2. Elute the chromatin antibody complexes by adding elution buffer to the beads and incubating at 37 °C for 1 h on a shaker.
3. Reverse cross-link by adding 0.05 volumes of 4 M NaCl and incubating for 4 h at 65 °C (input should also be treated the same way).
4. Treat the chromatin with (100 μg /ml) RNase at 37 °C for 1 h. Add 0.025 volume of 0.5 M EDTA (pH 8.0), 0.05 volume of 1 M Tris-HCl (pH 6.5), and (100 μg /ml) proteinase K. Incubate for 1 h at 45 °C.
5. Recover DNA by extraction using phenol–chloroform–isoamylalcohol. Precipitate by adding 0.1 volumes of 3 M sodium acetate (pH 5.2), 20 μg of glycogen/ml and 2.5 volumes of ethanol.
6. Wash the pellet with 70 % ethanol and air dry.
7. Dissolve precipitated DNA in milli-Q water. The DNA can be used for PCR, qPCR, and ChIPseq.

3.7 Fluorescence Recovery After Photobleaching (FRAP)

FRAP can be used to observe and compare the dynamics of the different core histones and their variants in a live cell [Fig. 3c (i) and (ii)]. The following protocol describes how to perform FRAP of histones, what are the controls which are required to be measured, and how to carry out data analysis.

3.7.1 Image Acquisition

1. Seed cells in live cell chamber and let cells reach 80 % confluency. Low confluency may lead to high cell movement. Select a region to bleach (*see Note 24*).
2. Capture two images before bleaching to serve as pre-bleach control.
3. Bleach with both the lasers of 488 nm and 514 nm at 100 % power so that the intensity of the bleach region falls ~20–25 % with minimum iterations to avoid photo-damage to the cells.
4. Acquire images initially at 30 s interval for the first 15 min and then at an interval of 5 min for the rest 45 min.

3.7.2 Data Analysis

1. Open the image file using ImageJ software and carry out Z projection.
2. Split the channels. Using the StackReg plugin in ImageJ software and carry out rigid body transformation of the fluorescent channel to correct for rotational and lateral movements.
3. Open the ROI Manager.
4. Three intensities need to be measured for every time point; ROI corresponding to whole nuclei, the bleach region and outside the nuclei to measure background. Add the three ROIs in ROI manager and select measure all.
5. Calculate the percentage recovery for each time point by the following way:
 - (a) Subtract background intensity from the intensity of whole nuclei and bleach region.
 - (b) To correct for photobleaching due to acquisition divide the intensity of the bleached region by the intensity of whole nuclei.
 - (c) The value obtained was then multiplied with the value obtained by dividing the background subtracted intensity of total nuclei by the intensity of bleach region of the bleaching time point for normalization.
6. Finally, the percentage recovery was calculated by expressing the values of all the time points relative to the value of pre-bleach intensity considered as 100 %.
7. Plot the graph of fluorescence recovery against time.

3.8 Histone Dimer Pull Down Assay

Histones exist inside the cell as an octamer in the nucleosome and as dimers in complex with the chaperones. Therefore, it is more logical to use H2A/H2B dimer or H3/H4 tetramers for in vitro identification or confirmation of histone binding partners (Fig. 3b). The following protocol describes how to reconstitute histone dimers/tetramers followed by their use in in vitro pull down experiments.

3.8.1 Preparation of Histone Dimer/Tetramer

1. Prepare the aliquots of lyophilized histone monomers in 10 mM HCl with a final concentration of 2 mg/ml.
2. Mix equal amounts of H2A and H2B or H3 and H4 and dilute in HD Buffer A to make final protein concentration 1 mg/ml (*see Note 25*).
3. Dialyze against HD buffer A for 2 h at 4 °C.
4. Dialyze for 6 h each stepwise against HD buffer A with increasing NaCl concentration: 250 mM, 500 mM, 750 mM, 1 M, and 2 M NaCl.

5. Dialyze finally against HD buffer B overnight at 4 °C. The dimer will remain in supernatant and excess histones will precipitate.
6. Centrifuge at $15,000\times g$ for 20 min at 4 °C. Transfer the supernatant to a fresh tube.
7. Load the protein onto the Superdex 200-16/60 filtration column equilibrated with HD buffer B.
8. Monitor the elution of protein by using 215 nm UV. H2A/H2B dimer will elute at about 84 ml, H3/H4 tetramers will elute at about 72 ml.
9. Pool the fractions containing the proteins. Check the proteins by loading on 18 % SDS-PAGE.

3.8.2 Preparation of Nuclear Lysate for Pull Down

1. Isolate the nuclei as described above from $\sim 4\times 10^6$ cells.
2. Lyse the nuclei in HD buffer C by vortexing briefly. Check lysis by observing under a microscope. If lysis is not complete then sonicate at low amplitude for 3 cycles.
3. Pellet down the chromatin by centrifuging at $13,000\times g$ for 20 min at 4 °C.
4. Collect the supernatant containing the nucleosolic proteins in a fresh tube.
5. Dialyze the supernatant against HD buffer D to lower the salt concentration to 150 mM.

3.8.3 Dimer/Tetramer Pull Down

1. To the nucleosolic fraction add 10 µg of dimer/tetramer (*see Note 26*).
2. Carry out overnight incubation at 4 °C on a constant rocker.
3. Add 10 µl of Ni-NTA beads (washed with HD buffer D) and further incubate for 4 h.
4. Wash extensively in HD buffer D.
5. Boil the beads in Laemmli buffer and load on 4–20 % gradient gel for analysis or perform immunoblotting against the anticipated binding partner.

4 Notes

1. Chilled buffers are to be used throughout the experiment as to avoid degradation of protein and loss of PTM's.
2. Incubation time required to lyse the cell may vary among different cell lines.
3. ~ 250 mg of tissue sample can also be used when working with human samples or not so easily available tissues.

4. The number of strokes can be varied depending upon the tissue-type and can be standardized by visualizing nuclei in homogenate under phase contrast microscope.
5. Acid extraction by H_2SO_4 is a common method for histone purification. For specifically examining histone phosphorylation one may consider a high salt-based extraction method instead. Phosphorylation is particularly acid labile but it works fine in our laboratory.
6. SDS or AUT-PAGE can be stained as per “silver staining” protocol or by “coomassie staining” and can be processed for mass spectrometry for identification of unidentified histone post-translational modification or histone variants. Amido Black is an alternate staining dye, it stains with less intensity and destains less slower than coomassie but staining is not compatible with mass spectrometry [21].
7. Coomassie-stained SDS or AUT-PAGE can be used for 2D-SDS-AUT or AUT-SDS PAGE for resolution of histones.
8. The light initiates gel polymerization, depending upon the thickness of gel the light intensity needs to be optimized. Acrylamide is photo-polymerized with riboflavin as initiator because the ions generated by ammonium persulfate-initiated gel polymerization, same as for SDS gels, interferes with stacking.
9. High concentration of urea in this gel system helps in denaturation of histone proteins without imparting any charge. Also, differential binding of Triton X-100 to histones seems to play a role. The non-ionic detergent Triton X-100 in this gel system binds to α -helices by a mechanism that is not clearly understood. Triton binding has been observed to be highest for histone H2A and least for histone H1 resulting in separation of histones on the basis of charge, mass, and hydrophobicity. The system can clearly separate histone major and minor variants (e.g., H2A.Z, H2A.X, H3.1, H3.2, H3.3, etc.) and post-translationally modified isoforms (e.g., mono, di-, tri-acetylated histone H4 species). Histones retain their native positive charge and migrate from anode to cathode in this electrophoresis system.
10. Like 2D-SDS-AUT-PAGE, the reverse 2D, i.e., AUT-SDS can also be carried out (Fig. 1d).
11. In transfer buffer methanol prevents polyacrylamide gel swelling, removes SDS from polypeptides, and also enhances the binding of protein to the membrane.
12. Overnight transfer can be done depending on the acrylamide concentration, gel buffer system, and molecular size of proteins. But since histones are small molecular weight protein, so preferred for ~3 h transfer.

13. The side of the cell at coverslip should be carefully monitored throughout the procedure.
14. For Permeabilization time and concentration of Triton X-100 can vary depending on the cell line.
15. Dilution of primary antibody in blocking buffer and the time of incubation with membrane can be standardized or used as per manufacturer's specification for the antibody to be used.
16. After DAPI counter-staining slides can be kept for 2–3 days at 4 °C in 1× PBS for further processing but after mounting image should be acquired as early as possible.
17. Carry out the process in the fumigation hood and the volumes of xylene, ethanol, and tap water should be enough to immerse the slide rack. Prepare Primary/secondary antibody solutions, vectastain solution, counter-staining solution, and DAB reagents at least one step before the incubation step.
18. Care should be taken with speed of running tap water otherwise it may lead to detachment of tissue sections from slides.
19. Antigen retrieving solution can be prepared at higher concentrations and stored at 4 °C.
20. Incubation conditions (dilution, temperature, and time) of primary and secondary antibody can be used as per manufacturer or as per individual standardization.
21. DAB treatment for more than 10 min increases the chance of background staining significantly.
22. The exact time of cross-linking varies among cell line and therefore needs to be standardized.
23. The exact time for MNase digestion needs to be established empirically. The chromatin preparation for ChIP typically should consist of mono-nucleosomes with some di-, tri-, and tetra-nucleosomes.
24. Use of slow migrating cell line and high confluency minimizes lateral movement by the cell.
25. The concentration of histones can be determined with their molar extinction coefficient. Proper quantification of histones is essential for high efficiency of reconstitution.
26. If non-specific binding of proteins to the beads is suspected then a control experiment should be set up in parallel in which only the beads will be added without adding the dimer/tetramer. Also, to minimise non-specific binding the imidazole concentration can be increased up to 75 mM in Buffer D. Beyond 100 mM histones start eluting from the beads. Incubation time also can be varied. Further, NaCl concentration of buffer D may be increased to remove non-specific binding.

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Chapter 14

Single Base Resolution Analysis of 5-Methylcytosine and 5-Hydroxymethylcytosine by RRBS and TAB-RRBS

Maria A. Hahn, Arthur X. Li, Xiwei Wu, and Gerd P. Pfeifer

Abstract

Sodium bisulfite-assisted deamination of cytosine forms the basis for conducting single base resolution analysis of 5-methylcytosine in DNA. The TET family of proteins represents a group of enzymes that can oxidize 5-methylcytosine to 5-hydroxymethylcytosine. A modification of the bisulfite-based DNA methylation mapping technique employs TET1-mediated oxidation of 5-methylcytosine (TET-assisted bisulfite sequencing) for single base analysis of 5-hydroxymethylcytosine. Whole genome analysis of cytosine modifications with bisulfite sequencing techniques still is challenging and expensive. Reduced representation bisulfite sequencing (RRBS) has been used to limit the complexity of the analysis to mostly CpG-rich genomic fragments flanked by restriction enzyme cleavage sites, for example MspI (5'CCGG). In this chapter, we describe detailed methods used in our laboratory for analysis of 5-methylcytosine and 5-hydroxymethylcytosine combined (RRBS) and for specific analysis of 5-hydroxymethylcytosine (TAB-RRBS).

Key words DNA methylation, 5-Methylcytosine, 5-Hydroxymethylcytosine, CpG islands, High throughput sequencing

1 Introduction

Epigenetic changes such as the establishment of cytosine modification patterns play a crucial role in development and cancer. Recently, in addition to 5-methylcytosine (5mC), a new cytosine modification, 5-hydroxymethylcytosine (5hmC) was discovered [1, 2]. This base is thought to be an intermediate formed during DNA demethylation processes [3]. For example, loss of 5mC and gain of 5hmC occurs in the paternal genome immediately after fertilization [4, 5]. The Tet protein family of 5mC dioxygenases, consisting of the mammalian members Tet1, Tet2, and Tet3, is responsible for the oxidation of 5mC to 5hmC [2, 6]. Recent studies suggest that 5hmC, due to its relatively high abundance, is not only a transient intermediate formed when 5mC is converted to unmodified cytosine but that it rather plays its own specific role in genome

regulation. 5hmC is particularly abundant in neuronal cells of the brain [1, 7]. According to recent studies, 5hmC is viewed as a stable base modification and substitutes a substantial portion of 5mC in the genome during neuronal differentiation [8] and during the aging process [9]. Similar to 5mC, changes in 5hmC are strongly linked to cancer development. The level of 5hmC is dramatically reduced in many cancer types compared to corresponding normal tissue [10]. It has been suggested that the loss of 5hmC in cancer is associated with DNA hypermethylation. In order to understand the role of 5hmC in cancer and during development and cell differentiation, this epigenetic mark needs to be mapped in mammalian tissues at high resolution. However, there are technical and cost limitations that have hampered these approaches. We can compare three major types of 5hmC detection methodologies for genome-wide applications: (1) methods which are based on restriction enzymes which, under certain conditions, are able to distinguish 5hmC from 5mC [11], (2) methods based on oxidation of modified cytosines [12, 13], and (3) methods based on enrichment of 5hmC or modified 5hmC [7, 14, 15]. 5hmC mapping in embryonic stem cells revealed that 5hmC is frequently found to be asymmetrical at the CpG sites of complementary DNA strands, which is in contrast to 5mC [12]. This fact suggests that some 5hmC detection methodologies based on restriction enzyme usage may provide limited information since efficient differential DNA digestion with enzymes such as MspI and HpaII requires presence of 5hmC on both DNA strands. At the same time, methods based on affinity capture of 5hmC or modified 5hmC are dependent on 5hmC density and in addition do not provide single DNA base resolution. In contrast to those approaches, methods based on modified cytosine oxidation provide single base resolution and are very sensitive. There are two types of 5hmC profiling methods involving oxidation: chemical oxidation of 5hmC, the so-called oxidative bisulfite sequencing (oxBS-Seq) [13] and 5mC oxidation by TetI protein, the so-called Tet-assisted bisulfite sequencing (TAB) [12]. Whole genome TAB sequencing of 5hmC requires high coverage due to the low frequency of 5hmC in many tissues and is associated with very high cost. In contrast to whole genome sequencing based on bisulfite conversion, the RRBS technique, which focuses on specific compartments of the genome, notably CpG islands, allows significant cost reduction due to sequencing of only a part of the genome [16, 17]. The combination of 5mC oxidation approaches with the RRBS method results in a high number of reads per single cytosine at reasonable cost [12].

The RRBS methodology was developed in Rudolf Jaenisch's laboratory and became popular due to its cost effectiveness and a relative high coverage of CpG sites in the genome [16]. This method became particularly applicable for samples with limited cell numbers such as germ cells or oocytes [18]. The method is based

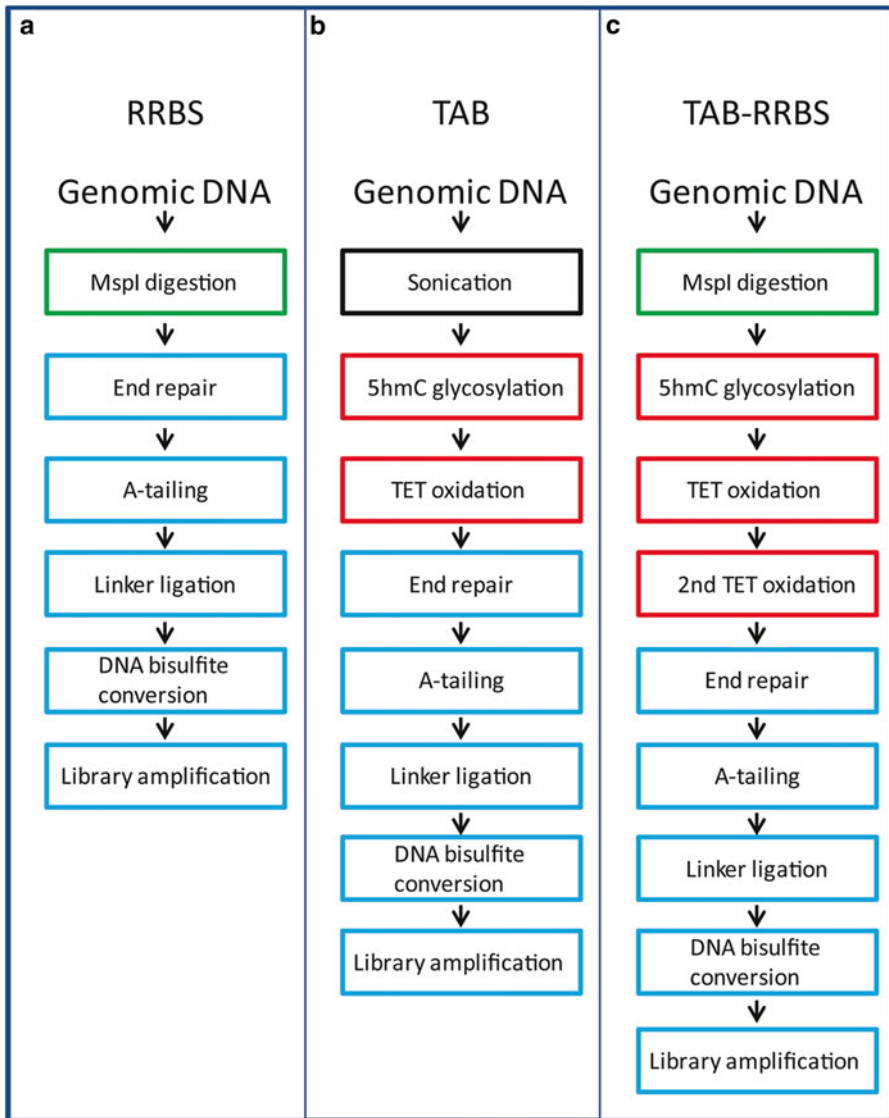


Fig. 1 Flow charts of RRBS, whole genome TAB sequencing and TAB-RRBS procedures. **(a)** RRBS library preparation. **(b)** TAB library preparation. **(c)** TAB-RRBS procedure as a combination of TAB and RRBS techniques

on DNA digestion with MspI (5'CCGG recognition site) followed by end repair, A-tailing, linker ligation, DNA bisulfite conversion, and library amplification (Fig. 1a). This methodology provides information on DNA fragments located between two MspI sites with distance of up to 350 bp. According to our data, the method can represent approximately 82 % of the CpG islands and approximately 70 % of the promoters in the human genome. It is important to note, however, that standard sodium bisulfite treatment does not distinguish 5mC from 5hmC [19, 20].

The TAB sequencing method was developed in Chuan He's laboratory [12]. This technique is based on the oxidation properties of the Tet1 protein, which is able to oxidize 5mC, 5hmC, and 5-formylcytosine (5fC) to 5-carboxylcytosine (5caC), and on inhibition of Tet1 oxidation by glycosylation of 5hmC (Fig. 1b). The 5caC formed by Tet1 oxidation is then decarboxylated to cytosine and deaminated in a solution of sodium bisulfite, and therefore can be read as cytosine in the sequencing data. The 5mC to C conversion rate by Tet1 oxidation and bisulfite treatment can be above 96 % [12]. The protocol includes a glycosylation protection step of 5hmC by T4 phage β -glucosyltransferase, which prevents further oxidation of 5hmC by the Tet1 protein fragment. As an end result, unmodified cytosine, 5mC, 5fC, and 5caC in the genome are all deaminated and read as T, whereas only 5hmC will be read as C. The method is outlined in Fig. 1. A 5hmC TAB-Seq Kit (Wisegene; Chicago, IL) has become available for general use. This kit provides researchers with high quality Tet1 protein fragment and eliminates difficulties of Tet1 purification in the lab.

In our laboratory, we combined and optimized the TAB and RBBS techniques, which resulted in increased 5mC to C conversion efficiency of over 99 % and a dramatic decrease of cost for 5hmC profiling. This approach allowed us to reach a ≥ 20 read coverage on average for each analyzed cytosine located on each separate DNA strand by using approximately 50–60 million paired-end sequence reads. The combined protocol consists of MspI digestion, DNA glycosylation, two consecutive rounds of oxidation by Tet1 fragment, end repair, A-tailing, linker ligation, DNA bisulfite conversion, and library amplification (Fig. 1c).

Several steps were done in order to achieve the highest conversion rate of 5mC and to keep high library diversity. We use the EZ DNA Methylation-Gold Kit (Zymo Research; Irvine, CA), which leaves no more than 0.5 % unconverted cytosines in the CHH context after regular RBBS. We used two rounds of Tet1 oxidation in order to achieve the highest oxidation rate. In order to evaluate Tet1 oxidation efficiency we used non-glycosylated control samples for each oxidation batch. Absence of glycosylation leads to de-protection of 5hmC from Tet1 oxidation. Theoretically, all modified cytosines should be converted after Tet1 oxidation in the absence of glycosylation. By using LC-MS/MS quantification of 5mdC in genomic DNA, we identified a sample with the highest 5mC level in the genome and performed regular TAB-RBBS and non-glycosylated TAB-RBBS with this sample. Our analysis of the non-glycosylated control revealed that Tet1 protein is inefficient at the ends of MspI restriction fragments (Fig. 2). The data indicate that the first six DNA bases next to the MspI site after digestion have a low conversion rate. The lowest Tet1 oxidation rate, only 81 %, was observed at the third DNA base next to the MspI site. This fact indicates a potential weakness of the Tet1 oxidation

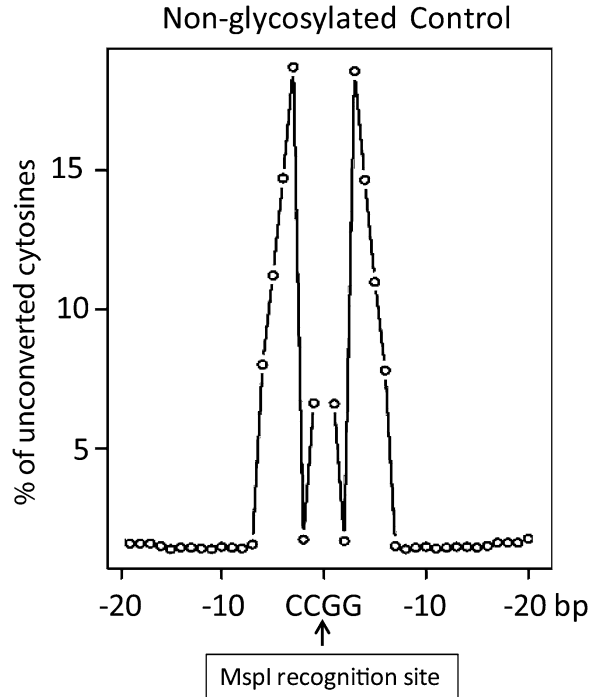


Fig. 2 The catalytic Tet1 protein fragment is inefficient at DNA ends generated by MspI digestion. A composite profile for levels of unconverted cytosine around MspI restriction sites was generated by calculating a mean of percentage of unconverted cytosines in both directions from the MspI sites

methodology in whole genome 5hmC mapping since Tet1 may oxidize poorly also DNA fragment ends after sonication, which may cause a decreased 5mC conversion rate (thus erroneously scoring as 5hmC) in samples. However, the RRBS approach allows us to exclude the first 6 bases located next to MspI sites in the genome. This is reflected in very high oxidation rates of 5mC, more than 99 %, when moving more than 6 bases away from the MspI sites (Fig. 2). If we take into account that bisulfite conversion efficiency is approximately 99.6 % then the number of unconverted cytosines remaining after Tet1 oxidation is extremely low, less than 0.5 % [Number of unconverted cytosines in the CHH context after regular RRBS is ~0.4–0.5 % (bisulfite conversion efficiency) and the number of unconverted cytosines in the CG context is ~0.9 % in the non-glycosylated control. $0.9 - 0.4 \% = \sim 0.5 \%$]. Further inspection of the data indicated very few specific genomic DNA regions, which have a poor Tet1 oxidation rate. In order to eliminate this potential artifact, we subtracted levels of unconverted cytosines in non-glycosylated control from the level of unconverted cytosines for each analyzed cytosine. Incorporation of all steps in the TAB-RRBS protocol resulted in a >99 % oxidation rate of 5mC. An example of final profiling of 5hmC in colon cancer

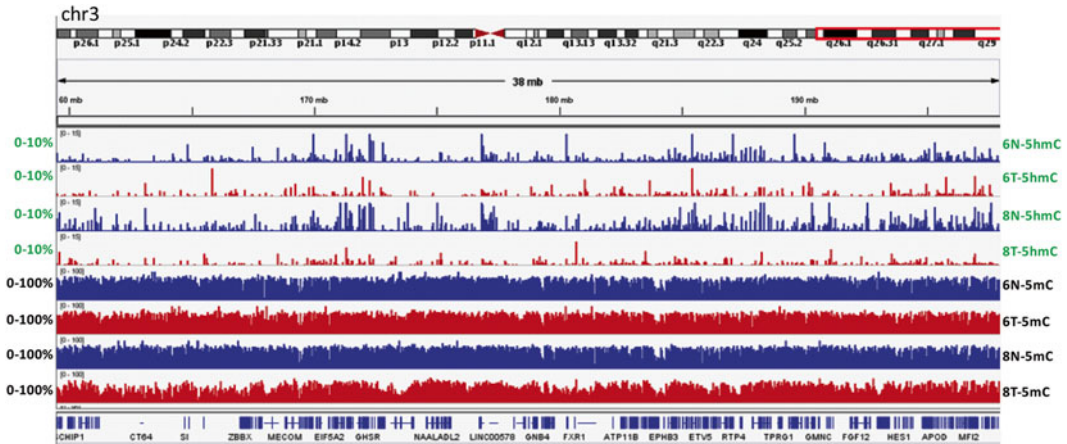


Fig. 3 Representative snap-shots of TAB-RRBS and RRBS results for colon tumors and adjacent nonmalignant tissues. The 5hmC profiles and 5mC (including 5hmC) profiles of a segment of chromosome 3 in two colon tumors (6T and 8T) and two adjacent nonmalignant colon tissues (6N and 8N) were generated by TAB-RRBS and RRBS, respectively. The analyzed ranges of unconverted cytosines remaining after the procedures are indicated on the left side of the profiles. Data are shown only for those CpG sites where the number of reads for each cytosine in the CpG context was at least 10 in all RRBS samples and at least 20 in all TAB-RRBS samples. Please note the decline of 5hmC in the tumors. Genes are indicated at the *bottom*

specimens is shown in Fig. 3. Our protocol provides an opportunity for cost-efficient profiling of 5hmC in DNA samples with relatively low 5hmC levels (~0.1 % of all cytosines are 5hmC). Here we describe our protocol for combination RRBS mapping of 5mC plus 5hmC and for TAB-RRBS mapping specific for 5hmC.

2 Materials

1. 500 ng double-stranded genomic DNA.
2. MspI 100 units/ μ l.
3. TE buffer: 10 mM Tris-Cl, pH 8.1, 1 mM EDTA.
4. Klenow Fragment (3' \rightarrow 5' exo-).
5. Deoxynucleoside triphosphates.
6. 20 mg/ml glycogen from mussels.
7. Phenol/chloroform/isoamyl alcohol (25:24:1 mixture, pH 6.7/8.0).
8. Chloroform.
9. Sodium acetate (NaOAc), 3 M solution, pH 5.5.
10. T4 DNA ligase.
11. Agencourt AMPure XP beads (Beckman Coulter Inc.; Fullerton, CA).

12. EZ DNA Methylation-Gold kit (Zymo Research; Irvine, CA).
13. MinElute PCR purification kit (Qiagen; Valencia, CA).
14. PfuTurbo Cx Hotstart DNA polymerase (Agilent; Santa Clara, CA; supplied with 10× reaction buffer).
15. Novex® TBE gel, 6 % (Life Technologies).
16. Novex® Hi-Density TBE sample buffer (5×) (Life Technologies).
17. SYBR® Safe DNA gel stain (Life Technologies).
18. Amicon Ultra-0.5, Ultracel-30 membrane, 30 kDa (Millipore; Billerica, MA).
19. 5hmC TAB-Seq kit (Wisegene; Chicago, IL, cat. no. K001).
20. 5hmC Tet1 Oxidation kit (Wisegene, cat. no. K003).

3 Methods

3.1 RRBS Protocol for Mapping of 5mC Plus 5hmC

3.1.1 MspI Digestion

Set up a 30 µl digestion reaction as follows:

	Amount	Final concentration
10× reaction buffer	3 µl	1×
MspI	100 u/µl	1 µl
DNA, 500 ng in TE buffer	26 µl	16.7 ng/µl

Mix the sample. Incubate overnight at 37 °C.

3.1.2 End Repair and A-Tailing

1. Set up an end-repair reaction as follows:

	Amount
DNA from Subheading 3.1.1	30 µl
dNTPs (10 mM dATP, 1 mM dCTP, 1 mM dGTP)	1 µl
Klenow Fragment (3' → 5' exo-)	5 u/µl

2. Incubate for 20 min at 30 °C.
3. Incubate for 20 min at 37 °C.
4. Perform phenol/chloroform purification followed by ethanol precipitation as follows:
 - (a) Add 370 µl of 1× TE buffer. Add 400 µl phenol/chloroform. Mix for 3 min.
 - (b) Centrifuge for 3 min at 14,000×g in an Eppendorf centrifuge at room temperature. Transfer supernatant to a new tube.
 - (c) Add 400 µl chloroform to the supernatant. Mix for 3 min.

5. Centrifuge for 3 min at $14,000\times g$ at room temperature. Transfer supernatant into new tube.
6. Add $0.5\ \mu\text{l}$ 20 mg/ml glycogen and $40\ \mu\text{l}$ of 3 M sodium acetate pH 5.5 to the supernatant. Vortex for 10 s.
7. Add 1.1 ml of 96 % ethanol. Mix sample. Incubate 15 min at $-70\ ^\circ\text{C}$.
8. Spin down the DNA at $14,000\times g$ for 20 min at room temperature.
9. Wash pellet twice with $700\ \mu\text{l}$ 75 % ethanol.
10. Remove residual ethanol. Dry DNA pellet.
11. Add $12\ \mu\text{l}$ TE buffer.

3.1.3 Linker Ligation

1. Set up a ligation reaction as follows:

	Amount	Final concentration
DNA from Subheading 3.1.2	$12\ \mu\text{l}$	
$10\times$ T4 ligase reaction buffer	$1.5\ \mu\text{l}$	$1\times$
$7.5\ \mu\text{M}$ methylated linker (Illumina)	$0.5\ \mu\text{l}$	$0.25\ \mu\text{M}$
T4 ligase $400\ \text{u}/\mu\text{l}$	$1\ \mu\text{l}$	$26.7\ \text{u}/\mu\text{l}$

2. Incubate overnight at $16\ ^\circ\text{C}$.
3. Incubate for 10 min at $65\ ^\circ\text{C}$.
4. Add $37.5\ \mu\text{l}$ Agencourt AMPure XP beads. Mix well.
5. Incubate for 15 min at room temperature.
6. By using a magnet, separate beads and remove all solution.
7. Wash beads by using a magnetic rack. Add $150\ \mu\text{l}$ of 80 % ethanol. Incubate for 1 min at room temperature. Remove ethanol.
8. Repeat **step 7**.
9. Dry beads.
10. Add $20\ \mu\text{l}$ TE buffer. Vortex. Incubate for 2 min at room temperature. Transfer DNA into a new tube.

3.1.4 Bisulfite Conversion

1. To DNA from Subheading 3.1.3, add $130\ \mu\text{l}$ of freshly prepared CT-mix (from EZ DNA Methylation-Gold Kit).
2. Incubate at $98\ ^\circ\text{C}$ for 10 min, then $64\ ^\circ\text{C}$ for 2.5 h, then put on hold at $4\ ^\circ\text{C}$ (maximum for 24 h).
3. Convert DNA according to the manufacturer's instructions (EZ DNA Methylation-Gold Kit).
4. Elute with $20\ \mu\text{l}$ elution buffer (EZ DNA Methylation-Gold Kit).

5. Add 100 µl of PB buffer (MinElute PCR Purification Kit) and purify DNA by using MinElute columns according to the manufacturer's instructions.
6. Elute DNA with 20 µl EB buffer (MinElute PCR Purification Kit).

3.1.5 Library Amplification

1. Set up a PCR reaction as follows:

	Amount	Final concentration
DNA from Subheading 3.1.4	20 µl	
10× PfuTurbo Cx Hotstart DNA polymerase reaction buffer	20 µl	1×
10 µM primers R+F (Illumina)	20 µl	1 µM
2.5 mM dNTPs	20 µl	0.25 mM
PfuTurbo Cx Hotstart DNA Pol. 2.5 u/µl	4 µl	0.05 u/µl
Water	116 µl	

Primer F: 5'CAAGCAGAAGACGGCATACG

Primer R: 5'AATGATACGGCGACCACCGA

2. Mix well. Amplify samples by using the following conditions:

95 °C	5 min	
98 °C	30 s	
98 °C	10 s	} 8–9 cycles
65 °C	30 s	
72 °C	30 s	
Hold at 4 °C		

3. Purify PCR products by using MinElute PCR purification kit according to the manufacturer's instructions and elute with 10 µl EB buffer.

3.1.6 Gel Purification

1. Add 2.5 µl Novex® Hi-Density TBE sample buffer (5×) to the samples from Subheading 3.1.5. Mix well.
2. Load samples into wells of a Novex® TBE Gel, 6 %.
3. Run gel for 40 min at 200 V.
4. Dye the gel with SYBR® Safe DNA gel stain according to the manufacturer's instructions.

5. Under UV light, cut DNA fragments ranging from 150 to 500 bp (insert size range should be from 30 to 380 bp).
6. Add 500 µl of EB (MinElute PCR Purification Kit). Smash gel in buffer.
7. Shake samples overnight at room temperature.
8. Concentrate the eluted DNA with an Amicon Ultra-0.5, Ultracel-30 membrane according to the manufacturer's instructions.
9. Quantify library with QPCR.

3.1.7 *Illumina Sequencing*

Perform Illumina paired-end sequencing.

3.2 TAB-RRBS
Protocol for Mapping of 5hmC

3.2.1 *MspI Digestion*

1. Set up a digestion reaction as follows:

		Amount	Final Concentration
10× NEBuffer 2		3 µl	1×
MspI	100 u/µl	1 µl	3.3 u/µl
500 ng DNA in TE buffer		26 µl	16.7 ng/µl

2. Mix the sample. Incubate overnight at 37 °C.
3. Perform phenol/chloroform purification followed by ethanol precipitation in the presence of glycogen as follows:
 - (a) Add 370 µl of TE buffer. Add 400 µl phenol/chloroform. Mix for 3 min.
 - (b) Centrifuge for 3 min at 14,000×g at room temperature. Transfer supernatant into a new tube.
 - (c) Add 400 µl chloroform to the supernatant. Mix for 3 min.
4. Centrifuge for 3 min at 14,000×g at room temperature. Transfer supernatant into a new tube.
5. Add 0.5 µl 20 mg/ml glycogen and 40 µl 3 M sodium acetate pH 5.5 to the supernatant. Vortex for 10 s.
6. Add 1.1 ml of 96 % ethanol. Mix the sample. Incubate for 15 min at −70 °C.
7. Collect the DNA by centrifugation at 14,000×g for 20 min at room temperature.
8. Wash pellet twice with 700 µl 75 % ethanol.
9. Remove residual ethanol. Dry DNA pellet.
10. Add 3 µl TE buffer.

3.2.2 Glycosylation

1. Set up a glycosylation reaction as follows (please *see* **Note 1**):

	Amount	Final concentration
Genomic DNA from Subheading 3.2.1	3 μ l	25 ng/ μ l
10 mM UDP-Glucose (Wisegene, K001)	1 μ l	500 μ M
10 \times β GT protection buffer (Wisegene, K001)	2 μ l	1 \times
T4- β GT (40 μ M) (Wisegene, K001)	0.5 μ l	1 μ M
Water	13.5 μ l	

2. Mix the sample. Incubate for 1 h at 37 $^{\circ}$ C.
3. Perform phenol/chloroform purification followed by ethanol precipitation in the presence of glycogen as described under Subheading 3.2.1.

3.2.3 First Oxidation

1. Set up an oxidation reaction as follows:

	Amount
Genomic DNA from Subheading 3.2.2	3 μ l
Tet1 oxidation reagent 1 (Wisegene, K001)	3.5 μ l
Tet1 oxidation reagent 2 (Wisegene, K001)	15 μ l
Tet1 protein (Wisegene, K001)	9 μ l*
Water	19.5 μ l

* *see* **Note 2**.

2. Incubate for 1 h at 37 $^{\circ}$ C.
3. Add 1 μ l 20 mg/ml proteinase K. Mix the sample. Incubate for 10 min at 50 $^{\circ}$ C.
4. Perform phenol/chloroform purification followed by ethanol precipitation in the presence of glycogen as described in Subheading 3.2.1.

3.2.4 Second Oxidation

1. Set up an oxidation reaction as follows (*see* **Note 3**):

	Amount
Genomic DNA from Subheading 3.2.3	3 μ l
Tet1 oxidation reagent 1, (Wisegene, K001)	3.5 μ l
Tet1 oxidation reagent 2, (Wisegene, K001)	15 μ l
Tet1 protein (Wisegene, K001)	9 μ l
Water	19.5 μ l

2. Incubate for 1 h at 37 °C.
3. Add 1 µl proteinase K (20 mg/ml). Mix the sample. Incubate for 10 min at 50 °C.
4. Perform phenol/chloroform purification followed by ethanol precipitation in the presence of glycogen as described under Subheading 3.2.1. After ethanol precipitation, dissolve the DNA in 18 µl of TE buffer.

3.2.5 End Repair
and A-Tailing

1. Set up an end-repair reaction as follows:

	Amount
DNA from Subheading 3.2.4	18 µl
10× NEBuffer 2	3 µl
dNTPs (10 mM dATP, 1 mM dCTP, 1 mM dGTP)	1 µl
Klenow fragment (3′ → 5′ exo-) 5 u/µl	1 µl
Water	9 µl

2. Incubate for 20 min at 30 °C.
3. Incubate for 20 min at 37 °C.
4. Perform phenol/chloroform purification followed by ethanol precipitation as described under Subheading 3.2.1. After ethanol precipitation, dissolve the DNA in 12 µl of TE buffer.

3.2.6 Linker Ligation

1. Set up a ligation reaction as follows:

	Amount	Final concentration
DNA from Subheading 3.2.5	12 µl	
10× T4 ligase reaction buffer	1.5 µl	1×
7.5 µM methylated linker (Illumina)	0.5 µl	0.25 µM
T4 ligase 400 u/µl	1 µl	26.7 u/µl

2. Incubate overnight at 16 °C.
3. Incubate for 10 min at 65 °C.
4. Add 37.5 µl Agencourt AMPure XP beads. Mix well.
5. Incubate for 15 min at room temperature.
6. By using a magnet, separate beads and remove all solution.
7. Wash beads by using a magnetic rack. Add 150 µl of 80 % ethanol. Incubate for 1 min at room temperature. Remove ethanol.
8. Repeat **step 7**.
9. Dry beads.
10. Add 20 µl TE buffer. Vortex. Incubate for 2 min at room temperature. Transfer DNA into a new tube.

3.2.7 Bisulfite Conversion

1. To DNA from Subheading 3.2.6 add 130 µl of freshly prepared CT-mix (from EZ DNA Methylation-Gold Kit).
2. Incubate at 98 °C for 10 min, then at 64 °C for 2.5 h, and hold at 4 °C.
3. Convert DNA according to the manufacturer’s instructions (EZ DNA Methylation-Gold kit).
4. Elute with 20 µl elution buffer (EZ DNA Methylation-Gold kit).
5. Add 100 µl of PB buffer (MinElute PCR purification kit) and purify DNA by using MinElute column according to the manufacturer’s instructions.
6. Elute DNA with 20 µl EB buffer (MinElute PCR purification kit).

3.2.8 Library Amplification

1. Set up a PCR reaction as follows:

	Amount	Final concentration
DNA from Subheading 3.2.7	20 µl	
10× PfuTurbo Cx Hotstart DNA pol. reaction buffer	20 µl	1×
10 µM Primer R+ F	20 µl	1 µM
2.5 mM dNTPs	20 µl	0.25 mM
PfuTurbo Cx Hotstart DNA polymerase 2.5 u/µl	4 µl	0.05 u/µl
Water	116 µl	

2. Mix well. Amplify samples by using the following conditions:

95 °C	5 min	
98 °C	30 s	
98 °C	10 s	} 10–12 cycles
65 °C	30 s	
72 °C	30 s	
Hold at 4 °C		

3. Purify PCR products by using MinElute PCR purification kit according to the manufacturer’s instructions and elute with 10 µl EB buffer.

3.2.9 Gel Purification

1. Add 2.5 µl Novex® Hi-Density TBE sample buffer (5×) to the samples. Mix well.
2. Load samples into the wells of a Novex® TBE Gel, 6 %.

3. Run gel for 40 min at 200 V.
4. Dye the gel with SYBR® Safe DNA gel stain according to the manufacturer's instructions.
5. Under UV light, cut DNA fragments ranging from 150 to 500 bp (insert size range should be from 30 to 380 bp).
6. Add 500 µl of EB (MinElute PCR purification kit). Smash gel in buffer.
7. Shake samples overnight at room temperature.
8. Concentrate the eluted DNA with an Amicon Ultra-0.5, Ultracel-30 membrane according to the manufacturer's instructions.
9. Quantify library with QPCR.

3.2.10 Illumina Sequencing

Perform Illumina paired-end sequencing.

4 Notes

1. In order to evaluate the 5mC conversion rate, we include a non-glycosylated control sample in every sample batch. This sample should have the highest DNA 5mC level among all oxidized samples in the batch. In contrast to the rest of the samples, the non-glycosylated control does not undergo the glycosylation step (Subheading 3.2.2).
2. The exact amount of added Tet1 protein varies from batch to batch. The manufacturer's protocol indicates the amount of protein, which is required for conversion of DNA with ~3 % 5mC in the genome (i.e., ~3 % of all cytosines are methylated, embryonic stem cell level). The protocol described here is adjusted for human colon tumor samples, which have approximately 4–5 % 5mC in the genome. Therefore we used the double amount of Tet1 protein as suggested by the manufacturer. Try to avoid repeated thawing and freezing of Tet1 protein. The activity of Tet1 protein is substantially dropping after a first thawing/freezing cycle.
3. The second oxidation step is necessary to convert the leftover unconverted 5mC. This step is required to achieve a 5mC conversion efficiency of over 99 %.

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Chapter 15

Quantitative DNA Methylation Analysis for Epigenotyping of Colorectal Cancer

Atsushi Kaneda and Koichi Yagi

Abstract

Accumulation of epigenetic alteration plays important roles in tumorigenesis. Aberrant DNA hypermethylation in gene promoter regions is a common epigenetic mechanism for silencing tumor suppressor genes in many types of cancer including colorectal cancer (CRC). By using quantitative methylation information, CRC can be classified into three distinct methylation epigenotypes with different genetic features, suggesting existence of at least three molecular pathways in genesis of CRC. We describe in this chapter, the methods for analyses of aberrant DNA methylation to epigenotype CRC.

Key words Colorectal cancer, DNA methylation, Epigenotype, MALDI-TOF-MS, CpG island methylator phenotype (CIMP)

1 Introduction

Cancer arises through accumulation of genetic alteration and epigenetic alteration [1–3], including colorectal cancer (CRC) [4]. Frequent mutations of genes, such as *KRAS*, *p53*, and *APC*, were detected in CRC; epigenetic alteration such as DNA methylation or loss of imprinting also plays an important role in colorectal carcinogenesis, and aberrant hypermethylation of gene promoter regions is a common epigenetic mechanism for gene silencing involved in the initiation and progression of cancer [5–9]. In 1999, a subgroup of CRC was reported to show significantly frequent CpG island methylation, the so-called CpG island methylator phenotype (CIMP) [10]. CIMP+ CRC significantly correlates to microsatellite instability and *BRAF* mutation [11].

In our previous studies, we epigenotyped colorectal cancer and adenoma by two-way hierarchical clustering method using highly

quantitative DNA methylation data, and identified three clusters of colorectal cancer and adenoma with distinct methylation epigenotypes [6, 12]. High-methylation epigenotype correlated to *BRAF*-mutation(+) and microsatellite instability [6], as CIMP was previously reported [11]. Serrated adenoma showed this epigenotype, indicating that high-methylation CRC arises through serrated pathway [12–14]. In microsatellite-stable colorectal cancer, intermediate-methylation epigenotype correlated to *KRAS*-mutation(+) and lack of *BRAF* mutation, and low-methylation epigenotype correlated to lack of *BRAF/KRAS* mutation. Conventional, non-serrated adenoma was clustered into these two epigenotypes, indicating that intermediate- and low-methylation cancers arise through conventional adenomas [12].

DNA methylation markers were also clustered into two groups, Group-1 and Group-2 markers [15]. Group-1 markers included most of previously established CIMP markers [10, 11, 16], and are methylated specifically in high-methylation/CIMP+colorectal cancer. Group-2 markers are methylated in both high- and intermediate-methylation epigenotypes, but not in low-methylation epigenotype. Therefore, CRC with methylation of both Group-1 and Group-2 markers is regarded as high-methylation, and CRC without Group-1 marker methylation but with methylation of Group-2 markers is regarded as intermediate-methylation epigenotype [6, 15].

We here introduce the methods to analyze the methylation markers quantitatively, by bisulfite-PCR based highly quantitative method, MALDI-TOF mass spectrometry (MassARRAY®).

2 Materials

2.1 Clinical Samples

Tissue samples of clinical colorectal cancer, adenoma, and normal mucosa are obtained with written informed consents and with approval by Ethics Committee of the institutes.

1. Keep tissues at -80°C until use.
2. Embed frozen tissues in OCT medium, and slice them using Criostat.
3. Stain a $5\text{ }\mu\text{m}$ -thick specimen with Giemsa and microscopically examine.
4. When the tumor cell content is at least 40 %, collect 10 consecutive $10\text{ }\mu\text{m}$ -thick slices and keep at -80°C . Stain a slide with hematoxylin–eosin (H/E) (*see Note 1*).
5. The H/E slides are microscopically examined for determination of tumor cell contents by two independent pathologists. When the tumor cell content is at least 40 %, the samples are selected for further study (*see Note 2*).

6. Extract genomic DNA from the frozen samples of 10 consecutive slices, using QIAamp DNA Micro Kit (QIAGEN, Hilden, Germany) or other DNA extraction kits (*see* **Note 3**).

2.2 Bisulfite Treatment

1. Freshly prepare the following three solutions (*see* **Note 4**):
 - (a) 25 mL of 6 M NaOH by dissolving 6 g of NaOH (pellet) in 24.2 mL of distilled water (DW).
 - (b) 5 mL of 4.04 M sodium bisulfite by dissolving 1.92 g of sodium metabisulfite ($\text{Na}_2\text{S}_2\text{O}_5$) in 4.4 mL of DW.
 - (c) 10 mL of 10 mM hydroquinone by dissolving 11 mg of hydroquinone in 10 mL of DW.
2. Freshly prepare 120 μL of '3.6 M sodium bisulfite, 0.6 mM HQ (pH 5.00)' solution, by mixing 107 μL of 4.04 M sodium bisulfite, 7 μL of 10 mM hydroquinone, and 6 μL of 6 M NaOH (*see* **Note 5**).
3. Also, prepare the following materials.
 - (a) Sonicator for fragmentation of genomic DNA.
 - (b) Wizard DNA Clean-Up System (Promega) or other desalting kits.
 - (c) 5 M NH_4OAc .

2.3 MassARRAY

Besides MALDI-TOF mass spectrometry (MassARRAY, Sequenom, San Diego, CA) and the Installation CD, prepare the following materials:

1. 384 microtiter PCR plate for sample reaction.
2. 384 thermocycler.
3. 96-well v-bottom plate for preparation of reaction solution.
4. Bisulfite-treated DNA for template.
5. Hot start Taq polymerase.
6. dNTPs.
7. Primers.
8. Clean Resin.
9. MassCLEAVE Reagent Kit (Sequenom).
 - T7 Polymerase (Sequenom).
 - T Cleavage Mix (Sequenom).
 - C Cleavage Mix (Sequenom).
 - SAP (Shrimp Alkaline Phosphatase, Sequenom).
 - 5 \times T7 R&DNA Polymerase Buffer (Sequenom).
 - RNaseA (Sequenom).
 - DTT 100 mM (Sequenom).
 - 4 Point Calibrant (Sequenom).
10. 384-element SpectroCHIP (Sequenom).

2.4 Hierarchical Clustering

1. GeneSpring 7.3.1 software (Agilent technology, Santa Clara, CA) or other software for clustering.

3 Methods

3.1 Bisulfite Treatment

By bisulfite treatment, cytosine (C) is converted to uracil (U), i.e., thymine (T) after PCR amplification. But methylcytosine (mC) is not converted, i.e., cytosine (C) after PCR amplification.

1. Fragment genomic DNA by sonication.
2. Suspend the 500 ng of fragmented DNA in DW in a volume of 19 μ L.
3. Mix the 19 μ L of DNA solution with 1 μ L of freshly prepared 6 M NaOH (*see Note 6*).
4. Incubate at 37 °C for 15 min for denaturation.
5. To the solution, add 120 μ L of '3.6 M sodium bisulfite, 0.6 mM HQ' solution, and the solution is subject to 15 cycles of denaturation at 95 °C for 30 s and incubation at 50 °C for 15 min (*see Note 7*).
6. Desalt the samples with Wizard DNA Clean-Up System or other desalting kits and elute in 50 μ L of TE.
7. To the 50 μ L sample solution, add 2.5 μ L of 6 M NaOH, and desulfonate by incubation at room temperature for 5 min.
8. Mix with 35 μ L of 5 M NH_4OAc and 220 μ L of 100 % ethanol, keep at -80 °C for 20 min or overnight, and centrifuge at 15,000 rpm (20,000 $\times g$) for 20 min to precipitate DNA. After washing with 70 % ethanol and drying, dissolve in 40 μ L of DW.

3.2 PCR Amplification

Design bisulfite PCR primers to include no or only one CpG site in a primer sequence, to amplify DNA region containing several CpG sites. PCR product will be 200–500 bp in length. For C at CpG site within a primer sequence, choose a nucleotide which does not anneal to C or U, such as adenosine (A). Primer sequences of Group-1 and Group-2 markers to epigenotype colorectal cancer were previously designed and shown [6]. All the primers were already validated for their quantitativity by calculating correlation coefficient (R^2) of standard curve using methylation control samples (0, 25, 50, 75, and 100 % methylation) at each CpG unit. Exclude the CpG units with $R^2 \leq 0.9$ for analysis. Use primer pairs whose amplicon contained 3 or more CpG units with $R^2 > 0.9$ [6].

1. Add 10-mer tag sequence (5'-aggaagagag-) to the 5' end of the forward primer, and add the T7 promoter sequence and insert sequence (5'-cagtaatagcactcactatagggagaaggct-) to the 5' end of the reverse primer (Fig. 1). Using these primers, perform PCR amplification as follows.

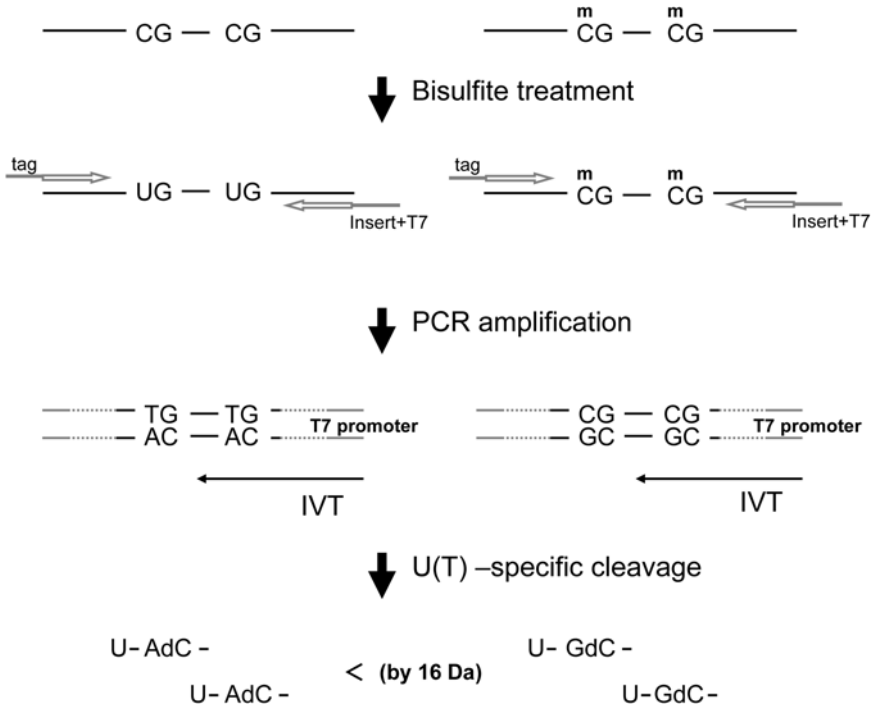


Fig. 1 Scheme of methylation analysis using MassARRAY. Bisulfite-treated DNA is amplified by PCR, and PCR product is transcribed by in vitro transcription (IVT) and the RNA was cleaved by RNaseA. Unmethylated cytosine (C) was converted to uracil (U) by bisulfite treatment, i.e., thymine (T) in PCR product, and finally adenine (A) in IVT product. Methylated cytosine (mC) was not converted, i.e., cytosine (C) in PCR product, and finally guanine (G) in IVT product. Methylation status was determined by 16-Da mass difference between A and G in a cleaved RNA product. When the product contains 2 or 3 CpG sites, the mass difference will be 32 or 48 Da. Methylation rate was calculated quantitatively for each cleaved product

2. Prepare the PCR reaction solution:

Distilled water	1.42 μ L
10 \times PCR Buffer	0.50 μ L
dNTP mix (25 mM each)	0.04 μ L
5 U/ μ L Hot start Taq	0.04 μ L
Primer mix (Forward and Reverse, 1 μ M each)	2.00 μ L
<i>Total</i>	4.00 μ L

- Put 4 μ L of the solution in each well of 384-well plate.
- Add 1 μ L of bisulfite-treated DNA as a template.
- The sample is subject to the following reaction.

Step 1	94 °C for 15 min
Step 2	94 °C for 20 s
Step 3	52 °C for 30 s
Step 4	72 °C for 1 min, Go to Step 2 (45 cycles)
Step 5	72 °C for 3 min
Step 6	4 °C forever

6. For SAP treatment, prepare the reaction solution:

RNase-free water	1.70 μ L
SAP enzyme	0.30 μ L
<i>Total</i>	2.00 μ L

7. Add the 2 μ L of SAP reaction solution to each of 5 μ L PCR solution, and the samples are subject to the following cycles:

Step 1	37 °C for 20 min
Step 2	85 °C for 5 min
Step 3	4 °C forever (<i>see Note 8</i>)

3.3 *In Vitro* Transcription and RNaseA Cleavage

Using the T7 promoter in the reverse primer sequence in PCR product, perform in vitro transcription (IVT) to obtain IVT product of reverse strand. The IVT product is subject to U(T)-specific cleavage using RNaseA (Fig. 2).

1. Prepare the following reaction solution.

RNase-free water	3.15 μ L
5 \times T7 R&DNA Polymerase Buffer	0.89 μ L
T Cleavage Mix	0.24 μ L
DTT 100 mM	0.22 μ L
T7 R/DNA polymerase	0.44 μ L
RNase A	0.06 μ L
<i>Total</i>	5.00 μ L

2. Put 5 μ L of the solution in each well of 384-well plate. Add 2 μ L of SAP-treated samples, and the samples are subject to the following cycle.

Step 1	37 °C for 3 h
Step 2	4 °C forever

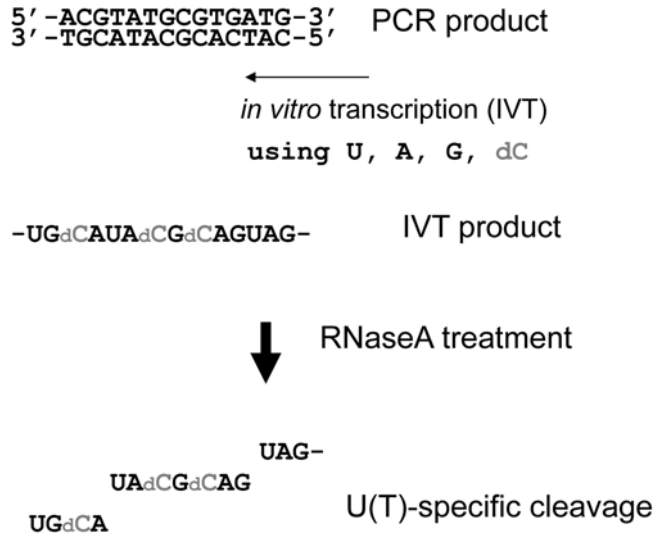


Fig. 2 U(T)-specific cleavage of IVT product. T Cleavage Mix contains dCTP instead of CTP. Since RNaseA cleaves RNA at the 3' site of both U(T) and C, and the IVT reaction mixture contains dC instead of C, U(T)-specific cleavage using RNaseA is possible

3.4 Purification by Resin and Mass Analysis Using MassARRAY

Perform quantitative analysis using MassARRAY [6, 17, 18].

1. Add approximately 6 mg of Clean Resin to the sample in each well of 384-well plate.
2. Add 20 μL of RNase-free water in each well, and rotate for 10 min at room temperature.
3. Transfer the resin-purified supernatant to 384-element SpectroCHIP bioarray, then the samples are subject to MALDI-TOF mass spectrometry.

For desorption and ionization of the oligonucleotide, a laser beam irradiates the matrix-oligonucleotide-cocrystal. The ionized product is then accelerated in an electrical field into the TOF device. The TOF device separates the accelerated analyte ions of different mass-to-charge (m/z) ratios by providing a field-free drift tube of defined length. After passing the tube, ions are detected and every signal is assigned to a specific molecular mass [19]. Using mass difference of 16 Da between A and G in a cleaved RNA product, mass for unmethylated allele and that for methylated allele is determined, and the ratio is calculated as methylation score (Fig. 3). This analytic unit containing several CpG sites in a cleaved product is called "CpG unit".

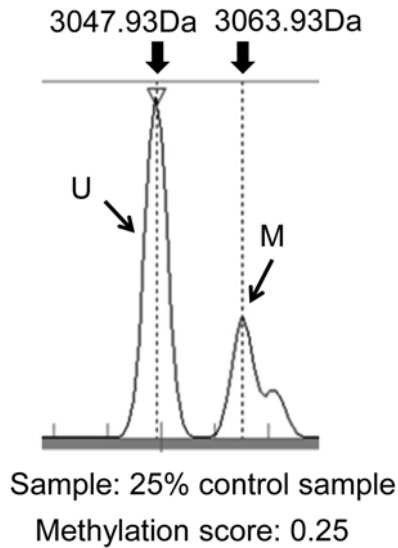


Fig. 3 Representative MALDI-TOF-MS image of methylation control sample. X-axis indicates mass-to-charge ratio (m/z) to distinguish 16-Da difference between A and G derived from unmethylated (U) and methylated (M) alleles, respectively. Y-axis indicates signal intensity. Using signal intensity, the methylation score is calculated by $M/(U + M)$, which generally gives quantitative results

3.5 Mutation Analysis

Mutation at *BRAF* 1799 and *KRAS* 34, 35, and 38 are analyzed by genotyping assay also on MassARRAY platform [19].

1. Design PCR amplification primers and a post-PCR extension primer using MassARRAY Assay Design 3.0 software. The primer sequences are listed in our previous report [12].
2. Perform PCR amplification in a similar manner to the above, in 5 μ L volumes containing 0.2 units of Taq polymerase, 5 ng of genomic DNA, 2.5 pmol of PCR primer, and 2.5 nmol of dNTPs. PCR Reactions are cycled at 94 °C for 15 min, followed by 45 cycles of 94 °C for 20 s, 56 °C for 30 s, and 72 °C for 1 min.
3. Perform SAP treatment to deactivate unincorporated dNTPs as above at 37 °C for 20 min and 85 °C for 5 min.
4. Perform post-PCR primer extension using 5.4 pmol of extension primer, 50 μ mol of the appropriate dNTP/ddNTP combination, and 0.5 units of Thermosequense DNA polymerase. Extension reaction are cycled at 94 °C for 30 s, followed by 40 cycles of 94 °C for 5 s, 5 cycles of 52 °C for 5 s and 80 °C for 5 s, and 72 °C for 3 min.
5. Add a cation exchange resin to remove residual salt from the reactions as above.

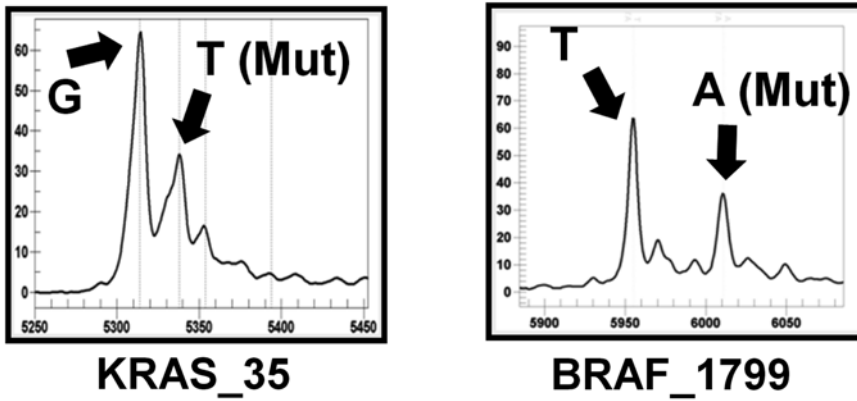


Fig. 4 Representative MALDI-TOF-MS image of *KRAS*/*BRAF* mutations. X-axis indicates mass-to-charge ratio (m/z) to distinguish wild-type and mutant allele, and Y-axis indicates signal intensity

6. Transfer the Resin-purified product to a SpectroCHIP and analyze mass difference using MassARRAY as above. By the difference of molecular mass of nucleotides, the extended base at the possible mutation site is determined (Fig. 4).

3.6 Hierarchical Clustering

Unsupervised two-way hierarchical clustering was performed based on Euclid distance correlation and average linkage clustering algorithm in sample and marker directions using GeneSpring 7.3.1 or other clustering software packages. The heatmap was drawn using Java TreeView software (<http://jtreeview.sourceforge.net/>). In the sample direction, CRC cases will be clustered into three groups: high-, intermediate-, and low-methylation epigenotypes. *BRAF*-mutation(+) cases are significantly enriched in high-methylation epigenotype, and *KRAS*-mutation(+) cases are significantly enriched in low-methylation epigenotype. In the marker direction, methylation markers will be clustered into two groups: Group-1 markers with methylation in high-methylation CRC cases, and Group-2 markers with methylation in high- and intermediate-methylation CRC cases (Fig. 5).

4 Notes

1. Tumor cells can be enriched by excluding necrotic or inflammatory lesions by macrodissection. Or laser capture microdissection can also be performed.
2. This step is critical for epigenotyping. Samples without enough tumor cells provide uninformative methylation results, which could interfere hierarchical clustering.

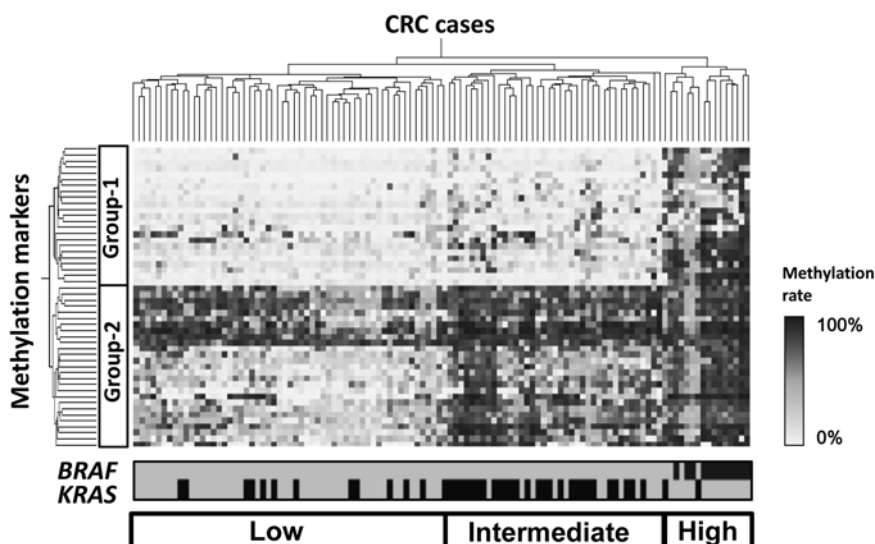


Fig. 5 Representative result of two-way hierarchical clustering of CRC using quantitative methylation data. CRC cases will be clustered into three epigenotypes, correlating with oncogene mutations

3. More slices can be collected. In that case, the last slice is recommended to be stained with H/E and examined for tumor cell contents.
4. These solutions must be prepared immediately before bisulfite treatment step. Especially, sodium bisulfite solution is very unstable.
5. Bisulfite conversion must be performed at pH 5.0–5.2. If the “3.6 M sodium bisulfite, 0.6 mM HQ” solution is prepared as shown, the pH will be 5.00, and be increased to 5.0–5.2 when mixed with denatured DNA solution at the **step 5** of Subheading 3.1.
6. It is more accurate to add 5 μ L of 1.2 M NaOH to 15 μ L of 500 ng DNA solution.
7. Due to the NaOH in the denaturation, the pH of the solution will increase to ~5.1.
8. It is recommended to check PCR product by electrophoresis of 1.5 μ L of the solution using 2 % agarose gel.

Acknowledgement

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Chapter 16

Histone Modifications Associated with Cancer Cell Migration and Invasion

Miki Hieda, Nariaki Matsuura, and Hiroshi Kimura

Abstract

Genome-wide aberrant histone modifications are present in a wide range of cancers, and they are associated with carcinogenesis and cancer progression. Aberrant histone modification patterns affect transcriptional regulation, chromosome stability, chromatin structure, chromatin remodeling, and DNA methylation; furthermore, these patterns can predict clinical outcome in many types of cancer. The main cause of poor clinical outcome is metastasis, which is strongly associated with tissue invasion at the primary tumor site. Invasion of cancer cells into surrounding tissue and the vasculature is an important initial step in tumor metastasis, and cell migration is a critical requirement for metastasis. Here, we describe the advantages of detecting global histone modifications by immunohistochemical analysis and provide a collection of protocols for assaying cell migration, invasion, and cell–extracellular matrix adhesion in vitro.

Key words Modification specific antibody, Matrigel invasion assay, Chemoinvasion assay, Cell migration assay, Transwell migration assay, Boyden chamber assay

Abbreviations

IF	Immunofluorescence microscopy
IP	Immunoprecipitation
ECM	Extracellular matrix
FFPE	Formalin-fixed, paraffin-embedded
Ac	Acetylated
me1	Mono-methylated
me2	Di-methylated
me3	Tri-methylated
HE	Hematoxylin and eosin

1 Introduction

1.1 Immunohistochemical Staining of Histone Modifications in Cancer Tissue

Epigenetic alterations such as DNA methylation and histone modifications are observed in many types of cancers (reviewed in refs. 1–4). As dysregulation of histone modifications is often associated with carcinogenesis and cancer progression, global histone modification profiles can be good markers to predict clinical outcome ([5, 6], and are reviewed in ref. 7). Immunohistochemical staining with histone modifications might be a powerful tool for cancer diagnostics, since histones are abundant proteins ($>3 \times 10^7$ molecules per cell) concentrated in the nucleus, sometimes with foci formation [8], making the detection sensitivity high. Even though a specific modification might occur only in 1 % of total histone, $>3 \times 10^5$ antigens are still higher than typical transcription factors. In addition, histone methylation and acetylation are relatively stable during sample preparation compared to protein phosphorylation, which can sometimes be difficult to detect because of dephosphorylation. Moreover, in our experience, histone tails are highly accessible in paraffin-embedded tissue.

Immunohistochemical staining can be used in conjunction with other histological stains and clinicopathological data (Fig. 1). Conventional histological stains provide information regarding the

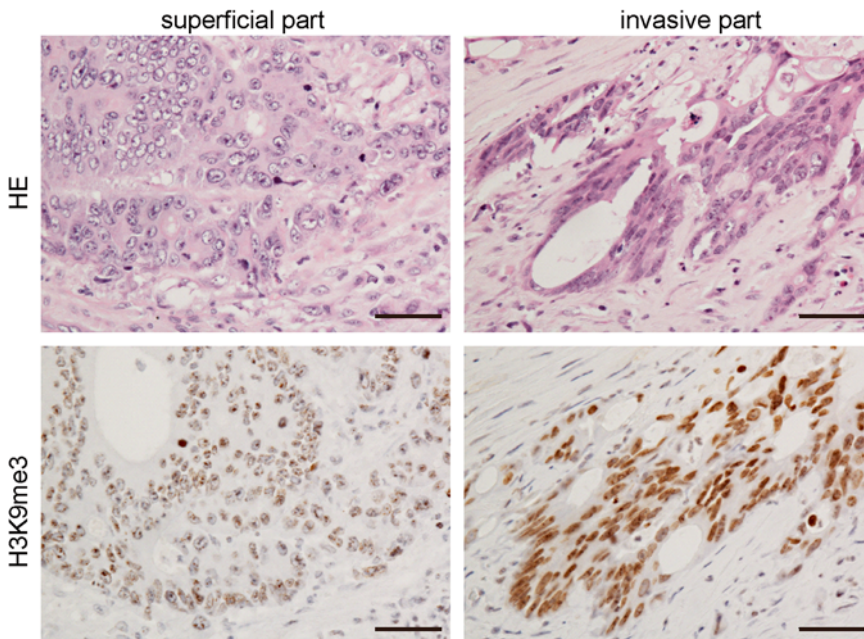


Fig. 1 H3K9 tri-methylation level in colorectal cancer tissue, examined by immunohistochemistry. Paraffin-embedded colorectal cancer tissues were stained using anti-H3K9me3 mAb. Representative overall staining patterns of colorectal cancer tissue are shown. Note the high level of H3K9me3 staining in malignant cells in invasive part, relative to the surrounding normal cells. Adapted from ref 16. Bar: 50 μ m

status of cells, e.g., cancerous region, the presence of inflammatory responses, and so forth. If clinical data are available for the patients, associations between aberrant histone modification and clinico-pathological data may suggest the underlying molecular mechanisms of the pathological effects of aberrant histone modification. Therefore, we can use immunohistochemistry not only to detect the existence of histone modification in single cells, but also to describe what kinds of cells are stained, which in turn will be useful to determine what has happened in patients.

For immunohistochemistry, needless to say, validation of the antibodies is essential for successful detection of histone modifications [8, 9]. The sequence similarity around the modification sites, e.g., histone H3 lysine 9 and lysine 27, makes discrimination by antibodies difficult, and some antibodies may recognize both sites. In addition, as histone tails contain multiple modifiable amino-acid residues, nearby modifications often affect antibody recognition. Therefore, the specificity and affinity of primary antibodies must be rigorously validated [9–11]. In light of these considerations, along with the limited supply of polyclonal antibodies, fully characterized monoclonal antibodies are particularly useful for immunohistochemical detection of histone modifications. Recently, ready-to-use tissue sections and well-validated monoclonal antibodies have become available from multiple commercial suppliers (*see below*).

1.2 Association Between Aberrant Histone Modification and Metastatic Potential

Malignancy of cancer, or clinical outcome, is often linked to metastasis. Metastatic activity is strongly associated with two parameters: initial tissue invasion at the primary tumor site and migratory activity. Multiple lines of evidence supports a correlation between aberrant histone modification and cancer invasion. For example, over-expression of EZH2, which catalyzes histone H3 lysine 27 trimethylation (H3K27me3), is associated with prostate and breast cancer aggressiveness [12–14]; and G9a, which catalyzes histone H3 lysine 9 mono- and di-methylation (H3K9me1 and H3K9me2), promotes lung cancer invasion [15]. In these contexts, promotion of cancer cell invasion has been attributed to repression of the cell adhesion molecules E-cadherin and Ep-CAM, respectively. We recently showed that over-expression of SUV39H1, an enzyme that mediates heterochromatin-associated histone H3 lysine 9 trimethylation (H3K9me3), activates cell migration through an unknown mechanism that is independent of transcriptional regulation. Moreover, we observed elevated H3K9me3 in the invasive region of colorectal cancers ([16]; *see Fig. 1*). In addition to these cancer-specific observations, heterochromatin formation has also been implicated in normal cell migration [17–21].

Cancer invasion requires several distinct cellular functions, including motility, proteolysis of extracellular matrix (ECM), and adhesion to ECM. Here we describe methods for monitoring each

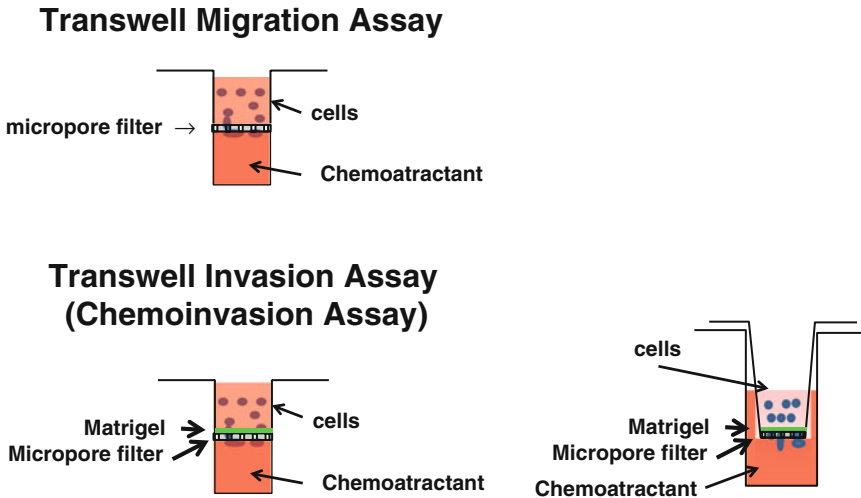


Fig. 2 Schematic presentation of in vitro transwell migration and invasion assays. Both the transwell migration assay (*upper panel*) and transwell invasion assay (*lower panel*) are based on two medium-containing upper and lower chambers separated by a porous membrane filter. Cells seeded in the upper chamber can migrate into the lower chamber, which contains chemoattractant, through the membrane pores. In the invasion assay (*lower panel*), the porous membrane is overlaid with a thin layer of ECM, which blockades pores. After incubation, cells that have migrated/invaded are counted. Disposable housing chambers with polycarbonate membrane inserts are commercially available (*lower right*). When using such a chamber, the levels of medium should be even

of these functions, i.e., assays for migration, invasion, and cell–ECM adhesion. These assays are commonly used to evaluate metastatic potential in vitro and can be performed using standard laboratory equipment.

Cell migration can be evaluated via several different methods including scratch assays, cell-exclusion zone assays, microfluidics-based assays, and transwell assays. The first assay we discuss is a transwell migration assay that was originally developed by Steven Boyden [22] for the analysis of leukocyte chemotaxis, and is therefore often called the Boyden chamber assay. This assay is based on a chamber of two medium-filled compartments separated by a micro porous membrane (Fig. 2). In general, cells are placed in the upper well, and the membrane serves as a barrier to discriminate migratory from non-migratory cells. Via reorganization of the actin cytoskeleton, migratory cells can extend protrusions towards chemoattractants and ultimately pass through the pores of the polycarbonate membrane. At the end of the migration period, the migratory cells can be stained and counted.

The second assay system we present is a transwell invasion assay, one of most common invasion assays used for assessment of invasive properties in vitro (Fig. 2). The technical setup is similar to that of the transwell migration assay, except that the porous filter is

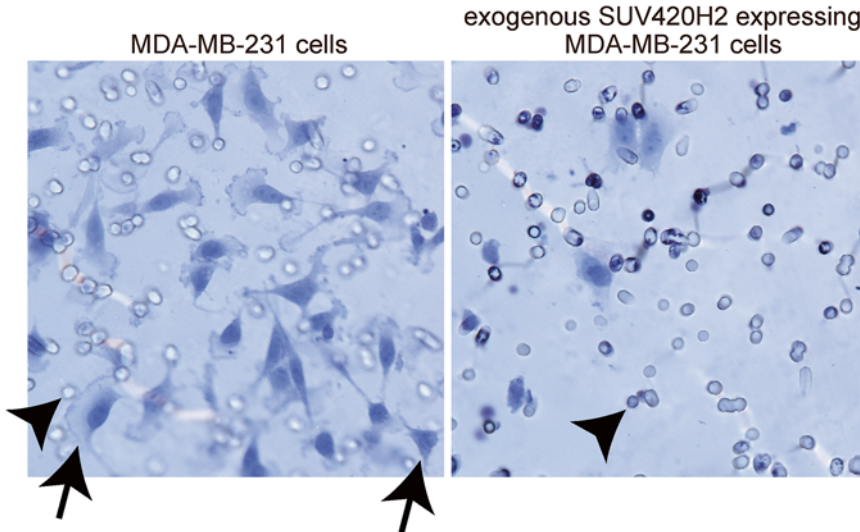


Fig. 3 Cell invasion assay to examine the effect of a histone methyltransferase. Over-expression of SUV420H2, an enzyme that mediates histone H4 lysine 20 trimethylation (H4K20me3), activated cell invasion (unpublished data). MDA-MB-231 invasive breast cancer cells (parental or stably over-expressing SUV420H2) were subjected in vitro invasion assays. Twenty-four hours after incubation, cells were fixed in paraformaldehyde and stained with hematoxylin. Arrows and arrowheads are invaded cells and pores of the polycarbonate membrane, respectively

overlaid with a thin layer of ECM before cells are seeded into the top chamber [23–25]; this is because, in pathology, invasion of carcinomas is defined as the penetration of tissue barriers such as basement membrane. The ECM occludes the membrane pores, blocking noninvasive cells from migrating through them. Invasive cells are able to degrade the matrix proteins in the layer and pass through the ECM layer and the pores of the polycarbonate membrane, ultimately adhering to the bottom of the filter (Fig. 3).

The third protocol we describe here is an assay for cell–ECM adhesion, which significantly affects invasive properties. The ECM is composed of a series of glycoproteins, including collagens, laminins, proteoglycans, and fibronectin. This assay can discriminate adhesion to each type of ECM component.

2 Materials

2.1 Immunohistochemical Staining of Histone Modifications

1. Formalin-fixed, paraffin-embedded (FFPE) specimens. Ready-to-use tissue sections are commercially available from several companies, as controls. We recommend the use of thin sections (~2 μm thick) in order to obtain high-quality images.
2. Primary antibodies specific for histone modifications. Excellent monoclonal and polyclonal antibodies have been developed and commercially available, although polyclonal antibodies suffer

from lot-to-lot variations. Many of monoclonal antibodies we have developed can be used for FFPE tissues (*see* **Note 1**).

3. Biotinylated secondary antibodies.
4. Peroxidase-conjugated streptavidin.
5. 3,3-diaminobenzidine (DAB).
6. 0.05 M Tris-HCl (pH 7.6).
7. 0.01 M citrate buffer.
8. H₂O₂.
9. Hematoxylin.
10. Xylene.
11. TBST buffer: 50 mM Tris-HCl (pH 7.6), 300 mM NaCl, 0.1 % Tween-20.
12. 5 % BSA/TBST.
13. Ethanol.
14. Methanol.
15. Mounting media, Malinol. Besides Malinol, a wide range of resin-based mounting media are commercially available, such as Diatex, Entellan, Eukitt, Rhenohistol and Depex. They differ in the refractive index. All of these mounting media require the specimen to be first dehydrated in alcohol and then transferred to xylene.

2.2 Transwell Migration Assay (Boyden Chamber Assay)

1. Cultured cells of interest. Results can vary depending on cell types.
2. Sterile 30 % BSA solution: Dissolve BSA in distilled water (dH₂O), or Milli-Q water, and filter the solution through a 0.2- μ m filter. Store in 1-ml aliquots at -20 °C.
3. Sterile serum-free cell-culture medium containing 0.1 % BSA. Culture medium should be supplemented with BSA instead of fetal calf serum (FCS) because the latter contains adhesion and chemoattractant factors.
4. Sterile complete cell-culture medium appropriate for cells of interest. In this protocol, FCS is used as chemoattractant. A defined chemoattractant can be added to the medium in place of FCS.
5. Boyden chamber (e.g., 48-well chamber, 3.2-mm diameter wells; Neuro Probe Inc.). Boyden chambers are one of commercially available housing chambers for cell migration and invasion assays (*see* **Note 2**).
6. Polycarbonate membrane inserts (e.g., 8- μ m pore, 25 \times 80 mm; Neuro Probe Inc.). Membranes are available with pore diameters ranging from 3 to 12 μ m. The 8- μ m pore sized membrane is optimal for migration assays using epithelial and

fibroblast cells. For leukocytes, a smaller pore size (3 μm) is optimal.

7. Sterile phosphate-buffered saline (PBS).
8. Sterile collagen Type IC. Stock should be stored at 4 °C; do not freeze (*see* **Note 3**).
9. Fixation solution: 4 % paraformaldehyde or formaldehyde in PBS.
10. Trypsin/EDTA, cell-culture grade.
11. Trypsin Neutralizing Solution (TNS; Lonza).
12. Hematoxylin solution. Trypan blue (0.4 %), crystal violet, or Giemsa stain can also be used.
13. Mounting media: Mix glycerol and PBS at a 1:3 ratio.
14. Cotton swab.
15. Sterile forceps.
16. Humidified tissue culture incubator at 37 °C, with an appropriate concentration of CO₂ in the atmosphere.

2.3 Transwell Invasion Assay (Chemoinvasion Assay)

1. Cultured cells (*see* Subheading 2.2, **item 1**).
2. Sterile 30 % BSA solution (*see* Subheading 2.2, **item 2**).
3. Sterile serum free medium containing 0.1 % BSA (*see* Subheading 2.2, **item 3**).
4. Sterile complete cell-culture medium (*see* Subheading 2.2, **item 4**).
5. Sterile basement membrane matrix: Matrigel (Matrigel™ Matrix; BD Biosciences). Matrigel is liquid at 4 °C, but rapidly polymerizes into gel when warmed at room temperature. For short-term storage, Matrigel can be stored at 4 °C. For longer storage, make aliquots of the desired volume using chilled pipette tips and tubes, and store the aliquots at –20 °C. Thaw overnight at 4 °C (*see* **Note 4**). Avoid repeated freeze–thaw cycles.
6. Chilled pipette tips and 1.5-ml tubes in the refrigerator, for handling Matrigel.
7. Housing chamber with polycarbonate membrane inserts (*see* **Note 2**). Different types of chambers are commercially available. Some are precoated with Matrigel. Here we describe a protocol using a transwell with polycarbonate membrane (Chemotaxicell, 0.48 cm²) that is not coated with Matrigel. Selection of ECM protein and concentration, as well as the pore size of the membrane, can be optimized for individual assays (*see* **Note 5**).
8. 24-well plate for the base of the housing chamber described above.

9. Fixation solution: 4 % paraformaldehyde or formaldehyde in PBS.
10. Hematoxylin solution. Trypan blue (0.4 %), crystal violet, or Giemsa stain can also be used.
11. Mounting media: Mix glycerol and PBS at a 1:3 ratio.
12. Cotton swab.
13. Sterile forceps.
14. Humidified tissue culture incubator at 37 °C, with an appropriate concentration of CO₂ in the atmosphere.

2.4 Cell-ECM Adhesion Assay

1. Cultured cells of interest.
2. Sterile serum free medium containing 0.1 % BSA (*see* Subheading 2.2, **item 3**).
3. Sterile collagen Type IC. Stock should be stored at 4 °C; do not freeze.
4. Sterile fibronectin. Stock should be stored at –80 °C.
5. Sterile vitronectin. Stock should be stored at –80 °C.
6. Sterile laminin. Laminin is a critical component of the basement membrane. Stock should be stored at –80 °C.
7. Sterile 30 % BSA solution.
8. 0.5 % BSA solution.
9. PBS.
10. Crystal violet solution.
11. DMSO.
12. 98-well flat-bottom plate.
13. Plate reader.

3 Methods

3.1 Immunohistochemical Staining of Histone Modifications

Methods for immunohistochemical staining can be divided into four steps: (1) preparation of tissue section on a slide, (2) antigen-antibody reaction, (3) detection, and (4) observation. We start this protocol from paraffin-embedded specimens on glass slides. Methods for fixation, sectioning, and mounting on slides are not described here because various established methods are available and these steps can considerably vary depending on the tissues of interest. Several detection procedures are also available. Methods involving fluorescence detection offer higher resolution, whereas enzyme-labeled chromogenic detection methods offer higher sensitivity and are compatible with most histological stains, like, hematoxylin/eosin (HE) staining, which can provide detailed information about the tissue. We here describe enzyme-labeled methods using biotin-streptavidin system for signal amplification.

3.1.1 Preparation of Tissue Sections on Slides

At this stage, antigen retrieval is the most critical step, and should be optimized for the target antigen and tissues [26], because many epitopes may be masked or altered by certain fixatives. Some antibodies may not be able to recognize epitopes that are altered by fixation or hidden within subcellular structures. Limited protease treatment sometimes, but not always, helps unmasking hidden epitopes.

1. To deparaffinize specimens on slides, incubate slides in xylene for 5 min.
2. Repeat **step 1** three times using three jars.
3. To remove xylene, wash the specimens in ethanol.
4. Repeat **step 3** four times using four jars.
5. Wash with running water for 5 min (tap water can be used).
6. Process samples for antigen retrieval, using an appropriate procedure. We usually use 0.01 M citrate buffer in a Pascal pressure chamber (DAKO) at 125 °C for 2 min and 90 °C for 10 s. If this equipment is not available, try a warmed water bath (95 °C for 20 min, followed by a 20-min cool-down). Various procedures can also be used, including limited protease treatment, microwaving, or autoclaving.
7. Block endogenous peroxidase in 3 % H₂O₂ in methanol for 10 min.
8. Wash with running water for 5 min.
9. Block the section with 5 % BSA/TBST for 30 min in a humid chamber to prevent drying.

3.1.2 Antibody Reaction

The second step involves choosing antibodies and antibody-binding conditions, including the concentration, incubation time, and temperature. Antibody validation has been described above. Even when well-validated antibody whose quality is guaranteed is used, optimization is still required to determine the best concentration and incubation period; these parameters depend on the tissue sample and antibody.

1. Incubate the slides with primary antibodies at room temperature (RT) for 1 h, or at 4 °C overnight, in a humid chamber. Incubation conditions should be optimized, but in most cases, 1-h incubation at RT is sufficient, but some antibodies require a longer incubation period.
2. Wash the slides three times with TBST for 5 min each.
3. Incubate the slides with biotinylated secondary antibody at RT for 30 min in a humid chamber.
4. Wash the slides three times with TBST for 5 min each.
5. Incubate the slides with peroxidase-conjugated streptavidin at RT for 30 min in a humid chamber.

6. During the incubation **step 5**, make the DAB solution:
Dissolve 20 mg of DAB in 100 ml of 0.05 M Tris-HCl (pH 7.6).
Let stand for 10–15 min.
Add 16.7 μ l of H_2O_2 .

3.1.3 Detection

1. Wash the slides three times with TBST for 5 min each.
2. Place the sections in DAB solution. Incubate for ~5 min to develop.
3. Place the sections in water or TBST to stop development.
4. Wash the sections with running water for 5 min.
5. Counterstain with hematoxylin for 2 min.
6. Wash the sections with running water for 10 min.
7. Dehydrate the sections three times in 100 % ethanol.
8. Incubate in xylene three times for 5 min each.
9. Mount with a coverslip using mounting media.

3.1.4 Observation

The assessment should be unbiased and the procedure requires adequate internal negative and positive controls. These considerations are all very important, because staining of different samples is often performed on different days. Non-cancerous regions, leukocytes, endothelial cells, or fibroblasts in the same tissue section can often be used as internal positive and/or negative controls. In order to achieve unbiased assessment, at least two people should evaluate each sample independently. HE staining allows pathologists to distinguish the difference between epithelial and stromal cells, but specific markers such as pan-cytokeratin of epithelial and stromal cells can be used to discriminate cancerous and non-cancerous regions.

3.2 Transwell Migration Assay (Boyden Chamber Assay)

The effect of overexpression or knockdown of histone-modifying enzymes, or other proteins, on cell migration can be investigated using this assay [16, 27]. Appropriate expression vector or siRNA should be transfected 1–2 day before the assay. Results can vary depending upon the cell type and the conditions (especially the medium, incubation time, cell-seeding density, and chemoattractant) under which the procedure is performed. Unless otherwise stated, all procedures described below should be performed at RT.

1. Cut one corner of the polycarbonate membrane to distinguish the front and back surfaces. There are two visibly different sides of the filter: one is shiny, and the other is dull, but the orientation does not affect the migration. Cells on the back surface will be counted. To avoid mixing up the surfaces, marking the same corner consistently is recommended.

2. Dilute the collagen solution with PBS to 100 $\mu\text{g}/\text{ml}$ (100–300 $\mu\text{g}/\text{ml}$; depends on cell types) in a 10-cm dish. Float the membrane in the diluted collagen for 30 min in order to coat only one side (*see Note 6*).
3. Dry the membrane in a tissue-culture hood (coated-face up).
4. During coating the membrane, prepare the wells and cells. Add 30 μl of culture medium, with or without chemoattractant, to each well of the lower chamber. Negative control wells, which do not contain chemoattractant, are important for subsequent analysis and interpretation. The volume should be chosen to fill the lower chamber of the housing. To avoid bubble formation, place the pipette close to the wall of the well. If bubbles appear, use a dry tip to remove them. To avoid drying, keep the housing in a 15-cm cell-culture dish with a lid.
5. Trypsinize cells and stop the reaction by adding TNS (*see Note 7*).
6. Dilute the cell suspension to 10 ml with serum-free media containing 0.1 % BSA. Count the cells.
7. Centrifuge at 1,000 rpm for 5 min.
8. Aspirate the supernatant, and resuspend the pellet at the desired concentration in serum-free media containing 0.1 % BSA. (In the case of MDA-MB-231 cells, the concentration should be $1\text{--}5 \times 10^6$ cells/ml). The number of cells depends on the cell type, so optimization is required.
9. Place the membrane (coated-side down) on to the lower chamber. Work carefully, using forceps, to avoid formation of air bubbles. Prepare the chamber in triplicate (i.e., on at least three independent membranes) for each condition.
10. Assemble the chamber and verify that no wells contain bubbles.
11. Add 50 μl of cell suspension to each upper chamber ($0.5\text{--}2.5 \times 10^5$ cells/well) using a single-channel pipette. The pipette tip should touch the wall of the chamber in order to avoid forming bubbles or breaking the membrane. Between each pipetting step, resuspend the cells in order to avoid settling and to keep the cell suspension homogenous.
12. Incubate for an appropriate period in a tissue-culture incubator at 37 °C (in most cases, 3–6 h). The incubation time should be determined experimentally (*see Note 8*).
13. To stop the reaction, remove the membrane, put it into the fixation solution (coated-side up), and leave it for 20 min.
14. Gently wash the membrane several times with dH₂O in a 10-cm dish. (Keep track of the upper and lower surfaces.)
15. Stain with hematoxylin solution for 20 min.

16. Gently wash the membrane several times with dH₂O in a 10-cm dish.
17. Remove the non-invading cells, i.e., those that stayed on the upper side of the polycarbonate membrane, using a cotton swab.
18. Cut the membrane in half (the whole membrane is too big to mount on a normal glass slide). Mount the membrane on a slide with mounting medium.
19. Place a cover slip on top of the membrane and apply gentle pressure to exclude any air bubbles.
20. Observe and collect images of the migrated cells under the microscope at approximately 20–100× magnification (depending on cell density).
21. Count the cells that have passed through the membrane on the bottom (lower surface) of the membrane. Several fields should be analyzed for each membrane. Other methods can also be used to quantitate the migrated cells (*see* **Note 9**).

3.3 Transwell Invasion (Chemoinvasion) Assays

Many experimental approaches have been developed for evaluation of invasive activity *in vitro*. The advantages and limitations of these procedures have been summarized in [28, 29]. Here we describe a transwell invasion assay, also called a chemoinvasion assay, using Matrigel-coated porous membranes. This method involves drying the coated Matrigel and reconstituting it with culture media. The drying/reconstitution process, which is used widely in chemoinvasion assays, creates an even more closely packed 3D matrix that is denser than comparable collagen gels and forms a barrier to cell migration [23, 30]. It should be noted that since metastasis involves multiple steps, the results of *in vitro* invasion assays may not always be consistent with *in vivo* metastatic potential [31]. Unless otherwise stated, all procedures described below should be performed at RT.

1. Place a transwell with polycarbonate membrane (Chemotaxicell) into empty wells of the companion plate (24-well plate) using sterile forceps.
2. Under sterile conditions, dilute the Matrigel on ice to 0.1–1 mg/ml (i.e., to your desired concentration) using cooled pipette tips and tubes to avoid gelation. Matrigel starts gelation above 10 °C, but does not gel at concentrations below 1 mg/ml.
3. Add 100 µl of diluted Matrigel (i.e., 10–100 µg/well) to the inner compartment of the Chemotaxicell, and let stand overnight to dry in the tissue-culture hood with the fan running. The upper surface of the insert membrane will be coated with a uniform layer of dried basement-membrane matrix solution. This basement-membrane layer serves as a barrier to discriminate

invasive cells from noninvasive cells. To confirm the barrier function of coated Matrigel, a well without coating can be used as the control.

4. On the next day, to rehydrate the basement membrane layer of the inserts, add 200 μ l of serum-free media containing 0.1 % BSA to the inner compartment. (When using ready-to-use transwell chambers with Matrigel-coated membranes, start at this step).
5. Stand for at least 1 h.
6. During rehydration, prepare cell suspension in serum-free media (in the case of MDA-MB-231 cells, use $2\text{--}10 \times 10^5$ cells/ml). The number of cells depends on the cell types, so optimization is required.
7. Continuing from **step 5**, carefully remove the rehydration medium from the inner compartment without disturbing the layer of Matrigel on the membrane. A small amount of rehydration medium may be left in the compartment.
8. Add 500–700 μ l of medium containing 10 % FCS or the desired chemoattractant(s) to a 24-well plate.
9. Use sterile forceps to transfer the Chemotaxicell to the 24-well plate without trapping air bubbles beneath the membranes.
10. Immediately add 200 μ l of cell suspension ($0.4\text{--}2 \times 10^5$ cells/well) to the inner compartment.
11. Incubate for 12–48 h in a cell-culture incubator. Incubation time depends on cell type and cell numbers (*see Note 10*).
12. Aliquot 0.5 ml of the fixation solution to another 24-well plate.
13. Discard the media inside the insert, and put the insert into the fixation solution. Incubate for 20 min.
14. Wash the insert three times with PBS.
15. Stain the insert with hematoxylin for 20 min.
16. Wash the insert three times with PBS.
17. Remove non-invading cells from the upper surface of the membrane by “scrubbing” using cotton swab. Scrubbing efficiently removes Matrigel and non-invading cells from the upper surface of membrane. Do not scrub too strongly. The attachment of the membrane to the insert housing is quite firm, and will not be dislodged during scrubbing, nor will cells be dislodged from the bottom surface of the membrane; however, excessively strong scrubbing can introduce creases that interfere with subsequent observation.
18. Invert the insert. Insert the tip of a sharp scalpel blade through the membrane at the edge adjacent to the housing wall to remove the membrane from the insert housing. Rotate the

insert housing against the stationary blade and the membrane will be released. (Alternatively, a membrane cutter for Chemotaxicell is also available from Kurabo.)

19. Peel the membrane from the remaining point of attachment and place it on a small drop of mounting agent on a glass slide. Place a second very small drop of mounting agent on top of the membrane, and then place a cover slip on top of the membrane. Apply gentle pressure to expel any air bubbles.
20. Observe and photograph migrated cells under the microscope and count the number of invaded cells (as described above for the transwell migration assay; *see* also **Note 9**).

3.4 Cell-ECM Adhesion Assay

1. Make a serial dilution of fibronectin/vitronectin/laminin/collagen in PBS (1–100 µg/ml).
2. Place 100 µl of fibronectin/vitronectin/laminin/collagen solution or 3 % BSA into a 96-well plate.
3. Dry the plate overnight in a cell-culture hood with the fan running.
4. Add 100 µl of 3 % BSA/PBS solution to each well, and incubate for ~1 h in the cell culture hood at RT.
5. During incubation, prepare the cell suspension ($3\text{--}10 \times 10^4$ /ml) in cell-culture media containing 0.1 % BSA, using the same methods as for the cell migration assay.
6. Completely remove the 3 % BSA/PBS from each well.
7. Add 100 µl of cell suspension to each well ($3\text{--}10 \times 10^3$ cells/well). Incubate in the incubator at 37 °C for 2–3 h.
8. Remove the medium from the wells and wash each well twice with PBS.
9. Add 100 µl crystal violet solution to each well and incubate for 10 min at RT.
10. Aspirate the crystal violet solution and wash three times with PBS.
11. Add 20 µl of DMSO and incubate for 10 min at RT.
12. Add 80 µl of dH₂O and measure absorbance at 590 and 630 nm.

4 Notes

1. Histone modification-specific monoclonal antibodies that are useful for immunohistochemistry have been described [9–11, 16, 26], and some are commercially available from several companies (e.g., Active Motif, Diagenode, MBL, TAKARA, and Wako).

2. Other disposable and membrane-attached housings are available from several companies (e.g., Corning, Kurabo, Falcon, BD Biosciences, and Iwaki).
3. Collagen is a major component of ECM and can be solubilized under acidic conditions (pH 3.0). Gelation is observed under physiological conditions, whereas diluted collagen does not polymerize. Fibronectin can be used instead of collagen.
4. Matrigel is a solubilized mixture of basement membrane proteins extracted from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma, a tumor rich in ECM proteins. Its major components are laminin, collagen IV, heparin sulfate proteoglycans, and entactin/nidogen [32]. In addition, it contains TGF- β , epidermal growth factor, insulin-like growth factor, fibroblast growth factor, and tissue plasminogen activator [33, 34].
5. Ready-to-use basement membrane-coated chambers with a wide range of pore diameters from 3 to 12 μm are available from several cell biology companies (e.g., BD, Merck, and Corning).
6. In most cases, coating is not essential. However, some cell types perform more consistently when used with coated membranes. For example, endothelial and smooth muscle cells are typically used with membranes coated with collagen, fibronectin, or a similar ECM protein. We usually coat the back side in order to promote attachment to the membrane after cells pass through the pore.
7. To neutralize trypsin, FCS-containing medium can also be used. When FCS-containing medium is used to neutralize trypsin, the medium should be washed out completely, because FCS contains chemoattractants like growth factors that affect migration activity.
8. When cells are incubated for longer period, it is important to maintain sterility. Follow the manufacturer's protocol for sterilization. In the case of Boyden chambers from Neuro Probe Inc., the gaskets can be autoclaved, but the Boyden chambers themselves cannot.
9. Cells can be counted directly under a microscope. However, it is easier to count cells after taking digital images using a camera. Fluorescent dye staining, or lysing the cells for spectrometry analysis can be used for quantification. The membrane may also be processed for electron microscopy.
10. For longer experiments, it is preferable to seed cells at lower densities. It is necessary to determine the growth rate for each cell line before performing transwell invasion assays.

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Part IV

Factors that Influence Epigenetic Changes in Cancer

Aberrant Epigenetic Modifications in Radiation-Resistant Head and Neck Cancers

Hon-Yi Lin, Tim Hui-Ming Huang, and Michael Wing-Yan Chan

Abstract

Head and neck cancers are characterized by both genetic and epigenetic aberrations. In treating head and neck cancers, ionizing radiation (IR) is an essential modality in either definitive or adjuvant setting. However, radiation-resistant head and neck cancers are not uncommon. The major biological determinant for IR resistance was previously considered at genetic level because DNA is the major target of irradiation damage. However, in head and neck cancers, recent evidence demonstrated epigenetic disturbance after IR, implicating its role in IR resistance. Hence, this chapter intends to establish an in vitro model for investigating DNA methylation changes in IR-resistant head and neck cancer cells. Bisulfite pyrosequencing is the main methodology it introduced.

Key words Head and neck cancers, Ionizing radiation, Radioresistance, Radiosensitivity, Epigenetics, DNA methylation, microRNA, Histone modification

1 Introduction

Ionizing radiation (IR) is an important modality in treating head and neck cancers [1]. However, resistance to IR is not uncommon. In radiobiology, ionizing irradiation eradicates cancer cells mainly via damaging DNA, resulting in double strand breaks and subsequent genetic disturbance [2, 3]. Therefore, the biological determinant of IR resistance is previously thought to be mainly decided at the post-irradiation genetic disturbance, such as mutation, deletion, and chromosome rearrangement. Recently, post-irradiation epigenetic changes have been suggested to also play a significant role in affecting IR resistance [4–9].

Several types of epigenetic alterations have been reported in head and neck cancers, including DNA methylation [10–13], histone modifications [14], and miRNA modulation [15–17]. Of these, DNA methylation on promoter regions of tumor suppressor gene or tumor-suppressing miRNA plays an essential role [18–23]. For example, we and the others have previously demonstrated that

promoter hypermethylation of *DAPK* was significantly associated with poor clinical outcomes, especially post-irradiation cancer recurrence [24, 25]. Moreover, a distinct DNA methylation pattern has been reported in HPV-infected patients [26, 27], who demonstrated better survival than that of non-HPV-infected patients [28]. These data suggested a significant role of DNA methylation in serving as a prognostic factor in head and neck cancer patients.

Beyond a potential application of prognostic factor, several lines of evidence suggested a therapeutic role of epigenetic modulation. First, epigenetic therapy of using demethylating agents has been observed to enhance radiosensitivity [29, 30]. Second, gene-specific targeted demethylation is suggested as a future therapeutic strategy to overcome cancers [31]. All of these data raise an interest in investigating the role of epigenetic aberrance/modulation in IR response.

Bisulfite sequencing has been considered as the gold standard to measure DNA methylation at single nucleotide resolution [32]. Recently, bisulfite conversion coupled with pyrosequencing has been demonstrated as an important tool for quantitative and high-throughput detection of DNA methylation on individual CpG sites [33]. When compared with conventional bisulfite sequencing, the major advantage of pyrosequencing is that there is no need to insert PCR product into a cloning vector [33]. Practically, it has been proven as a useful tool in deciphering DNA methylation in head and neck cancers [34–37].

Hence, this chapter focuses on establishing an in vitro model to investigate the role of DNA methylation in IR-resistant head and neck cancer cells. Bisulfite pyrosequencing is the main method it introduces for detecting DNA methylation.

2 Materials

2.1 Establishment of Irradiation-Resistant Sublines

2.1.1 Preparation of the Original Head and Neck Cancer Cell Lines

1. Original head and neck cancer cell lines: SCC15 (ATCC, CRL-1623), SCC4 (CRL-1624), SCC25 (CRL-1628), and SCC9 (CRL-1629) [38].
2. Cell culture dish, medium, and incubator: RPMI-1640 (Gibco, Cat. no. 11875), 10 % FBS, 50 units/ml of penicillin/streptomycin, and 5 % CO₂ incubator.

2.1.2 Establishment of Irradiation-Resistant Sublines

1. 1.5-cm biomaterial-equivalent bolus.
2. 6-MV linear accelerator (Elekta) or Co-60 irradiator.
3. 0.4 % crystal violet (Sigma) in 50 % methanol.

2.2 Methylation Analysis by Using Bisulfite Pyrosequencing

2.2.1 Genomic DNA Extraction

Genomic DNA Mini Kit (Geneaid, Cat. No. GT050).

1. GT Buffer.
2. Proteinase K.
3. 20 mg/ml RNase A (Invitrogen, Cat no. 12091-021).
4. Absolute ethanol.
5. W1 Buffer.
6. GD column.
7. 1.5 ml centrifugation tube.
8. ddH₂O.

2.2.2 Bisulfite Conversion for Extracted Genomic DNA

EZ DNA Methylation Kit (Zymo Research, Orange, CA).

1. CT conversion reagent.
2. PCR machine.
3. M-binding buffer.
4. Zymo-Spin IC™ column.
5. M-wash buffer.
6. M-desulfonation buffer.
7. M-wash buffer.
8. Sterile 1.5 ml microcentrifuge tube.

2.2.3 Bisulfite-Based Pyrosequencing

1. Validated PCR primers.
2. RBC Sensizyme Hotstart Taq premix (RBC Bioscience, Taiwan).
3. Sterile Dimethyl Sulfoxide (DMSO).
4. Pyrosequencer: PyroMark Q24 (Qiagen).
5. The Pyro Gold Reagents (Qiagen).
6. In vitro methylated DNA (Millipore).

3 Methods

3.1 Establishment of Irradiation-Resistant Cell Sublines

3.1.1 Preparation of the Original Head and Neck Cancer Cell Lines

Several head and neck cancer cell lines can be obtained as original lines for IR-resistant experiments, such as SCC15 (ATCC, CRL-1623), SCC4 (CRL-1624), SCC25 (CRL-1628), and SCC9 (CRL-1629) [38].

1. Culture the original head and neck cancer cell lines as their recommended conditions. For instance, propagate OML1 cancer cells in RPMI-1640 (Gibco, Cat. no. 11875), supplement with 10 % FBS and 50 units/ml of penicillin/streptomycin, and maintain at 37 °C, 5 % CO₂ incubator.

2. Prepare two sets of original head and neck cancer cells: the first set for establishing IR-resistant cell subline and the second set for control.

3.1.2 *Establishment of Radiation-Resistant Cell Sublines*

The first set of original cancer cells were used for establishing IR-resistant cell subline. Several types of established protocol have been reported, for instance, a protocol of 10-Gy by 10-fraction given biweekly with a total dose of 100 Gy [39]. However, after such a high-dose protocol, some types of cancer cell lines survived very poorly. Thus, in such a condition, a modified half-dose protocol could be used alternatively, i.e., 5-Gy by 10-fraction given weekly with a total dose of 50 Gy. Note that the next fraction of irradiation only delivered when irradiated cells regrow to about 80 % confluence of the incubated dishes (*see Note 1 and Fig. 1*).

1. Seed the original cancer cells to a sterile 6-cm cell plate, and then incubate until about 80 % confluence.
2. Place 1.5 cm biomaterial-equivalent bolus on the both up- and down-side of the culture plate—arrange just like a sandwich (*see Note 2*).
3. Put the bolus-covered culture plate on the table of 6-MV linear accelerator (Elekta) (*see Note 3*).
4. Rotate the gantry of linear accelerator to 180° for delivering ionizing radiation.
5. Irradiate the first fraction of 5 Gy.

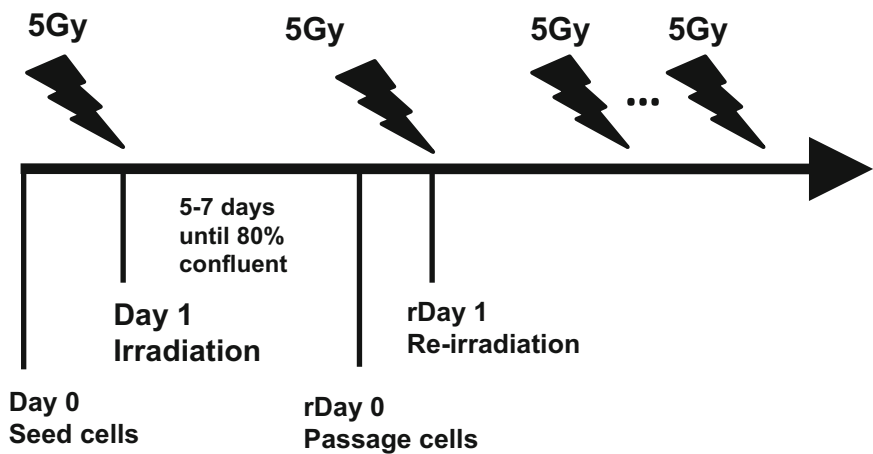


Fig. 1 Timeline for establishing irradiation-resistant cancer cell subline. Repeating 5-Gy irradiations to original head and neck cancer cells up to a total dose of 50 Gy is able to obtain irradiation-resistant cell subline. Note that before each fraction of 5-Gy irradiation, at least 5–7 days incubation is essential to allow cells to regrow to about 80 % confluent of the culture plates. Abbreviation: *Gy* gray, *rDay 0* re-irradiation day 0

- After irradiation, change the culture medium regularly at a time interval of 2–3 days.
- After 5–7 days, when cells regrow to about 80 % confluent, re-irradiate the second fraction of 5 Gy.
- Repeat the irradiating protocol up to 10 fractions with a total dose of 50 Gy.

3.2 Validation of the Radiation Resistance of Established Sublines

3.2.1 Establishment of a Stable Subline

- After completion of irradiation course, irradiated cancer cells suffered from substantial degrees of sublethal damage [2]. Thus, continuously culture the irradiated cancer cells for 2–4 weeks is essential before further experiments. If this procedure is omitted, biased data may be obtained.
- After a stable subline is obtained, several tubes of irradiated cells can be stored at liquid nitrogen.

3.2.2 Radiation Stress Treatment to Test the Level of Radiation Resistance

To test whether radiation resistance has been established, radiation stress treatment should be conducted. For stressing, a single shot irradiation of 10 Gy or higher dose was delivered. Note that original non-irradiated cancer cells were used for control.

- Seed 1×10^3 cells in 10-mm culture plate.
- Deliver a single shot of 10-Gy irradiation to both original and established cell subline. Use sandwich-type bolus as described above.
- Seven days after irradiation, stain cells by using 0.4 % crystal violet (Sigma) in 50 % methanol.
- Count visible colonies for cell survival analysis (*see* Fig. 2).

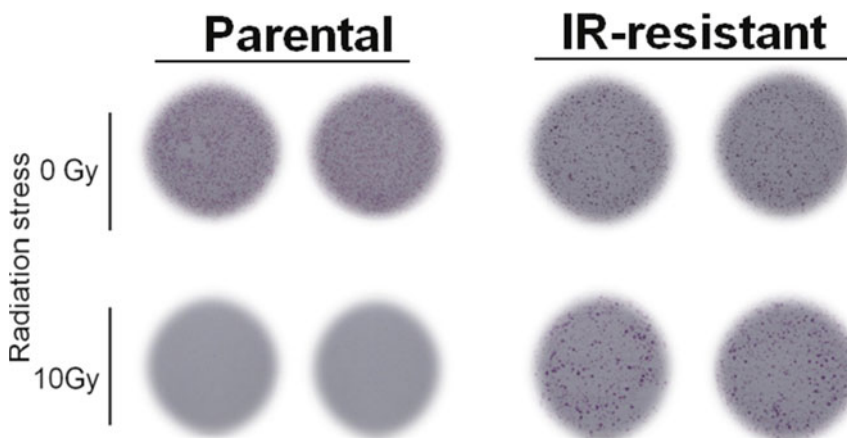


Fig. 2 Radiation stress test. A single shot of 10-Gy irradiation is used to induce a radiation stress. After irradiation of 10 Gy, IR-resistant cell subline showed a more survival colonies than that of the parental head and neck cancer cells. OML-1 cells were used. Abbreviation: *IR* irradiation

3.3 Methylation Analysis by Bisulfite Pyrosequencing

After an IR-resistant cell subline is obtained, differential DNA methylation profiles between these cells and their original parent cells can be compared by methylation microarray [26] or deep sequencing [40]. Genes of interests were then selected for validation by using various methods such as quantitative methylation-specific PCR (q-MSP) or bisulfite pyrosequencing [41]. Of these, bisulfite pyrosequencing has a major advantage of reliably detecting CpG methylation without cloning of PCR products into cloning vectors [33]. More notably, bisulfite pyrosequencing has been proven as a useful tool in investigating cancer samples [42], including head and neck cancers [34–37]. Thus, it is recommended for further methylation detection.

3.3.1 Genomic DNA Extraction and Bisulfite Conversion

Genomic DNA was extracted by using Genomic DNA Mini Kit (Geneaid, Cat. No. GT050), as follows:

1. Homogenize the cell pellet by adding 200 μ l GT buffer.
2. Add 20 μ l proteinase K and incubate them at 60 °C for 30 min for adequate protein degradation.
3. Add 200 μ l GT buffer and incubate at 60 °C for 20 min or until the cell lysate was clear. During incubation, invert the tube every 5 min.
4. Following a 60 °C incubation, add 2 μ l 20 mg/ml RNase A (Invitrogen, Cat no. 12091-021), and then incubate at room temperature for 5 min to remove RNA.
5. Add 200 μ l of absolute ethanol, then transferred to GD column in a collection tube, and then centrifuge at 20,000 $\times g$ for 1 min.
6. Add 400 μ l W1 buffer to the GD Column, and then centrifuge at 20,000 $\times g$ for 30 s.
7. Add 600 μ l wash buffer to the GD Column, and then centrifuge at 20,000 $\times g$ for 30 s.
8. Put the GD column back to the collection tube, and then centrifuge at 20,000 $\times g$ for 3 min.
9. Transfer the GD column to a sterile 1.5 ml centrifugation tube, and then add 50 μ l preheated ddH₂O to incubate at room temperature for 5 min.
10. Centrifuge sample at 20,000 $\times g$ for 30 s to elute DNA.

3.3.2 Bisulfite Conversion for Extracted Genomic DNA

Extracted genomic DNA was bisulfite modified by using EZ DNA Methylation Kit (Zymo Research, Orange, CA), as described previously [43].

1. For 500 ng DNA sample, add 5 μ l M-dilution buffer and adjust to a total volume of 50 μ l with ddH₂O.

2. Mix samples by flicking and incubate them at 37 °C for 15 min. After incubation, add 100 µl CT conversion reagent to each sample and mix thoroughly.
3. Incubate samples in PCR machine by using the following program: 50 °C for 2 h, 95 °C for 15 s, 50 °C for 4 h, 95 °C for 10 s, 50 °C for 9 h 30 min. Then, place samples on ice for 10 min.
4. Place samples in a 1.5 ml microcentrifuge tube.
5. Add 400 µl M-binding buffer and mix by inversion.
6. Load mixture into a Zymo-Spin IC™ column followed by centrifuge at 20,000 × *g* for 30 s.
7. Add 200 µl M-wash buffer to the column followed by centrifugation at 20,000 × *g* for 30 s.
8. Add 200 µl M-desulfonation buffer to the column and incubate at room temperature for 20 min.
9. Centrifuge the column at 20,000 × *g* for 30 s.
10. Wash the column twice by using 200 µl M-wash buffer and then centrifuge at 20,000 × *g* for 30 s.
11. Place the column into a new 1.5 ml microcentrifuge tube.
12. Elute DNA twice by adding 50 µl ddH₂O and incubate for 5 min
13. Centrifuge at 12,000 rpm for 2 min. Discard the flow through.

3.3.3 Bisulfite Pyrosequencing

1. Amplify bisulfite-modified DNA by using PCR. A tailed reverse primer in combination with a biotin-labeled universal primer should be used (*see* **Note 4**).
2. PCR was amplified in a 25 µl reaction, which contained 12.5 µl of 2× RBC Sensizyme Hotstart Taq premix (RBC Bioscience, Taiwan), 0.5 µM of each primer, 1 µl DMSO, and 4 µl of bisulfite-modified DNA.
3. Use PCR program as follows: 95 °C for 5 min; 50 cycles of 95 °C for 30 s, 56 °C for 1 min, 72 °C for 45 s; a final extension at 72 °C for 7 min. A total of 1.5 µl of each PCR products was analyzed on a 2 % agarose gel before pyrosequencing.
4. Conduct pyrosequencing by using PyroMark Q24 (Qiagen). Use Pyro Gold Reagents (Qiagen) according to the manufacturer's protocol (*see* Fig. 3 and **Notes 5–9**).

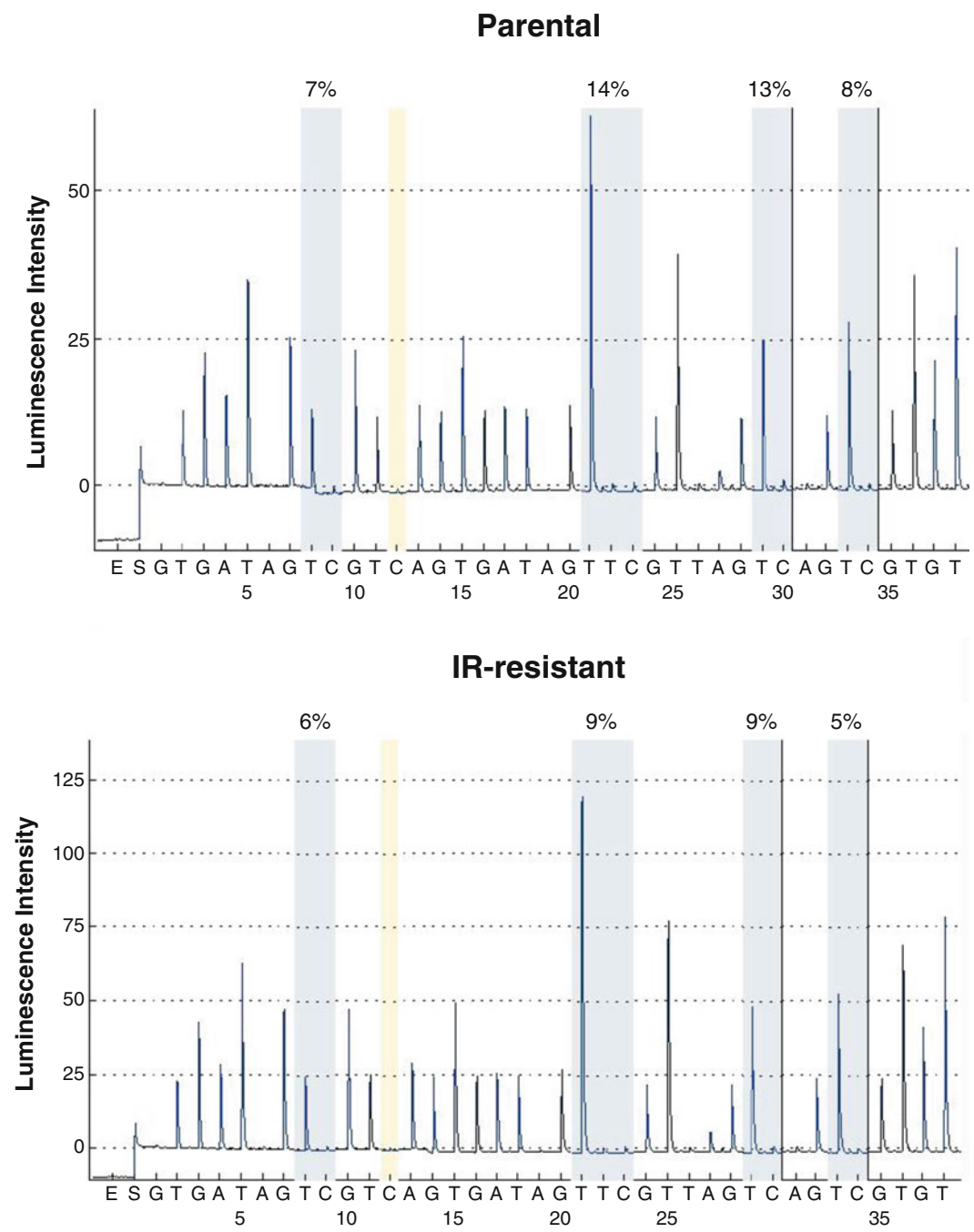


Fig. 3 Representative pyrograms of methylation analysis by bisulfite pyrosequencing in parental and IR-resistant OML-1 cells. Promoter region of *PTEN* were subjected to bisulfite pyrosequencing. The percentage of methylation of four CpG sites is indicated on *top of the shaded box*. X-axis indicates dispensation order (time). Hypomethylation of the CpG sites of *PTEN* were found in the IR-resistant cells

4 Notes

1. To establish the irradiation-resistant cancer cell sublines, the next fraction of irradiation can only be delivered when irradiated cells regrow to about 80 % confluence of the incubated plates, or at a time interval of 2 weeks before a higher dose is given [39]. This is an important procedure to allow some degree of repair for sublethal damages [2].
2. For irradiating cell dishes, biomaterial-equivalent bolus should be used to cover both up- and down-side of the cell dishes—just like sandwich—to provide adequate buildup of irradiation dose to the cultured cancer cells [44].
3. For irradiating culture plates, irradiation is delivered by using Co-60 irradiator or linear accelerator. However, delivering irradiation by using a linear accelerator is recommended due to a more even distribution of radiation dose.
4. In pyrosequencing, PCR and sequencing primers could be designed by using PyroMark Assay Design 2.0 (Qiagen GmbH, Hilden, Germany). Genes of interest were selected according to the result from microarray or deep sequencing. Biotin-labeled universal primer was used as described previously [45].
5. In pyrosequencing, in vitro methylated DNA (Millipore) or SSSI treated genomic DNA should be applied as a positive control of methylation.
6. Methylation percentage of individual cytosine was determined by the luminescence intensity of cytosines divided by the sum of luminescence intensity of cytosines and thymines at each CpG site (multiplied by 100).
7. After in vitro experiments, human samples should be conducted for in vivo validation. Thus, adequate statistics for time-to-event endpoints can be obtained. For instance, considering primary end points of local or locoregional disease control is suitable. Considering secondary end points of disease-free, disease-specific, and overall survival is encouraged. For comparing cancer control and survival rates between groups, Kaplan-Meier plot with log-rank test could be used. For conducting univariate or multivariate analysis, hazard ratio with 95 % confidence interval could be estimated by using Cox proportional hazard regression.
8. Study limitation: post-irradiation radioresistant cell sublines may gradually lose their acquired IR-resistant characteristics in continuous passages. Thus, all experiments should be conducted within ten passages after their establishment.

Reestablished new radioresistant cell subline should be considered if an interval stress test detects a significant loss of their radioresistance.

9. Conducting quantitative methylation-specific PCR (Q-MSP) is still useful for confirming the status of promoter methylation of the target genes [33, 43].

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Chapter 18

Cancer-Associated Infectious Agents and Epigenetic Regulation

Vidya Vedham and Mukesh Verma

Abstract

Infectious agents are one of the factors which contribute to cancer development. Few examples include human papilloma virus in cervical cancer, hepatitis virus in hepatocellular carcinoma, herpes virus in Kaposi's sarcoma, Epstein-Barr virus in nasopharyngeal carcinoma, human T-cell lymphotropic virus type-1 (HTLV-1) in T-cell leukemia and lymphoma, *Helicobacter pylori* in gastric cancer. These agents cause genomic instability in the host and most of them affect host immune system. Infectious agents may integrate in the host genome although their site of integration is not fixed. Expression of some infectious agents involves epigenetic regulation by DNA methylation, histone modification, miRNA level alteration, and chromatin condensation. This chapter provides examples where epigenetic regulation has been reported in cancer-associated infectious agents. Epigenetic inhibitors and their potential in cancer control and treatment are also discussed.

Key words Biomarker, Cancer, Epigenetics, Histone, Methylation, Risk assessment, Virus, Early-life exposure, Childhood cancer, Adult cancer, Cofactors, AIDS-related malignancies, Targeted therapy, Vaccines

Abbreviations

ATLL	Adult T-cell Leukemia/Lymphoma
BL	Burkitt's Lymphoma
EBV	Epstein-Barr Virus
EBVaGC	EBV-associated Gastric Carcinoma
<i>H. pylori</i>	<i>Helicobacter pylori</i>
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma
HCV	Hepatitis C Virus
HIV-1	Human Immunodeficiency Virus type 1
HL	Hodgkin Lymphoma
HTLV-1	Human T-Lymphotropic Virus type 1
IARC	International Agency for Cancer Research
NPC	Nasopharyngeal Carcinoma
PTLD	Post-Transplantation Lymphoproliferative Disease

1 Infectious Agents in Different Cancer Types

Infectious agents are responsible for about 18 % of malignancies around the world [1]. This amounts to approximately two million cancer cases each year that are attributed to infectious agents and the frequency is about three times higher in developing (26 %) than in developed (7 %) countries [2]. The mere presence of an infectious agent in tumor tissue is not sufficient to establish causality and often evidence from epidemiologic, clinical, and biological studies are used to categorize an infectious agent as carcinogenic. Seven major infectious agents were identified as carcinogenic by the International Agency for Research on Cancer (IARC): hepatitis B virus (HBV), hepatitis C virus (HCV), certain strains of the human papillomavirus (HPV), Epstein-Barr virus (EBV), human immunodeficiency virus type 1 (HIV-1), human T-cell lymphotropic virus type-1 (HTLV-1), and the gram-negative bacterium *Helicobacter pylori* (*H. pylori*) [3]. Estimates of infectious prevalence and risk of cancer was calculated from the literature.

Despite the high prevalence of many of these infectious agents (e.g., HPV, EBV, and *H. pylori*) in the world, not every infected individual will develop cancer and malignancies associated with infectious agents generally occur after prolonged latency and/or chronic infections in the host. Hence pathogenic infections are often necessary but are not sufficient for cancer development. Cancer initiation often requires one or more additional cofactors. Host cofactors such as genetic susceptibility, age, robustness of the immune system, dietary habits, and certain environmental factors tip the balance for these infections to become malignant [4, 5]. Since infectious diseases are often preventable and/or treatable, cancers associated with infections can be preventable, thereby constituting a vital strategy to decrease the global cancer burden.

1.1 Cervical Cancer

Cervical cancer ranks as the third most common cancer in women disproportionately affecting poor and under served population in developing countries [6]. Almost all cervical cancers are associated with persistent infection of high-risk HPV serotypes related to HPV-16 and HPV-18 [7, 8]. So far, over 100 HPV types have been identified, of which about 15 strains are categorized as high-risk serotypes [9, 10]. In addition to cervical cancer, HPV infection has also been associated with a subset of anal, oropharyngeal, vulvar, vaginal, and penile cancers [11]. Approximately 80–90 % of the HPV-associated non-cervical cancers are caused by HPV 16 and 18, thus asserting the need for preventing HPV infection in reducing the cancer burden [12, 13].

HPV are a group of over 130 double-stranded DNA viruses, of which over 40 types can be sexually transmitted. HPV infections are often cleared spontaneously in spite of a high probability

(~80 %) of acquiring a cervical infection in sexually active individuals [13]. Studies have shown that HPV infection contributes to the development of cervical intraepithelial neoplasia (CIN) and subsequent invasive carcinomas [14–16]. Although the percentage of infected individuals who develop low- to high-grade intraepithelial lesions is very low, additional cofactors such as immunosuppression, smoking, multiparity, and coinfection with herpes simplex virus, chlamydia, HIV, or other strains of HPV can lead to carcinogenesis in individuals with high-grade lesions [17].

Screening and primary prevention can be effective tools to alleviate the cancer burden associated with HPV. In the USA, there was significant reduction in cervical cancer incidence as a result of cervical cancer screening programs that involved detection and treatment of precancerous lesions [18]. In developing countries similar widespread implementation of cervical cancer screening programs has been limited due to availability of resources. However, recently the development of relatively inexpensive HPV-based tests has made screening programs more cost-effective and feasible [19, 11]. The US Food and Drug Administration have approved the development of HPV vaccines based on evidence that they protected women from persistent HPV infection and premalignant anogenital cancer [20]. This vaccine is recommended for females 9–26 years of age and is administered intramuscularly on a 3-dose schedule [21].

1.2 Nasopharyngeal Carcinoma

Nasopharyngeal carcinoma (NPC) is tumor in the epithelial cells of the nasopharynx and is endemic in southern China, south-east Asia, and North Africa [22]. NPC has three histological subtypes: keratinizing squamous cell carcinoma, nonkeratinizing carcinoma, and basaloid squamous cell carcinoma [23]. In the endemic areas, EBV is always associated with NPC [24]. Epstein-Barr virus (EBV) is a gamma herpes virus and was the first human virus linked to cancer [25]. EBV is generally acquired at a young age through saliva, and childhood infections are asymptomatic [26]. Plasma EBV-DNA levels are correlated to treatment outcome and survival in NPC patients [27]. The virus infects the epithelial cells of the posterior nasopharynx in Rosenmüller's fossa in Waldeyer's ring. This is located superior and posterior to the eustachian tubes and hence ear pain is a frequent presenting symptom in patients [25]. The tumor initially develops in the walls of the nasopharynx without any symptoms and NPC is frequently misdiagnosed in early stages prior to its spread to the regional lymph nodes [28]. Radiotherapy (RT) alone was the standard treatment for almost all stages of NPC until recently it was suggested that brachytherapy given after RT may significantly improve survival in patients with locally advanced disease [29]. Although survival is usually poor for metastatic patients, treatment of metastatic disease is by platinum-based chemotherapy [30]. More recently, molecular therapy

targets epidermal growth factor receptor (EGFR) and Immunotherapy using EBV latent membrane proteins are being tested [31]. Recent data suggests that EBV-DNA can be used as a tool to monitor NPC and recurrences were preceded by a rise in the plasma EBV-DNA load [32].

The high prevalence of EBV, its easy mode of transmission, and a lack of understanding of the triggers that lead to EBV-associated cancer complicate the development of targeted prevention strategies. Currently, broad-spectrum antiviral agents and immune-based therapies, including specific monoclonal antibodies, have shown promise in the treatment and management of EBV-related lymphoproliferative disorders.

1.3 Gastric Cancer

Gastric cancer is the second most common malignancy in the world and epidemiological studies have shown that a strong causal relationship between *H. pylori* infection and gastric cancer [33, 34]. *H. pylori* prevalence varies by geographic region with the overall prevalence of almost 80 % in developing countries in contrast to about 50 % in developed countries [35].

H. pylori is a gram-negative bacterium found on the luminal surface of the gastric epithelium [36]. *H. pylori* infection is associated with two gastrointestinal cancers: gastric non-Hodgkin lymphoma or mucosa-associated lymphoid-tissue (MALT) lymphoma and noncardiac gastric adenocarcinoma [37]. In *H. pylori*-infected individuals the risk of either of these cancers is approximately six times higher. Epidemiological studies have highlighted smoking, high intake of salt, and alcohol consumption as important cofactors in *H. pylori*-mediated gastric cancer [38].

H. pylori infection usually occurs during childhood by the oral–oral or fecal–oral route and the pathogen can persist indefinitely if untreated [39]. Differential pathogenic studies have found that certain strains of *H. pylori* that are more virulent [40]. The bacterium injects into the host epithelium and alters the epithelial cell function [41]. One of the most extensively studied virulence factors is a 140 kDa protein produced from a gene known as CagA (cytotoxin-associated gene A), which is, used a biomarker for gastric carcinoma [42–44].

In recent years, it has been observed that improvements in sanitation and hygiene have been responsible for dramatic declines in the transmission of *H. pylori* over the last few decades [39]. Hence, early prevention of infection by improving sanitary conditions and dietary modifications are the most important tool for the prevention of *H. pylori* infection.

The most effective treatment for *H. pylori* is a triple therapy that involves a combination of two antibiotics and a proton-pump inhibitor [45]. Antibiotic resistance is a potential problem in some individuals and often an increase in resistance can be observed over time [46, 47]. *H. pylori* changes its genetic program to circumvent

the antibiotic effect and these changes in the genetic program can be in the form of chromosomal mutations, acquisition of resistance plasmids, or through transposition of pathogenicity islands [48].

1.4 Liver Cancer

Liver cancer is the fifth most common cancer in the world. Liver cancer consists predominantly of hepatocellular carcinoma (HCC) is the third leading cause of cancer death worldwide [49]. Chronic infections with either hepatitis B virus (HBV) or hepatitis C (HCV) virus are linked to about 80 % of worldwide [50]. In 2008, the World Health Organization (WHO) estimated that about two billion people worldwide had been infected with HBV [51]. Both HBV and HCV have been categorized by the International Agency for Research on Cancer (IARC) as human carcinogens [52].

The prevalence of HBV and HCV infection varies significantly in different parts of the world [53]. In China almost 12 % of the population is positive for HBV, while most parts of Africa tests positive for the virus. In contrast less than 1 % of the populations in Western and Northern Europe and North America are HBV positive. Hence, more than 80 % of HCC cases occur in developing countries in south-east Asia, East Asia, and sub-Saharan Africa [54].

Exposure to both HBV and HCV occurs through bodily fluids (i.e., semen and blood) and can cause acute or chronic liver disease and cancer [55]. HBV infection in infants and children occurs either by perinatal transmission from infected mothers or horizontal transmission from infected household contacts [56–60]. HBV and HCV infections increase an individual's risk of HCC by 22.5- and 17.3-fold, respectively [61]. Other cofactors that contribute to hepatitis-mediated HCC risk include excessive alcohol intake, obesity and metabolic syndrome, HIV-associated immunodeficiency, and exposure to aflatoxins [62–68].

Preventing both childhood and adult liver cancers can be achieved by preventing HBV/HCV infections. Vaccines are the most effective ways for primary prevention of HBV infection in most populations [69]. Universal HBV-vaccination strategies developed by countries such as USA and China have high success rates in preventing [70, 71]. Similarly, the World Health Assembly recommended routine infant HBV vaccination in all countries since 1992 [72]. Currently, there is no vaccination for HCV and interferon treatment is effective in clearing acute HCV infections in about a third of patients [73, 74].

2 Multiple Infections and Cancer

Many pathogenic infections manifest into cancer when the host immune system is altered in response to multiple infections or coinfections with other pathogens. These multiple pathogenic infections often cause chronic inflammation in tissues and organs which may lead to the cancer initiation.

Recent studies have demonstrated the significance of coinfections in cancer initiation and progression. A connection between *Plasmodium falciparum* (*P. falciparum*)-mediated malaria contributing incrementally to the development of EBV-associated endemic Burkitt's lymphoma has been highlighted in African populations where these coinfections are common [75, 76]. Similarly, some studies have highlighted that previous exposure to infectious agents such as *Chlamydia trachomatis* (CT) and herpes simplex virus type 2 (HSV-2), increases the risk for cervical cancer in HPV-positive individuals [77, 78]. In addition, other studies have highlighted that individuals positive for multiple strains of HPV have a lower response to cancer treatments [9, 79, 80]. These studies have shown a correlation between microbial load and cancer manifestation. Similarly, HBV positive individuals have a higher chance of developing HCC when infected with *H. pylori*, HCV, or HDV [81, 82].

In HIV positive individuals, coinfection is an important factor for carcinogenesis. Coinfection with oncogenic viruses, EBV, HBV, HCV, KSHV, (Kaposi's sarcoma-associated herpesvirus), and HPV cause significant cancer-related morbidity and mortality [83, 84]. These viruses interact with HIV in unique ways to predispose HIV-positive individuals to malignant diseases [85, 86].

Another common cause of cancer is multiple malignant infections in transplant patients. The introduction of potent immunosuppressive therapy and the presence of latent infections in donors and recipients pose new challenges in transplantation medicine [87]. Post-transplant lymphoproliferative disorders (PTLD) are a group of lymphoproliferative diseases that occur in transplant patients, ranging from self-limited lymphoproliferation to aggressive lymphomas [88]. Similarly other viruses such as HBV, HCV, and HPV also infect immunocompromised patients and have been linked to the development of cancer in transplant patients [89, 90].

Despite the implication of coinfections as a risk factor for cancer, the relative risks of single versus multiple infections for specific cancer types are yet to be estimated. Exploring the effects of single and multiple infections on a specific cancer have design limitations and hence cannot yield complete information. Because multiple infections can cause a single cancer via different biological pathways, estimating the contribution of any individual agent is challenging and requires complex epidemiologic methods.

2.1 Epigenetic and Genetic Regulation

Pathogenic infections are known to induce a spectrum of genetic and epigenetic alterations that can lead to cancer development. Cancer genetics has been studied for decades and it is known that small nucleotide polymorphism, mutations, deletions, insertions, and translocations constitute genetic regulation of cancer. Epigenetics involves changes in gene expression without a change in gene sequence. Epigenetic changes are required for normal

growth and development but in cancer these regulations are disturbed. Below is the description of major components of epigenetic machinery often altered by infectious agents.

2.1.1 Methylation

DNA methylation is the covalent transfer of a methyl group from S-adenosyl-L-methionine to cytosines in CpG dinucleotides. Promoter region of genes contain high percentage of GC content which are called CpG islands. These CpG islands are usually unmethylated in normal cells. However, during abnormal expression of genes, cytosines of the promoter region get methylated at 5' position resulting in inactivation of tumor suppressor genes as seen in cancer. Several groups have shown that viral oncoproteins induce expression, interact with cellular DNA methyltransferases as well as histone-modifying enzymes such as histone deacetylases and histone acetyltransferases altering the host cell activity. The cyclin-dependent kinase inhibitor, p16INK4a has often been implied as a tumor-suppressor gene and is observed to be inactivated due to hypermethylation [91]. Studies have shown a high frequency of hyper-methylation of the p16INK4a in HBV-, EBV-, KSHV-, and HPV-related tumors. In a recent review on virus induced epigenetic mechanisms for cancer, Poreba et al. have listed the epigenetic interactions between oncogenic viral proteins and host epigenetic machinery [92]. Similarly, Chaiwongkot et al. reported differential methylation of E2 binding sites in preinvasive and invasive cervical lesions [93]. E2 is a transcriptional regulation factor for E6 and E7 genes of HPV. In HPV positive anal squamous cell cancer (SCC), host genome methylation was observed by using high throughput methylation profiling of clinical samples [94]. Other studies have reported an association of the aggressiveness of cervical carcinoma with the level of methylation of MLH1, MGMT, and p16INK4 [95].

The HBV genome was found to be hypermethylated in hepatocellular carcinoma (HCC) [96, 97] leading to the inactivation of the transcription factor PCDH10 in HBV positive hepatocellular cells [98]. Differential methylation patterns of RASSF1A, GSTP1, CHRNA3, and DOK1 were also observed in HCV and HBV infected cells in HCC [99]. Contribution of alcohol consumption and cancer progression was also correlated with methylation levels. One group of investigators reported no association of methyl transferase genes in HBX-induced androgen receptor expression with HBV-mediated HCC [100]. EBV induces hypermethylation of at least half dozen genes in B cells [101].

2.1.2 miRNA

MicroRNAs (miRNAs) are small noncoding RNA molecules that regulate gene expression pathways and are an important component of the cellular RNA interference system. MicroRNAs have been reported in different cancer types including those cancers where the etiological agent is an infectious agent and studies have

shown interactions between virus and host miRNAs [102–104]. More than 1,200 miRNAs have been reported to date and the number is still increasing [104]. Upregulation of miR-34a and downregulation of miR-203 was observed in cells transformed with HPV16 [105]. Other groups of investigators reported downregulation of miR-143 and miR-145 [106]. Abnormal expression of these two miRNAs has been observed glioblastoma and colorectal cancer. Probably a large number of miRNAs or genome-wide miRNA analysis will indicate cervical specific miRNAs. Although the mechanisms by which viral infections modulate the expression of specific cellular miRNAs is unknown, cancer-specific miRNA profiling is an excellent biomarker for screening high risk populations. Further research is needed concerning the regulatory dependencies between miRNAs and their mRNA targets in infectious agent-associated cancers.

2.1.3 Chromatin and Histone

At the molecular level, the nucleosome (unit of chromosome) constitutes the core of chromatin and consists of basic histone proteins. Each nucleosome has 146 base pairs of DNA and histones wrapped around the DNA to neutralize the negative charge. Histones exist as octamers with dimers of H3, H4, H2A, and H2B. Histone H1 is not the part of this complex but it is needed for the histone–DNA complex formation. During cancer histones get modified posttranscriptionally at different sites and the most common modifications are acetylation, phosphorylation, ubiquitination, and biotinylation. Histone modifications make chromatin active or inactive. Infectious agents can cause alteration in histone profiles of the host. Histone acetylation contributes in EBV micro RNA expression in B cells [107]. In case of HPV infected samples, the chromatin immunoprecipitation analysis indicated higher levels of H3K4me1, H3K4me3, H3K9Ac, H3K27Ac, and H3K36me3 [108]. In gastric cancer, a number of histone modifications have been reported where infection by *H. pylori* was the main event for transformation [109–113]. Xia et al. demonstrated p21 (WAF1) regulation by histone modification in *H. pylori* positive gastric cancer cells [113].

2.1.4 Genetic Regulation: Mutation, SNPs, Deletion

Two major types of genetic alterations are common in malignant cells. The first one is microsatellite instability that affects chromatin structure and gene expression; and the second one is called chromosome instability where loss of entire or portion of chromosome is observed. Genomic instability is a hallmark of most cancers. Recent discoveries in virology underscore the importance of chromatin dynamics in the regulation of essential viral processes, including entry, gene expression, replication, and encapsidation. Since viruses can integrate in the genome at random, very frequently they affect host genomic stability and may cause single nucleotide polymorphisms (SNPs), mutations, or deletions. In Burkitt lymphoma (BL), translocation was reported close to c-myc oncogene

Table 1
Epigenetic changes in cells infected by viruses and/or bacteria

Infectious agent	Comments
HPV	P16INK4a, MLH1, and MGMT hypermethylation contributes in cervical carcinogenesis [91, 95]
HPV	Methylation of E2 binding site [93]
HBV	PCDH10 transcription factor inactivation was caused due to hypermethylation in HBV positive hepatocellular cells [98].
HCV and HBV	Differential methylation patterns of RASSF1A, GSTP1, CHRNA3, and DOK1 were observed in HCV and HBV infected cells in HCC [99]
HBV	HBV genome was methylated in hepatocellular carcinoma (HCC) [96, 97].
HBV	TIPM-3 gets hypermethylated in HBV infected HCC [152]
EBV	Histone acetylation contributes in EBV micro RNA expression in B cells [107]
EBV and <i>H. pylori</i>	Methylation changes in gastric cancer patients positive for <i>H. pylori</i> and EBV [153]

in EBV positive patients [114]. The constitutive activation of c-myc is a defined feature of BL. Following translocation mutations start appearing with the passage of time. HPV-associated genomic stability has also been reported by several investigators [115–117]. Akagi et al. observed viral–host DNA concatemers as a result of recombination events [115]. Few examples of epigenetic changes during infection are summarized in Table 1.

3 Methods and Technologies Used to Study Epigenetic Regulation in Cancer Development Due to Infection

3.1 Technologies for Methylation Detection

Methylation specific PCR involves PCR based technology where primers are selected in the methylated region of the gene [91]. Microarray-based high throughput methylation is applied in analyzing a large number of samples [94]. Differential methylation pattern of selected genes can be obtained by methylation specific real-time PCR [99]. Infinium Human methylation 450 bead chip has been used for genome-wide methylation analysis [118].

3.2 Chip-on-Chip Immunoprecipitation for Histone Modifications

Chip-on-chip immunoprecipitation (ChIP) technology is the most common technology in histone characterization and it is used to enrich specific DNA fragments to discover differences between tested and control DNA. First cells are treated with formaldehyde to cross-link proteins and their bound DNA and then broken into small fragments of about 500 base pairs. Enrichment of certain fragments is achieved by using antibodies against histones.

DNA is released from these fragments and hybridized to genomic tiling microarray. Results indicate regions of DNA which are associated with histones.

3.3 miRNA Profiling

MicroRNA (miRNA) profiling is determined by microarray technology where a large number of samples from infected and uninfected cells can be analyzed [107]. Typically RNA is isolated from cells and quantitated, and the amount of targeted miRNA in the RNA sample is measured. Microarray technology and bioinformatics tools are then used to characterize miRNAs. With the advancement of sequencing methodologies, it became feasible to measure the expression of the entire microRNAome (or microbiome) of a sample. This gives us information about miR abundance with a larger dynamic range than microarray and allows us to discover new miRNAs.

3.4 Nucleosomal Mapping and Imaging Technologies for Chromatin Compaction and Relaxation

DNase I mapping is used to locate nucleosomes in chromatin. This technique is useful in identifying protein binding sites on DNA as well as distinguishing compact chromatin from relaxed chromatin [119]. First the nuclei are isolated from cells and then treated with DNase I. Deproteinization produces DNA fragments of different sizes which can be analyzed to extrapolate DNase I hypersensitivity sites. These size-selected DNAs can be ligated with adaptors and highly amplified to generate a library that can be subsequently sequenced on the Illumina sequencing platform.

4 Diagnosis, Prevention, and Treatment Approaches Against Infectious Agents

Epigenetic approaches have been used in different stages of cancer development starting from diagnosis to treatment. Few selected approaches are described below.

4.1 Candidate Gene vs. Epigenome-Wide Profiling for Screening and Diagnosis

Traditionally genes of infectious agents associated with cancer are identified and then different approaches are applied to target those genes. The disadvantage of this approach is that we are limited to few identified genes only and may miss majority of those genes which are very significant for viral mediated transformation. Epigenome-wide Association Studies (EWAS) might be useful in those cases where control and infected samples can be analyzed for all components and disease-associated profiles can be identified [118, 120]. Such an approach is useful in screening as well as disease diagnosis. Because epigenetic alteration in cancer appears much before pathological features, diagnosis of cancer can be done much earlier than by traditional methods.

In HBV infection the most common genes which get hypermethylated are p16INK4, GSTp1, CDH1, RASSF1A, and

p21WAF1/CP1 which play a major role in HCC development [121]. A different set of genes are regulated by methylation in HCV mediated HCC. These genes include SOCS-1, Gaad45beta, MGMT, STAT-1, and APC. Such information has helped us understanding HBV/HCV induced carcinogenesis of liver cells. Molecular profiling revealed that HCC precursors and tumors exhibit epigenetic specific signatures which correlated with risk factors and tumor stage [122].

miRNAs are known to regulate HBV replication and pathogenesis [123]. HCC specific miRNA profiling in HCC indicated DNMT-1 regulated miR-152 [124]. In another study, HBX protein of HBV was shown to downregulate miR-101 during HCC development [125]. Previously it was known that HBX protein is involved in HCC but the mechanism was not clearly understood.

In *H. pylori* and gastric cancer, Schneider et al. demonstrated virulence of *H. pylori* and intensity of mononuclear cell filtration association with levels of methylation [126]. They also distinguished precancerous lesions from cancer methylation marks which have implication in early diagnosis and progression of gastric cancer. The most significant genes observed in this study were PCDH10, PSP02, and ZIC1. In another study, Hong et al. demonstrated the correlation between growth of stomach epithelial cells and hypermethylation of housekeeping genes [127]. The progress of gastric cancer is dependent on the clinical stage at diagnosis and treatment.

4.2 Vaccine, Nutrients, or Epigenetic Inhibitors for Prevention

Vaccines have been very successful in preventing infections because they reduce the risk of infection by working with the body's natural defenses to help it safely develop immunity to disease. HPV and HBV are two examples where thousands of lives have been saved to date [128–132]. Diet has been recognized as a potential source of both useful food components (e.g., fiber, vitamins) and natural compounds which have demethylating and acetylating properties. Poor diet, alcohol consumption, and obesity are cofactors for several infection-associated cancers. Healthier food choices can help to reduce the risk of developing cancer, and some foods have been shown to help protect against selected cancers. Many natural food components have the properties of inhibiting methyltransferase (the enzyme involved in DNA hypermethylation) and acetyltransferase (the enzyme that transfers acetyl moieties of histone). For example, Epigallocatechin-3-gallate (EGCG), a green tea compound, inhibits DNMT1 activity, histone modifications, and/or specific miRNA (miRNA-210) expression in a variety of tumor types [133–137]. Cancer prevention approaches can be developed by evaluating dietary protective factors, such as regular consumption of soy, tea, and ginseng.

4.3 Epigenetic Inhibitors, Either Alone or in Combination for Cancer Treatment

Cancer is the first disease where epigenetics drugs were used [138]. Unlike genetic events epigenetic events are reversible; hence hold better promise in therapeutic approaches. Currently, tumor suppressor genes are the primary targets of epigenetic therapy because many cell cycles inhibitors and genes involved in tumor growth are regulated epigenetically [139–143]. Table 2 represents epigenetic inhibitors used in cancer treatment. Although a number of new

Table 2
Selected epigenetic inhibitors

Epigenetic inhibitor	Comments
NY-ESO-1	For treatment of endometrial cancer [154, 138]
SGI 110	DNA methylation inhibitor HCC (II); myelodysplastic syndromes (MDS) (I/II); acute myeloid leukemia (AML) (I/II); ovarian cancer (I/II) [155, 146]
Combination of demethylating agent and topoisomerase inhibitors	Beneficial in lung cancer survival [156]
OTX015	Bromodomain-containing protein inhibitor (proteins that bind to acetylated lysines); hematological malignancies (I) [155, 138]
Trichostatin	Methylation inhibitors for different cancers [157]
Panobinostat	HDAC inhibitor Myelofibrosis (exploratory); AML (I/II); MDS (I/II); multiple myeloma (III) [155]
Mocetinostat	Class I HDAC inhibitor Diffuse large B cell lymphoma (II); follicular lymphoma (II) ACY1215 HDAC6 inhibitor Multiple myeloma (I/II) [155, 146]
HDAC inhibitors with topoisomerase inhibitors	Beneficial in lung cancer treatment [158]
mTORC1/C2 and HDAC inhibitors	Breast cancer treatment [159]
Entinostat	Class I HDAC inhibitor Breast cancer (II); non-small-cell lung cancer (NSCLC) (II); colorectal cancer (CRC) (II); AML (II); Hodgkin's lymphoma (II); renal cell carcinoma (RCC) (I/II); ALL (I) [155, 146]
Sequential treatment of cytarabine and decitabine	Beneficial in leukemia treatment [160]
GSK 525762	Bromodomain-containing protein inhibitor (proteins that bind to acetylated lysines) carcinoma (I) [155, 146]
Valproic acid with nicotinamide	Combination therapy inducing apoptosis [161]
Retinoic acid with HDAC inhibitors	For the treatment of AML [162]

(continued)

Table 2
(continued)

Epigenetic inhibitor	Comments
Combination of methylation and HDAC inhibitors	For the treatment of cervical cancer [163]
DNAMT and HDAC inhibitors	Combination was beneficial in endometrial cancer [164]
Radiation with HDAC inhibitors	Beneficial in lung cancer therapy [165]
Depsipeptide in combination with HDAC inhibitors	Reduction in cancer cell viability was observed [166]
Combination of proteasomes with HDAC inhibitors	Responsive in cervical cancer treatment [165, 167]

Most of these inhibitors have not yet been tested for infectious agent-associated malignancies. However, they may be potential candidates

epigenetics drugs have been discovered, only four drugs with an epigenetic mode of action are currently approved by the US Food and Drug Administration (FDA): the DNA methylation inhibitor azacitidine (Vidaza; Celgene), which has been on the market for almost a decade; the HDAC inhibitor vorinostat (Zolinza; Merck), which was approved in 2006; the DNA methylation inhibitor decitabine (Dacogen; Eisai), also approved in 2006; and romidepsin (Istodax; Celgene), a second HDAC inhibitor, which was approved in 2010 [138]. No new epigenetic agents have been approved since 2010, but several are under study for cancer therapy [144–146]. About ten histone deacetylase inhibitors are being investigated [147]. Azacytidine and decitabine are the demethylating agents approved for specific cancer treatment. Since these compounds can be toxic at higher doses, mostly low doses are used either alone or in combination with HDAC inhibitors or poly(ADP-ribose) polymerase (PARP) inhibitors [148, 149]. Key epigenetic proteins are being targeted for cancer therapy [150]. Prediction models for response to epigenetic therapy have also been proposed [151]. It is possible that epigenetic alterations make some proteins susceptible to treatment by other drugs [150]. All inhibitors shown in Table 2 have not been used for treatment of infectious agent-associated cancers, but they have the potential for further evaluation in infectious agent-associated malignancies.

5 Challenges and Research Opportunities

Infectious agents and their contribution to cancer burden are tremendous. It would be of interest to investigate possible epigenetic mechanism by which infectious agents, such as HBV, integrate their genome in the host genome and initiate the

process of carcinogenesis. Another research topic is to demonstrate whether viral infection mediated carcinogenesis is a direct result of epigenetic regulation or the effect of inflammation due to infection. Epigenetic regulators should be targeted for cancer therapy which is an area of future research which should be explored more by developing collaboration among industrialists, researchers, physicians, and bioinformatics experts [151]. Some efforts in this direction have been initiated by the Structural Genomic Consortium (SGC) which enabled the public dissemination of epigenetic protein crystal structures and chemical probes for computational analysis [150]. Challenges also include developing pharmacodynamic and patient stratification markers which can be applied in personalized medicine.

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Chapter 19

Toxicoepigenomics and Cancer: Implications for Screening

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Abstract

Scientists have long considered genetics to be the key mechanism that alters gene expression because of exposure to the environment and toxic substances (toxicants). Recently, epigenetic mechanisms have emerged as an alternative explanation for alterations in gene expression resulting from such exposure. The fact that certain toxic substances that contribute to tumor development do not induce mutations probably results from underlying epigenetic mechanisms. The field of toxicoepigenomics emerged from the combination of epigenetics and classical toxicology. High-throughput technologies now enable evaluation of altered epigenomic profiling in response to toxins and environmental pollutants. Furthermore, differences in the epigenomic backgrounds of individuals may explain why, although whole populations are exposed to toxicants, only a few people in a population develop cancer. Metals in the environment and toxic substances not only alter DNA methylation patterns and histone modifications but also affect enzymes involved in posttranslational modifications of proteins and epigenetic regulation, and thereby contribute to carcinogenesis. This article describes different toxic substances and environmental pollutants that alter epigenetic profiling and discusses how this information can be used in screening populations at high risk of developing cancer. Research opportunities and challenges in the field also are discussed.

Key words Biomarkers, Epigenetics, Epidemiology, Liver cancer, miRNA, Treatment

Abbreviations

DNMTs	DNA methyltransferases
EWAS	Epigenome-wide association studies
GWAS	Genome-wide association studies
HAT	Histone acetyltransferase
HDACS	Histone deacetylases
LINE	Long integrated nuclear element
MDBPs	Methylated DNA-binding proteins
miRNA	microRNA
PFOS	Perfluorooctane sulfonate
SINE	Small integrated nuclear element

1 Introduction: Gene Regulation by Epigenetic Mechanisms in Cancer

Epigenetics can be defined as inheritable and reversible phenomena that affect gene expression without altering the underlying nucleotide sequences. Epigenomics is the study of genome-wide epigenetic modifications. Epigenetic modifications (DNA methylation, histone modifications, abnormal noncoding RNA expression, and chromatin remodeling) are needed for normal development, but these modifications can be altered by exposure to toxicants and lead to the development of cancer [1, 2]. Thus, cancer is considered a genetic and epigenetic disease. Genetic effects (mutations, polymorphisms, deletions) occur in all cells, whereas epigenetic changes are cell- and tissue-specific [1, 2].

The epigenome is dynamic and very susceptible to toxic substances and environmental exposure [3–7]. Genetic marks are static and remain the same at any point in time; epigenetic marks vary depending on exposure, and changes can be measured at different points in time. Epigenetic marks contribute to the phenotypic characteristics of cells, tissues, and individuals. Epigenetic programming occurs during development and reflects altered gene expression in disease states. Toxicogenomic studies are conducted to explain the epigenetic effects of environmental exposures [8]. Similar to genome-wide association studies (GWAS), epigenome-wide association studies (EWAS) involve measuring epigenetic changes that occur at the genome-wide level and their association with disease [9]. Compared with genetic changes, epigenetic alterations respond quickly to environmental changes. Technologies are available to measure these changes [8, 10].

Excellent progress has been made recently in the field of altered epigenomic profiling in response to toxins and environmental pollutants [1, 10, 11]. It now is possible to use high-throughput technologies to measure epigenetic patterns that are altered by toxin exposure [9]. Different environmental exposures, including toxins, affect different components of the epigenetic machinery and alter the methylation and acetylation equilibrium. Epigenetic alterations are similar to genomic variations (e.g., mutations, polymorphisms, deletions) that contribute to altered gene expression and cause genomic instability. Epigenetic mechanisms may influence toxicological data or toxicogenomic approaches to identifying the epigenetic effects of environmental exposures [12].

Four major components of epigenetic machinery are DNA methylation, histone modification, noncoding RNA expression (mainly microRNA [miRNA] and PIWI RNA), and chromatin remodeling [2]. Other epigenetic mechanisms that regulate gene expression are genomic imprinting, X-chromosome inactivation, and transposition [13–15]. Aberrant DNA methylation marks are a hallmark of cancer. During methylation, cytosine residues are

methyated covalently at the 5' position. CpGs (regions of DNA with adjacent cytosine and guanine nucleotides) are underrepresented regions of the genome that are located primarily in the promoter region [16–19]. Approximately 45,000 CpG islands are present in the human genome, and global hypomethylation and gene-specific hypermethylation have been reported for oncogenes and tumor suppressor genes, respectively [20]. Non-genotoxic substances may cause hypomethylation.

Methylation is conducted by specific enzymes present in all human cells. Along with 5-methylcytosine (5mC), 5'-hydroxymethylcytosine (5hmC) associated with ten-eleven-translocation-2 (TET-2) also has been reported in cancer tissue samples [21, 22]. The enzymes needed for methyl group transfer are called DNA methyltransferases (DNMTs). These enzymes maintain a methylation profile using S-adenosyl as the methylation donor. DNA demethylases are involved in reversing methylation and potentially are responsive to environmental exposures. The methylation process stops the expression of genes, either by binding to transcription factors or by recruiting methylated DNA-binding proteins (MDBPs). Methylated DNA attracts MDBPs, which in turn can contribute to chromatin condensation. DNA and histones form repetitive nucleoprotein units, the nucleosomal core particles. Each particle consists of 146 bp of DNA wrapped around an octamer of histones. The methyl-binding domain of MDBPs recruits histone deacetylases (HDACs), which modify histone tails. Gene-specific histone tail modifications have been identified in cancer and other diseases. Acetylation is not the only histone modification; histones are modified by phosphorylation, ubiquitination, sumoylation, and biotinylation [23–26]. The term “histone code” is used for these histone modifications [27–31]. The consensus among epigenetic scientists is that histone tail acetylation enhances the accessibility of a gene to transcription. In contrast, deacetylated tails are highly charged and tightly associated with the DNA backbone, thus limiting the accessibility of transcription factors and other proteins—including polycomb repressor proteins—involved in epigenetic regulation. The enzyme that transfers acetyl groups on histones is called histone acetyltransferase (HAT). When chromatin is condensed (heterochromatin), transcription stops; when chromatin opens (euchromatin), transcription starts. Both DNA methylation and histone modifications make chromatin active or inactive.

H3-histone acetylation at the K9 residue is a hallmark of active promoters and a consistent mark of active genes. A number of transcription factors recruit histone-modifying enzymes and specifically modify histones, resulting in altered gene expression. DNA methylation involves either *de novo* methylation or maintenance of the methylated form of DNA [32–34]. The most important prominent enzymes for methylation are DNMT1, DNMT3A, and

DNMT3b. DNMT activity is very high during hypermethylation. DNMT1, which is involved in maintaining methylation, is activated by toxins. According to recent theories, Polycomb-group proteins also are involved in activating DNMT1 [35].

The etiology of cancer involves complicated interactions between multiple environmental factors, including toxins, and individual genetic and epigenetic backgrounds, which are programmed very early in life (during fetal development and embryogenesis). Different individuals respond differently to toxins and other environmental insults. Scientists once thought that a person's genetic makeup or genetic background determined his or her susceptibility to toxins, and that toxins caused genetic alterations that led to the development of disease. After gaining an understanding of epigenetic-mediated gene regulation and interactions between toxins and epigenetic components, however, scientists realized that toxins can affect normal development without causing genetic changes. An individual's epigenomic makeup or background may be as important as his or her genetic background in determining disease susceptibility [36–42]. Furthermore, some toxins (non-genotoxic substances) may not affect the genome (non-mutagens) and may cause epigenetic damage only. For example, social exposures such as childhood adversity and abuse might impact an individual's response to drugs used for treatment later in life. These issues should be considered when assessing the adverse effects of potentially toxic agents. In addition, epigenetic programming due to exposure to different agents may be transgenerational, although only limited reports based on model systems support this idea [2, 10, 43–46]. Similarly, literature on toxicogenomics and cancer, a new scientific field, is limited and there is a need to conduct more studies that may provide opportunities to develop additional approaches to preventing cancer.

2 Epidemiology, Epigenetics, and Exposure to Environmental Pollutants and Toxic Substances

Even transient exposures to hazardous substances can have persistent, lifelong phenotypic effects, and epigenetic equilibrium can be altered by exposure to toxic substances [34, 47]. During toxicology analysis, careful attention should be paid to the possible epigenetic effects of the toxin(s) under study. Individual variability in response to toxicant exposure may result from different combinations of exposures to behavioral conditions, chemicals, diet, radiation throughout life, and genetic background. The dynamic nature of the epigenome and its involvement in different pathways makes it vulnerable to the effects of xenobiotics, not only during critical periods in development but also later in life.

Epidemiologic studies help to elucidate the etiology of a disease, especially by identifying factors that contribute to disease development. In the case of cancer, such factors include toxicants, radiation, infectious agents, specific dietary components, tobacco, alcohol, and environmental factors [5, 47]. Such studies should be conducted in cohorts and consortia of cohorts with large population sizes. By virtue of their greater scope, resources, population size, and opportunities to interact with experts in multiple disciplines, consortia can be used to address complex scientific questions that are difficult for a single investigator to answer. Large-scale studies including EWAS, with both non-exposed controls and exposed populations, are needed to test the validity of epigenetic biomarkers that are altered by exposure to toxic substances [9]. NCI-supported cohorts can provide data related to exposure history. Examples of well-established cohorts and cohort consortia include the Breast and Prostate Cancer Cohort Consortia (BPC3); Asian Cohort Consortium; Breast Cancer Association Consortium (BCAC); International Barrett's and Esophageal Adenocarcinoma Consortium (BEACON); International Childhood Cancer Cohort Consortium (I4C); Nurses' Health Study; Latin American Cancer Epidemiology Consortium (LACE); and Women's Environmental Cancer and Radiation Epidemiology Study (WECARE).

Complementary information can be obtained by conducting GWAS and EWAS. In GWAS, alleles that confer modest susceptibility to cancer are needed, rather than alleles with high penetrance. These less common, low-penetrance alleles are located mostly in noncoding regions. Many samples (from controls and subjects exposed to adverse conditions) are needed to provide sufficient statistical power to identify such alleles, which can be obtained from many institutes by pooling data and samples. Cohort consortia provide information about exposure history and sufficient numbers of samples for analysis. In addition, cohort consortia offer advantages to epigenetic approaches to identifying disease-associated rare variants. These resources may be useful for future studies involving potential correlations between genetic and epigenetic factors and their contributions to the development of cancer.

Pooling data from several cohorts offers additional advantages. For example, lifestyle and exposure-related data are collected before cancer diagnosis; therefore, the quality of information and reliability of data are not affected by the absence or presence of cancer. In addition, data-sharing policies and plans are developed for each consortium, and dedicated resources are allocated for the release of the public data set. Potential data- and biospecimen-sharing requirements and careful phrasing of informed consent should be given special attention. The use of common protocols among participating cohorts reduces the potential for substantial variation that can result when studies are conducted using different protocols.

Table 1
Key toxic substances affecting the epigenome

Toxic substance	Comments	References
Arsenic	Induces genetic and epigenetic changes in different cancer types	[8, 48, 49, 52–54]
Benzene	Benzene and its metabolic products contribute to gene-specific hypermethylation and global hypomethylation	[4–6, 56]
Cadmium	Induces hypermethylation of selected genes in lung cancer; cadmium can cross mother-fetus barrier and alter the epigenome of both mother and fetus; cadmium exposure may be associated with lower birth weight and altered methylation patterns in specific genes	[58, 59, 61]
Chromium	Induces hypermethylation in selected genes, contributing to lung cancer	[48, 60]
Nickel	Alters chromatin structure and induces histone acetylation and methylation, especially H3K9 methylation	[48, 70]
Perfluorooctane sulfonate (PFOS)	Contributes to disease development by altering methylation of <i>GSTPI</i> and LINE/SINE sequences	[71, 72]
Polycyclic aromatic and halogenated compounds	Altered histone H3 acetylation in a breast cancer model	[79]
Uranium and other environmental pollutants	Contributes to leukemia; low exposure of bisphenol, mercury, and lead in women undergoing in vitro fertilization	[12, 74, 75]

Exposure to metallic substances with potential carcinogenicity, including nickel, chromate, arsenite, and cadmium, has increased recently because of occupational exposures, the massive growth of manufacturing activities in developed and industrialized countries, increased consumption of non-ferrous materials, and the disposal of waste products (Table 1) [5, 34, 36–42]. These metals are weak carcinogens and do not damage DNA directly (as does radiation); but they exert their carcinogenic effects by epigenetic mechanisms, especially after chronic exposure [48]. Selected toxicants and their contributions to the development of cancer via epigenetic alterations are described below and listed in Table 1.

2.1 Arsenic

Arsenic is a metal, and exposure to it induces global and gene-specific methylation changes and histone modifications in different populations [8, 48, 49]. Arsenic in drinking water is a health hazard and Group I carcinogen, as determined by the International Agency for Research on Cancer (IARC). Investigators have demonstrated that arsenic causes genetic and epigenetic changes and

contributes to the development of lung cancer [8, 37, 41, 48, 49]. Changes in miRNA profiling have been shown to result from arsenic exposure [50, 51]. In addition, dimethylated arsenic may cause oxidative damage [52]. Arsenic exposure may lead to bladder, lung, and liver cancers [48, 53, 54]. The use of folate supplementation to prevent arsenic carcinogenicity has been reported [55].

2.2 Benzene

Benzene—an important industrial chemical that also is present in smoke, automobile exhaust, and gasoline—becomes carcinogenic when metabolized [4]. Xing et al. characterized the metabolomic enzyme genes *CYP1A1*, *EPHX1*, and *NQO1* by determining the polymorphisms and analyzed methylation of promoter regions in 11 genes causing hematotoxicity in samples collected from 77 benzene-exposed workers and 25 unexposed controls [5]. Hypermethylation of the *ERCC3* gene was observed only in samples that were collected from exposed workers. Benzene exposure also has been observed in petrochemical workers. When Alu and long interspersed nuclear element (LINE) methylation were evaluated in exposed and control populations, significant hypomethylation of Alu and LINE sequences was observed [6]. Benzene and its metabolite hydroquinone were reported to induce global hypomethylation and cytogenetic changes in leukemia [56]. Both genetic and epigenetic mechanisms were found to be involved in the development of cancer in this study. Global hypomethylation resulting from benzene exposure has been observed by other investigators as well [4]. Investigators now are studying the degree of DNA breakage and adduct formation that occurs during benzene exposure. Methylation profiling in different genes may help to identify high-risk individuals who are likely to develop cancer due to exposure to low levels of benzene [57].

2.3 Cadmium

Altered methylation patterns have been observed in mother-fetus pairs exposed to cadmium [58]. Cadmium exposure occurs in factories that produce certain batteries, in the electroplating process, and in metal industries. Such exposure has been linked to pulmonary diseases including lung cancer. In one study, cadmium exposure induced hypermethylation in tumor suppressor gene *p16* and DNA repair gene *hMLH1* [59, 60]. Methylation patterns in a cadmium-exposed population were found to be associated with lower birth-weight [61]. In cell line and animal model systems, exposure to cadmium resulted in altered methylation patterns in DNA repair genes that could be reversed by demethylating agents [62].

2.4 Chromium

Chromium and its compounds (disodium dichromium heptaoxide, potassium bichromate, chromic anhydride, chromic trioxide) are used in industries such as steel manufacturing and pressure-treated wood. About a half-million people are exposed to chromium worldwide every year. Investigators have reported toxicity

resulting from exposure to chromium and its by-products and their involvement in the development of cancer via epigenetic mechanisms [16, 63, 64]. In an epidemiology study, Ali et al. investigated chromium toxicity and its contribution to lung cancer via methylation [63] and reported methylation in the *APC* (86 %), *MGMT* (20 %), and *hMKH1* (28 %) genes. These investigators suggested the possibility of genomic instability resulting from chromium-induced epigenetic changes. Other investigators also have investigated chromium toxicity [8, 64].

2.5 Nickel

Nickel is a nonessential metal that is used in the production of jewelry, coins, stainless steel, batteries, and medical devices. Prolonged exposure to nickel may change chromatin to heterochromatin and acetylated histones [48, 65, 66]. Inactivation of RARBeta2, RASSF1A, and CDKN2A by hypermethylation was observed as a result of exposure to nickel (nickel subsulfide, Ni₃S₂) [67]. Nickel compounds alter methyltransferase activity and histone acetylation and methylation in model systems [68, 69]. In addition, nickel and hypoxia have been shown to decrease histone acetylation and increase methylation of H3K9 [70].

2.6 Perfluorooctane Sulfonate (PFOS)

Exposure to compounds such as PFOS before birth and during the life course affects health and may result in diseases such as cancer [71]. Investigators have studied PFOS exposure and determined methylation levels in specific genes such as *GSTP1*, and also studied global methylation and hypomethylation of the LINE and short interspersed nuclear element (SINE) regulatory sequences [71, 72]. Investigators also have observed that PFOS affected gene regulation both prenatally and after birth and contributed to carcinogenesis [73].

2.7 Uranium

Depleted uranium, a dense metal, is used in military applications. It emits alpha particles that can damage cellular processes. Along with genetic effects, uranium contributes to the causation of cancer by epigenetic mechanisms, mainly methylation. In a Chinese population exposed to radon and uranium, abnormal methylation was observed of *p16(INK4)* and a methyltransferase gene [12]. The involvement of uranium in leukemia via hypomethylation was demonstrated in a model system by analyzing blood and spleen samples [74]. Such studies may provide biomarkers of early exposure and can be used to screen high-risk populations and identify individuals who are likely to develop cancer and other diseases.

Among molecular targets for future research in cancer control, members of the iron- and oxoglutarate-dependent dioxygenase family of enzymes are important because they influence histone modifications (especially histone acetylation and methylation) [41]. In one study, altered methylation profiling was observed in lead-, mercury-, and bisphenol-exposed women undergoing in vitro fertilization [75].

Data generated in methylation profiling differ from data generated by histone-modification patterns in toxin-exposed and control samples. Similarly, different kinds of data are generated when samples are analyzed to determine miRNA profiling and in vivo imaging of chromatin. The key to success in using toxicoepigenomic data to assess cancer risk is to properly integrate the different types of data so that they can be accessed easily and stored in a common place. A central/national data repository would be ideal for such purposes. Another important aspect is large data cataloging and the development of analytical tools that can be accessed easily by the research community. This requires policies that maximize rapid data release while fully crediting investigators who generate data.

3 Challenges and Future Directions

As noted, it makes sense to identify epigenetic markers in both non-exposed normal controls and exposed populations. The advantages of identifying epigenetic biomarkers of exposure include improved exposure assessment, documentation of early alterations preceding cancer development, and identification of high-risk populations. Discovery-oriented as well as hypothesis-driven toxicoepigenomic studies can be conducted using additional information and resources.

Epigenetic marks are tissue-specific; genomic marks are not. This fact has implications for tissue-specific toxicity, pharmacokinetics, and pharmacotoxicity [76–78]. The information provided above indicates how altered epigenomic profiling may be important in chemical-induced toxicity that may contribute to cancer development, and how the status of methylation, histones, non-coding RNA, and chromatin can provide important information as a component of an overall risk-prediction and safety assessment. The biggest challenge in the toxicoepigenomic field is the analytical and clinical validation of epigenetic biomarker profiles that result from exposures to toxic substances. Success has been achieved with several methylation markers, but additional work is needed to determine the profiling of histones and miRNAs in control and exposed samples.

One of the most compelling challenges is identifying which CpG islands are functionally significant. All CpG islands are not involved equally in toxicoepigenomics. LINE and SINE sequences are hypomethylated in disease states, and their role in toxicoepigenomics is not well understood. The integration of data from toxicology and from methylation, histone, and miRNA profiles also requires further investigation. Another topic needing further study is determining the role of epigenetic plasticity with respect to toxicity and non-genotoxic carcinogenicity. Risk-prediction models based on toxin exposure and epigenetic mechanisms also should be developed. Also unknown is whether toxicity causes

epigenetic changes that lead to the recurrence of tumors in treated populations. In utero epigenetics and the exposure of mothers to toxic substances is another area that needs further study and evaluation. The correlation of CpG islands and genomic instability in a population exposed to a toxin versus an unexposed population is an additional topic for future research. The epigenetic and genetic predisposition of cancer will be predicted best in the context of environmental exposures.

Future research in both fields, genetics and epigenetics, may provide a better understanding of the mechanisms involved in regulating gene expression. Further research is needed to evaluate the possibility of transgenerationally inherited, chemically induced epigenetic changes associated with phenotypes and epigenetically induced adaptation to chemical stressors in cases of long-term chemical exposure. Additional areas of concern involve the causality of the relationships between epigenetic and phenotypic changes.

Finally, it is important that epigenetic concepts and related information be considered while evaluating the toxicity of different agents, because this information may be useful in identifying new toxic agents. In addition, the fact that epigenetic alterations can be reversed may be helpful in developing prevention strategies. Decreasing the amount of toxic agents in the environment likely will result in dramatic improvements in health as well as reduced medical costs.

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Human Papilloma Virus (HPV) Modulation of the HNSCC Epigenome

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Abstract

Currently, the human papilloma virus (HPV), in addition to tobacco and alcohol, is considered another independent risk factor for oropharyngeal squamous head and neck cancer (OPSCC), where the prevalence of HPV-16 increases to 50–90 % for the oropharynx. Also, incidence and mortality in head and neck SCC (HNSCC) continue to be higher in African Americans (AA) than in Caucasian Americans (CA). A recent study found that poorer survival outcomes for AA versus CA with oropharyngeal tumors were attributable to racial differences in the prevalence of HPV positive (+) tumors; HPV negative (–) AA and CA patients had similar outcomes (Settle et al., *Cancer Prev Res (Phila)* 2:776–781, 2009). Evidence indicates that a HPV+ diagnosis has significant prognostic implications; these patients have at least half the risk of death when compared with the HPV– patient, due in part to a better response to chemoradiotherapy (Fakhry et al., *J Natl Cancer Inst* 100:261–269, 2008).

Epigenetic events of promoter hypermethylation are emerging as promising molecular strategies for cancer detection, representing tumor-specific markers occurring early in tumor progression. HPV infection is now recognized to play a role in the pathogenesis of OPSCC, where HPV+ and HPV– patients appear to be clinically and biologically distinct with reported genome-wide hypomethylation and promoter hypermethylation in HPV+ HNSCC tumors. A recent study from our group applying pathway analysis to investigate the biological role of the differentially methylated genes in HPV+ and HPV– HNSCC reported 8 signal transduction pathways germane to HNSCC (Worsham et al., *Otolaryngol Head Neck Surg* 149:409–416, 2013).

Key words Human papilloma virus, Hypermethylation, Gene networks, Pathways, Signal transduction

1 Introduction

For squamous head and neck cancer (HNSCC), epidemiological and laboratory evidence now warrant the conclusion that the human papilloma virus (HPV) is a causative agent for some HNSCC [1, 2] and an independent risk factor for oropharyngeal SCC (OPSCC) [3–5]. While it is becoming more firmly established that HPV positive HNSCC have better survival outcomes

than HPV negative HNSCC, believed to be because of better response to chemoradiation [6], the mechanism for these improved prognosis outcomes remains underexplored. Our group [7] and others [8–10] have shown that HPV can modulate the HNSCC epigenome. The overall goal was to examine and assess an HPV-associated methylation phenotype identified from gene pathway analysis of high-throughput methylome data to infer biological mechanisms underlying HPV-associated HNSCC.

In African Americans (AA) with OPSCC, survival disparities are attributed to racial differences in the prevalence of HPV positive tumors [11–14]; AA had a significantly lower prevalence of HPV positive tumors as compared to Caucasian American (CA) with corresponding worse survival for AA. A recent study from our group [13] reported that HPV has a substantial impact on overall survival in AA OPSCC. Among AA, HPV positive patients had better survival than HPV negative (HR=3.44, $p<0.001$), a finding not previously reported presumably due to a paucity of multiethnic cohorts and limited number of AA patients. HPV negative AA also did worse than both, HPV positive CA and HPV negative CA.

Global characterization of the HNSCC methylome is beginning to uncover differential landscapes in HPV positive versus HPV negative tumors. HPV positive cells had higher CpG methylation both in non-repetitive regions (genic and non-genic) and in repetitive regions [8, 15]. Querying differentially methylated genes into the pathway analysis framework to identify distinct signaling pathway networks has the potential to provide a molecular basis for further exploration of these genes as differential targets in HPV positive and HPV negative HNSCC. In this type of analysis, a biological system is surveyed in the context of disease (or other interesting phenotypes) to identify gene groups associated with biological systems (bionetwork) [16, 17]. Bionetwork coupling affords a strategic knowledge base approach and has been used to better understand the systems biology of disease processes and identify potential therapeutic targets [18–20]. The major signaling systems have been conserved to a remarkable extent in all animals and signal transduction is the means by which cells respond to extracellular information.

While it is becoming more firmly established that HPV positive HNSCC have better survival outcomes than HPV negative HNSCC, believed to be because of better response to chemoradiation [6], the mechanism for these improved prognosis outcomes remains underexplored.

The objective was to identify differentially methylated genes in HPV positive vs HPV negative primary HNSCC genomes with clues to signaling networks. DNA from 4 HPV positive and 4 HPV negative freshly frozen primary HNSCC were subject to comprehensive genome-wide methylation profiling using the Illumina 27k platform (*see Note 2*). Differentially methylated gene lists were

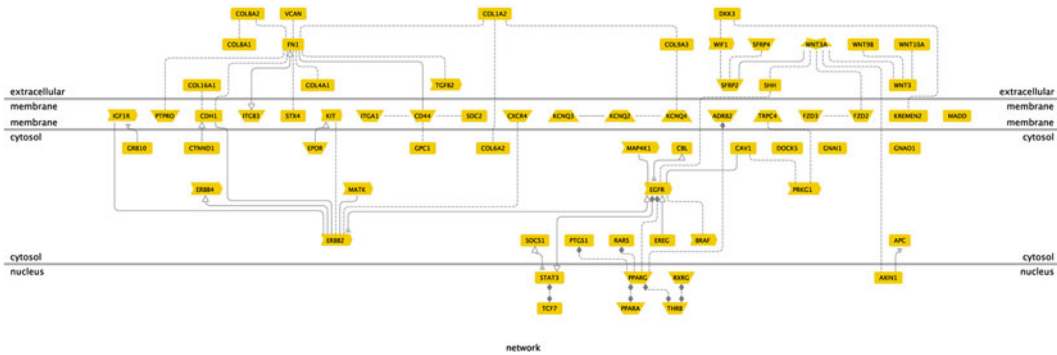


Fig. 1 Gene network encompassing 66 signal transduction genes within 8 pathways of which 62 % are hypermethylated (41 of the 66) and 25 (39 %) hypomethylated. Of the 66 genes, 62 were accounted for in 8 signal transduction canonical pathways ($p < 0.05$)

examined using the Signal Transduction Pathways (canonical) filter in the Genomatrix Pathway System (GePS) (*see Note 3*). Of the 26,486 autosomal CpG loci, the 1,355 (5 %) differentially methylated CpGs between HPV positive and HPV negative samples were assigned to 1,168 genes. Of the 1,168 genes, 686 (59 %) were hypermethylated, 467 (40 %) were hypomethylated, and 15 (1 %) had both hypo and hyper methylation (i.e., these 15 genes each had more than one CpG with a twofold change, and at least one of those was hyper and one was hypo).

Pathway analysis applied to investigate the biological role of the 1,168 differentially methylated genes using the Signal Transduction Pathways (canonical) filter in GePS, formed a network of 66 genes (Fig. 1), of which 62 % are hypermethylated (41 of the 66) and 25 (39 %) hypomethylated. Of the 66 genes, 62 were accounted for in 8 signal transduction canonical pathways ($p < 0.05$). These included the Wnt/Beta (β)-catenin degradation ($p = 0.003$, Fig. 2), basic mechanism of action of peroxisome-proliferator-activated receptor (PPAR) elements ($p = 0.006$, Fig. 3), E-cadherin ($p = 0.018$, Fig. 4), RXR (retinoid X receptor) and RAR (retinoic acid receptor) heterodimerization ($p = 0.036$, Fig. 5), ion channels and their functional role in vascular endothelium ($p = 0.036$, Fig. 6), ErbB receptor ($p = 0.04$, Fig. 7), signaling events mediated by stem cell factor receptor (c-Kit) ($p = 0.04$, Fig. 8), and Integrin signaling (Filopodium formation) ($p = 0.041$) signaling events. Of the 66 genes, 8 were represented in more than 1 pathway, and included *CAV1*, *CD44*, *EGFR*, *ERB2*, *GPC1*, *PPARA*, *PPARG*, and *SDC2*.

Our study revealed a predominant hypermethylation profile for genes in signal transduction pathways of HPV positive HNSCC tumor genomes. Because signaling events in the cell play a critical role in the execution of key biological functions, insights into how complex cellular signaling cascades and networks

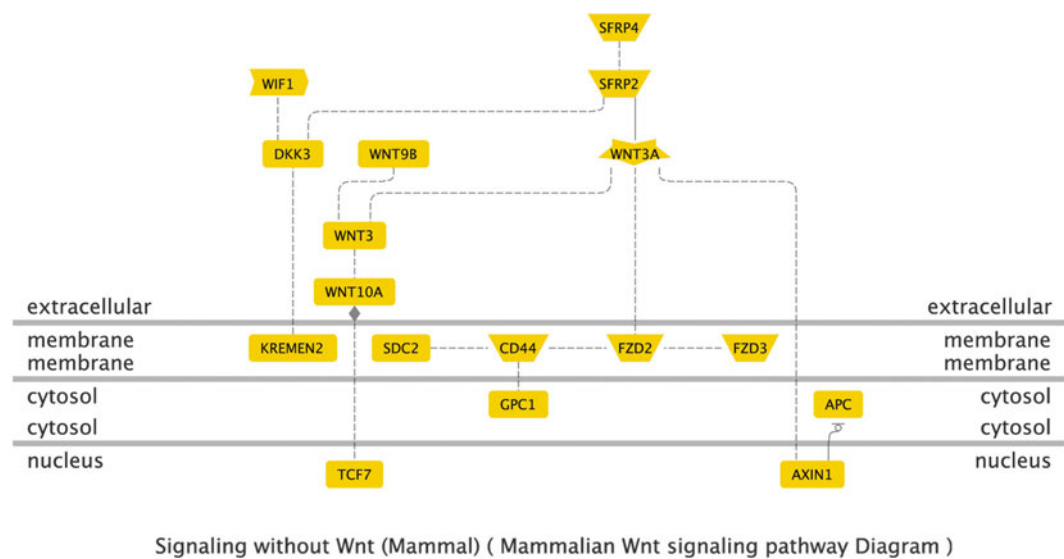
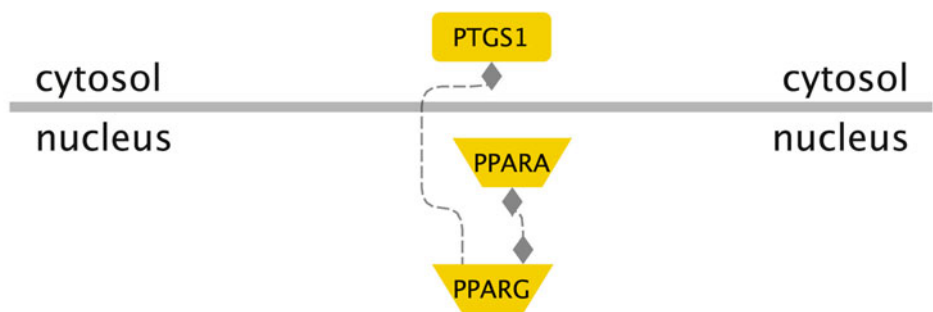


Fig. 2 The Wnt/Beta (β)-catenin degradation pathway



basic mechanism of action of ppara pparb(d) and pparg and effects on gene expression

Fig. 3 Basic mechanism of action of peroxisome-proliferator-activated receptor (PPAR) elements pathway

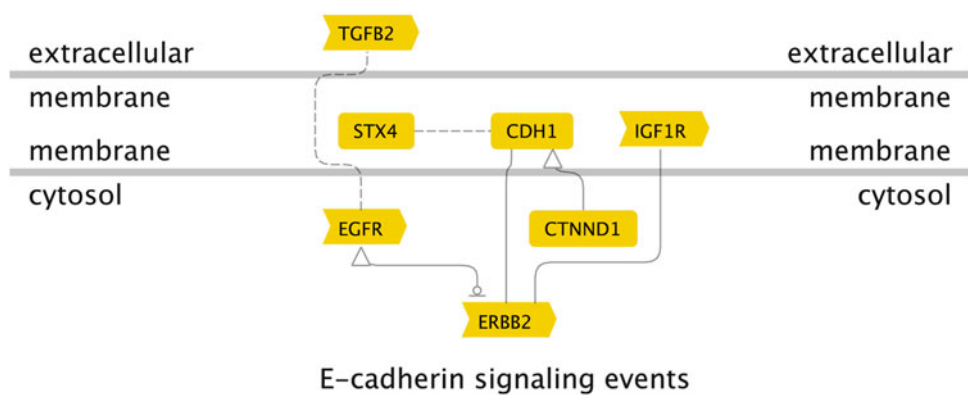
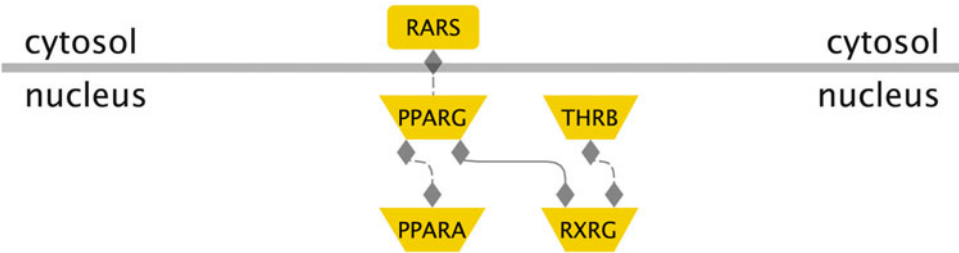
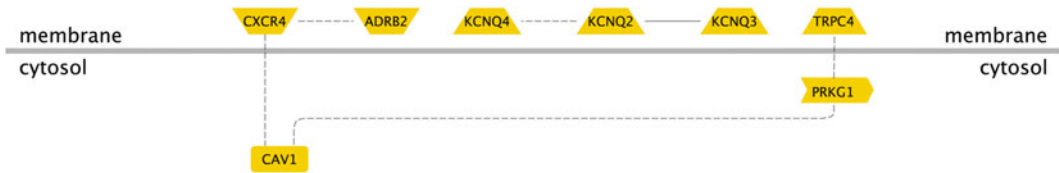


Fig. 4 E-cadherin pathway



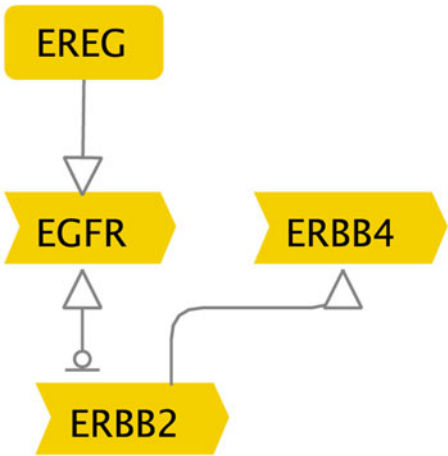
RXR and RAR heterodimerization with other nuclear receptor

Fig. 5 RXR (retinoid X receptor) and RAR (retinoic acid receptor) heterodimerization pathway



ion channels and their functional role in vascular endothelium

Fig. 6 Ion channels and their functional role in vascular endothelium pathway



ErbB receptor signaling network

Fig. 7 ErbB receptor pathway

may be programmed in HNSCC are likely to be critical in the development of new biological agents designed to hit multiple targets. Coupled with bionetwork analysis, this strategic comprehensive approach using the Illumina 27k and Genomatix pathway analysis, suggest a mechanistic underpinning for an HPV-associated methylation phenotype with the potential for insights into race-based survival outcomes of HNSCC patients as well as diagnostic and prognostic markers for improvements in treatment of HNSCC (*see Note 1*).

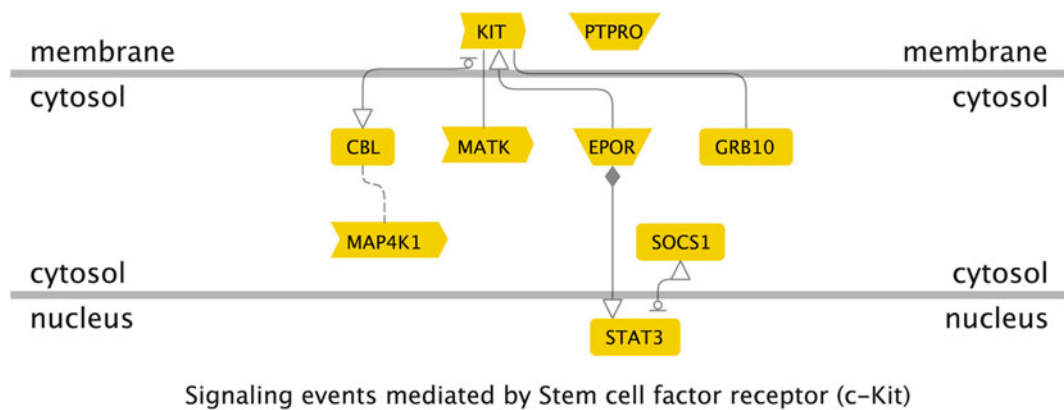


Fig. 8 Signaling events mediated by stem cell factor receptor (c-Kit)

2 Materials

Subjects: DNA from 4 HPV positive and 4 HPV negative freshly frozen primary HNSCC were subject to comprehensive genome-wide methylation profiling using the HumanMethylation27 BeadArray (San Diego, CA). Demographic characteristics and tumor site and are presented in Table 1. This study was approved by the Henry Ford Health System Institutional Review Board committee.

3 Methods

3.1 HPV Detection

DNA was extracted according to the manufacturer's protocol (Qiagen Inc, Chatsworth, CA). Tumor HPV DNA concentrations were measured using real-time quantitative PCR (qPCR) as previously described [14, 21].

3.2 HPV-16 Detection by Real- Time Quantitative PCR (qPCR)

DNA was extracted according to the manufacturer's protocol (Qiagen Inc, Chatsworth, CA). Real-time PCR reactions were set up in a reaction volume of 20 µl using the TaqMan Universal Master Mix II with UNG (Applied Biosystems, Foster City, CA). Specific primers and probes were designed to amplify a housekeeping gene, *β-globin* [22] (to standardize the input DNA), and the E6 region of HPV-16 [23]. The primers and probes used were as follows: *β-globin*: forward 5'-GTGCACCTGACTCCTGAGGAG A-3', reverse 5'-CCTTGATACCAACCTGCCAG-3', 6FAM-5'-AAGGTGAACGTGGATGAAGTTGGTGG-3'-TAMRA; HPV-16 E6 region: forward 5'-GAGAACTGCAATGTTTCAGGACC-3', reverse 5'-TGTATAGTTGTTTGCAGCTCTGTGC-3', 6FAM-5'-CAGGAGCGACCCAGAAAGTTACCCACAGTT-3'-TAMRA.

Table 1
Cohort characteristics

Sample ID	Site	Age	Race	Gender	HPV-16E6
31	Larynx (supraglottis)	51	CA	F	Negative
41	Tongue	41	CA	M	Negative
42	Oral cavity	54	CA	M	Negative
43	Tongue	71	CA	M	Positive
45	Tongue	49	CA	M	Positive
46	Tongue	46	CA	M	Negative
47	Tongue	54	CA	M	Positive
48	Oral cavity	52	AA	M	Positive

CA Caucasian American, AA African American, F female, M male

DNA amplifications were carried out in a 96-well reaction plate format in an Applied Biosystems 7900HT Sequence Detector (Applied Biosystems, Foster City, CA). HPV viral copy number was determined using the CaSki cell line genomic DNA reported to contain an integrated human papillomavirus type 16 genome (about 600 copies per cell). Multiple water blanks and HPV-16 positive controls (HPV+ HNSCC specimens) were included in every run. Both the HPV and β -globin PCR reactions were carried out in duplicate.

CaSki cell line genomic DNA is known to have 600 copies/genome equivalent (6.6 pg of DNA/genome). Standard curves were developed for HPV-16 viral copy number using serial dilutions of 50 ng, 5 ng, 0.5 ng, 0.05 ng, and 0.005 ng of DNA. Using the same serial dilutions, standard curves were also developed for the β -globin housekeeping gene (2 copies/genome). This additional step allowed for relative quantification of the input DNA level and attribution of the final quantity as the number of viral copies/genome/cell [24]. The cutoff value for HPV-16 positive status was ≥ 0.03 (≥ 3 HPV genome copy/100 cells) [13, 14, 21].

3.3 The Infinium 27k Assays

The Infinium 27k assays (*see Note 2*) were performed at the Applied Genomics Technology Center (AGTC), which is part of Wayne State University School of Medicine's Department of Obstetrics and Gynecology (Detroit, MI). The 27k platform measures methylation status of over 27,000 CpGs located in more than 14,000 gene promoters. The methylation score for each CpG is represented as a beta (β) value according to the fluorescent intensity ratio. Every β value is accompanied by a detection p -value. β

values may take any value between 0 (non-methylated) and 1 (completely methylated) and determined using the Genome Studio (Illumina, San Diego). Probes are discarded if this detection p -value is more than 0.05. The only corrections that are made to the data are background subtraction and normalization. The resulting beta values were exported into Microsoft Excel and JMP (SAS Institute, USA) for data analysis.

3.4 Pathway Analysis

The Genomatix Pathway System [25] (GePS) uses information extracted from public and proprietary databases to display canonical pathways and create and extend networks based on literature data (*see* **Note 3**). More than 400 human pathways can be displayed based on data from the NCI-Nature Pathway Interaction Database, Biocarta, and various other sources which are supplemented with proprietary database content from NetPro and Genomatix in-house curated annotations such as interaction annotation, gene information, sequence derived information, transcription factor information, and small molecule information. GePS also allows creation of networks from an arbitrary input gene list where connections are based on literature, i.e., co-citations. This gene list can be filtered by *GeneRanker* results, literature mining results and expression data. The resulting gene sets can be combined to new gene sets and serve as filters. Furthermore networks can be created from scratch without an input gene list. Genes, complexes, and interactions can be simply created by clicking and dragging with the mouse.

Genomatix Pathway System software was applied to build a network from differentially methylated genes. Biological processes enriched in the differentially methylated gene lists using the Signal Transduction Pathways (canonical) filter in GePS were identified and ranked starting with the lowest p -values determined using Genomatix analysis software (Fig. 1).

3.5 Statistical Methods

To avoid gender-specific methylation bias, sex chromosome loci ($n=1,092$) were excluded resulting in a final dataset that consisted of 26,486 autosomal loci associated with 13,890 genes. Criteria for differential methylation was set as CpG sites with a ratio of the mean β for the HPV positive samples to the mean β for the HPV negative samples of at least 2.0 or less than 0.5 (i.e., a twofold difference).

4 Notes

1. *Significance of DNA methylation markers*: When compared to the genetic genome, which is identical in every cell and tissue in the human body, the epigenome is highly variable over the life course, from tissue to tissue and from environment to environment [26]. Also, unlike genes that are inactivated by

nucleotide sequence variation, genes silenced by epigenetic mechanisms are still intact and, thus, retain the potential to be reactivated by environmental or medical intervention [26]. There are several current human therapeutic intervention trials to reverse deleterious epigenetic changes. Some examples include epigenetic therapeutic trials to treat T-cell lymphoma based on reactivation of tumor suppressor genes [27] and similar trials to prevent colorectal cancer by inhibiting the enzyme responsible for DNA methylation [28]. Such therapies have shown promise in halting tumor growth by reactivation of the tumor suppressor gene or by blocking progression of precancerous epigenetic lesions. Also, epigenomic biomarkers are quickly becoming far more practical than genomic biomarkers. Our group has shown that promoter hypermethylation is amenable to PCR-based methylation assays using whole genomic DNA from fresh/frozen tissue, cell lines, as well as formalin-fixed paraffin tissue DNA [13, 14, 24]. Methylation of CpG islands may serve as a relatively simple “yes–no” signal for the presence of tumor, and potentially the pathogenesis of HNSCC, when examined for under optimal assay conditions by sensitive polymerase chain reaction (PCR) techniques [13, 14, 24, 25]. Additionally, aberrant promoter hypermethylation always occurs in virtually the same location within an affected gene, allowing a single PCR primer to be applicable to all patients for examination of the methylation status of a specific gene. This sharply contrasts with genomic biomarkers such as DNA mutations in genes such as p53 or mitochondrial genes [25], which often involve myriad different base changes at many locations within the gene even in cancers of the same histologic types. Classification based on promoter methylation profiling may well be a more promising approach than expression profiling since these DNA-based techniques are not subject to the problems of tissue preservation and the potential pitfalls of tissue heterogeneity. Aberrant DNA methylation may represent a stable biomarker of HNSCC pathogenesis or a potential target and would be easily detected by PCR-based methods.

2. *Whole genome methylation profiling:* Whole genome methylation profiling using genomic DNA from fresh tissue samples as in the case of this study posed no problems with the Illumina 27k platform. The 27k is not recommended for DNA from formalin-fixed paraffin embedded (FFPE) samples, which this platform does not support. Though the 27k platform measures methylation status of over 27,000 CpGs located in more than 14,000 gene promoters, the more recent Illumina Infinium HumanMethylation450 BeadChip is far more comprehensive. The 450k platform includes 485,764 cytosine positions in the human genome and provides coverage of 99 % of Reference

Sequence (RefSeq) genes. The RefSeq collection aims to provide a comprehensive, integrated, nonredundant, well-annotated set of sequences, including genomic DNA, transcripts, and proteins. Also, unlike the 27k platform, the 450k supports profiling of DNA from FFPE tissue samples.

3. *Pathway analysis*: Alternatives to the Genomatix Pathway System software approach include more recent pathway analysis approaches such as Ingenuity Pathway Analysis (IPA). The IPA Integration Module enables access to the high quality, detail-rich biological and chemical knowledge in IPA directly from their internal websites, applications, and gene catalogues, as well as from internal research reports, e-mails, and other shared documents that are part of daily research workflows. IPA has been gaining traction over other pathway analysis software packages. The number of genes in a gene list for interrogation in pathway analysis software is a factor. For more robust and intuitive analysis, gene lists of at least over 500 are recommended.

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Chapter 21

Epigenetic Regulation of HIV, AIDS, and AIDS-Related Malignancies

Mukesh Verma

Abstract

Although epigenetics is not a new field, its implications for acquired immunodeficiency syndrome (AIDS) research have not been explored fully. To develop therapeutic and preventive approaches against the human immunodeficiency virus (HIV) and AIDS, it is essential to understand the mechanisms of interaction between the virus and the host, involvement of genetic and epigenetic mechanisms, characterization of viral reservoirs, and factors influencing the latency of the virus. Both methylation of viral genes and histone modifications contribute to initiating and maintaining latency and, depending on the context, triggering viral gene repression or expression. This chapter discusses progress made at the National Institutes of Health (NIH), recommendations from the International AIDS Society Scientific Working Group on HIV Cure, and underlying epigenetic regulation. A number of epigenetic inhibitors have shown potential in treating AIDS-related malignancies. Epigenetic drugs approved by the US Food and Drug Administration and their implications for the eradication of HIV/AIDS and AIDS-related malignancies also are discussed.

Past and current progress in developing treatments and understanding the molecular mechanisms of AIDS and HIV infection has greatly improved patient survival. However, increased survival has been coupled with the development of cancer at higher rates than those observed among the HIV/AIDS-negative population. During the early days of the AIDS epidemic, the most frequent AIDS-defining malignancies were Kaposi's sarcoma and non-Hodgkin lymphoma (NHL). Now, with increased survival as the result of widespread use in the developed world of highly active antiretroviral therapy (HAART), non-AIDS defining cancers (i.e., anal, skin, and lung cancers, and Hodgkin disease) are on the increase in HIV-infected populations. The current status of AIDS-related malignancies also is discussed.

Key words AIDS, Cancer, Epigenetics, HIV, Methylation, Prevention, Risk assessment treatment

1 Introduction: The Landscape of HIV, AIDS, and Epigenetics

Human immunodeficiency virus (HIV) infection results in active viral transcription, reduced immunity and reduced CD40 counts, and, finally, acquired immunodeficiency syndrome (AIDS) development. To advance our understanding of the risks, development, progression, diagnosis, and treatment of malignancies observed in individuals with an underlying HIV infection or AIDS, the published papers were analyzed (Table 1). The total number of

Table 1
Publications in the field of HIV, AIDS, and AIDS-related malignancies

Terms used for analysis	Number of publications
AIDS	191,384
AIDS + HIV	105,698
AIDS + Cancer	19,699
HIV + Cancer	20,527
HIV + AIDS + Malignancy	6,760
HIV + AIDS + Malignancy + HHV-8	568
HIV + AIDS + Malignancy + HHV-6	28
HIV + AIDS + Malignancy + HPV	274
HIV + AIDS + Malignancy + HPV16	25
HIV + AIDS + Malignancy + HPV18	7
HIV + AIDS + Kaposi's sarcoma	3,078

PubMed was used for the analysis using the terms shown in this table. The analysis was done up to January 2013. References included papers, reviews, and book chapters

papers published on AIDS was 191,284. Because HIV infection is the main cause of AIDS, 105,698 publications included HIV infection. Surprisingly, only one-tenth of the AIDS publications were associated with cancer when the term “AIDS + Cancer” was searched. Almost the same number of publications was reported when the search term was “HIV + Cancer.” The analysis depends on how the terms were used, because the term “HIV + AIDS + Malignancy” yielded only 6,760 publications. This number, although smaller than the previous numbers, shows that thousands of publications have reported HIV- and AIDS-related malignancies. We then sought to determine how many of these 6,760 publications included the terms “herpes virus” or “HHV-8.” This search resulted in 568 publications having referred to HHV-8 and 28 publications referring to HHV-6. The reduced number of publications using HHV-6 may be because HHV-6 is considered a co-pathogen only [1]. HPV is another virus that has been associated with HIV + AIDS + Malignancy, and a search using the term “HPV” resulted in 274 publications, and HPV16 was involved in one-tenth of these publications. The main cancer in AIDS patients is Kaposi's sarcoma (KS), and my analysis supported this idea as well (Table 1). A crucial step in the development of HIV/AIDS is the latency period and its regulation by epigenetic mechanisms. This involves methylation of an HIV promoter,

histone modifications, and the presence of inhibitory nucleosomes. New epigenetic regulators of HIV latency are of potential interest as candidates for intervention and therapeutic agents against HIV latency.

2 Epigenetic Components

HIV/AIDS development involves epigenetic regulation, especially during the latency period. Epigenetic regulation involves alterations in gene expression without any structural changes in the genomic structure. The major component of epigenetic regulation is chromatin, which is a complex structure consisting of DNA and proteins (Fig. 1). Chromatin provides genomic stability and a scaffold for the packaging of the entire genome. The nucleosome is the basic unit of chromatin and is comprised of 146 bp of DNA wrapped around a histone octamer. Four types of histones are present in chromatin: H2a, H2b, H3, and H4. Chromatin can change from euchromatin to heterochromatin. Heterochromatin represents condensed chromatin associated with transcriptionally inactive genes; euchromatin represents an open structure with access to transcription factors and is associated with active gene transcription (Fig. 2). Efforts to study the coordinated regulation of the nucleosome have demonstrated that all of its components are subject to covalent modifications. These modifications alter the function and organization of chromatin. DNA and histone modifications during epigenetic regulation are dynamically laid down and removed by chromatin-modifying enzymes in a highly organized

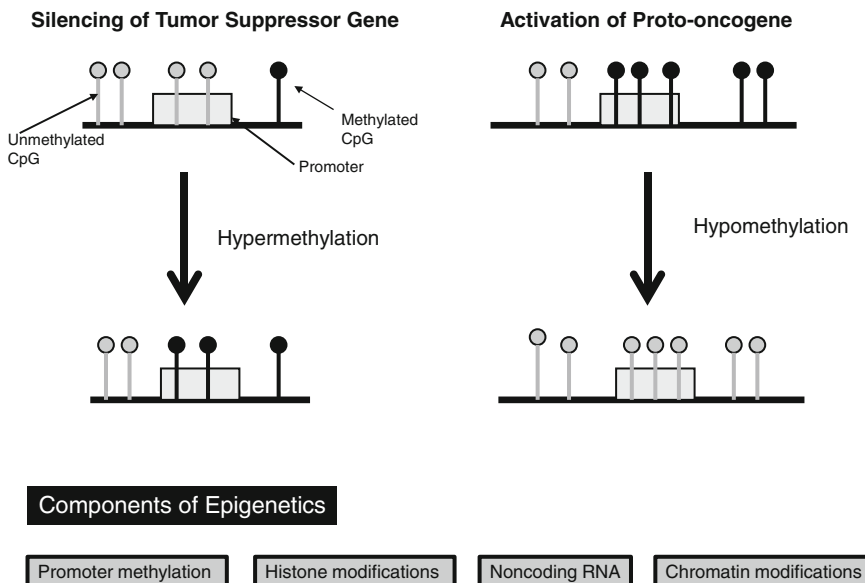


Fig. 1 Components of epigenetics

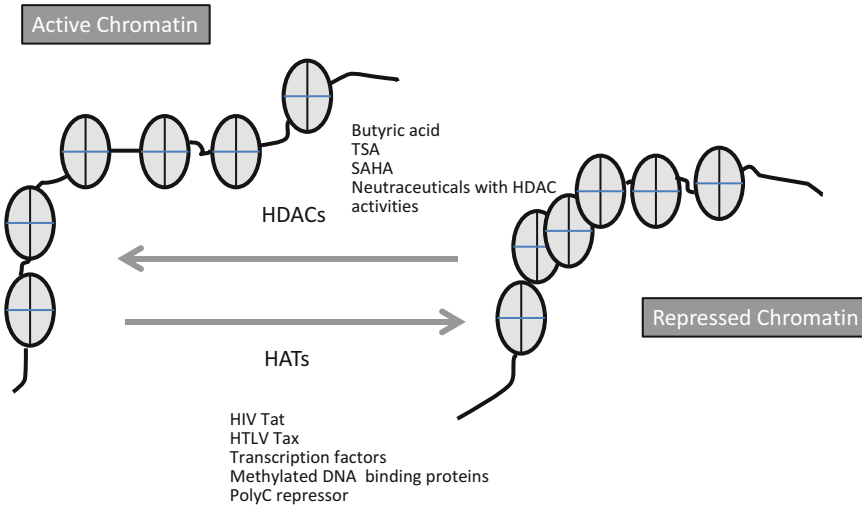


Fig. 2 Chromatin interaction with other components of the cell during infection

and regulated manner. A list of DNA and histone modifications and their functions is shown in Table 2. These modifications serve as docking sites for specialized proteins with unique domains that recognize these modifications. In turn, these chromatin readers recruit additional factors and modifying enzymes that serve as the effector molecules in gene regulation. Chromatin changes and gene expression are regulated simultaneously by histone deacetylases (HDACs) that deacetylate the histone and nonhistone proteins involved in gene expression. In mammals, a total of 18 HDACs have been identified and grouped into four classes: Class I (HDACs 1, 2, 3, and 8), Class II (HDACs 4, 5, 6, 7, 9, and 10), Class III (HDACs Sirt1–Sirt7), and Class IV (HDAC 11). HDACs work in multisubunit transcriptional corepressor complexes that are recruited by sequence-specific transcription factors to the promoter region.

Recent advancements in the field of epigenetics explain the role of noncoding RNAs, especially microRNAs (miRNAs) in gene activation and inactivation. The regulation of miRNA expression is tightly controlled, and often the same rules and regulations that govern coding gene expression apply to miRNAs. As is true for coding genes, the alterations of levels of the temporal and differential expressions of each of the miRNAs clearly affect the proper development and function of the tissue in which they are expressed [2, 3]. miRNAs are known to regulate at least one-third of all human genes. They serve as a physiological and intracellular response to detect small double-strand RNAs leading to silencing in a sequence-specific manner. More than 1,500 miRNAs have been identified to date. The earliest indications of epigenetic links to cancer were derived from gene expression analysis and methylation

Table 2
DNA and histone modifications involved in epigenetic regulation

Name of the epigenetic component	Comments
DNA modifications	
• 5-Methylcytosine	• Involved in transcription
• 5-Hydroxymethylcytosine	• Involved in transcription
• 5-Formylcytosine	• Function unknown
• 5-Carboxylcytosine	• Function unknown
Histone modifications	
• Acetylation	• Transcription, replication, repair, condensation
• Methylation (Lysine) (K-me1, K-me2, K-me3)	• Transcription and repair
• Methylation (Arginine)	• Transcription
• Phosphorylation (Serine or Threonine)	• Transcription, repair, and condensation
• Phosphorylation (Tyrosine), with SH2 domain	• Transcription and repair
• Ubiquitination	• Transcription and repair
• Sumoylation	• Transcription and repair
• ADP-ribosylation	• Transcription and repair
• Deimination	• Transcription and decondensation
• Proline isomerization	• Transcription
• Crotonylation	• Transcription
• Propionylation	• Unknown function
• Butyrylation	• Unknown function
• Formylation	• Unknown function
• Hydroxylation	• Unknown function

profiling studies. Hypermethylation of gene promoters causes gene inactivation (e.g., tumor suppressor genes), and hypomethylation causes gene activation (e.g., viral oncogenes).

A number of epigenetic inhibitors have been purified from natural resources or developed synthetically. Methyltransferase inhibitors azacytidine and decitabine have been licensed for clinical therapy for patients with myelodysplastic syndrome (MDS). Zebularine and isothiocyanate are among other potential inhibitors of methyltransferases. 5-Azacytidine forms a covalent complex

with cytosine (C-5)-specific DNA methyltransferases and inhibits their activity. This compound is also activated by uridine–cytidine kinase and thus can be incorporated in both RNA and DNA. HDAC inhibitors are a very promising class of compounds for cancer therapy. Histone acetylation is a reversible process in which histone acetyltransferase (HAT) transfers the acetyl moiety from acetyl coenzyme A to an amino acid, whereas an HDAC (histone deacetylase), as the name suggests, removes the acetyl group. Evidence shows that HDAC inhibitors are well tolerated and effective. Different forms of HDACs differ in their structure and have histones and nonhistones as their substrate. Normal cells are relatively resistant to HDAC inhibitors. Epigenetic inhibitors that have been identified so far belong to one of the following classes of agents: short-chain fatty acids, hydroxamic acids, benzamides, cyclic tetrapeptides containing S-2-amino-9, 10-epoxy-8-oxodecanoic acid (AOE) moiety, epoxides, and psammoplins. Epigenetic inhibitors, either alone or in combination, work effectively in cancer treatment. In Burkitt's lymphoma and nasopharyngeal carcinoma, infection with Epstein Barr virus (EBV) has been observed. It has been determined that hypermethylation of latency genes allows EBV to stay integrated in the host genome and contribute to these cancers. In myelodysplastic syndrome, with an incidence of 20,000 cases annually in the USA, the standard treatment is weekly blood transfusion, which is considered aggressive treatment. Treating these patients with Vidaza was successful, and most of the patients did not need transfusion. The survival time and quality of life of these patients increased substantially.

Molecular pathology includes epigenetic profiling and considered over genetic changes, such as mutations and genomic instability resulting in altered gene expression, (or only histopathological analysis). Some of these epigenetic changes (altered profiling) are global hypomethylation of tumor DNA, specific tumor suppressor gene hypermethylation that results in their inactivation, loss of imprinting, and chromatin alterations [4]. Epigenetic changes contribute to genomic instability and mutations, and the general concept is emerging that epigenetic changes precede genetic changes in tumor initiation [4–6]. Global hypomethylation induces genomic imprinting, transposon activation, and chromosomal instability. For example, in breast and ovarian cancer and Wilms tumor, global hypomethylation contributes to unbalanced chromosomal translocations with break points in the pericentromeric DNA of chromosome 1 and 16, which resulted in a loss of heterozygosity of markers on these chromosomes. Localized demethylation of LINE and SINE sequences causes breakage of chromosomes and induces abnormal recombinations. Excessive demethylation in centromeres may cause aneuploidy. In turn, mutations in DNA methyltransferase genes (which are involved in DNA methylation) cause chromosomal alterations. Epigenetically

silenced genes are distributed throughout the chromosome, and at least 40 % of these genes are regulated by epigenetic mechanisms. Hypomethylation of retrotransposons may result in their activation, leading to chromosomal disarrangements and making conditions favorable for tumorigenesis. In colon cancer, a transposon has been reported that resulted in chromosomal rearrangements [7]. Both genetic and epigenetic approaches should be considered for disease risk assessment, diagnosis, control, prognosis, and survival.

3 HIV Genes Regulated by Epigenetic Mechanisms

Both methylation and histone-mediated regulation of HIV have been studied. When the viral genome becomes integrated into the host genome, the viral genome is chromatinized and regulated epigenetically. The chromatin organization at the viral long terminal repeat (LTR) promoter has been characterized and showed precisely positioned nucleosomes. Nucleosome-free regions represent binding sites for multiple transcriptional regulators. Nuc-1 is positioned just after the transcription start site and blocks viral transcription because, for successful viral transcription, nuc-1 should be displaced by host factors. Binding of available transcription factors recruits chromatin-modifying enzymes that shape the microenvironment surrounding the chromatin, including the region near the LTR, and participate in viral transcription. Chromatin immunoprecipitation assays have indicated that the rate of histone modifications (acetylation and methylation) correlates with the level of viral transcription. In contrast, methylation in the promoter region is responsible for transcription repression. DNA methylation is currently thought of as the last step of repression of gene expression, which ultimately gives chromatin a compact conformation (heterochromatin). Most of the data support the view that specific transcription factors, histone modifications, and DNA methylation levels at the viral promoter determine the efficiency of viral gene expression. A few examples where such regulation was observed are described below. The clinical implications of these observations also are discussed.

3.1 *Methylation-Mediated Regulation of HIV*

HIV infection results in active viral transcription, reduced immunity and reduced CD40 counts, and, finally, AIDS development. HIV LTR undergoes hypermethylation and results in transcription depression and lower viral load in HIV patients [8]. In some infected individuals, these systems do not develop into the disease, and it is difficult to infer whether and when they will do so. These groups of individuals are referred to as “controllers.” “Elite controllers” are one category of controller and have undetectable viremia; other controllers are called “virus controllers” and have low levels of viremia. The status of both controller groups can

last up to 10 years. The underlying mechanism of low viremia and asymptomatic characteristics is not completely understood. The role of epigenetics has been proposed in such situations [8]. A restrictive chromatin and nucleosome remodeling affect viral transcription.

In human lymphotropic virus (HTLV), the pro-virus formation is reduced due to hypermethylation [9, 10]. In the cell line model, HIV-infected cells produce virus particles where LTR is hypermethylated. Recent evidence indicates that a reduction in LTR methylation levels correlates with a higher level of viral gene transcription. Methylation influences the stability of latency in HIV-infected cells. Two CpG islands are located in the 5'LTR and stop transcription factors from attaching to the promoter when these regions are hypermethylated. Binding sites for methylated DNA-binding protein 2 (MDB2) are located in the second CpG island.

Li et al. demonstrated a relationship between LTR methylation and *gag* expression of HIV-1 in human spermatozoa and sperm-derived embryos that helps to explain how HIV-1 gene expression is regulated in vertical transmission from sperm to embryo [11]. DNA methylation is very important in regulating the expression of viral genes entering into cells. In germ cells and in pre-implantation embryos, methylation patterns are reprogrammed genome wide. Methylation acquisition begins in prospermatogonia and persists throughout spermatogenesis. After fertilization, paternal demethylation of the whole genome starts in zygotes. De novo methylation occurs after implantation. HIV-1 can infect male germ cells, is present in all developmental stages of spermatogenesis, and is brought to the embryo by fertilization. These are the stages of methylation that were characterized by Li's group in exploring the significant role of *gag*. For the host genome, the preferential demethylation of the paternal genome in zygotes is very common. The importance of elongation factors such as Elp1, Elp3, and Elp4 has been proposed in paternal genome demethylation and reprogramming [12]. Whether these elongation factors also are associated with the demethylation mechanisms of HIV-1 genes in spermatozoa, zygotes, and pre-implantation embryos is being explored.

High expression of telomerase was shown to be associated with hypermethylation at the distal telomerase promoter and with hypomethylation at the proximal telomerase promoter in HIV-specific CD8-positive (+) cells in controllers. The reverse pattern of hypermethylation and hypomethylation was reported in progressors [13]. Methylation status at a resolution of each individual cytosine residue within the hTERT promoter in HIV-1-positive CD8+ T cell controllers clustered at cytosines located at 3–7, 10–14, 16, 18, 20, 23–24, and 28 of the distal promoter segment. These regions were found to be unmethylated in normal controls.

3.2 *Histone-Mediated Regulation of HIV*

It was observed about a decade ago that latent HIV could be transcriptionally reactivated by the treatment of latently infected cells by HDAC inhibitors, which suggested that one or several HDACs were bound to the HIV promoter under latency conditions and that deacetylation of histones played a role in the establishment and maintenance of HIV latency. The inhibitors used were trichostatin and trapoxin. These observations provided the clue that treating HIV-infected patients with HDAC inhibitors might be of therapeutic value by forcing the reactivation of HIV expression and the elimination of the latent HIV reservoir.

Along with methylation of the promoter region, histones also are modified by the methylation mechanism, which is mediated by histone methyltransferases. Histone methylation plays a significant role in the latent HIV promoter. The histone methyltransferase SUV39H1, an H3K9 methyltransferase, is recruited to the HIV promoter and methylates histones. The SUV39H1 methyltransferase initiates heterochromatin structure by recruiting specific factors such as HP1. In HeLa cells, latently infected U-1 cells, Jurkat cells, or peripheral mononuclear cells from healthy donors, SUV39H1-mediated deposition of H3K9 by providing a platform for HP1 and resulted in repression of the HIV LTR [14]. This effect can be reversed by knocking down HP1. The re-expression due to HP1 knockdown occurred via recruitment of the HIV transcription activators PCAF and pTEF-B. G9a is another methyltransferase that regulates HIV latency. G9a knockdown resulted in increased basal LTR transcription, increased TNF- α stimulated LTR transcription, increased viral replication, and reduced H3K9me2 (a dimethylated form of histone) [15]. G9a inhibitor treatment resulted in higher levels of H3k9me2. A third methyltransferase, H3k27me3, also is involved in HIV latency [16]. H3K27me3 works in coordination with the Polycomb Repressive Complex 2 (PRC2) with an enzymatically active component called EZH2. When EZH2 was knocked down, loss of H3K27me3 was observed, which reactivated HIV latency.

Mammalian cells generally develop a defense mechanism against retroviruses and other genetic parasitic elements, and epigenetic silencing of exogenous sequences is involved in the process [17]. At the same time, this process also can repress transcription of oncoretroviral and lentiviral vectors carrying therapeutic transgenes and influence results (as happened in early 1990s when gene therapy approaches were implemented using retroviral vectors). HIV-1 can integrate proviral DNA in a subset of infected cells to escape immunological and pharmacological surveillance. Experiments were conducted to understand the underlying mechanisms and pathways involved and it was observed that HDAC inhibitors could affect the process, which suggests that HDAC-mediated chromatin remodeling at the exogenous promoter might result in transcriptional induction [18]. Further investigation

identified HDAC4 as a potential player in the process as demonstrated by its association with the cytomegalovirus (CMV) promoter and by the use of pharmacological inhibitors and siRNA-mediated silencing. These experiments also demonstrated that both inhibition and knockdown of HDAC4 were sufficient to reactivate transgene expression. Note that HDAC4 is a class IIa HDAC that is highly expressed in the brain, chondrocytes, heart, and skeletal muscle and plays a major role in bone development. Class II HDACs lack significant HDAC activity per se and may control gene expression by acting as scaffolds for complexes containing catalytically active class I HDACs [19]. An alternative mechanism involving interaction of these HDACs with nucleoproteins, thereby modifying chromatin association with the nuclear membrane, also has been proposed [20].

Individuals respond differently to toxins and other environmental insults. Scientists used to think that genetic makeup or background determined a person's susceptibility to toxins, and that toxins caused genetic alterations that lead to the development of disease. After developing an understanding of epigenetic-mediated gene regulation and the interaction of toxins with epigenetic components, however, scientists realized that toxins could affect normal development without causing genetic changes, and that an individual's epigenomic makeup or background might be as important as his or her genetic background in determining disease susceptibility. Furthermore, some toxins (nongenotoxic substances) might not affect the genome and could cause epigenetic damage only. Social exposures such as childhood adversity and abuse might impact an individual's response to drugs used for treatment later in life; these issues should be considered when assessing the adverse effects of potentially toxic agents. In addition, epigenetic programming might be transgenerational, although few reports that support this idea exist in the literature [21, 22].

Chromatin remodeling via histone modifications was demonstrated in macrophages, which are HIV-1 reservoir sites [23]. HIV-1 virions gained entry into macrophages following ligation of surface CD4-CCR5 coreceptors and released two copies of single-stranded HIV-1 RNA. HIV-1 RNA caused tumor necrosis factor alpha release and required endocytosis through toll-like receptor8 (TLR8), MyD88, histone modifications, and chromatin remodeling. H3K4me3 was markedly increased, and H3K27me3 decreased, during chromatin modifications in macrophages. This information identified an important potential source of novel therapeutic targets to reduce HIV-1-mediated pathogenesis.

Histone modifications are involved in increased or decreased expression of interferon releasing factor 1 (IRF1). This factor helps in the transactivation process before viral oncogenes start their expression. In a cohort of sex workers, most of the participants who had been exposed to HIV-1 were found to be seropositive for

HIV-1; but a group was found to be seronegative for HIV-1, even after continuous exposure to HIV-1 for 3–5 years. Analysis of samples from both groups showed that the recruitment of histone deacetylases and histone modifications stopped IRF1 release in HIV-1-resistant groups and provided protection. This study is being validated in a large cohort.

4 AIDS-Related Malignancies

Multiple immune-related disorders are associated with viral infection. Viruses are etiological factors for both autoimmune diseases and secondary immune deficiencies. Viruses such as EBV and HIV target and infect immune system cells and alter their function. Although chronic HIV infection causes severe immune-suppression, EBV is immunologically contained after acute symptomatic infection [10]. Cooperation among different viruses during coinfection or multiple infections is important in the pathogenesis of virus-associated malignancies.

EBV is associated with a number of malignant tumors such as lymphomas (e.g., Burkitt lymphoma, Hodgkin disease, NK/T cell lymphoma, AIDS-associated lymphoma), carcinomas (e.g., nasopharyngeal carcinoma, gastric carcinoma, carcinomas of major salivary glands, thymic carcinoma, mammary carcinoma), and sarcomas (e.g., leiomyosarcoma). This suggests that the expression of latent EBV genes is site-specific and regulated epigenetically (because the genetic background of all cells is the same). The virus exists as an episome and replicates with the host genome. Nuclear antigene genes (EBNA1–EBNA6) and viral transmembrane proteins (LMP1, LMP2A, LMP2B) are regulated by methylation of the promoters of transcripts of these genes as well as by histone modifications during transcription. Some genes, such as EBER1 and EBER2, are transcribed during the latency period, but their promoters do not get methylated. Binding of EBNA1 on the host chromosome has been identified, and binding initiates local demethylation of the host genome and perhaps activation of host genes. Histone acetyltransferase interacts with EBNA5 of EBV and participates in gene transcription. LMP1 expression correlates with DNA methyltransferase and induces polycomb group protein Bmi-1. Furthermore, EB1 and HHV-8 infection contribute to the inactivation of DNA repair enzyme gene *O6-methylguanine-DNA methyltransferase* (MGMT) [24]. In short, EBV infection contributes to the epigenetic reprogramming of host cells and makes these cells susceptible for transformation.

EBV infects children and adults differently. When primary infection of B cells occurs in childhood, it is asymptomatic; but when the same virus infection occurs in adults, it results in mononucleosis. Only a limited number of infected viruses are eliminated

by cytotoxic T cells, whereas the majority of viruses evade immune response. Due to highly efficient latency, EBV may remain in the host throughout the life of the host by expressing the minimum number of genes and maintaining a low number of viral genomes.

In Nigeria, HIV-positive individuals frequently are infected with HTLV-1, especially pregnant women and sex workers. HTLV-1 infection has been linked with leukemia, lymphoma, tropical spastic paralysis, and other syndromes. *Helicobacter pylori* (*H. pylori*) resistance also has been reported in African populations, and *H. pylori* infection is linked with gastric cancer and ulcers.

Among AIDS-related malignancies, Kaposi's sarcoma (KS) is the most prominent. More than 100 years ago, Moriz Kaposi reported five cases (in middle-aged and elderly males) in Vienna, Austria, with idiopathic multiple-pigmented sarcomas of the skin [25]. All had purplish skin nodules on the lower extremities; this form of the disease was named Kaposi's sarcoma in 1981. KS occurs predominantly in elderly males of Southern European ancestry and also in Israel and other Middle Eastern countries. In Africa, KS has existed for many decades and long preceded HIV infection [26]. KS also is known to develop after organ transplantation, especially in patients of Mediterranean, Jewish, or Arabic ancestry. In 1981, the Centers for Disease Control and Prevention (CDC) reported an increased occurrence of two rare diseases, KS and *Pneumocystis carinii* pneumonia, in young gay men. This was the start of the AIDS epidemic and AIDS-KS. KS-associated herpesvirus (KSHV), also known as HHV-8, along with viral infection, is the cause of KS (AIDS-associated KS), the endemic form of KS, and renal transplant-related KS. KSHV also is associated with primary lymphoproliferative diseases, primary lymphoma, and Multicentric Castleman disease. The latent infection with KSHV is critical for tumorigenesis. Ganciclovir treatment inhibits KSHV replication and reduces lytic replication of KSHV, resulting in the reduced incidence of KS development in AIDS patients who are at high risk for KS. Proinflammatory cytokines and other factors are released during the lytic phase and affect disease progression in an autocrine or paracrine fashion.

HHV-8 has been detected in tumor tissues as well as in blood. Approaches based on CpG methylation status also have been used to distinguish tumor vs. cell-free DNA to follow up the viral load of HHV-8 in KS [27]. The procedure is based on MBD2 bead binding of viral DNA. This assay is very helpful for following up response to treatment.

KS is a multifocal angioproliferative disorder of the vascular endothelium. In most cases it affects mucocutaneous tissue, although other viscera also may be affected. Based on the natural history, site of predilection (bias site), and prognosis, different clinical variants of KS have been reported [28]. The general therapy is effective for KS patients but, in the absence of therapy, the clinical

course of KS varies from innocuous (not harmful) lesions seen in the classic variants to rapidly progressive and fatal lesions of epidemic disease. The classical type of KS generates vascular tumors that affect the lower extremities of elderly men. The male to female ratio of classic KS is about 17:1, and the reason for this difference is not completely understood. The lesions are multiple purple-red pigmented plaques on the skin of the arms, legs, and trunk of men (usually 50 years old or older) in the endemic areas. These lesions gradually start spreading to other parts of the body. Some patients with classic KS are at high risk of developing hematopoietic neoplasms.

Iatrogenic KS, another type of the disease, is associated with the use of steroids, immunosuppressive agents, and drugs with antitumor necrosis factor activity in patients with autoimmune disorders and other complications. AIDS-KS is the most common and aggressive form of KS, and its prognosis is difficult. The KS epidemic is correlated with the mode of HIV acquisition and, in the Western hemisphere, HIV-seropositive male homosexuals are at higher risk for KS than are other groups involved in high-risk behaviors.

In HIV-infected individuals, immunosuppression leads to a fulminant disease that begins with a few skin lesions and, if not treated, may lead to diseases affecting various organs, including the lung, liver, gut, and spleen. Although epidemic KS may develop throughout the entire spectrum of HIV disease, it is favored in the context of advanced immunosuppression and could represent the first manifestation of HIV infection in selected patients [29]. With decreased CD counts and increased deterioration of immunity, AIDS-KS lesions enlarge, multiply in number, and become nodular. Visceral involvement occurs in more than half of AIDS-KS patients [30]. The advancement of disease results in abdominal pain, diarrhea, weight loss, bleeding, and vomiting. Pulmonary KS results in cough, dyspnea, and hemoptysis.

HIV infection reduces immunity in human papillomavirus (HPV)-positive individuals. HPV infection can occur in men who practice anal sex. Although it is known that HPV DNA methylation causes a decrease in the oncogenicity of HPV, it was not known whether the oncogenic potential of HPV is reduced in HIV+ men. Methylation levels in the HPV16 promoter and enhancer were measured in a small number of individuals who were HIV+ and HPV+ [31]. Methylation levels of promoter and enhancer CpG islands were different in low- and high-grade anal intraepithelial neoplasia (AIN). In low-grade AIN, the enhancer was hypermethylated; in high-grade AIN, the promoter was hypermethylated. The viral origin of replication was never methylated. Further investigation indicated the replication of HPV but repressed transcription of viral genes. DNA methylation and viral load may be considered for diagnosis of AIN.

In triple infection by HIV, hepatitis B (HBV), and hepatitis C (HCV), which is common in drug users, antiretroviral therapy (ART) does not work [32]. Investigators attempted miRNA-mediated therapies in such situations and succeeded to some extent. Computer-based models were created to identify an effective miRNA that could prevent multiplication of these infectious agents. Thus far, few miRNAs have been identified that will be further characterized and tested in future. miRNAs considered likely promising because of their similarity with genomes of these three infectious agents are miR-3065-3p, miR-99, miR-548, and miR-122. miRNAs provide molecular immune defenses when the body is exposed and attacked by RNA or DNA viruses. These miRNAs control both DNA and RNA viruses by interfering with their replication. Investigators are exploring similarities between miRNA functions and the targets of related viruses [33].

5 Future Prospects

Future investigations are needed that will (1) provide information on the clinical outcomes of cancers in the HIV-infected population, and (2) identify specific contributions resulting from HIV infection and its potential interactions with other pathogens for the development and pathogenesis of these cancers. Ultimately, such efforts could inform screening approaches and therapies targeted to the HIV-infected population. Specific areas of study in the indicated categories may include but are not limited to the following examples:

5.1 Biomarkers, Diagnostics, and Therapeutics

- Discovery of reliable molecular and immunological diagnostic and prognostic biomarkers and pathogen markers, useful for early detection, progression, or response to treatment of non-AIDS-defining and AIDS-defining malignancies;
- Discovery and development of novel targets and efficacious new therapeutic agents, interventional strategies, or improved delivery systems for the treatment of persons afflicted with non-AIDS-defining and AIDS-defining malignancies;
- Development and evaluation of novel, selective chemical entities generated by combinatorial chemistry or combinatorial genomics methodologies for therapeutic agents;
- Studies to understand the pharmacokinetics of targeted therapies for AIDS-defining and non-AIDS defining malignancies in the context of (HAART);
- Studies utilizing the AIDS and Cancer Specimen Resource (ACSR) biorepository of human specimens from a wide spectrum of AIDS-defining and non-AIDS defining malignancies to develop biomarker or diagnostic assays.

5.2 Etiology, Pathogenesis, and Immunology

- Studies to determine the pathogenic or immunological mechanisms involved in HCV, HBV, KSHV/HHV8, EBV, HPV, Merkel cell polyomavirus, or other oncogenic infectious agent-mediated tumor initiation and promotion of malignancies, concurrent with underlying HIV infections;
- Development of animal and/or cell-based models for non-AIDS-defining and AIDS-defining malignancies;
- Studies to determine the cellular proteome and transcriptome of virally induced tumors in the context of HIV infections;
- Studies to determine the effects of prolonged moderate immunosuppression and/or incomplete or failed responses to HAART on the development of either non-AIDS-defining or AIDS-defining malignancies;
- Studies aimed at identifying the roles of HIV infection in the immunological and pathogenesis of non-AIDS-defining cancers;
- Studies to determine the effects of the concomitant prolonged exposure of HIV-infected patients to antiretroviral therapy and viruses with oncogenic potential on the development of either non-AIDS-defining or AIDS-defining malignancies;

5.3 Molecular Epidemiology and Prevention

- Studies to characterize the host genetic susceptibility to non-AIDS-defining and AIDS-defining malignancies; Assessment of risk factors that impact cancer in the context of HIV infection, in different geographic locations in domestic and international settings;
- Studies to characterize the immunologic, virologic, genetic, and epigenetic differences between those patients on HAART who develop preneoplastic and neoplastic conditions and those patients who resolve these conditions or do not develop them;
- Studies to identify host and/or lifestyle factors (e.g., alcohol, drug use, tobacco, energy balance, environmental, nutrition) that may affect immunocompetence, enhance immunosuppression, and exacerbate the pathogenesis of either non-AIDS-defining or AIDS-defining malignancies;
- Studies to elucidate the effects of routes of acquisition of oncogenic viruses and other host cofactors and concomitant pathogens on the molecular epidemiology and natural history of non-AIDS-defining or AIDS-defining malignancies;
- Studies to better determine the role of infectious pathogens in the development and clinical course of non-AIDS-defining or AIDS-defining malignancies;
- Studies that focus on the development of better prevention strategies that may reduce the burden of infectious-related malignancies in diverse HIV/AIDS populations;
- Studies involving multiple infections to address questions such as: What are the appropriate study designs to understand the

etiology of different cancers when multiple infections are reported? What is the effect of coinfection on cancer survivors? whether coinfection produces amongst a variable host response, a proliferative response of lymphoid elements to viral antigens and in combination with other factors: genetic, epigenetic, socioeconomic, and/or environmental, which contribute to increased risk of cancer;

- Studies to characterize the host genetic susceptibility to HIV-associated oral malignancies and other tumors in infected persons;
- Studies to determine the cellular proteome and transcriptome of virally induced tumors of the oral cavity in the context of HIV infection;
- Studies to determine the mode of entry, latency, reactivation, translocation, and transformation of mucosal cells of the oral cavity by oncogenic viruses during HIV infection;
- Studies to determine the immunologic, molecular, virologic, and pathologic mechanisms that are involved in EBV-, HPV-, and KSHV/HHV8-driven oral malignancies and tumors in the context of HIV infection;
- Studies to determine the effects of prolonged moderate immunosuppression and/or incomplete or failed responses to HAART on the development of oral malignancies and tumors;
- Studies to determine the molecular epidemiology of the viral strains that are implicated in the promotion of HIV-associated malignant transformations of the oral cavity;
- Studies that use novel or well-characterized animal-based models for HIV-associated oral malignancies;
- Studies to determine the similarities and differences between HIV-associated tumors of the oral mucosa and other anatomical (e.g., vaginal, gastrointestinal, rectal) mucosal surfaces;
- Studies that are geared toward identifying reliable diagnostic and prognostic host biomarkers and oral oncogenic pathogen markers useful for detecting HIV-associated oral malignancies and their progression and use of these markers in diagnostic assays;
- Studies that use novel immunotherapies or combinations of novel and standard therapies to modulate the immune response and control the oncogenesis of oral pathogens and oral AIDS malignancies through the use of cytokines, chemokines, adjuvants, antibodies, other molecules of the immune system, and other treatment modalities (e.g., chemotherapeutic agents);
- Studies that develop oral topical formulations with potentially combined effects (microbicidal, virucidal, analgesic, and anti-inflammatory) to enhance oral mucosal defenses and control or treat oral AIDS malignancies;

- Studies that use metagenomic approaches to characterize the oral virome associated with oral AIDS malignancies and compare it with that of healthy (non-HIV/AIDS cohorts); and
- Studies that address oral pathogen translocations with potential interactions with HIV in the oral cavity of HIV-infected/AIDS subjects to induce exacerbation of inflammation and oral AIDS malignancies.

5.4 Latent Reservoirs in HIV-Infected Infants and Children Undergoing Therapy

- Evaluate whether early ART in children affects proviral reservoir size, immune activation, and level of persistent viremia;
- Conduct studies to identify and quantify the tissues and cell types that constitute the persistent viral reservoir in children receiving early ART;
- Evaluate the impact of early infection and treatment on the developing brain;
- Develop tools to measure and quantify HIV in reservoirs such as novel imaging techniques or novel molecular and culture assays appropriate for use in pediatric populations;
- Investigate the role of immune activation, inflammation, and their mediators in various tissues on HIV persistence in infants with perinatal HIV infection;
- Evaluate whether the duration of viral suppression after ART, time from birth until viral suppression is achieved on ART, and/or timing of infection (in utero, intrapartum, postpartum) is associated with viral reservoir size, ongoing viremia, immune activation, and HIV-specific immune responses in HIV-infected children;
- Identify and validate viral and host cellular factors and functions involved in the persistence of HIV infection despite ART that could be targeted for eradication of persistent virus in HIV-infected children;
- Delineate the viral, host, and immune mechanisms involved in the persistence of HIV infection despite effective ART, and the establishment and maintenance of the latent viral reservoir in HIV-infected children;
- Host factors (including epigenetics) and viral genetic factors in the establishment and maintenance of HIV-1 persistence in the setting of suppressive ART in children.

5.5 HIV/AIDS and Epigenetics in Neurological Disorders, Including Brain Tumors

- Identify specific genetic, epigenetic, or proteomic signatures that impact initial immune or inflammatory responses to HIV infection, HAART-induced reconstitution of immune responses, inflammation resulting from immune reconstitution, or HIV-1-induced neuroinflammation and oxidative stress in the presence of drugs of abuse;

- Studies to explore the role of epigenetics in HIV-1 infection and drug abuse;
- Examination of changes in the epigenetic host response to HIV infection through DNA methylation, chromatin modification, and/or noncoding RNAs.

**5.6 Noncoding RNA
(Epigenetic
Regulation)
and HIV AIDS**

- Investigate convergent epigenomic changes caused by environmental exposures (infection, stress, drugs of abuse) that impact aspects of HIV infection and/or AIDS progression;
- Identify epigenomic changes to the host or viral genome that impact selection of integration sites, HIV gene expression, HIV replication, control of viral latency and reactivation, or copy number of integrated proviruses;
- Explore epigenetic processes that affect the host immune response to infection, particularly early responses to infection, and effects of drugs of abuse;
- Investigate the role of epigenetic modifications in the host response, response to treatment, or outcome of HIV infection and coinfections common in drug users;
- Studies exploring correlations between epigenetic changes in brain and “surrogate” cell types (monocytes, T cells or other cellular compartments) to identify useful biomarkers for disease progression in the central nervous system
- Assessment of the impact of epigenetic factors on disease phenotypes (e.g., long term non-progression, rapid progression, development of neurologic complications) or shifts in disease phenotypes
- Studies that address whether epigenetic processes influence high risk sexual behavior, thereby impacting vulnerability for acquiring or transmitting HIV
- Next generation sequencing studies to define host and viral noncoding regulatory RNAs, their function, and their relevance to HIV/AIDS progression
- The impact of epigenomic processes or noncoding RNA regulation on host copy number variation or viral copy number as it relates to HIV/AIDS infection/progression
- Comparisons of the epigenome/transcriptome/proteome/interactome of virus or host differences in different cell/tissue types
- Studies addressing the impact of epigenetic modifications associated with addiction or chronic drug abuse on HIV infection or host responses to infection
- Studies identifying epigenetic modifications associated with HIV infection that impact the host response to drugs of abuse;

- Identify fundamental stable differences in HIV infectivity that are influenced by aging, coinfection with other viruses, drugs of abuse, or hormonal factors;
- Explore small molecule modulators of epigenomic regulators as potential therapeutic strategies for HIV/AIDS treatment or prevention

Thus far, much less research has been conducted on HBV, HCV, and non-AIDS-defining cancers in the context of an underlying HIV infection. HIV coinfection with HCV is of particular interest, because it is the fastest rising coinfection in US urban populations among HIV-positive individuals. To continue to increase investigator interest in these areas, further research should be conducted in both non-AIDS-defining and AIDS-defining malignancies that are poorly represented.

The National Institutes of Health's (NIH) National Cancer Institute (NCI) has established the Office of HIV and AIDS Malignancies (OHIM), which extends across the spectrum of research, training, international studies, and clinical trials. OHIM's goal is to support research in clinically, socially, and demographically diverse populations of HIV/AIDS patients. One OHIM program, the AIDS Malignancy Consortium (AMC), supports clinical trials at the following sites: Boston Clinical Core Site, Memorial Sloan Kettering Cancer Center Core Site, Albert Einstein Core Site, University of California, Los Angeles (UCLA)/University of Southern California (USC) Core Site, University California, San Diego (USCD) Core Site, University of California, San Francisco (UCSF) Core Site, University of Miami Core Site, and Seattle Core Site. The consortium aims to foster the development of innovative research ideas designed to reduce the burden of malignancies among HIV-infected individuals. AMC is expanding its capacity to conduct trials in Africa and other resource-limited areas worldwide to address the heavy burden of HIV-associated malignancies in those areas. In 2010, four sites were selected to serve as International Core Sites with the hope that trials will be conducted successfully and more international sites will be identified. The four Core Sites in Africa are located at Eldoret, Kenya (Moi University School of Medicine); Johannesburg, South Africa (University of Witwatersrand); Kampala, Uganda (Uganda Cancer Institute); Harare, Zimbabwe (University of Zimbabwe Clinical Research Center, and Kaposi's Sarcoma Clinic, Parirenyatwa Hospital, Harare). AMC has additional collaboration sites in India and Brazil.

The International AIDS Society Scientific Working Group on HIV Cure meets regularly and evaluates progress in the field. Some of the recommendations made by this group are described below. Efforts should be made for parallel assessment of existing

in vitro latency models and patient-derived latently infected cells. Approaches such as deep sequencing of latently infected cells using latently infected cells from in vitro models and patient derived cells, and validation of the phenotypic expression profiles of the latently infected cells, should be pursued. Testing of expression modules and development of single cell assays also are essential. This will identify a short list of genes associated with the disease profile. Next, characterization of viral transcripts associated with latency or entry/exit should be done. Additionally, reservoir size should be precisely determined clinically and biologically. This could be assessed first using large, well-characterized cohort studies comparing reservoir size to a range of clinical parameters. Transcriptomics, genomics, epigenomics, metabolomics, and microbiome-profile based approaches should be implemented. Identification of host factors that determines the size of the reservoir should be given preference. Assays should be geared toward high-throughput, with reasonable sensitivity and specificity.

Following completion of genome-wide association studies (GWAS) in several cancers, it was observed that disease-associated single nucleotide polymorphisms (SNPs) are not localized near any genes in the pathways involved in these cancers. Perhaps now is the time to evaluate the status of alternative approaches such as epigenome-wide association studies (EWAS) to determine whether EWAS have evolved to the extent that they can be useful in identifying disease-associated marks for screening high-risk populations and in developing strategies for cancer control and treatment.

Despite intriguing leads that have resulted from epigenetic studies of cancer, most human studies of epigenetics are too small, focus on small genomic regions, and study only one component of the epigenome. To understand epigenetic regulation completely and to reduce sample-related variability, all components of epigenetic regulation (methylation, histone modification, and miRNA expression) should be studied in the same sample. In addition, GWAS in human populations have been successful in identifying previously unidentified genomic regions that are associated with the risk of certain cancers, validated findings for some cancers in regions associated with previously identified candidate genes, and helped to explain some racial disparities in cancer incidence. GWAS examine genetic variants in the germ lines of persons with and without cancer or in persons with cancer who experience different cancer outcomes. It seems likely that a similar agnostic approach to querying the epigenome might meet with similar success. However, GWAS findings have limitations, including that relative risks in association studies are modest. To date, GWAS of some cancers, such as breast and prostate cancers, have failed to identify any risk-associated SNPs that are located in the coding regions of genes

known to play a role in the development of these cancers. It will be important to correlate findings from EWAS with existing GWAS findings. EWAS may provide functional correlation of genes associated with the risk of cancer. Technologies are available to profile methylation, histone modifications, and miRNAs; however, epidemiologic studies have not been conducted using histone profiles, and miRNA profiles have been used in a limited number of studies. So what should be an ideal study in such situations? An ideal epidemiologic study must include thousands of study participants to address the problem of false-positive findings due to multiple comparisons being made. The costs of simultaneously conducting large-scale methylation profiles across the genome and of fully characterizing histone modifications and miRNAs (and possibly chromatin compaction and relaxation) should be reasonable, making it possible to use these technologies in studies that involve many thousands of cases and appropriate controls or cancer cases with different outcomes. Utilization of cohorts with information about life style, behavior, family history, and BMI of the participants should be considered in such studies. All components of the epigenome can be assessed in the relatively noninvasively collected biospecimens (such as saliva, blood, urine, and nipple aspirate) that typically are available in the large cancer epidemiology studies that seek to identify risk factors for cancer, cancer recurrence, or survival after cancer. EWAS enable the collection of information about epigenomic variations throughout the genome/epigenome. Another positive aspect of epigenomic studies is their therapeutic potential since FDA has approved at least four epigenetic inhibitors. Reagents have been discovered that, when used either alone or in combination, are effective anticancer agents. For example, oridonin, a tetracycline diterpenoid compound, has been effective against colorectal cancer. The mechanisms involved are the induction of histone (H3 and H4) hyperacetylation; activation of *p21*, *p27*, and *p16*; and suppression of *c-myc* expression.

In summary, significant progress has been made in studying AIDS/HIV and AIDS-associated malignancies in terms of basic biology and clinical implications. The information provided in this article may be helpful in understanding the etiology of the disease and developing intervention and therapeutic approaches.

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Epigenetics of Colorectal Cancer

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Abstract

Colorectal cancer (CRC) is one of most common malignancies and a leading cause of cancer related deaths worldwide. Epigenetic change is an important mechanism of colorectal carcinogenesis. Accumulation of epigenetic changes was found in colorectal cancer and other tumors. Aberrant changes in DNA methylation, histone modification, imprinting, and noncoding RNAs were frequently found in human colorectal cancer. Epigenetic changes may serve as a diagnostic, prognostic, and chemo-sensitive marker. It also becomes a cancer preventive or therapeutic target in some circumstances.

Key words Colorectal cancer, Epigenetic, Hypermethylation, Hypomethylation, Loss of imprinting (LOI), Field defect, Histone modification, MicroRNA, Long noncoding RNA

Abbreviations

CRC	Colorectal cancer
HMTs	Histone methyltransferases
HDMs	Histone demethylases
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
LOI	Loss of imprinting
LncRNA	Long noncoding RNA
TSA	Trichostatin A
5-aza-CR	5-azacytidine
5-aza-CdR	5-aza-2'-deoxycytidine

1 Introduction

Colorectal cancer (CRC) is the third and second most commonly diagnosed cancer in males and females respectively, with over 1.2 million new cases and 608,700 deaths in 2008 [1]. The accumulation of genetic and epigenetic alterations may drive the initiation and progression from adenoma to adenocarcinoma [2].

Epigenetics is defined as inheritable changes in gene expression without DNA sequence changes. Disruption of the epigenetic landscape is considered to be a common hallmark of all cancers, including colorectal cancer [3]. Most of researches were focused on promoter region hypermethylation in tumor suppressor genes in colorectal cancer. DNA methylation is regarded as a diagnostic, prognostic, and chemo-sensitive marker in different tumors [4–7], including CRC. Aberrant histone modification changes were found in colorectal cancer and histone modification may regulate gene expression directly or by interacting with DNA methylation [8]. MicroRNA (miRNA) may serve as a cancer detection marker or therapeutic target; it may perform tumor suppressor or oncogenic function [9, 10]. Long noncoding RNAs are a new kind of biomarker and player in human cancer, including colorectal cancer. Epigenetic changes are reversible under certain circumstances. This character has made it possible to treat tumors by epigenetic related reagents [11–13]. In this chapter, we mainly introduce abnormal changes and the roles of DNA methylation, histone modification, miRNA, and long non-coding RNAs in colorectal cancer.

2 DNA Methylation

In normal cells, 60 % of human genes contain CG dinucleotide cluster in the promoter region. Methylation of cytosine may protect genome integrity by keeping endoparasitic sequences inactive through heavy methylation [9] which is involved in the regulation of tissue-specific expression patterns, genomic imprinting, X-chromosome inactivation, and other biological activities [14, 15]. During colorectal carcinogenesis, unmethylated CpG sites in the gene promoter may become densely methylated, and normally methylated sporadic CpG sites in the gene body become unmethylation [2, 16, 17]. In general, global DNA hypomethylation is related to genome instability and chromosome fragility.

DNA methylation patterns are established and maintained by three DNA methyltransferases (DNMT): DNMT1, DNMT3A, and DNMT3B [18]. The function of DNMT1 is maintaining existing methylation patterns. DNMT3A and DNMT3B are responsible for de novo methylation [19]. DNA methylation is a covalent chemical modification through the addition of a methyl group at the 5' position of the cytosine base from S-adenosylmethionine by DNMT1, DNMT3A, and DNMT3B [20]. DNA methylation in gene promoter region represses gene transcription by recruiting proteins that bind methylated CpG sequences (Methyl-CpG binding proteins [MBPs]) [21]. MBPs, consisting of methyl-CpG binding protein 2 (MeCP2) and methyl-CpG binding domains (MBD1, MBD2, MBD3, and MBD4), may block transcription factors binding to DNA [21, 22].

2.1 DNA Methylation in Regulation of Tumor Suppressor Expression in CRC

Regional hypermethylation has been extensively analyzed in a variety of cancers because methylation of promoter CpG islands of different tumor-suppressor genes can cause their inactivation [23, 24]. These genes are usually involved in important biological behaviors, including proliferation, apoptosis, angiogenesis, invasion, and adhesion. Promoter hypermethylation of *MLH1*, *APC*, *RBI*, *VHL*, *MGMT*, *GSTP1*, and *BRCA1* genes represent paradigmatic cancer-related epigenetic silencing events [25, 26]. Interestingly, it was found that sporadic and inherited cancers may exhibit similar DNA methylation patterns [27].

A relative number of genes are key component of important signaling pathways. For example, the Wnt signaling is the most important pathway in CRC, which is usually aberrantly activated during colorectal carcinogenesis. The canonical pathway has a well-established role in colorectal oncogenesis, driving proliferation and dedifferentiation in 90 % of CRCs [28]. Several antagonists of Wnt signaling, including *SOX17*, *SFRP1*, *DKK1*, *DKK3*, and *WIF-1*, were found inactivated by promoter methylation, which may contribute to the aberrant activation of this pathway and promote the tumorigenesis [29–31]. Table 1 showed the frequently methylated tumor suppressor genes and the affected pathways in CRC.

2.2 DNA Methylation Serves as a Diagnostic, Prognostic, and Chemo-sensitive Marker in CRC

DNA hypermethylation in promoter region of genes has been the most studied epigenetic mark in human cancer [44, 45]. The application of DNA methylation for diagnostic purposes has several advantages when compared with other biomarkers, such as genetic mutations or gene-expression profiles. Alterations of DNA methylation are mainly found in exact regions (CpG islands) and can be detected by a wide range of sensitive and cost-efficient techniques [20, 46]. The stability of DNA allows the analysis of biomarkers in paraffin processed tissues opening for analysis the extensive archival tissue stores of most medical institutions. In addition, cancer patients show increased levels of free-circulating DNA in blood and lymph, providing an unlimited source in which DNA based indicators can be tested noninvasively [47]. For colorectal cancer, feces enriched in cancerous cells may offer another accessible non-invasive supply of DNA for diagnostic purposes.

DNA methylation in *SEPT9* promoter region by detecting peripheral blood and the stool DNA in different studies has been demonstrated to be a promising biomarker for detection of CRC [48–50]. The high sensitivity and specificity of *SEPT9* methylation in plasma for detection of CRC may improve values of current fecal occult blood and DNA testing [50, 51]. The detective effect was further validated in precancerous lesions [52, 53]. Methylation of *hMLH1*, *p16*, *DAP-kinase*, *APC*, *MGMT*, *RASSF2A*, and *Wif-1* is also regarded as a helpful plasma or serum detection marker [32].

Table 1
Hypermethylated tumor suppressor genes and affected pathways/effect in colorectal cancer

Genes	Description	Pathways/effect	References
<i>WIF1</i>	Wnt inhibitory factor-1	Wnt signaling	[32]
<i>APC</i>	Adenomatous polyposis coli	Wnt signaling	[32]
<i>DKK-1</i>	Dickkopf-1	Wnt signaling	[9, 33]
<i>DKK-3</i>	Dickkopf-3	Wnt signaling	[34]
<i>SFRP1, SFRP2</i>	Secreted frizzled-related protein	Wnt signaling	[35]
<i>SOX17</i>	SRY-box containing gene 17	Wnt signaling	[29]
<i>MGMT</i>	O6-methylguanine DNA methyltransferase	DNA repair	[9]
<i>hMLH1</i>	mutL homolog 1	Mismatch repair	[36]
<i>HIC1</i>	Hypermethylated in cancer-1	Transcriptional repressor	[37]
<i>p14, p15, p16</i>	Cyclin-dependent kinase inhibitor 2A	Cell cycle control	[9, 34, 38]
<i>GATA4, GATA5</i>	GATA binding protein	Transcription factor (multiple tumor suppressive function)	[39, 40]
<i>DAPK</i>	Death-associated protein kinase	Apoptosis	[41]
<i>BNIP3</i>	BCL2/adenovirus E1B 19-kDa interacting protein	Apoptosis	[34]
<i>CDH1</i>	E-cadherin	Cellular adhesion	[34]
<i>CDH13</i>	H-cadherin, T-cadherin	Cellular adhesion	[42]
<i>TSP1</i>	Thrombospondin-1	Angiogenesis inhibitor	[43]
<i>RASSF1A, RASSF2A</i>	Ras association (RalGDS/AF-6) domain family member	RAS signaling	[34]
<i>RARB2</i>	Retinoic acid receptor	MAPK signaling	[9, 34]

Hypermethylation of *SOX17* is an early event of CRC, which may serve as an early detection marker [29], whereas methylation of *ASC/TMS1* was reported to be a late-stage event in CRC [54]. DNA methylation can also predict the prognosis of CRC patients. For example, methylation of *DACH1* was associated with late tumor stage, poor differentiation, and lymph node metastasis [5]. In addition, CpG island methylation of *IGFBP3*, *EVL*, *FLNC*, and *CD109* is associated with an eightfold increase in mortality risk relative to that of CRC patients with no DNA methylation of these genes, and methylation of *IGFBP3* and *CD109* was also related to a high risk of CRC recurrence [55]. Another interesting report suggests that *LINE-1* hypomethylation is a prognostic marker [56].

Inactivation of some genes induced by promoter region hypermethylation may lead to chemo-resistance in CRC patients. Cells harboring a methylated *p16* gene are more resistant to irinotecan-induced cell cycle arrest [57]. Methylation of *DEXI* is associated with resistance to camptothecin and poor outcome to irinotecan-based chemotherapy in colon cancer [58]. Other heavily methylated genes in CRC, such as *BNIP3* and *MGMT*, were also found related to chemo-resistance [59, 60]. The potential diagnostic, prognostic, or chemo-sensitive markers are listed in Table 2.

Table 2
CpG island methylation markers in colorectal cancer

Genes	Potential value	Specimen type	Methylation status	Remarks	References
<i>MLH1</i>	Diagnosis	Serum	Hyper	Diagnosis of sporadic MSI CRC	[61]
<i>ALX4</i>	Diagnosis	Serum	Hyper	Diagnosis of	[62]
<i>Septin-9</i>	Diagnosis	Plasma	Hyper	colorectal	[51, 63]
<i>BMP3, NDRG4,</i>	Diagnosis	Stool	Hyper	adenomas and	[64]
<i>TFPI2, Vimentin</i>				cancers	
<i>BMP3</i>	Diagnosis	Tissue	Hyper		[65]
<i>P16</i>	Diagnosis	Serum	Hyper	Diagnosis of CRC	[66, 67]
<i>DAPK</i>	Diagnosis	Serum	Hyper		[68]
<i>RUNX3</i>	Diagnosis	Serum	Hyper		[69]
<i>TMEFF2, NGFR,</i>	Diagnosis	Plasma	Hyper		[70]
<i>SEPT9</i>					
<i>APC, MGMT,</i>	Diagnosis	Plasma	Hyper		[32]
<i>RASSF2A, WIF1</i>					
<i>GATA-5, GATA-5</i>	Diagnosis	Stool	Hyper		[39]
<i>HPPI, HLTF,</i>	Prognosis	Serum	Hyper	Poor prognosis	[71]
<i>MLH1</i>					
<i>LINE-1</i>	Prognosis	Tissue	Hypo	Associated with worse overall survival	[56]
<i>PPARG</i>	Prognosis	Tissue	Hyper	Poor prognosis	[72]
<i>CDH13, FIBN3</i>	Prognosis	Tissue	Hyper	Poor prognosis	[73]
<i>TFAP2E</i>	Predictive	Tissue	Hyper	Resistance to 5-FU-based chemotherapy	[74]
<i>GALR2</i>	predictive	Tissue	Hyper	Resistance to bevacizumab	[75]
<i>ALX4</i>	predictive	Tissue	Hyper	Resistance to cetuximab	[75]

Hyper hypermethylation, *hypo* hypomethylation

2.3 Hypomethylation, Field Defect, and Loss of Imprinting in CRC

Global DNA hypomethylation is one of the first recognized epigenetic alterations in colorectal adenomas and CRCs [76], which occurs gradually, age-dependently, and early in the process of CRC carcinogenesis [77]. Global DNA hypomethylation occurs predominantly at CpG sites in repetitive sequences, unique sequences including oncogenes and imprinted loci, less commonly in CpG islands and CpG island shores (highly conserved sequences up to 2 kb surrounding CpG islands) [25, 78, 79]. Hypomethylation was associated with oncogene activation in CRC, such as *P-cadherin* (*CDH3*) and *long interspersed nuclear element-1* (*LINE-1*). A significant association was found between aberrant demethylation of *CDH3* and tumor site or Dukes stage [80], and *LINE-1* hypomethylation is a potential biomarker for prediction survival benefit for fluoropyrimidines in CRC patients [81–84]. In addition, hypomethylation can result in increased chromosomal instability (CIN) [85]. Demethylation at pericentromeric sites may facilitate recombination and altered chromosome replication and is frequently seen in microsatellite stable (MSS), CIN CRCs [77, 86–88].

The term “field cancerization” was first used by Slaughter et al. to describe the presence of mucosae that are predisposed to cancer development for oral cancers [89]. It was also denoted as the presence of “field defect” and was used to describe the accumulation of genetic and epigenetic alterations in tissues with normal appearance [90]. It is recognized clinically because of the high propensity of survivors of certain cancers to develop other malignancies of the same tissue type, often in a nearby location [90–92]. In colorectal cancer, the field defect is also characterized by the simultaneous occurrence of multiple but distinct tumors, which are either separate malignancies or a single malignancy accompanied by multiple pre-neoplastic lesions [93]. Promoter region hypermethylation was considered as an evaluation marker for field defect in colonic cancer. The discovery of field defect markers could be of great use in mucosa that appears normal, both for early detection and risk assessment for colon cancer, such as *MGMT* methylation in CRC [93].

Loss of imprinting (LOI) refers to loss of monoallelic gene regulation conferred by parent-of-origin-specific DNA methylation, affecting at least the genes *IGF2*, *PEG1*, *p73*, and *LIT1* [94–98]. LOI of *insulin-like growth factor II* (*IGF2*), an important autocrine growth factor in cancer, was first identified in Wilms’ tumor, in which it is the most common molecular alteration [95]. Following that, similar observations were made in many other malignancies, including CRC [99–102]. Abnormal activation of the normally silent maternally inherited allele of *IGF2* gene was attributed to the hypomethylation of the proximal *IGF2* promoter in CRC [88]. Overexpression of *IGF2* induced by LOI activates the IGF1 receptor (IGFR), which autophosphorylates and activates the PI3K and GRB2/RAS/ERK pathway [103]. LOI of *IGF2* may be a valuable predictive marker of an individual’s risk for CRC [104].

2.4 CpG Island Methylator Phenotype (CIMP) in Colorectal Cancer

The existence of CpG island methylator phenotype (CIMP) was initially proposed by Toyota et al., who suggested that CRC could be classified into two categories: one group that shows rare methylation (CIMP negative) and the other group that shows aberrant methylation of several genes simultaneously (CIMP positive) [105]. This proposal emerged as a new pathway for colorectal carcinogenesis, in addition to the classic mutator or chromosomal instable (CIN) and microsatellite instable (MSI) categories, standing for a subset of sporadic colorectal cancer bearing excessive cancer-specific promoter region hypermethylation [9]. The five classic markers of CIMP were *CDKN2A*, *MINT1*, *MINT2*, *MINT31*, and *MLH1*, which provided a simplified and representative approach to define CIMP [2]. In addition to these 5 loci, CIMP marker panel has been extended to include *CACNA1G*, *CRABP1*, *IGF2*, *NEUROG1*, *RUNX3*, *SOC1*, *HIC1*, *IGFBP3*, and *WRN*. It is a major challenge in CIMP-based CRC subgroup classification that there is no general consensus of which specific methylated loci should be used to define CIMP subgroups [106]. CIMP CRC can be divided into two classes, CIMP1 and CIMP2, based on analysis of a large panel of methylation marks [107]. CIMP1 tumors are often microsatellite instable and have high *BRAF* mutations, in contrast, CIMP2 tumors have very high *KRAS* mutations, but rarely have MSI or *BRAF* or *TP53* mutations [108]. Tumors that do not have CIMP have a high frequency of *TP53* mutations and an intermediate frequency of *KRAS* mutations [108]. CIMP is still a controversial topic.

3 Histone Modification

In addition to DNA methylation-induced transcriptional silencing of genes, posttranslational covalent modifications, including acetylation, methylation, phosphorylation, ubiquitylation, SUMOylation, citrullination, and ADP-ribosylation of histone tails, constitute another kind of epigenetic modifications in regulating chromatin structure and gene expression in human cancers. Histone (de)acetylation and (de)methylation in CRC are best characterized. The global pattern of histone modifications may serve as a predictor for the risk of recurrence of human cancers [8, 109].

3.1 The Enzyme of Histone Modification

The status of histones acetylation is controlled by two kinds of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs) [110]. HATs and HDACs are responsible for the addition and removal of acetyl groups from lysine residues, acting as transcriptional co-activators or co-repressors, respectively. Of all the known modifications, acetylation has the most potential to unfold chromatin since it neutralizes the basic charge of the lysine [106]. HATs are divided into three main families: MOZ/YBF2/SAS2/TIP60 (MYST) family, GCN5 *N*-acetyltransferase (GNAT)

family, and the CREB binding protein (CBP)/p300 family [111]. The MYST family mainly targets histone H4, GNAT mainly targets histone H3, and the CBP/p300 family targets both H3 and H4 [112]. There are four distinct families of HDACs. Class I, II, and IV HDACs share homolog in sequence and structure, which is of great difference in the class III NAD-dependent enzymes of the Sir family [113]. Similar to histone acetylation, histone methylation is dynamically regulated by histone methyltransferases (HMTs) and histone demethylases (HDMs), which install and remove histone methylation marks, respectively, in a site-specific manner [114, 115].

Altered expression of histone modification enzymes was often found in cancer, which may disrupt the original balance and promote cancer progression. For example, High expression of HAT-related protein CREB binding protein (CBP) in CRC tissues correlated with long-term survival, while upregulation of p300 is related to poor prognosis [116]. Multiple class I HDACs, including HDAC1, HDAC2, and HDAC3, are upregulated in a subset of CRCs. Expression of these HDACs was significantly enhanced in strongly proliferating, dedifferentiated tumors, and increased expression of HDACs has been shown to be associated with short survival time of CRC patients [117]. Gradually increasing expression of HDAC2 was observed from normal tissue, colorectal adenoma to colorectal cancer, and the overexpression of HDAC2 is accompanied the hypoacetylation at H4K12 and H3K18 histones during adenoma to carcinoma progression [118]. In addition, increased expression of SIRT1, one class III HDAC, was found in CRCs, especially the cases with microsatellite instability and CpG island methylator phenotype [119]. Enhancer of zeste homolog 2 (EZH2), H3K27 specific HMT and catalytic subunit of polycomb-repressive complex 2 (PRC2), is frequently overexpressed in CRC [120, 121]. Intracellular depletion of EZH2 induced cell cycle arrest, inhibited CRC cell growth, and reduced the expression of several cancer-associated genes involved in proliferation or invasion [122]. The expression of KDM4C, regulating sphere formation by mediating the cross talk between Wnt and Notch pathways, was increased in spheres from CRC cells [123].

3.2 Histone Code and Gene Expression in Colorectal Cancer

Combination of histone modifications, such as acetylation, methylation, and phosphorylation, are thought to establish a dynamic and potentially reversible “histone code” that permits active transcription in a euchromatin configuration but inactive transcription when chromatin is in a heterochromatin state [34]. Among the seven modifications, acetylation and methylation are the most extensively characterized ones that are involved in CRC pathogenesis [106].

The best understood histone modifications are acetylation/deacetylation and methylation/demethylation of lysine and arginine residues within histone tails. The consensus is that hypoacetylated

loci typically silence gene expression whereas hyperacetylated histones contribute to gene activation. Acetylation of histone tail residues destabilizes the chromatin fiber, which permits enhanced mobility of nucleosomes along the chromosome and less impeded access of transcription factors to DNA [124]. Histone lysine methylation occurs on histones H3 and H4 and can be mono-, di-, or tri-methylated. Similarly, histone arginine methylation occurs in mono methyl, symmetrical di-methyl, or asymmetrical di-methyl state [125]. In general, methylations of H3K4, H3K36, and H3K79 are linked to gene expression activation, whereas H3K9me2, H3K9me3, H3K27me3, and H4K20me3 are associated with gene expression repression [2]. For example, lower level expression of Wnt5a (one of the WNT signaling factors) was found in the highly metastatic CRC cells, which corresponded to multiple histone modifications, including lower levels of acetylated histone H3, H4, and H3K4me2 and higher levels of H3K27me3 in the promoter region. Furthermore, the expression of Wnt5a was restored after treatment with HDAC inhibitor [126]. In addition, deacetylation of histone H3K9 is associated with transcriptional silencing of E-cadherin in colorectal cancer cells [127]. Whereas, transcription of some genes is associated with bivalent histone modifications, such as DACT3 (a regulator of Wnt signaling), expression of which was reduced in CRC cells, along with enrichments of both H3K27me3 (repressive) and H3K4me3 (activating) [128].

3.3 The Interaction of Histone Modification and DNA Methylation in Regulating Gene Expression

Although DNA methylation and histone modification are catalyzed by distinct enzymes, these epigenetic modifications are tightly coordinated in regulation of gene expression. For example, deacetylation (H3K9) and simultaneous methylation of histone H3 (H3K9me2) in the *MLH1* promoter was a key event mediating epigenetic inactivation of this gene, but treatment with DNMT inhibitor 5'-aza-cytidine (decitabine) lead to the complete reversal of *MLH1* methylation and the corresponding histone code [129]. It suggests that DNA methylation and histone modifications act in concert. A study showed that elimination of DNA methylation from a *p16* (*INK4a*) allele resulted in profound changes in surrounding histones. After continued passage of such cells, methylation of histone H3 lysine-9 occurred in conjunction with re-silencing in the absence of DNA methylation [130]. Furthermore, it was suggested that hypoacetylation of histone together with histone methylation may prior to DNA methylation in gene silencing process [131]. In addition, the epigenetic modification enzymes may directly interact with each other while associated with chromatin in a gene promoter region. It is known that DNMTs, MBDs, HATs, HDACs, HMTs, and HDMs can form protein complexes in the promoter regions of certain genes in CRC cells, blocking/inhibiting transcription factor (TF)-dependent transcription initiation by RNA polymerase (RNA Pol II) [106].

3.4 Noncoding RNA

Noncoding RNAs (ncRNAs) are functional RNA molecules that do not code for proteins. Based on size, they are divided into different classes, including long noncoding RNA (lncRNA), Piwi-interacting RNAs (piRNAs), small interfering RNAs (siRNAs), and microRNAs [132]. NcRNAs, particularly microRNAs (miRNAs), are mechanistically involved in controlling the expression of various cancer-related genes, and their expression may be altered in cancer. In addition, many roles are emerging for lncRNAs in ribonucleoprotein complexes that regulate various stages of gene expression [133].

3.5 MicroRNAs (miRNAs) in Colorectal Carcinogenesis

miRNAs are single stranded, evolutionarily conserved, small RNA molecules (19–25 ribonucleotides) that mediate posttranscriptional gene repression. Primary miRNA genes are transcribed in the nucleus, processed to precursor miRNAs by Drosha, and transported to the cytoplasm, where they undergo further processing by the ribonuclease III enzyme Dicer, resulting in mature miRNAs that are incorporated into the RNA-induced silencing complex. miRNAs act as endogenous suppressors of gene expression through imperfect binding of the RNA-induced silencing complex to 3' untranslated regions (3'UTR) of target mRNAs and induce either mRNA degradation or translational repression. Similar to genes, miRNAs can act either as tumor suppressors (tsmiRs) by inhibiting the expression of oncogenes or as tumor promoters (oncomiRs) by suppressing the expression of target tumor suppressor genes [34, 134]. Global miRNA expression patterns can distinguish different tissue and tumor types better than mRNA expression patterns, making them attractive targets for their development as cancer biomarkers [135].

MiR-143 and miR-145 are the initially found CRC related miRNAs, which were reported to be significantly downregulated in CRC and then were regarded as tsmiRs [34, 136]. The list of aberrantly expressed miRNAs in colorectal adenomas and cancers is continuously growing in recent years. Studies suggested that miRNAs were involved in many major signaling pathways and cellular processes in colorectal carcinogenesis, including β -catenin/WNT signaling (miR-135a/b, miR-139, miR-145, miR17-92) [137–142], p53 signaling (miR-34b/c) [143], cell cycle control (miR34a, miR-192, miR-215, miR-675) [140, 144, 145], proliferation (let-7 family, miR-18a, miR-21, miR-126, miR-143, miR-200c) [137, 140, 144], apoptosis (miR-34a, miR-133b, miR-195) [140, 144], differentiation (miR-141, miR-200c) [146, 147], and migration/invasion (miR-126, miR-143, miR-196a, miR-200a/b/c, miR-373, miR-520c) [140, 144, 146]. It is likely that the altered expression of many miRNAs may target hundreds of growth regulatory genes and pathways that are critical in multistep colorectal carcinogenesis.

3.6 Epigenetic Mechanisms for miRNA Dysregulation in CRC

The mechanisms responsible for dysregulation of miRNAs in human cancers are still poorly understood. One mechanism that has attracted much attention is aberrant methylation of the miRNA gene promoters. Studies have demonstrated that hypermethylation of miR-9-1, miR-129-2, and miR-137, promoters of which are enriched in CpG islands, was associated with reduced expression in CRC cell lines and tumor tissues [34]. MiRNA-34b and miRNA-34c, both targeting the TP53 pathway, undergo frequent promoter CpG island hypermethylation in CRC [148]. Similarly, the cell cycle regulating miRNA-124a was found to be associated with CpG island hypermethylation, repressive chromatin configuration, and occupancy by MeCP2 and MBD2 [149]. While expression of hsa-miR-342, a miRNA encoded in an intron of the Ena/Vasp-like gene and inducing apoptosis, is regulated by hypermethylation of the Ena/Vasplike CpG island [150].

By comparing miRNA expression and histone modifications (H3K4me3, H3K27me3, and H3K79me2) before and after DNA demethylation, 47 miRNAs were found to be potential targets of epigenetic silencing in early and advanced CRCs [151]. DNA demethylation at these miRNA promoters resulted in upregulation of H3K4me3 and H3K27me3 at the promoters of these miRNAs, providing additional insight into the association between hypermethylation, histone modifications, and miRNA dysregulation in cancer. These initial clues into the biology of miRNA provide a springboard for future studies.

3.7 Long Noncoding RNAs and CRC

The range of ncRNAs in eukaryotes is vast and exceeds the number of protein-coding genes (representing about 2 % of the total genome sequence). Besides the different families of small ncRNAs, a large proportion of transcriptome results in RNA transcripts that are longer than 200 nucleotides, which are often polyadenylated and are devoid of evident open reading frames (ORFs)—these are defined as long ncRNAs (lncRNAs) [133]. The function of LncRNAs was associated with a spectrum of biological processes (Fig. 1), including alternative splicing, modulation of protein activity, alternation of protein localization, and play structural and organizational role. LncRNAs can be also precursors of small RNAs and even tools for miRNAs silencing [152]. One of their primary tasks is regulation of protein-coding gene expression [153]. LncRNAs act mainly through four different mechanisms, which were supposed as signals, decoys, guides, and scaffolds [154]. LncRNAs are emerging as key molecules in human cancers, including colorectal cancer.

LncRNA may serve as a novel biomarker to predict the prognosis of colorectal cancer (CRC) or be involved in important signaling pathways related to carcinogenesis. For instance, the well-known lncRNA HOTAIR was upregulated in cancerous

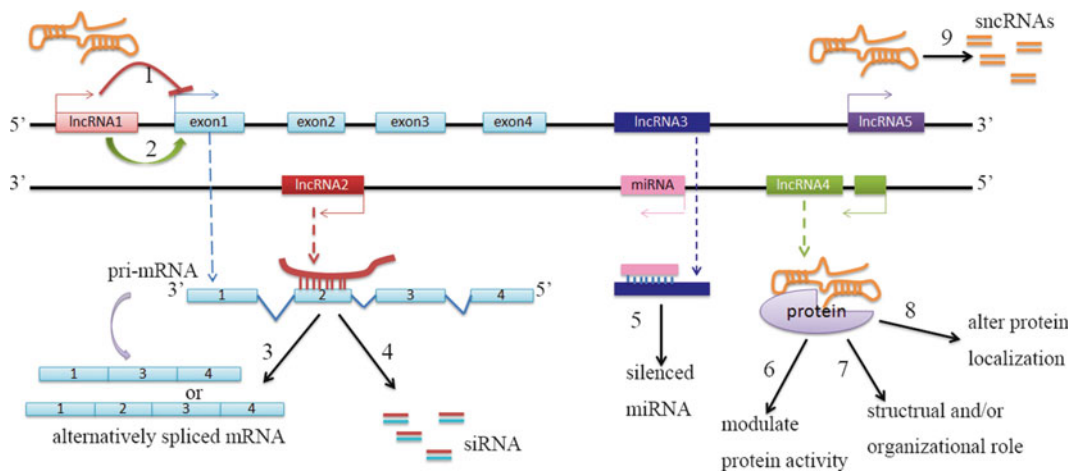


Fig. 1 Schematic illustration of lncRNAs functioning. LncRNA transcribed from an upstream noncoding promoter can negatively (1) or positively (2) regulate expression of the downstream gene by transcriptional interference or inducing chromatin remodeling/histone modifications, respectively. LncRNA is able to hybridize to the pre-mRNA and block recognition of the splice sites by the spliceosome, thus resulting in an alternatively spliced transcript (3). Alternatively, hybridization of the sense and antisense transcripts can allow Dicer to generate endogenous siRNAs (4). The binding of lncRNA to the miRNA results in the miRNA function silencing (5). The combination of lncRNA and specific protein partners can modulate the activity of the protein (6), play structural and organizational role (7), and alter the protein localization in the cell (8). Finally, long ncRNAs can be processed to the small RNAs (9)

tissues than in corresponding noncancerous tissues in CRC and high level expression of *HOTAIR* correlated tightly with the presence of liver metastasis and poor prognosis of CRC patients. Furthermore, *HOTAIR* induced genome-wide retargeting of PRC2 [155]. It was reported that expression of *prostate cancer-associated ncRNA transcripts 1 (PCAT-1)* in CRC tissues was significantly upregulated compared with the matched normal tissues. The increased expression was associated with distant metastasis and poorer overall survival [156]. LncRNA loc285194 is a novel p53 transcription target in CRC cells, which acts in part through repression of miR-211. Loc285194 was downregulated and ectopic expression of loc285194 inhibits tumor cell growth both in vitro and in vivo [157].

LncRNAs certainly possess structural versatility allowing them to bind and interact with a number of proteins, including epigenetic regulators. The field remains vastly open for the identification of additional lncRNA-protein partners implicated in cancer. In particular, there are tens of thousands of unspliced mono-exonic antisense lncRNAs expressed from intronic regions in the human genome that remain to be explored.

4 Epigenetic Therapy in Colorectal Cancer

It is now a well-established concept that epigenetic alterations can be the driver events in the pathogenesis of CRC. The reversibility of epigenetic modifications means that altered gene expression can be turned on or off. This makes them attractive candidates for therapeutic intervention. Furthermore, there is growing evidence supporting the hypothesis that epigenetic alterations may be a driving force of drug resistance in human cancers, including CRC [158, 159]. The extensively studied two classes of chemical compounds include DNMT and HDAC inhibitors, both of which have undergone major preclinical investigations and are currently being explored for efficacy in the treatment of various human cancers in several clinical trials. For instance, the DNA demethylating drugs 5-azacitidine and 5-aza-2'-deoxycytidine (decitabine) and azacytidine, two inhibitors of DNMT, are approved by FDA for use in patients with myelodysplastic syndrome, and are currently being tested extensively in human patients with solid cancers, including CRC [160]. Interestingly, studies have indicated that both decitabine and azacytidine at a dose that is much lower than their maximally tolerated doses (MTD) are effective in inhibiting tumor-specific DNA hypermethylation [161]. Blocking the enzymatic activity of DNMTs by using small molecule inhibitors is another strategy to achieve DNA demethylation [162].

HDAC inhibitor is another kind of extensively studied epigenetic drugs. There are at least 20 structurally different HDAC inhibitors that are currently in clinical trials as monotherapy or combinational therapy for human cancers, including CRC [163]. There is evidence from *in vitro* and *in vivo* studies that vorinostat (a drug has been used clinically) can downregulate thymidylate synthase expression, which results in synergistic antitumor activity when combined with 5-FU in CRC cells.

Considering the collaboration between DNA methylation and histone modifications in transcriptional regulation of tumor suppressor genes, another strategy of epigenetic therapy is to combine DNMT and HDAC inhibitors. As a result, combinational epigenetic therapy targeting both DNMT and HDAC activity synergistically reactivates gene expression and result in effective tumor suppression. In a phase I clinical trial of patients with refractory solid tumors (including CRC), combinational treatment with 5-azacitidine and valproic acid resulted in a significant decrease in global DNA methylation and induced histone acetylation with stable disease lasting up to 12 months in a subset of patients [164].

Overall, epigenetic agents, primarily DNMT and HDAC inhibitors, have undergone major preclinical investigations and extensive clinical trials, either alone or in combination with others or conventional chemotherapeutic agents in treatment of human

cancers, including CRC [106]. But it is still a long way to find more selective and effective epigenetic agents with minimum toxicity and good responsiveness.

5 Concluding Remarks

With the advancing studies in epigenetics, novel mechanisms will be understood, and more effective and less side-effective epigenetic therapy will be applied in the clinic in the near future.

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Epigenetics in Breast and Prostate Cancer

Yanyuan Wu, Marianna Sarkissyan, and Jaydutt V. Vadgama

Abstract

Most recent investigations into cancer etiology have identified a key role played by epigenetics. Specifically, aberrant DNA and histone modifications which silence tumor suppressor genes or promote oncogenes have been demonstrated in multiple cancer models. While the role of epigenetics in several solid tumor cancers such as colorectal cancer are well established, there is emerging evidence that epigenetics also plays a critical role in breast and prostate cancer. In breast cancer, DNA methylation profiles have been linked to hormone receptor status and tumor progression. Similarly in prostate cancer, epigenetic patterns have been associated with androgen receptor status and response to therapy. The regulation of key receptor pathways and activities which affect clinical therapy treatment options by epigenetics renders this field high priority for elucidating mechanisms and potential targets. A new set of methylation arrays are now available to screen epigenetic changes and provide the cutting-edge tools needed to perform such investigations. The role of nutritional interventions affecting epigenetic changes particularly holds promise. Ultimately, determining the causes and outcomes from epigenetic changes will inform translational applications for utilization as biomarkers for risk and prognosis as well as candidates for therapy.

Key words Breast cancer, Prostate cancer, CpG (cytosine–guanine), DNA methylation, Epigenetic changes, Disparities

1 Introduction

The etiology of cancer was long held as an aberration of the genome. However, over the past decade, the regulation of the genome through epigenetic change has added to the complexity. Epigenetic modification has long been known to play a key role in normal developmental functions such as imprinting and X-chromosome inactivation [1]. Only recently has the role of aberrant epigenetic modifications been revealed to play an important role in neoplasia. Formally, epigenetic changes are defined as heritable cellular information which are not genetic and can be transmitted through cell division [2]. Two major epigenetic modifications discussed in this chapter include DNA level methylation in which CpG islands in the genome undergo covalent bonding with a methyl group resulting in control of gene expression and histone

modification in which histone proteins undergo deacetylation/methylation resulting in regulation of chromosomal packing [2, 3].

DNA level methylation changes can be categorized into two broad types of hypomethylation and hypermethylation which significantly affect gene expression [4]. Hypomethylation results when previously methylated genes in adult DNA undergo demethylation resulting in expression of a gene. Expression of genes normally repressed, such as oncogenes *HRAS* and others, can lead to aberrant cellular activity and subsequent tumorigenesis [5]. Conversely, hypermethylation affecting gene transcription occurs when CpG islands in regulatory or promoter sites of a gene undergo silencing by methylation [4]. A complex series of steps take place for methylation and gene silencing to occur including recruitment of several regulatory proteins and biochemical reactions ultimately resulting in alterations in the histone state and chromosomal folding [4, 6, 7]. Among the most studied examples of hypermethylation in relation to cancer is silencing of *hMLH1* in colorectal cancer [8, 9] and *BRCA1* in breast cancer [9, 10]. Both result in the incapacitation of key tumor suppressor genes—in this case, proteins involved in DNA maintenance and repair [8, 10].

Histone modifications have increasingly gained importance in the context of epigenetics. Histone proteins can undergo acetylation, phosphorylation, and methylation which in turn regulate chromosomal stability and packing. Acetylation results in relaxing of the chromosomal packing, allowing for transcription factors to access and initiate transcription of genes [11]. Conversely, deacetylation by histone deacetylases (HDACS) and subsequent methylation of histone residues results in the tightening of the histones, reducing access of regulatory transcriptional proteins.

Several studies have identified that a complex relationship exists between DNA level epigenetic changes and histone level changes. There is data to suggest that DNA methylation changes may precipitate histone residue modifications and chromatin packing [9, 11, 12]. Data to support this conclusion came from studies demonstrating that histone demethylation through inhibition of HDACs was not sufficient to reverse methylation of DNA and result in gene expression [13]. Other studies conversely argue that histone mediated chromatic modification, not DNA methylation, is not the primary driver for epigenetic mediated gene silencing [14]. The supporting data for this hypothesis was derived from studies demonstrating DNA-methylation independent gene silencing through histone modification alone [15].

Additional studies are warranted to identify the exact mechanism which predominantly contributes to signaling. Most likely, a tissue-specific pattern with complexes composed of DNA methyltransferases (DNMTs), HDACS, and cofactors (methyl CpG binding proteins) will be ultimately responsible for gene silencing [11, 13]. Hence, it is paramount to examine epigenetics in multiple

organ systems. While significant strides have been made in the field for colorectal cancers, gliomas, and leukemias [9], there is still a need for further investigation in other high-incidence cancers such as breast and prostate cancer. To date, our group and others have identified that epigenetic modifications (both global and gene-specific) significantly contribute to tumorigenesis and progression in both breast and prostate cancers [16–20]. This chapter reports the findings from several studies on breast and prostate cancer as well as the methods and tools to conduct further investigation. Notably, the role of epigenetics in contributing to health disparities is highlighted. Furthermore, the most recent findings from the promise of natural compounds are also discussed. Lastly, future directions explore potential translational directions in the field of epigenetics.

2 Epigenetic Changes in Breast and Prostate Cancers

2.1 Breast Cancer

Breast cancer is the most frequently diagnosed cancer and the second leading cause of cancer death in women [21]. In addition to genetic alterations such mutations in oncogenes and tumor suppressor genes, epigenetic alternations such as promoter methylation and histone modification could also lead to initiation, promotion, and metastasis of breast cancer [9]. Epigenetics may further play a role in drug interventions and cancer therapy thereby meriting significant focus for research to improve breast cancer outcomes.

2.1.1 Methylation of Promoter CpG Islands of Genes in Breast Cancer

Hypermethylation of promoter CpG islands represents an alternative mechanism of gene inactivation and may occur early in breast cancer development. More than 100 genes have been reported to be hypermethylated in primary breast tumors or breast cancer cell lines [10, 22, 23]. Many of these aberrantly methylated genes play critical roles in tumor suppression, cell cycle regulation, apoptosis, angiogenesis, tissue invasion, and metastasis [9, 22].

CpG Island Hypermethylation and Breast Cancer Progression

Promoter CpG islands hypermethylation have been associated with breast cancer progression. Recent study assessed methylation levels of tumor suppressor genes, *RARβ2* and *RASSF1A*, *MINT17*, and *MINT13* during key steps of breast cancer development [24]. The study identified a significant increase in the expression levels of these genes during the breast cancer development [24]. Hypermethylation of promoter CpGs of *RARβ2* and *RASSF1A* have been confirmed to play a role in breast cancer in other studies and can be considered early epigenetic events in breast cancer. Jovanovic and colleagues found *RARβ2* and *RASSF1A* methylation within lesions from both in situ lobular (LCIS) and ductal carcinoma (DCIS) [18]. Another study screened 57 promoter

CpG loci in 20 invasive ductal carcinomas (IDC) and their paired normal breast tissues. The study demonstrated that methylation of 15 genes (*DLEC1*, *GRIN2B*, *HOXA1*, *MT1G*, *SFRP4*, *TMEFF2*, *APC*, *GSTP1*, *HOXA10*, *IGF2*, *RAR β* , *RASSF1A*, *RUNX3*, *HIN-1*, and *SFRP1*) increased stepwise from normal to atypical ductal hyperplasia (ADH)/flat epithelial atypia (FEA) to ductal carcinoma (DCIS) [25].

The association of promoter CpG gene hypermethylation and breast cancer progression can be assessed by comparing the methylation status in normal, pre-malignant breast lesions, and breast cancer tissues. DNA for these studies is best prepared by using formalin-fixed paraffin-embedded (FFPE) tissues with laser capture microdissection that ensures the type of tissues needed (normal, benign, tumor) is captured for use in DNA extraction and methylation assays. Figure 1 demonstrates an example schema of this process.

Aberrant DNA methylation has also been related to clinical and pathologic characteristics of breast cancer. Genome-wide DNA methylation analysis using methylation specific digital karyotyping (MSDK) of breast tumor tissues identified the presence of differentiation specific DNA methylation and gene expression patterns in breast carcinoma [18]. These epigenetic changes have subsequently been associated with tumor size, lymph node status, distant metastasis and hormone receptor status [18, 23, 26]. The MSDK is a

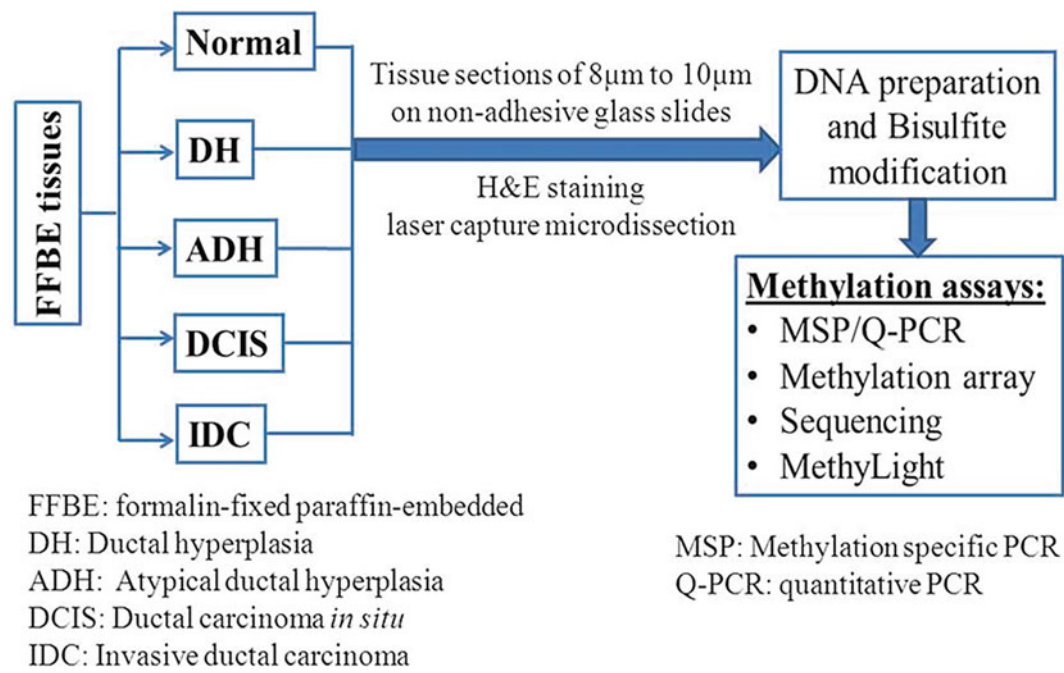
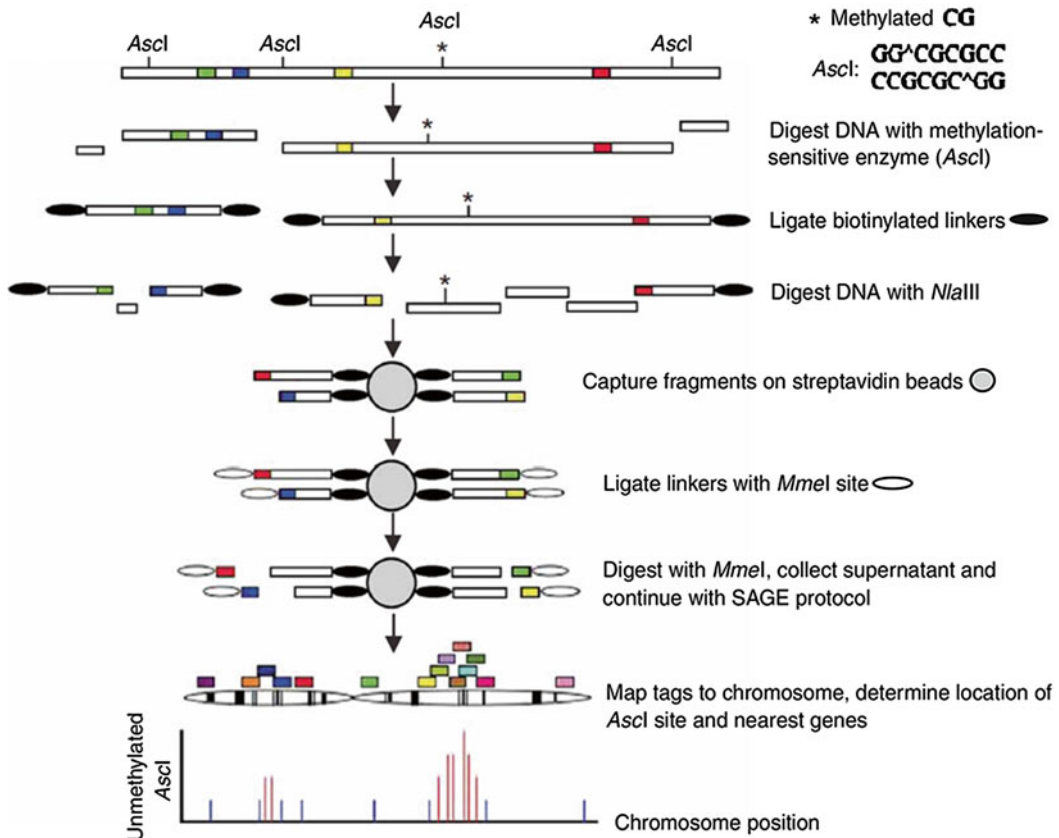


Fig. 1 Assessment of hypermethylation of CpG sites in gene promoters in breast cancer



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Fig. 2 Schematic of the MSDK approach. Reprinted by permission from Macmillan Publishers Ltd: [Nature Genetics] (37(8):899-905), copyright (2005)

comprehensive DNA methylation profiling technology and was developed by the K. Polyak lab at Dana Farber Cancer Institute and Harvard Medical School [27]. The MSDK method allows for comprehensive and unbiased methylation profiling. The principles and overview of this approach is provided in Fig. 2.

DNA Methylation in Hormone Receptor Positive and Negative Breast Cancer

Different epigenetic profiles have also been identified between hormone receptor positive and negative tumors [23, 28, 29]. Although array-based methylation analysis can measure large numbers of genes simultaneously, there are some shortcomings. Array-based analysis is not able to provide quantitative measurement of CpG methylation and requires subsequent experiments to confirm and validate findings. Hence the use of new techniques, such as pyrosequencing is warranted. Pyrosequencing is a sequencing-by-synthesis method which can quantify DNA methylation at specific CpG sites within the target region of interest. Feng et al. used pyrosequencing methylation analysis to identify

two panels of methylation profiles which correlated with hormone receptor expression in breast cancer [28]. Specifically, the study examined 12 tumor suppressor genes (*ARHI*, *RASSF1A*, *HIN-1*, *RAR β 2*, *hMLH1*, *14-3-3 σ* , *RIZ1*, *p16*, *E-cadherin*, *RIL*, *CDH13*, and *NKD2*) in 90 pairs of malignant/normal breast tissues. The data from this study showed that 5 (*RIL*, *HIN-1*, *RASSF1A*, *CDH13*, and *RAR β 2*) out of 12 genes examined were frequently methylated in breast cancer tissues, but not in normal breast tissues. The methylation of *HIN-1* and *RASSF1A* strongly correlated to the expression of ER and/or PR, while, the methylation of *RIL* and *CDH13* strongly correlated to negative ER and/or PR. Subsequent studies have shown that the differences of methylation profiles between hormone receptor positive and negative breast tumors can also influence tumor response to hormonal therapy such as tamoxifen [29, 30].

Approximately 70 % of breast tumors are positive for ER expression at the time of diagnosis. Patients with ER+ breast cancer are candidates for tamoxifen treatment which competes with estradiol for binding to ER. Postmenopausal women with ER+ breast tumors are also candidates for treatment with aromatase inhibitors (AIs). AIs reduce the production of estrogen through inhibition of the enzyme aromatase that synthesizes estrogen from testosterone and androstenedione [31, 32]. Despite the well documented benefits of hormone therapy for ER+ breast cancer patients, not all patients with ER+ tumors respond to the therapy. As many as one third of ER+ breast cancer patients experience relapse within 5 years and develop drug resistant tumors [33]. Lack of ER expression and/or PR status have been identified as major causes of resistance. Interestingly, there are a number of studies showing that the 5'UTR of the *ESR1* gene is methylated in ER-negative breast cancer cell lines [34, 35]. The methylation of ER CpG islands was specifically confirmed in breast tumor tissues [36]. However, some recent studies [29] have shown no significant relationship between *ESR1* expression levels and quantified *ESR1* methylation levels when the tumors were analyzed collectively. A trend between *ESR1* gene expression and *ESR1* methylation levels in PR-negative tumors was, however, observed in their study [29].

Recently, Pathiraja et al. reviewed several studies which utilized high-throughput DNA methylation profiling to identify DNA methylation of candidate genes that contribute to resistance [34]. Widschwendter and colleagues utilized MethyLight analysis to examine 148 tumors from patients who had received adjuvant tamoxifen therapy and found promoter methylation of *ESR1* and *CYP1B1* predicted response to tamoxifen treatment [29]. Another study by Martens and team examined 499 CpG sites from regulatory regions of 117 candidate genes from 200 ER and/or PR+ tumors [37]. The bisulfite treated DNA was hybridized to an array of immobilized oligonucleotides reflecting the methylated

(CG) and non-methylated (TG) status for each CpG position. The hybridization conditions allowed the detection of the single nucleotide differences. Of the genes analyzed, the team identified ten genes with promoter DNA methylation status that correlated with clinical outcome and endocrine therapy resistance: *PSAT1*, *STMN1*, *SI00A2*, *SFN*, *PRKCD*, *SYK*, *VTN*, *GRIN2D*, *TGFBR2*, and *COX7A2L*. Specifically, Martens et al. identified that phosphoserine aminotransferase (*PSAT1*) was strongly correlated with poor outcome from all genes screened, and hypermethylation of the *PSAT1* promoter was a good prognostic marker for outcome [37].

DNA Methylation and Molecular Subtypes of Breast Cancer

Molecular subtypes of breast cancer have also been associated with DNA methylation. Subtypes are determined by gene expression profiles and expression of ER/PR, HER2 (human epidermal growth factor receptor 2), cytokeratin 5/6 (CK5/6), and/or HER1 (EGFR, human epidermal growth factor receptor 1) expression. Breast cancer can be classified as five main subtypes, Luminal A (ER/PR+/HER2-), Luminal B (ER/PR+/HER2+), HER2-enriched type (ER/PR-/HER2+), basal-like (ER/PR-/HER2-, CK5/6+ and/or HER1+)/triple negative (TNBC), and normal breast like or unclassified (breast cancers that do not fall into these four subtypes) [38, 39]. Overall about 42–59 % of breast cancers are Luminal A subtype, Luminal B subtypes are 6–10 %, ER/PR-/HER2+ subtypes are 7–12 % and TNBCs are 15–20 % [40, 41].

Similar to gene profiling patterns, epigenetic patterns have been identified that correlate with breast cancer subtypes in multiple studies [23, 26, 42, 43]. Holms and colleagues used the methylation array (Illumina GoldenGate Methylation Cancer Panel I, from Illumina, San Diego, CA, USA) in their study of 1,505 CpG loci corresponding to 807 cancer-related genes [42]. High methylation frequency was found among Luminal type tumors compared to basal-like tumors by array-based methylation assays. The analysis included a total of 189 fresh frozen primary breast tumors from different molecular subtypes of breast cancer and identified that subtype-specific genes were often regulated by methylation. Basal-like, Luminal A and Luminal B tumors had different methylation profiles as shown in Fig. 3a. The frequency of methylation in Luminal B samples was significantly higher than in basal-like tumors (Fig. 3b). Breast tumors with amplification of the HER2 gene are less likely to respond to therapy and more likely to develop metastatic disease [44]. Trastuzumab (Herceptin), an antibody targeting the HER2 receptor, has been shown to successfully treat HER2+ breast cancer and prolong the survival of patients with metastatic HER2-overexpressing breast cancer [45, 46]. However, a significant portion of patients with HER2+ breast tumors will eventually become resistant to trastuzumab [47].

Methylation patterns in relation to subtype have been most studied in the basal-like or Triple Negative Receptor Breast Cancer

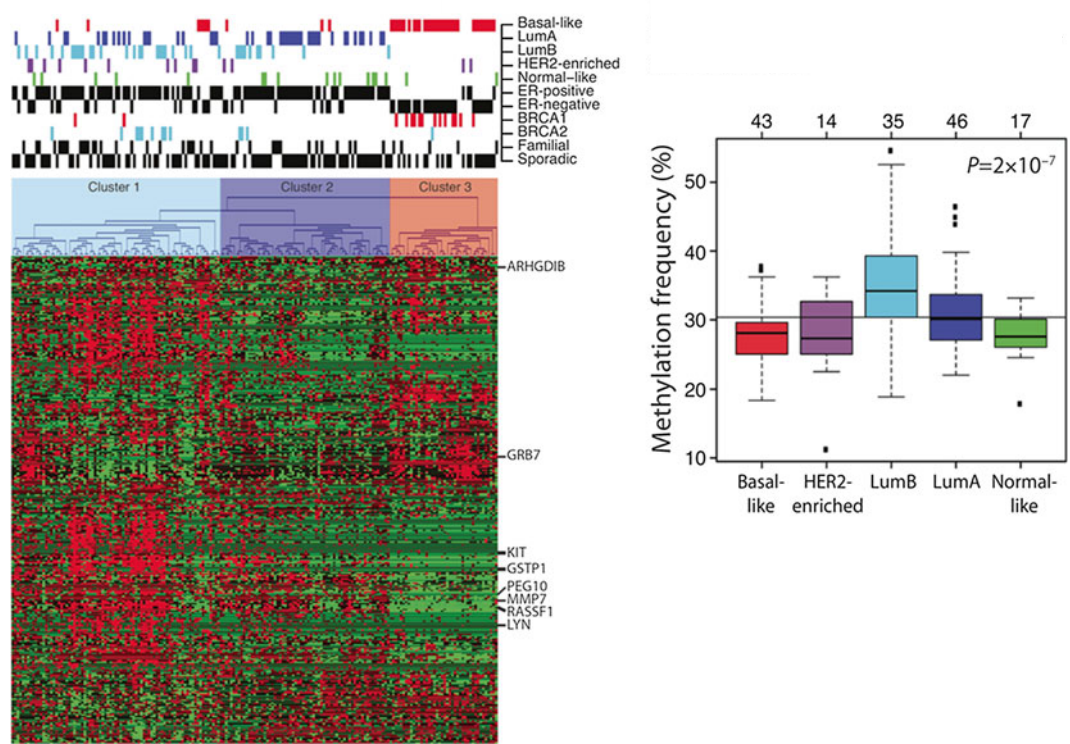


Fig. 3 Methylation clustering by subtype. (a) Hierarchical clustering of gene methylation. Heatmap shows relative methylations levels (*red*, more methylated; *green*, less methylated). Clustering resulted in three clusters: Cluster 1 (Luminal B), Cluster 2 (Luminal A), and Cluster 3 (Basal-Like). Adapted from [42]. (b) Boxplot stratified by subtype for methylation frequencies of the 196 subtype-associated CpGs. CpGs represented in the *plot* are more frequently methylated in Luminal B tumors and less methylated in basal-like tumors. *P*-value was calculated using analysis of variance. The number of tumors in each subtype is shown at the *top axis*. Adapted from [42]

(TNBC) subtype. Branham et al. analyzed methylation of CpG islands in 69 cancer-related genes in TNBC tumors and compared the methylation profile with non-TNBC tumors [48]. The assay used for characterization of the methylation profile was Methyl-specific multiplex ligation-dependent probe amplification assay (MS-MLPA) obtained from MRC-Holland, Amsterdam, The Netherlands (www.mrc-holland.com). The MS-MLPA assay extended restriction enzyme incubation time, separated ligation and restriction steps which reduced background signals. The PCR products were fluorescently labeled and separated by capillary electrophoresis (ABI-3130 sequencer, Applied Biosystems, Foster City, CA, USA). The resulting data was subsequently analyzed by GeneMarker v1.75 software (Softgenetics, State College, PA, USA) which normalized the data. The normalized peaks from the analyzed samples were compared with the normalized peaks from the control reaction. Through this method, a specific panel of genes including *CDKN2B*, *CD44*, *MGMT*, *RB*, and *p73* was found to be methylated in TNBC tumors, but not in non-TNBC tumors [48].

Promoter Hypermethylation of Tumor Suppressor Genes in Breast Cancer

Epigenetic inactivation of the tumor suppressor genes, such as *BRCA1*, has been implicated as important events in sporadic breast cancer [9, 10, 49]. Early reports showed that one of the key mechanisms of *BRCA1* expression loss was epigenetic silencing [10, 50]. More recently, loss of expression of *BRCA1* has been associated with basal-like/TNBC tumors [51–53]. Stefansson et al. examined *BRCA1* methylation in 111 sporadic breast tumors which had previously been screened for *BRCA1* germline mutations [53]. The study demonstrated that CpG island hypermethylation of *BRCA1* was significantly associated with basal-like/TNBC tumors [53]. Another recent study by Hsu et al. also examined methylation of *BRCA1* promoters from 139 early stage breast cancer tissues using Methylation-specific PCR (MSP) and their data also demonstrated an association of *BRCA1* promoter methylation and TNBC type of tumors [54].

Another key tumor repressor in breast cancer implicated in epigenetic mediated loss is *PTEN* [18, 26, 55–57]. The gene encodes PIP3 phosphatase and negatively regulates the PIP3-Akt pathway. Loss of *PTEN* can lead to activation of Akt pathway, suppression of apoptosis, and increased cell survival [58]. Expression of *PTEN* protein has been found to be lost or reduced in 38 % of invasive breast cancer [55]. Promoter hypermethylation has been implicated as a key mechanism of loss of the *PTEN* gene in breast cancer. Khan et al. examined *PTEN* promoter methylation from 44 invasive breast tumors using MSP. The study found that 34 % tumors had hypermethylation of *PTEN* genes and the *PTEN* promoter hypermethylated tumors had loss of *PTEN* protein in 60 % of samples [55]. Similarly, Garcia et al. analyzed promoter hypermethylation of the *PTEN* gene in 90 invasive breast tumors and found that the *PTEN* promoter was hypermethylated in 48 % of tumor tissues [56]. Subsequent studies have confirmed these findings and further associated *PTEN* hypermethylation with *ERBB2* overexpression, large tumor size, and higher histologic grade [26].

2.1.2 Methylation Profile and Breast Cancer Health Disparities

The incidence and mortality of breast cancer can vary significantly among racial and ethnic groups [21]. Although the overall survival from breast cancer has improved, African-American women still show the worse outcome and increased mortality at virtually all age groups [21]. In addition to socioeconomic factors, biological factors and lifestyles may contribute to breast cancer health disparity. Studies have suggested that differences in the frequency of tumor receptor-subtypes among African-American women may potentially play a role in the reduced cancer outcomes [59]. Specifically, younger African-American women are more often diagnosed with the TNBC subtype compared to European-American/Caucasian women [40, 59, 60–62]. Notably, women with TNBC have limited treatment options since targeted receptor therapies such as tamoxifen for HR+ tumors, and trastuzumab for HER2+ tumors would not be recommended [63].

In addition to clinicopathological factors, varying epigenetic patterns may also contribute to breast cancer disparities. For example, Mehrotra and colleagues examined the frequency of promoter hypermethylation in five key genes implicated in breast cancer (*HIN-1*, *Twist*, *Cyclin D2*, *RAR- β* , and *RASSF1A*) from African-American and Caucasian patient tumors. Among the cohort of women with ER/PR- tumors and <50 years old, the study identified a higher methylation frequency of *HIN-1*, *Twist*, *Cyclin D2* and *RASSF1A* in African-American women compared with Caucasians [64]. Another study conducted by Dumitrescu et al. investigated tumor suppressor gene promoter hypermethylation in breast tissue from healthy African-American and Caucasian women. The study found *p16^{INK4}* promoter hypermethylation was more frequently observed among Caucasian women with family history and *BRCA1* promoter hypermethylation was more frequently observed among African American women with family history [65]. Hence, there is data to suggest that differences in the frequency of gene promoter methylation may influence the disease outcome of breast cancer among African-American and Caucasian women. Studies investigating the role of epigenetics on other populations including the Hispanic/Latino, Asian, and Native American/Pacific Islander women are needed to better understand the implications of epigenetics on disparities.

2.1.3 DNA Methylation and Histone Modification in Breast Cancer

DNA (5-cytosine)-methyltransferases (DNMTs) are enzymes that methylate the cytosine residue of CpGs. Four major types of DNMTs have been identified (DNMT1, 2, 3a, and 3b), among which DNMT1, DNMT3a, and DNMT3b are primarily active [66]. The demethylating drugs 5-azacytidine (VidazaTM) and 5-aza-2'-deoxycytidine (decitabine) are the most studied DNMT inhibitors [9]. These compounds incorporate into the DNA replacing cytosine during DNA replication and result in covalent trapping of DNMTs. The trapped DNMTs cannot continue methylation, hence, the cell loses DNMT activity resulting in DNA demethylation [67]. Although DNA methylation inhibition alone can reduce gene silencing, abnormal histone modification in combination with DNA hypermethylation can frequently compound gene silencing and complicate reversal of expression. Figure 4 shows a schematic of epigenetic modification that regulates chromatin organization and gene expression. The interrelated activity of DNA level and histone level epigenetic modification suggests effective therapies to reverse silencing must apply dual targeting of both the DNA and histone level modifications [2].

The use of a 5-aza-2'-deoxycytidine and HDAC inhibitors (such as trichostatin A) separately have been evaluated clinically in multiple cancer systems, with greatest focus on leukemias and myelodysplastic syndromes [68, 69]. Further investigation is warranted in solid tumor systems such as breast, prostate, and colorectal cancer. In breast cancer, there is substantial preclinical data to

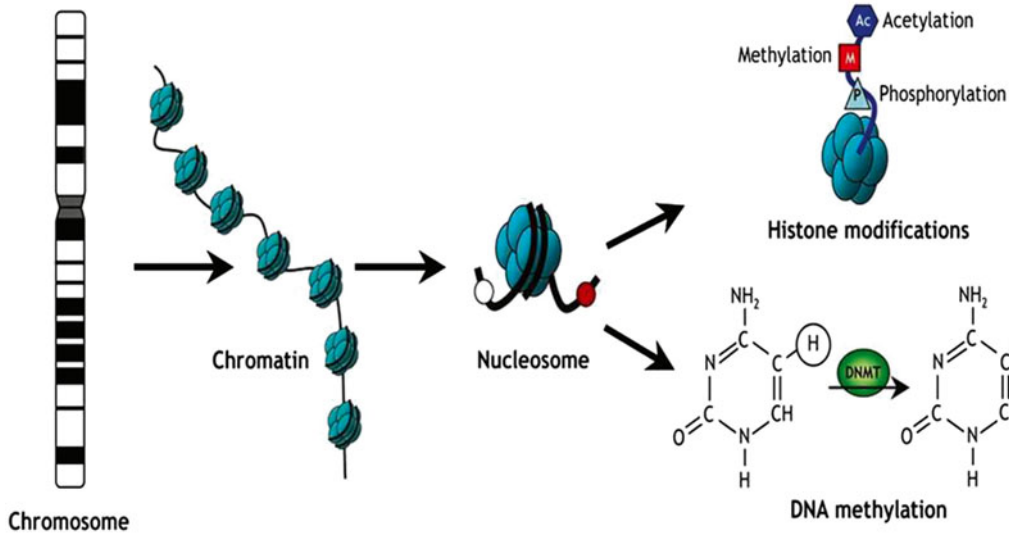


Fig. 4 Schematic of epigenetic modifications that regulate chromatin organization and gene expression. Strands of DNA are wrapped around histone octamers, forming nucleosomes, which organize chromatin. Reversible and site-specific histone modifications occur at multiple sites through acetylation, methylation, and phosphorylation. DNA methylation occurs at 5-position of cytosine residues in a reaction catalyzed by DNA methyltransferases (DNMTs). Together, these modifications provide a unique epigenetic signature that regulates chromatin organization and gene expression. Adapted from Luong P., Basic Principles of Genetics [Connexions Web site]. March 2, 2014. Available at: <http://cnx.org/content/m26565/1.1/>

suggest combined therapy may be efficacious. For example, a study by Wu et al. investigated Caspase 8 (an apoptosis related gene) which is methylated in human breast cancer cell lines, MDA-MB231 (TNBC line), SKBR3 (HER2+), BT474 (HER2-positive), and MCF-7 (ER-positive) [16]. Treatment with 5-aza-2'-deoxycytidine inhibited DNMT3a and DNMT3b and reactivated *CASP8* gene in MDA-MB231, SKBR3, and BT474, but not in MCF-7 cells. Chromatin Immunoprecipitation (ChIP) assay identified that the silencing of the *CASP8* gene in MCF7 involved methylation of histone H3 (Lys27). Only through combined treatment with 5-aza-2'-deoxycytidine and the HDAC inhibitor, trichostatin A, was the gene silencing reversed and Caspase 8 expression significantly restored [16]. This approach can potentially be utilized to restore other silenced genes implicated in breast cancer tumorigenesis and resistance to disease. An excellent target would be the *ESR1* gene which can be demethylated to restore expression of ER and subsequent tumor response to targeted therapy such as tamoxifen. Indeed, Fan and colleagues have confirmed that combined treatment does restore ER expression in ER- breast cancer cell lines and restores response to ER targeted therapy [70]. Additional studies to confirm these findings in primary TNBC cells lines and additional preclinical models is warranted particularly with the new sets of histone methyltransferase and demethylase inhibitors available for testing (see Table 1).

Table 1

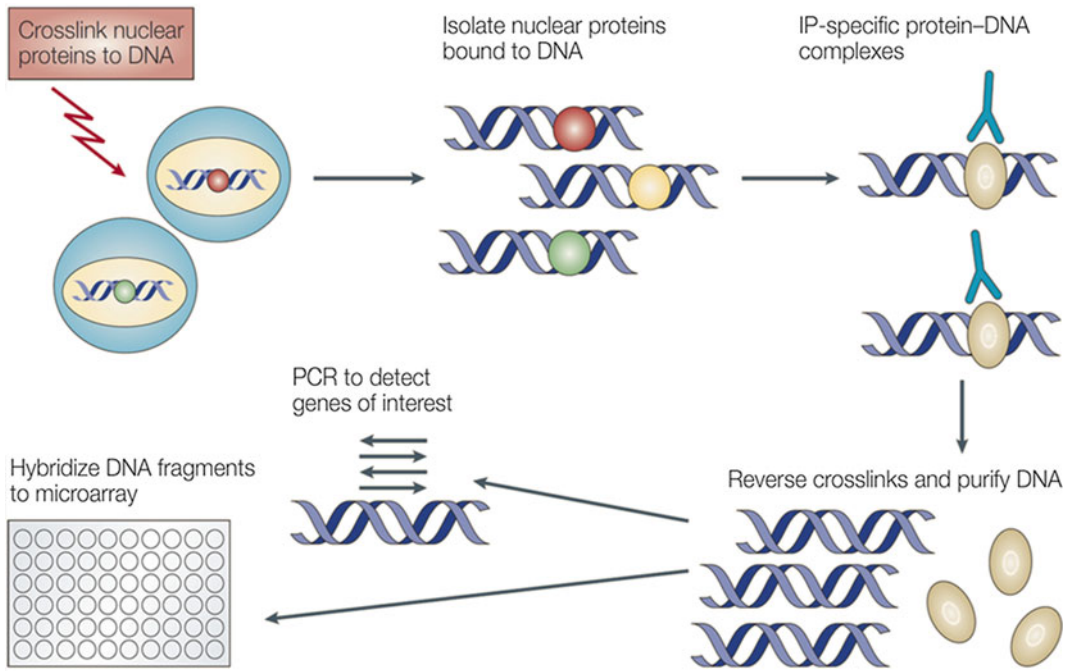
Examples of histone methyltransferases and demethylase inhibitors [116]

Inhibitor name	Major targeted enzyme	Histone targets
Chaetocin1	Suv39h1, G9a	H3K9
Clorgyline	LSD1	H3K4
DZNep	EZh1	H3K27
N-oxalylglycine	JMJD2A, JMJD2C	H3K9, H3K36
Novobiocin	SMYD3	H3K4
Pargyline	LSD1	H3K4
Polyamine analogs	LSD1	H3K4
Tranilcypromine	LSD1	H3K4

Further studies investigating the efficacy of these compounds could benefit from the use of the ChIP assay. The ChIP assay is a powerful tool for determining histone modification status. Figure 5 shows the assay principles and major steps. When performing the ChIP assay, cells are first reversibly fixed (such as with formaldehyde) which serves to cross-link or “preserve” the protein–DNA interactions occurring in the cell nucleus. Cells are then lysed and chromatin is harvested and fragmented using either sonication or enzymatic digestion. The chromatin is then subjected to immunoprecipitation using antibodies specific to a particular protein or histone modification. Any DNA sequences that are associated with the protein or histone modification of interest will co-precipitate as part of the cross-linked chromatin complex and the relative amount of that DNA sequence will be enriched by the immunoselection process. After immunoprecipitation, the protein–DNA cross-links are reversed and the DNA is purified. The enrichment of a particular DNA sequence or sequences can then be detected by a number of different methods, such as PCR, q-PCR, immunoblotting, or assessing on DNA hybridized array.

2.2 Summary

Aberrant epigenetic modifications in breast cancer impact a multitude of cell functions including cell cycle regulation, DNA repair, hormone regulation (*ER* α , *PR*), cell adhesion, invasion, angiogenesis, and cellular growth-inhibitory signaling related genes. A summary of genes with CpG hypermethylation in breast cancer and methods used for identification is summarized in Table 2. Although breast tumors are also frequently hypomethylated on genome-wide scale, the number of genes reported as hypomethylated in breast cancer is relative small. When assessing epigenetic changes, both DNA and histone level modifications must be examined, particularly if modified genes are therapeutic targets.



Patel J, et al. *Nat Rev Cancer*. 2004 Jul;4(7):562-8.

Fig. 5 Principles of Chromatin Immunoprecipitation assay (ChIP). Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Cancer] (Jul;4(7):562-8), copyright (2004)

3 Prostate Cancer

3.1 DNA Methylation in Prostate Cancer

Prostate cancer is the most frequently diagnosed cancer and second leading cause of cancer death in men in the USA and Western countries [21]. Prostate cancer is one of the most common human malignancies that arises through genetic and epigenetic alterations. Overall there are numerous genes that undergo aberrant hypermethylation in prostate cancer. These genes include classic and putative tumor-suppressor genes involved in a number of cellular pathways such as hormonal responses, tumor-cell invasion and/or tumor architecture, cell cycle control, and DNA damage repair [71–73]. For many of these genes, promoter hypermethylation is often the main mechanism underlying their functional loss. Silencing of these genes can contribute to cancer initiation, progression, invasion, and metastasis. Hypermethylation of genes in prostate cancer has been correlated in several studies with pathologic grade, clinical stage, androgen independence, as well as outcome [74, 75]. A better understanding of the epigenetic changes in prostate cancer is likely to contribute to improved diagnosis, clinical management, and better outcome.

Table 2
Summary of genes hypermethylated in breast cancer

Gene	Official gene name	Methods of identification
14-3-3sigma	Stratifin (SFN)	MSP + Bisulfite sequencing
ABCB1	ATP-binding cassette, sub-family B, member 1	MethyLight
AK5	AK5 adenylate kinase 5	MSP + Bisulfite sequencing
AMN	Amnion associated transmembrane protein	MSP + Bisulfite sequencing
APC	Adenomatous Polyposis Coli	MSP
BCL2	B-cell CLL/lymphoma 2	MethyLight
BRCA1	Breast Cancer 1, early onset	MSP + Bisulfite sequencing
CALCA	Calcitonin-related polypeptide alpha	MethyLight
CASP8	Caspase-8, Apoptosis-related cysteine	MSP
CCND2	Cyclin D2	MSP
CDCP1	CUB domain containing protein 1	Bisulfite sequencing
CDH1	Cadherin 1, E-cadherin	MSP + Bisulfite sequencing
CDH13	Cadherin 13, H-cadherin	MSP
CDKN1C	P57	MSP
CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16, p14ARF)	MethyLight
CEBPD	CCAAT/enhancer binding protein	MSP
CLCA2	Chloride channel, calcium activated, family member 2	Bisulfite sequencing
CST6	Cystatin E/M	MSP + Bisulfite sequencing
Cx26	Connexin 26	MSP
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	MethyLight
DAB2	Disabled homolog 2, mitogen-responsive phosphoprotein	Bisulfite sequencing
DAL1	Erythrocyte membrane protein band 4.1-like 3	MSP
DAPK	Death Associated Protein Kinase 1	MethyLight
DCC	Deleted in colorectal carcinoma	MSP + Bisulfite sequencing
DLC1	Deleted in liver cancer 1	MSP + Bisulfite sequencing
DSC3	Desmocollin 3	Bisulfite sequencing
ESR1	Estrogen receptor 1	MethyLight
ESR2	Estrogen receptor 2	MethyLight
FHIT	Fragile histidine triad gene	MSP
FOXA2	Forkhead box A2	MSP + Bisulfite sequencing

(continued)

Table 2
(continued)

Gene	Official gene name	Methods of identification
GPC3	Glypican 3	MSR + Southern Blot
GREM1	Gremlin 1	MSP
GSTP1	Glutathione S-transferase pi	MethyLight; MSP
HIC-1	Hypermethylated in cancer 1	MSR + Southern Blot
HOXA5	Homeobox A5	MSP + Bisulfite sequencing
HOXD11	Homeobox D11	MSP + Bisulfite sequencing
HRAS	Harvey rat sarcoma viral oncogene homolog	MethyLight
HS3ST2	Heparan sulfate 3-O-sulfotransferase 2	MSP
HSD17B4	Hydroxysteroid (17-beta) dehydrogenase 4	MethyLight
hTERT	Telomerase reverse transcriptase	MethyLight; MSP
ID4	Inhibitor of DNA binding 4	MSP
IGFBP3	Insulin-like growth factor binding protein 3	MSP
KLK10	Kallikrein-related peptidase 10 (NES1)	MSP + Bisulfite sequencing
KLK6	Kallikrein-related peptidase 6	Bisulfite sequencing
LAMA3	Laminin, alpha 3	MSP
LAMB3	Laminin, beta 3	MSP
LAMC2	Laminin, gamma 2	MSP
LATS1/LATS2	Large tumor suppressor, homolog 1/2	MSP
NKD2	Naked cuticle homolog 2	Pyrosequencing
SERPINB5	Maspin, mammary serine protease inhibitor	MSP
MGMT	O-6-methylguanine-DNA methyltransferase	MethyLight
MLH1	MutL homolog 1, colon cancer, nonpolyposis type 2	MethyLight
MYOD1	Myogenic differentiation	MethyLight
PAX5	Paired box 5	MSP + Bisulfite sequencing
PCDH10	Protocadherin 10	MSP + Bisulfite sequencing
PGR	Progesterone receptor	MSR + Southern Blot
PLAGL1	Pleiomorphic adenoma gene-like 1	MSP
PTEN	Phosphatase and tensin homolog	MSP
PTGS2	Prostaglandin-endoperoxide synthase 2 (Cox-2)	MethyLight
RAD9	RAD9 homolog A	Bisulfite sequencing

(continued)

Table 2
(continued)

Gene	Official gene name	Methods of identification
RAR- β	Retinoic acid receptor, beta	MSP + Bisulfite sequencing
RARRES1	Retinoic acid receptor responder 1	MSP
RASSF1A	Ras associated domain family 1	MSP
RBP1	Retinol binding protein 1	MSP
RIZ1	PR domain containing 2, with ZNF domain (PRDM2)	MSP
RNR1	Mitochondrially encoded 12S RNA	MSP + Bisulfite sequencing
ROBO1	Roundabout, axon guidance receptor, homolog 1	Bisulfite sequencing
RUNX3	Runt-related transcription factor 3	MSP + Bisulfite sequencing
SCGB3A1	Secretoglogin, family 3A, member 1 (HIN-1)	MSP
SERPINB5	Serpin peptidase inhibitor, clade B, member 5	Bisulfite sequencing
SFRP1	Secreted frizzled-related protein 1	MSP + Bisulfite sequencing
SIM1	Single-minded homolog 1	MSP + Bisulfite sequencing
SLIT2	Slit homolog 2	MSP
SOCS1	Suppressor of cytokine signaling 1	MSP + Bisulfite sequencing
SRBC	Protein kinase C, delta binding protein (PRKCDBP)	Bisulfite sequencing
SULT1A1	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1	Bisulfite sequencing
SYK	Spleen tyrosine kinase	MSP
TERT	TERT telomerase reverse transcriptase	MethyLight
TDH	L-threonine dehydrogenase	MSP + Bisulfite sequencing
TFF1	Trefoil factor 1	MSP + Bisulfite sequencing
TGF- β R2	Transforming growth factor β receptor 2	MSP + Bisulfite sequencing
THBS1	Thrombospondin 1	MSP + Bisulfite sequencing
TIMP3	TIMP metalloproteinase inhibitor 3	MSP + Bisulfite sequencing
TMEFF2	Transmembrane with EGF-like and two follistatin-like domains 2	MSP
TMS1	PYD and CARD domain containing (PYCARD)	MSR + Southern Blot
TNFRSF12	Tumor necrosis factor receptor superfamily, member 25	MSP + Bisulfite sequencing
TPM1	Tropomyosin 1	Bisulfite sequencing
TSC1	Tuberous sclerosis 1	MSP

(continued)

Table 2
(continued)

Gene	Official gene name	Methods of identification
TSC2	Tuberous sclerosis 2	MSP
TSLC1	tumor suppressor in lung cancer 1	MSP
TSPAN-2	Tetraspanin 2	MSP + Bisulfite sequencing
TWIST1	Twist homolog 1	MSP
TYMS	Thymidylate synthetase	MSP + Bisulfite sequencing
WIF1	WNT inhibitory factor 1	MSP + Bisulfite sequencing
WRN	Werner syndrome	MSP
XT3	Solute carrier family 6, member 20 (SLC6A20)	MSP + Bisulfite sequencing

3.1.1 Hypermethylation of CpG Islands of Genes for Risk Prediction of Prostate Cancer

Aberrant DNA methylation patterns may be the earliest somatic genome changes in prostate cancer. Studies have suggested methylation of CpG sites of gene promoters may predict risk of prostate cancer [72]. Studies assessing predictive methylation patterns compared methylation status between cancer tissues and non-cancer tissues. One study by Yegnasubramanian et al. using real-time methylation-specific PCR (Q-MSP) assessed CpG island hypermethylation of 16 genes from seven prostate cancer cell lines (LNCaP, PC-3, DU-145, LAPC-4, CWR22Rv1, VCaP, and C42B), normal prostate epithelial cells, normal prostate tumor cells, 73 primary prostate cancers, 91 metastatic prostate cancers, and 25 noncancerous prostate tissues [76]. The study identified that CpG islands at *GSTP1*, *APC*, *RASSF1a*, *PTGS2*, and *MDR1* were hypermethylated in more than 85 % of prostate cancers and cancer cell lines. CpG islands at *EDNRB*, *ESR1*, *CDKN2a*, and *hMLH1* exhibited low to moderate rates of hypermethylation in prostate cancer tissues and cancer cell lines but were entirely unmethylated in normal tissues. Furthermore, hypermethylation of the CpG island in *EDNRB* was correlated with the grade and stage of primary prostate cancers and *PTGS2* CpG island hypermethylation portended an increased risk of recurrence [76].

Another study conducted by Vanaja et al. investigated use of CpG sites as molecular markers to distinguish indolent and aggressive prostate tumors [77]. The study examined the methylation status of 8 genes, including *FLNC*, *EFS*, *ECRG4*, *RARB2*, *PITX2*, *GSTP1*, *PDLIM4*, and *KCNMA1* in 32 non-recurrent, 32 recurrent primary prostate tumors, and 32 benign prostate tissues using EpiTYPER™ technology (Sequenom, Inc, San Diego, CA). CpG site hypermethylation of *FLNC*, *EFS*, *ECRG4*, *PITX2*, *PDLIM4*, and *KCNMA1* genes were found to predict local and systemic recurrence of prostate cancer. Specific CpG site hypermethylation of

RARβ2 and *GSTP1* CpG sites were found to be useful for diagnosis of prostate cancer [77].

Additional studies conducted by Mahapatra and colleagues used microarray analysis to compare promoter global hypermethylation profiles from prostate cancer vs. normal tissues. They identified 25 genes which were significantly methylated in prostate cancer tissues compared with normal tissues: *AOX1*, *CYBA*, *EDG3*, *ELF4*, *EPB41L3*, *FLJ12056*, *FLJ90650*, *FLT4*, *GAS6*, *GRASP*, *GSTP1*, *HAAO*, *HIF3A*, *HOXC11*, *LEP*, *MGC39606*, *MOBK2B*, *RAB34*, *RARβ2*, *RHCG*, *RND2*, *SLC34A2*, *SPATA6*, *TPM4*, and *ZNF154*. The results were confirmed by pyrosequencing. The distinct profiles identified by this study could be used as biomarkers to identify subjects who may be at high risk to develop aggressive prostate cancer [78].

3.1.2 Methylation Profile in Localized and Advanced Prostate Cancer

Mahapatra and colleagues also investigated the role of epigenetics on progression of prostate cancer. Specifically, the study utilized microarray analysis with DNA samples from prostate cancer and normal adjacent tissues from 238 patients. DNA methylation analysis was conducted using the Illumina™ Infinium™ Human Methylation 27 Bead Chip. The bead chip allowed the interrogation of 27,578 CpG sites representing 14,495 protein-coding gene promoters, which includes about 1,000 cancer-associated genes. Four distinct methylation profiles emerged for: (a) normal vs. tumors, (b) recurrence vs. non-recurrence, (c) clinical recurrence vs. biochemical recurrence, and (d) systemic recurrence vs. local recurrence. The normal vs. tumors profile was described earlier above. Notably, a 25 gene profile was identified which was associated with recurrent prostate cancer including the following genes: *ACTL6B*, *AEBP1*, *AMID*, *CD8A*, *CRIP1*, *FLJ30934*, *FLNC*, *FMOD*, *FOXE3*, *GAS7*, *GDPD5*, *HS3ST2*, *LOC349136*, *NEUROG1*, *PLTP*, *PTGER2*, *RASGRF2*, *RUNX3*, *SIX6*, *SLC9A3*, *SPSB4*, *SRD5A2*, *SUSD3*, *SYT10*, and *TMEM74*. Validation of genes by pyrosequencing from group 1 (*GSTP1*, *HIF3A*, *HAAO*, and *RARβ2*), group 2 (*CRIP1*, *FLNC*, *RASGRF2*, *RUNX3*, and *HS3ST2*), group 3 (*PHLDA3*, *RASGRF2*, and *TNFRSF10D*), and group 4 (*BCL11B*, *POU3F3*, and *RASGRF2*) confirmed the microarray results.

The study results revealed an expanded list of genes as which can be used both for biomarkers for diagnosis and prognosis of prostate cancer. Interestingly, alterations in methylation that were identified in early stage tumors were very homogeneous whereas methylation patterns in tumors from recurrent patients were more heterogeneous. This can be explained by the increasing number of epigenetic aberrations that accumulate during tumor progression, and tumor cells acquire increasingly unique epigenetic changes. Therefore, no single gene can likely predict the progression of all advanced prostate tumors. The altered methylation of

genes associated with cancer recurrence should be combined with histopathologic workup for a more robust prediction of prostate cancer progression.

In a similar study, Lin and colleagues examined 20 benign prostate tissues, 16 prostate cancers and 8 advanced prostate cancers (both metastatic and castration-resistant) using MassARRAY™ EpiTYPER™ assay (Sequenom, San Diego, CA). Matrix-assisted laser desorption ionization/time-of light mass spectrometry was utilized. Their data suggested that most DNA methylation changes occurred in the context of allele-specific methylation and the variations in tumor epigenetic landscape of individuals may be partly mediated by genetic differences that may affect prostate cancer disease progression [79].

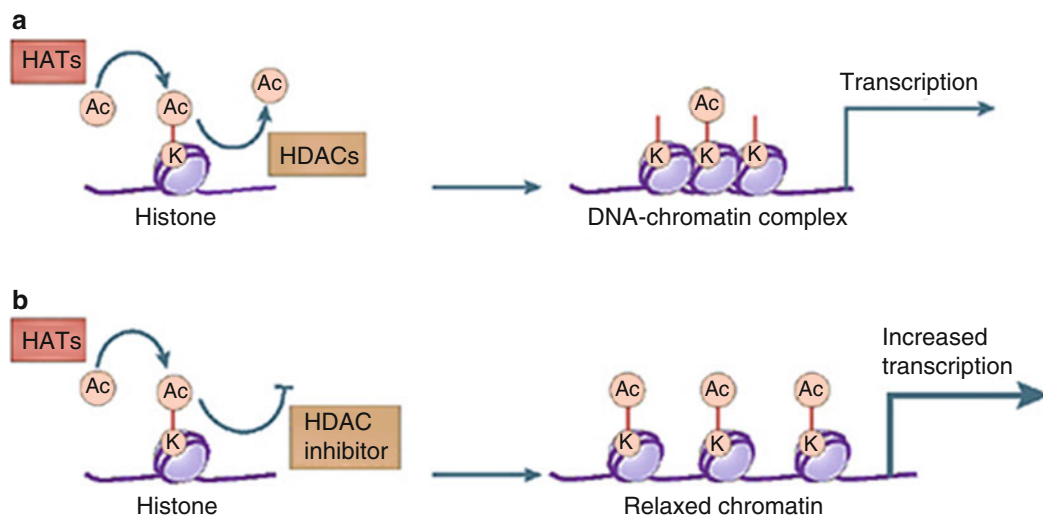
3.1.3 Methylation Patterns Between Androgen Receptor Positive and Androgen Receptor Negative Prostate Cancer

A study from the Vadgama lab characterized the promoter methylation profile of 82 genes in three prostate cancer cell lines (LNCaP, PC3, and DU145) and two normal prostate cell lines (RWPE1 and RWPE2) using TranSignal Promoter Methylation Array (Panomics Inc., CA, USA) [17]. The data showed that >50 % of the genes were hypermethylated in prostate cancer cells compared with 13 % in normal cell lines. Among hypermethylated genes were genes for tumor suppressors (*RB*, *TMS1*, *DAPK*, *RBL1*, *PAX6*, and *FHIT*), cell cycle (*p27KIP1* and *CDKN2A*), transporters (*MDR1*, *MLC1*, and *IGRP*), and transcription factors (*STAT1*, *CIITA*, *MYOD*, and *NPAT*) genes. Relative methylation patterns show that most of these genes were methylated from 5-fold to >10-fold compared with normal prostate cells. In addition, the study identified promoter methylation for the first time in genes such as *RIOK3*, *STAT5*, *CASP8*, *SRBC*, *GAGE1*, and *NPAT*. Furthermore, a significant difference in methylation pattern was observed between AR-sensitive versus AR-negative cancer cells for the following genes: *CASP8*, *GPC3*, *CD14*, *MGMT*, *IGRP*, *MDR1*, *CDKN2A*, *GATA3*, and *IFN*. Identification of differences in methylation pattern between androgen-sensitive and androgen-independent prostate cells provided further insight into potential target genes relevant in the diagnosis, prognosis, and treatment of prostate cancers.

The use of the TranSignal Promoter Methylation Array from Panomics was key for screening multiple gene promoters. The TranSignal Array consists of immobilized probes of known gene promoters on a nitrocellulose membrane. Methylation binding protein-purified methylated DNA was hybridized on the membrane and detected by the chemiluminescence method.

3.1.4 Histone Modification and Androgen Receptor Activity

Chromatin remodeling and histone post-translational modifications also play an important role in deregulation of gene expression in prostate cancer [80]. Figure 6 shows an example of histone acetylation at specific lysine (K) residues and the effect of chromatin modification on gene transcription [81]. Korkmaz and colleagues



Kazantsev AG, et al. *Nat Rev Drug Discov.* 2008 Oct;7(10):854-68.

Fig. 6 Example of histone acetylation. **(a)** Levels of histone acetylation at specific lysine (K) residues are determined by concurrent reactions of acetylation (AC) and deacetylation, which are mediated by histone acetylases (HATs) and histone deacetylases (HDACs). This histone acetylation is vital for establishing the conformational structure of DNA-chromatin complexes, and subsequently transcriptional gene expression. **(b)** By blocking the deacetylation reaction, HDAC inhibitors change the equilibrium of histone acetylation levels, leading to increased acetylation, chromatin modification to relax confirmation and transcription upregulation. Reprinted by permission from Macmillan Publishers Ltd: [Nat Rev Drug Discov] (Oct;7(10):854-68), copyright (2008)

investigated the role of histone modification in relation to androgen receptor status [82]. They examined whether histone acetylation can influence AR transcriptional activity by using histone deacetylase (HDAC) inhibitors (HDACIs) trichostatin A (TSA), sodium butyrate (Na-But) and depsipeptide (FR901228). The study found that inhibition of HDAC activity significantly increased endogenous AR activity, as well as the prostate-specific antigen (PSA) in LNCaP cells. An increase in histone acetylation of target genes, such as CREB-binding protein may be essential for mediating AR transcriptional activity in prostate cancer cells. Histone acetylation seems to be intimately involved in AR activity regulation [82].

3.2 Methylation Profile and Health Disparities in Prostate Cancer

3.2.1 Methylation Profiles Differ Among African American, Caucasian and Asian Men with Prostate Cancer

The incidence and mortality of prostate cancer is twofold higher in African-American than that in Caucasian men in the USA [21]. A complex combination of environment, socioeconomic and genetic variations may all contribute to the disparities of incidence and mortality in prostate cancer. Aberrant hypermethylation in regulatory genes in prostate tissues may precede and predispose to developed malignancy [73]. Differences in the distribution of aberrant methylation may contribute to differential cancer health disparities among varying cohorts. An early study from Woodson and colleagues investigated differences in DNA hypermethylation

of *GSTP1*, *CD44* and *E-cadherin* in archival tumor tissues from African-American ($n=47$) and Caucasian ($n=64$) men using real-time methylation-sensitive PCR [83]. The study identified *GSTP1* hypermethylation in 84 % of prostate cancer tissues, but no difference was identified between African American and Caucasian men. *E-cadherin* was not methylated in any of the tumors. The data did, however, show that the frequency of *CD44* methylation was higher in African-American men compared with Caucasian even though the *CD44* methylation was less prevalent overall (in 32 % of tumors) [83].

A study by Enokida et al. used a similar approach with methylation-specific PCR to analyze 291 prostate cancer (African American = 44, Caucasian = 77, and Asian = 170) and 172 benign prostate hypertrophy samples (African American = 38, Caucasian = 38, and Asian = 96) [84]. CpG methylation of *GSTP1* was found in 65.6 % of prostate cancer tissues, and 24.5 % of benign prostate hypertrophy samples. The frequency of *GSTP1* methylation was significantly higher in prostate cancer in each ethnic group. Compared to Caucasian and Asian men, African-American men had a higher hazard ratio of *GSTP1* hypermethylation based on logistic regression. The methylation of *GSTP1* was significantly associated with higher Gleason score in Asian men, but not in African-American men—possibly due to small sample size of African-Americans in their study [84].

Recently another study by Kwabi-Addo and colleagues used pyrosequencing to quantitatively measure the methylation status of *GSTP1*, *AR*, *RAR β 2*, *SPARC*, *TIMP3*, and *NKX2-5* in prostate cancer and matched normal tissues from 39 African American and 67 Caucasian men [85]. Their data showed that overall there was significant methylation in prostate cancer tissues from African-American compared to Caucasian men. In agreement with Enokida et al., Kwabi-Addo and colleagues found that the higher frequency of methylation in African-American samples was not correlated to disease aggressiveness since Gleason score was highest in tissues from Caucasian men. In addition, data from Kwabi-Addo's study demonstrated higher methylation of *NKX2-5* and *TIMP3* in normal prostate tissues from African-American compared to Caucasian men. Hence, analysis of gene methylation may increase sensitivity for detecting prostate cancer tumor activity in different ethnic groups [85].

3.3 Summary-Gene Frequently Methylated in Prostate Cancer

Promoter hypermethylation is a significant mechanism of gene silencing. To date more than 50 genes with common aberrant hypermethylation have been identified in prostate cancer (Table 3). These genes encompass many cellular functions including cell cycle control, apoptosis, hormone response, DNA repair and damage prevention, signal transduction, tumor invasion and tumor suppression. Frequent promoter methylation of genes such as *APC*,

Table 3
Summary of genes hypermethylated in prostate cancer

Pathway	Gene	Official gene name	Methods of identification
Hormonal response	AR	Androgen receptor	MSP
	ESR1	Estrogen receptor 1	MSP
	ESR2	Estrogen receptor 2	MSP, Bisulfite sequencing
	RAR β 2	Retinoic acid receptor β 2	MSP, MethyLight PCR
	RARRES1	Retinoic acid receptor responder 1	MSP,QMSP
Cell cycle control	CCND2	Cyclin D2	MSP, QMSP
	CDKN2A	Cyclin-dependent kinase inhibitor 2A (p16)	MSP, QMSP
	RPRM	Reprimo	QMSP
	SFN	Stratifin (14-3-3 sigma)	QMSP
	CDC2	Cell division cycle 2	TranSignal array
	CDKN1B	Cyclin-dependent kinase inhibitor 1B (p27 ^{kip})	TranSignal array
	CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21)	TranSignal array
Signal transduction	DKK3	Dickkopf 3	MSP + Bisulfite sequencing
	EDNRB	Endothelin receptor type B	MSP, QMSP, Southern blot, MethyLight
	RASSF1A	Ras association domain family protein 1 isoform A	MSP, QMSP
	RUNX3	Runt-related transcription factor 3	MSP
	SFRP1	Secreted frizzled-related protein 1	MSP + Bisulfite sequencing
Tumor invasion	APC	Familial adenomatous polyposis	MSP, Bisulfite sequencing, MethyLight
	CAV1	Caveolin 1	MSP, Bisulfite sequencing
	CHD1	E-cadherin	MSP, COBRA
	CHD13	Cadherin 13	MSP, QMSP
	CD44	Cluster differentiation antigen 44	MSP
	LAMA3	α -3 laminin	MSP
	LAM C2	γ -3 laminin	MSP, QMSP, TranSignal array
Tumor suppressor	TIMP3	TIMP metalloproteinase inhibitor 3	MSP
	RB	Retinoblastoma	TranSignal array
	TMS1	Target of methylation-induced silencing 1	TranSignal array
	DAPK	Death-associated protein kinase	TranSignal array
	RBL1	Retinoblastoma-like 1	TranSignal array
	PAX6	Paired box gene 6	TranSignal array
	FHIT	Fragile histidine triad	TranSignal array
DNA damage repair	GSTM1	Glutathione S-transferase M1	MSP + Bisulfite sequencing
	GSTP1	Glutathione S-transferase P1	MSP, QMSP, MethyLight
	GPX3	Glutathione peroxidase 3	Bisulfite sequencing, MSP, QMSP
	MGMT	O-6-methylguanine DNA methyltransferase	MSP, QMSP
	ASC	Apoptosis-associated Speck-like protein containing a CARD	

(continued)

Table 3
(continued)

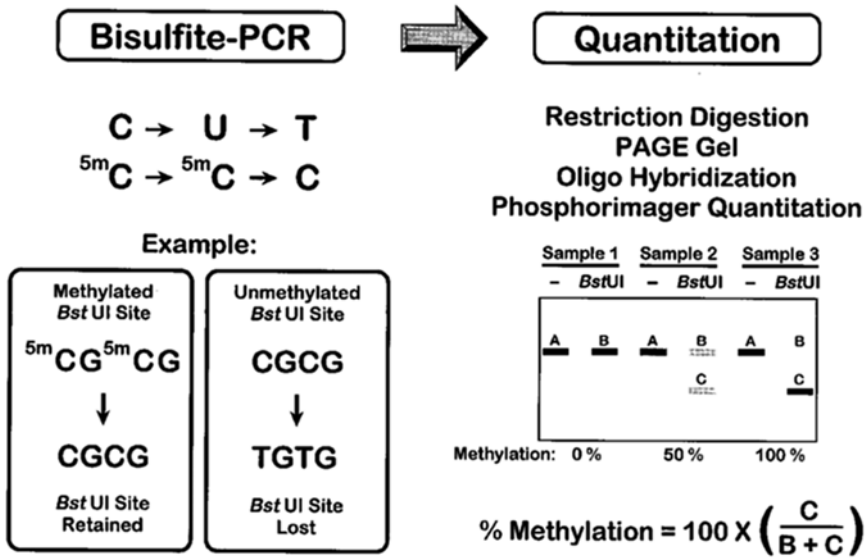
Pathway	Gene	Official gene name	Methods of identification
Apoptosis	BCL2	B cell lymphoma 2	MSP, COBRA, QMSP
	DAPK	Death-associated kinase	MSP, QMSP
	CASP8	Caspase 8, Apoptosis-Related Cysteine	TranSignal array, MSP
	CD14	Monocyte differentiation antigen CD14	TranSignal array
	MDR1	Multidrug resistance receptor 1	QMSP, MSP + COBRA, COMPARE, TranSignal array MSP, MethyLight MSP
Others	PTGS2	Prostaglandin endoperoxidase synthase 2	TranSignal array
	HIC	Hypermethylated in cancer	TranSignal array
	GLUT4	Solute carrier insulin transport	TranSignal array
	GATA3	Gata binding protein 3	TranSignal array
	SYBL1	Synaptobrevin-like 1	TranSignal array
	ATF2	Activating transcription factor 2	TranSignal array
	MYOD	Myogenic differentiation 1	TranSignal array
	SIM2	Single-minded homologue 1	TranSignal array
	WT1	Wilms' tumor 1	TranSignal array
	CIITA	Class 2 MHC transcription 5A	TranSignal array

MSP methylation sensitive PCP, *QMSP* quantitative methylation sensitive PCP, *COBRA* combined bisulfite restriction analysis, *COMPARE* combination of methylated DNA precipitation and restriction enzyme digestion

CCND2, *GSTP1*, *RARβ2*, *RASSF1A* and *PTGS2*, were found. The frequency of *RARβ2* methylation has been reported in 60–95 % of tissues and the frequency of *MDR1* methylation has been reported at 51–100 % in tissues. Methods for the identification of epigenetic changes implicated in prostate cancer included MSP, quantitative-MSP (QMSP), MethyLight PCR, Combined Bisulfite Restriction Analysis (COBRA), and Combination of methylated DNA precipitation and restriction enzyme digestion (COMPARE) assay. The MSP and MethyLight analysis have been introduced previously. The principles of COBRA and COMPARE analysis should also be noted for future utility.

The COBRA assay has been described by Xiong and Laird in 1997 [86]. COBRA consists of a standard sodium bisulfite PCR treatment followed by restriction digestion and a quantitation step. The purified bisulfite-PCR products are further digested with a restriction enzyme with a recognition sequence containing a CpG site in the original unconverted DNA. Cleavage will occur only if the CpG sequence has been retained during the bisulfite conversion by a methylation of the cytosine residue. It is essential to ensure that the bisulfite conversion is complete. Therefore, a control digest is

COBRA - Combined Bisulfite Restriction Analysis

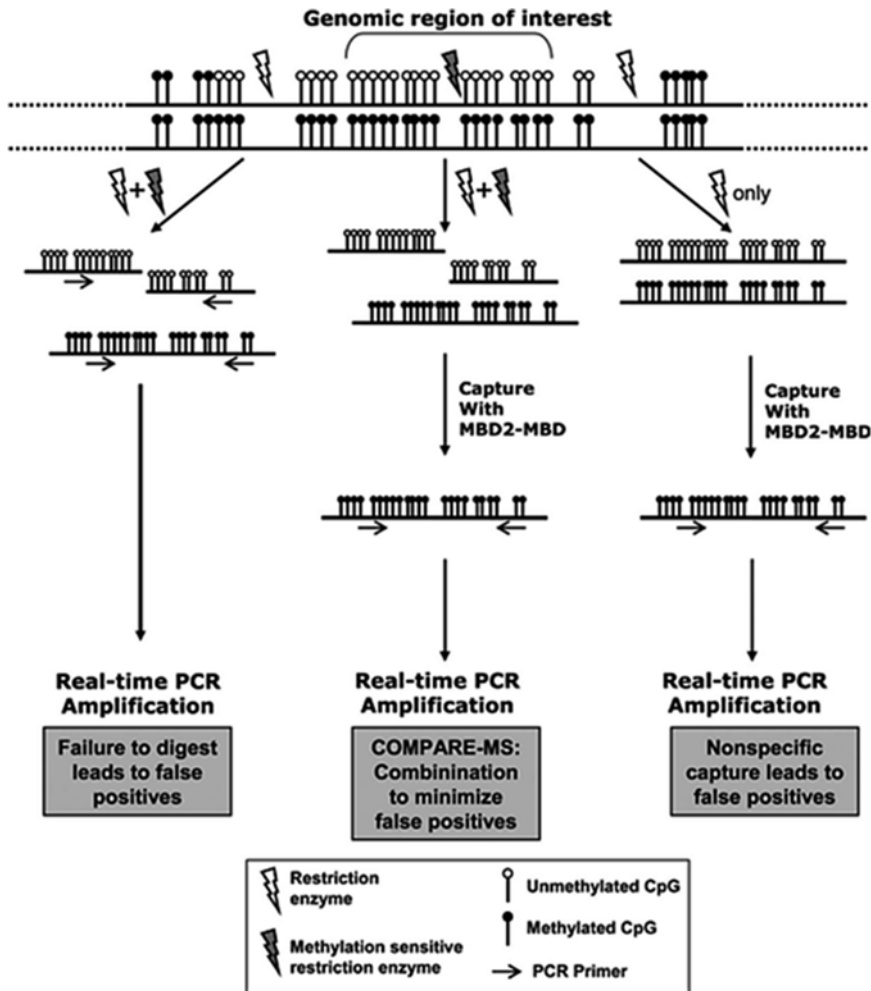


Xiong Z, et al. *Nucleic Acids Res.* 1997 Jun 15;25(12):2532-4.

Fig. 7 Workflow of COBRA assay. Reprinted by permission from Oxford University Press: [Nucleic Acids Res] (Jun 15;25(12):2532-4), copyright (1997)

performed with an enzyme such as *Hsp92II*, which has a recognition sequence (CATG) that should be destroyed by the bisulfite conversion. Any cleavage by *Hsp92II* would indicate either non-CpG DNA methylation or incomplete sodium bisulfite conversion. The digested PCR products are then separated on an 8 % denaturing polyacrylamide gel and transferred to Zetabind charged membrane (American Bioanalytical) by electroblotting. The membranes are hybridized with 5'-end-labeled oligonucleotides and quantified with a Molecular Dynamics PhosphorImager. The principle and workflow of COBRA is shown in Fig. 7.

Details of the COMPARE assay are described by Yegnabramanian and colleagues [87]. The key of the COMPARE assay is that the methylated genomic DNA fragments are captured and enriched by magnetic-bead immobilized recombinant methyl-binding domain polypeptides. SYBR Green-based real-time PCR is subsequently performed. The Methylation Index (MI) for each sample is determined as follows: $MI_{sample} = (Q_{untreated}) / (QM.SssI)$, where $Q_{untreated}$ is the amount of methylation at a given locus for the sample of interest, $QM.SssI$ is the amount of methylation at the given locus for the equivalent amount of M.SssI methylated sample of interest. The MI is an estimate of the fraction of alleles that are methylated in a given sample. Figure 8 is the workflow of COMPARE.



Yegnasubramanian S, et al. *Nucleic Acids Res.* 2006 Feb 9;34(3):e19.

Fig. 8 Workflow of COMPARE assay. Reprinted by permission from Oxford University Press: [*Nucleic Acids Res*] (Feb 9;34(3):e19.), copyright (2006)

In summary evidence suggests that epigenetic alternations could be an early event in prostate carcinogenesis and many factors such as diet and environmental factors may all contribute to the alternations.

4 Methods and Technologies Used for Epigenetic Changes Detection

The sodium bisulfite modification method was established to investigate methylation patterns in DNA in the 1990s. The method used uses sodium bisulfite modification which converts unmethylated cytosines to uracil, with methylated cytosines remaining unchanged [88, 89]. This method sensitized detection of methylation patterns

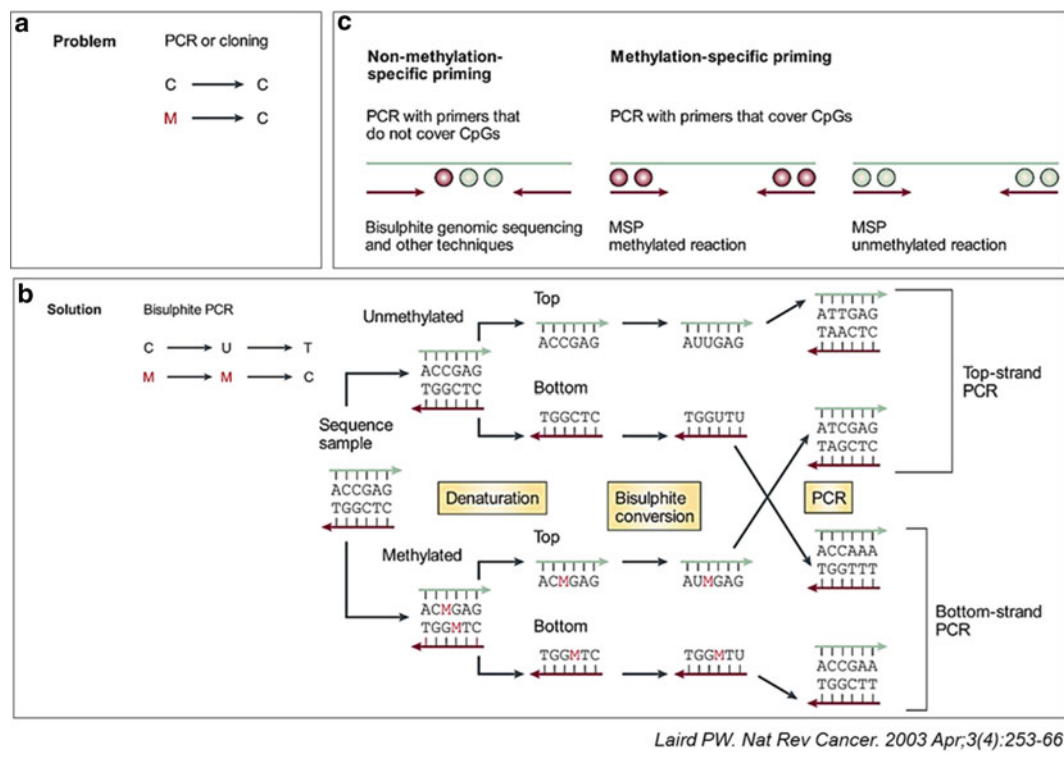


Fig. 9 Principles of bisulfite conversion and methylation specific PCR. Standard molecular biology techniques to analyze individual gene loci, such as polymerase chain reaction (PCR) and biological cloning, erase DNA methylation information, leaving the investigator oblivious to the epigenetic information that was present in the original genomic DNA (panel **a**). 5-Methylcytosine residues are indicated as *red Ms*. The solution to this problem is to modify the DNA in a methylation-dependent way before amplification. This can be achieved either by digestion with a methylation-sensitive restriction enzyme (not shown), or by treating the genomic DNA with sodium bisulfite (panel **b**), which converts unmethylated cytosines to uracil residues. As a consequence, the converted DNA is no longer self-complementary, and amplification of either the *top* or *bottom* DNA strand requires different primers. Priming can be either universal, or methylation specific (panel **c**). MSP, methylation-specific PCR. Reprinted by permission from Macmillan Publishers Ltd: [Nat Rev Cancer] (Apr;3(4):253-66), copyright (2003)

from only small quantities of DNA and allows one to distinguish methylated from unmethylated DNA via PCR amplification. During PCR amplification, unmethylated cytosines are amplified as thymine and methylated cytosines are amplified as cytosines (Fig. 9) [89, 90]. Most of the methods for DNA methylation analysis at specific loci are based on this approach.

To determine DNA promoter CpG methylation, the modified DNA can be further analyzed using different approaches according to the goals of studies: global or locus-specific methylation analysis. High-performance liquid chromatography (HPLC) is a classical method to quantify global methylation [89]. This method is highly reproducible, but it requires large amount of high quality

genomic DNA and is not suitable for high-throughput analysis. More recently, new methods have been developed for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements [91]. This approach requires little DNA and can be applied to paraffin-embedded tissues [92].

Global methylation could provide an overall view of methylation profile, however, it will need to have gene-specific methylation analysis to characterize candidate genes and link such changes to functional outcomes. The gene-specific methylation analysis can be achieved by (1) methylation sensitive PCR (MSP); (2) bisulfite sequencing or bisulfite-pyrosequencing; (3) matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). The most frequent used methods are MSP and bisulfite-sequencing/ pyrosequencing. The keys for those methods are (1) bisulfite modification of DNA; (2) primer design. Recently more high-throughput array base analysis have been developed by different companies. These arrays are designed upon promoter and CpG islands and are described in further detail below.

4.1 Methylation Arrays

The following array based analytical methods are currently in use for measuring DNA methylation.

4.1.1 Human-Methylation450 BeadChip™ (Illumina Inc, San Diego, CA USA)

This is a beadchip based array and it offers a unique combination of comprehensive, expert-selected coverage, including 99 % of RefSeq genes, 96 % of CpG islands, and other content categories selected by methylation experts. The Illumina's Infinium Methylation Assay™ provides quantitative methylation measurement at the single-CpG-site level which offers the highest resolution for understanding epigenetic changes. With Infinium Methylation Assays™, researchers can quantitatively interrogate methylation sites at single-nucleotide resolution, profiling 12 samples in parallel to deliver high-throughput power while minimizing the cost per sample. However, the array requires an additional module to run. The analysis of Infinium methylation data requires the GenomeStudio Methylation Module™ (Illumina Inc). This program enables two basic types of methylation data analysis: calculating methylation levels within an individual sample, and determining whether methylation levels have changed between a reference group and another experimental group.

4.1.2 Human DNA Methylation Microarray™ (Agilent Technologies, Inc. Santa Clara, CA, USA)

Agilent's Human DNA Methylation Microarrays™ consist of 27,627 expanded CpG islands and 5,081 UMR regions CpG islands. The array enables comprehensive analysis of the relative methylation levels in a genomic DNA sample. Each glass slide is formatted with one high-definition 244 K array and costs ~\$629. However, it requires Agilent equipment to conduct and analyze the array.

**4.1.3 EpiTect Methyl II
PCR Arrays™
(QIAGEN, USA)**

EpiTect Methyl II PCR Arrays™ is a qPCR based array. Genes are selected based on their reported methylation status in a variety of experimental settings. These arrays allow correlation of CpG island methylation status with biological phenotypes and disease outcomes. The array allows the simultaneous DNA methylation profiling of a panel of 22 or 94 gene promoters in either disease or pathway focused genes. The method is based on the detection of remaining input DNA after cleavage with a methylation-sensitive restriction enzyme (MSRE) and/or a methylation-dependent (MDRE) restriction enzyme. These enzymes will digest unmethylated and methylated DNA, respectively. Following digestion, the remaining DNA is quantified by real-time PCR in each individual enzyme reaction using primers that flank a promoter (gene) region of interest. The relative fractions of methylated and unmethylated DNA are subsequently determined by comparing the amount in each digest with that of a mock (no enzymes added) digest using the ΔC_t method. The reliability and simplicity of the procedure make this technology highly suited for semi-high-throughput DNA methylation profiling and biomarker discovery for various research fields, such as stem cell differentiation and development. The EpiTect Methyl II Signature PCR Array™ (22 genes) is available in 96-well format and EpiTect Methyl II Complete PCR Array™ (94 genes) is available in 384-well formats. The EpiTect Methyl II PCR Arrays™ from QIAGEN are affordable and easy to perform since it is a qPCR based assay. These arrays are useful for obtaining methylation profiles in different diseases or specific pathways quickly, but no quantified information. In addition, since the genes arranged in the array are not duplicated and qPCR is extremely sensitive, the slightest mistakes can have significant influence on the final results.

**4.1.4 TaqMan Human
DNA Methylation Array™
(Applied Biosystems, USA)**

The Applied Biosystems TaqMan Array Human DNA Methylation™ and Transcriptional Repression 96-well Plate™ contain 28 assays for DNA methylation and transcriptional repression-associated genes and 4 assays for candidate endogenous control genes. All assays are plated in triplicate. The Gene Signature Plates are 96-well plates that are pre-configured with the most appropriate TaqMan Gene Expression Assays™ (PCR primers and TaqMan® probe sets) for a specific biological process, pathway, or disease state. Each plate contains predefined assays and endogenous controls dried-down in the wells, ready for accurate assessment of an entire gene signature in one simple experiment. Since this array is a TaqMan based detection assay, it is therefore an improved assay in terms of sensitivity and specificity compared to EpiTect Methyl II PCR Arrays™ (SYBR-green based detection) from QIAGEN. However, this array is at a slightly higher cost compared to EpiTect Methyl II PCR Array™ and the plates have been only validated for use on Applied Biosystems® 7000, 7300, 7500 and 7900HT Fast Real-Time PCR Systems.

**4.1.5 TranSignal
Promoter Methylation
Arrays (Panomics Inc, USA)**

Panomics now is part of Affymetrix Inc (Huston TX, USA). The TranSignal Promoter Methylation Arrays™ designed by Panomics use genomic DNA that is digested with a restriction enzyme to produce small fragments of methylated DNA. The digests are purified and annealed to linkers and subsequently incubated with MBP (methylation binding protein) to form protein–DNA complexes. These complexes are isolated, purified, and subsequently labeled with biotin-dCTP. The final step is hybridization of the labeled DNA on the array. The signals are detected by using Streptavidin-HRP, which eliminates the use of hazardous radioactive chemicals associated with traditional Southern blotting techniques. A single experiment using the Panomics array takes two days to complete and permits analysis of 82 different promoter regions from each sample. The Panomics TranSignal Promoter Methylation Arrays™ are relatively easy to use since the assay uses enzyme digestion to produce methylation DNA instead bisulfide modification. The data obtained from these arrays are relatively consistent and reproducible, but the exposure time for visualizing the chemiluminescent signals is very critical for maintaining the consistent results from array to array. The arrays are also reusable; however, the stripping step is quite important, and background signals may significantly affect results.

4.2 Summary

Continued rapid improvement of the current technology makes the study of DNA methylation more and more accessible. The method or approach should be selected based upon the goal, scope, and design of the individual study. Therefore, it is important to understand the type of information provided by different approaches, and the potential for bias associated with the different methods. In addition, the cost, availability of sample, and supplemental instruments needed for analysis should be considered when selecting a method for methylation studies.

5 Factors That Influence the Epigenetic Changes in Cancer

There is growing evidence that environmental and dietary factors can affect the epigenome. These events can start as early as prenatal or early postnatal development. Moreover, the changes can be sustained throughout life, leading to long term modification of phenotypes and contribute to development of abnormalities including cancers.

**5.1 Nutritional
Factors That Influence
the Methylation
Outcomes in Cancer**

Nutrients such as folic acid, Vitamin B, and S-adenosylmethionine (SAM) are key components of the methyl-metabolism pathway. DNA methylation occurs at 5' position of the cytosine residues within CpG dinucleotides through addition of a methyl group to form 5-methylcytosine. Dietary factors can modify DNA methylation

by influencing the supply of methyl groups for the formation of SAM; or by modifying the utilization of methyl groups through processes inducing shifts in DNA methyltransferase activity [66].

As indicated before, the DNA methyltransferase (DNMTs) family of enzymes catalyze the transfer of a methyl group to DNA. The DNMTs use SAM as the methyl donor. Three active DNA methyltransferases have been identified in mammals: DNMT1, DNMT3a and DNMT3b. DNMT1 is primarily involved in the maintenance of DNA methylation after replication and DNMT3a and DNMT3b are the *de novo* methyltransferases that set up DNA methylation patterns early in development. Dietary factors can modify the availability of methyl donors, including folate, choline, and methionine, as well as the activity of DNMTs. An early animal study from the Poirier group showed that dietary methyl deficiency of folate, choline and methionine altered hepatic DNA methylation patterns and induced liver cancer in the absence of carcinogen [93]. In addition, dietary factors may be also able to regulate DNA demethylation activity [66]. The hypermethylation of CpGs in promoters of tumor suppressor genes can lead to gene transcriptional silencing and cause malignant transformation in cancers including breast, colon, lung, and prostate cancers.

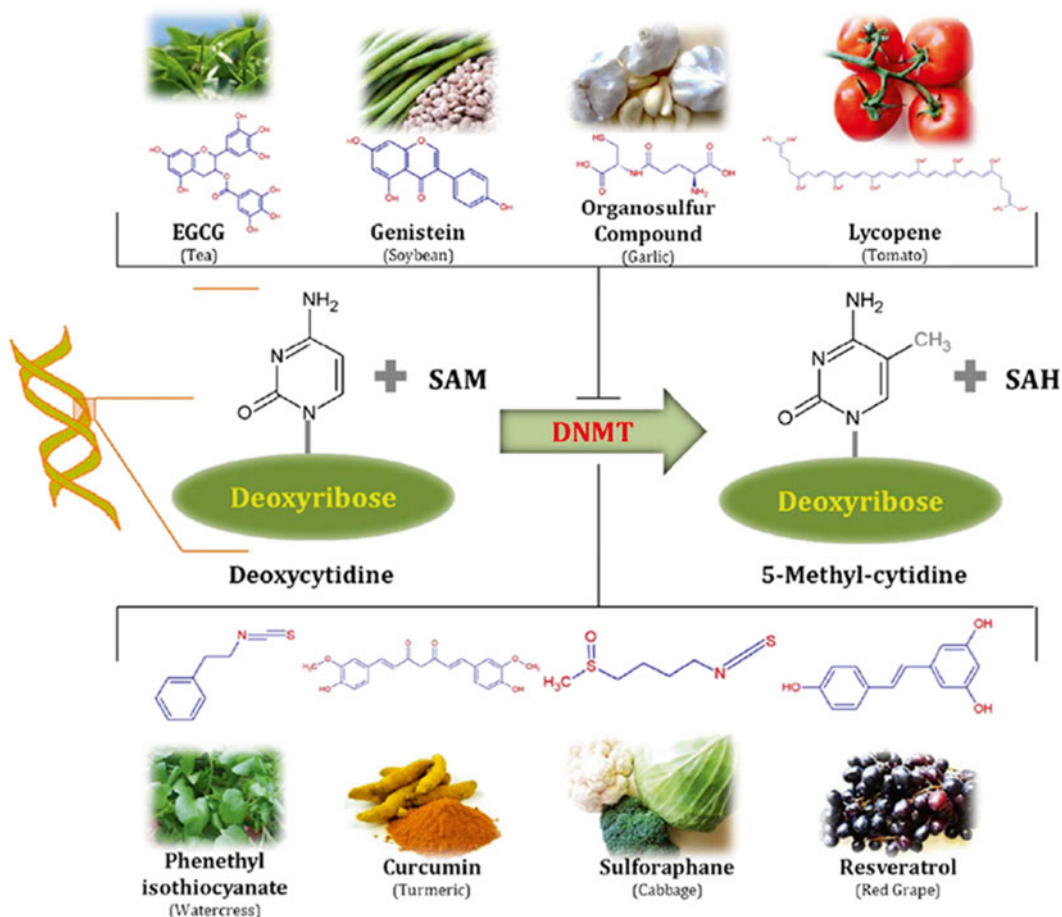
Moreover, recent studies have linked obesity overall with epigenetic modifications associated with cancer. A recent study by Uriarte and colleagues have found that high-fat, high-sucrose diet intake induces epigenetic changes in retroperitoneal adipocytes of Wistar rats [94]. Adipocytes act as regulators of energy balance and glucose homeostasis, and aberrations lead to the emergence of obesity. In addition, epigenetic modifications of obesogenic genes such as leptin may further exacerbate the risk of obesity [95]. It is well established that obesity is associated with increased risk of the multiple types of cancers, including postmenopausal breast cancer, pancreatic cancer, and colorectal cancer to name a few [96].

5.2 Role of Nutrition on Epigenetic Alternations: Bioactive Dietary Components for Cancer Prevention by Modification of Epigenetics

Epigenetics has been shown to play a role in cancer development and can be modified by nutritional factors [66]. Hence, utilization of dietary compounds in addition to pharmacological drugs to target epigenetic modification may be a valuable strategy for cancer prevention and treatment [97]. Several decades of studies have provided evidence that bioactive dietary components play important roles in regulation of epigenetics changes predominantly by: (1) inhibition of DNMTs (Fig. 10); and, (2) inhibition of histone modifications (Fig. 11). These bioactive dietary components may modify cancer risk as well as tumor progression. Below, the role of some common bioactive dietary compounds and their effects on epigenetics are described in detail.

5.2.1 Tea Polyphenols

Tea is consumed worldwide. Epidemiologic and laboratory studies have indicated that polyphenolic compounds present in tea and green tea may play a role in cancer prevention [97] and progression [98].



Shankar S et al. *Pharmacol Ther.* 2013 Apr;138(1):1-17

Fig. 10 Dietary inhibitors of DNA methylation. DNA methylation is a biochemical process that is essential for development. Some dietary phytochemicals are reported to inhibit the methylation of cytosine. Hypermethylation of cytidine by DNMTs usually results in transcriptional gene silencing and gene inactivation. Several phytochemicals derived from different food source such as: resveratrol from grapes and berries, curcumin from turmeric, tea phenols from tea leaves, genistein from soybeans, sulforaphane from broccoli, phenethyl isothiocyanate from cauliflower, organosulfur compounds from garlic, quercetin from citrus fruits, and lycopene from tomato act as dietary inhibitors of DNA methyltransferases. These compounds also alter gene expression via epigenetic mechanisms. Reprinted from *Pharmacol Ther.* 2013 Apr;138(1):1–17. Sharmila Shankar, Dhruv Kumar, Rakesh K. Srivastava, Epigenetic modifications by dietary phytochemicals: Implications for personalized nutrition, Pages 1–17, Copyright (2013), with permission from Elsevier

The most abundant chemical compounds are catechins, which consist of (–)-epigallocatechin (EC), (–)-epicatechin-3-gallate (ECG), (–)-epigallocatechin (EGC), and (–)-epigallocatechin-3-gallate (EGCG) [97]. EGCG is the most abundant and accounts for more than 50 % of total polyphenols. EGCG has been identified as most effective constituent of green tea [99] and has been extensively studied as a potential demethylating agent. Early studies have shown that EGCG can inhibit DNMT activity by forming hydrogen

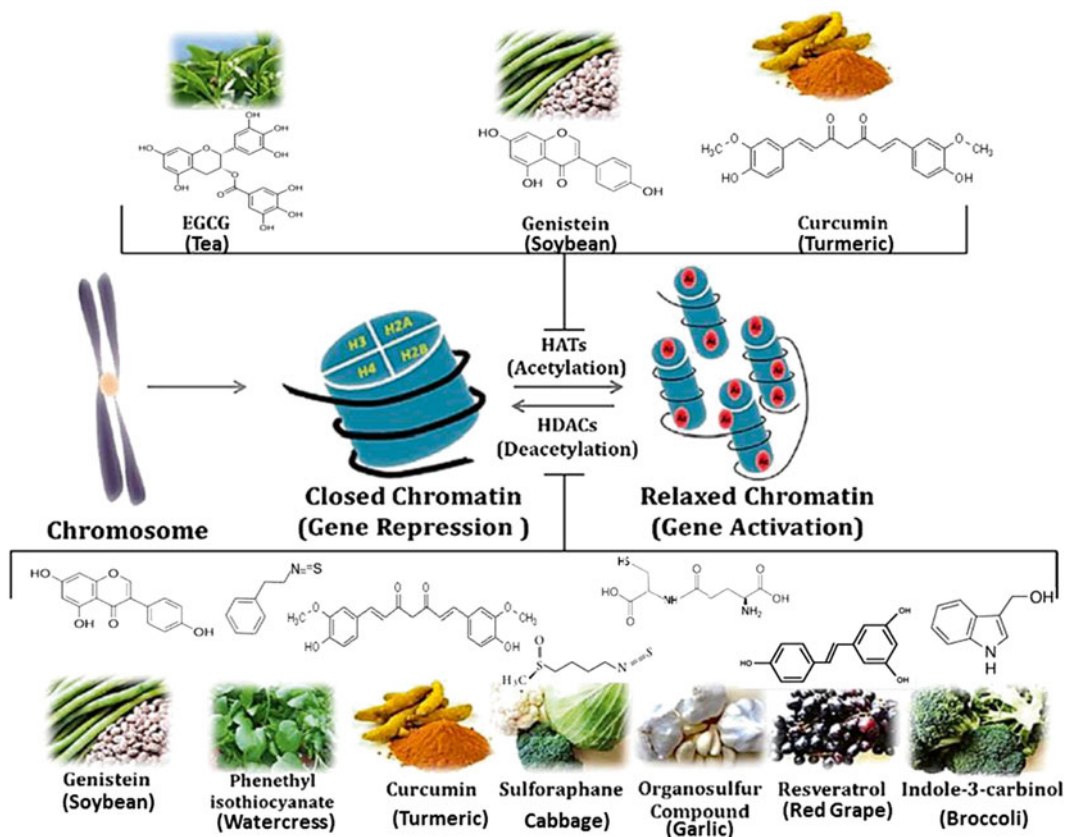


Fig. 11 Examples of dietary inhibitors of histone modifications. Representation of histone modifications (acetylation and deacetylation) by the phytochemicals derived from different food sources. Phytochemicals like EGCG, genistein, and curcumin play important role in inhibition of histone acetylation by inactivating histone acetyl transferase enzyme. Some other phytochemicals like sulforaphane, curcumin, genistein, phenyl isothiocyanate, organosulfur compound, resveratrol, and indol-3-carbinol inhibits the deacetylation of relaxed chromatin by inactivating histone deacetylase enzyme. Reprinted from Pharmacol Ther. 2013 Apr;138(1):1–17. Sharmila Shankar, Dhruv Kumar, Rakesh K. Srivastava, Epigenetic modifications by dietary phytochemicals: Implications for personalized nutrition, Pages 1–17, Copyright (2013), with permission from Elsevier

bonds with Pro¹²²³, Glu¹²⁶⁵, Cys¹²²⁵, Ser¹²²⁹, and Arg¹³⁰⁹ in the catalytic pocket of DNMT [100]. The inhibition of DNMTs by EGCG can lead to the reactivation *RARβ*, *p16^{INK4a}*, *MGMT* (o⁶-methylguanine methyltransferase), and *bMLH1* genes in colon, esophageal, and prostate cancers cells. Besides direct inhibition of DNMT by EGCG, it has been reported that EGCG could decrease S-adenosyl-L-methionine (SAM) and increase S-adenosyl-L-homocysteine (SAH), a potent inhibitor of DNA methylation. Together, these data provide evidence of indirect inhibition of DNA methylation by EGCG [97].

Recently, EGCG has also been discovered to have strong HAT inhibitory activity. Choi et al. found that EGCG reversed the acetylation of histone H3 and H4 on chromatin and suppressed p300/CBP-mediated p65 acetylation. This resulted in inhibition of

lymphoma. However, in this study EGCG did not change HDACs [101]. Combination of green tea polyphenol and histone deacetylase inhibitor has been showed synergistic epigenetic reactivate estrogen receptor- α (ER α) in the ER-negative breast cancer cell line, MBA-MB231 [102]. Inhibition of tumorigenesis by EGCG or green tea polyphenols has also been showed in vivo studies [97]. However, the effects on epigenetic mechanisms and epigenome in vivo have not been well defined yet.

5.2.2 Curcumin

Curcumin is a yellow pigment present in the spice turmeric. It has been shown to have anti-inflammatory, anti-angiogenic, wound-healing, antioxidant, and anticancer properties. Specifically, curcumin has been shown to inhibit DNMT activity by covalently blocking the catalytic thiolate of C1226 of DNMT1 [103]. Evidence also shows that curcumin may be a potent DNA hypomethylating agent. Besides, curcumin also can function as a HDAC and HAT inhibitor and play a role in histone modification in cancer cell models. However, the issue with using curcumin is its insolubility and instability in water that results in low bioavailability. It has been suggested that the bioavailability of curcumin can be enhanced by utilizing rubusoside (found in Chinese blackberry extract) or phosphatidylcholine (found in soy and egg yolks) [97].

5.2.3 Selenium (Se)

Se is a nutrient found in Brazil nuts, chicken, game meat, and beef [97]. Se is an essential element with antioxidant, proapoptotic, DNA repair, and anticancer properties. An early study from Vadgama et al. showed that supplementation of Selenium enhanced chemotherapeutic effect of Taxol and Doxorubicin in different cancers cell lines beyond that seen with the chemotherapeutic drugs used alone [104]. The role of Se in epigenetics has been investigated by several groups. Xiang and colleagues showed that selenite reactivates a silenced gene, *GSTP1*, by decreasing DNMT1 and DNMT3a, and modulating histones in human prostate cancer cells [105]. The histone modification by Se involves decreasing histone deacetylase activity and increasing acetylation of histone H3. In vivo studies from Davis et al. showed that Se deficiency caused global hypomethylation of liver and colon [106]. In rodents, the depletion of methyl groups by dietary restriction leads to a decrease in SAM those results in liver carcinogenesis and in liver hypomethylation, which precedes tumor development. Overall further studies in vitro and in vivo need to be conducted for validating the effect of Se on the epigenome.

5.2.4 Genistein

Genistein is an isoflavone belonging to the flavonoid group of compounds and is found in plants including fava beans, soybeans, lupin, kudzu, and psoralea. Genistein has found to have anticancer and antiangiogenic properties in many cancers. It has been indicated to inhibit prostate, cervix, brain, breast, and colon cancers [97].

The mechanisms of the anticancer effect of genistein may involve regulation of gene transcription or silencing activity by modulating epigenetic changes, such as DNA methylation and/or chromatin modifications. It has been suggested that genistein can act as a DNMT inhibitor. Within this role, it can cause demethylation of CpG islands in the promoter of genes and subsequently activate tumor suppressor genes, *p16*, *RAR β 2* and *MGMT* at least in renal cancer [107], breast cancer [108], and prostate cancers cells [109]. Specifically, genistein can mediate histone acetylation and induce tumor suppressor genes *p16*, *p21*, *PTEN*, *CYLD*, *p53* and *FOXO3* in prostate cancer cells [110]. Mechanistically, genistein can modulate histone H3-Lysine 9 (H3-K9) methylation and deacetylation [97] hence its use as a dietary compound may have significant effects on global epigenetic changes.

5.2.5 Resveratrol

Resveratrol is a dietary polyphenol derived from grapes and is most commonly consumed in the form of red wine. The anticancer properties of resveratrol have been supported by its ability to inhibit proliferation of a wide variety of human cancer cells [111]. Its best characterized epigenetic roles include affecting the histone H3K9 acetylation which significantly regulates activity of the *BRCA1* promoter [97]. Resveratrol has less DNMT inhibitory activity compared to EGCG and was unable to reverse the methylation of certain tumor suppressor genes. However, resveratrol has been associated with activation of the type III HDAC inhibitors, sirtuin 1 (SIRT1) and p300 [112]. The SIRT1 has been reported to mediate *BRCA1* signaling in human breast cancer cells by altering H3 acetylation [112].

5.3 Summary

There is substantial evidence in the literature to demonstrate that dietary factors may play an important role in the epigenetic process, including histone modification. Certain bioactive dietary components such as tea phenols, curcumin, selenium, genistein, and resveratrol may have great potential not only in the prevention but also in the therapy of a wide variety of cancers by altering various epigenetic modifications. Furthermore, additional in vivo studies are needed to validate the data obtained from in vitro studies. Specifically, clinical studies evaluating the efficacy of these interventions should analyze the safety profile of doses, route of administration, organ specificity, as well as bioavailability of these bioactive components in human subjects. Epigenetic alterations affected by these compounds should also be assessed.

6 Future Directions

In this chapter, the role of epigenetics as key mediators of gene expression has been discussed. Specifically, evidence was presented to highlight the role of epigenetic aberrations in cancer with an

emphasis in breast and prostate cancer. Studies have shown that epigenetic signatures can serve as biomarkers for early detection as well as prognosis and outcomes [71, 89].

In breast, this chapter gave example of methylation of the estrogen receptor gene (*ESR1*) as a potential mechanism for both emergence of triple-negative breast cancer and tumors that eventually form resistance to standard targeted therapy [18]. Studies identified that *ESR1* promoter methylation was correlated with both ER and PgR protein expression levels [113]. Loss of the ER receptor, a key target for treatment of 80 % of breast cancers by well-established therapies like tamoxifen, can result in more aggressive breast phenotype and reduced treatment options.

Another key biomarker in breast cancer implicated by aberrant epigenetic changes is *BRCA1*. Although, *BRCA1* promoter methylation was not common in overall breast cancers, there was a significantly higher frequency in basal-like and/or TNBCs. This pattern was further correlated with reduced breast cancer outcome implicating *BRCA1* methylation in both the development of TNBC/aggressive tumors as well as patient survival [49, 53, 54]. These data warrant clearer confirmation in breast tumors from larger and more diverse ethnic cohorts.

A detailed list of well-established as well as new biomarkers in breast cancer epigenetics are shown in Table 2 and includes *CASP8*, *CCND2*, *CDH3* (E-cadherin), *CDKN2A*, *RAR β* , *RASSF1A*, and *SERPINB5*. Combined, these biomarkers represent regulation of crucial cellular processes in breast cancer development and progression including tumor suppression, proliferation, and cell adhesion. Accumulating preclinical evidence points to potential future utilization of these biomarkers in standard tumor evaluation, and ultimately may inform treatment options in breast cancer. Particularly, evaluation through immunohistochemical methods as performed in many of the studies reviewed in this chapter show efficacy in detection. Careful validation in additional preclinical and clinical models is necessary prior to use; however, the long term promise of identifying high risk patients for more vigilant clinical evaluation is highly necessary [18].

Studies investigating the epigenetics in prostate cancer have similarly revealed several biomarkers which have been associated with both risk and outcome. The methylation of the glutathione *S*-transferase PI (*GSTPI*) in particular has yielded promise [71]. The differential methylation patterns of *GSTPI* in prostate cancer vs. normal prostate tissue, combined with the vital role in detoxification that glutathione *S*-transferase plays renders this a high-priority biomarker. Preclinical studies have already shown that *GSTPI* closely correlated with prostate cancer risk as well as reoccurrence [77]. Evaluation of *GSTPI* in a standard clinical context has yet to be established, however, several groups are in the process of developing and testing screens for hypermethylation from both

tumor samples [114] as well as urine samples for less invasive assessment [115]. A more detailed list of genes is provided in Table 3. Key genes include *GSTP1*, *AR*, *RAR β 2*, *SPARC*, *TIMP3* and *NKX2-5*. Future studies in prostate cancer and epigenetics should also investigate the role of epigenetic patterns as biomarkers in diverse populations. The presence of a new series of high resolution and high through-put tools will facilitate larger screening and association studies needed to understand the mechanisms and targets for epigenetics in prostate cancer.

Preclinical studies to date have yielded promising response to DNA and histone level reversal of modifications. As the complex interplay of DNA and histone modifications and their accompanying enzymes proteins (DNMTs, HDACs, etc) are better understood, targeting of aberrant epigenetic activity along various points in these pathways offer options for potential intervention. Ofnote, combined demethylating agents (5-aza-2'-deoxycytidine) with HDAC inhibitors (trichostatin A) conceptually offer the most rational approach to tackling both DNA level and histone level aberrations [13, 69]. Although these approaches have resulted in some progress in clinical application (such as with leukemias), the application in solid tumors must still be explored and clarified further. Combined translational approaches investigating both preclinical and clinical applications must include larger and more diverse cohorts to reduce variances from disparities and produce results more globally applicable. Furthermore, earlier investigations ruling out efficacies of epigenetic biomarkers and/or anti-methylation treatment merit some reconsideration in light of new, more specific assays available to carry out rigorous epigenetic study.

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Part V

Future Directions: Risk Assessment, Diagnosis, Treatment, and Prognosis

Epigenetic Inhibitors

Mukesh Verma and Hirendra Nath Banerjee

Abstract

Traditional treatments for cancer include chemotherapy, radiation therapy, and surgery. Recently, epigenetic inhibitors have been found to be very effective in cancer treatment. Epigenetic changes such as DNA methylation, histone deacetylation, and microRNA (miRNA) expression are capable of silencing the expression of tumor suppressor genes and inducing oncogenes, leading to clonal proliferation of tumor cells. Methyltransferase inhibitors and histone deacetylase inhibitors have attracted the attention of researchers and clinicians because they provide an alternative therapeutic regime in some diseases, including cancer.

Epigenetic changes are characterized by altered gene expression without any changes in the nucleotide sequences of DNA. In addition, epigenetic changes are dynamic and can be reversed by epigenetic inhibitors. Drugs that inhibit DNA methylation or histone deacetylation have been studied for the reactivation of tumor suppressor genes and repression of cancer cell growth. Epigenetic inhibitors work alone or in combination with other therapeutic agents. To date, several epigenetic inhibitors have been approved for cancer treatment. The main challenge in the field of epigenetic inhibitors is their lack of specificity. Their mechanisms of action and potential in treating cancer are described in this article.

Key words Cancer, Chromatin, Epigenetics, Histone deacetylase, Histone inhibitors, Methylation, Methyltransferase, microRNAs

1 Introduction: Epigenetics and Gene Regulation

Human diseases often arise as a result of alterations in gene expression. The genome encodes hereditary information that ultimately directs all cellular functions. The epigenome provides an additional layer of genetic regulation that affects the expression of functional gene products. The epigenome consists of chromatin modifications and covalent modifications of cytosine residues in guanine-cytosine (GC)-rich regions, called CpG islands, which are prominent in the promoter regions of genes. Gene silencing resulting from epigenetic mechanisms has been well established in cancer and other diseases. Multiple genes involved in cell cycle regulation, differentiation, tumor suppression, DNA repair, and apoptosis are regulated epigenetically. Unlike mutations and other genetic changes, epigenetic

alterations do not cause DNA sequence changes in the target gene. Research has shown that dysregulated epigenetic modifications, especially in early neoplastic development, may be as significant as genetic mutations in driving cancer development and growth [1–6].

Chromatin is a highly ordered structure made up of units known as “nucleosomes.” Chromatin is composed of histone and non-histone proteins and DNA and exists as a condensed nucleoprotein complex. Two conformational states of chromatin, euchromatin and heterochromatin, have been identified and represent transcriptionally active and inactive states, respectively. The N-terminal region of histones can protrude from a nucleosome and be modified by posttranslational processes. These posttranslational histone changes govern transcriptional regulation via chromatin conformational changes. Promoter methylation also contributes to gene regulation. The process is complex, and these two processes (promoter methylation and histone modification) do not necessarily occur as mutually exclusive processes. In fact, they are more likely to be dynamic processes in which other proteins and enzymes also participate. Furthermore, noncoding RNAs also contribute to epigenetic gene regulation by altering the stability of the proteins involved in transcriptional regulation [7–21]. Methylation requires a DNA methyltransferase (DNMT), and three forms—DNMT1, DNMT2, and DNMT3—have been reported. Histone deacetylases (HDACs) have several forms, and their specific modifications are being investigated so that HDACs can become targeted to a specific gene. The following section discusses epigenetic inhibitors, especially their potential in treating cancer.

2 Epigenetic Inhibitors in Clinical Trials and FDA-Approved Inhibitors

A number of epigenetic inhibitors have been purified, either from natural sources or synthetically developed. The methyltransferase inhibitors azacitidine and decitabine have been approved by the US Food and Drug Administration (FDA) for clinical therapy in patients with myelodysplastic syndrome (MDS) (Table 1). Among others, zebularine and isothiocyanate also are potential methyltransferase inhibitors. 5-azacytidine (5-AzaC; Vidaza) forms a covalent complex with cytosine (C-5)-specific DNMTs and inhibits their activity. This compound also is activated by uridine–cytidine kinase and thus can be incorporated into both RNA and DNA.

HDAC inhibitors are another promising class of compound for use in cancer therapy. Histone acetylation is a reversible process in which a histone acetyltransferase (HAT) transfers the acetyl moiety from acetyl coenzyme A to an amino acid. In contrast, an HDAC inhibitor, as the name suggests, removes the acetyl group.

Table 1
Epigenetic inhibitors

Inhibitor	Other names	Comments
DNA methyltransferase inhibitors (DNMTs)		
5-azacytidine	Vidaza	FDA approved. (Vidaza by Celgene was approved for treatment of myeloplastic syndrome). One limitation of nucleotide analogs such as 5-azacytidine is that they require active DNA synthesis, which limits the activity of the drug in hyperproliferating cells, including potential cancer stem cells
Isothiocyanate		The potential mechanism may vary in different patients and likely includes a combination of induction of senescence, differentiation, apoptosis, and perhaps clearance by immune activation
Zebularine		More soluble than other inhibitors. Inhibitory activity toward cytidine deaminase
Decitabine (5-aza-2'-deoxycytidine)		FDA approved. Although protection against cancer remains for months to years, resistance to treatment has been observed in some cases. The mechanism of this resistance is not completely understood
Arabinosyl-5-azacytidine	Fazarabine	In phase I/II trials
5,6-dihydro-5-azacytidine	DHAC	In phase I/II trials.
Hydrazine		In phase I trials
Histone deacetylase (HDAC) inhibitors		
Vorinostat	Zolinza, suberoylanilide hydroxamic acid (SAHA)	FDA approved for treatment of CTCL. (SAHA is sold by the Merck pharmaceutical company.) Targets class I, II, and IV HDACs. Data collected from multiple trials evaluating SAHA as a single therapy in patients with hematologic malignancies ($n=341$) showed that this agent in general is well tolerated. The most commonly observed low-grade adverse effects were myelosuppression, anorexia, nausea, fatigue, and vomiting

(continued)

Table 1
(continued)

Inhibitor	Other names	Comments
Romidepsin	Istodax, depsipeptide	Approved for T-cell lymphoma. Did not show any significant clinical efficacy in patients with lymphocytic leukemia, AML, or MDS. Treatment results in toxicity, fatigue, nausea, and anorexia
Valproic acid		FDA approved. Is an aliphatic short-branched chain fatty acid that is actively transported across the blood–brain barrier. Very effective against AML and MDS as a single therapy. In combination therapy with decitabine, in patients with AML resulted in minimal clinical benefit compared to treatment of patients with decitabine alone. Side effects when these two drugs were administered together included neurotoxicity
FK228	Depsipeptide	In phase I/II trials
Panobinostat	LBH589	In phase I/II trials. Is a hydroxamic acid HDAC inhibitor with high potency. Has exhibited promising results for treatment of several hematologic diseases, including CTCL, Hodgkin lymphoma, and leukemia. Has been used in combination with idarubicin, bortezomib, melphalan, and everolimus for the treatment of hematologic malignancies
Belinostat	PXD101	In phase I/II trials. Several ongoing trials are examining the efficacy of this hydroxamic acid-type HDAC inhibitor, either alone or in combination with idarubicin and bortezomib, for the treatment of hematologic malignancies
Entinostat	MS-275	Has a very long half-life (52 h). Shows significant biological activity in patients with hematologic malignancies; however, its efficacy as a single-agent therapy is limited
Butyrates (phenylbutyrate)		For urea cycle disorders

Table 1 content comes from references [172, 178, 182, 187, 189, 190, 193–195]

At least 18 forms of HDAC inhibitors have been reported; the forms differ in their structure and whether they have histones or non-histones as their substrates [22–42]. Evidence exists that these compounds are well tolerated and effective. Normal cells are relatively resistant to HDAC inhibitors. Epigenetic inhibitors that

have been identified thus far belong to one of the following classes of agents: short-chain fatty acids, hydroxamic acids, benzamides, cyclic tetrapeptides containing S-2-amino-9, 10-epoxy-8-oxodecanoic acid (AOE) moiety, epoxides, and psammaplins [43–64].

3 DNMT Inhibitors in Cancer Therapy

After several clinical trials, two DNMT inhibitors were approved by the FDA for use in treating MDS [64–70]. A randomized, controlled phase III study was performed that compared 191 patients with MDS who were treated with 75 mg/m²/day of 5-AzaC (Vidaza) given subcutaneously for 7 days every 28 days with a control group of 95 individuals treated with supportive care [71]. Compared with those receiving supportive care, the patients receiving 5-AzaC treatment showed significantly higher response rates, improved quality of life, reduced risk of leukemic transformation, and improved survival (18 months vs. 11 months, $p=0.03$). Based on these results, the FDA granted approval of 5-AzaC as a monotherapy for MDS and chronic myelomonocytic leukemia [71–134].

Decitabine (5-AzaC) also was tested in a phase III clinical trial of 170 MDS patients [135]. Eighty-nine individuals received intravenous decitabine (15 mg/m² every 8 h for 3 days every 6 weeks), and 81 patients received only supportive care (transfusions, growth factors, and antibiotics). The median age was 70 years, and about 14 % of individuals in both groups had secondary MDS.

One limitation in using the above inhibitors is that three separate DNMT genes (encoding *DNMT1*, *DNMT2*, and *DNMT3*) exist, and DNMT1 is the most abundant methyltransferase in cancer cells. Evidence indicates that inhibiting DNMT1 is not sufficient to stop cancerous growth. Additional inhibitors that target the remaining two DNMTs are required for successful treatment.

4 Clinical Trials of 5-AzaC (Vidaza) or 5-Aza-2'-Deoxycytidine (Decitabine) for Hematologic Malignancies

The mechanism of the antiproliferative effect of methylation inhibitors may be associated with reactivation of tumor suppressor genes by hypomethylation. Methylation inhibitor decitabine (5-aza-2'-deoxycytidine) showed promising results in patients with metastatic non-small-cell lung cancer [136].

In addition to global methylation changes, specific *p15INK4B* and *p21WAF1* methylations have been examined in clinical studies. The promoter region of *p15INK4B* frequently is hypermethylated in hematologic malignancies, including acute lymphoblastic leukemia, acute myeloid leukemia (AML), and MDS [110, 137, 138]. Investigators have attempted to correlate malignancies with

demethylation of *p15INK4B* or other methylated target genes. For example, one study showed that, in 9 of 12 MDS patients with *p15INK4B* hypermethylation, *p15INK4B* methylation levels had decreased after more than one course of decitabine treatment, and the decrease was correlated with the clinical response [139].

The expression of p21WAF1, another cyclin-dependent kinase inhibitor, has been explored in several decitabine studies. One study found that p21WAF1 messenger RNA (mRNA) was undetectable in 6 of 24 AML patient samples, irrespective of the levels of p21WAF1 promoter methylation [140]. Furthermore, decitabine induced p21WAF1 expression in the AML cell line KG-1 and subline KG-1a, even though the p21WAF1 promoter was not methylated; another study provided similar results [141–152]. To date, firm evidence that clinical responses are dependent on the demethylation of target genes is lacking.

5 HDAC Inhibitors

Histone deacetylation is another repressive epigenetic modification that removes acetyl groups from positively charged histone lysines and facilitates the binding of nucleosomes to the negatively charged DNA phosphate backbone. Deacetylation closes the chromatin, which is associated with gene repression, mainly by obstructing access of transcription complexes to coding sequences [153–158]. Inhibition of HDACs is one approach to the re-expression of epigenetically silenced genes, including tumor suppressor genes. Several HDAC inhibitors currently are being studied in preclinical trails designed to show their effects on the induction of differentiation and apoptosis in tumor cells [145, 159, 160]. The FDA approved vorinostat (Zolinza) for the treatment of cutaneous manifestations of T-cell lymphoma (CTCL) [161].

The specific anticancer targets of HDAC inhibitors are not entirely clear, but they appear to act via p53-independent, death receptor-dependent pathways [162] and/or a defective G2 checkpoint [163, 164]. Several classes of HDAC inhibitors are categorized by chemical family. These include short-chain fatty acids, hydroxamic acids, cyclic peptides, benzamides, epoxides, and hybrids [160].

The best-known HDAC inhibitor to date, hydroxamate suberoylanilide hydroxamic acid (SAHA, vorinostat) has shown promising results in cancer treatment. It suppresses the growth of lymphomas as well as ovarian, endometrial, and pancreatic cancers in vitro and in vivo [165–170]. SAHA also has been shown to induce growth arrest and differentiation in acute promyelocytic leukemia (APL) [171], breast cancer [172, 173], and multiple myeloma cells. This drug was approved by the FDA for the treatment of CTCL.

6 Chemical Classes of HDAC Inhibitors

HDAC inhibitors have differing structures and various effects on gene re-expression. However, they commonly suppress the cyclin-dependent kinase inhibitor p21WAF1 [165–170, 174–179]. Several HDAC inhibitors also have been shown to induce the expression of gelsolin, an actin-binding protein [180–182] that may be a tumor suppressor [183]. SAHA induces possible tumor suppressor genes, including 15-lipoxygenase-1 [184], thioredoxin-binding protein-2 (TBP-2) [185], and semaphoring III [186]. It also downregulates the gene expression associated with proliferation, including that of *cyclin A* [187, 188], *cyclin D* [188, 189], *hTERT* [190], and *her2/neu* [191, 192]. HDAC inhibitors may inhibit angiogenesis and metastasis by downregulating bFGF [193], HIF1- α [194], VEGF [193], and RECK (a regulator of metalloproteinases) [195] in cancer.

Several studies have focused on the specificity of activity of various HDAC inhibitors and patterns of gene re-expression, including tumor suppressor genes. For example, the DNA repair enzyme hMLH1 is re-expressed in ovarian cancer cells after their treatment with an HDAC inhibitor known as PXD101. Because hMLH1 expression has been associated with response to cisplatin, re-expression of this gene might resensitize these cancers to cisplatin.

In addition to their effects on histone acetylation, several HDAC inhibitors affect the acetylation of non-histone proteins such as the tumor suppressor gene *p53*. Cellular stress such as DNA damage induces *p53*, a transcriptional activator; *p53* also plays an important role in chemotherapy response, as tumors with a mutant *p53* often have a poor prognosis. The transactivation activity of *p53* is increased due to lysine acetylation by the histone acetyltransferase p300; conversely, *p53* is deacetylated by HDAC1, with a subsequent decrease in transactivation. Correspondingly, the HDAC inhibitors sodium butyrate and trichostatin A have been shown to elicit hyperacetylation of *p53*, providing another possible pathway for the suppression of tumor growth. The effects of HDAC inhibitors on other non-histone proteins also may be important for their anticancer activity.

Accumulating in vitro data suggest that inhibitors of both DNMT and HDAC may have synergistic antiproliferative activity against cancer cells, and clinical trials of this approach have commenced. For example, a combination of decitabine and valproic acid has shown promising activity in patients with AML and MDS, with a response rate of 22 %. In addition, azacytidine combined with phenylbutyrate (an HDAC inhibitor) showed promising efficacy in myeloid malignancies. Laboratory data suggest that DNMT inhibitors may increase the sensitivity of cancer cells to

chemotherapeutic agents, partly by regulating the expression of genes associated with drug resistance. Combinations of drugs with different epigenetic targets require further clinical attention.

7 Other Epigenetic Therapies Under Development

Demethylating agents and HDAC inhibitors are two classes of epigenetic therapy that currently are undergoing considerable pre-clinical study. Other drugs repress epigenetic modifications as well. For example, in addition to inhibitors of class I and II (zinc [Zn]-dependent) HDACs, inhibitors of class III (nicotinamide adenine dinucleotide [NAD]-dependent) deacetylases have been studied. One drug was reported to activate HATs, resulting in an increase of histone acetylation, although this compound's ability to permeate the cell membrane was poor. Inhibitors of histone methyltransferase such as EZH2 and SMYD3, which are overexpressed in many cancers, have been developed and patented. Other epigenetic targets include the methyl-binding domain proteins (MBDs), which play an important role in suppressing tumor suppressor genes. Antisense inhibitors of MBD2 suppressed tumor growth in both lung cancer cell lines and xenografts; these inhibitors were not significantly toxic to normal cells. Such specific effects may be due to MBD assistance in the recruitment of repressive complexes to specific genes. Another study showed that *chromodomain* antibodies, which target methylated Lys 9 of histone H3, could inhibit binding of the repressive protein HP-1, resulting in apoptosis of cultured fibroblasts. One such targeted therapy is double-stranded RNA (siRNA). siRNA against CpG island sequences mediated gene-specific, targeted methylation of the genes *E-cadherin* and *erbB* in normal human mammary and breast cancer cells [91].

Zn-finger proteins also have been demonstrated to specifically target distinct sequences; thus these proteins could be used as fusion partners for enzymes/inhibitors of epigenetic processes. For example, a Zn-finger protein was used to target the histone methyltransferases G9A and SUV39H1 to specific genes, including *MDR1*, *erythropoietin*, *erbB2/B3*, *VEGF*, and *PPAR-gamma*, resulting in epigenetic silencing [194]. Similarly, it was found that Zn-finger proteins could target a DNA methylase (*SssI*) to specific sequences in yeast, demonstrating the feasibility of gene-specific silencing by DNA methylation. Human experiments with these Zn-finger proteins have yet to be completed.

8 Advantages of Using Epigenetic Inhibitors

Epigenetic inhibitors, either alone or in combination, work effectively in cancer treatment. In Burkitt lymphoma and nasopharyngeal carcinoma, infection with Epstein-Barr virus (EBV) has been observed,

and it has been determined that hypermethylation of latency genes allows EBV to stay integrated in the main genome and contribute to these cancers. In MDS, with an incidence rate of 20,000 new cases per year in the USA, the standard treatment is weekly blood transfusion, which is considered aggressive treatment. Treatment with Vidaza, an epigenetic inhibitor, was successful in these patients, and most of them did not need transfusion. The survival time and quality of life of these patients increased substantially.

9 Combination Therapies (Methylation Inhibitors and HDAC Inhibitors with Other Drugs and Treatments)

Vorinostat, an HDAC inhibitor (also called Zolinza), was effective when combined with pelvic palliative radiotherapy for gastrointestinal carcinoma [193]. Vorinostat originally was approved by the FDA for the treatment of T-cell lymphoma [194]. In more than 400 patients with solid and hematologic malignancies, this agent was well tolerated and showed therapeutic response. Studies are planned to combine vorinostat with radiation therapy to treat non-small-cell lung cancer, glioblastoma multiforme (GBM), multiple myeloma, and MDS (a set of bone marrow conditions that often result in terminal blood cancer).

HDAC inhibitors also have been used in combination therapy. These inhibitors, in conjunction with tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), were capable of inducing apoptosis in breast, prostate, lung, and other cancer cells [185, 194]. Reports of epigenetic inhibitors functioning as radiosensitizers in breast, prostate, and glioma cells further suggest the importance of these inhibitors [184]. The interaction of inhibitors with DNA repair also has been proposed [189]. The combination of a methylation inhibitor (azacytidine), an HDAC (valproic acid), and carboplatin resulted in decreased death receptor 4 (DR4) methylation in patients with heavily treated advanced malignancies [196].

10 Future Directions and Conclusion

Azacytidine and decitabine are the most successful methyltransferase inhibitors used in the clinic. SAHA is the most widely used HDAC inhibitor. Several new inhibitors currently are being investigated and characterized. Both academia and industry have expressed interest in these new types of drugs for the treatment of cancer and other diseases. The problem of gene-specific targeting remains, however, and further research is needed to make these inhibitors gene-specific. The wrong kind of epigenetic intervention may result in the silencing of genes that should not be silenced. Nevertheless, biological responses can be distinguished from

responses that those resulting from deleterious mutational and other genetic changes. Overcoming problems that arose during the discovery of first-generation epigenetic inhibitors is another issue. Cytotoxicity is problematic for most of the newly identified inhibitors, and some compounds present problems relating to their solubility in aqueous solutions. However, drugs that reverse epigenetic changes to reactivate tumor suppressor genes may be a promising cancer therapy and can uncover masked tumor suppressor genes that can become new molecular targets for cancer therapy. Another limitation of epigenetic inhibitors is their relatively narrow therapeutic window. HDAC inhibitor effects on biological targets sometimes are reversible upon drug removal, and achieving a continuous effect of the drug could require long-term administration. Future research is needed to identify epigenetic drugs for different grades and stages of tumors.

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Chapter 25

Use of Epigenetic Modulators as a Powerful Adjuvant for Breast Cancer Therapies

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Abstract

Breast cancer (BC) is one of the five most frequent cancers in the world. Despite earlier diagnosis and development of specific treatments, mortality has only declined of about 30 % during the past two decades. Two of the main reasons are the emergence of drug resistance and the absence of specific therapy for triple negative breast cancers (TNBC), which are characterized by a poor prognosis due to high proliferation rate. Therefore, the future goal of the fight against BC will be to find new therapeutic approaches to overcome drug resistances and cure TNBC. Recent research on gene expression profiles linked to the different types of BC cells have led to consider the use of epigenetic modulators to modulate the expression of genes deregulated in cancer. The preliminary encouraging results have demonstrated a positive effect of DNA Methyl Transferase (DNMT) and Histone DeAcetylase (HDAC) inhibitors on different types of BC, as well as drug-resistant cells, with low side effects. In this review, we will describe the different epigenetic modulators currently used or investigated in BC therapy research in vitro as well as preclinical and clinical trials, and promising compounds, which might be used in future BC therapies.

Key words Breast cancer, DNA, DNMT, DNMTi, Epigenetic, HDAC, HDACi, Histone, Methylation, Therapy

Abbreviations

ATF	Artificial transcription factor
BC	Breast cancer
DCIS	Ductal carcinoma in situ
DNMT	DNA methyl transferase
ER	Estrogen receptor
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
PR	Progesterone receptor
SAHA	Suberoylanilide hydroxamic acid
TNBC	Triple negative breast cancer

TSA	Trichostatin A
TSG	Tumor suppressor gene
VPA	Valproic acid

1 Introduction

According to the World Health organization, breast cancer (BC) is one of the five most frequent cancer observed in the World, with 1.4 million new cases and 458,000 deaths in 2008 [1]. Thanks to earlier detection and more adequate treatments, mortality linked to this pathology has declined about 30 % over the past two decades. Nevertheless, the American Cancer Society estimated 64,640 new cases of breast carcinoma in situ (the first stage of cancer, without invasion of BC cells) in the USA in 2013 [2]. Apart from pediatric cancers, most cancers are linked to aging populations. Indeed, more than one cellular event is required to induce BC, and the probability to observe these events increase with age: nearly half of BC are diagnosed for women between 50 and 69 years of age in the UK. Various risk factors have been correlated to BC such as age, gender, race, ethnicity, or lifestyle but it is now well described that the most important risk factor for BC is genetic and more precisely, heredity. For example, more than 1,000 mutations of Breast Cancer 1 (*BRCA1*) and *BRCA2* genes have been reported in high-risk families (reviewed in [3]).

1.1 BC Subtypes

Therefore, it is now widely accepted that cancer development is a multistep process, which originates from atypical ductal hyperplasia to become ductal carcinoma in situ (DCIS). DCIS is a noninvasive pathology, but if not treated, could grow, spread, and become an invasive and more aggressive BC [4]. Many genetic and epigenetic alterations are linked to the evolution of BC, such as both mutations and hypermethylation of the tumor suppressor gene Breast Cancer 1 (*BRCA1*) [5], hypermethylation of *RASSF1A* [6] or hypomethylation of the oncogene *SYNUCLEIN γ* [7].

BC is not just one disease. Indeed, the various molecular alterations, which have been associated with the initiation of the invasive process, are heterogeneous, and reflect numerous subtypes (Fig. 1). The current BC classification is based on two different approaches: the immunohistochemical detection of cellular markers (estrogen receptor (ER), progesterone receptor (PR), and human epidermal growth receptor 2 (HER2)) or the molecular characterization of gene expression profiles. Using the latter classification, the most currently used in clinical studies, five different BC subgroups have been characterized and it has been described that these subtypes respond differently to treatments and are associated with different clinical outcomes [4] (we will use this classification, based on gene expression profiles, throughout this review). Today, many different primary therapeutic protocols are

Breast cancers molecular subtypes

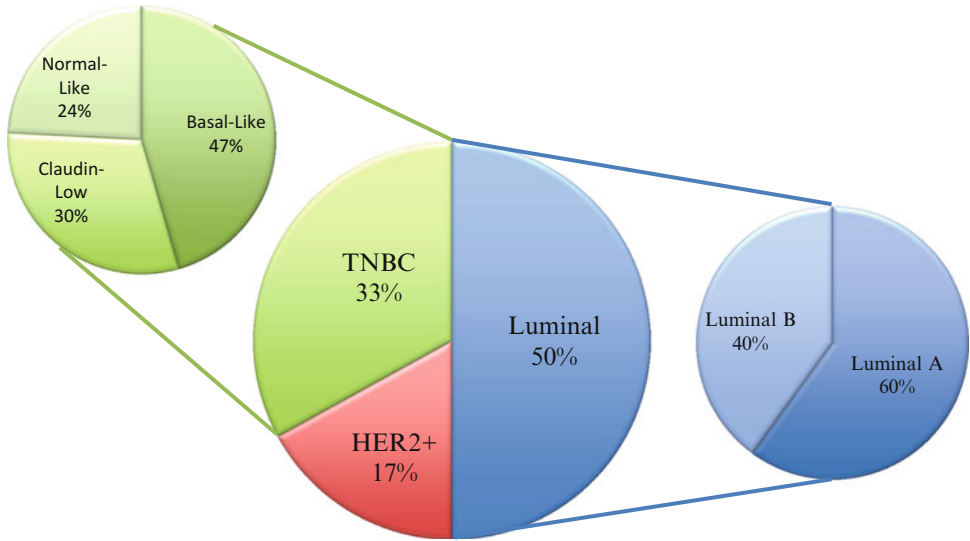


Fig. 1 Histogram describing the percentage of the breast cancers molecular subtypes in the overall population. The classification used for this study is based on the analysis of gene expression profiles. Adapted from ref. [3]. *TNBC* Triple negative breast cancers, *HER2* Human epidermal growth factor receptor 2.

used to treat the different BC subtypes (as described later), but in spite of an increasing interest for epigenetics in BC tumorigenesis, none of them involves epigenetic drugs.

1.1.1 Luminal Subgroups

Luminal A and B, which represent 50 % of total BC are generally associated with a good prognosis. These two types of BC are characterized by the expression of the estrogen receptor ER (ER⁺) that is involved in cell proliferation, survival, and invasion of BC cells. Nevertheless, endocrine therapy targeting the ER pathway has proven to be a really efficient way to treat this type of cancer and several molecules targeting this pathway are currently used: Tamoxifen, which antagonizes ER, Fulvestrant that decreases the level of ER, or Letrozole, an aromatase inhibitor (AI) that decreases estrogens levels via the inhibition of androgen metabolism [8].

BC patients with luminal B tumors frequently present a worse prognosis than luminal A patients. This might be explained by the presence of an amplification of the *HER2* gene, associated with a strong proliferation phenotype. As luminal B patients are both positive for ER and HER2, therapies combine the protocol used for luminal A BC supplemented with an HER2 inhibitor (e.g., Letrozole combined with Lapatinib, a synthetic HER2/1 inhibitor, a combination which has already been approved by FDA).

1.1.2 HER2⁺ Subgroup

HER2⁺ patients are characterized by the amplification of the onco-gene *HER2*. Due to the absence of ER expression, these tumors

Table 1
Chemotherapies used in treatment of BC cited in this review and their mechanism of action

Drug	Target	Mechanism of action
5-fluorouracil	DNA	Inhibition of DNA and RNA synthesis
Cisplatin		Inhibition of DNA synthesis, DNA damage, mutations
Doxorubicin (=Adriamycin)		Intercalation between base pairs, inhibition topoisomerase II
Epirubicin		Inhibition of DNA, RNA, and protein synthesis
Paclitaxel	Microtubules	Binds to tubulin β , therefore inducing the stablilization of microtubules (dynamic is stopped)

differ from luminal B cancers and hormonotherapies have been proven to be inefficient. HER2 overexpression was first associated with poor clinical outcomes but, during the last 15 years, the development of a growing number of new drugs targeting HER2 (e.g., Trastuzumab, Lapatinib, or Pertuzumab) and their application in combination with already existing cytotoxic chemotherapies (described in Table 1) have improved the long-term survival of these patients.

1.1.3 Triple Negative
Subgroup (TNBC)

Triple negative BC (ER⁻/PR⁻/HER2⁻), which is associated with a poor prognosis, is characterized by the absence of expression of both estrogen and progesterone receptors, but HER2 protein as well. These tumors are divided in three subgroups: (a) *Basal-Like*: these tumors exhibit some characteristics of breast myoepithelial cells, associated with both a high rate of proliferation and an extremely poor prognosis. (b) *Claudin-Low*: these tumors, which are associated with a poor prognosis, present an epithelial-to-mesenchymal transition (EMT) and stem-cell-like and/or tumor initiating cell features [9]. (c) *Normal-Like*: since these tumors present all features of non-pathological epithelial breast tissue, their classification is particularly difficult to establish and still under discussion mainly because of the absence of specific markers.

TNBC can however be responsive to preoperative Anthracycline/Taxane-based chemotherapies (e.g., docetaxel, doxorubicin, and cyclophosphamide) but a poor prognosis is given to patients who failed to be sensitive.

1.1.4 New Therapeutic
Strategies: A Real
Necessity

As described above, in spite of the existence of specific therapies for several BC subgroups, numerous studies have reported the apparition of acquired and de novo resistances during the course of treatments. Moreover, a specific therapy for TNBC is still lacking. Nevertheless, the extensive research that has been conducted on BC subtypes tumorigenesis during the last 20 years led to the emergence of new potential therapeutical strategies to fight BC.

Numerous approaches have been focused on finding inhibitors of the apparition of endocrine resistances.

For example, inhibitors of the phosphatidylinositol 3-kinase (PI3K)/AKT/mammalian target of rapamycin (mTOR) signaling pathway, which presents an essential role in growth and survival signal transduction, have been developed. Since cyclin expression is also increased in ER⁺ resistant BC, CDK4/6 inhibitors have been developed to inhibit cell cycle [10]. Antiangiogenic agents (e.g., Bevacizumab, anti-vascular endothelial growth factor-A (VEGF-A)) have also been developed since TNBC have been described to present higher levels of VEGF-A than other BC subtypes [11]. We can also cite poly(ADP-ribose) polymerases (PARPs) inhibitors which have been developed in order to induce cell death in deficient homologous repair cells, such as BRCA1/BRCA2 mutated BC. These compounds gave promising results in TNBC *BRCA1* mutated patients but the benefits of using PARPs inhibitors have not been proven in unselected TNBC clinical studies. Despite the potential effects on tumorigenesis of these new molecules, a specific TNBC therapy has yet to be approved.

Moreover, while genetic changes in BC are often correlated to subgroups, response to treatment and clinical outcomes, some epigenetic events were also reported to be related to the apparition of hormonotherapy resistances [12] and numerous studies have developed new epigenetic approaches to restore ER expression in resistant BC cells [13]. Indeed, as soon as 1999, Soares et al. [12] reported a global DNA hypomethylation in breast carcinomas compared to normal cells suggesting a role of epigenetics in BC carcinogenesis. So, even if genetic or epigenetic alterations are some of the most promising targets to treat BC, none of the many existing primary therapeutic protocols currently used to treat BC actually target epigenetic mechanisms.

In this review, we therefore describe the different epigenetic modulators used in BC therapies research and clinical trials, and the novel epigenetic strategies that might be used in the future in clinic. This review focuses on DNA methylation and posttranslational histone modifications, which are the major epigenetic modifications that control gene expression.

2 DNMT Inhibitors in BC Therapy

DNA methylation consists of the transfer of methyl residues on CpG dinucleotide-rich regions, called CpG islands, located in the 5' untranslated region of genes. This promoter methylation is accepted to be associated with gene repression. These methylation events are catalyzed by three DNA methyl transferases (DNMT): DNMT1 mainly catalyzes the DNA methylation of maintenance, while DNMT3a and DNMT3b are de novo methyltransferases.

Since promoter methylation is described to inhibit gene transcription, DNMT inhibitors (DNMTi) could be used to reverse the repression of tumor suppressor genes (TSG) or Estrogen Receptor 1 (*ESR1*) gene (which encodes ER α) in BC cells. Nucleoside analogues, a class of DNMTi, have already been used for a long time in vitro studies. The main compounds used in these studies are 5-azacytidine and 5-aza-2'-deoxycytidine (Decitabine), which are incorporated into DNA in replacement of cytosines during the replication process. Then, when DNMTs are recruited on the DNA to methylate cytosines, the enzymes are trapped by the analogue and cannot exert their function anymore, therefore inducing a passive DNA demethylation following DNA replication and cell division [14].

**2.1 Preclinical Data
Obtained Using DNMT
Inhibitors**

The classical BC cell lines (described in Table 2), used for preclinical studies are representative of the different molecular subtypes of BC. These models are the closest in vitro models available to predict BC cells response to the new treatments tested.

2.1.1 Decitabine

Numerous studies have described beneficial effects of the DNMTi Decitabine (1–10 μ M) on both viability and TSG expression in different BC cell lines, including TNBC models in vitro.

These preclinical studies revealed a large panel of changes in phenotype linked to Decitabine: (a) inhibition of cell growth [15–17]; (b) re-expression of TSG such as *HER4* in BT20 cells (an inhibitor of proliferation which promotes differentiation and/or apoptosis [18]), Dishevelled-Binding Antagonist Of Beta-Catenin 1 (*DACT1*), an antagonist to the Wnt/ β -catenin signaling, in MCF-7 and MDA-MB-231 cells [19], Protein Kinase D1 (*PRKDI*), which maintains epithelial phenotype, in MDA-MB-231 cells [20], Ras-association domain family 1 gene (*RASSF1A*), which blocks

Table 2
Breast cancer cell lines linked to the different subtypes of breast cancers

Classification	Immunoprofile	Breast cancer cell line
Luminal A	ER ⁺ , PR ⁺ , HER2 ⁻	MCF-7, T47D
Luminal B	ER ⁺ , PR ⁺ , HER2 ⁺	BT-474, ZR-75
HER2	ER ⁻ , PR ⁻ , HER2 ⁺	MDA-MB-453
Claudin-low	ER ⁻ , PR ⁻ , HER2 ⁻	BT549, Hs578T, MDA-MB-231
Basal-like	ER ⁻ , PR ⁻ , HER2 ⁻	BT-20, MDA-MB-468

ER Estrogen receptor, PR Progesterone receptor, HER2 Human epidermal growth factor receptor 2
Adapted from refs. [95, 96]

cell-cycle progression and inhibits cyclin D1 accumulation, in MCF-7 [21], Mismatch Repair Genes in MCF-7 cells [22], or *CLAUDIN-1* (implicated in tight junctions, whose downregulation is associated with metastasis in BC) in MDA-MB-453 cells [23]; (c) induction of apoptosis in BT-20 cells [18]; and/or (d) induction of the expression of ER in MDA-MB-231 cells [24, 25].

The reactivation of expression of TSG in BC cells is required to reestablish a non-cancerous phenotype and a response to treatments. Therefore, it is an important finding that Decitabine can reinduce the expression of TSG in different subtypes of BC, in particular in TNBC, for which specific therapies are not yet available, or in luminal subtypes of BC to avoid the apparition of resistances to hormonotherapies.

However, the effect of Decitabine might be different in ER⁺ and ER⁻ BC cell lines. Indeed, a 48 h treatment with 10 μ M Decitabine induces the invasiveness of MCF-7 cells (ER⁺), but decreases the invasive capacities of MDA-MB-231 cells (ER⁻) [26]. Treatment of MCF-7 with Decitabine also reduces ER protein levels [24]. Indeed, it has been reported that a decrease of ER mRNA stability, through the modulation of the RNA Binding protein HuR, is observed after a Decitabine treatment of ER⁺ cell lines [27]. These data show the importance of the selection of patients according to their genetic profiles. If an ER⁺ BC is pretreated with Decitabine, it would not be sensitive to future hormonotherapy anymore due to the loss of ER expression, therefore increasing the risk of the apparition of resistance to treatments. This might explain why conflicting results have been obtained while using Decitabine alone.

Conflicting results obtained by Jawaïd et al. have shown no growth inhibition of Decitabine on MCF-7 and MDA-MB-231 cells after a 10 μ M treatment during 72 h, but only a weak effect on MCF-7 growth with lower concentrations (100 and 500 nM) [28].

Due to the high Decitabine concentration (doses > 500 nM) required to observe a significant response in BC cells, the use of this compound in BC patients is not recommended since negative side effects will more likely be induced [29]. In order to limit cytotoxicity, low doses of Decitabine were tested in combination with other chemotherapeutic molecules. For example, Sandhu et al. [29] used up to 0.5 μ M of Decitabine in MDA-MB-453, BT-549, and Hs578T cells before treatment with Doxorubicin, Paclitaxel or 5-fluorouracil, combinations which effectively enhance chemotherapies efficiency following DNMT inhibition for 7 days. Similar results have also been obtained with MCF-7 and MDA-MB-231 cells but for higher doses of Decitabine (10 μ M for MCF-7 cells and 12 μ M for MDA-MB-231 cells for 72 h), demonstrating that synergistic effects are observed when MDA-MB-231 cells are pretreated with Decitabine before the use of Paclitaxel, Adriamycin, or 5-fluorouracil. In MCF-7 cells, semi-additive effects were only

observed with the 5-fluorouracil chemotherapy [30]. It is noteworthy that low doses of Decitabine during 24 or 72 h (0.2 μM) efficiently restore chemotherapy-resistant MCF-7 cells sensitivity to Doxorubicin [31, 32]. However, these results were moderated by Ari et al. in 2011 [26]. In their study, they showed that Decitabine stimulated invasiveness features of MCF-7 cells (10 μM for 48 h), reversed by a standard polychemotherapy (5-fluorouracil, Epirubicine, and Cyclophosphamide), while decreasing the invasive capacity of MDA-MB-231 cells. Induction of invasiveness by Decitabine was supported by another team which indicated that a concentration of 2.5 μM of Decitabine induced human *CRIPTO-1* (*CR-1*) mRNA expression, a gene involved in invasion and tumor progression [33]. Moreover, Ateeq et al. [21] showed that treatment of 7 days of MCF-7 cells with 5 μM Decitabine in vivo pre-clinical models decreased the growth of tumors but also induced the expression of prometastatic genes, such as *Heparanase*.

These results clearly demonstrate the critical role of Decitabine in cancer cells. Decitabine can induce the death of numerous types of BC cells and therefore inhibits cancer cell proliferation, but can also increase invasiveness features of cells. We therefore must be cautious when using this type of drugs because only one invasive cell is sufficient to induce metastasis. However, until now, the ability of Decitabine to induce tumor metastases has not yet been proven.

2.1.2 5-Azacytidine

The phenotypes of BC observed after treatment with 5-azacytidine alone are similar to those obtained with Decitabine. As described above for Decitabine: a decrease of BC cell growth, an increase of expression of tumor suppressor genes or the *ESR1* gene and an increase of apoptosis in tamoxifen-resistant MCF-7 cells (1 μM 5-azacytidine and Estradiol) have been shown after 5-azacytidine treatment during 6 days [34]. 5-azacytidine can also restore or amplify effects of chemotherapies. Indeed, different in vivo studies have shown a limitation of tumor growth in mice (5 mg/kg a day, three times a week during 5 weeks) [35], or potentiate Cisplatin effects while decreasing its nephrotoxicity in rats (2.5 mg/kg at days zero and three) [36].

Taken together these preclinical results, obtained using nucleoside analogues, have attracted an increasing interest to use these molecules in BC treatment and to be able to restore the cytotoxic effects of chemotherapies already used in clinical trials.

2.2 Clinical Trials Using DNMT Inhibitors-Based Protocols

A phase I study was performed in 2003 in order to test the side effects and determine the optimal dose of Decitabine to use to treat BC patients diagnosed with advanced solid tumors [37]. The results obtained showed that a 72 h intravenous infusion of Decitabine (30 mg/m² per day) was tolerated by the patients. Moreover, after samples analysis, changes in gene methylation were detected [38]. Indeed, the results showed a decrease of the

methylation of the calcitonin gene-related peptide (*CALCA*), a gene implicated in cell metabolism and often hypermethylated in BC. Decitabine has also been shown to be safely combined with chemotherapy, such as Carboplatin, in patients with solid tumors, as shown in 2007 during a phase I study [39]. A dose of Decitabine (90 mg/m²) followed by a dose of Carboplatin 8 days after (treatments renewed every 28 days) has been shown to be well tolerated. Since preclinical studies revealed the increased efficiency of treatments combining DNMTi with other drugs, a phase I/II study is currently recruiting patients with advanced solid tumors to evaluate the effects of 5-azacytidine combined with Abraxane, a Paclitaxel albumin-stabilized nanoparticle [40].

To our knowledge, there are few clinical trials using DNMTi in BC treatments. However, DNMTi are largely used in clinical trials in other types of cancers, such as acute myeloid leukemia or lung cancer. Furthermore, the first results obtained in clinical trials are encouraging, showing few side effects and its efficiency to modulate gene expression in patients.

3 HDACs Inhibitors

The degree of chromatin compaction is controlled by histones compaction, which is regulated by posttranslational modifications such as methylations, ubiquitylations, or acetylations. Histone acetylations are catalyzed by Histone Acetyl Transferases (HATs) and these modifications are associated with opened active chromatin allowing gene transcription, while Histone Deacetylases (HDACs) remove these acetylations, close the chromatin and inhibit transcription. In BC, activity of Histone deacetylases (HDACs) has been correlated with inhibition of the expression of critical genes, such as TSG, and therefore HDACi have been tested in BC in order to determine whether they could improve the response to treatments or decrease tumor aggressiveness [41, 42].

Until now, five classes of HDACs inhibitors have been described (Table 3) and an increasing number of new molecules have been developed [14]. Since HDACs are specifically targeted to genes, the use of HDACi will only affect genes modified by the HDACs class targeted. Trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) are actually the most extensively studied because they were first discovered, present a broad range of activity and inhibit HDACs class I and II.

3.1 Preclinical Data Obtained Using HDAC Inhibitors

3.1.1 TSA

Several studies have described growth inhibition and cell death in TNBC model cell lines (such as MDA-MB-231) treated with TSA alone (500 nM, 48 h [28] or 100 μM, 72 h [43]). These results point out that TSA can decrease the growth of ER⁻ BC cells, as it has been described for ER⁺ BC cells (like MCF-7 cells).

Table 3
HDAC inhibitor families and their targets

HDACi families	Names	HDACi target
Hydroxamate based	TSA SAHA LBH 589	HDAC class I and II inhibition HDAC class I, II, and IV inhibition
Aliphatic based	VPA	HDAC class I and II inhibition
Cyclic peptide based	FK228	HDAC class I
Benzamide based	MGCD0103	HDAC class I, II, and III inhibition
	SNDX-275	HDAC class I inhibition

Based on refs.[14] and [97]

However, these doses are elevated and could be toxic for patients. Lower doses of TSA (0.1–1 μM) can also efficiently induce the expression of TSG within 14 h of treatment and increase the stability of ER in T47D cells after only 3 h of treatment [19, 44]. However, surprisingly, an other study using longer time of exposure (16 h, 0.3 μM TSA) have shown a decrease of ER protein expression in MCF-7 cells [45]. Moreover, it has been shown that TSA treatments sensitize TNBC cells to Tamoxifen [46], induce apoptosis [47], or decrease MCF-7 cell proliferation when used in combination with Raloxifen [48]. As seen above for DNMTi, drastic effects on BC features were only observed with high doses of TSA, but patients could better tolerate combinations of low doses of TSA and additional antitumoral drugs.

In most cases, epigenetic-mediated gene silencing is mediated by both DNA methylation and posttranslational modifications of histones associated with chromatin condensation. Co-treatments with TSA and DNMTi could then improve the re-expression of these epigenetic regulated genes. Indeed, studies using TSA combined with Decitabine have restored Tamoxifen sensitivity in MCF-7 cells [49]. However, authors did not present any data showing that TSA/Decitabine pretreatment was more beneficial than each drug used alone. It is noteworthy that the sequence of administration of these drugs seems to be crucial since better results were obtained when Decitabine treatment preceded TSA/Tamoxifen co-treatment in MDA-MB-231 cells [50]. Indeed, DNMT and HDAC inhibitors synergize to induce re-expression of ER in ER⁻ BC cells, critical to induce sensitivity to Tamoxifen. When Decitabine was used before TSA/Tamoxifen treatment, the

ER mRNA levels observed were higher than the ER quantified with Decitabine used in the same time with TSA and Tamoxifen. Moreover, the restoration of sensitivity to Tamoxifen in ER⁻ cells treated with both Decitabine and TSA was confirmed in *in vivo* studies, suggesting the powerful use of epigenetic compounds to facilitate endocrine therapy [51]. Indeed, it has been demonstrated that the growth of xenografts performed with MDA-MB-435 pre-treated with Decitabine (96 h, 2.5 μ M) and TSA (12 h, 100 ng/mL) decreased compared to the control, and *in vitro* experiments have shown that these epigenetic modulators can enhance the Tamoxifen-driven growth inhibition of MDA-MB-435.

3.1.2 SAHA

Many studies have shown beneficial effects of SAHA in both *in vitro* and *in vivo* models. In BC cell lines, including ER⁺ BC (MCF-7), ER⁺ HER2⁺ (BT-474), HER2⁺ (MDA-MB-453), and TNBC (MDA-MB-231 and 4 T1), antitumoral properties of SAHA (1–10 μ M, 24 h) have been reported, like inhibition of tumor growth and migratory/invasive features [52–54]. Indeed, SAHA can induce apoptosis in MDA-MB-231 cells [55], restore sensitivity to classical drugs in resistant MCF-7 cells [56], improve radiosensitivity in MCF-7 and MDA-MB-231 cells [53], or overcome Doxorubicin resistance in MCF-7 resistant cells [32]. SAHA treatments have also been shown to improve survival of mice bearing MDA-MB-231 xenografts [57]. Furthermore, it has been shown that SAHA (25 mg/kg, three times a week, during three weeks) can inhibit 4 T1 cell metastasis *in vivo* [53].

As shown earlier, combination of different epigenetic compounds could also provide novel therapeutic solutions to decrease drug concentrations but also to limit acquired resistances to chemical compounds. It is known that HDACs interact with other chromatin modifier proteins, such as lysine-specific demethylase (LSD1), which control histone demethylation. The HDACs/LSD1 complex has been shown to be implicated in BC tumorigenesis [58]. Indeed, the histone demethylation activity of LSD1 is dependent on its interaction with HDACs, and is often associated with inhibition of gene expression. A combination of SAHA with Pargyline, a LSD1 inhibitor, has recently shown promising results in TNBC and HER2⁺ BC by inhibiting their growth [58, 59].

3.1.3 LBH589

LBH589, also known as Panobinostat, is another HDAC inhibitor, efficient against class I, II, and IV within the nanomolar range of concentrations (100 nM, 72 h). It can increase cell death in estrogen sensitive and insensitive BC cells [60], increase the expression of numerous antitumoral genes, and improve survival of mice injected with aggressive TNBC MDA-MB-231 cells [57, 61]. Furthermore, LBH589 can also inhibit the expression of aromatases (implicated in androgen metabolism), and synergize with Letrozole to decrease estrogen levels [62]. These results indicate

that the use of LBH589 in patients presenting aggressive BC might be of particular interest since it can be efficient at low concentrations and present very few side effects.

3.1.4 SNDX-275

SNDX-275, also known as Entinostat or MS-275, is a class I HDAC inhibitor. Its use has shown promising preclinical results at low concentrations (0.2–0.5 μM). Indeed, it can induce apoptosis in HER2⁺ BC cell lines (MDA-MB-453 and BT474) with a 1.5 μM treatment, during 24 h [63], but the maximum tolerated doses in patients were about 0.4 μM . The use of SNDX-275 in combination with other compounds might be a hopeful way to use it at lower concentrations. For example, it has been shown that the sensitivity to Trastuzumab in Trastuzumab-resistant HER2⁺ BC cell lines is enhanced with a treatment of 0.2 μM of SNDX-275 during 96 h while using half the concentration of Trastuzumab [64]. SNDX-275 can also restore the sensitivity to the aromatase inhibitor Letrozole, in ER⁻ tumors (MDA-MB-231) in vivo (2.5 mg/kg/day), leading to the inhibition of tumor progression and lung metastasis [65, 66], the reversion of epithelial–mesenchymal transition and the inhibition of tumor progression, metastasis, and angiogenesis in the apoptosis-resistant cell line MDA-MB-468 when SNDX-275 (6 mg/kg/day given 4 times with doses of 35 mg/kg) was followed by TRAIL injections [67].

These preclinical data have shown that SNDX-275 might have a strong impact on different resistant BC cell lines and therefore could be considered as a future promising tool to treat the most resistant cancers.

3.1.5 Valproic Acid

Valproic acid (VPA) is an inhibitor of class I and II HDAC activity and can inhibit the growth of both ER⁺ and ER⁻ BC cells (MCF-7 and MDA-MB-231) with incubation during 72 h with 10 mM VPA or during 24 h with 250–500 mM VPA [28, 68]. VPA can enhance Tamoxifen efficiency in ER⁺ cell lines (MCF-7, ZR-75-1, T47D) with a 750 μM therapeutic dose for 6–7 days [69]. However, at low-doses (3 μM), VPA could also induce migration and invasion of BC cells [33]. Indeed, VPA alone or in combination with Decitabine induced the expression of *CR-1*, which plays an important role in embryonic development while also regulating various stages of tumor progression, and enhancing MCF-7 invasion and migration abilities, compared to MCF-7 control.

3.2 Clinical Trials Using HDAC Inhibitors- Based Protocols

Based on the very interesting results observed in preclinical studies using HDACi, many clinical studies in BC patients have been designed with several of these compounds. They are described in the Table 4.

3.2.1 TSA

In spite of very promising results obtained in preclinical studies, too many side effects were observed with TSA treatments in clinical trials. This compound would then not be used in clinic [70].

Table 4
Clinical trials designed with HDAC inhibitors-based protocols

Drug	Type of BC targeted	Phase	Status	In combination with	Ref.
Panobinostat (LBH 589)	HER2 ⁺	I	Completed	Trastuzumab + Paclitaxel	[98]
	All	I	Completed	Capecitabine, ± Lapatinib	[99]
	All	I/II	Suspension of participants recruitment	Letrozole	[100]
	HER2 ⁺ trastuzumab resistant	I/II	Completed	Trastuzumab	[101]
	TNBC	I/II	Participants recruitment	Decitabine, Tamoxifen	[81]
	HER2 ⁺	II	Terminated = 4 patients		[79]
	HER2 ⁻ ER ⁺ tamoxifen resistant	II 0	Ongoing Terminated		[102] [103]
Entinostat (SNDX-275)	All types of cancers, BC included	I	Completed		[104]
	HER2 ⁺ trastuzumab resistant	I	Participants recruitment	Lapatinib + Trastuzumab	[105]
	ER ⁺ and lung cancers	I	Ongoing	± Exemestane ± Erlotinib	[106]
	ER ⁺	II	Ongoing	± Exemestane	[107]
	AI resistant	II	Completed	Aromatase inhibitors	[108]
	AI resistant	II	Participants recruitment	Azacytidine	[78]
	All TNBC Surgery removable	II	Terminated	Anastrozole	[109]
Vorinostat (SAHA)	All	0	Participants recruitment	Hormone therapy (Anastrozole, Letrozole, Exemestane)	[110]
	AI resistant	0	Completed	Hormone therapy	[111]
	All	I	Ongoing	Ixabepilone	[112]
	DCIS	I	Ongoing		[113]
	All	I/II	Ongoing	Paclitaxel + Bevacizumab	[114]
	HER2 ⁺	I/II	Completed	Trastuzumab	[115]
	Newly diagnosed	II	Ongoing	surgery	[116]
	Surgery removable	II	Ongoing	Paclitaxel albumin-stabilized nanoparticle formulation + Carboplatin	[117]
	All	II	Completed		[118]
	All	II	Completed	Tamoxifen	[119]
	All	II	Participants recruitment	Lapatinib	[120]
Valproic acid (VPA)	All	II	Participants recruitment	FEC100	[82]
CUDC-101	All	I	Completed		[84]

AI Aromatase inhibitor, ER Estrogen receptor, Status completed: All participants have been recruited, Status terminated: the study has been stopped early and will not start again

Adapted from clinicaltrials.org

3.2.2 Vorinostat=SAHA

For historical reason (first approved by FDA), Vorinostat has been used in the majority of BC clinical studies using epigenetic drugs. But until now, only few studies have been completed and published. First studies reported various adverse effects of Vorinostat when used alone [71, 72], confirming that its combination with other compounds and a decrease of its concentration would be a better option for its use in therapeutic approaches. Indeed, a phase I trial combining Vorinostat with Doxorubicin, showed that a maximum of 800 mg/day of Vorinostat should be administered to limit side effects, in combination with a weekly dose of Doxorubicin (20 mg/m^2) [73]. Another phase I/II trial indicated that Vorinostat could also be combined with Paclitaxel and Bevacizumab. The aim of the phase II of this clinical trial will be to improve the response rate from 40 to 60 % [74]. The study is ongoing. A third study reported that Vorinostat (400 mg/day) combined with Tamoxifen, in ER⁺ endocrine resistant BC, was well tolerated and induced a 40 % clinical benefit after 24 weeks [75]. Finally, a phase I study, combining Decitabine and Vorinostat, has shown prolonged disease stabilization in different solid tumors [76].

3.2.3 Entinostat =SNDX- 275

A randomized, placebo-controlled, phase II study has evaluated the Entinostat (5 mg weekly) effect in combination with the aromatase inhibitor Exemestane (25 mg daily) versus Exemestane alone [77]. The results have demonstrated that combining Entinostat and Exemestane increased overall survival from 19.8 to 28.1 months.

Furthermore, a phase II study is currently evaluating the combination of two epigenetic modulators: SNDX-275 and Azacytidine in metastatic BC [78].

3.2.4 Panobinostat =LBH589

Until now, only one clinical trial using Panobinostat alone (with only four patients included) has been completed and results published [79]. Panobinostat seems to be a promising tool because of its easy way of administration (oral) and its use at very low concentrations. The first results showed that this drug presents limiting side effects and can be combined with other drugs (Panobinostat 10 mg orally three times weekly in combination with Paclitaxel 175 mg/m^2 and Carboplatin administered intravenously on day 1 of every 21-day cycle) [80]. Another phase I/II clinical trial is currently evaluating the combination of Panobinostat with the DNMTi Decitabine and Tamoxifen, in TNBC [81].

3.2.5 Other Clinical Trials Using HDAC Inhibitors

Valproic acid (VPA) has been approved by FDA and can inhibit class I and II HDACs. A phase II study is currently recruiting patients to evaluate whether VPA could enhance chemotherapy sensitivity of BC [82], since a phase I study has showed that VPA (140 mg/kg/day for 48 h) could be associated with Epirubicin (100 mg/m^2) [83]. Furthermore, another completed phase I study

has evaluated effects and doses to be used with a compound called CUDC-101, which can inhibit EGFR, HER2, and HDACs [84]. The use of multi-target agents could solve problems of resistances often met in single-target therapies. For the moment, multi-target therapies are performed with the combination of different drugs, presenting different pharmacokinetics and toxicity. The use of a single drug with more than one target is a good alternative to prevent the different side effects of multidrug therapies. CUDC-101, contain the structural elements to inhibit HDACs (like Vorinostat) and to inhibit EGFR and HER2 (like Lapatinib). The inhibition of HDACs could modulate various targets, and one of them is the HER family receptor tyrosine kinase pathway, to counteract molecular heterogeneity of cancers and resistances against anti EGFR and HER2 treatments [85].

4 Promising Compounds

The current epigenetic modulators used nowadays are not specific and induce global epigenetic inhibition. Indeed, these compounds usually inhibit for example all the DNMTs for DNMTi, inducing the re-expression of TSG, but can also induce as a consequence the expression of oncogenes, as described above (using Decitabine on MCF-7 cells can induce expression of angiogenic genes in vitro and in vivo [21]). These indirect effects cannot be controlled, because their mechanisms of action are still unknown. First data on DNA methylation have shown that interactions of DNMTs with transcription factors are crucial to induce targeted methylation [86]. In other words, inhibition of a unique DNMT could inhibit an important number of methylated promoters. Therefore, a better knowledge and understanding of the regulation of epigenetics will be needed in the future to improve BC therapy. One of the promising ways of treatment will be personalized medicine, the theranostic. The objective will be in the future to extensively characterize protein, epigenetic and genetic markers of a specific tumor. Therefore to use the best available combination of drugs inducing the best course of treatment but the lowest toxicity and the fewest side effects, as well.

4.1 *Natural Compounds*

As described earlier, different chemical epigenetic modulators are currently in use in BC clinical trials. However, they present most of the time a high toxicity and many side effects. To reduce toxicity, natural compounds with epigenetic properties (found in food or plants), are currently under research (reviewed in [87]). For example, we can cite curcumin and genistein which are inhibitors of expression of DNMT1, HDAC1 and MeCP2 (a protein that binds specifically to methylated DNA, inhibiting gene expression) [88]. Curcumin has been shown to enhance Tamoxifen sensitivity at

2.5 μM concentration in Tamoxifen-resistant MCF-7 cells [89] and genistein purified from soya can inhibit growth of BC xenografts [90].

4.2 Adenosine/ Methionine Cycle

Montenegro MF et al. [91], have reactivated the expression of *RASSF1A*, a tumor suppressor gene, by inhibiting both DNA methylation and the transcription factor E2F1; To do so, the authors used DIPY, an inhibitor of adenosine elimination, in combination with TMEG, an antifolate to inhibit methionine production. By inhibiting methionine production (substrate of methylases), methylation of proteins and DNA is impossible. Furthermore, adenosine accumulation in the cell results in the production of an inhibitor of cellular methyltransferases. This treatment has been shown to induce apoptosis of MDA-MB-231 cells.

4.3 miRNA

miRNAs are classified as epigenetic modulators since they specifically inhibit the expression of proteins, by interacting with the 3' UTR of pre-mRNA. Numerous studies have shown that miRNA expression pattern is altered in cancers and that they can be considered as biomarkers of BC grades (reviewed in [92]). Most of the time, miRNA are downregulated in BC compared to normal tissues. The delivery of lacked miRNAs in specific patients by different vectors such as adenoviral vectors, nanoparticles, and lipid-based materials might therefore be a promising new therapeutic protocol to specifically treat BC.

4.4 Artificial Transcription Factors (ATF)

The major flow of drugs used in BC treatment is the fact that they are not specific enough. Indeed, HDACs and DNMTs inhibitors induce a general histone acetylation or gene demethylation, without being able to control which promoters would be modified. The benefit to use ATF would be to specifically control which promoters would be demethylated. ATF used the principle of Zinc-Finger proteins, which could recognize specific sequences. Indeed, ATF contain DNA-Binding Domains (DBD). Each DBD recognizes specifically 3 bp, and ATF comprise 6 DBD, to target a highly specific 18 bp sequence. Rivenbark et al. have proven that inducing a specific recruitment of DNMT3a on a promoter is achievable [93], and can induce expression of the targeted gene. Another team using ATF has demonstrated that *Maspin*, a TSG which silencing is correlating in BC cells with invasive and metastatic behavior, could be re-expressed in MDA-MB-231 cells, to reduce tumor growth [94].

5 Conclusion

The development of new therapies to fight BC will be essential in the future to overcome the apparition of resistances to already existing therapies, such as hormonotherapies, but also to find new

compounds that might be efficient to treat TNBC. Epigenetic-based therapies seem to be a good option since the clinical trials already performed present better results than the ones performed with the current existing therapies while presenting very little side effects. Their way of administration is also easier and less invasive for patients (orally for Vorinostat), and they seem to be also efficient on drug-resistant BC cells. Moreover, recent intensive research on gene expression profiles linked to BC cells has led to the development of new emerging patient-specific therapies, such as ATF. These compounds seem to be even more efficient than DNMT and HDAC inhibitors actually used in clinical trials.

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Epigenetic Approaches in Glioblastoma Multiforme and Their Implication in Screening and Diagnosis

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Abstract

Epigenetic modifications have been reported in a number of non-germ-line tumor types. Epigenetic modifications to the genome, especially DNA methylation and histone modifications, affect gene expression causing increased risk for cancers and other diseases. We have summarized information about DNA methylation percentages in Glioblastoma multiforme (GBM) line HTB-12, alveolar cell carcinoma, and acute lymphocytic leukemia samples and determined H3 (K27) methyltransferase activity in GBM and leukemia cells and made comparisons to H3 (K27) methyltransferase activity in normal astrocyte, lung, and lymphocyte cells. GBM and alveolar cell carcinoma gDNA possessed lower gDNA methylation percentages compared to normal cells. Methyl-sensitive cut counting analysis (MSCC) showed fold decreases in GBM CpG methylation sites for genes PBK, KIF23, COL6A3, and LOX. There was no significant difference in CpG DNA methylation, but less histone methyltransferase activity in acute lymphocytic leukemia compared to normal cells. GBM possessed increased histone methyltransferase activity compared to normal samples. Challenges in the field in diagnosis and prognosis for cancer risk especially with regard to the results of this work are discussed.

Key words Biomarker, Epigenetics, Glioblastoma multiforme, Histone, Methyl transferase, Methylation

1 Introduction

Epigenetics is the study of changes in gene expression caused by mechanisms other than changes in the germ line DNA sequence [1–9]. Epigenetics is a rapidly advancing field with increasing impact on biological and medical research [5, 10, 11]. The DNA molecule contains within its chemical structure two layers of information. The DNA sequence that bears the ancestral genetic information and the pattern of distribution of covalently bound methyl groups to cytosines mainly found within the sequence C-phosphodiester bond-G (CpG). While an individual's genetic information is similar in all tissues, the distribution of CpG methylation across the genome is cell-type specific [12, 13]. The major

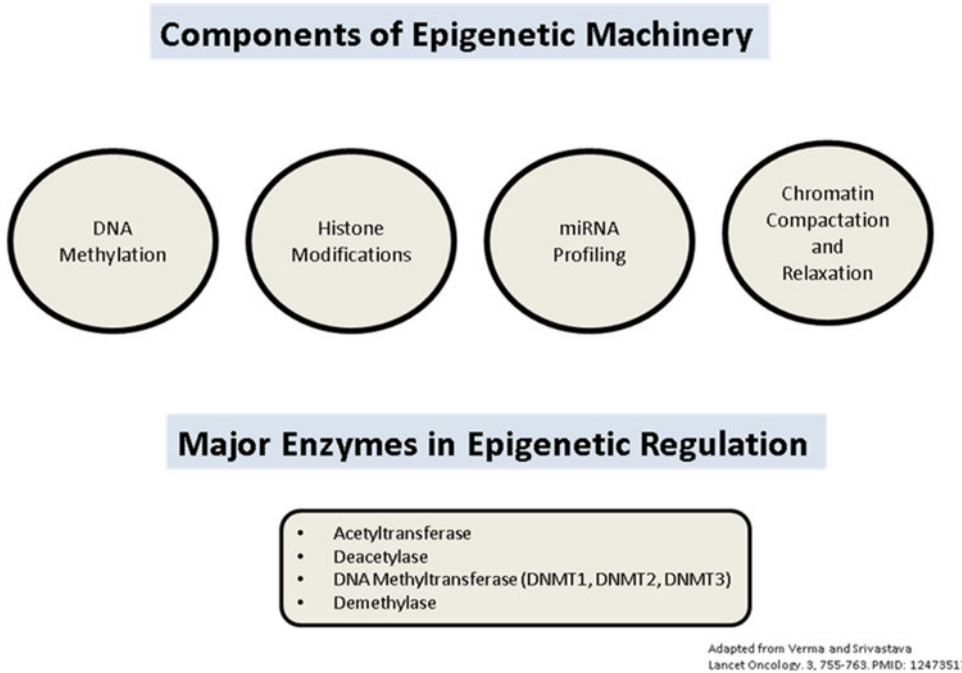


Fig. 1 Components of epigenetics and major enzymes involved in gene regulation

components of epigenetics are shown in Fig. 1. DNA methylation is an important regulator of gene function [14, 15]. This expands the potential role of DNA methylation beyond embryogenesis to other time-points in life and to postmitotic tissues such as the brain [16–18]. DNA methylation is proposed to act as a genomic response to both physical and social signals from the environment at different time points in life and to serve as a genomic memory of these exposures at different time scales, stably altering gene expression programming and thus modulating the physical and behavioral phenotypes to respond to these environments. It is hypothesized that DNA methylation provides within the structure of the DNA a dynamic interface between the changing world around us and the relatively fixed and stable genome.

2 Epigenetic Regulation in Glioblastoma Multiforme and Potential Biomarkers of Screening and Diagnosis

Glioblastoma multiforme (GBM) is an aggressive and lethal cancer, accounting for the majority of primary brain tumors in adults [19, 20]. GBMs are characterized by genetic alterations large and small, affecting genes that control cell growth, apoptosis, angiogenesis, and invasion [21, 22]. Epigenetic alterations also affect the expression of cancer genes alone, or in combination with other

genetic mechanisms [23–27]. For example, in each GBM, hundreds of genes are subject to DNA hypermethylation at their CpG island promoters. A subset of GBMs is also characterized by locus-specific and genome-wide decrease in DNA methylation, or DNA hypomethylation. Other epigenetic alterations, such as changes in the position of histone variants and changes in histone modifications are also likely important in the molecular pathology of GBM, but somewhat surprisingly there are very limited data about these in GBM. Alterations in histone modifications are especially important to understand, given that histone deacetylases are targets for drugs that are in clinical trial for GBMs [28–30]. The technological wave of next-generation sequencing will accelerate GBM epigenome profiling, allowing the direct integration of DNA methylation, histone modification, miRNA profiling, and gene expression profiles. Ultimately, genomic and epigenomic data should provide new predictive markers of response and lead to more effective therapies for GBM.

3 Methods and Technologies in Epigenetics

3.1 *Gene Expression and Methylation*

Genomic DNA methylation, or the addition of a methyl group to the nucleotide base cytosine, is one of two main ways epigenetics can affect gene expression [31–33]. This cell type specific mechanism works by regulating gene transcription and DNA methylation patterns. Methylation persists over cellular generations and thus continues on in the epigenome. There are two ways in which DNA methylation can alter gene expression: hypermethylation and hypomethylation. Hypermethylation entails higher levels of methylation than occur normally and has been associated with gene silencing and can lead to the development of cancers, especially if those genes are tumor suppressor genes. Conversely, hypomethylation involves the loss of normal methylation potentially leading to activation of formally suppressed genes, such as oncogenes, which can cause a normal cell to become cancerous [6, 34–40].

The addition of a methyl group prevents the transcription of the gene segment and ultimately the expression of the protein encoded by the gene segment [41–46]. Methyl groups are transferred to the 5-carbon position of a cytosine from S-adenosylmethionine (SAM) by DNA methyltransferases (DNMTs) and are linked via a covalent bond.

3.2 *DNA Methylation Sites*

DNA methylation primarily occurs on cytosines located next to a guanine on the same strand of DNA and linked by a phosphodiester bond, commonly referred to as cytosine–phosphate–guanine (CpG) island [44]. This is not to be confused with cytosine–guanine hydrogen bonding between two complementary strands of DNA. For a site to be deemed a CpG island it must possess two

characteristics: a sequence GC content greater than 55 % and a minimum 0.65 ratio of CpG to GpC [47, 48]. Most CpG islands are found in the 5' promoter regions of genes, with roughly 1 % occurring outside of the promoter regions. Most of the CpG islands located in the promoter regions remain unmethylated, a state needed to maintain gene expression. However 70 % of CpG islands found outside the promoter region and in the coding region have been found to be methylated [49].

3.3 Enzymes and Methylation

Methyl groups from SAM are transferred to cytosines by DNMTs. There are three main DNMTs that are involved in DNA methylation: DNMT1, DNMT3a, and DNMT3b. DNMT3a and DNMT3b are mainly active during early development and work de novo to establish cell type specific DNA methylation patterns [50]. Another enzyme, DNMT3L, does not work directly on the DNA, but regulates the activity of DNMT3a and DNMT3b. DNMT1 primarily works by maintaining already established DNA methylation patterns [50]. The enzyme is able to recognize methylated CpG sites on the parent DNA strand and will catalyze the addition of a methyl group onto the corresponding daughter strand during DNA replication. It should be noted that, though each DNMT has a preferential role, they have been found to contribute to multiple pathways. Though certain enzymes have been found to passively remove DNA methylation by preventing the addition of methyl groups to newly synthesized DNA by DNMTs, there is little evidence to suggest there are enzymes that act as a DNA demethylase [51].

3.4 Stem Cell Methylation

Methylation of DNA can also occur on CHG or CHH sites, where H represents an adenine, cytosine, or thymine. Found almost exclusively on embryonic stem cells, CHG and CHH methylation sites account for 25 % of all methylation sites on embryonic stem cells [52]. Interestingly, these methylation sites disappear after the stem cells differentiate. This has led scientists to believe that the loss of CHG and CHH methylation plays an important role in embryonic stem cell differentiation [52].

3.5 Gene Expression and Histone Modification

Modification of the histone proteins is another method by which epigenetics can affect gene expression. The core histone complex is comprised of two sets of four different subclasses of histone proteins: H2A, H2B, H3, and H4 [53–55]. The proteins range between 103 and 136 amino acids in length, with the H3 histone comprising 135 amino acids [56]. About 147 base pairs of double-stranded DNA wraps around the core histone complex to form a nucleosome. Nucleosomes are condensed and packaged into chromatin which comprises chromosomes. There are two forms of chromatin, euchromatin and heterochromatin. Euchromatin is less condensed than heterochromatin and contains genes that will be actively transcribed. Heterochromatin contains genes that are not actively transcribed and are considered silent.

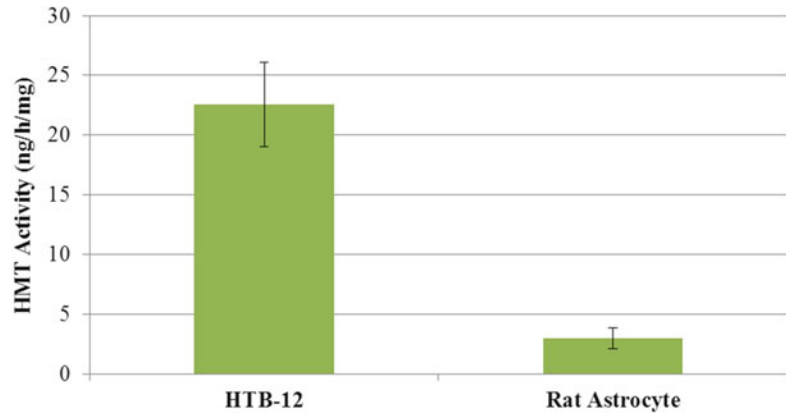


Fig. 2 Histone methyltransferase H3 (K27) activity quantification of HTB-12 and rat astrocyte nuclear protein extracts. Samples were tested using an EpiSeeker Histone Methyltransferase H3 (K27) Activity Quantification Assay Kit. HTB-12 was tested using 4.64 μg and rat astrocyte using 12.82 μg of nuclear protein extract. HTB-12 had an HMT activity of 22.54 ng/h/mg, while rat astrocyte had an HMT activity of 2.96. The purpose was to compare histone methyltransferase H3 (K27) activity between cancerous and normal cells

Histone proteins in nucleosomes are situated so the N-terminus is exposed, while the C-termini are folded together and interact with one another. It is at the N-terminus where histone modification occurs; which can include methylation, acetylation or phosphorylation. Methylation of lysine occurs on two different histone proteins (H3 and H4) and at six different sites between the two (H3K4, H3K9, H3K27, H3K36, H3K79, and H4K20). These methylated sites can be mono-, di-, or tri-methylated [54, 57–61]. Histone methyltransferase H3 (K27) activity quantification of HTB-12 and rat astrocyte nuclear protein extracts are shown in Fig. 2.

4 Techniques Used in Our Laboratory for Brain Cancer Epigenetics Research

4.1 Methyl-Sensitive Cut Counting (MSCC) Assay

A number of assays have been reported for global methylation analysis [62–65]. Global methylation analysis was performed at the University of Nebraska Epigenomics Core Facility. Four genes which in previous laboratory findings had been shown to be upregulated in GBM (data not shown) were selected (PBK, KIF23, COL6A3, and LOX). The MSCC protocol steps are shown in Fig. 3. DNA was digested using the restriction endonuclease HpaII, which is methyl-sensitive and will cut DNA at unmethylated CCGG sites. It is estimated that generally 90 % of CpG islands in genomic DNA have at least one HpaII site. The MSCC assay [66] requires that a HpaII site must be greater than 40 bases from another HpaII site to be a valid measurable CpG. In this case, the selected gene sequences used for this assay were considered to be a single CCGG site.

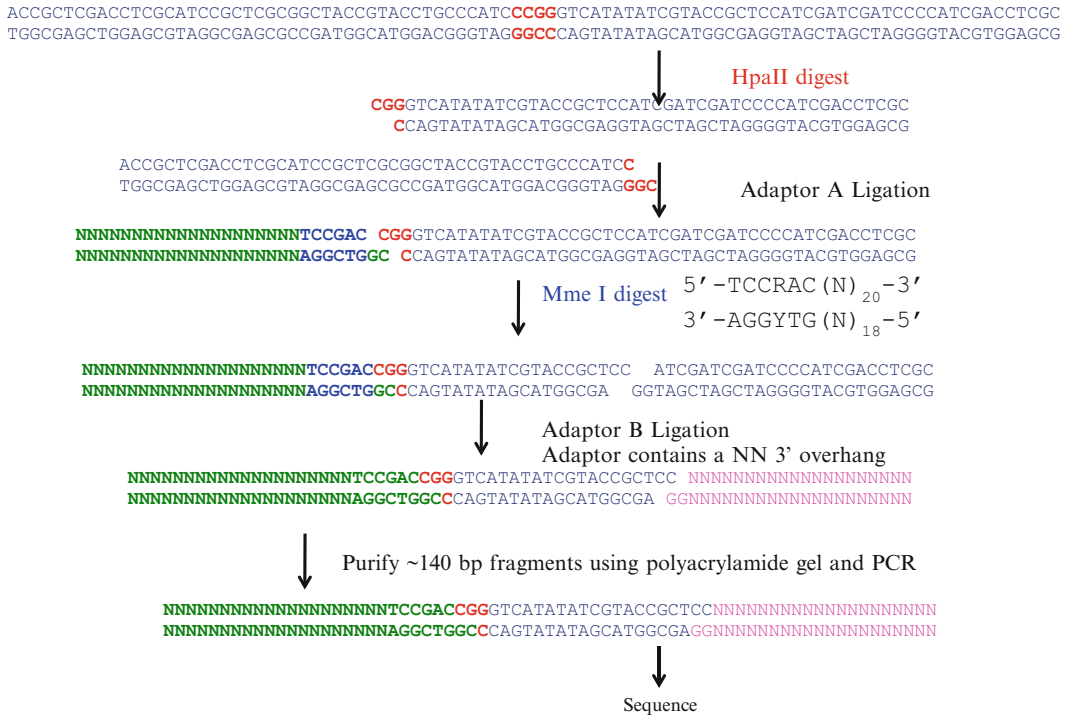


Fig. 3 MSCI procedural diagram depicting the digestion of gDNA by restriction enzymes, amplification of gDNA segments by PCR, and sequencing. gDNA was digested with the restriction enzyme HpaII, adaptors with the recognition site for the restriction enzyme MmeI were ligated to the gDNA fragments. gDNA fragments were then digested with MmeI and those segments were PCR amplified and sequenced

Randomly selected gDNA samples from astrocytes of stored tissues from a brain cancer patient (Preston Robert Tisch Brain Tumor Center, Duke University, Durham, NC) and normal astrocyte from healthy brain were used for comparison. DNA (2 µg) was digested using 20 U of HpaII. An adaptor with the recognition site for the MmeI restriction enzyme was added using T4 DNA ligase. The DNA was then ethanol precipitated and nicks were repaired using a 8 U Bst DNA polymerase. MmeI (2 U) was added to the DNA, which will cleave 18 bases adjacent to the HpaII sites. These sequences were then ligated to another adapter for PCR amplification and sequencing. Tag sizes were purified using a 10 % PAGE gel and amplified utilizing quantitative PCR to reduce tag over-amplification using Bio-Rad iProof high-fidelity polymerase. An Illumina HiSeq 2000 Genome Analyzer at the University of Nebraska Medical Center Sequencing Core Facility was used for tag sequencing. Tag sequences were paired against the human genome (h19) library using Bowtie, a short sequence aligner. Sequence tags, representing an unmethylated HpaII site, were counted in GBM and normal astrocyte DNA. The change in

Table 1

Differences in fold change in HpaII sites between GBM #1747 and normal astrocyte #1 corresponding to the gene associated with the HpaII site

Gene symbol	Description	Chromosome	Reference sequence	Fold meth average change
PBK	PDZ binding kinase	Chr 08	NM-018492	-3.33
KIF23	Kinesin family member 23	Chr 15	NM-138555	-0.25
COL6A3	Collagen, type VI, alpha 3	Chr 02	NM-004369	-1.61
LOX	Lysyl oxidase	Chr 05	NM-002317	-0.91

Negative numbers represent a decrease in DNA methylated sites in GBM. Results were generated from MSCC analysis data. Fold changes ranged between 0.25 and 3.33. PBK showed the largest decrease in DNA methylated sites in the GBM compared to the normal at 3.33, while KIF23 showed the least, but still notable decrease at 0.25. Results indicate that all four genes were hypomethylated in GBM when compared to normal astrocyte

tag counts between the two samples represented a change in the methylation status of the HpaII site. The results of the experiment are shown in Table 1.

4.2 Histone Methyltransferase H3 (K27) Assay

Histone methyltransferase activity (HMT) was determined using the EpiSeeker Histone Methyltransferase H3 (K27) Activity Quantification Assay Kit (Catalog # ab113454, Abcam®, Cambridge, MA) in GBM HTB-12 (4.64 µg) and rat astrocyte (12.82 µg) to determine differences in this activity between cancer and normal cells. Absorbance was determined after conjugation with antibodies at 450 nm using a 96-well plate reader. Adomet served as a methyl donor. The capture antibody was diluted with 1× wash buffer at a 1:100 parts and the detection antibody at a 1:1,000 parts. A standard curve was created using HMT standards at 2.5, 5, and 10 µg HMT/µl.

Assay plate wells were coated with streptavidin. For the blank well, 27 µl of histone assay buffer, 1.5 µl of diluted Adomet, and 2 µl of biotinylated substrate was added. The HMT standard wells contained 27 µl of histone assay buffer, 1.5 µl of diluted Adomet, and 2 µl of the HMT standard. Nuclear extract wells contained 24 µl of histone assay buffer, 1.5 µl of diluted Adomet, 2 µl of biotinylated substrate, and 3 µl of nuclear extracts. Wells were covered and incubated at 37 °C, 75 min. They were then washed three times with 1× wash buffer. Diluted capture antibody (50 µl) was added to each well and incubated at room temperature, 60 min. Wells were washed four times with 150 µl of 1× wash buffer. Diluted detection antibody was added at 50 µl per well. Strips were incubated at room temperature, 30 min. Three additional washes using 150 µl 1× wash buffer were performed with a final wash of 3 min duration prior to discarding buffer.

Developing solution (100 μ l) was added to each well, incubated in the dark at room temperature, 7 min. Stop solution (50 μ l) was added and the absorbance determined. Results from the HMT assay were compared against the HMT standard curve. The following formula was used for the determination of HMT activity in samples: Activity (ng/h/mg)=[absorbance/(protein amount(μ g) \times hour of original incubation \times slope of standard curve)] \times 1,000. The HMT analysis was repeated three times for every sample.

4.3 Statistical Analysis of the HMT Assay

Statistical significance was determined using one-way between subjects ANOVA ($\alpha=0.05$), and post hoc Tukey's HSD to determine specific difference in means of treatments in cells mean may have.

The results of the GBM Histone Methyltransferase H3 (K27) Assay are shown in Fig. 2.

5 Future Directions: Requirements and Challenges in Bringing Epigenetic Biomarkers in Clinic for Cancer Diagnosis

The most important requirement of using epigenetic biomarkers of GBM in diagnostics is proper clinical validation. A number of epigenetic biomarker assays and technological challenges have been reviewed recently [67]. If the objective is to use these markers for screening high risk populations, then high-throughput technologies should be adopted. Conversely, if personalized medicine is required then very specific biomarkers with high sensitivity should be utilized. Combining epigenetic biomarkers with genetic, proteomic, and glycomic biomarkers should also be considered.

It is not feasible for an individual investigator to develop an epigenetic biomarker for diagnostic use. This must be conducted as a team effort involving several investigators varied skills. Furthermore, availability of resources, including representative samples, equipment, reagents, and funds) should be committed to the purpose. After few promising biomarkers are identified, they should be tested independently in different sets of populations to help guarantee their validity and reliability. The National Cancer Institute (NCI) of the National Institutes of Health (NIH) initiated Early Detection Research Network program in which multiple investigators from more than 30 institutes collaborated on identifying and validating biomarkers. Should there be similar efforts in the private sector, the availability of many reliable biomarkers for diagnostic work would be more quickly realized. Integration of genomic and proteomic biomarkers with epigenetic biomarkers may help to subtype disease stages [68, 69]. Many times results of methylation profiling from blood and tissues are inconsistent. Koestler et al. (2012) conducted a systematic epigenome-wide methylation analysis and demonstrated that shifts in leukocyte subpopulations might account for a considerable proportion of

variability in these patterns [70]. The location of miRs in any specific organ should be carefully determined. In a tissue biopsy the local concentration (number of miRs) may be low or high. Determining the accurate level of miRs is very critical to maintain high sensitivity and specificity of the miRNA assay. Biomarkers identified in this article have promise in diagnosis and prognosis of GBM in future.

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Chapter 27

Detection of Circulatory MicroRNAs in Prostate Cancer

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Abstract

Prostate cancer (PCa) is one of the most common cancer worldwide and accounts for 14.4 % of all new cancer cases. The clinical outcome and management of PCa can be significantly improved by use of biomarker assays for early detection, prognosis and also for prediction and monitoring of treatment response. MiRNAs are short, endogenous, single-stranded RNA molecules that play important role in regulation of gene expression and can modulate a number of cellular processes. Discovery of miRNAs in circulation has not only facilitated understanding their role in various diseases but also paved new avenues for biomarker discovery due to their ease of access and stability. The fact that a minimally invasive test based on miRNAs profiles can distinguish the presence or absence of disease illustrates immense potential of these molecules as predictive biomarkers.

In this chapter, we have summarized the presumed mechanisms of miRNA release into the circulation and systematically summarized the studies of circulatory miRNAs in PCa. Also, we have mainly focused on the methodology of identification of circulatory miRNAs from biofluids.

Key words MicroRNA, Prostate cancer, Serum, Plasma, Urine

1 Introduction

Prostate cancer (PCa) is a major health concern in the USA with an estimated ~238,590 new cases and ~29,720 deaths in 2013 [1]. African American (AA) men have a 60 % higher incidence rate and twice the death rate as of Caucasian American men (CA). Prostate Specific Antigen (PSA) is the gold standard for the diagnosis of PCa. PSA testing is nonspecific, as elevated PSA levels due to benign prostatic hyperplasia (BPH), infection, and/or chronic inflammation may lead to confounding outcomes. Also, the PSA testing results in remarkable over-diagnosis and over-treatment, especially in cases where $PSA < 4$ ng/ml, justifying the need for new biomarkers. Therefore, identification of biomarkers that can predict the disease at an early stage is needed for optimizing management and treatment strategies.

Epigenetic mechanisms may help us identify novel markers for prostate cancer and we have described below the potential of micro-RNAs (miRNAs), which are part of epigenetic machinery, in diagnosing prostate cancer. MiRNAs are small 21–23 nt RNAs that represent a new class of universal gene regulatory mechanism and act primarily by binding to target mRNAs by imperfect Watson–Crick pairing in the 3'UTR region. Deregulation of microRNAs has been linked to cancer initiation and progression where miRNAs act as tumor suppressors or oncogenes, regulating multiple pathways including cell proliferation, differentiation, apoptosis, metastasis, and angiogenesis [2]. It is estimated that miRNAs regulate approximately 60 % of all protein-coding genes [3]. Recently, miRNA expression profiles have been shown as potential tools for cancer diagnosis and prognosis that can help in clinical decision making about the timing of biopsy and the necessity of treatment. Moreover, circulatory microRNAs represent an unprecedented opportunity to develop minimally invasive assays [4–6]. Although over 50 miRNAs alterations have been identified in PCa, only 10 have been characterized yet [7]. Because of their small size and secondary structure, mature miRNAs are highly stable for their utility as biomarkers, for prediction, diagnosis/prognosis, and disease progression (including survival and recurrence).

The first description of the presence of circulating miRNAs and their potential as cancer markers was reported by Lawrie et al. [8]. These extracellular miRNAs in serum, plasma, saliva, urine, and other body fluids have recently been shown to be associated with various pathological conditions including cancer. Discovery of stable miRNAs in serum and urine has stirred researchers to identify reliable miRNAs for early detection and classification. Circulatory miRNAs are more resistant to RNase digestion when compared to tissue or cellular miRNAs [9]. High stability in body fluids and robust nature of miRNAs to external insults such as enzymatic degradation, freezing/thawing or resistance to pH (pH = 1–13) variability make miRNAs ideal candidate biomarkers [10]. Due to the presence of miRNAs in circulation in a highly stable form, they may be used as biomarkers for cancer and other diseases.

There are three forms in which circulatory microRNAs are found in body fluids [11]. (a) They can either be encapsulated by lipids, and this form exists as either lipid vesicles or microvesicles or apoptotic bodies. The lipid vesicles include the exosomes which range from 30 to 100 nm, arise from the endosome, and they get released when these multicellular bodies fuse with the plasma membrane [12]. The microvesicles are membrane bound and larger than the exosomes, and they get released through membrane blebbing of the plasma membrane. The apoptotic bodies are shed during apoptosis. (b) The second form in which circulatory microRNAs exist is as complexes with nucleoproteins [13, 14]. These are the greatest majority of circulatory microRNAs that are found linked to

Table 1
Forms in which miRNA exits the cell

1. Lipid encapsulations	2. RNA-binding proteins	3. High density lipoprotein
<i>Lipid vesicles:</i> <ul style="list-style-type: none">• Exosomes• Released when the multicellular bodies fuse with the plasma membrane <i>Microvesicles:</i> <ul style="list-style-type: none">• Membrane bound• Released through membrane blebbing of the plasma membrane <i>Apoptotic Bodies:</i> <ul style="list-style-type: none">• Shed during apoptosis	<ul style="list-style-type: none">• Nucleophosmin 1 (NPM1) may be involved in transport of complexes• Might be released by death or apoptotic cells and are stable because of Ago2 or Ago1• May have special channels that allow their release	<ul style="list-style-type: none">• How they get released is not exactly known• Hypothesized that the HDLs may bind to the miRNA in circulation

RNA-binding proteins, i.e., in the form of nucleoprotein complexes Argonaute2 (Ago2)-miRNA or Ago1-miRNA. How these complexes get released is not exactly known, but it is thought that nucleophosmin (NPM1) might be involved [15] by transporting miRNA from the nucleus to the cytosol, and some studies have shown that NPM1 can be released into the extracellular space [16]. (c) The third form in which circulatory microRNAs exist are when they are bound to High Density Lipoproteins (HDLs) [17]. They are smaller in size than the exosomes, and have been shown to be different than all the other forms. It is not sure how they are released, but it has been suggested that the high density lipoproteins (HDL) may bind to the circulating miRNAs. Of these three types, the lipid encapsulations are categorized as vesicle bound, whereas the RNA bound or HDL bound are not vesicle bound [10]. A summary of the forms in which miRNAs are known to exit the cells and come in circulation is summarized in Table 1.

Although the precise cellular release mechanisms of miRNAs remain largely unknown, the initial studies revealed that these circulating miRNAs may be delivered to recipient cells, where they can regulate translation of target genes [18].

The stability of miRNAs in circulation and the ease by which miRNAs can be detected in a quantitative manner by methods such as real-time PCR and microarrays have sparked great interest in the use of circulating miRNAs as clinical biomarkers. Emphasizing the importance of circulatory miRNAs as biomarkers we recently demonstrated that the expressions of miR-205 and miR-214 were significantly decreased in prostate cancer (PCa), compared to controls. By estimating the levels of these miRNAs in urine, we were able to discriminate patients from controls with 89 % sensitivity and 80 % specificity, as judged by the use of a receiver operating characteristic (ROC) curve [19].

Expression profiles of circulating miRNAs can be obtained by using high-throughput profiling techniques such as Solexa sequencing, miRNA microarray, and bead-based miRNA profiling. These high-throughput techniques are very effective and greatly facilitate the circulating miRNA expression profiling process. Due to the requirement of relatively large volume of starting material in order to obtain the expression profile of miRNAs, these high-throughput techniques are usually used in initial screening phases. The most widely applied methodology in miRNA research is Quantitative real-time PCR, which has been widely applied especially in assessing the low level of certain circulatory miRNAs in individual samples. To date, the most widely used and successful approach, in terms of specificity and sensitivity, is a two-step approach using looped miRNA-specific reverse transcription primers and TaqMan probes. So far, both high- and low-throughput techniques are extremely useful in miRNA studies and are responsible for the majority of findings regarding miRNAs expression profile, including circulating miRNAs [20].

In the following section, we describe the methodology used for isolation of microRNAs from serum and urine and their quantitation. The methods described can be adapted for other body fluids as well.

2 Materials and Methods

2.1 *Materials for RNA Isolation*

1. mirVana™ Paris™ Kit (LifeTechnologies, Carlsbad, CA).
This kit contains:
30 mL miRNA Wash Solution 1 (21 mL of 100 % ethanol needs to be added before use).
50 mL Wash Solution 2/3 (40 mL 100 % ethanol needs to be added before use).
80 Collection Tubes.
40 Filter Cartridges.
25 mL Cell Disruption Buffer.
25 mL 2× Denaturing Solution (375 µL 2-mercaptoethanol needs to be added before use).
2×25 mL Acid-Phenol–Chloroform.
5 mL Elution Solution.
2. 2-Mercaptoethanol (14.3 M).
3. 100 % Ethanol, ACS grade or better.
4. Heat block.
5. Centrifuge (set at room temperature) (Sorvall Instruments, Wilmington, DE).

6. Spectrophotometer (NanoDrop 2000C, Thermo Scientific, Waltham, MA).
7. Up to 625 μ L of serum or urine sample.
8. Nuclease-free water.
9. Microcentrifuge tubes.
10. RNase decontamination solution.
11. Vortex mixer.

2.2 Methods

for microRNA Isolation

1. Clean work surface areas and pipettes carefully with an RNase decontamination solution, such as RNaseZap or RNaseAway. These reagents inactivate RNases immediately upon contact. RNases, stemming from bacteria, fungi, and the human body, are present everywhere in the environment, including work benches and laboratory equipment. If RNases are accidentally introduced into the RNA isolation process, they can cause RNA degradation and problems in downstream applications.
2. Thaw urine or serum samples on ice. Gently vortex and then briefly spin down the samples.
3. Label three microtubes and three collection tubes per each urine/serum sample.
4. Prepare the 2 \times Denaturing Solution, miRNA Wash Solution 1 and Wash Solution 2/3 supplied with the *mirVana* kit as described above.
5. Transfer up to 625 μ L of sample to a microtube. Then add an equal volume of 2 \times Denaturing Solution (kept at room temperature) and vortex the mixture briefly.
6. Add a volume of Acid Phenol: Chloroform equal to the combined total volume of sample plus 2 \times Denaturing Solution (for example, if the original sample volume was 300 μ L and it was mixed with 300 μ L of 2 \times Denaturing Solution in **step 1**, add 600 μ L Acid-Phenol:Chloroform). (Make sure to withdraw *bottom* phase from the Acid-Phenol: Chloroform bottle).
7. Vortex the mixture thoroughly for 30–60 s.
8. Centrifuge for 5 min at 13,000 rpm at room temperature. Repeat if interphase is not compact.
9. Carefully recover the aqueous (upper) phase without disturbing the lower phase or the interphase and transfer it to a new microtube. Measure the volume recovered.
10. Add 1/3 volume of room temperature 100 % ethanol to the recovered aqueous phase (e.g., if 600 μ L was recovered, add 200 μ L 100 % ethanol) and vortex thoroughly.
11. Place filter cartridge into a collection tube. Both are provided with the *mirVana* Paris kit.

12. Pipet the sample–ethanol mixture onto the filter cartridge. Up to 700 μL can be applied at a time. For volumes greater than 700 μL , apply the mixture in successive applications to the same filter.
13. Centrifuge for about 30 s at $12,851\times g$ making sure that the whole mixture went through the filter.
14. Transfer filtrate to a new microtube and repeat the application, as mentioned, if the mixture volume is greater than 700 μL . Combine the filtrate and measure the total volume.
15. Discard filter. (The filter now contains only large RNA which can be recovered if needed for other purposes.)
16. Add 2/3 volume of room temperature 100 % ethanol to filtrate. (For example, if 600 μL was recovered, add 400 μL of ethanol.) Vortex thoroughly.
17. Pass filtrate through a new filter cartridge added to a new collection tube. For volumes greater than 700 μL apply the mixture in successive applications to the same filter.
18. Centrifuge for 30 s at $12,851\times g$ and then discard flow-through. Make sure to keep filter and collection tube.
19. Add 700 μL of supplied Wash Solution 1 to the filter cartridge and centrifuge for 15 s at $12,851\times g$.
20. Discard flow-through, but keep filter and collection tube.
21. Add 500 μL of supplied Wash Solution 2/3 to the filter cartridge and centrifuge for 15 s at $12,851\times g$.
22. Repeat the last step by adding a second 500 μL of Wash Solution 2/3 to the filter cartridge and centrifuging for 15 s at $12,851\times g$.
23. Discard flow-through, but keep filter and collection tube.
24. Centrifuge the filter and collection tube for 1 min. to dry the filter. Transfer the filter to a new collection tube.
25. Add 100 μL preheated (95 $^{\circ}\text{C}$) nuclease-free water (or use the supplied Elution Solution) to the center of the filter. If the starting volume was low, it may be necessary to add less water (or Elution Solution) to obtain a sufficiently high concentration.
26. Centrifuge for 30 s at $12,851\times g$. Discard the filter and label the collection tube now containing the isolated microRNA.

2.3 Determining RNA Concentration and Quality

Determine the RNA concentration using a NanoDrop[®] ND-1000 Spectrophotometer (Thermo Scientific, Waltham, MA). The recommended sample amount for testing is 1.5 μL . Also notate the A260/A280 ratio and the A260/A230 ratio indicating the purity of the sample. For pure RNA the 260/280 absorbance ratio should be 1.8–2.1 and the A260/A230 ratio should be close to 2.

Store the RNA samples at -80°C or continue with reverse transcription.

3 Reverse Transcription of microRNA

3.1 Choosing Appropriate TaqMan Assays

TaqMan microRNA assays consist of a primer plus probe set designed to detect and quantify primary and mature microRNAs in various species. TaqMan assays for most miRNAs found in the miRBase sequence repository are available, either predesigned or custom made from Life Technologies. Carefully choose the Taqman assay for the miRNA of interest and its desired form (primary or mature) in the chosen species.

It is also necessary to choose an appropriate endogenous control (TaqMan Small RNA Control) in order to normalize the expression levels of the target gene. The control needs to be validated for the urine/serum samples in question.

3.2 Materials for Reverse Transcription of microRNA

1. TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). This kit contains the following reagents:
 - 1 mL 10× RT Buffer.
 - 200 µL dNTP mix (100 mM total).
 - 100 µL RNase Inhibitor (20 U/µL).
 - 100 µL Multiscribe Reverse Transcriptase (50 U/µL).The kit should be stored at -15 to -25 °C.
2. Components of this kit are used with the RT primer provided with the TaqMan® MicroRNA assay to convert miRNA to cDNA. TaqMan® MicroRNA Assays (Primers) (Applied Biosystems, Foster City, CA). Store assays at -15 to -25 °C.
3. Polymerase Chain Reaction (PCR) machine (GeneAmp® PCR System 9700, Applied Biosystems, Foster City, CA).
4. 0.2 mL PCR tubes.
5. RNA sample of known concentration.
6. Nuclease free water.
7. Vortex Mixer.
8. Centrifuge.

3.3 Methods for Reverse Transcription of microRNA

1. Reverse transcription is performed using TaqMan® MicroRNA Reverse Transcription Kit and TaqMan® MicroRNA Assays.
2. Each reaction will contain 5 µL of RNA sample, 7 µL of master mix and 3 µL of primer (TaqMan Assay).
3. For each RNA sample calculate ratio of RNA sample to water based on the prior NanoDrop readings to obtain the desired sample concentration of 0.2–2 ng/µL for PCR.
4. Thaw all reagents on ice and spin them down briefly.

5. Prepare master mix as follows figuring in 10–15 % overage:

Reagent	Master mix volume (per 15- μ L reaction)
Nuclease free water	4.16 μ L
10 \times RT buffer	1.5 μ L
Multiscribe™ reverse transcriptase (50 U/ μ L)	1.0 μ L
RNase inhibitor (20 U/ μ L)	0.19 μ L
100 mM dNTP mix (100 mM total)	0.15 μ L
Total	7.0 μ L

Each reaction will consist of 7 μ L master mix, 3 μ L RT primer, and 5 μ L RNA sample.

6. Include one no-template-control (NTC) reaction per miRNA validated.
7. Gently vortex and spin down primer-master mix. Store mix on ice.
8. Add 10 μ L of the primer-master mix to each PCR tube.
9. Gently spin down the thawed RNA samples and store them on ice.
10. Add a combined total of 5 μ L RNA sample and nuclease free water in the above calculated amounts to each PCR tube. 5 μ L of water is added to the NTC controls.
11. Spin down PCR tubes and incubate on ice for 5 min.
12. Place PCR tubes in thermal cycler and start the run using the following cycling conditions:

Step	Time (min)	Temperature ($^{\circ}$ C)
Hold	30	16
Hold	30	42
Hold	5	85
Hold	∞	4

13. Set the reaction volume to 15.0 μ L.
14. After the run is over, proceed to Real Time Quantification as soon as possible. Brief storage of RT products at -20° C is possible.

4 Real Time Quantitative PCR

4.1 Materials for Real Time Quantitative PCR

1. TaqMan® Universal Master Mix II, no UNG (Applied Biosystems, Foster City, CA). Store at $2-8^{\circ}$ C.
2. TaqMan® MicroRNA Assays (probes) (Applied Biosystems, Foster City, CA). Store assays at -15 to -25° C.

3. PCR plate (MicroAmp® Optical 96-Well Reaction Plate, Applied Biosystems, Foster City, CA).
4. Optical Adhesive (MicroAmp Optical Adhesive, Applied Biosystems, Foster City, CA).
5. Mini Plate Spinner (Labnet MPS 1000, Labnet Int., Edison, NJ).
6. Real-Time PCR System, including analysis software (Applied Biosystems, Foster City, CA).
7. Reverse Transcription (RT) Product.
8. Vortex Mixer.
9. Centrifuge.

4.2 Methods for Real Time Quantitative PCR

1. Draw out reaction plate diagram. All samples and controls are run in triplicates. An RT-product control and a NO-RT-product control are needed per miRNA validated.
2. Calculate the amount of reagents needed for each probe-master mix as shown (figure in about 10 % overage):

Reagent	Volume per 20 μ L Reaction
TaqMan Assay (probe)	1.0 μ L
TaqMan Universal Master Mix II	10.0 μ L
Nuclease free water	6.5 μ L
Total volume	17.5 μ L

Each 20 μ L reaction will consist of 6.5 μ L nuclease free water, 10 μ L TaqMan Universal Master Mix, 1.0 μ L probe, and 2.5 μ L RT product.

3. Thaw the probe on ice. Make sure the probes have minimum exposure to light as it will cause them to deteriorate.
4. Shake Universal Master Mix bottle gently and store on ice.
5. Label reaction plate.
6. Make probe-master mix, adding water, Universal Master Mix and TaqMan probe as detailed above. Mix with pipette.
7. Vortex mixture gently and spin down briefly.
8. Spin down PCR tubes containing RT products.
9. Fill reaction plate with the probe-master mix, adding 17.5 μ L per well.
10. Add 2.5 μ L of RT product per well. Also, for the RT controls add 2.5 μ L of NTC-RT product per well and for the No-RT controls add 2.5 μ L of nuclease free water per well.
11. Cover the reaction plate with adhesive plastic film making sure it fits tightly around all wells.

- 12. Spin reaction plate down in mini plate spinner for 30 s. Check that all air bubbles have been eliminated from the wells.
- 13. Place the reaction plate in the real-time PCR system drawer. Close the drawer and turn on the PCR system machine. Create a plate document using the instrument's user manual. Use a sample value of 20 μ L and the following program cycling conditions:

Enzyme activation		PCR	
		40 cycles	
Step	Hold	Denature	Anneal/extend
Time	10 min	15 s	60 s
Temperature ($^{\circ}$ C)	95	95	60

Run the plate and analyze the data according to the user's manual using the comparative Ct method.

5 Use of Internal Control: An Important Aspect of the miRNA Quantitation

Use of proper internal control is an important aspect of the miRNA quantitation and is a major challenge when quantitating circulatory microRNAs. Although miRNAs are stable in serum and plasma, their levels may alter under various conditions and, furthermore, circulating miRNA quantification has to deal with a lack of internal control for proper normalization.

In case of quantification of circulating miRNA the literature-based tissue endogenous controls have been employed in most of the studies. For example, miR-16, which has been shown to be consistently expressed in different human tissues and is also detectable in serum, was used as a reference for serum miRNA analyses in several studies [21]. A list of normalizing controls used in various circulatory miRNA profiling studies in prostate cancer is provided in Table 2.

Another useful strategy to identify best suited normalizing control is the use of the specialized algorithms, such as GeNorm [22], and NormFinder [23] which are designed to identify the optimal normalization control among a set of candidates. It ranks the set of candidate normalization controls according to their expression stability in a given sample set and given experimental design. This strategy is innovative, straightforward and universally applicable and enables a more accurate assessment of relevant biological variations from a miRNA qRT-PCR experiment.

Normalizing the level of circulating miRNAs by the volume of serum/plasma sample is another one of the most feasible ways of standardization. Currently, the optimal way for experimental miRNA data normalization is probably the spike-in normalization

Table 2
Summary of the circulatory miRNA studies in prostate cancer

Upregulated	Downregulated	Control used	Methodology employed	Study design	Body fluid type	Reference
miR-100, miR-125b, miR-141, miR-143, miR-296	–	Three synthetic <i>C. elegans</i> miRNAs: cel-miR-39, cel-miR-54, and cel-miR-238	qRT-PCR	25 metastatic PCa vs. 25 controls	Serum	Mitchell et al. [24]
miR-16, miR-92a, miR-103, miR-107, miR-197, miR-34b, miR-328, miR-485-3p, miR-486-5p, miR-92b, miR-574-3p, miR-636, miR-640, miR-766, miR- 885-5p	–	Negative control probes (for each miRNA an antisense wild type version, a double mutant control, and sense control probe); cel-miR-39, cel-miR-54, and cel-miR-238; four sheep controls; 2 humans	Mircoarray	5 PCa vs. 8 controls	Serum	Lodes et al. [25]
miR-9, miR-141, miR-200b, miR-375, miR-516a-3p		cel-miR-39, cel-miR-54, and cel-miR-238	qRT-PCR	7 metastatic PCa vs. 14 PCa	Serum	Brase et al. [26]
let-7a	miR-145, miR-155	miR-16	qRT-PCR	20 PCa vs. 63 controls	Whole blood	Heneghan et al. [21]
miR-21	–	U6	qRT-PCR	20 localized PCa, 20 Androgen Dependent PCa, 10 Hormone refractory PCa, and 6 BPH	Serum	Zhang et al. [6]
miR-21, miR-221	–	RNU1A	qRT-PCR	51 PCa vs. 20 controls	Plasma	Yaman Agaoglu et al., 2011 [27]
miR-93, miR-106a, miR-874, miR-1207-5p, miR-1274a	miR-24, miR-26b, miR-30c, miR-223	Global median normalization	qRT-PCR	36 PCa vs. 12 controls	Serum	Moltzahn et al. [28]

(continued)

Table 2
(continued)

Upregulated	Downregulated	Control used	Methodology employed	Study design	Body fluid type	Reference
miR-221		RNU6B	qRT-PCR	28 PCa vs. 20 controls	Plasma	Zheng et al. [29]
miR-26a, miR-195, let7i	–	cel-miR-39	qRT-PCR	37 localized cancer, 8 metastatic PCa, 18 BPH, and 20 healthy controls	Serum	Mahn et al. [4]
miR-141	–	NA	qRT-PCR	21 metastatic PCa patients	Plasma	Gonzales et al. [30]
miR-141, miR-298, miR-346, miR-375	–	cel-miR-39	qRT-PCR	25 metastatic PCa vs. 25 controls	Serum	Selth et al. [31]
miR-20a, miR-21, miR-145, miR-221	–	Chemically synthesized RNA oligonucleotides	qRT-PCR	82 PCa assessing association with CAPRA score	Plasma	Shen et al. [32]
miR-622, miR1285	Let 7c, Let 7e	RNU6B		80 CaP, 44 BPH, and 54 healthy controls	Plasma	Chen et al. [33]
miR-375, miR-378, miR-141	miR-409-3p	RNU6B, cel-miR-39, miR-54, and cel-miR-238	qRT-PCR	28 patients of low-risk localized disease, 30 of high risk localized disease and 26 of metastatic CRPC	Serum	Nguyen et al. [34]
miR-21	–	cel-miR-39, SNORD43, and RNU1-4	qRT-PCR	24 PCa patients vs. 48 patients with nonmalignant urological disease without history of cancer	Serum	Sanders et al. [5]

miR-141, miR-200a, miR-200c, miR-210, miR-375	–	<i>C. elegans</i> oligonucleotides	qRT-PCR	25 PCa vs. 25 controls	Serum	Cheng et al. [35]
miR-141, miR-146b-3p, miR-194	–	cel-miR-39	qRT-PCR	8 with Biochemical recurrence (BCR) of PCa and 8 without BCR	Serum	Selth et al. [36]
miR-16, miR-141, miR-151-3p, miR-141, miR-375, miR-21, miR-423-3p, miR-205	–	miR-30e	qRT-PCR	25 mCRPC patients and 25 patients with localized cancer	Plasma	Watahiki et al. [37]
miR-107, miR-141, miR-375, miR-574-3p, miR-200b	–	cel-miR-39	Microarray and qRT-PCR	78 PCa vs. 28 controls	Plasma	Bryant et al. [38]
–	–	cel-miR-39	qRT-PCR	47 recurrent PCa and 72 non recurrent patients	Serum	
–	–	RNU44 and RNU48	qRT-PCR	48 advanced PCa, 70 local cancer, and 17 controls	Urine	
–	miR-205, miR-214	RNU48	qRT-PCR	36 PCa vs. 12 controls	Urine	Srivastava et al. [19]

approach to control the technical variances during the purification step. This approach is based on the use of exogenous synthetic nonhuman mature miRNA from *C. elegans* (cel-miR-39, -54, and -238) or plants as controls, which are spiked into the serum samples prior to RNA extraction.

6 Concluding Remarks and Future Directions

The circulatory miRNAs emerge as an ideal biomarker as they fulfill a number of criteria, such as noninvasive accessibility, a high specificity and sensitivity, ability to differentiate pathologies, early detection, sensitivity to relevant changes in the disease, and capability for rapid and accurate detection, and therefore emerge as a highly valuable tool for diagnosis or prognosis.

The growing use of powerful detection methods such as digital PCR and next generation sequencing methods have given a significant boost to the search for minimally invasive disease indicators with microRNAs being at the forefront due to their resilience and abundance. Although rapidly evolving technologies have promised great advances in the area of miRNAs as biomarkers, there are still several important issues which need to be addressed in order to establish circulating miRNAs as relevant novel noninvasive cancer biomarkers. The foremost need is to gain a better understanding of the origin/source and the underlying mechanisms by which miRNAs are released into the circulation. Also, it needs to be established, whether freely circulating miRNA molecules have any functional role other than reflecting the presence and pathological features of disease. The other major challenge is the lack of consensus on the use of normalization controls for systemic miRNA analysis. While individual miRNAs have been described as biomarkers of prostate cancer diagnosis and prognosis, it is highly unlikely that one miRNA will serve as recipe for either. Larger independent studies are necessary in order to establish a well-characterized panel of miRNAs specific to each type of tumor, early or advanced cancer stage, response to treatment, patient outcome, recurrence test and the specificity and sensitivity of circulating miRNAs.

Furthermore, prospective studies in a large cohort and clinical trials are needed to verify the results obtained in retrospective exploratory cohorts, to determine whether circulating miRNAs can serve as a diagnostic and screening tool to detect cancer at its early stage.

As the functional roles of miRNAs in cancer biology are further uncovered and the methods of circulating miRNA detection and analysis are improved, circulating miRNAs will serve as novel minimally invasive or noninvasive biomarkers for various types of cancer. The wide applicability and potential importance of circulatory miRNAs will surely initiate a revolution in clinical management,

including estimating prognosis, predicting therapeutic efficacy, maintaining surveillance, and forecasting disease reoccurrence and will imply them from bench to bedside.

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Identification and Characterization of Small-Molecule Inhibitors of Lysine Acetyltransferases

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Abstract

Lysine acetyltransferases (KATs) acetylate various proteins including histones, transcription factors, metabolic enzymes, and other cellular substrates. Protein acetylation significantly impacts protein stability and function. Certain KATs such as p300 (KAT3B) are overexpressed in cancer cells and are linked to tumor progression and drug resistance. Thus, pharmacologic inhibition of KATs represents a new strategy for cancer therapy. Quantitative biochemical assays of KAT enzymatic activity have been developed and adapted for high-throughput screens of small-molecule compounds to discover specific KAT inhibitors. Such compounds are useful probes for understanding the cellular functions of these critical enzymes and importantly, they may be further developed as anticancer therapeutics. Here we describe a fluorescence-based KAT activity assay and cell-based validation of KAT inhibition by small-molecule compounds.

Key words Lysine acetyltransferases (KAT), p300/CBP, Acetylation, High-throughput screening, Small-molecule inhibitors

1 Introduction

Recent proteomic studies have shown that over 2,000 proteins in mammalian cells undergo posttranslational acetylation [1–4]. Acetylation impacts enzymatic activity, protein stability, subcellular distribution, and protein-protein and protein-DNA interactions. Acetylation of proteins involved in nuclear functions such as chromatin structure and transcriptional regulation has been extensively studied [5]. In general, global lysine acetylation of core histones leads to the remodeling of chromatin structure to promote DNA replication, DNA repair and gene transcription, which is required for robust cell proliferation. Many DNA-binding transcription factors (TFs) such as the androgen receptor (AR), NF- κ B, STAT3 and p53 are acetylated, and in general, acetylation of these TFs augments their functions. More recent studies revealed that many proteins in the cytoplasm and organelles are also acetylated [2–4, 1, 6]. In particular, many enzymes involved in intermediate

metabolism are acetylated, and acetylation appears to have a critical role in regulating their enzymatic activity [4, 7].

Protein acetylation is actively regulated by lysine acetyltransferases (KATs) and deacetylases (HDACs; the widely accepted acronym for deacetylases is used here). KATs catalyze the covalent attachment of the acetyl group to the ϵ -amine of a substrate lysine side-chain. There are several diverse families of KATs including GNAT [GCN5-related *N*-acetyltransferases, e.g., GCN5 (KAT2A) and PCAF (KAT2B)], p300 (KAT3B)/CBP (KAT3A) and MYST (named after the founding members MOZ, Ybf2/Sas3, Sas2, and Tip60) that display minimal sequence similarity but recognizable structural homology of their catalytic cores [8, 9]. Other proteins such as TAFII250 (TAF1), nuclear steroid receptor coactivators and CLOCK have also been reported to have acetyltransferase activity, but their enzymatic properties are less well characterized. Acetyl-CoA is the universal acetyl donor in the acetylation reaction catalyzed by these KATs. Despite the remarkable divergence of their amino acid sequences, structural similarities of the acetyl-CoA-binding pockets of these diverse KATs have been described [9].

HDACs catalyze the removal of the acetyl group from protein substrates. There are four well-characterized classes of HDACs. The so-called classical HDACs are zinc-dependent enzymes, and their catalytic domains share sequence and structural similarity [10]. Class III HDACs are related to yeast Sir2 protein and possess NAD⁺-dependent deacetylase activity. Chemical inhibitors to the classical HDACs have shown potent anticancer activity. Importantly, two HDAC inhibitors (HDACIs), suberoylanilide hydroxamic acid (SAHA, vorinostat or Zolinsa) and romidepsin (FK228, depsipeptide, or Istodax), have been approved by the Food and Drug Administration (FDA) for treating cutaneous T-cell lymphoma. Many other HDAC inhibitors are actively being tested in clinical trials for advanced solid cancers [11]. However, one noted limitation of these FDA-approved HDACIs is that they are effective only against certain types of hematological malignancies with inconsistent efficacy for treating solid cancers. Although the reasons for the lack of efficacy of these drugs against solid cancers remain to be resolved, it is important to note that HDACIs are broadly active in inhibiting different zinc-dependent HDACs, resulting in hyperacetylation of not only histones, but also perhaps many other cellular proteins that are subject to acetylation such as heat-shock protein 90 (Hsp90) [12] and β -catenin [13–15]. The broad inhibition of protein acetylation could provide an explanation for the pleiotropic effects of the so-called pan HDACIs.

Accumulating evidence indicates that pharmacologic inhibition of KATs could have anticancer therapeutic effects. Overexpression of specific KATs has been observed in diverse types of human cancer. For example, p300 overexpression has been observed in breast [16, 17], liver [18, 19], esophageal squamous cell carcinoma [20]

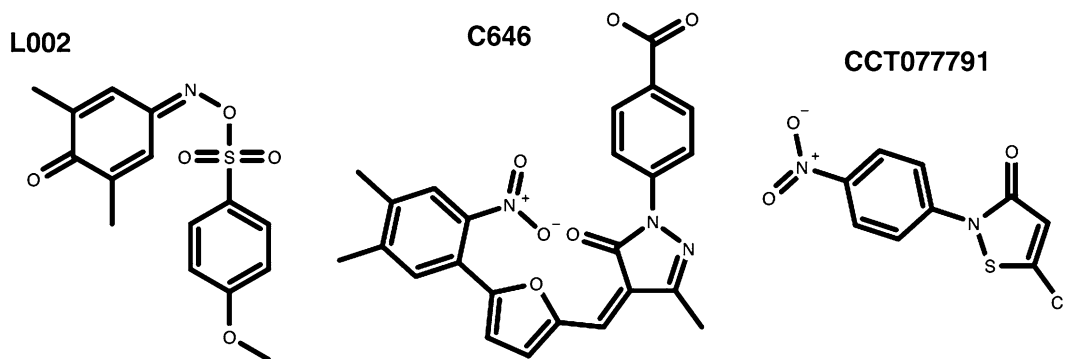


Fig. 1 Small-molecule KAT inhibitors identified in high throughput screens. L002 and structurally related compounds were identified as p300 inhibitors using the fluorescence-based KAT activity assay in a high throughput screen [32]. C646 was identified as a p300-selective inhibitor through a structure-based virtual screen [29]. CCT077791 was identified as an isothiazolone-based PCAF (also known as KAT2B) inhibitor [35]

and prostate cancer [21–24]. In prostate cancer, p300 expression levels correlate with tumor grades, the Gleason scores and a poor prognosis [23]. p300 serves as a critical coactivator of AR-mediated transcription of genes required for cancer cell survival and proliferation [21, 25, 22]. p300 expression is increased in castration-resistant prostate cancer, when compared to the levels of p300 observed in androgen-sensitive prostate cancer [25, 24]. Genetic and pharmacologic inhibition of p300 in prostate cancer cells induces growth arrest and apoptosis [26].

Because of the considerable therapeutic potential of pharmacologic inhibition of KATs, small-molecule KAT inhibitors including synthetic compounds and natural products have been identified and characterized [27] (Fig. 1). Bisubstrate inhibitors, which are synthetic peptide-acetyl-CoA conjugates, have also been shown to potently inhibit p300/CBP and other KATs [28]. More recent efforts include the discovery of the p300-specific inhibitor C646 through a structure-based virtual screen [29]. This compound is effective in suppressing cell proliferation and in eliciting apoptosis in prostate cancer [26], melanoma [30], and leukemia cells [31]. A high-throughput screen has identified the lead KAT inhibitor L002 and its analogs [32]. These compounds displayed inhibitory effects on several KATs, including p300, CBP, PCAF, and GCN5 [32]. L002 potently suppressed the growth of breast cancer xenografts in vivo [32]. Thus, KAT inhibitors may lead to novel anticancer therapies. Various biochemical methods including radioactivity-based filter-binding assay, spectroscopic, enzyme-coupled assay, and fluorescence-based assays have been developed for detecting and quantifying KAT enzymatic activities [33, 34]. Biochemical assays amenable for high-throughput screens will be continuously developed and used for discovering new highly specific KAT probes and therapeutic lead compounds for cancer therapy. Here we describe

protocols for the identification and characterization of small-molecule KAT inhibitors in conventional low-throughput assays, which has been successfully adapted and miniaturized in a 1,536-well format for a high-throughput screen [32].

2 Materials

2.1 Fluorescence-Based KAT Activity Assay

1. Purified p300 catalytic domain (BPS Bioscience, catalog number: 500071, or ENZO Life Sciences, catalog number: BML-SE451-0100).
2. 5× KAT assay buffer: 500 mM HEPES, pH 7.5, 0.4 % Triton X-100. Store at 4 °C.
3. Peptide substrates (the N-terminal amino group is acetylated): histone H3: QTARKSTGGKAPRKQLATK; histone H4: SGRGKGGKGLGKGGAKRHR. These peptides can be custom synthesized at various vendors (e.g., GenScript and Biopeptide). Store the peptide solutions at −20 °C.
4. Acetyl-CoA (Sigma-Aldrich, A2056): 6 mM stock in distilled and deionized water. Store aliquots at −80 °C.
5. Developer solution: CPM (7-diethylamino-3-(4-maleimido phenyl)-4-methylcoumarin) (Sigma-Aldrich, C1484); Make 3 mM in DMSO. Store aliquots at −20 °C.
6. 1 % Triton X-100 solution.
7. Stop solution: isopropanol (Fisher Scientific).
8. Black 96-well plate (Perkin Elmer, part number 6005270).
9. Fluorescence plate reader (e.g., POLARstar OMEGA multi-plate reader, BMG Labtech, Germany).

2.2 Evaluation of p300 Inhibition by a Small-Molecule Inhibitor in Cells

1. HCT 116 human colorectal carcinoma cell line (CCL-247; American Type Culture Collection, Rockville, MD).
2. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10 % bovine calf serum, 10 units/ml penicillin, and 10 µg/ml streptomycin sulfate.
3. Etoposide (Sigma-Aldrich, catalog number E1383). A stock solution at 20 mM in DMSO is stored at −20 °C.
4. Trichostatin A (TSA, Sigma-Aldrich, catalog number T8552). A stock solution at 3.3 mM in DMSO is stored at −20 °C.
5. Antibodies recognizing p53 acetylated at K382 (Abcam, catalog number ab75754).
6. Antibodies against p53 (Santa Cruz Biotechnology, catalog number SC-126).
7. Antibodies against proliferating nuclear antigen (PCNA) (Abcam, catalog number ab92552) (*see Note 1*).

8. Passive Lysis Buffer (Promega, catalog number E1941).
9. Reagents for SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis, Western blotting, and enhanced chemiluminescence (ECL) detection.

3 Methods

3.1 Fluorescence-Based KAT Activity Assay

1. Do threefold serial dilutions of a small-molecule inhibitor in 1.5 ml microcentrifuge tubes. First, prepare 37.5 μ l of 200 μ M solution of the inhibitor in 1 \times KAT assay buffer. This is solution #1 with the highest concentration of the inhibitor (*see Note 2*). Pipet 25 μ l of 1 \times KAT assay buffer to each of the nine other tubes. Number the tubes #2 to #10 sequentially. Pipet 12.5 μ l of solution #1 to tube #2, and mix well. Dilute the inhibitor similarly to complete the serial dilutions (total 10 points). Remove 12.5 μ l from tube #10 so that the total volume is 25 μ l in each tube. For a no inhibitor control tube (#11), add 25 μ l of 1 \times KAT assay buffer. For the no enzyme control tube (#12), add 25 μ l of 1 \times KAT assay buffer.
2. Dilute p300 appropriately in 1 \times KAT assay buffer (*see Note 3*). Mix well and centrifuge briefly with a microcentrifuge. Place the diluted enzyme on ice.
3. Add 1 μ l of the diluted enzyme to each of tubes #1 to #11 prepared in **step 1**. Mix well and spin briefly with a microcentrifuge. Incubate the tubes at room temperature for 30 min. Do not add enzyme to tube #12.
4. While the drug–enzyme solution is being incubated, prepare the following cocktail in a 1.5 ml tube:
5 \times KAT assay buffer: 70 μ l.
1.25 mM histone peptide substrate: 14 μ l.
Acetyl-CoA (6 mM): 1.1 μ l.
Add distilled and deionized water to a total of 350 μ l.
Mix gently and place the solution on ice.
5. Dispense 25 μ l of the solution above to each of tubes #1 to #12 and mix well.
6. Add 50 μ l of isopropanol (stop solution) to each tube.
7. Prepare sufficient amount of developing solution by diluting CPM stock with 1 % Triton X-100.
8. Add 100 μ l of the developing solution to each tubes and incubate at room temperature for 15 min in dark.
9. Pipet the reactions to a black 96-well plate.
10. Read fluorescence with the BMG plate reader (POLARstar OMEGA multiplate reader; excitation: 355 nm, emission: 460 nm).

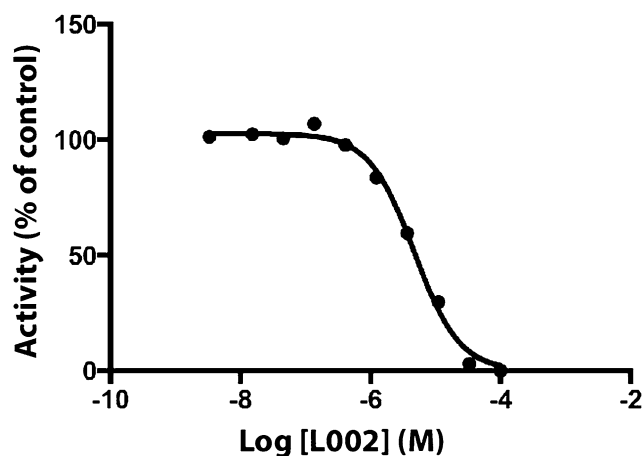


Fig. 2 Concentration–response curve of L002 against p300. The purified p300 catalytic domain from BPS Bioscience was diluted in 1× KAT assay buffer. The final assay concentration for p300 was 2.3 μg/ml. L002 was serially diluted in 1× KAT assay buffer and then incubated with p300. The histone H4 peptide was used as the substrate. A fluorescence-based p300 activity detection and curve-fitting were performed as described in this chapter. The IC_{50} is determined to be 4.731 μM

11. For data analysis, subtract background fluorescence reading (#12) from all other readouts. Normalize data against the no inhibitor control (#11). The inhibitory potency (IC_{50}) can be obtained using nonlinear regression with a proper computer program (*see* Fig. 2 and **Note 4**).

3.2 Evaluation of p300 inhibition by a Small-Molecule Inhibitor in Cells (*See Note 5*)

1. Seed 100,000 HCT 116 cells per well in 200 μl of the complete DMEM in 13 wells of a 48-well plate. Number the wells #1 to #13. Culture the cells at 37 °C in an incubator supplied with 5 % CO_2 .
2. At 24 h after cell seeding, etoposide is added to 1 μM in wells #3, #6, #9, and #12, and to 10 μM in wells #4, #7, #10, and #13. In wells #2 and #8, add an equal volume of the solvent DMSO as controls (*see Note 6*).
3. At 15 h after cells are exposed to etoposide, add a p300 inhibitor such as L002 [32] to a predefined final concentration to wells #5 to #7 and #11 to #13. Add an equal volume of the solvent DMSO to other wells (*see Fig. 3*).
4. At 17 h after cells are exposed to etoposide, add TSA to 0.2 μM in wells #8 to #13 (*see Fig. 3*).
5. At 18 h after cells are exposed to etoposide, remove the medium and add 50 μl of 1× Passive Lysis Buffer to each well. Freeze the plate at −80 °C for 2 h to overnight.

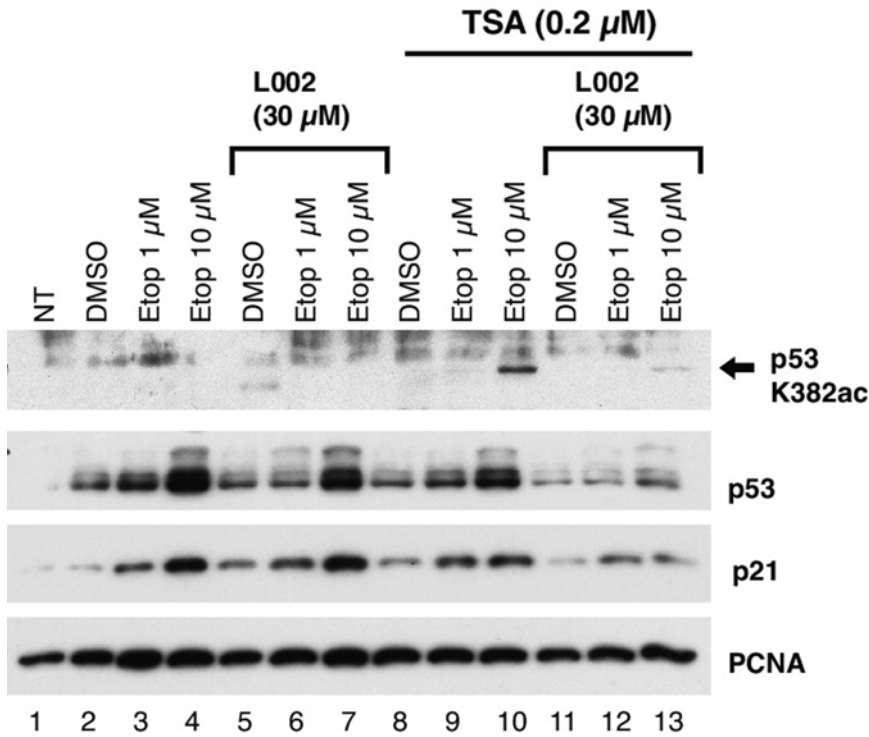


Fig. 3 Inhibition of p300-mediated p53 acetylation by L002. HCT 116 cells were exposed to the solvent (DMSO), etoposide (Etop), L002, TSA or combinations thereof as depicted. The cells were exposed to etoposide for 18 h, L002 for 3 h, and TSA for 1 h. The treated cells were lysed and subjected to Western blotting with antibodies against the indicated proteins. Note that p21 is the product of the p53 target gene CDKN1A. Both p53 and p21 were induced on etoposide (a DNA-damage agent) treatment. p53 acetylated at Lys382 was accumulated in the presence of both etoposide (10 μ M) and TSA (0.2 μ M) (lane 10 of the *top panel*). The presence of L002 reduced the levels of acetylated p53 (lane 13)

6. Pipet the lysates to 1.5 ml microcentrifuge tubes. Add 10 μ l of 6 \times SDS sample buffer to the lysates. Heat the samples at 95 $^{\circ}$ C for 5 min, and then place them on ice. Centrifuge the samples at 9,300 $\times g$ with a microcentrifuge for 1 min.
7. Load 10 μ l of the samples per lane and protein size-markers on a mini-polyacrylamide gel (12 % acrylamide in the resolving gel), and run at 200 V until the dye front has just left the gel.
8. Transfer the proteins to a PVDF membrane (e.g., Immobilon-PSQ from Millipore). Follow a conventional Western blotting and ECL detection protocol to probe the membrane for acetylated p53 with an antibody against p53 acetylated at lysine 382. The membrane is stripped of antibodies and sequentially probed for total p53 and PCNA, respectively. An example of the results from such experiments is shown in Fig. 3.

4 Notes

1. Antibodies recognizing other housekeeping gene products can also be used for verifying sample loading in Western blotting experiments.
2. Although we typically use 200 μM as the starting inhibitor concentration, this can vary, depending on the inhibitory potency of an inhibitor. If a potential inhibitor is not very potent, the starting concentration can be higher.
3. For a new source of p300, conduct an assay of the enzyme in a range of concentrations using the same protocol in the absence of an inhibitor to determine an optimal p300 concentration that is within the linear response range.
4. We use Prism 6 (GraphPad Software Inc.) to derive IC_{50} values through a nonlinear regression method (Log [inhibitor] vs. response, four parameters, variable slope). Other software packages with similar capacity are also available for data analysis.
5. There are numerous acetylation substrates of p300. Therefore, the assessment of p300 inhibition by a small-molecule inhibitor could be done through detecting the levels of acetylation of a specific substrate in the absence and the presence of the inhibitor in cell cultures. Histones are well-known p300 substrates. The effects of KAT inhibitors on histone acetylation have been examined in the literature [29, 32]. Nonetheless, a specific lysine residue in core histones is often acetylated by a number of different KATs, which could complicate data interpretation. Alternatively, an examination of the impact of an inhibitor on the acetylation of other p300 substrates such as p53 may generate more interpretable data. The availability of a high-quality antibody against a substrate acetylated at a specific lysine is critical for such experiments.
6. The duration of drug exposure can vary. For example, the exposure of the HCT 116 cells to etoposide for 7 h resulted in the accumulation of acetylated p53 in the absence of an HDAC inhibitor [32]. Similarly, the exposure time of the cells to a KAT and an HDAC inhibitor should also be adjusted to allow an optimal detection of protein acetylation.

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Epigenetic Regulation in Biopsychosocial Pathways

Kristin Litzelman and Mukesh Verma

Abstract

Theory and empirical evidence suggest that psychological stress and other adverse psychosocial experiences can contribute to cancer progression. Research has begun to explore the potential role of epigenetic changes in these pathways. In basic, animal and human models, exposure to stressors or to the products of the physiological stress response (e.g., cortisol) has been associated with epigenetic changes, such as DNA methylation and microRNA (miR) expression, which may influence tumor growth, progression, metastasis, or chemoresistance. However, the specific biological pathways linking stress, epigenetic changes, and cancer outcomes remain unclear. Numerous opportunities exist to extend the preliminary evidence for the role of epigenetic mechanisms in the biopsychosocial pathways contributing to cancer progression. Such work will improve our understanding of how the psychosocial environment influences cancer risk and survival, potentially leading to improved prevention and treatment strategies.

Key words Psychological stress, Psychosocial factors, Epigenetics, Cancer

1 Introduction to Biopsychosocial Pathways in Cancer

Increasing interest has focused on the role of stress and psychosocial factors, such as social isolation, in the development and progression of cancer. While the evidence for a role of stress in the initiation of cancer is equivocal [1], increasing evidence suggests that stress and psychosocial factors, and the resultant activation of stress response pathways, may play a role in promoting tumor growth [2] and the progression and metastasis of cancer [1, 3, 4]. Further, stress has also been implicated in reduced response to cancer treatment (e.g., chemoresistance) [5–8]. Understanding the pathways by which stress influences the biological microenvironment of a tumor will aid in the development and administration of interventions and treatment. However much work remains to be done in order to understand what role epigenetic effects may have in these pathways.

A recent search of HuGE Navigator's [9] Phenopedia identified 261 genes that were reported with psychological stress. Many of these genes were associated with the biological response to acute

or chronic psychosocial stress, or were shown to interact with stress exposure to influence mental health, physical health, or health behaviors (e.g., refs. 10–14). Certain stress-related genes have also been associated with cancer outcomes, such as shorter survival in colon cancer patients [15]. However, stress phenotypes and health outcomes can also be influenced by changes in gene expression triggered by epigenetic mechanisms [16]. This chapter explores epigenetic regulation in the associations between psychosocial stress, physiological stress response pathways, and cancer.

1.1 Evidence for the Role of Stress in Cancer Development and Progression

When exposure to a potentially stressful environment is appraised as threatening [17], this triggers a physiological stress response characterized by activation of the hypothalamic–pituitary–adrenal (HPA) axis and sympathetic nervous system (SNS) (i.e., “fight or flight”) [18, 19]. The result is a flood of stress-related hormones and catecholamines, including cortisol, epinephrine/adrenaline, and norepinephrine/noradrenaline. Although protective in the short term, repeated or prolonged stress may lead to dysfunction of the physiological mechanisms controlling this response [20] potentially leading to chronically elevated levels of stress hormones that can adversely impact other biological systems.

In the clinical and epidemiological literature psychosocial factors have been associated with cancer progression, metastasis, reduced survival, and increased risk of mortality [1, 4, 21–26]. Such associations have been seen for a variety of cancers, including breast (reviewed in ref. 25), prostate (e.g., ref. 27; *see* ref. 28 for a theoretical model), lung (e.g., ref. 29), skin (e.g., refs. 30–32), gastrointestinal (e.g., ref. 33), hepatobiliary (e.g., ref. 34), endometrial (e.g., ref. 35), and hematopoietic (e.g., ref. 36) cancers (reviewed in ref. 26), although some results are tentative or conflicting. Basic research and studies in animal models have supported these findings and provided evidence for potential pathways, including immunological dysregulation and inflammation [1, 3, 25, 37–40]. For example, animal models of breast cancer have revealed that social isolation in mice and rats was associated with greater mammary tumor burden and worse tumor histology [41, 42].

1.2 Stress, Epigenetics, and Cancer

Epigenetics involves alterations in gene expression without changing the DNA sequences [43]. The process is mediated by altered DNA methylation, histone modification, noncoding RNA (especially microRNA expression), and chromatin remodeling [44, 45]. Little research has explicitly examined psychological stress, epigenetic mechanisms, and cancer (Table 1). However, emerging evidence suggests that psychosocial and environmental factors may contribute to cancer development and progression through epigenetic mechanisms [46]. Such epigenetic changes may occur in genes directly implicated in tumor suppression or progression. Alternatively, such epigenetic changes may regulate other physiological systems,

Table 1
Publications in the field of stress and epigenetics

Term used to search PubMed ^a	Number of publications
Stress, psychological ^b OR psychosocial	159,291
Cancer AND (stress, psychological ^b OR psychosocial)	13,118
Cancer AND (stress, psychological ^b OR psychosocial) AND epigen*	9
Cancer AND (stress, psychological ^b OR psychosocial) AND methylation	2
Cancer AND (stress, psychological ^b OR psychosocial) AND histone	2
Cancer AND (stress, psychological ^b OR psychosocial) AND miRNA	3
Cancer AND (stress, psychological ^b OR psychosocial) AND epigen* AND prevention	1
Cancer AND (stress, psychological ^b OR psychosocial) AND epigen* AND treatment	4

^aAs of 12/17/2013

^b“Stress, psychological” is a MeSH (Medical Subject Headings) term defined as “stress wherein emotional factors predominate.” Incorporates concepts such as life stress and emotional stress and differentiates from strictly physiological stressors, such as oxidative stress

which in turn contribute to the development or progression of cancer. For example, there is considerable evidence that exposure to stressful environments, especially during the sensitive prenatal or infancy periods, contributes to epigenetic programming of the stress response pathways, resulting in greater stress reactivity (i.e., larger physiological reactions to a stressor, such as a more greatly increased heart rate) [47–49]. This contributes to greater exposure to stress hormones and catecholamines over the life course, which may contribute to a more permissive microenvironment by influencing tumor proliferation, angiogenesis, invasion, or anoikis or by inhibiting the immune response [4, 39].

1.2.1 Evidence for Associations Between Psychosocial Factors and Epigenetic Changes to Genes Relevant to Cancer

Basic research provides evidence that exposure to stress hormones influences epigenetic regulation of tumor suppressor and oncogenes. In one such study, Shi and colleagues [50] demonstrated that cortisol downregulated the expression of miR-145 (a putative tumor suppressor in some types of cancer [51]) in HPV positive cervical cancer cells. Importantly, cortisol's impact on miR-145 was associated with chemoresistance of the cervical cancer cells. Similarly, Pu et al. [6] recently reported that adrenaline increased the expression of miR-155 (which is highly expressed in a number of cancers and may play an oncogenic role [52]) in colon cancer cells; further, cells overexpressing this miRNA had a higher growth rate and exhibited chemoresistance [6].

Studies in human populations have also implicated epigenetic mechanisms in the association between stress and cancer. In one such study, Yang et al. [53] examined genome-wide methylation

patterns among children who had experienced severe maltreatment and recent out-of-home placement by social services, compared to control children who did not have any history of maltreatment. They found that maltreated children had significantly different methylation levels on nearly 3,000 CpG sites.¹ Of particular importance, a substantial number of the genes with methylation differences were associated with lung, colorectal, prostatic, breast, colon, and ovarian neoplasms, suggesting a potential mechanistic link between childhood adversity and future cancer onset or progression. This recent study has yet to be replicated, but provides compelling preliminary evidence for a direct pathway between childhood adversity and cancer via epigenetic programming.

In addition, a number of studies have implicated socioeconomic status (SES) in the development of epigenetic patterns that may contribute to cancer. For example, McGuinness et al. [54] recently showed that those living in the most deprived neighborhoods had global DNA methylation levels 17 % lower than those in the least deprived areas. In this study, manual workers and those with less education also had lower global methylation levels (although occupational or environmental exposures were not examined), and lower global methylation was in turn associated with higher levels of IL-6 and fibrinogen (both of which have been associated with cancer progression [e.g., refs. 55, 56]). This was supported by a study conducted in the Multi-Ethnic Study of Atherosclerosis [57], which found that lower levels of wealth were associated with lower *Alu* methylation, but higher Long Interspersed Nucleotide Element-1 (LINE-1) methylation,² although no associations were observed with other indicators of SES such as income, education, or childhood SES. Greater job seniority, a potential proxy for higher social status, has also been associated with decreased methylation in *Alu* and *IFN-γ* (a gene associated with tumor control) [59]. Interestingly, in another study [60], early life socioeconomic factors were associated with *greater* methylation of the *Sat2* and *Alu* in adulthood. Other work suggests that epigenetic changes associated with child and adulthood SES may differ, potentially explaining this contradictory finding [61].

Finally, there is evidence that more proximal or short-term stressors may influence epigenetic programming of cancer-related pathways. In a small sample of university students, Gidron and

¹ Interestingly, maltreated children tended to have elevated methylation levels at sites that had low to moderate methylation, but reduced methylation levels at sites that typically had high methylation, suggesting that maltreatment may contribute to a “flattened” or less variable global methylation pattern.

² Methylation levels of *Alu*, LINE-1, and *Sat2* are correlated with overall DNA methylation [58] and are therefore used as markers of global methylation levels, although they are not always highly correlated and may interrogate different cellular processes [57].

colleagues [62] showed that exposure to a brief stressor (i.e., an academic exam) was associated with decreased expression of let-7b, a miRNA with tumor suppressor properties [63]. In those with poor health behaviors, the stressor was also associated with an unexpected decrease in expression of miR-21, which has been shown to be upregulated in cancer [64]; the authors suggest that in healthy individuals miR-21 expression may be required to prevent cell death or mediate cellular repair processes, and may protect cells from stress-induced apoptosis [62].

*1.2.2 Evidence
for Epigenetic Changes
Indirectly Linking Stress
and Cancer*

Stress and psychosocial adversity may also influence cancer risk or progression indirectly by changing epigenetic programming of other physiological systems that may influence cancer risk and progression. For example, stress in all periods of the life course from prenatal stress to acute and chronic stress in adulthood has been associated with changes in the physiological stress response pathway [65]. Many solid epithelial tumors express receptors for stress hormones and catecholamines produced in response to stress [2, 66–73], which have been shown to increase tumor cell proliferation [74–76], adhesion [77, 78], migration [79–81], and invasion [82]. One recent study [83] found that exposure in utero to maternal depression or anxiety was associated with increased methylation in two placental genes, *NR3C1* and *11β-HSD-2*, which protect the fetus from excess exposure to maternal stress hormones. Elevated methylation of these genes has been associated with increased physiological stress reactivity in young offspring [47, 49, 84], which could contribute to greater long-term exposure to stress hormones and increase or accelerate cancer progression.

Evidence for this proposed pathway is illustrated in a study by Hasen et al. [85]. In a mouse model of breast cancer, Hasen and colleagues [85] showed that socially isolated mice had reduced hypothalamic expression of the kisspeptin receptor (*Kiss1r*), which is known to modulate the hypothalamic–pituitary–gonadal axis and regulate cancer metastasis [86, 87]. Further, socially isolated mice had reduced mammary gland expression of DNA methyltransferase (*DNMT3b*) and methyl CpG binding protein 2 (*Mecp2*), both known epigenetic regulators [88]. The stressor also interacted with *p53* genotype of the mice, such that the effect was stronger in *p53* heterozygotes. These results suggest that a stressor like social isolation can modify the HPA axis and influence epigenetic mechanisms in cancer.³

³ Interestingly, socially isolated mice in this study were actually less likely to develop mammary tumors, possibly because social isolation was associated with less well-developed mammary tissue [85]. Future work is needed to replicate and extend these findings among mice with normative mammary tissue development, and in humans.

Stress may also influence immune dysfunction pathways to cancer. In a study of women with ductal carcinoma in situ (DCIS) [89], Mathews et al. observed a pattern in which high stress was associated with less global histone acetylation in lymphocytes. Although the correlations between the psychosocial measures and the epigenetic markers did not reach statistical significance, the authors suggest that epigenetic modification of immune cells may contribute to immune dysfunction in women experiencing stress [89], which may in turn influence susceptibility to secondary or recurring cancers or metastases [90]. For example, immune dysfunction may contribute to inflammation, which serves as a tumor initiator and promoter [39]. Further, a blunted immune response may allow tumor cells to escape detection and elimination by the immune system [4].

1.2.3 Studies of Gene Expression Provide Additional Evidence of Associations That May Have Epigenetic Underpinnings

A number of studies have identified stress-related changes in the expression of genes that could influence the development or progression of cancer. Evidence from basic (in vitro) research and animal models suggests that stress (e.g., exposure to catecholamines among in vitro studies; social isolation in mouse models) increased the expression of genes associated with tumor growth, progression and metastasis [91, 92], cancer risk [93], and chemoresistance [5, 7], and decreased the expression of genes associated with tumor suppression, recognition, or elimination [94, 95]. For example, in an in vitro model of breast cancer, hydrocortisone exposure decreased expression of *BRCA1*, a tumor suppressor gene [95]. There is also evidence that chronic stress may influence the expression of genes associated with the immune response [94], metabolic changes prevalent in cancer cells [96], or β -adrenergic signaling [36, 97], which may indirectly influence cancer progression via the tumor microenvironment. The role of epigenetic mechanisms in these and other relationships could be tested with the application of appropriate epigenetic approaches.

2 Methodologies

Global alterations in the epigenetics landscape are the common feature of cancer. High throughput sequencing technologies have become very common in characterizing the genome, transcriptome, and epigenome. These techniques are capable of sequencing multiple DNA molecules simultaneously. While selecting a method, consider the characteristics of each technology, such as the required amount of DNA, flexibility in selection of CpG sites to analyze (for methylation analysis), how quantitative the technique is, technical complexity, and cost. If the aim is for cancer diagnosis, a method that is highly accurate should be chosen whereas if the aim is to

analyze DNA methylation as a cause of gene silencing, a specific region that controls gene expression should be focused. A few common technologies used in epigenetics field are described below.

2.1 DNase Hypersensitivity Assay

Those regions of the chromatin which are free of histone proteins provide access to DNase I activity. These sites are called DNase hypersensitivity sites (DHSs) and mostly located in the gene promoter region. DNase hypersensitivity assay (DHA) is a method to identify regulatory domains but can also suggest their function. It can map gene loci in small increments of about 20 kb. Some other methods can extend this range up to 100 kb which helps in mapping distal gene regions [98]. DHSs have been mapped to cis-acting regulatory regions including promoters, enhancers, silencers, and insulators. The map reflects transient remodeling of chromatin as seen in the case of transcriptional activation or epigenetic inheritance of programmed changes to a locus during differentiation.

After collecting cells, they are suspended in lysis buffer and treated with Nonidet P-40 at different concentrations followed by centrifugation to isolate nuclei. The next step is to suspend nuclei in another lysis buffer followed by DNase I treatment for different periods of time and analyzing the product by gel electrophoresis. Blots of the gel are hybridized with different probes and images are analyzed to locate DHSs [99].

2.2 Histone Modifications

Chromatin immunoprecipitation. Genome-wide histone modifications are detected by coupling immunoprecipitation and DNA microarray and the combined technology is called ChIP-on-chip (chromatin immunoprecipitation) [100]. The binding proteins are histones and antibodies are used to precipitate chromatin (DNA) lightly treated with formaldehyde (or sheared chromatin). Sometimes micrococcal nuclease, a nonspecific endonuclease, is also used to break chromatin. For immunoprecipitation of sheared chromatin, antibodies should be of high quality. Sheared DNA is separated from precipitated chromatin DNA complex and amplified by PCR and labeled with fluorescence tags (generally Cy3 and Cy5) [101]. Genomic DNA immobilized on microarray is hybridized with labeled immunoprecipitated and naked DNA (non-immunoprecipitated) and results showing differential intensity of fluorophore determine location of DNA covered/protected by histones. A more recent method, called competition ChIP, has been developed where genome-wide temporal effect of different chromatin binding proteins can be determined [102].

2.3 Methylation

Different methods are adopted for methylation assay such as bisulfite sequencing, combined bisulfite restriction analysis (COBRA), methylation specific PCR (MSP), real time MSP or MethyLight, pyrosequencing, MassArray. These methods are based on different

principles that differentially recognize 5-methylcytosine (C^m) from cytosine (C); or bisulfite-mediated DNA conversion. In the first category a restriction enzyme is selected which recognizes C^m , whereas in the second category bisulfite treatment converts unmethylated C into uracil (U). One more method involves recognition of methylated cytosine by an anti-methylcytosine antibody or a methylated DNA binding (MBD) protein. After appropriate shearing of DNA, methylated DNA can be collected using affinity methods. The latter method is more appropriate for genome-wide screening of samples. Lastly, the fraction of methylcytosine in the entire genome can be measured by mass spectrometry but this method is suitable for qualitative analysis only.

In bisulfite sequencing, the converted DNA is amplified by PCR using primers located in genomic regions without CpG islands and the product sequenced. Results of this technique show the methylation status of every single CpG site between the primers and how multiple CpG sites in a single DNA molecule are methylated. The disadvantage of this technique is that it is labor intensive and requires at least ten clones sequenced to get reasonably accepted results.

The COBRA technique is applied after the bisulfite treatment and the presence or absence of specific restriction enzyme decides results. Quantitation is possible in this technique which is achieved by determining the ratio of PCR product from the digested and undigested DNA. The procedure is simple and inexpensive. The disadvantage is that CpG sites that can be analyzed by COBRA are limited.

The flexibility of MSP in selecting a genomic region is very high because PCR primers can be designed at any positions. This technique is very simple and can interrogate methylation status of several CpGs at primer sites by performing PCR with primers specific to unmethylated or methylated sequences. The disadvantage of the technique is the high rate of false negative or positive results and requires careful determination of the number of PCR cycles performed.

Another technique, methylation-sensitive representational differential analysis (MS-RDA), is a genome-wide methylation analysis method which distinguishes DNA fragments differentially methylated between two samples. This approach enriches the unmethylated CpG-rich fraction of the genome. As a first step of this method, genomic DNA is digested with a methylation-sensitive restriction enzyme (such as HpaII), and DNA fragments are amplified by PCR using a universal adaptor primer. As a result, DNA fragments differentially methylated between samples will be present in one amplicon but not in the other.

2.4 miRNA Profiling

miRNAs can be hybridized to microarray slides or chips with probes to hundreds or thousands of miRNA targets so that the relative amount of miRNA can be calculated for quantitation purpose.

For transcriptomics and miRNA profiling GeneChipR Human Exon ST Array (Affymetrix) and TaqManR Array MicroRNA Cards (Life Technologies) are used [103]. miRNAs can be characterized by high throughput sequencing methods using miRNA Sequencing. The functional characterization of miRNAs can be done by using oligomiRs (locked nucleic acid, morpholino oligo, 2'-O-methyl RNA oligo [104]. For cancer epidemiology studies, either miRNA profiling or mutations/SNPs in the genes coding for miRNA can be used for risk assessment and screening clinical samples [105].

3 Challenges in the Field

3.1 *Technological Challenges*

Epigenetic markers have been measured in a number of tissue types, most frequently tumor tissue and blood, but also in normal tissue, buccal cells, urine, and saliva, among others [44]. Recent work has suggested that blood samples can be used as a surrogate for tumor tissue in epigenetic analyses [106]. However, there is also clear evidence that methylation patterns differ considerably by tissue type [107]. It therefore remains uncertain whether or in what circumstances readily accessible biospecimens, such as blood, can be reliably used to measure overall or gene-specific changes in epigenetic patterns attributable to psychosocial factors as a proxy for other tissue types [106]. Similarly, it is not yet clear whether global methylation is a useful marker of cancer risk or evaluating biopsychosocial pathways [108, 109], or whether methylation of repetitive elements (e.g., LINE-1) are useful proxies for global methylation in blood and/or other tissues [110]. This is particularly important for clinical and epidemiological studies, especially studies of healthy populations, as noninvasive or minimally invasive specimen collection is preferred or even necessary and collection of certain tissue types may not be feasible. Finally, it is not yet clear what adverse effects, if any, long-term tissue storage and tissue handling (e.g., repeated freeze-thaw cycles) may have on marker detection.

Another challenge is the quantitation of methylation. Quantification of the methylated DNA is very critical. The way it is achieved is that the exact number of DNA molecules is calculated in a standard DNA and extrapolation of the PCR amplified methylated DNA is achieved by comparison with standard DNA. Standard DNA can be prepared by gel purification or by cloning into a known plasmid followed by calculating the amount of recombinant DNA followed by calculating the number of DNA molecules in the recombinant plasmid. Precaution is also taken while preparing the template DNA for methylation specific PCR. Generally bisulfite treatment reduces the amount of DNA by 10 %, therefore calculations are adjusted accordingly.

Many methods exist for studying psychosocial stress. In vitro studies often rely on exposing cell lines to stress hormones such as

adrenaline/epinephrine (e.g., ref. 6). Animal models typically utilize physical stressors such as restraint or forced swim, or social stressors such as social isolation or social defeat [111]. In humans, observational studies examine three classes of stress [112, 113]: exposure to chronic or acute stressful experiences (e.g., early childhood adversity, low SES, or stressful life events); psychological stress such as perceived stress or symptoms of stress; and biological markers of the stress response, such as blood pressure, vagal tone, or salivary cortisol (*see* refs. 114–116 for questionnaires and resources). Experimental studies induce stress in their human subjects through a number of methods, including the Trier Social Stress Test [117], the Cold Pressor Test [118], the Matt Stress Reactivity Protocol [119], among many others. “Natural experiments” are also frequently leveraged to better understand the stress response in humans, such as exposure to the Dutch Hunger Winter [120] or the September 11th terrorist attacks in New York [121, 122].

3.2 Research Challenges

In order to evaluate whether a causal or mechanistic chain exists between psychosocial factors, epigenetic profiling, and cancer in humans, rigorous, thorough, longitudinal studies will be needed. Reviews and meta-analyses of studies examining the association between stress and breast cancer, for example, have pointed towards the importance of study design and measurement of the exposure (summarized by Antonova et al. [25]). As basic research and retrospective longitudinal studies provide evidence for these associations, large-scale, prospective epidemiological studies will be necessary to better understand these mechanisms in generalizable populations.

In addition, emerging hypotheses suggest that bidirectional relationships may exist among psychological factors, epigenetic markers, and cancer. For example, Lyon and colleagues recently presented their hypothesis that tumor- and inflammation-related epigenetic changes may contribute to persistent psychoneurological symptoms (including depression) among breast cancer patients [123]. This is supported by research showing that the presence of a tumor in a mouse model was sufficient to trigger depression- and anxiety-like behaviors [124, 125]. Researchers will need to carefully consider the possibility of bidirectional relationships in order to determine the causal pathways that may link psychosocial factors to cancer.

Numerous hypotheses exist in the current literature about the plausible biological mechanisms connecting psychosocial factors, epigenetic changes, and cancer. For example, stress may influence breast cancer risk via the role of stress hormones in mammary gland development, by altering the generation or activity of estrogen, via immune dysfunction, or by other pathways [25]. While these mechanisms are not mutually exclusive, systematically identifying and testing such hypotheses will be necessary to develop a comprehensive understanding of these relationships.

Finally, publication bias is and will likely remain a challenge for this emerging field, as researchers may not attempt to publish early null results, or null results may not be accepted for publication by journals. As such, interpretation of the cumulative research findings should be cautious, as the studies that are successfully published may show aberrantly strong associations. Diligence on the part of scientists and editors alike will be necessary to garner a true and balanced understanding of these associations. Further, replication of early results will be crucial.

4 Research Opportunities

Opportunities abound for future research in the epigenetics of stress and cancer. The opportunities and challenges faced by the field are summarized in Table 2.

Compared to the relative paucity of studies directly evaluating the relationships among stress, epigenetic markers, and cancer, numerous studies have examined stress, gene expression, and cancer. Applying epigenetic methodologies to the relationships identified in studies of gene expression will help to determine whether epigenetic mechanisms mediate these relationships.

Research *in vitro* has suggested that stress hormone or catecholamine exposure can directly influence the expression of miRNAs that contribute to tumor growth and/or chemoresistance [6, 50]. If and to what extent these pathways are observable in humans remains to be examined. While observational studies in humans have suggested that psychosocial factors contribute to global methylation patterns, future studies will need to determine if and to what extent these pathways directly to contribute cancer risk, progression, or mortality in human populations. Further, research in human populations is necessary to determine how such findings can be applied to clinical and research settings.

There is also a clear opportunity for the development and testing of interventions (i.e., clinical trials) based on this research. In animal models, beta blockers have been shown to prevent the adverse gene expression associated with stress (e.g., Shahzad et al. [91]). Research is needed to determine if epigenetic mechanisms may play a role in these associations, and whether such interventions would effectively prevent or reduce cancer progression among human populations. In addition, some trials have indicated that psychosocial support interventions are associated with improved cancer survival [31, 126–128], although those results were controversial (for a review, *see* refs. 1, 129). One recent study has shown that a cognitive-behavioral stress management intervention reverses anxiety-related leukocyte gene expression in early stage breast cancer patients, potentially improving their outcomes (although the sample size in this study was small) [130].

Table 2

Research opportunities and challenges in the field of stress, epigenetics, and cancer

Opportunities	Challenges
Apply epigenetic approaches to the relationships identified in gene expression studies, to determine whether epigenetic mechanisms may mediate these relationships	Measurement of the type and timing of stress
Examine understudied epigenetic markers, such as histone modification, chromatin remodeling, and noncoding RNAs	Measurement of confounders in human studies
Explore whether epigenetic changes associated with psychosocial factors contribute to cancer risk, progression, or survival	Differences in epigenetic patterns in different types of tissue; use of minimally invasive biospecimens, such as blood, as a proxy for other tissue types
Evaluate how findings from basic, animal, and human studies can be applied in clinical and research settings	Use of global methylation to examine cancer risk; use of repetitive element (e.g., LINE-1) methylation as a proxy for global methylation
Develop and test pharmacological and psychosocial interventions that may modify epigenetic patterns and improve cancer treatment and outcomes	Potential bidirectional relationships
Explore whether epigenetic changes mediate the observed associations between psychosocial support interventions and cancer survival	Evaluation of causality, especially in human studies
Explore whether immunoresponsive cancers, (like squamous cell carcinoma) or other classes of cancers are differentially susceptible to stress-epigenetic mechanisms	Development and maintenance of rigorous, prospective, population-based studies
Examine whether the putative associations between stress, epigenetic markers, and cancer differ by cancer site	Potential for publication bias and overestimating of effects
Explore the impact of the type and timing of the stress exposure (i.e., over the life course) on epigenetic patterning and cancer outcomes	Reconciling and testing the numerous potential biological pathways (e.g., catecholamine exposure, inflammation, immunosuppression)
Explore potential differential effects of chronic and acute stress	Selection of tissue type for analysis, and consistency of epigenetic markers among different types of tissues
Determine whether adverse epigenetic patterns are reversible and the factors or interventions that can achieve this effect	Effects of tissue storage (especially long-term storage) and handling

Future work should examine whether epigenetic changes mediate improvements in health outcomes associated with such biopsychosocial interventions.

Numerous outstanding questions remain about the epigenetic pathways potentially linking stress and cancer. First, how do these

effects and pathways differ by cancer type and site? For example, it has been suggested that immunoresponsive cancers [131] like squamous cell carcinoma [132] may be more responsive to psychosocial assaults. Additional research is needed to determine to what extent the pathways linking stress and cancer are consistent within and among different disease categories. Similarly, it will be important to determine whether susceptibility to stress and the role of epigenetics are consistent among cancer types.

Examination of the timing of the exposure will also be an important objective of future research. Previous epigenetic evaluation of stress has highlighted the impact of exposure to prenatal stress and adverse childhood events. Other periods of the life course, including childhood, adolescence, and adulthood, have been less well-studied but may play an important role in cancer outcomes. Critical or sensitive periods of the life course may be particularly important for certain types of cancers and may differ by cancer type. Further, different epigenetic pathways may be at work during different time periods [65, 133–135]. Research assessing the potential differential effects of psychosocial factors at each time period may reveal important associations. Similarly, there is some evidence that the effects of chronic versus acute stress may differ [132], and future research into these nuanced associations will be critical for the development of well-controlled studies and appropriate interventions. Careful measurement of the type and timing of stress exposure will be necessary to identify the causal pathways and the best periods for intervention.

Finally, research is needed exploring the reversibility of epigenetic markers. For example, if an individual is exposed to adverse childhood events that influence the epigenetic patterning of cancer-related genes, can later familial and psychosocial exposures reverse or mitigate these effects? If so, what are the most salient determinants or predictors of the cumulative epigenetic “risk load”?

5 Conclusions

Emerging research suggests that epigenetic mechanisms may play a role in the biopsychosocial pathways contributing to cancer risk and progression. However, the biological pathways leading from adverse psychosocial exposures to cancer progression, metastasis, and mortality remain unclear. Further research will be needed to confirm and clarify these pathways and the strength of the associations linking psychosocial factors to cancer outcomes. As these emerging mechanisms are elucidated, their application in the clinical and research settings will need to be carefully explored.

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Viral Epigenetics

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Abstract

DNA tumor viruses including members of the polyomavirus, adenovirus, papillomavirus, and herpes virus families are presently the subject of intense interest with respect to the role that epigenetics plays in control of the virus life cycle and the transformation of a normal cell to a cancer cell. To date, these studies have primarily focused on the role of histone modification, nucleosome location, and DNA methylation in regulating the biological consequences of infection. Using a wide variety of strategies and techniques ranging from simple ChIP to ChIP-chip and ChIP-seq to identify histone modifications, nuclease digestion to genome wide next generation sequencing to identify nucleosome location, and bisulfite treatment to MeDIP to identify DNA methylation sites, the epigenetic regulation of these viruses is slowly becoming better understood. While the viruses may differ in significant ways from each other and cellular chromatin, the role of epigenetics appears to be relatively similar. Within the viral genome nucleosomes are organized for the expression of appropriate genes with relevant histone modifications particularly histone acetylation. DNA methylation occurs as part of the typical gene silencing during latent infection by herpesviruses. In the simple tumor viruses like the polyomaviruses, adenoviruses, and papillomaviruses, transformation of the cell occurs via integration of the virus genome such that the virus's normal regulation is disrupted. This results in the unregulated expression of critical viral genes capable of redirecting cellular gene expression. The redirected cellular expression is a consequence of either indirect epigenetic regulation where cellular signaling or transcriptional dysregulation occurs or direct epigenetic regulation where epigenetic cofactors such as histone deacetylases are targeted. In the more complex herpesviruses transformation is a consequence of the expression of the viral latency proteins and RNAs which again can have either a direct or indirect effect on epigenetic regulation of cellular expression. Nevertheless, many questions still remain with respect to the specific mechanisms underlying epigenetic regulation of the viruses and transformation.

Key words Cancer, Epigenetics, Methylation, Transformation, Virus

1 Epigenetics

Epigenetic regulation consists of those changes in gene regulation resulting from environmental cues which can be passed from mother to daughter cells during replication which are not a result of changes in DNA sequence. Because the biologically functional state of DNA in eukaryotes is in the form of chromatin in which the DNA is wrapped around a histone core, the major contributors to epigenetic regulation are DNA and the histones H2A, H2B, H3,

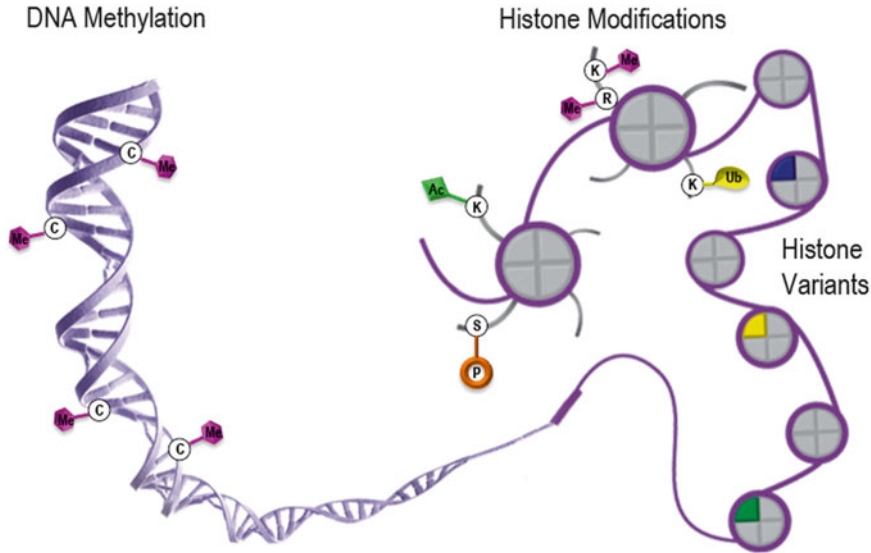


Fig. 1 Major forms of epigenetic regulation

and H4. The major forms of epigenetic regulation are indicated in Fig. 1.

DNA can be epigenetically regulated through the introduction of a methyl group onto the cytosine in the sequence CpG [1]. There are three known mammalian DNA methyltransferases. One of which, Dnmt1 prefers hemi-methylated substrates and may be responsible for maintenance of the methylation state of DNA during replication. The other two methyltransferases, Dnmt3a and Dnmt3b appear to be responsible for the de novo introduction of methylated cytosine into DNA [2]. Methylation of DNA is usually thought to be associated with repression of gene expression [1].

Histones are also known to contribute to epigenetic regulation. Histones can affect epigenetic regulation either through the presence of different variants or by posttranslational modification of amino acids in the histones. Histone H2A and H3 are known to exist in multiple forms which differ in their primary amino acid sequence at a limited number of sites in the histone [3, 4]. Generally the changes in amino acid sequence consist of replacement of serines by valine or alanine in regions other than the amino terminal tails.

There are four well known variants of H3 and three of H2A. The variants of H3 include H3.1, H3.2, H3.3, and CENP-A. H3.1 and H3.2 are primarily associated with newly replicated chromatin in higher eukaryotes in contrast to H3.3 which is associated with actively transcribing chromatin and CENH3 which is typically found in the centromeres of chromosomes [5, 6]. Interestingly, the specificity of association of the H3 variants in chromatin appears to be based primarily upon how the variant is introduced into the chromatin. Interactions between the histones and DNA in nucleosomes

are typically disrupted during processes in which DNA sequence information is read such as replication and transcription and regenerated after the process is complete. The regeneration of nucleosomes typically involves the interaction between histones, specific chaperones, and proteins involved in replication or transcription. For example, H3.1 and H3.2 interact with CAF-1 and ASF-1 through interaction with PCNA at replication forks [7], while H3.3 interacts with HIRA during transcription and CENP-A interacts with HJURP which recognizes centromeric region [7].

The variants of H2A consist of H2A.Z, H2A.X, and macroH2A. H2A.X appears to be primarily associated with DNA repair, macroH2A appears to be associated with repressed regions, while H2A.Z appears to be located at the flanks of nucleosome-free regions [8–10]. This may be due to the fact that H2A appears to be dynamically regulated with the histone constantly being displaced from and reassociated with nucleosomes [5].

Posttranslational histone modifications are also thought to play a major role in epigenetic regulation. A number of modifications are known to occur in histones including acetylation, methylation, phosphorylation, ADP ribosylation, and ubiquitylation. However, of these, acetylation and methylation are arguably the most studied. Acetylation of H3 (lysines 9 and 14) and H4 (lysines 5, 8, 12, 16, and 20) is generally thought to be primarily associated with transcriptionally active chromatin. In its role associated with transcription, acetylation appears to be a very dynamic process in which H3 and H4 undergo continuous acetylation and deacetylation along with movement of RNA Polymerase II through the transcribed chromatin [11]. There are a number of well-characterized cellular acetyl-transferases responsible for histone acetylation including p300 and others [12]. Similarly, there are at least 18 known histone de-acetylases, divided into four classes, Class I (HDAC1, 2, 3, and 8), Class II (HDAC4, 5, 6, 7, 9, and 10), Class III (the Sirtuin family), and Class IV (HDAC 11) [13, 14].

The other major form of well-studied histone posttranslational modification is methylation of H3 and H4. This form of regulation appears to be more complex in part because a particular lysine can be either mono-, di-, or tri-methylated. As a consequence while there are general relationships between methylation of a particular lysine, there are also major exceptions to many of these general relationships depending upon cell type, differentiation status and location within the genome. Nevertheless, methylation, particularly tri-methylation of H3K4, H3K36, and H3K79 are generally associated with transcriptional activation while tri-methylation of H3K9, H3K27, and H4K20 are generally associated with transcriptional repression [15]. Our own studies, to be discussed below in the SV40 section, suggests that in particular the mono-methylated form of many of these modifications can have a significantly different regulatory effect than the tri-methylated form [16, 17]. At the present

there are a number of histone methylases known and in general each form of histone methylation has its own enzyme [15]. Similarly, enzymes capable of demethylating each of the forms of methylated histones with the present exception of H4K20me3 and H3K79 have been identified [15]. The latter suggests that none of the modifications with the possible exception of methylation on H3K79 or H4K20me3 are stable. However, histone methylation is not thought to be as dynamic as histone acetylation.

1.1 Life Cycle of Tumor Causing Viruses

Because epigenetic regulation occurs in the context of a virus infection, it is necessary to first consider the typical biological events which occur during an infection. Infection by DNA tumor viruses can either result in productive infections in which progeny virus is obtained, transformation of the infected cells in which case tumor cells arise, or latent infections in which the virus exists as a semi-functional episome. The initial steps of a typical virus infection includes binding to an external cellular receptor, internalization into the infected cell, and transport to the nucleus of the cell usually with concomitant uncoating of the genetic information. Once delivered to the nucleus the viral genome then undergoes initiation of certain genes required for beginning the infection usually described as early genes or immediate early genes. As discussed in detail in subsequent chapters the initiation of transcription is dependent upon the virus. Some viruses like SV40 are present in virions as chromatin and capable of carrying transgenerational epigenetic information [18]. Schematic genomic structures of different viruses are shown in Figs. 2, 3, 4, and 5. Other DNA viruses are like herpes virus and enter the cell as DNA and then become organized into chromatin

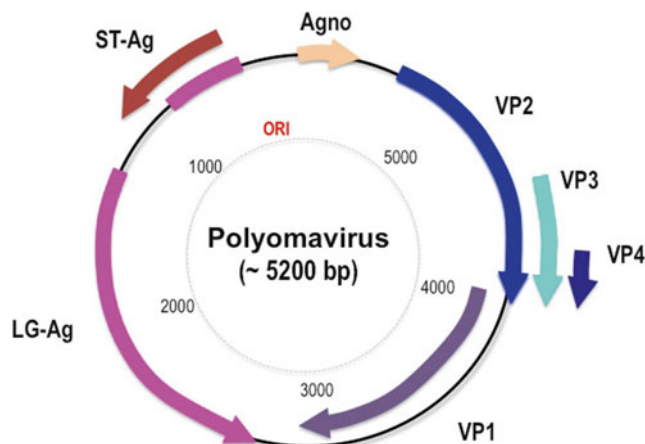


Fig. 2 Schematic representation of the polyomavirus genome. The organization of a typical polyomavirus (SV40) is shown. The early proteins T-antigen and t-antigen, the late proteins VP1-4 and the agnogene protein, and the origin of replication (ori) within the regulatory region are indicated

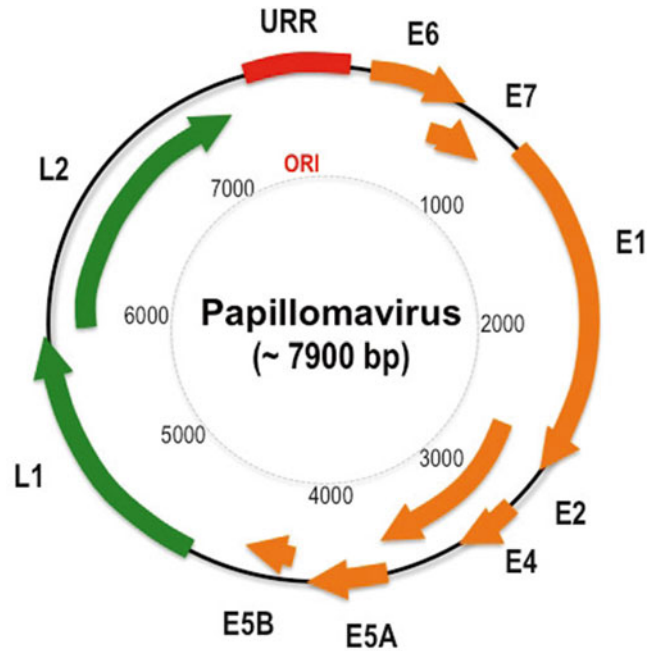


Fig. 3 Schematic representation of the papillomavirus genome. The organization of a typical oncogenic human papillomavirus (HPV16) is shown. The early proteins E1–7, the late proteins L1 and L2, and the origin of replication (ori) within the upstream regulatory region (URR) are indicated

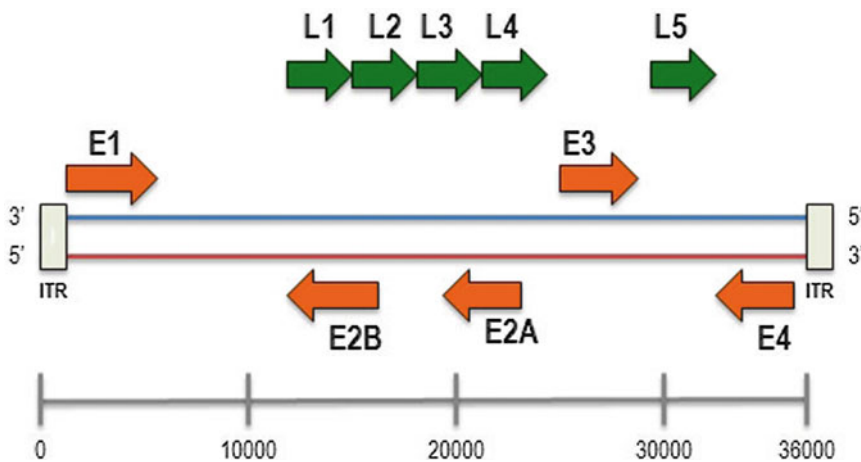


Fig. 4 Schematic representation of the adenovirus genome. The organization of a typical adenovirus (adenovirus 5) genome including the early proteins E1–4, the late proteins L1–5, and the terminal repeats (ITR) are indicated

upon transport to the nucleus [19] (Fig. 5). These differences are significant with respect to epigenetic regulation.

Following the initiation of early transcription there is generally a series of regulatory events leading to the repression or activation of both cellular and viral genes frequently in a cascade which ends

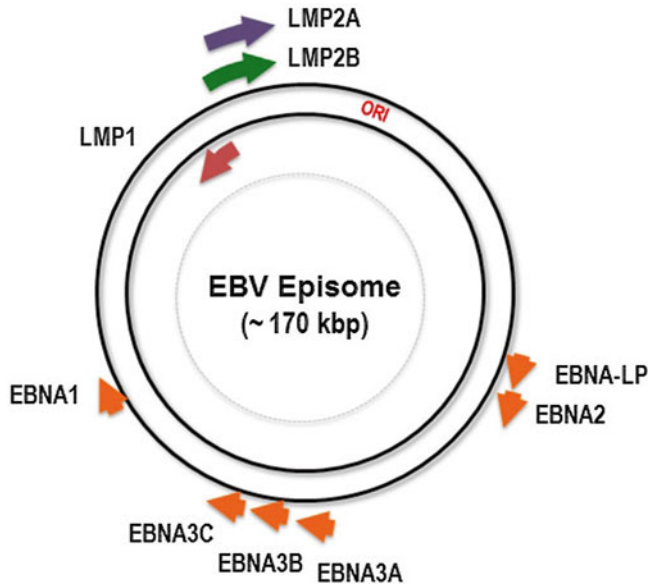


Fig. 5 Schematic representation of the herpesvirus genome. The organization of the episomal form of a typical oncogenic human herpesvirus (EBV) is shown. The location of the latency proteins EBNA1–3, EBNA-LP, LMP1, and LMP2 are indicated. The lytic origin of replication is also indicated (ori)

with two events: the initiation of late transcription which generally includes the viral proteins required for generation of the virus coat and the initiation of replication. The order of these events and whether they actually occur during infection can vary by particular virus. For example, certain viruses including members of the Herpes virus family may go into a latent state in which there is minimal transcription along with long term stability of the chromatin containing the genome as an episome. At other times the viruses integrate into the host genome generally resulting in transformation of the infected cell and subsequent generation of a tumor.

In a productive infection when an appropriate level of replication is reached along with the necessary amounts of coat proteins from late transcription, the viral chromosome begins the encapsidation process. Ultimately, this leads to the formation of new viable virus ready for a subsequent round of infection. Most if not all of these biological processes are likely to be regulated through epigenetic mechanisms. The role that epigenetics plays in regulation of each viral life cycle will be discussed in detail in subsequent sections.

1.2 Special Epigenetic Problems with Viruses

While the epigenetic regulation of the chromatin from cells and tumor-causing viruses appears to be very similar with respect to the mechanisms involved, there is a major difference between the epigenetics of cellular and viral chromatin. Typically it is thought that cells whether undergoing differentiation or terminally

differentiated contain only a single epigenetic state or epigenome defined by the particular set of modifications present in the chromatin. Viral chromatin, on the other hand, must exist as multiple epigenomes at the same time in the same cell in order to complete its life cycle. For example, following infection with SV40 and similar viruses, minichromosomes capable of early transcription, repressed early transcription, late transcription, replication, and encapsidation all must coexist within a single infected cell at late times in infection. Moreover, the relative proportion of each of the species must also be tightly controlled. One would expect that if one of the above processes was deregulated there would be a profound effect on the amount of virus ultimately generated by the infection. For example, in the SV40 system if late proteins were over-produced and no mechanism was present to ensure that a pool of chromosomes capable of replication was maintained, one might expect the infection to end with only a small amount of actual virus being produced. However, what is clear is that the levels of transcribing, replicating, and encapsidating SV40 chromosomes are optimized for viral yield by the infection. This control is most likely mediated through epigenetic regulation since all the minichromosomes still retain the same genomic information. In the SV40 system we have shown that at these late times a minimum of five distinct SV40 epigenomes are present in infected cells [18] based upon the combinations of histone modifications present in H3 and H4. As discussed in more detail below we believe that at least some of these epigenomes represent the biologically functional minichromosomes which are expected to be present. While the presence of multiple tightly regulated epigenomes within an infected cell appears to be only associated with these viral infections at this time, it would not be surprising to see similar mechanisms involved in regulation of cellular genes present in multiple copies on one chromosome or on diploid chromosomes.

1.3 Overview of Epigenetic Consequences of Viral Infection on Cellular Genes

The small DNA tumor viruses including polyomaviruses, adenoviruses, and papillomaviruses promote their productive infections and cellular transformation events by a common strategy in which one or more virus-specific early proteins disrupt normal cellular control processes by binding to critical cellular regulatory factors. For example, p53 which is a checkpoint regulator and transcription factor is a common target of a number of these early gene products [20–22]. The binding of the viral protein can then lead to either specific activation or repression of critical cellular genes or global effects on cellular expression patterns. Not surprisingly, factors involved in epigenetic regulation can be targets of the viral early proteins. For example, e1a of adenovirus binds to p130 and the related lysine acetyltransferases, EP300 and CREBBP [23–26]. The role of the viral early proteins in global modification of gene expression will be discussed in more detail with respect to each relevant individual virus.

**1.4 Overview
of Transformation by
Relevant Viruses
with Emphasis
on Latency Regulation**

Under certain circumstances related to regulation of viral gene expression and cellular context, the small DNA tumor viruses can transform infected cells. When this occurs, all or critical portions of the viral genome become integrated into the host cell genome. The integration event then results in both dysregulation of the viral gene expression and certain cellular genes. Typically the viral genes involved in reordering cellular gene expression to allow for replication of the virus such as p53 are over-expressed. This leads ultimately to the increased growth rate and dysregulation of the cellular control genes. For the larger herpesviruses transformation is a consequence of gene expression associated with latency. Presumably epigenetics could play a critical role in this process in a number of ways. Chromatin structure could affect the sites of DNA integration and subsequent expression of viral genes. In addition, viral proteins such as latency factors could directly modulate the activities of global epigenetic regulators. The influence of the different viruses on epigenetic regulation during integration or latency will be discussed below. An overview of the viruses to be discussed and the forms of epigenetic regulation and methods of detection are shown in Table 1.

2 Polyomaviruses

2.1 Overview

The polyomavirus family consists of a number of related small DNA viruses including mouse polyomavirus, Simian Virus 40 (SV40), human Merkel cell virus, human JC virus, and human BK virus as well as others. The viruses are characterized by circular double strand genomes of approximately 5,000 basepairs organized with a regulatory region containing the origin of DNA replication and transcriptional regulatory sequences controlling the early and late genes located on either side of the regulatory region (Fig. 2). SV40 is not considered a human pathogen although SV40 DNA sequences are occasionally found in human tumors. Merkel cell virus, JC virus, and BK virus were all isolated from clinical samples. The latter viruses appear to be present in human hosts without causing disease. The viral DNA present in virions and infected cells is organized with cellular histones in typical chromatin structures and is known as a minichromosome [27]. With respect to epigenetic regulation, SV40 is the most extensively characterized polyomavirus and will be discussed first in detail. Because of the overall similarity of the viruses in this family, it is likely that the other polyomaviruses will show similar patterns of regulation.

**2.2 Epigenetic
Regulation of an SV40
Lytic Infection**

The relationship between histone modification and the SV40 lytic infection has been studied using a number of variations of chromatin immunoprecipitation (ChIP) followed by PCR amplification. The histone modifications most extensively studied have been

Table 1
Forms of viral epigenetic regulation and methods of detection

Virus	Form	Method of detection		
		DNA methylation	Histone variants	Histone modifications
Polyoma Virus—SV40	Virus	NA	ND	ChIP [16, 18] ISFIP/RcChIP [11, 30] DAM ChIP [31]
	Cell	Methylation-specific PCR [44]	ND	ND
Polyoma Virus—other	Virus	NA	ND	Gel electrophoresis [41] Gel electrophoresis and Western blotting [39]
	Cell	Methylation-specific PCR [45]	ND	ND
Adenoviruses	Virus	Radioactivity based [70] Restriction endonuclease [71] Bisulfite [72, 73]	ChIP [62]	ChIP [61, 63]
	Cell	Restriction endonuclease [74–76]	ND	ChIP-ChIP [25] ChIP-Seq [63]
Human papilloma virus	Virus	Bisulfite [78, 79]	ND	ChIP [80]
	Cell	Bead-arrays [86] MeDIP-Seq [85]	ND	ChIP-Seq [81]
Herpesvirus—EBV	Virus	Restriction endonuclease [93]	ND	ChIP-Seq [91]
	Cell	Bisulfite [98, 99, 101] Bead-ChIP arrays [97]	ND	ChIP [96, 98, 100]
Herpesvirus—KHSV	Virus	MeDIP [103]	ND	ChIP-ChIP [102, 103]
	Cell	ND	ND	ChIP-Seq [112]

acetylation of H3 and H4 and methylation of H3 on lysine 4 and 9, and H4 on lysine 20. Because of the small size of the SV40 minichromosome and the ability to obtain fairly large quantities of material, one technique which has been particularly useful for these studies was immune selection and fragmentation followed by immunoprecipitation (ISFIP). In this modified ChIP procedure SV40 minichromosomes containing a particular modified histone or bound protein are immune selected with an antibody to the target protein and bound through the antibody to agarose. Following removal of all the nonspecifically bound minichromosomes the immune selected minichromosomes are fragmented by sonication and the minichromosome fragments remaining bound to the agarose separated from the unbound fragments. The chromatin bound to agarose is gently eluted and both forms of fragmented chromatin then subjected to a second ChIP using a second antibody targeting a protein or modification of interest. For example, to study

the histone modifications associated with transcribing SV40 minichromosomes antibody to RNAPII can be used to select the transcribing minichromosomes. Importantly, because the immune selected minichromosomes were fragmented, it is possible to distinguish changes in histone modification occurring in regions where the RNAPII or protein of interest is bound compared to regions which do not contain RNAPII or the other protein. Again because of the small size of the minichromosome and quantities available, ChIP-SEQ and ChIP on chip would be very useful to identify the location of histone modifications related to the phasing of nucleosomes in immune selected minichromosomes.

In virus particles (virions) the SV40 DNA is organized into chromatin [27]. For many years it has been known that the histones H3 and H4 in present in these virions can be found acetylated [27]. More recently, it has been shown that the SV40 chromatin in virions can also contain H3K9me1, H3K9me2, H3K9me3, and H4K20me1 along with the hyperacetylated H3 and H4 based upon analyses of minichromosomes from disrupted purified virions using standard ChIP techniques [18]. Moreover, we have found that the presence of histone modifications on the SV40 minichromosomes present in virions is variable. We have identified distinct classes of minichromosomes in infected cells and virions which we have referred to as epigenomes which contain specific combinations of histone modifications [18]. In virions we have identified at least three classes including one that consists primarily of H3K9me3 with a little H4K20me1, H3K9me2 and hyperacetylated H4, a second class containing H3K9me1 along with H3K9me2, H4K20me1, and hyperacetylated H4, and a third class containing hyperacetylated H3 and H4 along with H4K20me1 (Milavetz, unpublished observations). Importantly, the pattern of histone modifications present in the epigenomes of virions reflects the circumstances of the infection in which the virions were produced and thus carry trans-generational epigenetic information which can profoundly impact the initiation of a subsequent infection [18]. The epigenetic heterogeneity of the minichromosomes present in SV40 virions raises a number of interesting questions. Is there a specific epigenome responsible for the initiation of early transcription, or can any epigenome serve as a substrate for early transcription? The answer to this question will help to clarify the mechanism of early transcription. If one or more of the epigenomes is preferentially involved in the initiation of early transcription what is the function of the other epigenomes? How do they contribute to the infection?

All of the histone modifications observed in the minichromosomes in virions are also present in the minichromosomes that are transported to the nucleus during the early stages of an infection although the relative proportion of each modification may change [17, 18]. In particular from 30 min post-infection until approximately 10 h post-infection there is an increase in the relative level of H4K20me3 and a decrease in the levels of H3K9me1

and hyperacetylated H4 along with an overall decrease in the amount of the SV40 chromosomes present in the cell [17, 18, 28]. It was proposed that the reduction in SV40 chromatin along with changes in histone modification represented an inherent ability of the infected cell to recognize foreign chromatin and degrade that chromatin [17].

Transcription of the incoming SV40 minichromosomes from virions occurs very quickly during an infection. Within an hour of exposure to the virus early messenger RNA can be detected by RT-PCR and RNAPII can be found bound to SV40 minichromosomes using ChIP analyses [29]. However, it has not yet been determined whether any of the incoming SV40 epigenomes can serve as the substrate for the initiation of transcription or if only a subset can bind RNAPII. The RNAPII can be found in the promoter, early region and late region at this time with highest occupancy on the early region, less on the promoter, and least on the late region [28]. These results are consistent with the physical organization of the SV40 genome and an absence of an insulator sequence between the early and late transcription units.

Hyperacetylated H3 and H4 are also found preferentially associated with the early region during the initial phases of an infection [28]. However, the presence of hyperacetylated H3 and H4 was found to be a dynamic process. Using the ISFIP/ReChIP procedure hyperacetylated H3 and H4 were shown to be preferentially associated with regions of the SV40 minichromosome which also contained RNAPII. In fact no unacetylated H3 or H4 were found in chromatin along with RNAPII. In regions where RNAPII was not present the levels of hyperacetylated H3 and H4 were much lower [11]. Moreover, the histone acetyltransferase p300 was also found closely associated with RNAPII [11]. This led to the suggestion that p300 was an integral component of the RNAPII transcription complex responsible for hyperacetylating H3 and H4 in front of the transcription complex.

During the course of an SV40 infection early transcription is repressed by binding of the product of transcription, T-antigen, to a regulatory sequence found in the early promoter [27]. Thus, the SV40 system can serve as a useful model to study the epigenetic regulation associated with transcriptional repression. The epigenetics of early repression has been extensively studied using a mutant of SV40 which contains a deletion of the T-antigen binding site known as cs1085.

Repression of early transcription is associated with a significant reduction in the fraction of RNAPII bound to the promoter which is reversed in the mutant unable to repress early transcription [29]. Correspondingly repression also results in a significant reduction in the presence of hyperacetylated H3 and H4 located in the early region. Not surprisingly this reduction is reversed in the mutant confirming the direct relationship between the presence of RNAPII and hyperacetylated histones [30].

T-antigen mediated repression of transcription at early times also affects histone methylation. Based upon a comparison between the wild-type and mutant viruses, the only effect of T-antigen mediated repression during active transcription was to inhibit the introduction of H3K9me2. No effects were observed on H3K9me1 or H3K9me3. Methylation of H3K4 was not studied since the levels of H3K4 methylation at this time were very low [16].

Between 12 and 24 h post-infection late transcription and DNA replication both begin with late transcription preceding replication by a few hours [27]. Like early transcription, late transcription is also closely linked to histone hyperacetylation with the hyperacetylated histones preferentially associated with regions of chromatin which contain RNAPII [11, 30]. Also like early transcription, late transcription is dynamic with p300 bound to RNAPII and responsible for histone hyperacetylation [30]. P300 was shown to be required to direct histone hyperacetylation for active late transcription to occur using siRNA knockdown of p300 [30].

Following the initiation of late transcription and DNA replication there is an increase in all forms of methylated H3K4 and H3K9 [18] and an increase in H4K20me1 [17]. Using the ISF/ReChIP technique the relationship between the different forms of modified histones was investigated at the late times [18]. The modified histones were shown to be nonrandomly associated with certain modified histones typically found together. Based upon the association between histone modifications, it was suggested that the SV40 minichromosomes present at this time were heterogeneous and consisted of at least five different epigenomes defined by the presence of specific combinations of modified histones. One epigenome consisted of H3K4me2 with H3K9me1, H3K9me2, H3K9me3, and HH4. A second also contained H3K4me2 but with HH4 and HH3. A third contained H3K4me3 with HH4, while a fourth contained H3K9me1 with some H3K9me2, H4K20me1, and HH4. Finally a fifth epigenome appeared to consist essentially of only H3K9me3. ISF/ReChIP was also used to show that H4K20me1 was present at low levels on transcribing minichromosomes in regions devoid of RNAPII at late times but not early times [17]. None of the methylated forms of H3 were found in association with transcribing minichromosomes at late times (Milavetz, unpublished observations).

The relationship between DNA replication and the introduction of methylated H3 has been directly investigated. Using a novel two step strategy with one antibody bound to agarose beads and a second antibody bound to magnetic beads called DAM chips, H3K9me1 was found to be present in actively replicating minichromosomes that contain the DNA replication protein RPA70 [31]. The role of DNA replication in the introduction of methylation was also investigated using the DNA replication specific inhibitor aphidicolin. Inhibition of replication by aphidicolin completely blocked the introduction of H3K4me1 and H3K9me1 into SV40

minichromosomes, but had little or no effect on the introduction of the di-methylated or tri-methylated forms of H3K4 and H3K9 [16]. This indicated that only H3K4me1 and H3K9me1 were introduced along with active replication, while the di- and tri-methylated forms of H3K4 and H3K9 were being introduced without the necessity of active replication. Presumably other minichromosomes besides the actively replicating minichromosomes were acting as substrates for these modifications.

Repression of early transcription by T-antigen was also investigated at late times in infection using the mutant cs1085 [18]. Surprisingly repression at late times during active replication did not yield the same result as repression at early times when only transcription was occurring described above. During active replication repression resulted in the introduction of H3K9me1 and to a lesser extent H3K4me1 [18]. Instead of replication resulting in the conservation of preexisting chromatin structure, in the SV40 system replication appeared to serve as a switch in which new epigenetic information could be introduced [16, 18].

In the final stage of a lytic infection SV40 minichromosomes are bound by the coat proteins VP1, VP2, and VP3 and a new virion is produced. Comparing the histones modifications present at late times to those present in virions, it is clear that encapsidation is a selective process. There is enrichment for hyperacetylated histones [27] and very little if any methylated H3K4 [18]. The reasons for the selective encapsidation of minichromosomes containing either hyperacetylated H3 and H4 or methylated H3 and hyperacetylated H4 are not known at this time but seem likely to be related to the epigenetic consequences of these modifications.

DNA methylation does not appear to play a role in the epigenetic regulation of SV40 or the other polyoma viruses. The simplest explanation for the lack of DNA methylation regulation comes from an analysis of the DNA sequence of polyomaviruses. CpG which is the typical substrate for methylation in eukaryotic cells is very significantly under-represented in the genomes of the polyomaviruses which have been analyzed including SV40, JC virus, and BK virus with only 27 CpGs present in SV40, 16 present in JC virus, and 12 present in BK virus [32, 33]. The absence of DNA methylation during a lytic infection with BK virus has been confirmed [34].

2.3 Epigenetic Effects on Cellular Expression and Cellular Transformation

There have not been any specific studies investigating the genome-wide epigenetic consequences of an SV40 infection. However, T-antigen is known to interact with modulators known to be involved in cellular epigenetic regulation including p300 [20, 35], CBP [20, 36, 37], HDAC1 [36], HDAC3 [37], and lysine methyltransferase [38] and it is reasonable to expect that dysregulation of these modulators would have genome-wide consequences. Recently it has been shown that T-antigen's interaction with CBP/p300 results in an increase in the activity of the acetylase and a corresponding increase in the levels of acetylation of H3K56 and

H4K12 [35]. This result confirms that T-antigen's interaction with histone acetylases can result in a cellular genome-wide effect on histone acetylation.

SV40 transforms cells through the interaction of its large T-antigen with p53 and Rb and the interaction of its small t antigen with PP2A [20]. As noted above large T-antigen binds to a number of potential epigenetic regulators and would be expected to dysregulate a number of cellular genes through modulation of the levels of the epigenetic regulators. The increase in histone acetylase activity by T-antigen could account at least in part for the increase in gene transcription for many genes typically seen in transformed cells.

2.4 Epigenetics of Other Polyomaviruses

The other polyoma viruses have been much less studied with respect to epigenetic regulation. Nevertheless, one epigenetic process which appears to be consistent across the family of polyomaviruses is histone acetylation. The virions of mouse polyoma virus like SV40 contain hyperacetylated forms of H3 and H4 [39]. In addition, mouse polyoma virus large T antigen interacts with the histone acetyltransferases PCAF and GCN5 suggesting one possible way that the polyoma virus histones become methylated [40]. Polyoma virus small T also has been shown to play a critical role in regulating histone hyperacetylation presumably through control of the lytic infection and dysregulation of the cell cycle [41].

JC virus also appears to be regulated by acetylation. Both early transcription and late transcription appear to be stimulated by inhibitors of histone deacetylase and co-transfection with a vector expressing p300 results in a stimulation in the level of transcription [42]. The other human polyoma viruses Merkel and BK have not been investigated yet with respect to their epigenetic regulation.

2.5 DNA Methylation Effects of Polyomavirus Integration

In non-permissive cells, the polyomaviruses frequently integrate into the host cell genome with resulting dysregulation of both viral and cellular genes and transformation of the cells. Surprisingly there have been only a few reports on the role that DNA methylation plays in this transformation process. SV40 integration in fibroblasts has been shown to result in a marked increase in the level of DNA methyltransferase activity and corresponding increase in the level of methylation of the promoter of alpha2 collagen VI [43]. In human mesothelial cells the integration of SV40 was shown to have minimal effects on methylation in early passage cells but showed a significant increase in methylation on late passage when morphological changes in the cell were also observed. The morphological changes were correlated with an increased methylation of the RASSF-1 gene which is frequently found repressed in mesotheliomas [44]. The RASSF-1 has also been shown to be hypermethylated in Merkel cell carcinoma in conjunction with integration of the Merkel cell virus [45].

3 Adenoviruses

3.1 Overview

The adenoviruses (Ad) are a family of nonenveloped viruses whose genome consists of a small linear double strand of DNA of approximately 30–40 kb in size. Like the polyomaviruses the Ads have a similar lytic life cycle consisting of binding to a cell, transport of the genome to the nucleus, early transcription, replication, late transcription, and generation of new packaged virus. Adenoviruses are associated with human respiratory infections [46]. Within the virion Ad DNA is complexed with three virus coded polypeptides, V, VII and Mu [47–51]. Protein VII a highly basic protein similar to protamine is thought to condense the Ad DNA [52]. The interaction between Ad DNA and these proteins has been reported to result in a chromatin-like repeating structure [53]. However, since histones are not involved it is unlikely that the complexes in virions will be regulated through the same epigenetic pathways associated with chromatin containing histones.

Because Ad DNA was associated with viral proteins in the virion, there was significant interest in determining the corresponding organization of the DNA in the nucleus of infected cells. The initial reports using available technology of the 1970s and 1980s were conflicting, with some laboratories reporting that intracellular DNA remained bound to the viral protein VII and others reporting that the Ad DNA was associated with histones into nucleosomes [54–58]. More recently the question has been reexamined using ChIP technology with the result that there is general consensus that at least some of the Ad DNA is organized as chromatin during the course of the infection [59–63].

During the initial stages of an infection the Ad genome is thought to be transported to the nucleus in association with VII [64–66]. Within approximately 1 h of infection the Ad DNA becomes associated with cellular histones although there is also evidence that the VII remains associated as well [60]. As noted above there are two major variants of histone H3, a replication dependent variant, H3.1, and a replication independent variant, H3.3. Based upon ChIP analysis it is the replication independent variant H3.3 that is deposited onto the incoming Ad genome during the initiation of infection [62]. Interestingly, when Ad DNA is transfected into cells as an expression vector it also becomes associated with histones very rapidly [61]. The organization of the infecting Ad genome with histones appears to be biologically significant, since siRNA knockdown of the H3.3 chaperone HIRA leads to both a reduction in the introduction of H3.3 and a reduction in expression of early genes [61]. Moreover, acetylation of H3 on early genes increases for the first hours of infection consistent with the general observation that histone acetylation is associated with genes undergoing active transcription at early times [61, 63].

Recently, CTCF has been shown to play a critical role in the replication dependent shift to late transcription [67]. However, the status of the histone modifications on the Ad DNA was not addressed.

At late times in infection there appears to be a shift in the protein complement associated with the newly replicated Ad DNA from histones back to protein VII in order to compact the DNA for virion formation. This has been hypothesized to be due in part to the ability of the virus at late times to inhibit cellular histone synthesis [57, 68, 69].

With the exception of the ChIP studies, demonstrating that intracellular Ad DNA is organized as chromatin and can be acetylated described above, there has not been an extensive characterization of the role of histone modifications in the regulation of the genes of Ad. However, it seems that this is an area that is likely to see much growth since like the polyomaviruses the Ad viral life cycle is very likely to be regulated through epigenetic mechanisms.

3.2 Epigenetic Effects of Adenoviruses on Cellular Expression and Cellular Transformation

One hallmark of infection by Ad is the relatively rapid dysregulation of cellular gene expression. Within 24 h of infection the expression of most cellular genes is repressed while a few genes required for cellular replication are induced [46]. Since such an extensive change in expression was likely to be associated with corresponding changes in epigenetic regulation, the organization of certain histone modifications and transcription cofactors within infected cells has been analyzed by ChIP-chip and ChIP-seq strategies [25, 63]. In these genome-wide studies acetylation of H3K9 and H3K18 was followed along with certain transcription factors known to be affected by the ϵ 1a protein of Ad during the course of infection including retinoblastoma (Rb) family proteins and EP300 histone acetyltransferase. Consistent with the dysregulation of expression, many of the genes observed to be repressed during infection demonstrated a significant reduction in the levels of acetylated H3K18 with less effect on acetylation of H3K9. Moreover, the replication associated genes which were induced became associated with acetylated H3K18 following infection. Interestingly, the acetylated H3K18 was found more or less throughout the genes induced for expression [63].

3.3 Epigenetic Consequences of Integration of Ad

Ad causes tumors in appropriate hosts in part by integrating its genome into the genome of the host cell [46]. Insertion of a foreign DNA into the cell genome might be expected to have significant epigenetic consequences and for this reason the effects of integration on the extent of DNA methylation in the Ad and the cellular DNA has been investigated. Ad DNA in virions and in the nucleus of a lytically infected cell is not methylated [70, 71].

The effects of Ad integration on DNA methylation of the integrated Ad DNA, proximal cellular DNA, and distal cellular DNA have all been analyzed using bisulfate sequencing. Not unexpectedly,

the Ad DNA is organized like the rest of the cellular DNA in typical chromatin structure [72]. Within the integrated Ad DNA there tends to be significant methylation of most of the Ad DNA with the exception of parts of the early region [72, 73]. The regions of Ad DNA which are methylated are also characterized by the presence of hypoacetylated H3 and H4 in the chromatin associated with the DNA.

Integration of Ad DNA also has effects on the methylation of cellular DNA located adjacent to the integration site. In comparison to the methylation patterns present in the DNA of cells prior to integration of Ad, there was a significant reduction in the levels of methylated DNA adjacent to the Ad DNA at the site of integration [74, 75].

Finally, integration of Ad DNA has effects on cellular DNA at sites distal to the site of integration [76]. For example, within a region of DNA known as the intracisternal A particle retrotransposon there was a significant increase in DNA methylation following integration of Ad DNA. However, it should be noted that changes in DNA methylation were also observed when non-transforming DNAs were integrated into cellular DNA so that the latter effect may be more a result of a general phenomenon associated with integration of foreign DNA than one specific to Ad DNA [77]. What is most interesting from the epigenetic perspective is the observation that the changes in DNA methylation in the cellular chromatin persist even after the integrated Ad is lost. Revertants which had lost the integrated Ad DNA continued to maintain the DNA methylation patterns which resulted from the original introduction of the Ad DNA [76].

4 Human Papillomavirus

4.1 Overview

The human papillomaviruses (HPVs) are a family of nonenveloped viruses whose genome consists of approximately 8 kb of double strand closed circular DNA [46]. The family is quite large with a number of members capable of causing human disease from warts to cancer. The high-risk papillomaviruses are now thought to be the causative agent for most of the cervical cancers in the world, many anogenital cancers, and head and neck cancers. The life cycle of papillomaviruses is very similar to the other small tumor viruses with one major exception. The lytic cycle is tied very closely to the differentiation status of the infected cell. The virus infects the basal layer of epithelium where it expresses its early genes and undergoes replication. As the cell differentiates the virus switches to late genes and new virions are generated in the terminally differentiated cells. This complex lytic cycle has made the epigenetic characterization of the virus challenging.

4.2 Epigenetic Regulation of a Papillomavirus Lytic Infection

There have been two reports concerning the role of DNA methylation in the epigenetic regulation of a lytic HPV16 infection [78, 79]. Both of these reports have focused on methylation within the long control region (LCR) which regulates transcription of early genes in non-integrated episomal HPV. In the former study using bisulfite treatment to distinguish methylated from unmethylated DNA, the LCR was found to go from a methylated to an unmethylated state during the course of cell differentiation and corresponding virus production. The latter study used laser microdissection to isolate cells at various stages of differentiation including transformed cells, and tested for the presence of methylated HPV16 in the various cells using bisulfite treatment and sequencing. This study also demonstrated the shift from methylated to unmethylated in the LCR. But this study also showed that one specific region of the LCR, the E2 viral protein binding site became hypermethylated during this transition, despite the overall reduction in methylation. The status of histone modifications in HPV 31 during a lytic infection has also been addressed. Hyperacetylated H3 and H4 have been found in both the early and late regions prior to and following differentiation of the host cells [80]. Because significant differences were observed in this study for transcription factor binding, it was concluded that the factor binding was likely playing a larger role in control of gene expression than the acetylation of H3 and H4.

4.3 Epigenetic Consequences of HPV Integration

HPV causes cancer usually through the integration of one or more copies of the virus into the host cell genome and subsequent dysregulation of viral and cellular gene expression. Because the change from a lytic infection to cancer goes through a series of pathologically defined stages it is possible to analyze the different stages of the disease progression for specific forms of DNA methylation in the virus genome. A number of laboratories have studied the role of DNA methylation within the viral genome and the cellular genome following the establishment of a neoplastic transformation (reviewed in ref. 81). Almost all of these studies have relied on bisulfite treatment followed by PCR, cloning, and sequencing. There appears to be consensus that disease progression and deteriorating prognosis is associated with increased methylation of regions of the late structural proteins [81]. For example, in a set of samples taken from women with increasing severity of disease there was increased methylation of L1, L2, and the regulatory protein E2 [82]. Analysis of the LCR for methylation has led to less consistent results. In some cases the LCR appeared to be hypermethylated while in other studies the LCR was hypomethylated, for example [83, 84].

The effect of HPV integration on methylation of host genome genes has also been analyzed [85, 86]. Using either laser capture dissection of HPV positive and negative head and neck cancer

samples in conjunction with either arrays or MeDip-seq technology, 453 differentially methylated regions were identified many of which were located in gene regulatory regions. Of potentially greatest significance was the dysregulation by methylation seen in the cell adhesion genes for cadherin.

While no studies to date have specifically focused on the effect of HPV integration on changes in histone modifications during disease progression, ChIP-seq data generated in other studies using HeLa cells which are transformed with HPV18 have been reanalyzed to identify the histone modifications associated with the viral genome [81]. Histone modifications generally thought to be associated with gene activation including H3K4me1, H3K4me2, H3K4me3, H3K9ac, H3K27ac, H3K36me3, and H3K79me2 were all associated with the HPV18 LCR. In addition, a number of transcription binding factors appeared to be present within the LCR. Taken together these results suggest that the integrated copies of HPV18 in HeLa cells are transcriptionally active [81].

5 Herpesviruses

5.1 Overview

The herpesviruses are a family of enveloped viruses characterized by a double strand of linear DNA between approximately 150 and 250 kb in size. The viral genome is organized as a series of genes with terminal repeats at each of the ends of the linear DNA. Following infection the terminal repeats serve to circularize the genome and allow the virus to exist in the nucleus of an infected cell as a circular episome. Interestingly, the viral DNA in the infecting virion is not chromatinized but upon episome formation becomes chromatinized like cellular chromatin. The episome can either exist as a fully functional virus capable of replication and the generation of daughter virus, or the episome can exist in a latent form in which only a limited number of viral control elements coming from the early regions of the virus are produced with no replication or subsequent daughter virus production. Unlike the other viruses discussed which integrate as part of the transformation of normal cells into cancer cells, the cancer causing herpesviruses transform as a consequence of the latent infection. Because cells can be either lytically infected or latently infected with subsequent reactivation of the virus, there has been a great deal of interest in investigating the role that epigenetics plays in control of these biological processes.

Importantly, two members of the herpesvirus family, Epstein-Barr virus (EBV) and Kaposi's sarcoma herpes virus (KSHV) have been shown to be at least partially responsible for human cancers. EBV appears to be the causative agent for Burkitt's lymphoma and is associated with other lymphoproliferative diseases such as Hodgkin's lymphoma and nasopharyngeal carcinoma (reviewed in ref. 87, 88). KSHV appears to be the causative agent for Kaposi's sarcoma and is linked to other B cell lymphomas [89, 90].

5.2 Epigenetic Regulation of an EBV Infection

The role of epigenetic modification in the regulation of EBV latency has recently been analyzed using a newly developed software program and existing high-throughput ChIP-derived sequencing data from many laboratories [91]. This extensive analysis has focused on histone modifications, location of transcription factors and cofactors, nucleosome locations, and DNA methylation yielding a fairly complete picture of the organization of epigenetic regulators during EBV latency. During EBV latency 9 proteins, 21 microRNAs, and several noncoding RNAs are expressed which are associated with B-cell proliferation and survival. The proteins involved in lytic replication and subsequent virion formation are repressed.

Not surprisingly, RNAP II and RNAPIII, responsible for transcription of many of the latency protein and RNA genes, are located at the appropriate sites in the EBV genome along with H3K4me1, me2 and me3. Similarly, acetylated H3K9 and H3K27 are also located at these genes consistent with transcription. EBV does not appear to contain any regions with bivalent histone modifications as observed in KHSV (*see below*) [91] or specific regions enriched with the potential repressive mark H3K9me3. Significantly, certain latency genes containing RNA polymerase also contain a number of transcription cofactors including TAFs, GCN5, and P300 with potential epigenetic consequences [91].

DNA methylation is not found in the EBV genome derived from virions but is present in EBV episomes during latent infection. DNA methylation is found throughout the genome with the exception of certain of the latency genes [92, 93].

5.3 Epigenetic Consequences of EBV on the Cell

Because of the well-known ability of EBV to transform cells during a latent infection, there has been a great deal of interest in determining how the virus redirects the normal cellular expression patterns to that of a transformed cell. Increasingly epigenetics has been implicated in this process. A number of genome-wide and gene specific studies have investigated the binding partners of the latency proteins [94, 95], changes in gene expression [96, 97], changes in histone modifications [96, 98, 99], and changes in DNA methylation [97, 100, 101]. Latent infection by EBV appears to cause a significant increase in transcription of a number of cellular genes. This increase is associated with an overall reduction in the levels of putative repressive histone modifications including H3K9me3 and H3K27me3 as well as H3K4me3 [98]. Transformation is also associated with hypomethylation of the activated cellular genes [96, 97, 100] and hypermethylation of certain tumor suppressor genes [101]. These changes in gene methylation are consistent with the changes in the levels of gene expression. While a number of questions remain unanswered concerning the specific mechanisms by which EBV directs these epigenetic modifications the picture is becoming clearer how the changes resulting from infection can be maintained epigenetically even when the virus itself is lost from the infected cell.

5.4 Epigenetic Regulation of a KHSV Infection

The role that epigenetics plays in a KHSV infection has been recently reviewed [102]. Since this review is quite extensive, in this section we will focus only on some of the major aspects of epigenetic regulation. The role of histone modification in KHSV infection has been assessed in two cell lines using a ChIP-on chip strategy [102, 103] focusing on acetylated H3K9/14, H3K4me3, H3K9me3, and H3K27me3. In general the early genes responsible for establishing latency have H3K4me3 and acetylated H3K9/14 suggesting transcription while the silent genes have H3K9me3 and H3K27me3 suggesting repression. The genes associated with reactivation tend to contain bivalent epigenetic marks including H3K4me3 and H3K27me3.

Not surprisingly, acetylated H3K9/14 was found to be enriched on certain genes during latency and to be increased at these genes upon reactivation based upon ChIP-chip analyses [102, 103]. These results are consistent with a low level of transcription during latency and increased transcription during reactivation. When latently infected cells are treated with HDAC inhibitors reactivation of the virus is typically observed presumably as a consequence of the increased transcription at these genes. Two specific latency associated genes have been studied with respect to the role of histone acetylation during reactivation. The RTA promoter was shown to be hypoacetylated and associated with HDAC1, 5, and 7 during latency, while upon reactivation the HDACs are lost and acetylation occurs [104]. Along with the change in acetylation, a disruption of a nucleosome within the promoter was also observed. Histone acetylation was also observed in the origin of latent replication region of the genome where recruitment of DNA replication factors during latency occurs [105].

Interestingly the presence of H3K9me3 was inversely correlated with the presence of JMJD2A and KDM3A/JMJD1A the demethylases responsible for removal of methyl groups from H3K9 [106]. Instead, the demethylases were co-located at sites containing H3K4me3 and acetylated H3. This observation suggested that the role of the demethylases might be to prevent the introduction of H3K9me3 at locations which would be subsequently involved in reactivation [106]. LANA which is involved in reactivation was shown to collocate with JMJD1A at sites depleted for H3K9me2 [107]. Together these results suggest that histone demethylases may be directed to critical gene locations to prevent the introduction of H3K9 potentially repressive histone modifications.

H3K4me3 also appears to play a critical role in the regulation of KHSV gene expression. H3K4me3 is located on critical genes during latency and the amount of H3K4me3 present increases during reactivation [102, 103].

DNA methylation has also been implicated in the regulation of latency. First, treatment of cells latently infected with methylation inhibitors results in reactivation of virus [108]. Second, MeDIP

analysis indicates that DNA methylation occurs extensively throughout the KHSV genome during latency with the exception of the latency-associated locus [103]. However, the DNA methylation was found to occur slowly during the establishment of a latent infection unlike the changes described above for histone modifications which occurred rapidly [103]. The slower time course for the introduction of DNA methylation compared to histone modifications suggests that methylation is unlikely to be involved in establishing the latent chromatin structures but instead serve to ensure that when established that they are maintained.

5.5 Epigenetic Consequences of KHSV on the Cell

KHSV is also capable of reprogramming the epigenetics of cellular genes. LANA an early KHSV gene product associated with latency of the viral episome [109] has been implicated in modulating the expression of certain master transcriptional factors [110]. LANA is also capable of interacting with a large number of cellular chromatin modifiers including the histone acetyltransferase CBP [111]. Consistent with this capability LANA has been found bound to a number of sites in cellular chromatin using ChIP-seq [112].

6 Conclusions

Since the development of simple and rapid procedures like ChIP, MeDIP, and deep sequencing to analyze chromatin for nucleosome and binding factor location, histone modifications, and DNA modifications, there has been a huge increase in our understanding of the relationship between chromatin structure and the life cycle of tumor causing DNA viruses. Nevertheless, the field remains at a very early stage of development. Most of the published studies to date are primarily associative. That is, epigenetic changes are correlated to biological events such as the regulation of specific gene expression. Because of the emphasis on correlations at this time, there have been conflicting results where a particular epigenetic mark results in different biological consequences depending upon the system being studied. These conflicts are likely to be resolved in the next few years as the focus of research shifts to the mechanisms which signal the introduction of epigenetic marks and the reading of existing marks.

In addition to the new emphasis on mechanisms defining epigenetic regulation, there are a number of specific questions that are likely to be answered in the coming years. For those viruses like adenovirus and herpesvirus which become chromatinized upon infection, it is still not known how chromatin is formed and how the newly formed chromatin influences the initiation of appropriate early transcription. Similarly, for the viruses that exist as chromatin throughout their life cycle like the polyomaviruses and HPVs it is not clear how selective activation for early transcription occurs in the context of infecting chromatin. Does activation occur on a

particular epigenome or can any infecting epigenome regardless of the epigenetic marks which it carries serve as a substrate for the initiation of transcription? Later in infection, when multiple genes may be transcribed along with replication and encapsidation, how does epigenetics contribute to the control of the various pool sizes of the functional chromosomes? In SV40 we find that the pool of chromosomes containing RNAPII is fairly constant for a given set of conditions. How is this regulated? Finally, the role of DNA replication in epigenetic regulation needs to be further characterized. To what extent does replication play a role in conserving existing epigenetic marks compared to our demonstration that replication can serve to switch epigenetic marks? As these questions are answered it is likely that a much better understanding of the role that epigenetics plays in the regulation of biological function will become evident.

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Chapter 31

P53 Tumor Suppression Network in Cancer Epigenetics

Alok Mishra, Daniel J. Brat, and Mukesh Verma

Abstract

The tumor suppressor p53 is one of the most complex and widely studied genes in cancer biology. In spite of the vast on literature the transcriptional regulation of p53, aspects of its especially epigenetic regulation are not completely understood. This chapter presents a concise overview of p53-related epigenetic events involved in oncogenesis and tumor suppression. We limit the scope to epigenetic modifications of the p53 promoter per se as well as its well-established downstream targets. The indirect role of p53 affecting the epigenetic machinery of cancer cells via specific proteins and transcription factors is discussed. Current concepts of p53-related cancer epigenetics offer myriad avenues for cancer therapies. Challenges in the field are also discussed.

Key words p53, Acetylation, Cancer, Chromatin, Epigenetic, ChIP, Histones, Methylation, Micro RNA

Abbreviations

ChIP	Chromatin immunoprecipitation
HATs	Histone acetyl transferases
HMTs	Histone methyl transferases
HDACs	Histone deacetylase
HDMs	Histone demethylases
EMSA	Electromobility Shift assays
TRANSFAC	Transcription factor database 7.0
ENCODE	Encyclopedia of DNA elements

1 Introduction

P53 is involved in cell cycle regulation and functions as a tumor suppressor. In the late 1970s, several groups independently discovered the now-famous p53—a 53KD phosphor-protein that was co-immunoprecipitated with viral SV40 large T-antigen [1]. It is noteworthy that until the next decade, p53 was interpreted as an oncoprotein because of its viral homolog and overexpression pattern

Epigenetics in p53 Tumor Suppression Network

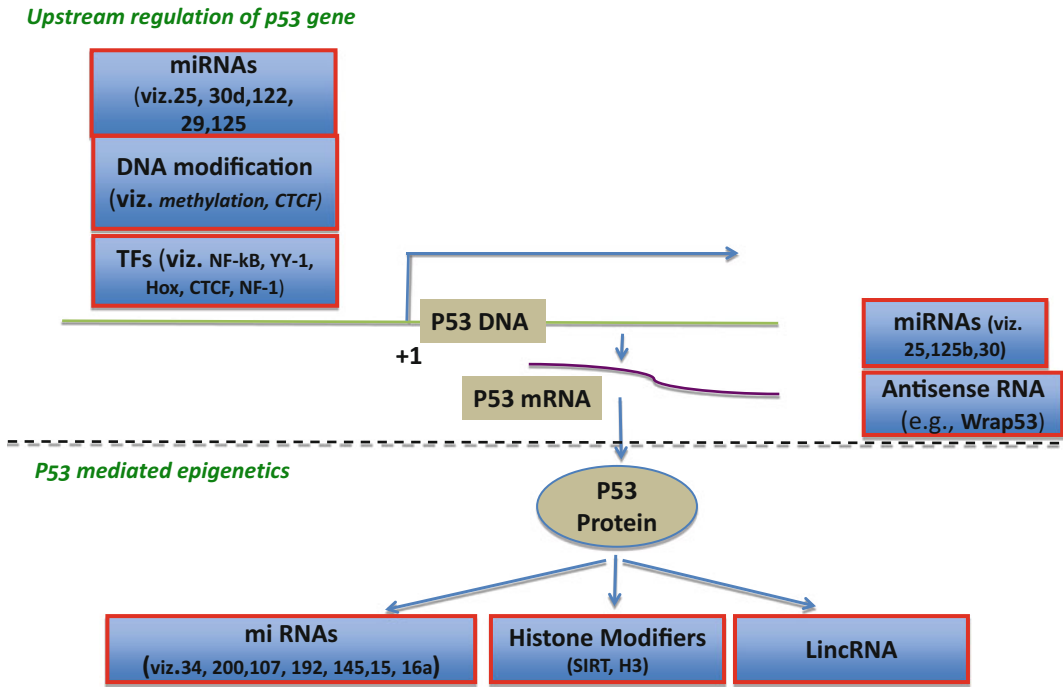


Fig. 1 Integrated signaling cascades of p53 axis implicated in epigenetic regulation during tumor suppression in humans. *TF* transcription factor (described in text). +1 denotes transcriptional start site of p53 gene

in many human tumors. Later on, based on Knudson's Two-hit hypothesis, DNA sequencing, knock out/in and epidemiological studies, the tumor suppressor role of p53 was established and appreciated. The International Agency of Research on Cancer (IARC) database shows that more than 25,000 mutations are present in p53 gene (<http://p53.iarc.fr>) and these are found in the majority of tumors. We will discuss the upstream and downstream epigenetic phenomena affecting the p53 gene in cancer-related phenotypes in the following sections. A schematic diagram is presented in Fig. 1.

2 The Epigenetic Regulation of p53 Gene Promoter: Upstream Regulation

Epigenetic genome-wide association studies (EWAS) of US National Institutes of Health (NIH) under the Roadmap Epigenomics Program cover several diseases which are regulated by epigenetic mechanism (<http://commonfund.nih.gov/epigenomics/index.asp>). The US-NIH Roadmap Consortium is a coordinated effort to make the global scale Epigenome data available in the public domain [2]. As any other promoter DNA regulatory element, chromatin of p53 gene, consisting of DNA and protein components, is an extremely

dynamic structure. Promoter modifications are orchestrated by specific mechanisms in the spatial, temporal, and contextual manner leading to p53 gene regulation.

Although discovery of mdm2-p53 auto-regulatory loop is the most important mechanism of p53 gene regulation, restructured promoter of p53 gene is also becoming rapidly recognized as one of the critical modes of p53 gene regulation [3, 4]. So far very few reports explain the details of epigenetic regulation of p53 gene in relation to oncogenesis.

2.1 Promoter Methylation

Epigenetics refers to heritable and modifiable markers that regulate gene expression without changing the underlying DNA sequence. Epigenetic regulatory complex consists mainly of four components: DNA methylation (primarily in the promoter region), histone modification, altered microRNA (miRNA) expression, and chromatin condensation and relaxation. Methylation of promoter regions at the CpG island is the main epigenetic regulation.

Aberrant methylation spectrum of the p53 gene promoters has been examined in many cancers viz. gliomas [5], breast cancers [6], adrenal cancer [7], head and neck cancer [8] including hematological malignancies—ALL [9], and CML [10]. In many glioma cell lines, transcriptional insulator element binding protein—CTCF—distantly dictates the methylation pattern of promoter region of p53 gene [11]. Interestingly, by synthetic DNA reporter construct studies, even before the discovery of endogenous hypermethylated p53 promoter, evidence of promoter methylation-specific transcriptional suppression of p53 gene were reported from the laboratories of Mass and James [12, 13].

2.2 Regulatory RNAs on p53 Promoter

MicroRNAs (miRNAs) are one of the four components of epigenetic regulation. These small molecules can be utilized for disease screening and risk assessment and may serve as the potential targets for intervention and therapy. Several studies have shown that miRNA-25 and miRNA-30d were able to repress p53 transcription [14, 15]. miRNA-125b and miRNA-504 regulate p53 by directly binding in its untranslated region (UTR) and further modulating downstream cellular process such as apoptosis [16]. However, some of the miRNA-generated signals also regulate the signaling pathways that affect the p53 activity via upstream mediators such as SIRT1, mdm-2, and AKT proteins. These are indirectly regulated by miRNA-34a, miRNA-122, and miRNA-29 respectively [17]. Harris and Ryan have reviewed extensively on the role of genetic variation in miRNA sequences implicated in carcinogenesis [18]. In ES cells, p53 gene is regulated by miRNA-125a and miRNA-125b for the regulation of stemness [19]. The *in silico* computational approaches (such as TRANSFAC®7.0 and ENCODE) and wet bench experimental analysis together for human p53 promoter have indicated that many important nuclear transcription factors

such as YY-1 [20], NF- κ B, YY-1, Pax, and E2F [21] are also recruited in modular fashion. Thus, remodeling of p53 promoter chromatin can be easily speculated through occupancy of several transcription factors.

Surprisingly, Farnebo's group has reported a natural rare antisense transcript of p53, designated Wrap53, which regulates endogenous p53 mRNA levels by generating head on-RNA duplex and targeting the 5' UTR of p53 mRNA [22]. Thus conventional ChIP, EMSA, enrichment of transcriptional machinery proteins (RNA PolII), methylated-acetylated base residues identity, and single nucleosome resolution based ChIP-seq databases have presented a wired snapshot of promoter-based regulation of p53 gene by combinatorial participation of methylation, miRNAs, antisense, and TFs.

3 The Epigenetic Regulation by p53: Downstream Regulations

The chromatin binding of p53 gene was mapped by genome wide using tiling microarray/chromatin immunoprecipitation paired end tag (ChIP-PET). Chromosome Conformation Capture Carbon Cop (5C) and ChIA-PET assays also confirmed that p53 binds to promoters and also within gene bodies. Among target genes, many are for noncoding RNAs including microRNAs (miRNAs) and large intergenic noncoding RNAs (lincRNAs) [23]. As evident from ENCODE and GENCODE databases one can understand that p53 regulated miRNA are growing in number.

The completely different nature of the promoter occupancy spectrum of p53 in primary, cancer, and fibroblast cells together with modified epigenetic markers due to multiple passaging numbers have been some of the known major complexities and road blocks in exploring the discreet landscape of p53-dictated epigenetic phenomena [24]. It is worth noting that aberrant p53 signaling does not necessarily imply loss or mutation of the p53 gene. For example, around 90 % glioblastoma (GBM) cases have aberrant p53 signaling pathways whereas less than 50 % harbor mutant p53. Different phenotypic manifestation of p53 mutation-related events confirmed that cell type-specific p53 signals might be a manifestation of epigenetic regulation.

3.1 p53-Mediated Chromatin Remodeling and Epigenetic Alterations During Oncogenesis

One of the most exciting areas of p53 biology is how p53 affects chromatin remodeling of its downstream targets. It is difficult to elucidate the heterogeneous landscape of specific types of epigenetic modifications and their intra-residue cross talk during p53-mediated restructuring of chromatin. This is emphasized here that p53-mediated chromatin remodeling is inducible as well as repressive in nature as per the physiological context. Histone

Acetyl Transferase/Histone Deacetylase (HAT/HDAC) enzymes target p53 for acetylation/deacetylation, representing a seminal observation confirming p53 as an important chromatin remodeler [25]. Histone acetylation of p21 promoter is mediated by p300:p53 [26]. P53 can also displace HDAC1 from the p21 promoter [27]. SWI/SNF complex—a major chromatin remodeling complex protein—also interacts with tumor suppressor p53 protein for the activation of p53-mediated transcription [28]. Rubbi and Milner have shown the role of p53 in Nuclear Excision Repair (NER). In vitro studies of many tumor cell lines have also shown direct interaction of p53 with basic transcriptional machinery subunit TFIIH while regulating transcriptional targets. P53 protein also facilitates phosphorylation of Serine-10 of Histone 3 (H3S10). P53-dependent acetylation of Lys-9 of histone H3 (H3K9) induces global chromatin accession for several interactors [29]. UV-mediated H3 acetylation profiling has clearly shown the role of p53 in epigenetic regulation with its target, as demonstrated by time and dose of UV exposure. Pint, a long noncoding RNA long intergenic noncoding RNAs (lincRNAs), is transcriptionally regulated by p53 [30]. Altogether, these findings establish that p53 plays a critical functional role in executing epigenetic programming of cancer cells.

3.2 miRNAs Regulated by p53 in Cancers

p53 is a major regulator of miRNA-107, miRNA-145, miRNA-34, miRNA15a/16-1, miRNA-194, miRNA-195, and miRNA-215, which, in turn, regulate cell cycle and proliferation via myc, cyclins, E2F, etc. in different cancers [17, 31]. At the same time, miRNA-34 and miRNA-200 are direct targets of p53 in the cascade of EMT pathways directed by SNAIL/SLUG. The p53 inducible *mir-200c/141* and *mir-200a/200b/429* antagonize EMT programs by negatively regulating the EMT-inducing transcription factors ZEBs [32–34]. The three important regulatory miRNAs, miRNA-34, miRNA-145, and miRNA-200, regulate stemness in induced pluripotent cells (iPSC), hematopoietic stem cells, as well as in cancer stem cells. miRNA-192 and miRNA-194 are known to indirectly regulate p53 activity by modulating mdm-2 levels. miRNA-145 is involved in prostate cancers and suppresses many Yamanaka factors—SOX, OCT, KLF-4 [35]. P53 also activates miRNA34 and subsequently oncometabolic switches involved in the Warburg effect and HIF pathways [36]. At the same time, p53 mediates CD44 regulation by repressing miRNA-34a. p53 inducible miRNA-107 is related to DICER dysregulation during metastatic progression. Mir-15a and mir-16-1 deficient mice lead to the development of chronic lymphocytic leukemia (CLL) and dysregulated cell cycle entry [37].

4 P53 in Cancer Stem Cell Epigenetics

In 2009, five articles were published back-to-back in nature emphasizing that p53—a tumor suppressor—does operate as an indispensable negative regulator for reprogramming efficiency towards induction of pluripotency [38–42]. These publications introduced the p53 protein as a new and central player in the field of stemness and differentiation. Interestingly, it is also observed that depletion of p53 leads to acquisition of bipolar epigenetic machinery in fibroblasts that promotes stemness [43]. In humans, p53-mediated induction of miR-34a and miR-145 represses the stemness-associated transcription factors Oct4, Sox2, Lin28a, and Klf4 [44]. In the context of mouse somatic cell reprogramming, p53 also induces the expression of miR-34a, b, and c and eventually downregulates Sox2, Nanog, and nMyc [45]. According to one of the most accepted epigenetic progenitor models of oncogenesis by Andrew Weinberg and colleagues, epigenetic changes are the earliest genetic events that arise in the cancer stem cell population [46].

5 Epigenetics in Cancer Therapeutics

Epigenetic inhibitors are capable of reversing the alterations caused by specific mechanisms and these agents are capable of reactivating the tumor suppressor genes. Great potential lies in the development of “epigenetic therapies”—several inhibitors of enzymes controlling epigenetic modifications, specifically DNA methyltransferases and histone deacetylases, have already shown promising anti-tumorigenic effects. According to the American Association of Cancer Research (AACR), the Dream Team Project amounting \$ 9.1M, under the leadership of Drs. Baylin and Jones, is a collaborative task to bring the promise of epigenetic therapy to clinical practice. In general, in this therapeutic regimen, a combination of inhibitors of DNA methylating agents and HDAC is used simultaneously at very low dose. Breast, Colon, lung, and leukemia are some of the cancers being investigated by the Dream Team.

The most common p53-based therapies rely on either disruption of p53:mdm interaction or rescuing the wild-type p53 gene in cancer cells [47]. Currently two drugs—Nutlin and JNJ-26854165—are under clinical investigation for improving the status of wild-type p53 expression [48]. High-throughput small-molecule libraries screening and cell-based assays of several compounds have fished out compounds that can restore p53 activity in cells harboring mutant proteins. The earliest reported compounds to reactivate mutant p53 were CP-3139822 and SCH529074. The p53 agonist nutlin in combination with VPA has

demonstrated a synergistic induction of p53-dependent apoptosis in AML. Although the mode of actions of these drugs has not focused on epigenetic mechanisms as such and exploited the drug-protein-protein interactions, it is tempting to speculate that these chemical compounds also act as epigenetic modifiers via p53. Needless to say, the redundancy in structure and function among the p53 superfamily members, such as p63 and p73, is one of the main considerations for failure of p53-based therapies [49].

6 Conclusion and Future Perspective

Myriad upstream and downstream epigenetic signals are involved in p53-related oncogenic events in solid cancers and hematopoietic malignancies. Current understanding of the epigenetic regulation of p53 gene promoter is limited due to its complexity. At the same, time, the knowledge of p53 targets, which are regulated directly or indirectly by p53 protein and ultimately execute the program of the cancer epigenetic network, is growing rapidly. In purview of the available data, we are tempted to speculate that epigenetic therapies of cancer are indirectly related with p53 pathways. Even after making significant progress in the last four decades, because of the multiple isoforms of p53, multiple nodes of regulation, redundancy in family members' function, and several upstream and downstream regulators for transcription—the universal epigenetic network is complex and in need of further research. Depending on the specific gene, transcriptional restoration or upregulation may prevent the development of cancer, halt disease progression, or delay the appearance of metastases. Well-designed clinical trials are therefore needed to explore this therapeutic strategy.

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Promoter Hypermethylation as a Biomarker in Prostate Adenocarcinoma

Jong Y. Park

Abstract

There is increasing evidence that DNA methylation is a critical source of gene regulation. In addition, interindividual differences in DNA methylation status in cancer related genes are associated with risk and progression of prostate cancer. Therefore, DNA methylation in cancer related genes can be potential biomarkers and therapeutic targets for prostate cancer.

In this chapter, current information on frequently hypermethylated genes associated with the carcinogenesis and progression of prostate cancer was updated. The potential biological role of hypermethylated genes in prostate cancer is discussed. These findings may provide new information of the pathogenesis, the exciting potential to be predictive and to provide strategies for personalized treatment of prostate cancer.

Key words Cancer, Epigenetics, Gene regulation, DNA methylation, Risk assessment, Personalized treatment, Prostate cancer, Epigenetic variation, Biomarker

1 Introduction

Prostate cancer is the most common type of cancer and the second leading cause of cancer mortality in the US men [1]. In 2013, it is estimated that 238,590 new cases will be diagnosed in the USA, and 29,720 men will die from the disease [1]. The low mortality rate and gradual decrease of incidence rates between 2000 and 2009 suggest that public awareness of early detection and advanced treatments of prostate cancer has begun to affect prostate cancer outcomes. However, the aging of the current population means that the disease will become an even greater public health problem in the future.

Carcinogenesis and progression of prostate cancer are often results of the accumulation of genetic and epigenetic alterations. Most genetic changes are involved in the inactivation of tumor suppressor genes. However, DNA methylation in a promoter region is often associated with downregulation of expression of these genes. DNA methylation in tumor suppressor genes appears

to be an early event of carcinogenesis and occurs with various frequencies. Therefore, DNA methylation alternations have the potential to be a new generation of biomarkers.

CpG islands are CpG-rich areas of 200 bp to several kb in length, usually located near the promoters of highly expressed genes, and are the sites of common methylation in human tumors [2, 3], including the prostate. A common molecular feature associated with tumorigenesis is hypermethylation of cytosines 5' to guanosines (CpG) within the regulatory (promoter) region of suppressor gene genomic DNA [3–7]. 5-methyl cytosine is unstable and mutates to thymine and methylated CpG sites degrade to TpG/CpA. In tumors, many CpG islands exhibit aberrant hypermethylation resulting in gene silencing. Many tumor suppressor genes, which are involved in tumorigenesis and progression, are downregulated by hypermethylation in the promoter region.

It is firmly established that an increase of methylation across the promoter region affects transcription of genes. However, the extent of methylation and exact CpG site(s) in the CpG islands required for gene silencing are not completely established yet except handful genes in prostate cancer [8–17]. However, recent ENCODE project provided information on impact of altered DNA methylation in promoter region based on over 100 cancer cell lines [3, 18].

The majority of previous publications in epigenetic research in prostate cancer focused on DNA hypermethylation with candidate gene approach. Indeed, a downregulation by DNA hypermethylation in the promoter region is a more common event than by DNA mutations in carcinogenesis. Numerous studies on various hypermethylated genes in different cancers suggest that this is a key part of the carcinogenesis and progression of cancer.

With recent advanced technology and bioinformatic, especially next generation sequencing, over 100 genes have been identified hypermethylated genes as a potential biomarkers in prostate tumor. The functions of these candidate genes were not established yet. Most genes can be categorized into tumor suppressor, tumor cell invasion/metastasis, metabolism, and DNA repair pathways. Defected function of these genes by promoter hypermethylation can contribute to carcinogenesis and progression of prostate cancer. Ten hypermethylated genes, which are most frequently investigated in prostate cancers, are discussed (Table 1).

2 Adenomatous Polyposis Coli (APC)

Metastasis is an extremely complicated process, which occurs through a series of sequential steps that include the invasion, transport, adhesion at a distant site, and outgrowth into a secondary organ. Although metastases are the cause of 90 % of human cancer mortality, little is known about the genetic and biochemical

Table 1**Characteristics of methylation studies and frequencies of methylated genes in prostate tumor and biosamples**

Gene/location	Country	Year	Sample	Method	Frequency	References
APC/5p21	USA	2002	Tissue	MSP	27 % (27/101)	[29]
	USA	2004	Tissue	QMSP	79 % (48/61)	[28]
	Korea	2004	Tissue	MSP	57 % (21/37)	[24]
	USA	2004	Tissue	QMSP	90 % (66/73)	[22]
	Portugal	2004	Tissue	QMSP	100 % (118/118)	[23]
	Germany	2004	Tissue	MSP	78 % (88/113)	[33]
	Germany	2005	Tissue	QMSP	83 % (44/53)	[35]
	USA	2005	Tissue	MSP	64 % (109/170)	[30]
	USA	2005	Urine	QMSP	48 % (25/52)	[45]
	USA	2006	Urine	MSP	17 % (2/12)	[48]
	Portugal	2006	Tissue	QMSP	93 % (28/30)	[36]
	Korea	2007	Tissue	MSP	65 % (117/179)	[25]
	Portugal	2007	Tissue	QMSP	25 % (21/83)	[27]
	Germany	2007	Tissue	QMSP	83 % (65/78)	[37]
	France	2007	Urine	QMSP	51 % (48/95)	[46]
	Germany	2008	Tissue	QMSP	80 % (64/80)	[38]
	France	2008	Blood	QMSP	92 % (39/42)	[49]
	Mixed	2008	Urine	QMSP	43 % (48/111)	[47]
	Italy	2009	Tissue	MSP	41 % (182/447)	[39]
	Korea	2009	Tissue	QMSP	51 % (18/35)	[34]
	Germany	2010	Tissue	QMSP	84 % (21/25)	[40]
	Japan	2010	Blood	QMSP	14 % (11/76)	[50]
	UK	2011	Tissue	PyroSeq	96 % (46/48)	[41]
	Pakistan	2013	Tissue	MSP	58 % (15/27)	[42]
	Germany	2013	Tissue	QMSP	95 % (80/84)	[43]
	USA	2013	Tissue	Nested MSP	22 % (100/453)	[44]
AR/Xq12	Japan	2000	Tissue	MSP	25 % (6/24)	[64]
	USA	2000	Tissue	MS-SNuPE	9 % (2/22)	[62]
	USA	2002	Tissue	MSP	8 % (3/38)	[63]
	Japan	2003	Tissue	MSP	15 % (16/109)	[61]
	Austria	2007	Serum	MSP	39 % (30/76)	[65]
CD44/11p13	USA	1999	Tissue	MS-SNuPE	77 % (34/44)	[72]
	Japan	2001	Tissue	MS-SNuPE	68 % (27/40)	[71]
	USA	2003	Tissue	QMSP	32 % (36/111)	[69]
	USA	2004	Tissue	MSP	72 % (58/81)	[73]
	USA	2004	Tissue	QMSP	33 % (30/90)	[74]
	Korea	2007	Tissue	MSP	22 % (39/179)	[25]
	Portugal	2008	Serum	MSP	0 % (0/18)	[75]
CDH1/16q22.1	USA	2001	Tissue	BiSeq	54 % (19/35)	[77]
	USA	2002	Tissue	MSP	27 % (27/101)	[29]
	USA	2003	Tissue	QMSP	0 % (0/111)	[69]
	USA	2004	Tissue	QMSP	0 % (0/73)	[22]
	Germany	2004	Tissue	MSP	4 % (5/114)	[33]
	USA	2004	Tissue	MSP	61 % (49/81)	[73]
	USA	2004	Tissue	QMSP	24 % (22/90)	[74]

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Table 1
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Gene/location	Country	Year	Sample	Method	Frequency	References
	USA	2005	Urine	QMSP	77 % (40/52)	[45]
	China	2006	Tissue	MSP	30 % (6/20)	[78]
	France	2007	Urine	QMSP	31 % (29/95)	[46]
	Korea	2007	Tissue	MSP	21 % (38/179)	[25]
	France	2008	Blood	QMSP	29 % (12/42)	[49]
	USA	2008	Tissue	MSP	40 % (14/35)	[79]
	UK	2011	Tissue	PyroSeq	33 % (16/48)	[41]
CDKN2A (p16 ^{INK4a})/9p21	USA	1997	Tissue	COBRA	11 % (4/36)	[83]
	Canada	1998	Tissue	COBRA	5 % (1/21)	[82]
	USA	2000	Tissue	MS-SNuPE	73 % (8/11)	[81]
	Japan	2002	Tissue	MSP	69 % (11/16)	[80]
	USA	2002	Tissue	MSP	36 % (36/101)	[29]
	Japan	2002	Tissue	MSP	66 % (21/32)	[84]
	USA	2004	Tissue	QMSP	6 % (4/73)	[22]
	Portugal	2004	Tissue	QMSP	77 % (91/118)	[23]
	Germany	2004	Tissue	MSP	4 % (5/113)	[33]
	USA	2005	Urine	QMSP	37 % (19/52)	[45]
	China	2006	Tissue	MSP	25 % (4/20)	[78]
	Korea	2007	Tissue	MSP	20 % (36/179)	[25]
	France	2007	Urine	QMSP	12 % (11/95)	[46]
	France	2008	Blood	QMSP	14 % (6/42)	[49]
	Japan	2008	Tissue	MSP	15 % (8/53)	[85]
	UK	2011	Tissue	PyroSeq	40 % (19/48)	[41]
	Pakistan	2013	Tissue	MSP	0 % (0/27)	[42]
CDKN2A (p14 ^{ARF})/9p21	USA	2002	Tissue	MSP	36 % (36/101)	[29]
	Japan	2002	Tissue	MSP	3 % (1/32)	[84]
	Portugal	2004	Tissue	QMSP	4 % (5/118)	[23]
	USA	2004	Tissue	QMSP	0 % (0/73)	[22]
	USA	2005	Urine	QMSP	37 % (19/52)	[45]
	France	2007	Urine	QMSP	6 % (6/95)	[46]
	Korea	2007	Tissue	MSP	10 % (17/179)	[25]
	France	2008	Blood	QMSP	14 % (6/42)	[49]
	Japan	2008	Tissue	MSP	4 % (2/53)	[85]
GSTP1/11q13	USA	1994	Tissue	Southern Blot	100 % (20/20)	[95]
	USA	1997	Tissue	RE-PCR	91 % (52/57)	[96]
	Germany	1999	Tissue	RE-PCR	75 % (24/32)	[97]
	USA	2000	Ejaculates	RE-PCR	44 % (4/9)	[120]
	USA	2001	Tissue	QMSP	91 % (63/69)	[98]
	USA	2001	Urine	MSP	79 % (22/28)	[116]
	Germany	2001	Tissue	MSP	90 % (18/20)	[112]
	Germany	2001	Serum	MSP	72 % (23/32)	[112]
	Germany	2001	Ejaculate	MSP	50 % (4/8)	[112]
	Germany	2001	Urine	MSP	76 % (22/29)	[112]
	USA	2001	Tissue	RE-PCR	95 % (40/42)	[100]
	Germany	2002	Biopsy washing	MSP	100 % (10/10)	[114]
	Portugal	2002	Urine	QMSP	19 % (13/69)	[113]
	Portugal	2002	Tissue	QMSP	91 % (63/69)	[113]

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Table 1
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Gene/location	Country	Year	Sample	Method	Frequency	References
	USA	2002	Tissue	MSP	36 % (36/101)	[29]
	Japan	2002	Tissue	MSP	75 % (24/32)	[84]
	China	2002	Tissue	QMSP	100 % (21/21)	[101]
	USA	2003	Urine	MSP	58 % (7/12)	[117]
	USA	2003	Tissue	QMSP	71 % (43/61)	[144]
	Japan	2003	Tissue	MSP	88 % (96/109)	[61]
	USA	2003	Tissue	QMSP	84 % (99/118)	[69]
	Germany	2003	Tissue	MSP	100 % (18/18)	[99]
	USA	2004	Tissue	QMSP	83 % (75/90)	[74]
	USA	2004	Tissue	QMSP	95 % (69/73)	[22]
	Korea	2004	Tissue	MSP	87 % (32/37)	[24]
	Portugal	2004	Tissue	QMSP	95 % (112/118)	[23]
	USA	2004	Tissue	MSP	72 % (58/81)	[73]
	Germany	2004	Tissue	MSP	79 % (89/113)	[33]
	USA	2004	Tissue	QMSP	75 % (46/61)	[28]
	USA	2004	Tissue	QMSP	87 % (83/96)	[102]
	Italy	2004	Tissue	RE-PCR	74 % (31/42)	[103]
	USA	2005	Urine	QMSP	48 % (25/52)	[45]
	USA	2005	Tissue	MSP	54 % (92/170)	[30]
	Germany	2005	Tissue	QMSP	91 % (48/53)	[104]
	USA	2006	Tissue	COMPARE-MS	99 % (129/130)	[105]
	USA	2006	Tissue	QMSP	90 % (56/62)	[106]
	Portugal	2006	Tissue	QMSP	80 % (24/30)	[36]
	USA	2006	Urine	MSP	12 % (3/12)	[48]
	Austria	2007	Serum	MSP	30 % (23/76)	[65]
	Portugal	2007	Tissue	QMSP	25 % (21/83)	[27]
	Korea	2007	Tissue	MSP	73 % (131/179)	[25]
	France	2007	Urine	QMSP	83 % (79/95)	[46]
	Germany	2007	Tissue	QMSP	92 % (24/26)	[107]
	Ireland	2007	Tissue	QMSP	95 % (75/79)	[108]
	Germany	2007	Tissue	QMSP	33 % (26/78)	[37]
	Japan	2008	Tissue	MSP	57 % (30/53)	[85]
	France	2008	Blood	QMSP	95 % (40/42)	[49]
	Germany	2008	Serum	QMSP	42 % (71/168)	[119]
	Portugal	2008	Serum	MSP	28 % (5/18)	[75]
	Germany	2008	Tissue	QMSP	93 % (74/80)	[38]
	Mixed	2008	Urine	QMSP	35 % (39/111)	[47]
	Korea	2009	Tissue	QMSP	63 % (22/35)	[34]
	Italy	2009	Tissue	MSP	69 % (309/448)	[39]
	USA	2009	Tissue	BiSeq	86 % (37/43)	[109]
	Germany	2009	Urine	QMSP	81 % (74/91)	[118]
	German	2009	Plasma	QMSP	39 % (28/91)	[118]
	USA	2009	Tissue	MSP	87 % (27/31)	[110]
	Germany	2010	Tissue	QMSP	84 % (21/25)	[40]
	Japan	2010	Blood	QMSP	29 % (22/76)	[50]
	UK	2011	Tissue	PyroSeq	98 % (47/48)	[41]
	Australia	2011	Tissue	MSH-PCR	88 % (172/195)	[111]
	Germany	2013	Tissue	QMSP	85 % (71/84)	[43]

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Table 1
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Gene/location	Country	Year	Sample	Method	Frequency	References
MGMT/10q26	USA	2002	Tissue	MSP	36 % (36/101)	[29]
	Japan	2002	Tissue	MSP	25 % (8/32)	[84]
	Japan	2003	Tissue	MSP	2 % (2/109)	[61]
	Portugal	2004	Tissue	QMSP	19 % (22/118)	[23]
	Korea	2004	Tissue	MSP	76 % (28/37)	[24]
	USA	2004	Tissue	QMSP	1 % (1/73)	[22]
	USA	2005	Urine	QMSP	19 % (10/52)	[45]
	France	2007	Urine	QMSP	15 % (14/95)	[46]
	France	2008	Blood	QMSP	12 % (5/42)	[49]
	Japan	2008	Tissue	MSP	26 % (14/53)	[85]
	USA	2013	Tissue	Nested MSP	12 % (49/414)	[44]
RARβ/3p24.2	Japan	2001	Tissue	MSP	79 % (11/14)	[127]
	USA	2002	Tissue	MSP	53 % (54/101)	[29]
	Japan	2003	Tissue	MSP	78 % (85/109)	[61]
	Portugal	2004	Tissue	QMSP	98 % (115/118)	[129]
	USA	2004	Tissue	MSP	84 % (44/52)	[128]
	Germany	2004	Tissue	MSP	70 % (79/113)	[33]
	USA	2004	Tissue	QMSP	64 % (58/90)	[74]
	USA	2004	Tissue	MSP	40 % (32/81)	[73]
	USA	2004	Tissue	QMSP	89 % (54/61)	[28]
	USA	2005	Urine	QMSP	35 % (18/52)	[45]
	Portugal	2006	Tissue	QMSP	97 % (29/30)	[36]
	Germany	2007	Tissue	QMSP	71 % (55/78)	[37]
	Portugal	2007	Tissue	QMSP	25 % (21/83)	[27]
	Korea	2007	Tissue	MSP	69 % (123/179)	[25]
	France	2007	Urine	QMSP	62 % (59/95)	[46]
	Germany	2008	Tissue	QMSP	95 % (76/80)	[38]
	Portugal	2008	Serum	MSP	39 % (7/18)	[75]
	Mixed	2008	Urine	QMSP	34 % (38/111)	[47]
	France	2008	Blood	QMSP	79 % (33/42)	[49]
	Korea	2009	Tissue	QMSP	71 % (25/35)	[34]
	USA	2009	Tissue	BiSeq	91 % (39/43)	[109]
	Germany	2010	Tissue	QMSP	96 % (24/25)	[40]
	UK	2011	Tissue	PyroSeq	100 % (48/48)	[41]
	Germany	2013	Tissue	QMSP	81 % (68/84)	[43]
	USA	2013	Tissue	Nested MSP	22 % (97/442)	[44]
RASSF1A/3p21.3	USA	2002	Tissue	COBRA	71 % (37/52)	[132]
	USA	2002	Tissue	MSP	53 % (54/101)	[29]
	USA	2004	Tissue	MSP	73 % (38/52)	[128]
	Portugal	2004	Tissue	QMSP	99 % (117/118)	[23]
	USA	2004	Tissue	QMSP	66 % (59/90)	[68]
	USA	2004	Tissue	QMSP	96 % (70/73)	[22]
	Korea	2004	Tissue	MSP	84 % (31/37)	[24]
	USA	2004	Tissue	MSP	49 % (40/81)	[73]
	Germany	2004	Tissue	MSP	78 % (88/113)	[33]
	Germany	2005	Tissue	QMSP	68 % (36/53)	[35]
	USA	2005	Urine	QMSP	73 % (38/52)	[45]

(continued)

Table 1
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Gene/location	Country	Year	Sample	Method	Frequency	References
	Korea	2007	Tissue	MSP	87 % (155/179)	[25]
	Japan	2007	Tissue	MSP	74 % (97/131)	[133]
	Portugal	2007	Tissue	QMSP	25 % (21/83)	[27]
	France	2007	Urine	QMSP	78 % (74/95)	[46]
	UK	2007	Tissue	BiSeq	50 % (7/14)	[134]
	France	2008	Blood	QMSP	98 % (41/42)	[49]
	Portugal	2008	Serum	MSP	1 % (3/207)	[75]
	Korea	2009	Tissue	QMSP	89 % (31/35)	[34]
	India	2010	Tissue	MSP	34 % (17/50)	[135]
	Japan	2010	Blood	QMSP	24 % (18/76)	[50]
	Pakistan	2013	Tissue	MSP	100 % (27/27)	[42]
	UK	2011	Tissue	PyroSeq	96 % (46/48)	[41]
	USA	2013	Tissue	Nested MSP	23 % (97/426)	[44]

determinants of metastasis. The methylated adenomatous polyposis coli (*APC*) gene is responsible for familial adenomatous polyposis, which is an inherited disorder characterized by extensive colon polyps and the development of colorectal cancer in early adulthood. *APC* is a well-characterized tumor suppressor gene that is involved in the Wnt signal transduction pathway and intercellular adhesion [19]. The *APC* complex is known to function as a gatekeeper in the cell, preventing the transcription of gene products that promote cell proliferation and survival rather than differentiation and apoptosis [20]. Hypermethylation of *APC* implies silencing of this gatekeeper, making the cell vulnerable to further epigenetic and genetic changes and, thus, progression toward the development of invasive cancer.

APC promoter methylation is common in various human tumors, especially colon [21]. Most studies found a prevalence of 22–100 % in prostate cancer tissue but only 5–6 % in noncancerous tissue [22–44]. Previous studies found that methylation in *APC* are associated with progression of prostate cancer [26, 27, 39]. In two small cohorts of prostate cancer patients, a threefold statistically significantly increased HR for promoter methylation in *APC* has been reported among the patients who experienced PSA recurrence, metastasis, or death [26, 27]. Richiardi et al. found that hypermethylation in the promoter of the *APC* gene is involved in prostate cancer progression using large survival analysis of two independent series of unselected prostate cancer patients [39]. Several studies reported 17–51 % methylation frequency of *APC* in urine [45–48] and 14–92 % in blood [49, 50]. A multiplex urine assay study for prostate cancer diagnosis [47] showed that the sensitivities of *APC* (43 %) in the urine sediments were similar to those

seen by other investigators, who demonstrated a similar sensitivity for APC [46].

Recently, Okegawa et al. investigated whether methylated APC in blood has potential in tumor detection in prostate cancer. They observed that 15 % of serum samples from prostate cancer patients were methylated in APC while all samples from control were negative [50].

3 Androgen Receptor (AR)

The specific causes of prostate cancer are not known, but multiple etiological factors, including genetics, hormones, diet, infection, and environmental exposures are thought to play significant roles. Although the precise role of androgens and their receptors in carcinogenesis and progression of prostate cancer has not been fully studied, previous studies suggest that these processes are important [51, 52]. The production of estrogens from androgens is mediated by the aromatase enzyme, the aberrant expression of which plays a critical role in the development of malignancy in a number of tissues [53]. Differences in the activities of these enzymes are determined to a large extent by genetic and epigenetic changes in the genes encoding them.

It had been known that androgens stimulate the growth of prostate cells through the androgen receptor (AR) [54]. There are two well-known AR target genes, PSA and TMPRSS2-ETS fusion genes. The exact roles of PSA and TMPRSS2-ETS in prostate cancer are not fully defined yet. While silencing of AR expression leads to decrease growth and induce apoptosis in vitro [55–57], over-expression of AR also induces growth inhibition and apoptosis [58]. In addition to prostatectomy and radiation therapy, androgen deprivation is one of the most effective treatments for prostate cancer. However, many advanced prostate cancers turn into castrate resistant cases. Prostate tumor cells in this stage grow aggressively without stimulation of androgens. Androgen receptor is one of the most frequently over-expressed proteins in castrate resistant cases [59]. Jarrard et al. reported a significant association between AR promoter methylation and its expression in vitro using prostate cancer cell lines [60].

Several groups found AR promoter methylation in 8–25 % of the prostate tumor tissues [61–64] and 39 % in serum [65]. Frequencies of AR promoter methylation are higher in castrate resistant cases than ones in primary prostate tumor tissues [62, 64]. The biological significance of AR silencing by promoter methylation in castrate resistant prostate cancer is not clear yet. Recently, Wang et al. reported that AR selectively upregulates M-phase cell-cycle genes in castrate resistant cells, including ubiquitin-conjugating enzyme E2C (UBE2C), a gene that inactivates the M-phase checkpoint.

They also found that epigenetic marks at the UBE2C enhancer are present in castrate resistant cells and direct AR-enhancer binding and UBE2C activation [54]. On the other hand, Schayek et al. found progression to metastatic stage in a cellular model of prostate cancer is associated with methylation of AR and AR suppresses the insulin-like growth factor-I receptor (IGF), therefore, suggest roles of IGF for stimulating AR signal in castrate resistant prostate cancer [66].

4 CD44

CD44 is a transmembrane glycoprotein that is involved in signal transduction and cell–cell and cell–matrix interactions by serving as a receptor. It codes a lipid raft protein like CAV1 or E-cadherin. Lipid rafts are also involved in angiogenesis and local invasion [67]. The CD44 expression in prostate tumor tissues is lower than ones in adjacent normal tissues. This low expression is correlated with CD44 promoter methylation [68, 69]. Gao et al. reported that decreased CD44 expression is correlated with Gleason score and the distant metastatic progression of prostate cancer [70]. Therefore, CD44 is considered as a metastasis suppressor gene. Furthermore, CD44 expression and its promoter methylation may correlate with not only tumorigenesis but also progression of prostate cancer [71]. CD44 promoter methylation frequencies were reported 22–77 % in tissues [25, 69, 71–74]. Interestingly, methylation at *CD44* was not detected in serum samples from prostate cancer patients [75]. These data warrant further study since CD44 methylation status in tissue is associated with aggressiveness and recurrence of prostate cancer [37, 70, 71].

5 E-Cadherin (CDH1)

The E-cadherin (*CDH1*) belongs to the family of cell–cell adhesion molecules and one of the key proteins in the maintenance of cell differentiation and the normal architecture of epithelial tissues [76]. DNA methylation-induced *CDH1* silencing was observed in prostate tumor and was associated with tumorigenesis, metastasis, and poor patient outcome [29]. Treatment with the demethylating agent 5-aza restored E-cadherin expression in the E-cadherin negative prostate cancer cell lines [77]. The prevalence of methylation varies from 0 to 61 % in tissues [22, 25, 29, 33, 41, 69, 73, 74, 77–79], 29 % in serum [49], and 31–77 % in urine [45, 46] samples from prostate cancer patients.

The reason for the discrepancy among these studies may come from technical issues, e.g., different CpG targets, detection methods, and samples, but also tumor status issues. Li et al. reported

that the overall methylation frequencies of E-cadherin promoter were high in advanced stage samples (70 %) and low in early stage (33 %) prostate tumors [77]. In addition, a previous study reported methylated and unmethylated E-cadherin gene expression are dominant in primary prostate cancer and bone metastasis, respectively [79]. These data suggested that CDH1 methylation might be a useful biomarker to assess progression of prostate cancer [77].

6 Cyclin-Dependent Kinase Inhibitors (CDKIs)

The tumor suppressor gene CDKN2 (p16) is one of the cyclin-dependent kinase inhibitors (CDKIs). CDKN2A (p16^{INK4a}) is a key protein in the signaling pathway, which can be damaged by a variety of genetic and epigenetic changes including hypermethylation in prostate tumors. Aberrant CDKI expression is observed in many tumor tissues including prostate [23, 80, 81]. The reported frequencies of p16^{INK4a} promoter methylation are inconsistent in prostate tumors, ranging from 0 to 77 % [22, 23, 25, 29, 33, 41, 42, 45, 46, 78, 80–85], 12–37 % in urine [45, 46], and 14 % in serum [49]. A recent study suggested that DNA methylomes may be different among racial groups [42]. However, carefully designed large studies are needed to demonstrate a population-specific methylome hypothesis. Perhaps these inconsistent results are due to different detection methods and/or different targets of methylated loci.

For example, Gu et al. identified DNA methylation at the *SmaI* site for 21 of 30 samples while found only one sample had an altered methylation pattern at the exon 1 of p16^{INK4a} [82]. Since Herman et al. first reported inactivation of CDKN/p16 by DNA methylation in prostate tumors [86], many other researchers have investigated the role of hypermethylated CDKN2A in carcinogenesis and progression of prostate cancer [22, 23, 25, 29, 33, 41, 42, 45, 46, 49, 78, 80–85]. Although methylation at exon 2 in p16^{INK4a} may not be functional, this methylation site in exon 2 may be a potential biomarker for prostate tumor because of a high prevalence of methylation in tumor tissues [23, 80, 81, 84]. These inconsistent methylation data due to different targets were confirmed by another groups, who reported that methylation in the promoter region occurred in 9 %, and 15 % of tumors in exon 1 [80, 85] and 66 % in exon 2 [80]. Jeronimo et al. found that the p16^{INK4a} gene was frequently methylated in tumor tissues (77 %). However, the high frequency of methylation was also found in benign prostatic hyperplasias (BPH) [23]. These data suggested that p16^{INK4a} methylation may be a potential biomarker for an early detection of prostate cancer.

Another CDKI, the CDKN2A/p14^{ARF}, generated from an alternative splicing process that replaces the first exon of p16^{INK4a}, has been known as a growth suppressor. Therefore, epigenetic

alterations of p14^{ARF} may affect p16^{INK4a}/RB1 pathways in the tumorigenesis and progression of prostate cancer. The p14^{ARF} promoter has been methylated in various cancers, glioma [87], bladder [88], leukemia [89], head and neck [90], and prostate cancer [22, 23, 25, 29, 45, 46, 49, 80, 81, 84, 85]. Based upon six independent studies, frequencies of p14^{ARF} methylation range from 0 % to 36 % in prostate tissues [22, 23, 25, 29, 45, 46, 80, 81, 84, 85], 6–37 % in urine [45, 46], and 14 % in serum [49]. With the exception of one study [22, 23, 25, 29, 45, 46, 80, 81, 84, 85], most studies reported low methylation frequencies that ranged from 0 to 10 %. The p16^{INK4a} and p14^{ARF} are frequently co-methylated, which may deregulate the RB1 or p53 pathway [80]. However, promoter methylation in p14^{ARF} is relatively rare in prostate tumors. Therefore, methylation in p16^{INK4a} rather than p14^{ARF} may be the predominant event in the INK4a/ARF loci in tumor tissues.

7 Glutathione S Transferase P1 (GSTP1)

Methylation of Glutathione S transferase P1 (GSTP1) promoter region is the most extensively investigated epigenetic change in prostate cancer. Methylation in GSTP1 was detected consistently more than 80 % of prostate cancers. GSTP1 is involved in the detoxifying process and elimination of potentially genotoxic foreign compounds by conjugating glutathione into toxic chemicals [91]. These processes protect prostate cells from DNA adducts and carcinogenesis [92]. Thus, defective GSTP1 activity may increase DNA mutations, therefore, may increase the prostate cancer risk [93, 94].

Lee et al. first reported a high frequency of GSTP1 hypermethylation in prostate tumor tissues [95]. Since then, numerous studies confirmed similar results consistently. Methylation of the GSTP1 promoter region occurs in 25–100 % of tumor tissues [22–25, 27–30, 33, 34, 36–41, 43, 45, 50, 61, 69, 73, 74, 84, 85, 95–114]. However, this methylation is detected with a low prevalence in normal prostate, BPH tissues, or adjacent tissue [115]. GSTP1 methylation was also detected consistently 12–83 % in urine [45–48, 112, 113, 116–118], 28–95 % in blood [49, 50, 65, 75, 112, 118, 119], and 44–50 % in ejaculates [112, 120] from prostate cancer patients, while either low or no methylation was detected in the samples from healthy controls [38, 45–50, 65, 75, 112, 113, 116–120]. Different frequencies of GSTP1 promoter hypermethylation between tumor and normal prostate tissues make an ideal biomarker for prostate cancer. Therefore, to increase the accuracy of model, some investigators who used a multiple gene panel approach, had commonly chosen GSTP1 and studied its promoter hypermethylation as a biomarker for prostate cancer incidence, progress, recurrence, or survival [45–47, 109].

8 Methylguanine-Methyl Transferase (MGMT)

Although the specific causes of prostate cancer are not known, androgen and estrogen abnormalities, inflammation, and DNA repair capacity have been implicated. DNA is constantly damaged by endogenous oxygen free radicals and exogenous chemicals. DNA mutations are estimated to spontaneously occur 20,000–40,000 times every day [121, 122]. The DNA repair process is important to the survival of cell; therefore, different repair pathways are available to reverse the different types of DNA damage. In fact, over 250 DNA repair enzymes participate in this process [123, 124]. Defects in these DNA repair pathways may increase persistent mutations in daughter cell generations, genomic instability, and ultimately prostate cancer risk.

DNA repair genes can be classified into several distinct pathways, including the direct reversal (DR) pathway. The only known enzyme in the DR pathway is Methylguanine-methyltransferase (MGMT). MGMT transfers the alkyl group at the O⁶ position of guanine to a cysteine residue within its active site, leading to the direct restoration of the natural chemical composition of DNA without the need for genomic reconstruction. Therefore, defective MGMT activity is associated with an increased mutation rate [125].

While two studies reported a low frequency of MGMT promoter hypermethylation (1–2 %) in prostate tumor tissues [22, 29, 61], others observed higher prevalence of hypermethylation (12–76 %) [23, 24, 29, 44–46, 84, 85]. Other groups reported 15–19 % MGMT hypermethylation frequencies in urine sediment samples [45, 46] and 12 % in blood [49] from prostate cancer patients. These data suggest that MGMT promoter methylation can be a potential biomarker for early detection and surveillance of prostate cancer. However, larger studies will be necessary to resolve these inconsistent results.

9 Retinoic Acid Receptor B (RARβ)

Retinoic acid receptor B (RARβ) is known as a tumor suppressor gene by interacting with retinoic acid. Expression of retinoic acid receptor B (RARβ) is reported to be absent or downregulated in tumor tissues [126]. The RARβ2 promoter is aberrantly methylated in many cancers, including prostate cancer [127]. Several groups reported that frequencies of methylation of the RARβ2 promoter range from 22 to 98 % of primary prostate cancers but rarely in normal prostate tissues or benign prostatic hyperplasia (BPH) samples [25, 27–29, 33, 34, 36–38, 40, 41, 43, 44, 49, 61, 73, 74, 109, 127–129]. Moderate or high frequencies of RARβ promoter methylation were also observed in 34–62 % of urine [45–47]

and 39–70 % in blood samples [45, 46, 49, 75]. Therefore, RARB2 gene methylation may be an ideal biomarker candidate for early detection of prostate cancer. Recently, Moritz et al. investigated a role of RARB methylation as a possible prognostic marker in patients with Gleason score ≤ 7 [43]. They observed that DNA hypermethylation was detected 81 % of 84 prostate tumor tissues and was correlated with lymph node involvement. Further, high methylation level was a significant predictor of biochemical recurrence following radical prostatectomy.

10 RAS Association Domain Family Protein 1 Isoform A (RASSF1A)

The RAS family of proto-oncogenes plays a key role in signal transduction pathways involved in cellular proliferation and survival, interacting with other regulatory circuits of cell growth and death. Over-expression of RAS family gene may cause reduction of growth factor dependency, resistance to apoptosis, or other features of the tumor phenotype. However, RAS association domain family protein 1 isoform A (*RASSF1A*), a tumor suppressor gene, was known to be associated with the DNA repair proteins and with the apoptotic effect [130]. Inactivation by methylation in promoter region of *RASSF1A* may deregulate the DNA repair pathway and cell cycle control in the tumor. Methylation in *RASSF1A* promoter was found in a large fraction of various tumors including prostate tissue [131]. In prostate tumors, *RASSF1A* promoter methylation is a common event, occurring in 23–99 % of tumor tissues [22–25, 27, 29, 33–35, 41, 42, 44–46, 49, 50, 68, 73, 128, 130, 132–135], 73–78 % in urine [45, 46], and 1–98 % in blood samples [45, 46, 49, 50, 75]. *RASSF1A* promoter methylation is also positively associated with aggressiveness of prostate cancer [23, 24, 29, 132]. These findings indicate that *RASSF1A* promoter methylation may be associated with early event of carcinogenesis and progression. Recently, Pan et al. performed a meta-analysis with 16 studies with 1,431 cases and 565 controls. The odds ratio (OR) of *RASSF1A* methylation in prostate cancer case as compared to controls, was 14.73 (95 % CI = 7.58–28.61). Further, *RASSF1A* methylation was significantly associated with aggressiveness based on Gleason score (OR = 2.35, 95 % CI: 1.56–3.53) [136]. Therefore, *RASSF1A* may be a potential epigenetic biomarker for screening and identifying aggressive prostate cancer with the PSA test.

11 Conclusions

Although a few large scale genome-wide analyses of epigenetic variations are currently ongoing, most published studies are small scale with a retrospective design. Therefore, meta-analysis or large

studies should be performed to obtain the complete extent and pattern of differential DNA methylation in the promoter region in the critical genes. Since epigenetic changes are involved in carcinogenesis and progression of prostate cancer, information of these epigenetic changes may provide clues for better diagnostic, prognostic, and predictive modalities than existing ones. The ultimate goals of these epigenetic studies are to improve patients' outcomes, and enhance quality of life. Therefore, future studies may evaluate whether testing DNA hypermethylation in bodily fluids at hypermethylated genes improve diagnostic sensitivity/specificity and prognosis for prostate cancer.

A number of clinical trials and therapies are targeting methylated genes. Unlike DNA somatic mutations, DNA methylations are reversible. Thus, hypermethylated tumor-suppressor genes can be reactivated with drugs. Several demethylating agents such as 5-azacytidine (Vidaza) and 5-aza-2'-deoxycytidine (decitabine) have been approved as treatments for the myelodysplastic syndrome (MDS) and leukemia [137–141]. Some MDS patients treated with 5-azacytidine showed a significant survival benefit [141, 142]. However, standard treatment with these drugs has not been optimized, including the effective dose, treatment duration, and management of adverse effects, and the identification of candidate patients, thus a patient who may benefit from treatment. Recently Kim et al. reviewed 14 clinical trials on low-risk MDS treated with 5-aza [143]. Most studies found the favorable outcomes. However, up to 50 % of patients did not respond and most responders experienced disease progression within 2 years of a response. Further, a major limitation of these therapies is their nonspecific target approach, which may induce unintended side effects. Therefore, not only tumor suppressor genes but also silenced oncogenes by methylation can be reactivated. Future studies should focus on developing drugs that can target specific genes.

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Sequencing the Cancer Methylome

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Abstract

DNA methylation is the most studied epigenetic event in cancer, with focus being placed on studying the entire DNA methylation landscape in specific cancers. Due to the recent advances of next-generation sequencing technology, several effective methods have been developed for high-throughput analysis of DNA methylation, enabling DNA methylation markers to be innovative diagnostic and therapeutic strategies in cancer. In this review, we discuss various current and emerging technologies in DNA methylation analysis that integrate next-generation sequencing with the basic principles of methylation detections including methylation sensitive restriction enzyme digestion, affinity purification with antibody or binding proteins, and bisulfite treatment. Variations to these described methods have also allowed for detection of 5-hydroxymethylcytosine marks on a genome-wide scale. We also describe several of the bioinformatic tools used to properly analyze methylome-sequencing data. Finally, recently developed artificial transcription-factor (ATF) targeting tools may provide flexible manipulation of DNA methylation events in specific gene regions, revealing the functional consequences of particular DNA methylation events.

Key words Cancer, Epigenetics, 5-Hydroxymethylation, Marker, Methylation, Methylome, Next-generation sequencing

1 Introduction

DNA methylation is a regulated epigenetic event essential for certain molecular functions including gene expression, modification and compaction of chromatin, genomic stability, X-chromosome inactivation, silencing of retroviruses, and control of tumorigenesis [1]. Methylation of the fifth carbon on cytosine residues (5-mC) is regulated by three highly conserved DNA methyltransferase (DNMT) proteins: DNMT1, DNMT3A, and DNMT3B. DNMT1 unerringly replicates the methylation pattern on nascent DNA strand from hemi-methylated parental DNA strand during mitosis, whereas DNMT3A and DNMT3B function as de novo methyltransferases, both critical for normal and disease development [2]. Together, these enzymes establish and maintain the DNA methylation landscape within the genome. Typically, DNA methylation

changes occur in highly dense CpG dinucleotide regions called CpG islands (CGIs) located near transcriptional start sites (TSS). This DNA methylation event interrupts the ability for the transcriptional machinery to activate gene expression and is commonly observed in tumor suppressor gene promoters of certain cancers. On the other hand, hypomethylated CGIs allow for gene activation and are typical markers for oncogenic gene expression in cancer phenotypes [3].

Within recent years, 5-hydroxymethylcytosine (5-hmC), an intermediate to demethylation of 5-mC, has become an increasingly studied event among epigenetic researchers [4]. 5-hmC was found to be abundant in human and mouse brains, as well as in embryonic stem cells [5, 6]. 5-mC can be oxidized to 5-hmC by the ten-eleven translocation (TET) family of DNA hydroxylase, including TET1, 2, and 3 [5, 6]. As recently shown by several studies, 5-hmC can be further oxidized by TET enzymes to 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) [7, 8]. The oxidation pathway from 5-mC to 5-fC or 5-caC followed by base excision by thymine-DNA glycosylase (TDG) was proposed as a plausible mechanism of active DNA demethylation [7]. Hydroxymethylation was originally discovered at the blastocyst stage of development where a decrease in global DNA methylation takes place and is then reestablished by *de novo* methyltransferases DNMT3A and DNMT3B after the implantation stage [9]. While it is reported that TET1 of the TET family maintains the pluripotency of embryonic stem cells (ESC) and inner cell mass specification [4], TET2 regulates cell lineage commitment by modulating the balance between self-renewal and differentiation [10]. TET3 is important in regulating the global DNA demethylation of zygotes [11]. Although the biological function of 5-hmC is not fully understood, it has been implicated in transcriptional regulation [12, 13]. Loss of 5-hmC has been reported as an epigenetic hallmark of melanoma, with diagnostic and prognostic implications [14].

DNA methylation can be analyzed by many different methods. Nevertheless, most methods typically utilize one of three basic strategies: (1) digestion of unmethylated or methylated DNA with methylation sensitive restriction enzymes; (2) use of anti-methylcytosine antibodies or methyl-binding domain (MBD) proteins to enrich methylated DNA; (3) bisulfite treatment of genomic DNA to convert unmethylated cytosine into uracil while leaving methylated cytosine unchanged. Tools to study DNA methylation have steadily progressed over time from using low throughput PCR and gel-based platforms to current high-throughput microarray and next-generation sequencing (NGS) technologies. Several excellent reviews describe the development of genome-wide DNA methylation analysis technologies [15–18]. The representative microarray-based methods include DMH [19], HELP [20], MCA [21] (restriction enzyme based), MeDIP [22], and MIRA [23]

(affinity enrichment based), which utilize genome-wide promoter or CpG island tiling arrays to identify differentially methylated genes. The Illumina Infinium beads array, which analyzes bisulfite-converted DNA, has become a popular platform for methylome analysis. The current version of Infinium 450K BeadChip is capable of measuring more than 450,000 CpG sites in promoters, CGIs, CGI shores, enhancer regions, and DNase I hypersensitive sites, all of which have been curated from published literature. The chip has been proven to be highly accurate and reproducible, and it requires a relatively small amount of input DNA [24]. Most importantly, the data analysis is relatively simple, making the Infinium array the best approach currently available for population-based epigenetic studies [25]. One of the disadvantages of microarray-based analyses, particularly those using enrichment of methylated DNA, is that they can survey for the presence or absence of methylated DNA, yet give little detail about the extent and pattern of CpG methylation within a given region. Often, extensive follow-up studies must be conducted on a single gene basis to confirm the results of such microarray experiments. Therefore, many of these approaches have been moved to the NGS platforms (i.e., HELP-tagging [26], DREAM [27], MRE-seq [28], MeDIP-seq [28], and MIRA-seq [29]). The whole genome bisulfite sequencing (WGS), reduced representation bisulfite sequencing (RRBS), and targeted bisulfite sequencing (TBS) approaches have also been widely used for single cytosine resolution and single molecule level methylation analysis. Modifications to these methods have also allowed genome-wide quantitative mapping of 5-hmC. The objective of this review is to provide insights about various sequencing-based methodologies that are currently available and apply them to understand the role of 5-mC and 5-hmC in cancer. Recent advancements for using DNA methylation and demethylation machinery for targeted methylation or demethylation are also discussed.

2 Methylome Sequencing Based on Restriction Enzyme Digestion

2.1 5-mC Sequencing Through Restriction Enzyme Digestion Enrichment

The enzymatic activities of restriction enzymes like *HpaII*, *BstUI*, *SmaI*, and many others can be blocked by the presence of 5-mC in their restriction sites (Fig. 1). The site-specificity and methylation dependence of these restriction enzymes allow separation of methylated DNA from unmethylated DNA using PCR-based enrichment procedures. For example, *HpaII* tiny fragment enrichment by ligation-mediated PCR assay (HELP) uses both *HpaII* and its methylation insensitive isochizomer *MspI* to distinguish methylated DNA from unmethylated DNA [20]. *MspI* digests every possible *MspI/HpaII* restriction site (CCGG) across the genome, while *HpaII* only cuts the unmethylated sites. By sequencing the libraries generated by this pair of enzymes and comparing the

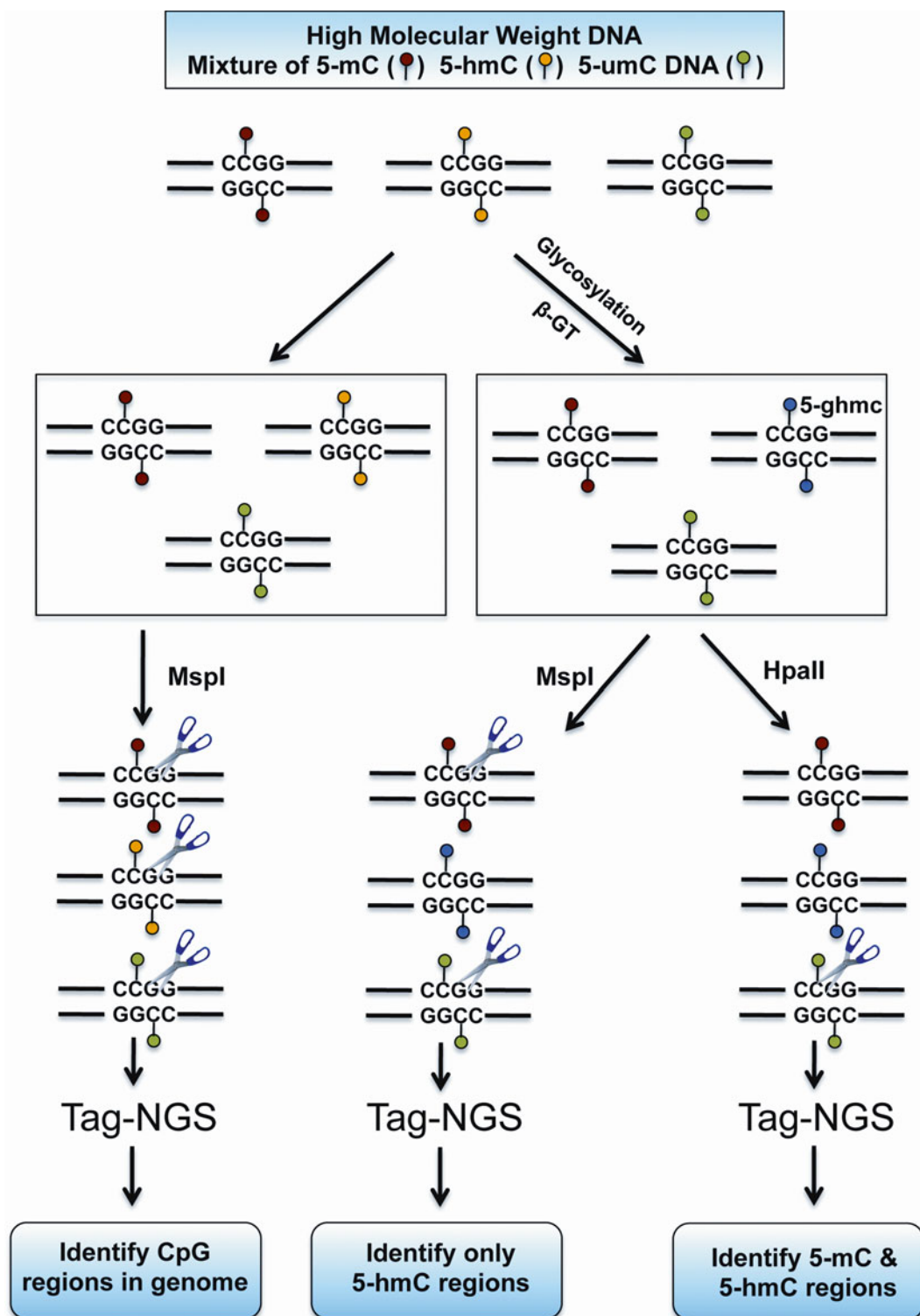


Fig. 1 An overview of restriction enzyme-based methods for genome-wide analysis of 5-mC and 5-hmC. The underlying principle is that HpaII only cuts at 5-umCs while MspI cuts at 5mCs and 5hmCs. β-GT can add a glucose molecule to 5-hmC to produce 5-ghmC, which cannot be cut by MspI. Analyzing three genomic libraries consisting of a regular MspI digestion and MspI and HpaII digestions of β-GT-treated DNA through NGS instrument can identify DNA contains both 5-mC and 5-hmC

enrichment profiles of *HpaII* and *MspI* tiny fragments, the HELP-seq method allows accurate quantification of methylation at >1.8 million loci in the human genome [26]. Digital Restriction Enzyme Analysis of Methylation (DREAM) is a similar method based on NGS analysis of methylation-specific signatures created by sequential digestion of genomic DNA with *SmaI* and *XmaI* enzymes, which recognize CCCGGG sequences [27]. The *XmaI* digestion produces sticky ends that can be ligated to linkers, while *SmaI* digestion results in blunt ends that are unable to be ligated to linkers. After a sequential digestion of genomic DNA with *SmaI* and *XmaI*, a ligation-mediated PCR only enriches methylated DNA, which can then be sequenced. DREAM provides information on 150,000 unique CpG sites, of which 39,000 are in CpG islands and 30,000 are at transcription start sites of 13,000 RefSeq genes. To take advantages of multiple methylation-sensitive restriction enzymes, Costello and colleagues developed a method called MRE-seq [28], which uses three enzymes (*HpaII*, *Hin6I*, *AciI*) to digest the genomic DNA and then size-select the smaller fragments (50–300 bp) for NGS. MRE-seq enriches the unmethylated DNA and can cover 1.7 million CpG sites across the human genome [28]. Another group of enzymes that digests only methylated DNA, such as *McrBC*, can also be used for DNA methylation analysis. This method was described as the CHARM (comprehensive high-throughput array for relative methylation) method by Andrew Feinberg and colleagues [30]. The CHARM method uses *McrBC* to digest the genomic DNA and is followed by ligation-mediated PCR to enrich smaller size DNA fragments, which are presumably methylated at each end of the DNA fragment. Due to a higher frequency of *McrBC* recognition sites ((G/A)^mCN_{40–300}(G/A)^mC) in the human genome, the CHARM method is expected to survey 5.6 millions mCpGs in the human genome. However, no report of combining CHARM with NGS has currently been published yet.

2.2 5-hmC Sequencing Through Restriction Enzyme Digestion Enrichment

The differential digestion approaches described above can also be used to study 5-hmC. Most of these methods utilize β -glucosyltransferase (β -GT), an enzyme that adds a glucose molecule to the hydroxyl group of 5-hmC in order to create β -gluoxyl-5-hmC (5-ghmC). The presence of 5-ghmC can block the enzyme activities of many restriction enzymes such as *MspI*, therefore creating a distinction between 5-hmC from the unmethylated cytosine and 5-mC. The HELP method has been modified to include the β -GT treatment (HELP-GT) for generating genome-wide 5-hmC profiles [31]. After restriction digestion by *HpaII*, *MspI* or *MspI*, β -GT conversion, and next-generation library construction and sequencing, HELP-GT can determine genome-wide distribution of 5-mC and 5-hmC at a single base pair resolution within *MspI* restriction sites. Figure 1 summarized the basic principle of utilizing the restriction enzyme-based method to differentiate 5-mC

and 5-hmC at genome scale. Theoretically, the same approach can be applied to other restriction enzymes, therefore expanding the CpG sites that can be interrogated.

3 Enrichment Based Methylome Sequencing

3.1 Methylated DNA Immunoprecipitation Sequencing (MeDIP-seq)

To study DNA methylation with affinity-based technologies, one of the common techniques used is MeDIP-seq (Fig. 2). This approach utilizes anti-mC or anti-mCG antibodies to probe for 5-methylcytosines within sheered genomic DNA. The typical process involves sheering and denaturing the DNA in order to create short ssDNA fragments, then immunoprecipitating the methylated DNA fragments from the ssDNA pool using the specified anti-mC antibody. The isolated methylated regions can then be ligated with sequencing adaptors for subsequent NGS library preparation. By this approach, the global DNA methylation landscape for the probed DNA sample can be determined with an approximate 100–300 base pair resolution [32–36].

Unlike restriction enzyme based methods that cut at specific CG-based palindromic regions and create a bias enrichment towards CGIs, MeDIP-seq provides the ability to investigate DNA methylation events not only within CpG islands, but also throughout the entire genome in an equal and unbiased fashion. Based on the advantage MeDIP provides for DNA methylation profiling, important DNA methylation events that occur in non-CGIs can potentially be discovered which previously would have been difficult to identify using restriction enzyme based enrichment. For example, Feber et al. demonstrate using MeDIP-seq that the majority of the differentially methylated regions (DMRs) observed in nerve sheath tumors lie within non-CGI regions such as non-CGI promoters, CGI shores, SINES, LINES, and LTRs [37]. Such a result would go undiscovered using restriction enzyme based approaches to study global DNA methylation changes.

Nevertheless, there are several aspects that limit MeDIP-seq as the best approach to analyzing the cancer methylome. MeDIP-seq traditionally requires a relatively large amount of input DNA for the immunoprecipitation process (typically 5–20 µg). This large requirement of DNA can be a critical factor when analyzing primary tumor DNA due to the limited amount of samples one can obtain from clinical settings. Recent developments, however, have improved on the input DNA required for MeDIP-seq by reporting that as little as 50 ng of DNA can be used successfully [35]. Another limitation of this approach is that it does not offer a single base pair resolution of DNA methylation changes, and absence of DNA methylation can only be inferred based on the lack of genomic reads during sequencing. With the absence of DNA methylation being an inferred assumption in MeDIP-seq, sequencing at the

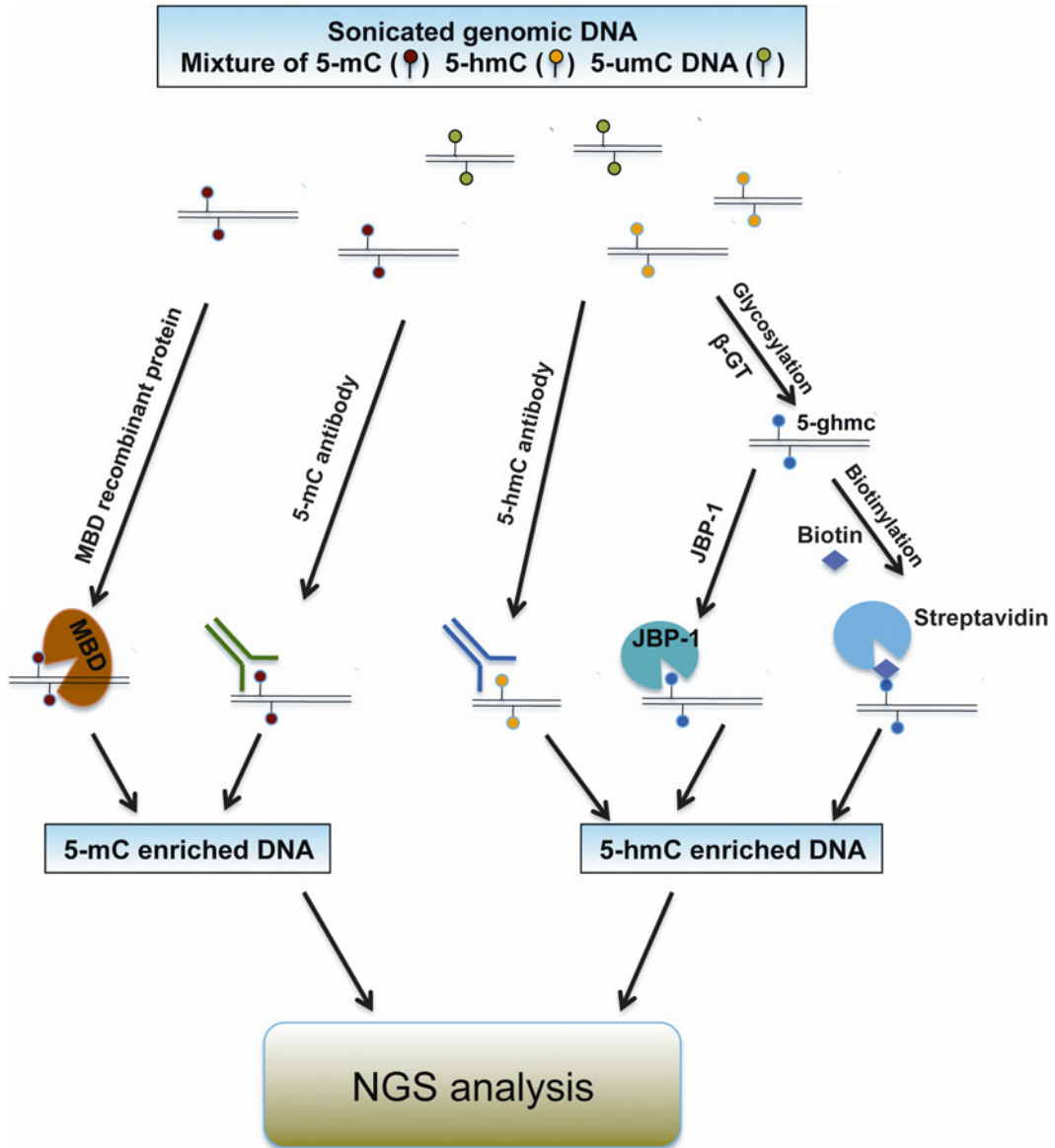


Fig. 2 An overview of affinity-enrichment based methods for genome-wide analysis of 5-mC and 5-hmC. The diagram illustrates the principles of MBD-seq, MeDIP-seq, hMeDIP-seq and two other methods specific for 5hmC enrichment through glycosylation. The DNA enriched by these techniques are then converted into sequencing libraries and sequenced by NGS instruments

proper read depth becomes a crucial element in order to avoid interpreting low-density methylated regions as unmethylated.

3.2 Methyl-Binding Domain Sequencing (MBD-seq)

MBD-seq (also known as Methyl-CpG Island Recovery Assay; MIRA) incorporates the same affinity based concept used in MeDIP-seq. However, instead of using an anti-mC/mCG antibody to perform a pull-down enrichment of methylated cytosine regions,

the MBD-seq approach utilizes the high biological affinity that the methyl-binding domain 2/methyl-binding domain 3-like 1 (MBD2/MBD3L1) heterodimer complex has for methylated CpG nucleotides. Incubated with a GST-tagged MBD2 and His-tagged MBD3L recombinant protein, methylated CpG nucleotides from sonicated gDNA will bind with the MBD2/MBD3L complex. The protein-DNA complex can then be extracted using glutathione-coated magnetic beads. After enrichment and separation from the protein-DNA heterocomplex, the isolated DNA can then be prepared as a genomic library and sequenced to determine DNA methylation regions at an approximate 100 base pair resolution.

Like MeDIP-seq, MBD-seq offers the ability to study DNA methylation events throughout the entire genome in a cost effective manner without having to use restriction enzyme based enrichment, which creates an overwhelming bias towards CGIs. However, MIRA-seq only requires approximately 250–500 ng per reaction, which is less input DNA than what MeDIP-seq traditionally requires. Also, unlike MeDIP-seq, denaturing the DNA into single strand fragments is not necessary, providing a more stable product after the pull-down enrichment of the MBD-methylated DNA complex [38]. These advantages enabled Jin et al. to compare the NCCIT embryonic carcinoma cellline [39]. However, MBD-seq has similar limitations to that of MeDIP-seq in regard to variability in CpG densities at genomic regions and the relatively large amount of DNA needed for proper isolation. Remarkably, MBD protein does not bind 5-hmC and, therefore, 5-hmC marks do not contaminate the DNA methylation data obtained from MBD-seq.

3.3 Genome-Wide 5-hmC Sequencing Based on Enrichment

The MeDIP-seq approach can also be used to study 5-hmC events in the genome by using an anti-hmC antibody (hMeDIP-seq). Interestingly, the anti-hmC antibody can bind double strand DNA, and therefore is more convenient to use. The coverage and resolution of hMeDIP-seq is very comparable to MeDIP-seq and has been used in several publications to document the genome-wide 5-hmC profiles of both human and mouse embryonic stem (ES) cells [40, 41]. As mentioned earlier, 5-hmC can be distinguished from 5-mC by β -GT treatment. By including a chemically altered glucose group within this reaction so that subsequently modified glucosyl-hmC base can bind biotin, a technique was developed whereby streptavidin purification would result in extremely efficient purification of DNA fragments (termed hydroxymethyl selective chemical labeling or hMeSeal) [42]. In a third approach [43], Robertson et al. have found that J-binding protein 1 (JBP1) has strong affinity to 5-ghmC, which leads to the development of another 5-hmC enrichment assay without using an antibody. In this case, 5-hmC in the genomic DNA is first converted to 5-ghmC by β -GT. The genomic DNA containing 5-ghmC is then pulled down by JBP1 and sequenced using NGS. Commercial products have

been developed by ZymoResearch and ActiveMotif based on the affinity purification methods discussed above. Theomson et al. conducted comparative analysis affinity-based 5-hmC enrichment techniques mentioned above [44]. They found that both the antibody (hMeDIP) and chemical capture (hMeSeal)-based techniques, but not the JBP-1 affinity-based methods, are accurate at reproducibly detecting genome-wide patterns of 5-hmC [44]. Figure 2 summarizes the principles of identifying 5-mC and 5-hmC at genome scale using affinity-based enrichment methods.

4 Methylome Sequencing Based on Bisulfite Conversion (Fig. 3)

4.1 *Whole Genome Bisulfite Sequencing*

Whole genome bisulfite sequencing (WGBS-seq), which combines bisulfite conversion with NGS, has been successfully used to sequence entire methylomes of several human cell types at a single-base resolution [45–51]. To perform WGBS-seq, the genomic DNA is first randomly fragmented to the desired size (200 bp). The fragmented DNA is converted into a sequencing library by ligation to adaptors that contain 5-mCs. The sequencing library is then treated with bisulfite to convert all unmethylated cytosines into uracil, leaving the 5-mC in the library as well as the ligated adaptor sequences unchanged. After amplifying the bisulfite treated library by PCR, the library is then sequenced using NGS. This shotgun approach is capable of surveying >90 % of cytosines in human genomes. WGBS-seq is instrumental in the identification of non-CG methylation in human ES cells [45, 46] and the identification of large partially methylated domains (up to megabases in length) in cancer genomes [52, 53]. However, despite its advantages, the WGBS-seq method remains too expensive to be applied to a large number of samples. In order to achieve higher sensitivity capable of detecting methylation differences between samples, a greater sequencing depth is required, ultimately leading to a significant increase in sequencing cost.

4.2 *Targeted Bisulfite Sequencing*

To help reduce sequencing cost, a number of targeted bisulfite sequencing (TBS-seq) methods have been developed [54]. Most of these methods are developed based on several sequence capture methods designed for exome sequencing such as array-based platforms, solution-based platforms, and padlock probes. The bisulfite padlock probes (BSPPs) method was independently developed by Deng et al. and Ball et al. [55–57] and follows the same principle of previously designed padlock probes [57]. However, the 150 bp single stranded BSPPs hybridize to bisulfite-converted genomic DNA instead of normal genomic DNA. Capture then occurs by filling in sequences between the probe-targeting arms with polymerase and is followed by a ligation step to form circularized DNA. After removing linear DNA with nucleases, the remaining circularized,

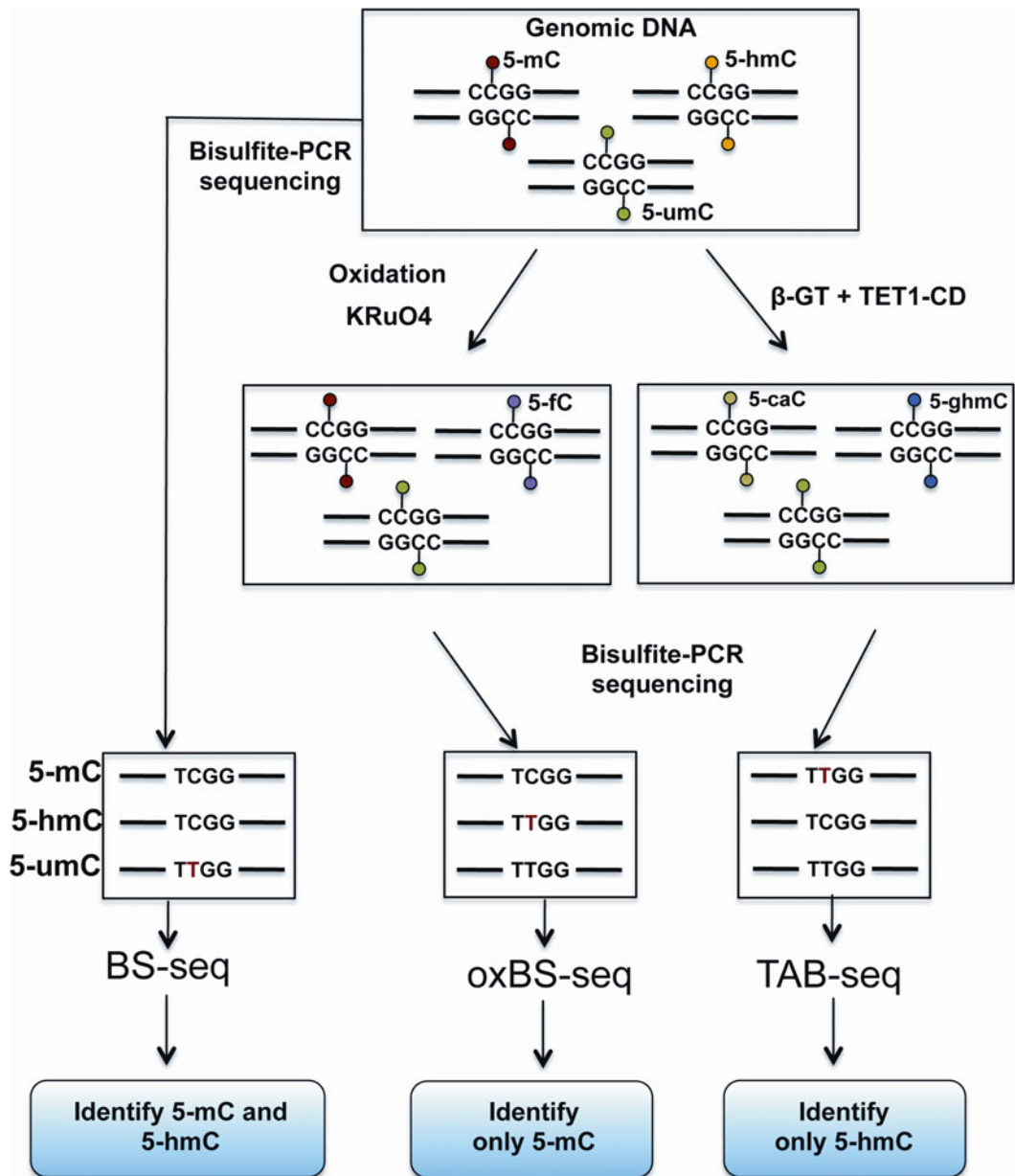


Fig. 3 An overview of bisulfite-based methods for genome-wide analysis of 5-mC and 5-hmC. BS-seq is based on the fact that bisulfite treatment results in the conversion of 5-umC to uracil, but will not affect 5-mC. After PCR and sequencing, 5-umC will be read as “T”, while 5-mC will be read as “C”. During bisulfite treatment, 5-hmC is converted to 5-CMS, which will be read as “C” after PCR and sequencing. Therefore BS-seq cannot differentiate 5-mC from 5-hmC. oxBS-seq first utilizes KRuO4 to oxidize 5-hmC to 5-fC while leaving 5-umC and 5-mC unchanged. Then, standard bisulfite treatment will convert what was originally 5-hmC (now 5-fC) and 5-umC to uracil and leave 5-mC unchanged. After PCR and sequencing, 5-umC and 5-hmC will be read as “T”, while 5-mC will remain as “C”. When the oxBS-seq results are subtracted from the standard BS-seq results, the difference between 5-mC and 5-hmC can be identified. TAB-seq tackles the same problem from a different angle. In TAB-seq, 5-hmC is converted to 5-ghmC by β-GT. Then the DNA is further treated with Tet protein to convert all 5-mC into 5-caC. Then, standard bisulfite treatment will convert what was originally 5-mC (now 5-caC) and 5-umC to uracil and leave 5-ghmC unchanged. Sequencing these bases will read 5-mC (now 5-caC) and 5-umC as “T”, while 5-hmC (now 5-ghmC) is read as “C”. Comparing the TAB-seq results with the standard BS-seq results allows single molecule mapping of 5-hmC and 5-mC

captured targets are amplified by PCR for NGS preparation. Using the most current BSPP protocol, Diep and colleagues accurately and reproducibly determine the methylation status of 500,000 CpG sites from 200 ng of bisulfite-converted DNA.

Hodge et al. first demonstrated the feasibility of using oligonucleotide microarray to capture DNA of interest for TBS-seq analysis [58]. The sequencing library is prepared from genomic DNA in the same way as WGBS-seq, treated with bisulfite and amplified by PCR. The amplified library is hybridized to an oligonucleotide capture array that is designed based on the bisulfite-converted DNA sequences. The capture DNA is further amplified and sequenced using NGS instruments. Hodges and colleagues demonstrated that their approach allowed for the capture of 25,044 CpGs in 324 CGIs spanning 300 kb of sequence [58]. However, due to the significant reduction in DNA sequences complexity after bisulfite treatment, it is predicted to be difficult to design capture probes that target bisulfite-converted DNA. Only 6–12 % of sequencing reads mapped to the targeted regions suggesting that the efficiency and specificity of the array-based capture for bisulfite-treated DNA is relatively low.

Lee et al. first combined the solution-based sequence capture method with bisulfite sequencing for TBS-seq analysis [59]. Contrary to the two approaches mentioned above, the sequencing library is prepared from fragmented native genomic DNA by ligation to the methylated sequencing adaptors. The library is then hybridized to biotinylated RNA probes in solution and recovered with streptavidin beads. The captured DNA is treated with bisulfite and amplified by PCR prior to sequencing. Lee et al. designed over 50,000 capture probes that target eight million base pairs in the human genome and demonstrated that up to one million CpG sites can be analyzed quantitatively using this solution capture approach. The capture specificity is comparable with exome sequencing [60] with an average on-target rate of ~80 % [59]. One of the limitations of this method is the initially reported requirement of a large amount of input DNA (20–30 µg), which limits the application of this method to cultured cell lines. However, a commercial kit based on the same principle has been developed by Agilent Technologies, allowing users to analyze over 3.7 million individual CpG sites in the human genome with 84 Mb of genomic sequences in the human genome being targeted, including a comprehensive collection of features including CGIs, CGI shores/shelves, cancer- and tissue-specific DMRs, GENCODE promoters [61], enhancers, conserved undermethylated regions, and DNase I hypersensitive regions. Due to the increase in size of target sequences and an optimized protocol, input DNA was reduced to 2–3 µg while still maintaining a better specificity and evenness of capture targets [59].

The enrichment of genomic targets can also be achieved by PCR amplification. Varley and Mitra developed a multiplex format of bisulfite-PCR approach called Bisulfite-Patch PCR.

Bisulfite-Patch PCR first uses a restriction enzyme to digest human genomic DNA and defines the ends of the fragments that will be selected. Targeted loci are then selected from the genomic restriction fragments by annealing patch oligos to the ends of the targeted genomic fragments [62]. This method enables highly multiplexed PCR amplification of DNA for 94 amplicons across a large number of clinical samples. By incorporating NGS, they have successfully sequenced 94 targeted gene promoters simultaneously in the same reaction. The method requires small amounts of starting DNA (250 ng), does not require a shotgun library construction, and is highly specific (90 % on-target rate). Although this method is relatively straightforward and easy to implement, the throughput still needs improvement. Microdroplet PCR amplification system developed by RainDance Technologies [63] allows the user to set up 1.5 million parallel PCR amplifications in a single reaction in less than an hour. Komori and colleagues first showcased the use of this method for TBS-seq analysis. Nearly 3,500 amplicons covering 77,674 CpGs in 2,127 genes were analyzed in primary CD4+ T cells using the RainDance technology [64]. If it is combined with 454-sequencing's long read ability, the amplicon-based bisulfite sequencing could reveal single molecule methylation patterns better than the short-read sequencing platforms.

4.3 Reduced Representation Bisulfite Sequencing (RRBS)

The upfront cost of developing capture probes and primers are still quite high for most of the TBS-seq platforms. With this in mind, RRBS (Reduced Representation Bisulfite Sequencing) is a cost-effective method for sub-genome scale DNA methylation analysis. For RRBS, the high molecular weight genomic DNA is first digested with a methylation-insensitive restriction enzyme (*MspI*). A smaller fraction of the digested DNA (40–220 bp) is then extracted. After end-repair and ligation to the methylated adaptors, the library is bisulfite treated, amplified by PCR and sequenced by NGS [65]. This method has been successfully applied to a variety of mouse and human genomic DNA. A wide range of input genomic DNA ranges from 1 µg to 30 ng can be used for RRBS analysis, although high quality genomic DNA is required for RRBS analysis. For lower quality DNA like DNA from FFPE (formalin-fixed, paraffin-embedded) specimens, removing the degraded, smaller DNA fragments (<500 bp) is required [66]. Recently, the RRBS method has been upgraded by the development of a gel-free and multiplexed-based protocol, which requires less hands-on preparation and allows for processing of 96 or more samples in a week [67]. Boyle et al. [68] showed that the new protocol has similar CpG coverage to the original RRBS protocols, while delivering higher throughput and lower costs. Due to its lower cost and higher throughput, RRBS is well suited for large-scale DNA methylation mapping studies, like those studying cancer samples [67].

One of the limitations is that the CpG sites analyzed by RRBS highly depend on the *MspI* restriction sites (CCGG) in the human genome. CpG sites outside of the *MspI* fragment will not be analyzed by this method. Overall, only 8–14 % of CpG sites in the human and mouse genome can be analyzed by RRBS. However, *MspI* restriction sites are biased towards CpG rich regions (CpG island); therefore, more than 80 % of CpG island and over half of the promoters in the human and mouse genome can be surveyed using RRBS. In addition, RRBS can also analyze a variety of genomic regions including introns, exons, intergenic regions, enhancers, and highly conserved regions including various repetitive sequences.

4.4 5-hmC Analysis Using Bisulfite Sequencing

Although the enrichment and digestion-based approaches for 5-hmC analyses are very useful, single-base resolution analyses are still highly desired for some applications. Unfortunately, the bisulfite treatment alone cannot differentiate 5-mC from 5-hmC because it efficiently converts 5-hmC to cytosine-5-methylsulfonate (CMS) [69]. CMS is read as ‘C’ during sequencing. Recently, two novel approaches, Oxidative Bisulfite Sequencing (oxBS-Seq) and TET-assisted bisulfite sequencing (TAB-Seq), have been developed to provide quantitative 5-hmC mapping with single-base resolution. Both methods use oxidation to create distinct populations within samples, but they accomplish this in different ways. Booth et al. developed the oxBS-Seq by slightly modifying the standard bisulfite conversion protocol [70]. The team found that treating DNA with potassium perruthenate (K₂RuO₄) selectively oxidizes 5-hmC into 5-fC, which after bisulfite conversion is read as uracil, just like unmethylated cytosine. By subtracting out an oxBS-Seq data set from regular bisulfite sequencing results, oxBS-seq can generate high-resolution 5-hmC map in samples [71]. TAB-Seq, developed by Yu et al. uses a different approach for single-nucleotide 5-hmC mapping [72]. It takes advantage of the fact that Tet proteins can oxidize 5-mC to 5-hmC [5, 6], but also 5-hmC to 5-caC, which behaves like unmodified cytosines during bisulfite conversion. In TAB-Seq, 5-hmC is first converted to 5-ghmC using β -GT as is done with enrichment approaches. However, in this case the goal is to prevent the 5-hmC from being oxidized by Tet recombinant enzyme. After β -GT treatment, DNA samples are modified with recombinant mouse Tet1 enzyme during which all 5-mC is converted to 5-caC. The subsequent bisulfite treatment converts unmodified cytosines and 5-caC to uracil and 5-caU respectively, leaving 5-ghmC unchanged. Once sequencing is complete the modified 5-hmCs (5-ghmC) are presented as cytosines. By comparing the TAB-seq dataset with standard bisulfite sequencing results, precise mapping of both 5-hmC and 5-mC modifications can be achieved. Figure 3 highlights the different

approaches used by oxBS-seq and TAB-seq to differentiate 5-mC from 5-hmC. These two methods have not been easily replicated in different laboratories. However, both of them have been commercialized and are currently available for purchase.

4.5 Bisulfite Sequencing of Immunoprecipitated DNA

Choi et al. first reported bisulfite sequencing of a MBD pull-down DNA library using 454-sequencing [29]. In this study, genomic DNA from a lymphoma cell line was digested with three restriction enzymes (*Csp6I*, *BfaI*, and *MseI*) and ligated with adapters. The methylated DNA fragments were isolated and enriched from the adaptor-ligated genomic DNA using MIRA assay. The methylation-enriched genomic library was then treated with bisulfite and amplified by PCR with primers designed to amplify molecules carrying bisulfite-modified adapter sequences at both ends. The PCR amplicons were then sequenced using the GS FLX sequencer. Choi et al. demonstrated this method to be very effective at identifying hypermethylated regions in the cancer genome [29]. However, the drawback of this approach is that the quantitative nature of the bisulfite sequencing is lost due to the selection bias towards the methylated DNA. Bisulfite sequencing of chromatin immunoprecipitated DNA (BisChIP-seq) was also applied to directly interrogate the genomic relationship between allele-specific DNA methylation, histone modification, or other important epigenetic regulators [73, 74]. In this case, a ChIP-capturing step is used to obtain a restricted representation of the genome occupied by the epigenetic feature of interest, for which a single-base resolution DNA methylation map is then generated. When applied to H3 lysine 27 trimethylation (H3K27me3), Brinkman et al. found that H3K27me3 and DNA methylation are compatible throughout most of the genome, except for CpG islands, where these two marks are mutually exclusive [73]. As mentioned above, bisulfite treatment results in the conversion of 5-hmC to CMS. Huang et al. raised an antibody specific for CMS-containing DNA fragments, which can be used to immunoprecipitate CMS-containing DNA after bisulfite treatment [75]. The anti-CMS technique is highly specific with a low background and is much less dependent on 5-hmC density than hMeDIP assay. However, the anti-CMS antibody is still not commercially available and therefore limited in its application.

4.6 Analysis of Chromatin Accessibility Using Bisulfite Sequencing

DNA methylation and nucleosome positioning work together to generate chromatin structures that regulate gene expression. Kelly et al. developed a method called NOME-seq (Nucleosome Occupancy and Methylome Sequencing) that uses a GpC methyltransferase (M.CviPI) and NGS to generate a high resolution map of genome-wide nucleosome positioning while retaining endogenous DNA methylation information from the same DNA strand. This M. CviPI-based chromatin accessibility assay was first

developed by Dr. Michael Kladde [76]. The principle of the method comes from the observation that CpG sites within DNA wrapped around nucleosomes are protected from in vitro methylation by CpG methylase (*M.Sss I*) and GpC methylase (*M.CviPI*). Probing of the nucleosomes with these cytosine methylases followed by bisulfite sequencing is an effective technique for mapping the nucleosome positioning at a single molecule level. It was used to study the role of nucleosomal occupancy in epigenetic silencing of tumor suppressor gene *MLH1* [77, 78]. However, the traditional bisulfite genomic sequencing (BGS) used for this type of analysis is very laborious and low-throughput. Recently, WGBS-seq [79] and Bisulfite-Patch PCR [80] have been used for NOME-seq analysis, which enables high-throughput mapping of chromatin structure at a single molecule level. Kelly et al. demonstrated that NOME-seq obtains DNA methylation and nucleosome positioning information from the same DNA molecule, giving a comprehensive genome-wide DNA methylation and nucleosome positioning correlation at the single molecule [79]. Nabili et al. recently showed that analysis of chromatin profiles using NOME-seq identified subpopulation of cells with distinct epigenetic profiles with the complex brain tumor stem cell populations, illustrating the biological relevance of epigenetically distinct subpopulations that in part underlie the phenotypic heterogeneity of tumor cell populations [80].

5 Bioinformatics Analysis

From the data analysis point of view, methylome sequencing can be divided into two types: enrichment-based sequencing and bisulfite sequencing. The former enriches genomic DNA fragments with methylated CpGs using various kinds of technologies. Sequence reads can then be mapped to the genome using general mappers like BWA [81], Bowtie [82], and SOAP [83]. To eliminate PCR and sequencing duplicates, reads mapped to the same location should be filtered out and retain a certain number of copies, typically one copy. Mapped reads also lengthen toward the 3' end up to the original length of fragments. DNA methylation is defined based on the depth of reads. To measure absolute DNA methylation or compare multiple samples, read depths are normalized by the total number of mapped reads similar to RNA-seq analysis for gene expression. Like ChIP-seq, an input sequencing sample is used to take into account genomic differences (e.g., copy number variants) of a sample in regard to the reference genome. To identify significantly enriched regions with statistical models, peak-calling programs for transcription factors and histone marks such as MACS, SICER, F-seq, and Useq have been utilized for the analysis of capture-based methylome sequencing data. Recently Batman [84], MEDIPS [85], and BALM [86] have been released to

specifically take methylome sequencing into account. Differential methylation between two samples can be simply defined as log2 fold change of average read depth for a given window or genomic feature. An alternate way is to use the enriched regions (peaks) so that the mutually exclusive peaks in two samples determine differential methylation. However, these methods are limited on statistical assessment and sensitivity, respectively. The best way is to apply statistical models to methylation changes and measure the significance of differentially methylated regions in terms of p-values. Programs like SICER, ChIPDiff, DiffBind, DBChIP, edgeR, and DESeq use this particular model. Several pipelines for MeDIP-seq analysis have been developed. MEDUSA performs read mapping using BWA, quality control using FASTQC and MEDIPS, DMR identification using USeq and DESeq, and DMR annotation using BEDTools [87]. MeQA supports preprocessing, quality assessment, read distribution, methylation estimation, and DMR annotation using BWA, SAMTools, SAMStat, BEDTools, MEDIPS, CEAS, and in-house scripts [88].

In contrast, bisulfite sequencing reads need specially designed mapping algorithms due to the technicality of bisulfite converting unmethylated cytosines to thymines without changing methylated cytosines. Several programs have been developed for mapping: BSMAP [58], BS-Seeker [59], Bismark [60], BRAT [59], RMAP-BS [61], MethylCoder [62], SOCS-B [62], and B-SOLANA [63]. After mapping, the methylation level of CpGs is defined as a beta value, i.e., the fraction of the number of methylated reads to the total number of mapped reads. For high quality of methylation profile, we filter out reads based on the base quality score of a cytosine, the mapping quality score of a read, and the read depth of a CpG site. Differential methylation is defined as the difference between the beta values of two samples so that a positive and negative values represent hypermethylation and hypomethylation, respectively. For statistical assessment of differential methylation for two samples without replicates, a Fisher's exact test is used with a 2 by 2 contingency table for the number of methylated and unmethylated reads for two samples. If replicates for each sample or two or more samples for each group exist, then the Student *T* test, Wilcoxon test, Rao-Scott test, and Kolmogorov-Smirnov test identify DMRs. Analysis of Variance (ANOVA) can be used for three or more samples with replicates or groups with multiple samples. In contrast to applying traditional statistical tests for differential methylation, DMEAS has been developed to measure methylation entropy that represents the distribution of DNA methylation patterns for the quantification of epigenetic heterogeneity [89]. CpG_MPs quantifies the methylation difference and identifies DMRs from genome-wide methylation profiles by adapting Shannon entropy, and BSmooth employs smoothing to estimate the methylation level in a genomic region for a single sample to identify DMRs based on a

statistic that appropriately summarizes consistent differences by accounting for biological variability [90]. AMP-methylKit and BSpice were developed for an end-to-end analysis of bisulfite treated sequencing reads. AMP maps RRBS reads using Bismark and calls per base methylation scores [91]. AMP-methylKit performs summarizing methylation over predefined regions or tiling windows, measuring and visualizing similarity between samples, principal component analysis (PCA), unsupervised clustering, and DMR detection and annotation [92]. As a comprehensive pipeline, BSpice has the same functions as AMP and methylKit, and also supports different mappers, various visualization methods, and external programs such as CpG_MPs and BSmooth [93]. It also provides a user-friendly environment by being connected to GenePattern, a web-based workflow system for microarray and next-gen sequencing data analyses.

Methylation and differential methylation of CpGs, tiling windows, or genomic features can be visualized on the UCSC genome browser [94], IGV [95], or IGB [96] through the BAM, BED, or wiggle format. The Identified DMRs are also classified by their genomic features. In comparison with genes, DMRs are classified into gene promoters, gene bodies, and downstream intergenic regions. DMRs in gene bodies are further stratified into exons and introns. DMRs are also compared to CGIs and are stratified into north CpG shelves and shores, inside of CGIs, or south shelves and shores. Other features such as repeats and noncoding RNAs can be compared. To perform functional analysis with GO terms, pathways, protein-protein interactions, a set of genes where DMRs locate in promoters or bodies is fed into PANTHER [97], DAVID [98], or IPA [99].

6 Efforts to Functionally Mimic the Cancer Methylome

Based on the overview of next generation sequencing techniques previously described, these large-scale cancer methylome studies have allowed the discovery of numerous epigenetic events that are strong prognostic predictors of cancer progression and severity, as well as provide several epigenetic events as candidates of cancer-drivers. Nevertheless, a great deal is still unknown regarding the mechanistic effects of tumor-induced DNA methylation due to the current inability to successfully manipulate specific DNA methylation events within a laboratory setting. Currently, many researchers rely on methyltransferase inhibitors like 5-aza-cytidine or 5-aza-deoxycytidine to study the epigenetic control of genes of interest [68, 100]. However, these inhibitors suppress the methyltransferases on a global level and do not provide complete clarity to whether the specific DNA methylation events in question are necessary or sufficient in causing the cancerous phenotype.

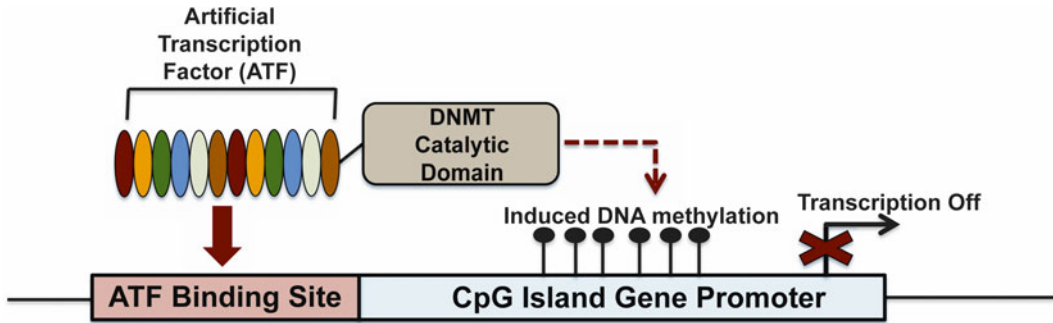


Fig. 4 Implementing artificial transcription factors (ATFs) to induce site-specific DNA methylation. The illustration demonstrates how ATFs, like zinc-finger DNA binding domains and transcription activator-like effectors (TALENs), can be coupled with DNA methylase domains in order to induce site-specific DNA methylation. This same targeting concept can also be used to demethylate genomic regions by fusing a TET catalytic domain to the desired ATF. Further development of ATF technology will enable functional study of particular DNA methylation events discovered by correlation-based, high-throughput methylome studies

To overcome this technical limitation, several approaches have been published that focus on revealing the functional consequences of specific DNA methylation events. One method performed by Li et al. demonstrates the ability to target and methylate predetermined regions by fusing the catalytic domain of *Dnmt3a* to zinc-finger DNA binding domains [101]. This particular method was able to achieve DNA methylation levels as high as 96 % for the targeted region and cause a threefold gene repression. These results were even further improved by using a *Dnmt3a*/*Dnmt3L* fusion with zinc finger DNA binding domains to induce targeted DNA methylation [102]. In a separate study, the counteractive result occurred when targeting the TET2 hydroxymethylase to the methylated ICAM-1 promoter in ovarian cancer cells in order to induce ICAM-1 specific hydroxymethylation [103]. These results are very promising in regard to the ability to target *de novo* methylase activity and induce the desired DNA methylation event. Nevertheless, the difficulty of constructing zinc-finger domains, as well as the limited genomic regions in which zinc fingers can exclusively bind, still make targeted DNA methylation a difficult endeavor. However, the limitations created by zinc fingers are now being overcome by the recently developed transcription-activator like effectors (TALEs) approach for specific genomic targeting, which allows for more specific DNA binding and easier design (Fig. 4). Mader et al. demonstrates this capability by binding the TET1 catalytic domain to TALE repeats in order to induce hydroxymethylation in specific gene regions like the HBB and the RHOXF2 locus [104]. As optimization of these artificial transcription factor based systems continues to improve, the ability to alter

specific DNA methylation events will become much easier, allowing increased understanding of the functional effects in particular epigenetic events.

7 Conclusions and Future Directions

As advancement in next-generation sequencing technology and the fine-tuning of methylome sequencing protocols continues the understanding of DNA methylation at a genomic level will exponentially grow and provide a greater understanding of how cancer cells utilize this molecular mechanism for oncogenic initiation and growth. Though a great deal of information has been gathered regarding oncogenesis through DNA methylation sequencing, the current cost of NGS has made most methylome-sequencing studies a somewhat static pursuit. Currently, the majority of studies focus on surveying the DNA methylation landscape of primary tumors or tumor cell lines without any other experimental variable. However, DNA methylation is a dynamic molecular process that can vary based on tissue types and be affected by numerous external variables. Simply, the full effects of DNA methylation cannot be determined from one-dimensional studies. Nevertheless, as sequencing costs continue to decrease, the methylome sequencing techniques described throughout this review can be utilized in much more intrinsically designed experiments. For example, one recent manuscript from Gifford et al. incorporated WGBS-seq to study the dynamics of genome-wide DNA methylation changes in embryonic stem cells by comparing the DNA methylation landscapes of each induced differentiated cell stage. Such a study allowed Gifford et al. to investigate the active changes of DNA methylation within one connecting experimental design [105]. Another example of using NGS DNA methylation profiling to study the dynamics of DNA methylation changes is by Sharma et al. who used RRBS to compare the DNA methylation differences of FoxP3+ CD4+ T-cells that are characterized by their location in mice and their ability to express the transcription factor Eos [106]. Such examples demonstrate the potential of just how these methylome-sequencing techniques can be further used to understand the complexities of DNA methylation events in a dynamically changing tumor. With this overarching goal, cancer methylome sequencing will progress to provide clearer detail of how DNA methylation operates within cancer.

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Global DNA Methylation Profiling Technologies and the Ovarian Cancer Methylome

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Abstract

Cytosine methylation in DNA constitutes an important epigenetic layer of transcriptional and regulatory control in many eukaryotes. Profiling DNA methylation across the genome is critical to understanding the influence of epigenetics in normal biology and disease, such as cancer. Genome-wide analyses such as arrays and next-generation sequencing (NGS) technologies have been used to assess large fractions of the methylome at a single-base-pair resolution. However, the range of DNA methylation profiling techniques can make selecting the appropriate protocol a challenge. This chapter discusses the advantages and disadvantages of various methylome detection approaches to assess which is appropriate for the question at hand. Here, we focus on four prominent genome-wide approaches: whole-genome bisulfite sequencing (WGBS); methyl-binding domain capture sequencing (MBDCap-Seq); reduced-representation-bisulfite-sequencing (RRBS); and Infinium Methylation450 BeadChips (450 K, Illumina). We discuss some of the requirements, merits, and challenges that should be considered when choosing a methylome technology to ensure that it will be informative. In addition, we show how genome-wide methylation detection arrays and high-throughput sequencing have provided immense insight into ovarian cancer-specific methylation signatures that may serve as diagnostic biomarkers or predict patient response to epigenetic therapy.

Key words Epigenetics, Methylation, Next-generation sequencing, Ovarian cancer, Whole-genome bisulfite sequencing

1 Introduction

Epigenetic regulation requires a complex interplay of DNA methylation, nucleosome positions, histone modifications, and expression of noncoding RNA to drive normal cellular processes in a cell type-dependent manner. Higher order chromatin structures and gene expression patterns during differentiation and development are established by several epigenetic mechanisms. Methylation of DNA cytosine residues at the carbon 5 position (5^{mc}C) is the best studied epigenetic mark that constitutes an important epigenetic layer of transcriptional and regulatory control in many eukaryotes.

DNA methylation patterns are established during embryonic development, mitotically inherited through multiple cellular divisions, and susceptible to the environment [1, 2], diet, and aging [3, 4], and disrupted in diseases [5]. DNA methylation is known to play important roles in several key physiological processes, including regulation of X-chromosome inactivation, gene expression, imprinting, and maintenance of chromosomal stability. DNA methyltransferases have a highly conserved catalytic motif that lays down DNA methylation patterns on target sequences of the genome. The distribution of methyl marks demarcates regions of transcriptional silence or transcriptional potential, creating a stable, epigenetic signature.

Often found in the sequence context CpG or CpHpG (H=A, T, C), DNA methylation is usually a repressive mark when located at gene promoters. However, CpG DNA methylation is increased in the gene bodies of actively transcribed genes in plants [6–8] and mammals [9–11]. There are approximately 28 million CpG sites [5] where the majority of CpG dinucleotides (70–80 %) are fully methylated in normal human cells [12]. Clusters of CpG sites, CpG islands, are 0.2–3 kb stretches of CpG rich (>50 % GC percentage) DNA that appear to be protected from the modification in normal somatic cells [13]. Nearly half of all known human genes are associated with a CpG island in the 5'-end regulatory region. Typically unmethylated in normal cells, promoter CpG islands are associated with active gene expression during differentiation. By contrast, methylated CpG island promoters are strongly associated with gene repression. Flanking the CpG islands are “shores,” regions of comparatively low CpG density that are located approximately 2 kb from CpG islands [14]. Beyond CpG islands and shores are “shelves,” the 2 kb region flanking the shores. Tissue- and cancer-specific differential methylation is exhibited in shores and has been associated with gene repression [15].

The role of cytosine methylation is essential to gene regulation which has profound implications in developmental processes, normal biology, and disease conditions in many eukaryotic organisms. The underlying biology of DNA methylation remains largely unknown, particularly the mechanism by which it is altered in pathological diseased states, such as cancer [16, 17] where a disruption of global DNA methylation patterns is typically associated. For example, global hypomethylation of CpG-poor regions leads to chromosomal rearrangement and oncogene activation [18], resulting in an overall decrease in total genomic 5^{mc}C in cancer cells. Loss of imprinting, or loss of parent of origin-specific gene silencing, appears to increase colorectal cancer risk in humans and mouse models [19–22]. Global hypomethylation (primarily in repeat elements and pericentromeric regions) in cancer is thought to contribute to genomic instability and aberrant expression of some oncogenes, such as *MYC* [23], which results in deregulation

of cellular processes. However, chromatin modification and CpG islands are susceptible to DNA methyltransferase activity so that hypermethylation of CpG island in the 5'-end regulatory region is commonly observed in cancer and is associated with silencing of tumor-suppressor genes and downstream signaling pathways [24]. Typically in normal cells, CpG-poor, distal enhancers are unmethylated but often gain methylation in cancer cells. The key to understanding why certain regions of the genome can be expressed in specific developmental contexts and how epigenetic changes might enable aberrant expression patterns and disease remains locked in the delineation of regional and broader DNA methylation patterns.

2 Methods to Map DNA Methylation on a Genomic Scale

Vital to understanding the influence of epigenetics is the ability to profile DNA methylation across the genome to provide a more comprehensive map of cancer DNA methylomes using whole-genome-based technologies. Previous analyses were restricted to specific loci through Sanger sequencing of bisulfite-converted and PCR-amplified genomic DNA fragments. Over the past decade, there has been a revolution in DNA methylation analysis technology where entire methylomes can be characterized at a single-base pair resolution on a genome-wide scale. Greater insight into the underlying mechanism and location of cancer-specific methylation changes at individual CpG residues will be gained through new technologies which may aid in further identification of potential epigenetic-based cancer biomarkers. Many methods and approaches exist for its identification, quantification, and mapping within the genome, contributing to our comprehension of the role of DNA methylation in both development and disease states. The earliest approaches were nonspecific and were at best useful for quantification of total methylated cytosines in a sample. However, over the past few decades, this field has seen considerable progress and development where there is such a diversity of DNA methylation profiling techniques that it can be challenging to select one.

Several methods have now been developed to map DNA methylation on a genomic scale. DNA methylation detection is based on the ability to distinguish cytosine from 5^{me}C in the DNA sequence. Different methods of analyzing methylation status are all based on three different principles: (1) digestion of DNA with methylation-sensitive restriction enzymes, (2) enrichment of methylated genomic DNA fragments using anti-methylcytosine antibody or methyl-binding domain (MBD) proteins, and (3) sequencing of bisulfite-converted DNA. These methods differ in their coverage and sensitivity, and the method of choice depends on the intended application and desired level of information. Results include global

methyl cytosine content, degree of methylation at specific loci, or genome-wide methylation maps. Recently, these principles have been scaled with high-throughput analytical platforms such as microarray and next-generation sequencing platforms, bringing us closer to exposing the whole methylome. In addition to DNA methylation pattern profiling, expression profiling of cells treated with DNA methyltransferase inhibitors and/or histone deacetylase inhibitors has also been used as a discovery tool for epigenetically silenced genes. Older, decreasingly used methods such as locus-specific analyses and gel-based analyses will not be covered here but can be briefly referred to in an excellent review by Peter Laird [25]. Also, Laird’s review, plus an exhaustive reference manual by Marcel Coolen and Susan Clark [26], covers many adaptations and variations of major genome-wide DNA methylation analysis techniques that have been used in the past to detect 5^{mc}C and will not be discussed here.

Here, we focus on four prominent genome-wide approaches (Table 1): whole-genome bisulfite sequencing (WGBS);

Table 1
Summary of whole-genome DNA methylation detection techniques

WGBS	MeDIP-Seq	MethylCap-Seq	RRBS	450 K
1. Sonication of DNA 2. Library preparation 3. Gel-size selection 4. Bisulfite treatment 5. Library amplification	1. Sonication of DNA 2. Library preparation 3. Denaturation and enrichment with 5-methylcytosine antibody 4. Library amplification	1. Sonication of DNA 2. Enrichment with methyl-binding domain protein 3. Washing and elution 4. Library preparation and amplification	1. Digestion with <i>MspI</i> 2. Library preparation 3. Gel-based size selection 4. Bisulfite treatment 5. Library amplification	1. Bisulfite treatment 2. Hybridization 3. Single-base extension 4. Stain Bead Chip 5. Hi Scan Bead Chip
Pros: <ul style="list-style-type: none">• >500 million reads per sample• 5 % coverage Cons: <ul style="list-style-type: none">• Large quantity of DNA required (1–5 µg)• Bioinformatics expertise• Cost	Pros: <ul style="list-style-type: none">• 0.05–5 µg DNA required• Efficient• Cost Cons: <ul style="list-style-type: none">• Limited resolution• Enrichment not linearly related to actual methylation	Pros: <ul style="list-style-type: none">• 0.2–1 µg DNA required• Efficient• Cost Cons: <ul style="list-style-type: none">• 17.8 % coverage• Marginally quantitative• Bioinformatics expertise• No information on individual CpG dinucleotides	Pros: <ul style="list-style-type: none">• 0.01–0.03 µg DNA required• High throughput• Less expensive than WGBS• Single-nucleotide resolution Cons: <ul style="list-style-type: none">• 3.7 % coverage• Lack of coverage at intergenic regions• Bioinformatics expertise	Pros: <ul style="list-style-type: none">• 0.5–1 µg DNA required• High throughput• Suitable for fresh-frozen and FFPE tissue• Quantitative measurement• Cost Cons: <ul style="list-style-type: none">• 1.7 % coverage• Preselection of probes• Probe bias

methyl-binding domain capture sequencing (MBDCap-Seq); reduced-representation-bisulfite-sequencing (RRBS); and Infinium Methylation450 BeadChips (450 K, Illumina). We discuss some of the requirements, merits, and challenges that should be considered when choosing a methylome technology to ensure that it will be informative.

3 Whole-Genome Bisulfite Sequencing (WGBS)

In 1992–1994, Frommer and Clark developed bisulfite sequencing where sodium bisulfite specifically converts cytosine to uracil but has no effect on 5^{mc}C, and during PCR amplification of bisulfite-treated DNA, uracil is replaced with thymine [27, 28]. After chemical modification, CpG methylation can be measured at single base resolution. Formerly, researchers performed clonal sequencing of bisulfite-converted PCR products from a restricted, relatively localized genomic region [27, 29]. The current revolution of high-throughput sequencing pioneers the generation of genome-wide, single-base resolution DNA methylation maps from bisulfite-converted DNA. WGBS is considered the gold standard in DNA methylation analysis due to its high accuracy and single cytosine measurement resolution. WGBS was first demonstrated by sequencing the smaller *Arabidopsis thaliana* genome in 2008 [30, 31]. Since then, WGBS has been used to successfully map the complete methylomes of several embryonic stem cell lines [32–34], IMR90 fibroblasts [33], human peripheral mononuclear cells [35], hematopoietic progenitor cells [36], mouse primordial germ cells [37], and small intestine cells [38]. Relatively few human cancer methylomes have been assessed by WGBS most likely due to the overall cost of the assay, technical expertise, and bioinformatics challenges. Nonetheless, a growing number of cancers such as colorectal cancer [39], prostate cancer [40], pediatric high-grade gliomas [41], breast cancer [42], and others [43, 44] have been assessed thus far using WGBS.

WGBS consists of shearing 1–5 µg genomic DNA, ligating to methylated adaptors before size selection and bisulfite conversion, followed by library construction and high-throughput sequencing (Table 1). Typically, approximately 95 % of all CpG sites in the genome can be assessed using WGBS. In order to achieve approximately 30-fold coverage of the 28 million CpG sites on autosomes and sex chromosomes, more than 500 million paired-end reads are required. Single-nucleotide resolution and whole-genome coverage are advantages of WGBS however, large quantities are required (1–5 µg) and this process requires complex computational and bioinformatics expertise. In-house adaptations of genome sequencing pipelines to bisulfite data were initially used to interpret data [33, 42, 45, 46]; however, public tools for the analysis of WGBS data are being developed as the technique becomes more

accessible. Also, WGBS is costly and thus difficult to be applied to large numbers of samples. Standard WGBS protocols or library preparation methods are just beginning to emerge, but commercial bisulfite conversion reagents kits exist. Although the Illumina sequencer GAII or HiSeq 2000 are mostly used, some have used the SOLiD sequencer [43] or 454-sequencing [47] to conduct WGBS. Most sequencing providers use customized in-house methods and the technique has yet to be utilized in a high-throughput clinical setting, partly due to the extensive hands-on and depth of sequencing required. Increased read lengths and paired-end sequencing strategies have aided the implementation of WGBS [48–50], although approximately a tenth of the CpG dinucleotides in the mammalian genome remain refractory to alignment of bisulfite-converted reads. However, Ziller et al. analyzed 42 WGBS data sets across 30 diverse human cell and tissue types and observed only 21.8 % of autosomal CpGs, highlighting the general inefficiency of WGBS [44]. Therefore, more work needs to be done to improve WGBS efficiency to yield relevant information about CpG methylation. Tagmentation-based WGBS (T-WGBS) addresses the problems of WGBS of requiring large DNA quantities, substantial bioinformatics resources, and high sequencing costs by using a hyperactive Tn5 transposase to fragment the DNA and append sequencing adapters in a single step [51, 52]. T-WGBS reliably compares to WGBS, and requires not more than 20 ng of input DNA, and library preparation takes 2 days.

4 Affinity Enrichment-Based Technologies

Two of the most common enrichment approaches include methyl-DNA immunoprecipitation (MeDIP), which uses a monoclonal antibody specific for 5-methylcytosine in the context of denatured DNA [53] and affinity capture of methylated native genomic DNA with MBDCap proteins (Table 1) [54, 55]. Both are proving to be particularly powerful tools for comprehensive profiling of DNA methylation of complex genomes. MECP2 was the first methyl-binding protein used for affinity purification of methylated DNA [56]. MeDIP is the enrichment of methylated regions of immunoprecipitation of denatured, single-stranded genomic DNA fragments with an antibody specific for methylated cytosine and targets regions of low CpG density (e.g., intergenic regions) [57], followed by hybridization to either a tiling array or to a feature microarray, such as a CpG island array [53, 58]. MeDIP is relatively inexpensive and provides reasonably comprehensive genome-wide data, but the resolution is limited and the enrichment is not linearly related to the actual methylation [59]. Array hybridization has mostly been the tool of choice for affinity-enrichment methods in which the input DNA and enriched DNA are labeled with different

fluorescent dyes. However, next-generation sequencing technique is rapidly utilized and preferred as the partner to methylcytosine affinity-enrichment techniques [60].

In contrast to MeDIP, MBDCap strategy captures double-stranded methylated DNA fragments and favors enrichment of CpG-dense regions (e.g., CpG islands) [61]. Higher affinity methyl-binding proteins including multimerized MBD1 domains [62] and protein complexes that contain the short isoform of MBD2 (MBD2b) and MBD311 (the latter approach is called the methylated CpG island recovery assay (MIRA) [54, 63, 64]. Furthermore, MBD family proteins including disease-relevant mutants, in regards to binding preference to methylated regulatory regions, are currently being characterized by several groups [65–67]. More commonly, MeDIP and MBDCap are increasingly coupled to next-generation sequencing. Distinctive genomic regions are commonly interrogated due to bias in the different capture technologies [68]. MBDCap-Seq workflow is similar to WGBS but does not include a bisulfite conversion step. The workflow is as follows. Methylated DNA is captured with MBD protein coupled to streptavidin beads after sonication of genomic DNA (0.2–1 µg). Following capture, a single fraction of bound methylated DNA is eluted at once in a stepwise elution series to enrich different CpG densities. A library is prepared from enriched DNA and it then subjected to high-throughput sequencing. One benefit to using MBDCap-Seq is the relatively low quantities of DNA required that can be obtained from fresh-frozen tissue and formaldehyde-fixed paraffin-embedded tissue [FFPE] [5, 69]. For an accurate interpretation of data, approximately 30 million single-end reads are required. Because MBDCap-Seq captures approximately 5 million methylated CpG sites on fully methylated DNA, it can yield approximately 18 % coverage of the genome. Unfortunately, MBDCap-Seq does not provide single-nucleotide resolution; however, it identifies regions containing multiple methylated CpG sites typically at CpG-rich regions in a readout similar to chromatin immunoprecipitation (ChIP-Seq) [5]. The number of reads mapping to a particular region of the genome depends on the density of methylated CpG sites and thus, MBDCap-Seq is only marginally quantitative [61].

MeDIP and MBDCap, partnered with either array hybridization or sequencing, has been widely used to explore the methylomes of *Arabidopsis thaliana* [7, 8, 70, 71], mouse [72–75], and human cells [68, 76–79]. Another enrichment technique is Agilent's SureSelect Human Methyl-Seq Kit that is based on the solution hybrid selection bisulfite sequencing (SHBS-Seq) approach that allows researchers to analyze over 3.7 million individual CpG sites for their methylation status by enriching genomic sequences with the use of a predesigned SureSelectXT library. Although not discussed here, other target enrichment techniques include

bisulfite padlock probes (BSPPs), SHBS-Seq, array capture bisulfite sequencing (ACBS-Seq), and bisulfite-patch PCR which are briefly described in Lee et al.'s mini review [80]. Overall, affinity-based methods do not result in information on individual CpG dinucleotides and require substantial experimental or bioinformatics adjustment for varying CpG density at different regions of the genome. However, affinity-based methods allow for rapid and efficient genome-wide assessment of DNA methylation.

5 Reduced Representative Bisulfite Sequencing (RRBS)

Reduced representative bisulfite sequencing is an efficient and high-throughput technique that is suitable for obtaining information from regions of high CpG content (e.g., CpG islands) and information about sequences outside CpG-rich regions [81, 82]. Despite being less comprehensive than WGBS [83], RRBS will generate a single-base pair resolution DNA methylation map nonetheless. RRBS utilizes the cutting pattern of methylation-insensitive restriction enzyme, MspI (C[^]CGG), to systematically digest and enrich genomic DNA (0.01–0.03 µg) at regions with moderate to high CpG density, such as CpG islands, followed by DNA size fractionation (Table 1). Every fragment produced by MspI digestion will contain DNA methylation information for at least one CpG dinucleotide, as opposed to whole-genome bisulfite sequencing [84]. Initially, RRBS relied on preparative gel electrophoresis for size fractionation but this is laborious and difficult to automate. However, a new “gel-free” multiplexed RRBS protocol, called mRRBS, has been developed where MspI fragments <40 bp are removed by a single clean-up protocol, followed by bisulfite conversion, PCR, and cluster amplification on an Illumina flowcell (all of which select against large fragments) which results in a similar size distribution of MspI fragments [85]. mRRBS takes advantage of increased sequencing throughput and the ability to barcode sequencing libraries, allowing for processing of samples in batches of 96 or more. Approximately 10 million sequencing reads are required for the downstream analysis of RRBS data sets, leading to approximately 3.7 % actual coverage of the approximately 28 million CpG dinucleotides genome wide [5]. Furthermore, the mRRBS protocol was simplified and streamlined by eliminating several steps of the original RRBS protocol. For example, the addition of Klenow fragment (3' → 5' exo-) directly to the post-digested MspI/DNA mixture aids in end repair, and adding the A-tail minimizes the cleanup steps and loss of material [85]. The replacement of multiple phenol:chloroform steps described in the original RRBS method [81, 82] with a single solid-phase reversible immobilization (SPRI) bead cleanup after adapter ligation also helps improve the ease and efficiency of the library generation process [85].

Another issue with RRBS is the high apparent cluster density, poor DNA cluster localization, and significant data loss during sequencing on the Illumina HiSeq 2000 due to the fact that the MspI recognition cut site (C[^]CGG) creates fragments that will make the first three bases of every read nonrandom. Illumina addresses this issue with a sequencing protocol called “dark sequencing” in which imaging and cluster localization are delayed until the fourth cycle of sequencing chemistry, beyond the extent of bias from the MspI cut site to improve performance and increase coverage [85]. Without dark sequencing, RRBS libraries cannot be multiplexed with other RRBS libraries but must be multiplexed with high-complexity libraries such as RNA-seq. However, one drawback of dark sequencing is that it requires custom programming on the Illumina machine.

RRBS has been used to provide detailed information about lung cancer [86], zebrafish brain [87], muscle cells [88], 82 different human cell lines and tissues [89], embryonic stem cells [90], and induced pluripotent stem cells [90]. RRBS targets bisulfite sequencing to an enriched population of the genome while retaining single-nucleotide resolution. One of the main advantages of RRBS is that it is more cost effective than WGBS. One disadvantage of RRBS is a lack of coverage at intergenic and distal regulatory elements since RRBS data are restricted to regions with moderate to high CpG density and enriched for promoter-associated CpG islands [5]. Similar challenges to WGBS are present in RRBS data analysis with a similar level of expertise and extensive bioinformatic work; however, RRBS data can be processed using similar WGBS pipelines [91, 92].

6 Infinium HumanMethylation450 BeadChip (450 K)

What makes the Infinium HumanMethylation450 BeadChip distinct from the other methylation technologies described above is that it does not depend on capture or enrichment, use of restriction enzymes or high-throughput sequencing for data generation. The 450 K assay detects cytosine methylation at CpG islands based on highly multiplexed genotyping of bisulfite-converted genomic DNA (0.5–1 µg). Converted genomic DNA is hybridized to arrays that contain two site-specific probes, one designed for the methylated locus (cytosine) and another for the unmethylated locus (converted to uracil). Single-base extension of the probes incorporates a labeled ddNTP that is fluorescently stained. The array is scanned for the detection of the ratio of fluorescent signal arising from the unmethylated probe compared with the methylated probe, allowing the level of methylation to be determined (Table 1). 450 K is based on the previous 27 K Illumina Infinium Methylation BeadChip that allows for the analyses of methylation states for 27,000 CpGs which is limited to only 1 % of all CpGs. Now, 450 K

allows researchers to interrogate 482,422 cytosines across the human genome, which represents only approximately 1.7 % of all CpG sites in the human genome, substantially less than other methods [5]. However, 450 K covers 99 % of RefSeq genes, with an average of 17 CpG sites per gene region distributed across the promoter, 5'UTR, first exon, gene body, and 3'UTR. Also, these sites are enriched for CpG (99.3 %) residues and almost half (>41 %, approximately 197,790 CpG sites) of the probes on the array cover intergenic regions, such as bioinformatically predicted enhancers, DNaseI hypersensitive sites, and validated differentially methylated regions (DMR) [93, 94]. Besides being able to use a small amount of DNA (~0.5 µg), 450 K can be performed on both fresh-frozen and FFPE tissue, making it suitable for clinical samples. Currently, methods are now being optimized to enable smaller amounts (0.2 µg) to be profiled efficiently [95]. This BeadChip enables up to 96 samples to be run in parallel because it has a 12-sample per array format compatible with automation. 450 K has become the preferred method of choice for genome-wide DNA methylation analyses of large studies because Illumina's assay provides quantitative methylation measurement at the single-CpG-site level, requires a low amount of input material, and is cost effective. However, there are drawbacks to using 450 K technology. First, the design is not hypothesis neutral in that the design is heavily biased due to preselection of probes that interrogate only certain CpG sites that have been previously identified in methylation-based assays [5]. Second, co-methylation is assumed where CpG sites located adjacent to those interrogated by the probes will be similarly un/methylated [96]. Third, the methylome platform is only available for humans. Finally, the two types of probes on the array have behavioral differences and single nucleotide polymorphisms may be affected by the filtering of probes, which need to be factored into the data analysis platform [97]. Furthermore, technical problems with the probe design and analysis methods used by the Illumina 450 K array have been reported [98, 99] and a recent review addressed common problems associated with using the 450 K array for epigenetics research [100].

7 Genome-Wide Methylation and Ovarian Cancer

Changes in transcriptional output and altered genomic stability are commonly due to aberrant DNA methylation in cancer. From proliferation rates, response to extracellular signals, and response to DNA damage, cancer cells undergo a multitude of stepwise and cumulative methylation changes that affect a host of biological functions. A complete understanding of the role of methylation in the mechanisms responsible for its establishment and maintenance, and its cross talk with other components of cellular machinery

remains crucial to understanding how epigenetic changes contribute to cancer progression. The Cancer Genome Atlas consortium (TCGA; <http://cancergenome.nih.gov>) harbors massive data sets for over 200 human cancer types. Out of the approximately 8,000 cancer methylomes generated thus far, 7,500 samples of these used the 450 K methodology [101–105]. Only two deeply sequenced WGBS of primary tumors have been completed to date [39, 44], three shallowly sequenced WGBS tumors (all colon) [43], and approximately 55 RRBS analyses, most of which investigate primary blood cancers [5]. The number of WGBS cancer methylomes is expected to increase as sequencing costs decrease and bioinformatics analyses improve.

One of the over 200 human cancer types listed in the TCGA is epithelial ovarian cancer (EOC), the most lethal gynecologic and fifth most deadly, malignancy in US women, having an average 5-year survival rate of 46 % which is unchanged since several decades [106, 107]. The failure to detect early-stage tumors (with less than 10 % of initial diagnosis being for localized disease) is the major cause of fatality, plus the eventual near-universal onset of resistance to standard chemotherapies (typically platinum combined with taxane [108, 109]). Most ovarian cancer patients respond to surgical debulking [110], followed by chemotherapy (typically a platinum/taxane combination regimen) despite late-stage diagnoses [111]. Unfortunately, the majority of initially responsive, late-stage ovarian cancer patients relapse to a fully drug-resistant condition [112]. The need for both prognostic and predictive biomarkers is urgent to assign adjuvant treatment strategies, enable patient stratification, and improve outcome.

Researchers have analyzed several EOC marker classes including copy number alterations [113], gene expression signatures [114] or values [115], protein expression [116–123], mutations [124], and functional assays [125]. Combinations of methylated DNA markers (i.e., “methylation signatures”), in addition to serum proteins, are highly stable, amenable to very sensitive detection by fluorescence-based PCR assays, and represent another approach for enhancing sensitivity and specificity of detection/prognostication [126, 127]. Due to the frequent association of distinct patterns of aberrant epigenetic modifications with ovarian cancer etiology, these sequences represent potential disease biomarkers. Interestingly, distinct methylation patterns delineate specific ovarian cancer subtypes (e.g., serous adenocarcinomas, clear cell tumors) or disease with high vs. low malignant potential [128, 129]. Consequently, such methylation biomarkers will likely be valuable adjuvants to histopathological methods of evaluating ovarian tumor grade and subtype. Single-methylation markers have been shown prognostic value including *HOXA11* [130], *ASS1* [131], *TUSC3* [132], and *DLEC1* [133]. Recently, methylation of specific CpGs in the *ZIC1/4* (hedgehog pathway) promoter [134]

and 7 members of the Wnt-pathway [135] have been shown to be associated with poor progression free survival and progression free survival, respectively.

With the advent of microarray technology and high-throughput DNA sequencing, those global, genome-wide studies are now possible. By performing genome-wide analysis of DNA methylation, gene expression, and histone modifications, it is feasible to identify specific biological pathways or processes responsible for advanced tumor progression [136]. Toward that objective, in 2001, our group pioneered one such method, differential methylation hybridization, demonstrating specific methylation “signatures” distinct to ovarian cancer [137]; in later studies, we successfully identified two distinct 182- and 112- loci panels associated with disease recurrence and short vs. long progression-free survival [138, 139]. Likewise, a panel of 198 CG dinucleotides (with 47 hypermethylated and 151 hypomethylated) was found to be significantly correlated with age-associated ovarian cancer [140]. Similar approaches were used to identify 66 methylated genes distinctive to serous EOC tumors and cells and a DNA methylation profile for doxorubicin resistance [141]. Also, microarray technology was used to identify 397 genes differentially methylated between high-grade and low-grade EOC [142, 143]. Using the previously mentioned 27 K array, promoter methylation of one marker, IFFO1, was shown to be frequently methylated in ovarian tumors but rarely detected in the blood of normal controls [144]. We have also used the 27 K array to examine CpG island DNA methylation from ovarian cancer tumor biopsies and found that the number of demethylated genes (in patients responding to a DNA methylation inhibitor) was greater in core biopsies obtained from patients with progression-free survival (PFS) more than 6 versus less than 6 months (311 vs. 244 genes) [145]. In order to analyze the methylomic profiles of ovarian tumor-initiating cells in ovarian cancer cell lines and patient tumor samples, Liao et al. utilized the 27 K array and found *ATG4A* and *HIST1H2BN* to be hypomethylated in ovarian tumor-initiating cells [146], and we further demonstrated that *HIST1H2BN* methylation was a significant and independent predictor of PFS and overall survival.

Other high-throughput, global DNA methylation assessments have been used to determine widespread genome-wide de novo methylation in ovarian cancer cells [147]. For example, 68 methylated genes were associated with disease subtypes and tumor histology in ovarian cancer cell lines and tumors [141]. In agreement with another study [148], our group performed a chromatin immunoprecipitation-hybridized microarray (ChIP-chip) to examine suppression of the TGF-beta pathway in ovarian cancer, successfully identifying *FBXO32* as a tumor-suppressor gene down-regulated by that specific cascade [149, 150]. Global assessments of

multiple epigenetic or genomic determinations can be integrated to achieve a larger perspective on cancer cell pathways, thus allowing greater insight into cellular processes impacted by aberrant chromatin structure. In one such study, using a cell culture model of EOC drug resistance, our group integrated both DNA methylation and gene expression assessments of sensitive vs. resistant clones of the EOC cell line A2780 to identify a number of pathways associated with the chemoresistant phenotype [151]. Another group similarly derived computationally, epigenetically associated “networks” involved in protein-protein interactions and pathways associated with EOC [152]. Our lab is currently using NGS approaches to examine epigenetic changes in EOC [134, 153].

As mentioned earlier, the field of methylome interrogation is moving from away from locus-specific analyses and microarray technology to whole-genome approaches that integrate next-generation sequencing. To date, no studies using WGBS have been completed to explore ovarian cancer. However, many others including our lab are currently pursuing WGBS in ovarian cancer. Like WGBS, RRBS is also a technique that has yet to be applied in ovarian cancer. As for affinity enrichment-based technologies, three studies have recently been published to date, using MeDIP-Chip and MBDCap-Seq in ovarian cancer cells. The first study by Gloss et al. used MeDIP-Chip to identify epigenetic-based biomarkers in A2780 and CaOV3 ovarian cancer cell lines [154]. They confirmed a panel of six gene promoters (*ARMCX1*, *ICAM4*, *LOC134466*, *PEG3*, *PYCARD*, and *SGNE1*) where hypermethylation discriminated 27 serous ovarian cancer clinical samples versus 12 normal ovarian surface epithelial cells. In the second study, Yu et al. used MBDCap-Seq to analyze the genome-wide DNA methylation profile of cisplatin sensitive ovarian cancer cell line A2780 and its isogenic derivative resistant line A2780CP [155]. They identified 1224 hypermethylated and 1216 hypomethylated DMRs in A2780CP compared to A2780. More specifically, methylation specific PCR and bisulfite sequencing confirmed hypermethylation of *PTK6*, *PRKCE*, and *BCL2L1* in A2780 compared with A2780CP. In the third study, we examined DNA methylation in ovarian cancer using MBDCap-Seq to screen 75 malignant and 26 normal or benign ovarian tissues. More specifically, we examined the association between DMRs and PFS by analyzing methylation regions closer to the transcription start site and found 63 DMRs significantly associated with PFS [134]. All of these studies have provided important information about potential ovarian cancer diagnostic biomarkers and differing methylation signatures that can possibly predict a patient’s response to demethylating chemotherapy and the development of platinum resistance.

Thus far, three recent studies have been published using the 450 K technique to profile the ovarian cancer methylome.

In the first study, Zeller et al. used isogenic, cisplatin-sensitive/resistant ovarian cancer cell lines and induced resensitization with demethylating agents to successfully identify 4092 hypermethylated genes in chemoresistant A2780 compared with the parental-sensitive A2780 cell line [156]. More specifically, they found nine genes (*ARHGDIB*, *ARMCX2*, *COL1A*, *FLNA*, *FLNC*, *MEST*, *MLH1*, *NTS*, and *PSMB9*) to have acquired methylation in ovarian tumors at relapse following chemotherapy or chemoresistant cell lines derived at the time of patient relapse. In the second study, Yamaguchi et al. used 450 K to obtain genome-wide methylation data for 14 clear cell ovarian carcinoma, 32 non-clear cell carcinoma, and 4 corresponding normal cell lines to determine how methylation profiles differ between cells of different histological derivations of ovarian cancer [157]. Categorical and pathway analyses indicated that the clear cell carcinoma-specific hypomethylated genes were involved in response to stress and many contain hepatocyte nuclear factor 1 binding sites, while the clear cell carcinoma-specific hypermethylated genes included members of the estrogen receptor alpha network and genes involved in tumor development. In the last study, Cicek et al. assayed fresh frozen tumor DNA from 485 clear cell ovarian cancer samples on 450 K BeadChips [158]. They identified a clear cell ovarian cancer tumor methylation profile, highlighting 22 CpG loci associated with nine genes (*VWA1*, *FOXP1*, *FGFRL1*, *LINC00340*, *KCNH2*, *ANK1*, *ATXN2*, *NDRG21*, and *SLC16A11*). Similar to the vast number of TCGA studies, 450 K proves to be the most accessible and successful genome-wide methylation interrogation technique in the field of ovarian cancer. Currently, we are using WGBS, MBDCap-Seq, RRBS, and 450 K on the same samples to compare and contrast between these technologies in order to determine variations among methylation signatures in platinum-sensitive and platinum-resistant ovarian cancer cells. These results are being validated using bisulfite pyrosequencing, a quantitative method for DNA methylation analysis.

Malignant cells exhibit overall genomic hypomethylation (primarily in repeat elements and pericentromeric regions) and simultaneously hypermethylation of normally protected CpG islands [159, 160], as compared to normal cells. These aberrant methylation patterns may be used as biomarkers for diagnosis and prognosis of cancer [161]. Epigenetically silenced genes can be reactivated with treatment of inhibitors of DNA methylation and histone deacetylation to restore normal gene function [162, 163]. The demethylating agent azacitidine and its deoxy derivative, decitabine, have been approved for treating hematological malignancies including MDS and AML [164]. We have recently shown that low-dose decitabine administered before carboplatin in

platinum-resistant ovarian cancer patients induced a 35 % objective response rate and PFS of 10.2 months [145]. Currently, we are testing SGI-110 (Astex Pharmaceuticals, Inc.), a second-generation DNA-hypomethylating drug in vitro, in vivo, and in ovarian cancer clinical trials [165]. SGI-110 benefits from having a longer pharmacokinetic half-life than decitabine, due to its prodrug form, prohibiting SGI-110 from cytidine deamination.

8 Summary

Next-generation sequencing (NGS) technologies are currently being used to map the changes in DNA methylation across many cancer types. As DNA methylation changes are not restricted to CpG island promoters, but occur genome wide, including genic and intergenic regions which house distal regulatory elements like enhancers and noncoding RNA genes, NGS allows us to interrogate all these regions. NGS can be used to stratify tumor types by the methylome map because most cancer subtypes do not behave as a single entity in response to current therapies. The methylome map will be informative to identify patients that would most benefit from epigenetic therapy. Challenges to genome-wide methylation detection technology include sensitive quantification of DNA methylation from degraded and minute quantities of DNA, like from FFPE tissue, and high-throughput DNA methylation mapping of single cells. The choice of which whole-genome methylation approach to use will depend on the quantity and quality of DNA available, bioinformatics expertise, cost, regard of the question being asked, and the required coverage of the genome. Future analyses will reveal the specific DNA methylation signatures that are either associated or drive the survival capacity of cancer cells. Also, methods for identification and mapping of 5-hydroxymethylcytosine and developments in DNA sequencing technologies are expected to augment our current understanding of epigenomics.

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Recent Progress in the Discovery of Epigenetic Inhibitors for the Treatment of Cancer

Sharad K. Verma

Abstract

Epigenetics investigates heritable changes in gene transcription that do not involve a change in DNA sequence, and an increased understanding in the role of epigenetic misregulation as a key contributor to cancer has triggered the development of epigenetic targeted cancer therapies. Among these include efforts around a class of enzymes known as histone methyltransferases (HMTs). The level of interest in the development of HMT inhibitors as a class of anticancer agents has significantly grown beyond academic settings, and in the last 5 years whole research groups from biotech and big pharma have been dedicated to this area. There are now multiple reports describing small-molecule HMT inhibitors, including chemical probes and drug candidates entering the clinic as first-in-class agents. Recent progress in this emerging area is the topic of this chapter.

Key words Epigenetics, Histone methyltransferases, Nucleosome, Chromatin, Posttranslational modification, *S*-Adenosylmethionine (SAM)

1 Introduction

As cancer is the leading cause of death worldwide (and second leading cause in the USA, exceeded only by heart disease) there remains much work to do with respect to the identification and development of new and more effective cancer therapies [1]. In recent years, there has been much progress in understanding the role of epigenetic misregulation as a key contributor to cancerous phenotypes, and this now provides another opportunity for the development of targeted cancer therapies. Investigations in this emerging area have already identified several drug targets that can lead to the selective activation and inhibition of target genes, and so epigenetics has become a rapidly growing field. Recent advances have uncovered the roles of epigenetic regulatory factors including, but not limited to, DNA methylation, histone modification, chromatin remodeling, and gene transcription, with respect to the onset and progression of cancer. Numerous studies suggest that aberrant,

posttranslational, histone modifications play an important role in the initiation and progression of several diseases, and accordingly enzymes which promote these posttranslational modifications have become targets of high interest [2–5]. Among these is a class of enzymes called histone methyltransferases (HMTs), which are a group of enzymes that catalyze the transfer of a methyl group from the cofactor *S*-adenosylmethionine (SAM) to the lysine and arginine residues of histone tails, and this posttranslational modification is a key event in maintaining gene expression patterns [6, 7]. The level of interest in the development of HMT inhibitors as anti-cancer agents has significantly grown beyond academic settings, as in the last 5 years whole research groups from biotech and big pharma have been dedicated to this area. There are now multiple reports from these groups describing small molecule HMT inhibitors, including chemical probes and drug candidates entering the clinic as first-in-class agents. The most advanced programs are those reporting small-molecule inhibitors of the lysine histone methyltransferases Enhancer of Zeste Homolog 2 (EZH2) and Disruptor of Telomeric Silencing-1 (DOT1L), which are the subject of this chapter.

2 EZH2 as a Target

The histone lysine methyltransferase EZH2 (Enhancer of Zeste Homolog 2) has been implicated as a key component in the progression of cancer, and inhibition of aberrant EZH2 activity is expected to attenuate tumorigenesis resulting from misregulated gene transcription [8]. From a mechanistic standpoint, EZH2 is the catalytic component of the multi-protein complex PRC2 (polycomb repressive complex 2) and catalyzes the methylation of the ϵ -NH₂ group of histone H3 lysine 27 (H3K27) in the nucleosome substrate via transfer of a methyl group from the cofactor *S*-(*S'*-adenosyl)-L-methionine (SAM). Repetition of this process in duplicate leads to trimethylation of H3K27 (H3K27me₃) and the formation of *S*-(*S'*-adenosyl)-L-homocysteine (SAH), and subsequent transcriptional silencing of target genes (Fig. 1). From a structural standpoint, the EZH2 subunit lacks enzyme function on its own but instead must be complexed with at least two of its noncatalytic partners (EED and SUZ12) to confer robust methyltransferase activity (Fig. 2). Within EZH2, methyl transfer occurs in a conserved catalytic subunit called the SET (Suppressor of Variegation Enhancer of Zeste Trithorax) domain, which provides the methyltransferase-active site [9, 10]. Several studies show that elevated levels of EZH2 correlate with silencing of EZH2 target genes and poor prognosis in solid tumors including prostate, breast, kidney, and lung [11–16]. Somatic activating mutations in the catalytic domain of EZH2 have been identified in follicular lymphoma (FL),

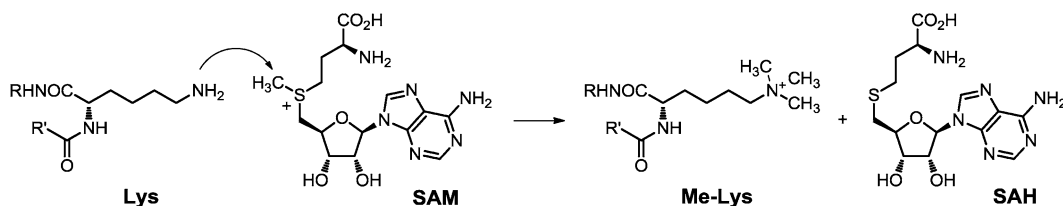


Fig. 1 Lysine methylation and oncogenesis. Methylation of histone-bound lysine by SAM (*S*-(*S'*-adenosyl)-L-methionine) is catalyzed by a histone lysine methyltransferase and generates a methylated lysine and the by-product SAH (*S*-(*S'*-adenosyl)-L-homocysteine) (SAH). EZH2 as part of the PRC2 complex for example catalyzes the methylation of histone 3 lysine 27 (H3K27) using SAM as the cofactor to generate H3K27me and SAH. Subsequent methylations can generate H3K27me₃. Similarly, DOT1L catalyzes trimethylation of H3K79 and can generate the H3K79me₃ phenotype. High levels of H3K79me₃ are a driver of proliferation in mixed-lineage leukemia 1 protein (MLL)-rearranged leukemia (MLL-r)



Fig. 2 The PRC2 complex. EZH2 is part of the PRC2 (polycomb repressive 2 complex) and must partner with the protein subunits EED, and SUZ12, to confer robust methyltransferase activity

and GCB diffuse large B cell lymphoma (DLBCL), leading to increased H3K27me₃ [17–23]. Accordingly, EZH2 has become an epigenetic target of high interest, with multiple research groups dedicating efforts towards identifying potent, selective, small-molecule inhibitors.

In reviewing small-molecule inhibitors of EZH2, it is important to first devote some discussion to 3-deazaneplanocin (DZNep, **1**; Fig. 3), which has been reported as an EZH2 inhibitor tool compound [24]. While DZNep has been reported as a tool compound and shown to deplete PRC2 subunits in cancer cell lines and

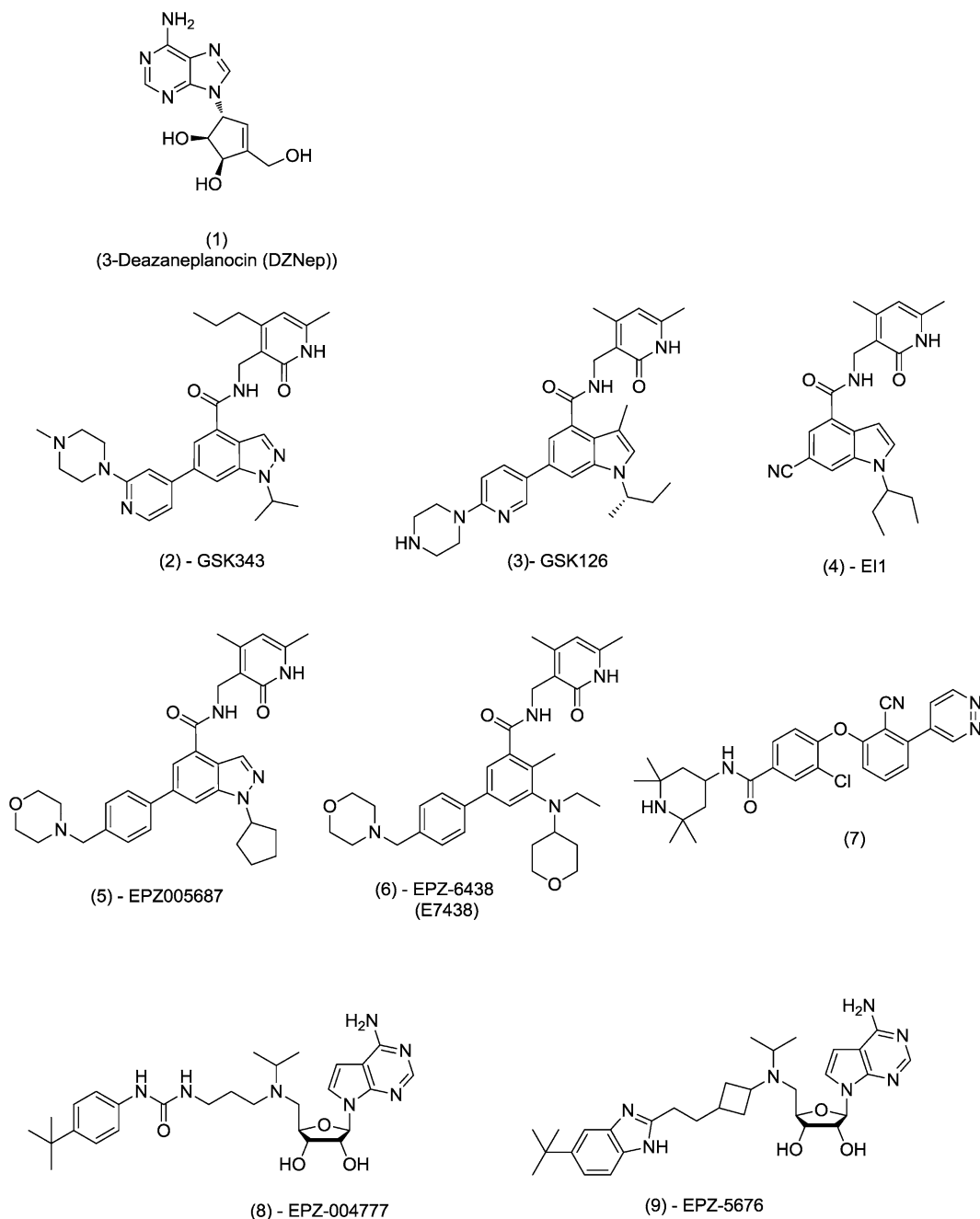


Fig. 3 Inhibitors of EZH2 (2–7) and (8–9) DOT1L

reactivate PRC2-silenced genes, it is in fact not a direct, on-target inhibitor of EZH2. Rather, DZNep is an inhibitor of *S*-adenosylhomocysteine (SAH) hydrolase and is thought to inhibit and cause the degradation of the PRC2 complex by way of an

indirect mechanism as a result of increased intracellular concentrations of SAH, an inhibitory byproduct of cellular methyltransferase reactions [25, 26]. Given the ability of DZNep to reduce methylation at multiple histone residues targeted by protein methyltransferases other than EZH2, the interpretation of cellular phenotypes resulting from DZNep-mediated inhibition is severely complicated. Furthermore, given the nonspecific nature of DZNep-mediated target inhibition, there are limitations to its utility as a clinical agent.

Over the last 2 years, research groups at GSK, Epizyme, Novartis, and Constellation have reported the structures of on-target small-molecule inhibitors of EZH2 (Fig. 3). Immediately apparent is the commonality of a pyridone amide scaffold. For each compound shown, high-throughput screening was the source of the initial compound hit that was subsequently optimized, though not necessarily the same hit in each case. This may indicate both the importance of this scaffold in EZH2 inhibition, and the challenge in finding tractable, chemically diverse starting points. Researchers at GSK reported GSK343 (compound 2) to demonstrate highly potent inhibition of EZH2 in both biochemical and cellular activity assays (EZH2 K_i app = 1.2 nM, H3K27me3 cell IC_{50} = 174 nM (HCC1806 cells)), as well as antiproliferative effects [27]. Further analysis of GSK343 indicated that this compound was competitive with the cofactor SAM, and noncompetitive with peptide or nucleosome substrates, and displayed high selectivity (>1,000 fold) for EZH2 when tested against a panel of methyltransferases, and against a panel of non-epigenetic targets including kinases, ion channels, 7-TMs, etc. The only exception to high selectivity was histone lysine methyltransferase EZH1, which is highly homologous to EZH2, having 76 % sequence identity overall, and 96 % sequence identity in the catalytic SET domain. GSK343 displayed 60-fold selectivity against EZH1, and this level of selectivity against EZH1 was generally representative for compounds reported from this class. A homology model of EZH2 was described with a proposed binding mode of GSK343 in the SET domain. However the specific structural factors responsible for the selectivity over EZH1 are unclear. GSK343 is a true on-target EZH2 inhibitor which can be used as a chemical probe to help decipher the biology around EZH2. GSK343 has been made available to the larger research community through an agreement with the Structural Genomics Consortium (SGC) Toronto, and can be obtained from this group [28]. A more advanced compound, GSK126 (compound 3), in addition to being highly potent and selective for EZH2, was reported to display robust activity in mouse xenograft models of diffuse large B cell lymphoma (DLBCL) bearing EZH2-activating mutations [29]. GSK126 demonstrated a decrease in global H3K27me3 and concomitant up-regulation of EZH2 target genes in a dose-dependent fashion (daily (QD) intraperitoneal (IP) dosing for 10 days). In 2013 researchers at Novartis reported a closely

related compound, referred to as EI1 (compound **4**), as a chemical probe for EZH2 [30]. A high-throughput screen (using recombinant 5-component PRC2 complex) was reported to provide the chemical scaffold used for the design of EI1. Similar to GSK343 and GSK126, EI1 is also a highly potent (EZH2 K_i = 13 nM), selective (>10,000-fold for EZH2 over other histone methyltransferases, and ~90-fold selectivity for EZH1) small-molecule inhibitor which displays a competitive mode of inhibition with the cofactor SAM. In biological studies, EI1 was also reported to inhibit cellular H3K27 methylation, activate EZH2 target gene expression, and selectively inhibit the growth of DLBCL cell lines carrying EZH2-activating mutations, in a dose-dependent manner. Researchers at Epizyme reported EPZ005687 (compound **5**) as a potent inhibitor of EZH2 (K_i = 24 nM). Biochemical mechanism of inhibition studies showed that EPZ005687 was competitive with the cofactor SAM, and non-competitive with oligonucleosome substrate [31]. EPZ005687 was reported to have >500-fold selectivity against 15 other protein methyltransferases, and 50-fold selectivity against EZH1. In addition, EPZ005687 was evaluated against 77 human ion channels and G protein-coupled receptors (GPCRs) in radioligand displacement assays, and displayed high selectivity overall. EPZ005687 specifically and potently inhibited H3K27me3 in various lymphoma cells, and this translated into apoptotic cell killing in heterozygous mutant cells, but with minimal effects on the proliferation of wild-type cells. Furthermore, EPZ005687 induced transcriptional activation of EZH2 target genes in the same EZH2 mutant cell lines (WSU-DLCL2), further indicating that small-molecule inhibition of EZH2 can lead to increased expression of known repressed targets of EZH2 and subsequent inhibition of EZH2 promoted tumorigenesis. Accordingly, EPZ005687 represents another valuable in vitro chemical probe. The same group reported EPZ-6438 (compound **6**) as a highly potent, selective, inhibitor of EZH2, which demonstrated robust in vivo activity in human tumor xenograft models of DLBCL in mice following either oral or intraperitoneal dosing. Researchers at Constellation Pharmaceuticals have published work detailing a novel series of EZH2 inhibitors [32]. Specifically, the series entailed a linear template containing 2–3 aromatics rings terminating in, for example, a tetramethylpiperidine or tetramethyltetrahydropyran moiety. Among the compounds in this series was compound **7** (Fig. 3), which was reported as a highly potent (EZH2_{wt} IC₅₀ = 0.032 nM), selective, inhibitor of EZH2 [33]. Compound **7** was obtained as a result of a lead optimization campaign stemming from a hit from a high-throughput screen (HTS) of an unbiased library of 150,000 compounds. The authors describe in great detail the hit triage process following the HTS screen, and optimization campaign, which culminated in the identification of compound **7**. While compound

7 strongly inhibited EZH2 (with concomitant global reduction of H3K27me3 and selectively affected cell growth and gene expression in GCB-DLBCL models), cellular potency was notably weaker. The disparity between the biochemical and cellular potency is attributed to a combination of poor permeability ($\text{Caco-2 } P_{\text{app}} = 0.4 \times 10^{-6} \text{ cm/s}$) and high endogenous SAM concentrations. However, given that the previously reported small-molecule EZH2 inhibitors occupy a narrow chemical space, the identification of compound 7 represents a highly notable achievement. Overall, there has been substantial progress in the identification and development of potent, selective, on target inhibitors of EZH2. The most advanced of these are E7438 and GSK126, which are currently in Phase 1 clinical trials [34, 35]. It will be of interest to follow the progress of these compounds, as they will serve to clinically validate EZH2 as a target, and the methyltransferase target class in general.

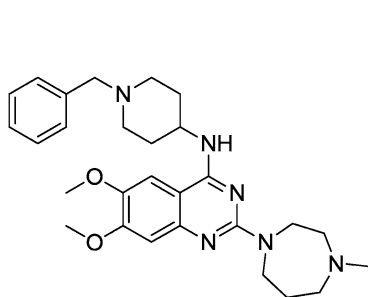
3 DOT1L as a Target

DOT1L is a lysine methyltransferase which catalyzes the mono-, di-, and tri-methylation of H3K79, and methylated H3K79 is associated with active gene transcription (Fig. 1). In terms of biological activity, DOT1L has been reported to be a required driver of proliferation in mixed lineage leukemia 1 protein (MLL)-rearranged leukemia (MLL-r) [36, 37]. From a structural standpoint, DOT1L is unique in that it does not contain the SET domain, which is the common catalytic domain of lysine methyltransferases [38]. In 2011, researchers at Epizyme reported EPZ004777 (compound 8, Fig. 4) as a highly potent ($\text{Ki } 300 \text{ pM}$), selective (1,200-fold over other HMTs), inhibitor of DOT1L [37]. EPZ004777 selectively blocked global H3K79 methylation in leukemia cells and demonstrated robust activity in a mouse model of mixed-lineage leukemia. In this *in vivo* study, EPZ004777 was administered by continuous administration at three concentrations (50, 100, and 150 mg/mL) for 14 days, and survival was monitored over the course of 30 days. Results indicated that statistically significant survival advantages over the vehicle-dosed animals were achieved at all doses administered, and the extent of survival increased with increasing dose of the compound. More notably, these results represented the first *in vivo* evidence that selective inhibition of an HMT enzyme could lead to antitumor efficacy. Based on this, and other data, a phase 1, open-label, dose-escalation, and expanded cohort study with a related compound, EPZ-5676 (compound 9), was initiated in treatment relapsed/refractory patients with leukemias involving translocation of the MLL gene (at 11q23) for advanced hematologic malignancies [39, 40].

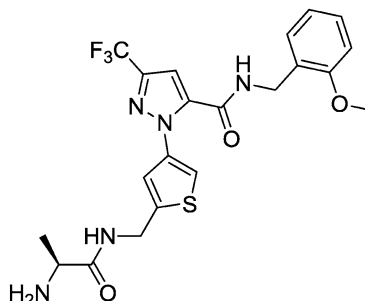
PRC2 targeting stapled peptides :

(10), $\text{SN}_L\text{FSSNRXKILXRTEILNQEWKQRRRIQPV}$

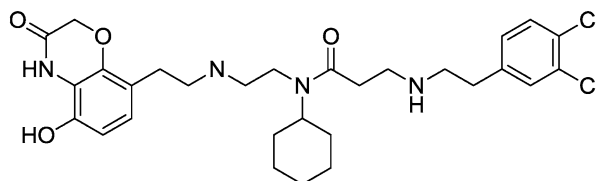
(11), $\text{SN}_L\text{FSSNRQKILERTXILNXEWKQRRRIQPV}$



(12), BIX-01294
G9a IC_{50} = 0.2-1.7 μM



(13), Methylgene compound
CARM1 IC_{50} = 60 nM



(14), AZ505
SMYD2 IC_{50} = 120 nM

Fig. 4 Sequence of stapled peptides reported to inhibit PRC2 activity, and chemical structures of examples of direct, small-molecule inhibitors of HMTs

4 Challenges and Future Directions

There are certainly notable challenges to consider with respect to the development of small molecule inhibitors of HMTs. In the case of EZH2, chief among these is a lack of structural information to guide design efforts. As EZH2 is active only as part of a multi-component complex, this creates considerable challenges with respect to characterization by X-ray crystallography and/or biophysical methods. Accordingly, structure-based design approaches for small-molecule EZH2 inhibitors have thus far relied on homology models, which have inherent drawbacks. The small-molecule EZH2 inhibitors presented herein are proposed to bind in the SAM pocket of the EZH2 SET domain based on cumulative data from mode of inhibition studies (which indicate they are SAM-competitive), binding studies, and SAR results. However, it is certainly possible that these inhibitors bind elsewhere and compete

with SAM for binding by perturbation of the pocket. Although reports of highly potent, selective, on-target EZH2 inhibitors have only begun to appear during the last year, it is interesting to note that the most advanced compounds produced from three different laboratories have the same pyridone-amide pharmacophore. This privileged inhibitor template may indeed lead to clinically useful anticancer agents, but its prevalence in the literature may be indicative of the challenge to discover novel entities going forward. However the recent result reported by Constellation provides promise that small-molecule inhibitors of EZH2 may not be restricted to compounds bearing the pyridone-amide scaffold present in compounds **2–6**. An alternative strategy for selective targeting of EZH2 methyltransferase activity by disrupting the interaction of EZH2 and EED with a hydrocarbon-stapled peptide that mimics the α -helical EED-binding domain of EZH2 was recently reported by researchers at Harvard University [41]. By targeting the interaction between EZH2 and EED, which is required for enzymatic activity, this approach would disable the PRC2 complex in cancer and thereby inhibit unrestrained cell proliferation. Specifically, stapled peptides of variable length were designed on the basis of the α -helical, EED-binding domain of EZH2. The essential α -helical domain of EZH2 (residues 40–68) that engages EED established the basis for designing hydrocarbon-stapled derivatives to disrupt the specific protein interaction. From these efforts, cell-permeable analogs which effectively targeted EED in situ and dissociated the EZH2–EED complex were reported (compounds **10** and **11**, binding affinities (K_D) of 333 nM and 264 nM, respectively). Likewise, impaired function of PRC2 (as reflected by reduction of the H3K27me3 and EZH2 protein levels in MLL-AF9 leukemia cells) and increased expression of PRC2-regulated cell differentiation marker genes were also reported. Accordingly, dismantling the PRC2 complex itself through disruption of protein interactions may offer a complementary approach for epigenetic targeting.

In summary, the recent results in targeting histone methyltransferases (HMTs) as a class of epigenetic enzymes indicate the potential for the development of targeted therapeutic agents. In this context, the recent molecules reported are perceived as being analogous to the kinase inhibitor “tool” that were made available to the chemical biology community over 20 years ago, which demonstrated the impact of selective kinase inhibition on cancer [7]. As deregulation of H3K27 seems to be a unifying component in a number of malignancies, the development of EZH2-targeting agents will play a prominent role in validating the HMT target class and perhaps epigenetic based therapies in general. The recent progress made with the identification and evaluation of the compounds shown in Fig. 3 as highly potent, selective, on-target inhibitors of EZH2 [42] and DOT1L, respectively, clearly distinguish them from compounds like DZNep and other natural

product-based compounds. These results have laid the foundation for the exploration of HMT inhibitors in general, as a viable therapeutic approach for the treatment of specific cancers. Reports which disclose small-molecule inhibitors of several HMTs including CARM1, G9a/GLP, and Smyd2 (compounds **12–14**) are perceived to serve as fertile areas for future development [43–47]. It is anticipated that epigenetic targeted therapies will continue to emerge as a key therapeutic modality in “big pharma,” wherein protein methyltransferases (as well as demethylases, and bromo-domain readers) will be targeted alongside other broadly acting epigenetic therapies such as HDAC and DNMT inhibitors.

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At the Crossroad Between Obesity and Gastric Cancer

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Abstract

Obesity has reached epidemic proportions worldwide with disproportionate prevalence in different communities and ethnic groups. Recently, the American Medical Association recognized obesity as a disease, which is a significant milestone that opens the possibilities of treating obesity under standardized health plans. Obesity is an inflammatory disease characterized by elevated levels of biomarkers associated with abnormal lipid profiles, glucose levels, and blood pressure that lead to the onset of metabolic syndrome. Interestingly, inflammatory biomarkers, in particular, have been implicated in the risk of developing several types of cancer. Likewise, obesity has been linked to esophageal, breast, gallbladder, kidney, pancreatic, and colorectal cancers. Thus, there exists a link between obesity status and tumor appearance, which may be associated to the differential levels and the circulating profiles of several inflammatory molecules. For example, mediators of the inflammatory responses in both obesity and gastric cancer risk are the same: pro-inflammatory molecules produced by the activated cells infiltrating the inflamed tissues. These molecules trigger pathways of activation shared by obesity and cancer. Therefore, understanding how these different pathways are modulated would help reduce the impact that both diseases, and their concomitant existence, have on society.

Key words Obesity, Inflammation, Gastric cancer, Epigenetics, Cancer

1 Introduction

1.1 A View on Obesity

It has been estimated by the World Health Organization (WHO) that within the next 5 years the number of overweight/obese individuals will be close to one-third of the world's population [1]. According to WHO, both increased consumption of food rich in fat and changes in physical activity patterns associated with sedentary lifestyles are creating an imbalance between the levels of calories consumed and calories expended [1]. The perspective of the disease is troublesome. More than 50 % of obese children will become obese adults; however, these unsettling patterns can be altered with tailored interventions [2–5]. Some estimations state that in 2011 about forty million children under the age of 5 were overweight [1]. In the USA 17 % of children and adolescents were

overweight/obese in 2009–2010. Although no gender specific increases in obesity prevalence were seen in that year, the data representing the decade from 2000 to 2010 indicated a significant increase in the prevalence in males aged 2–19 years [3, 6]. According to the Heart, Lung, and Blood Institute (NHLBI) of the National Institutes of Health (NIH) the degree of obesity/overweight can be estimated in adults with the body mass index (BMI) according to the ratio between weight (in kg) and height (in m²): underweight, BMI < 18.5; normal weight, BMI 18.5–24.9; overweight, BMI 25–29.9; obese, BMI, >30 (available at <http://www.nhlbi.nih.gov/guidelines/obesity/BMI/bmicalc.htm>).

Obesity has recently been recognized as a disease by the American Medical Association, changing the treatment of this disease and how it is monitored. Current research indicates that obesity is an inflammatory disease with increased intra-hepatic lipids (IHL) and visceral adipose tissue (VAT) that lead to increased levels of circulating lipids, altered blood pressure, and insulin resistance (IR), collectively referred to as metabolic syndrome [7–11] (MetS). This metabolic condition is characterized by elevated levels of IL6, IL8, TNF α , and IL1 β , among others, which in turn, have been linked to cardiovascular disease (CVD), asthma and type II diabetes [12–17]. Recently published studies help to clarify the mechanisms underlying the relationship between obesity and its associated-diseases. Adipose tissue secretes adipokines (adiponectin, leptin, resistin) associated with the appearance of the metabolic abnormalities observed in MetS [18–23]. Certain regions of adipose tissue, especially IHL, are associated with the metabolic dysfunction observed during obesity [24–27]. In addition, cytokines are produced by cells, both myeloid and lymphoid, infiltrating the adipose tissue [28–31]. It is estimated that about 65 % of the cells contained in the adipose tissue are leukocytes (reviewed in [32]).

The type of response mounted during an insult or challenge will vary in accordance with the varied modes of immune cell activation. According to Weisberg et al. [28], adipose tissue of obese mice have increased levels of macrophages (F4/80+) when compared to lean mice and produce increased levels of pro-inflammatory molecules. In addition, M1 macrophages respond to bacterial components, like lipopolysaccharide (LPS) and activated T cells products like interferon-gamma (IFN- γ) and induce a type 1 response (Th1) characterized by pro-inflammatory products like interleukin (IL) 6 (IL6), IL12, IL1 β , TNF α , among others, as well as reactive nitrogen and oxygen species (RNI and ROS, respectively) (reviewed in [33, 34]). Conversely, alternatively activated macrophages (M2 macrophages) are induced by IL13, IL4, IL10, and glucocorticoid hormones, which are associated with tissue remodeling and angiogenesis [33, 34]. Notably, an imbalance between M1 and M2 in the adipose tissue influences the appearance of insulin resistance, and the advent of pro-inflammatory

responses associated with obesity [35–39]. As such, promoting M2 development may help reduce the obesity-induced inflammatory responses observed in obese people.

1.2 Epigenetics and Obesity

Methylation of the DNA is a normal process during development and differentiation and has been described as a key mechanism of gene expression regulation and involves the transfer of methyl groups to the carbon in position five of the cytosine ring, as part of the CpG dinucleotide by DNA methyl transferases [40–43]. Recent publications show that the methylation status of DNA of lean individuals is different to that of obese subjects [44]. Others have suggested that profiles of differentially methylated CpG could predict the obese status [45]. In addition, several studies have shown that diet intervention and caloric restriction diets induce changes in the methylation of the promoters of genes involved in inflammatory responses [46–48]. Interestingly, some CpG in the *ATP10A* and *CD44* genes show differential methylation levels when comparing individuals with high and low response to diet intervention while CpGs on the *ATP10A* and *WTL* genes change their methylation status as a results of the diet intervention [48]. Additional studies have shown that exercise can modulate the metylation “landscape” of more than 7,500 genes, one third of which results in differential expression [49]. Thirty nine of those genes, associated with obesity and type 2 diabetes had a profile of methylation specifically modified by exercise in adipocytes [49].

Mature microRNA (miR), small noncoding RNA molecules (18 to 22 nucleotide long) generated by the action of two RNase III enzymes, Drosha and Dicer, have been associated with inhibition of translation and degradation of mRNA, having the potential to modulate inflammatory responses [50–52]. Specific miRs patterns have been associated with obesity. One study stated that miR-146b was highly expressed in mature adipocytes due to the response of these cells to proinflammatory cytokines in an attempt to regulate inflammation [53]. In fact, the dysfunction of the adipocytes seems to be a result of the continuous stimulation by proinflammatory cytokines. Kim et al. [54] found that miR-130 expression was increased and positively correlated with TNF α stimulation. This finding lead the researchers to hypothesize miR-130 may be responsible for adipocyte dysfunction and negative outcomes associated with obesity. Interestingly, miR-130b has been shown to inhibit adipogenesis and lipogenesis by targeting PPAR- γ [55]. On the contrary, Chou et al. [56] found increased adipogenesis when miR221 was downregulated and presented lower levels in adipocytes of obese individuals when compared to lean subjects. In some cases, families of miRs have similar functions and similar results.

Understanding the association of epigenetic markers with obesity is of capital importance because of several reasons. First, obesity has been classified as an inflammatory disease, so, modifying the

inflammatory environment by easily followed diets interventions would lead to a better compliance of obesity treatments and would reduce the use of medications and the secondary effects they may have; second, epigenetic profiles may help identify individuals who may benefit from specific treatments reducing over-treatment and unnecessary treatments. Third, these profiles may help developing personalized obesity treatments.

2 A View on Gastric Cancer

Based on the GLOBOCAN report, there were 14.1 million new cancer cases and 8.2 million cancer deaths worldwide in 2012; whereas, almost thirteen million cases of cancer were documented in 2008, of which approximately 60 % resulted in death [57, 58]. Comparing the incidence of cancer over that 5 year period shows that cancer diagnosis has increased and the percentage of cancer-related mortality remains unchanged. There is an international variation on the incidence of cancer worldwide [59], which is thought to be due to behavioral and environmental determinants. It is clear that behavioral factors, in particular, play a significant role in modulating the risk of cancer, including the type of diet we consume and the use of tobacco products [60].

Gastric cancer is one of the most common cancers worldwide. Despite the declining prevalence over the past 50 years, it is still the second leading cause of death due to cancer [57, 59, 61–63]. Worldwide, approximately 70 % of gastric cancer cases are reported from less developed countries such as Middle and Eastern Asia, South America, and Eastern Europe [62, 63]. Recent projections to the year 2030 suggest that the mortality due to gastric cancer will continue to increase [64]. According to the Laurén classification [65], gastric adenocarcinomas are divided into the diffuse type, which is diagnosed at earlier ages, involves several areas of the stomach without atrophy, and has similar male-to-female ratio distribution and have a uniform geographic distribution [65–67]. In contrast, the intestinal-type gastric cancer (well-differentiated) is more common in men and in older ages and is predominant in areas with high incidence of the disease [68]. A steady rise in the frequency of gastric cancer of the cardia in men between 4 and 10 % and a reduction of cancer in the distal parts of the stomach was noted between 1976 and 1987 [69]. These results have recently been validated in the USA using data from the US National Cancer Institute's (NCI) Surveillance, Epidemiology, and End Results (SEER) Program and the US Centers for Disease Control (CDC) and Prevention's National Program of Cancer Registries (NPCR) [70]. These registries, covering more than 89 % of the US population, demonstrate that proximal gastric cancer has increased in the last two decades [70]. In addition, a Japanese study of the

Japan Gastric Cancer Registry, which included more than 150,000 cases, showed a lowered incidence of the intestinal-type gastric cancer but a stable to higher frequency of the diffuse-type [71]. Of the approximately one million incident cases of gastric adenocarcinomas in 2008, about 90 % were classified as intestinal-type with an estimated 75 % attributed to infection with *Helicobacter pylori* (*H. pylori*) [62, 63]. The association of *H. pylori* with gastric cancer is so well-documented that the International Agency for Research in Cancer (IARC) classified the bacterium as a class I pathogen [72]. Infection with *H. pylori* occurs early in life [73] and may last without causing major problems [74–81]. However, it is estimated that between 1 and 3 % of individuals infected with *H. pylori* will develop intestinal-type gastric cancer [82–84]. In addition to the genetic background of the host [85–90], which modifies the risk of having more advanced gastric lesions and gastric cancer, virulent *H. pylori* strains have also been associated with increased risk of advanced gastritis and cancer [74–81, 85–90]. Infection with *H. pylori* triggers an inflammatory cascade that leads from a normal gastric epithelium to multifocal atrophic gastritis (MAG), intestinal metaplasia (IM), dysplasia and cancer [67, 91]. As in all inflammatory diseases, the immune system plays a crucial role in the development of gastric lesions and gastric cancer. Studies show mice lacking or having dysfunctional T and B cells do not develop gastritis when infected with *Helicobacter felis*. Additionally, Th1 responses in mice, characterized by the production of IFN- γ , have been associated with more aggressive lesions, as compared to Th2 responses in which IL4, IL10, and IL13 are predominant [92, 93]. In humans, Th1 responses are also linked to the mucosal damage seen in patients with *H. pylori* infection [94, 95]. However, similar to gastric cancer incidence, *H. pylori* infection is less prevalent in developed countries with improved socioeconomic conditions [62].

2.1 Epigenetics and Gastric Cancer

Both methylation and miR profiles have been associated with risk of gastric cancer (reviewed in [96, 97]). miRs that promote tumor formation are called oncomiRs and are typically over-expressed in the cell lines that precede the formation of the tumor. miR-181a is over-expressed in human gastric cancer tissues and when over-expressed in gastric cancer cell lines promotes its proliferation, colony formation, migration, and invasion and inhibits the apoptosis of cancer cells by neutralizing the tumor suppressor gene *KLF6* [98]. Similarly, miR-21 is significantly over-expressed in human gastric cancer tissues, gastric cancer cell lines and in patients infected with *H. pylori* [99]. Inhibition of miR-21 resulted in increased apoptosis, reduced migration and invasion of the gastric cancer cell line AGS [99]. On the contrary, miR-22 seems to act as a tumor suppressor miR by inhibiting the tumor activity of the CD151 molecule. CD151 has been associated with poor prognosis

and invasiveness of gastric cancer [100–102]. Recent studies show that the expression of miR-22 is significantly reduced in gastric cancer tissues and cell lines when compared to normal counterparts [103]. Interestingly, over-expression of miR-22 resulted in reduced expression of CD151 and reduced proliferation, invasion and migration of gastric cancer cell lines [103]. Another example of tumor suppressor miR is miR-148 which targets DNA methyltransferase I (DNMT1), which in turn inactivates the long noncoding RNA MEG3, the downregulation of which has been correlated with bad prognosis in gastric cancer [104, 105].

3 The Crossroad

3.1 Epidemiological Evidence of the Relationship Between Obesity and Cancer

It is estimated that about 4 % of all cancers in men and 7 % in women may be attributed to obesity [106]. Several lines of evidence show that obesity is a risk factor for several cancers, including endometrial, pancreas, esophagus, kidney, breast, prostate, colorectal, gallbladder, and thyroid [107–114]. These previous reports were confirmed in a recent meta-analysis of 141 publications including 282,137 cases of cancer [115]. Results from this meta-analysis indicated that an increment of 5 kg/m² in the body mass index (BMI), an estimator of body fatness, was associated with a significant relative risk (RR) of developing thyroid and colon cancer in men, endometrial and gallbladder in women and esophageal and renal cancer in both sexes [115]. Gastric cancer has also been associated with BMI, especially adenocarcinoma of the gastric cardia. Chow et al. [116] reported a significant trend in the relationship between BMI and risk of esophageal and cardia gastric cancer, especially in younger Japanese individuals. Similar results were reported by Ji et al. [117] in more than 1,000 cases of newly diagnosed gastric cancer individuals in Shanghai, China, between 1988 and 1989. A significant trend in cardia gastric cancer was observed in younger males with higher BMI, which was driven especially by the weight of the individuals [117]. In Japanese individuals a significant increase in BMI, especially in men, has been associated with increased number of gastric cancer cases in a period between 1971 and 2001 [118]. These trends have been replicated in several studies [70, 119–122].

4 Diet and Gastric Cancer

As pointed out above, obesity is the result of an imbalance between calories consumed and calories spent [1]. A recent report shows that mice infected with *H. felis* and fed a high fat diet (HFD) for 15 months have increased levels of gastric and circulating markers of inflammation paralleled with an increased infiltration of

immature myeloid cells (GR1+/CD11b+) and circulating levels of IL6 [123]. In humans, several diets are shown to be differentially associated with risk of gastric cancer. Fruit, vegetable, β -carotene, and ascorbic acid consumption protects against gastric cancer in several studies and populations, while consumption of salted foods, red meat, and starchy foods is associated with increased risk [124–132]. These same factors seem to modify the risk equally in cardia gastric cancer [133]. The amount of energy intake provided by food [134] is associated with differential risk of gastric cancer, suggesting that gastric cancer risk and any other cancer type could be modified by diet.

4.1 Cholesterol and Triglycerides Levels and Gastric Cancer

Evidence suggests that increased levels of circulating cholesterol and triglycerides are linked to increased risk of gastric cancer [135–137] especially of the non-cardia type [138]. Individuals, especially men, with elevated levels of triglycerides and cholesterol not only have a higher risk of developing the disease but also of presenting with lymph node metastasis [135, 136]. Interestingly, the levels of visceral adipose tissue and subcutaneous adipose tissue (SAT), measured by computerized tomography (CT), are significantly higher in cases, both males and females, of differentiated gastric malignancies [136].

Remarkably, a recent publication by Lai et al. [139] suggested that translocation and phosphorylation of the *H. pylori* virulence marker CagA into the membranes of the gastric adenocarcinoma cell line AGS is affected when these cells are depleted of cholesterol [139]. Furthermore, depletion of cholesterol in AGS cells was associated with reduced levels of IL8 production suggesting that cholesterol levels may be associated with increased inflammatory responses. We recently demonstrated that *H. pylori* infection of AGS cells was associated with increased levels of IL8 and that this effect was more than 40-fold higher in the absence of the *H. pylori* arginase gene, *rocF* [140].

Although the link between RocF activity, CagA phosphorylation and cholesterol metabolism is not well-established, recent research suggests that *H. pylori* has evolved strategies to generate compensatory mechanisms to limit the tissue damage and keep under control the immune response. Contrary to this line of thought, other researchers propose that cholesterol, at controlled levels, may act as an inducer of apoptosis in several gastric cell lines [141]. In general, research results from numerous studies are incongruent with some reporting that cholesterol levels are inversely associated with risk of cancer, while others report no association [142–148]. A recent prospective cohort study of 2,604 Japanese individuals followed for 14 years demonstrated that low levels of cholesterol were associated with the appearance of intestinal-type gastric cancer, especially in men [149]. According to this study a decrease in 1 mmol/L in the levels of cholesterol resulted in a 22 % increase in the risk of developing the malignancy in both men and women [149].

Metabolism of cholesterol is critical to regulating the levels of circulating lipids. Apolipoprotein E (ApoE) is an essential component in the abstraction of cholesterol-bound particles from the circulation [150]. Notably, the ApoE gene has several alleles, with the $\epsilon 3$ allele occurring most frequently. Individuals carrying the $\epsilon 2$ allele have reduced circulating levels of triglycerides and cholesterol. Conversely the levels are significantly higher in individuals with the allele $\epsilon 4$ [151, 152]. Carriers of $\epsilon 2$ alleles also have a significantly reduced risk of gastric cancer as compared to those individuals carrying the most common allele, $\epsilon 3$, especially in intestinal-type gastric cancer [153].

In summary, the association of cholesterol levels with the development of gastric cancer remains controversial; however, several epidemiological and laboratory reports suggest that the level of circulating cholesterol modifies the risk of developing gastric malignancies, especially cardia gastric cancer in men.

4.2 Glucose and Diabetes and Gastric Cancer

Otto Warburg [154] initially proposed that, compared to normal cells, cancer cells utilized more glycolysis than oxidative phosphorylation to produce energy in the form of adenosine tri-phosphate (ATP), generating more by-products essential for cell proliferation. As expected, the expression of receptors for glucose uptake is increased in tumor cells. The transport of glucose into the cells is mediated through a facilitative transport by a family of glucose transport proteins called glucose transporters (GLUT), many of which are kept in intracellular vesicles under normal circumstances and mobilized to the membrane during disease stages like diabetes and obesity (reviewed in [155, 156]). In gastric cancer the expression of these GLUT proteins is markedly regulated (reviewed in [156]). For example, Noguchi et al. [157] have shown that 80 % of normal gastric mucosa express GLUT2, while only approximately 50 % express GLUT4. In contrast, expression of GLUT1 is not observed in normal gastric mucosa [157, 158]. However, 95 % of malignant gastric tissues express GLUT1; its presence is associated with tumor infiltration to the deep gastric glands and gastric walls and lymph node metastasis [157], especially in intestinal-type gastric cancer [158]. Interestingly, in the gastric cancer cell line, MKN28, insulin stimulation results in more than a 20 % increase in levels of the insulin responsive GLUT4 receptor [159]. This suggests a potential link between obesity and risk of gastric malignancy, which may be mediated through elevated levels of insulin. Furthermore, *H. pylori* infection, which is a potent risk factor of gastric cancer, is associated with diabetes and its eradication may help improve levels of insulin resistance [160, 161]. It is important to note, however, that some reports reveal no association between diabetes and *H. pylori* infection [162, 163].

Several other metabolic enzymes have been implicated in risk of gastric cancer. Increased levels of expression of the enzyme

glucose-6-phosphate dehydrogenase (G6PD), an essential molecule for the conversion of glucose into ribose-5-phosphate and the first-limiting step in glycolysis, are associated with the size, invasion and local and distant metastases of gastric tumors in patients [164]. This biochemical reaction is accompanied with production of NADPH which protects cells from reactive oxygen species (ROS). Therefore, it is expected that cancer cells would have increased levels of G6PD and be more resistant to the DNA damage induced by ROS.

Treatment of gastric cancer is associated with an improved metabolic profile in some individuals. The relationship between glucose metabolism, diabetes and risk of gastric cancer is evidenced by a South Korean study showing that after 12 months of gastrectomy surgery to remove gastric cancer, patients previously diagnosed with type 2 diabetes mellitus (DM-2) had a significant improvement in their homeostasis model assessment-estimated insulin resistance (HOMA-IR) (81 % reduction), glucose (21 % reduction), insulin (73 % reduction), and BMI (10 % reduction) [165].

As stated earlier, dietary behaviors significantly impact gastric cancer risk. In particular, foods rich in starch are associated with increased risk of gastric cancer in several populations [124–132] which may be related to the level of consumption and utilization of carbohydrates and the demand for insulin, expressed as the glycemic load (GL). Glycemic load is associated with an increased risk of several cancers including oral, esophageal and breast [166–168]. Increased levels of GL are linked to an increased risk of gastric cancer, regardless of age, which is significantly higher in females and individuals with a higher BMI [169]. These findings were recently replicated by Bertuccio et al. [170] However, a subsequent study of a cohort of more than 60,000 women in whom 156 incident cases of gastric cancer were recorded over a period of 7 years did not report any correlation between GL, glycemic index (GI) or intake of carbohydrates [171].

4.3 Insulin and Gastric Cancer

The Insulin sensitive tissues of the human body, which include the liver, muscle, and adipose tissues, have a lower observable number of reported malignancies, a finding that suggests that insulin regulation of metabolic processes is protective against carcinogenesis [172]. The adipocytes associated with high fat areas may also have an association with cancer development. In tumors growing in adipose tissue-rich areas of the body such as the abdomen, adipocytes disappear and fibroblast-like cells accumulate near the invasive front of the tumor [173]. This finding may link dysfunctional adipocyte activity to the preliminary stages of tumorigenesis, especially in the presence of *H. pylori* infection.

The first evidence of the relationship between *H. pylori* infection and insulin resistance was presented by Aydemir et al. [174] in 2005 showing that despite no difference in BMI and glucose levels,

patients with *H. pylori* infection had a significantly higher HOMA-IR value (<0.05) when compared to those without the infection. This is in agreement with previous reports showing that the prevalence of *H. pylori* infection, measured in terms of the level of antibodies against the bacterium, was higher in insulin-dependent diabetes mellitus (IDDM) patients than in individuals without IDDM, especially in those less than 24 years of age [175]. The same study documented that the seroprevalence of *H. pylori* in IDDM was inversely associated with the time of evolution of the IDDM [175]. In a study examining more than 600,000 health records in Taiwan, incidence of *H. pylori* was significantly higher in diabetic patients (unadjusted OR 2.376 (95 % CI 2.275–2.482; $p < 0.0001$) and in those using insulin ($p < 0.0001$) [176].

Insulin is also implicated in cellular activation and angiogenesis mediated by the activation and signaling of the insulin receptor (IR), insulin growth factor (IGF)-1 and 2 (IGF-1 and IGF-2, respectively) and the IGF-1 receptor, IGF-1R [177, 178]. There is consensus in the current scientific literature that three major alterations in the body's insulin levels (insulin resistance, hyperinsulinemia, and increased levels of IGF-1) are responsible for linking type 2 diabetes, obesity, and cancer. Insulin resistance and hyperinsulinemia lead to increased concentrations of various insulin-like growth factors; specifically, signaling through IGF-1R can contribute to tumorigenesis [179–181]. In both hyperglycemic cancer patients as well as those with type 2 diabetes, the rate of tumor recurrence, metastatic spread, and degree of lethality of tumors is higher as compared with the tumor patients without an insulin-altering metabolic disease [182].

Recent studies demonstrate that higher survival rates of patients with gastric adenocarcinoma are associated with IGF-1 signaling. As the levels of IGF-1 increase, tumors progress from benign to cancerous, an observation that may link IGF-1 to lethality of obesity-associated cancers [183]. Tumors positive for the insulin receptor (IGF-IR) are also positively associated with lethality of tumor [184]. Since IGF-1 effects are mediated by signaling through the IGF-1R, it is expected that inhibition of this receptor would nullify the effects of the increased levels of IGF-1. Current research also indicates that blockade of IGF-1R results in reduced gastric tumor growth in vivo and in vitro. Furthermore, IGF-1R blockade also increases the level of apoptosis, blocks AKT-1 signaling and increases the effectiveness of chemotherapy and radiotherapy against gastric tumors in mice [185]. These inhibitory effects of IGF-1R blockage may be due to the capacity of inhibiting the IGF-1 induced tumor vascularization through the induction of vascular endothelial growth factor (VEGF) [179]. The last two approaches are promising adjuvant therapies to improve current treatments of gastric malignancies.

4.4 Metabolic Syndrome and Gastric Cancer

As described above, MetS is defined as the concurrent presence of high blood lipids, high glucose or insulin resistance, and high blood pressure [7–11]. In the previous sections we have shown that, for the majority of studies cited, all these factors that are markers of obesity are also associated with increased risk of gastric malignancies. Recent studies further support the conclusion that MetS is a bridge that links the two entities. A recent European study of close to 600,000 individuals, determined the association among levels of glucose, lipids, blood pressure and BMI with gastric adenocarcinoma [186]. In this study, 1,210 cases of gastric cancer were identified and found to be associated with glucose, triglyceride and composite MetS score (the sum of all the values transformed into Z-scores) levels in women but not in men. In contrast, BMI was associated with increased risk of gastric cancer in men but not in women [186]. Other publications support the idea that MetS is associated with gastric cancer [137].

4.5 Metabolism and Epigenetics

Several miR have been implicated with the control of metabolism. For example, down-regulation of miR-122 resulted in 25–30 % reduction in the levels of circulating cholesterol (reviewed in [187]). Similarly, miR-33a and miR-33b are strongly linked to the metabolism of cholesterol, regulating the levels of the very low-density lipoprotein (VLDL) and the high density lipoprotein (HDL), which are crucial biomarkers of risk of cardiovascular disease (reviewed in [187]). Horie et al. [188] have shown that reduced levels of miR-33 are associated with increased levels of HDL and prevention of the progression of atherosclerosis in mice by increasing the activity of the ATP-binding cassette transporter A1 (ABCA1), a rate-limiting factor in the generation of HDL. Another recent study [189] found that miR-200a, miR-200b, and miR-429 were upregulated in leptin-deficient obese (*ob/ob*) mice; however, leptin treatment normalizes the levels of these miRs. Interestingly, treatment to reduce the levels of miR-200a resulted in reduced body weight gain and normalization of blood glucose by increasing the expression level of leptin receptor and insulin receptor substrate 2. Furthermore, in mice with silenced miR-200a, liver insulin responsiveness was restored. miR-200a shows an association with impaired leptin and insulin signaling [189].

5 Concluding Remarks

Obesity, now well-known as an inflammatory state, is as expected, also associated with numerous chronic diseases such as asthma, diabetes, cardiovascular disease, and cancer. All of these diseases possess a major inflammatory component. If the World Health Organization's predictions regarding the effect of the obesity

epidemic become reality, an increased rate in all of these diseases associated with obesity is probable. Obesity is a complex disease defined by social, behavioral, environmental, and biological factors. Some of these factors are modifiable; others are not. Modifiable behavioral factors such as diet and physical activity, and environmental factors such as access to safe outdoor areas for recreation, and fresh fruits and vegetables significantly modulate the risk of many diseases regardless of obesity status. These modifiable determinants underline a common path for the development of obesity-related illnesses and cancer. Therefore, strategies to enhance nutrition and increase physical activity, and thereby reduce obesity and its inflammatory status, would likely also result in a reduction in the incidence of gastric cancer. As such, solution-oriented research, which examines the effect of diet and physical activity behavior change on inflammatory and metabolic factors related to obesity and gastric cancer, especially in those at greatest risk, should be a primary focus of future research. More important, as a society, we should strive to educate ourselves and others to influence positive behavioral and environmental changes, which are known to reduce the incidence of all obesity-related, life-altering preventable diseases.

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Detection of Epigenetic Aberrations in the Development of Hepatocellular Carcinoma

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Abstract

Hepatocellular carcinoma (HCC) is the third most common cause of cancer death worldwide. Hepatocarcinogenesis is a complex, multistep process. It is now recognized that HCC is a both genetic and epigenetic disease; genetic and epigenetic components cooperate at all stages of hepatocarcinogenesis. Epigenetic changes involve aberrant DNA methylation, posttranslational histone modifications and aberrant expression of microRNAs all of which can affect the expression of oncogenes, tumor suppressor genes and other tumor-related genes and alter the pathways in cancer development. Several risk factors for HCC, including hepatitis B and C virus infections and exposure to the chemical carcinogen aflatoxin B₁ have been found to influence epigenetic changes. Their interactions could play an important role in the initiation and progression of HCC. Discovery and detection of biomarkers for epigenetic changes is a promising area for early diagnosis and risk prediction of HCC.

Key words Hepatocellular carcinoma, Epigenetics, DNA methylation, Histone modification, microRNA, Biomarkers, Hepatocarcinogenesis, Early detection

1 Introduction

Hepatocellular carcinoma (HCC) is one of the most common and rapidly fatal human malignancies. More than 600,000 new cases are diagnosed annually worldwide with a nearly equivalent number of fatalities showing the lack of effective therapeutic alternatives for this disease that is largely diagnosed at an advanced stage [1, 2]. HCC has considerable geographic variation; its incidence is highest in East Asia and sub-Saharan Africa. In Western developed countries including the USA, HCC incidence is increasing [2, 3]. Chronic hepatitis B virus (HBV) and hepatitis C virus (HCV) infections are well-documented risk factors for the development of HCC. Other factors include chronic alcohol intake and several environmental factors, including aflatoxin B₁ (AFB₁), a dietary mold contaminant and polycyclic aromatic hydrocarbons (PAHs), ubiquitous environmental contaminants [4–6].

As with other cancers, the development of HCC is a complex, multistep process, involving multiple genetic aberrations in the molecular control of hepatocyte proliferation, differentiation, apoptosis, and the maintenance of genomic integrity. This process is influenced by the cumulative activation and inactivation of oncogenes, tumor suppressor genes (TSGs), and other cancer-related genes [7, 8]. Over the past decade, it has been recognized that epigenetic changes are also involved in cancer development and progression [9, 10] including in HCC [11]. More and more studies have indicated that HCC is both a genetic and epigenetic disease with genetic and epigenetic components cooperate at all stages of hepatocarcinogenesis [7, 12]. Investigating the profile of epigenetic changes in HCC and elucidating the mechanisms of these molecular events should help tailor approaches that explore new methods for the early detections and therapy of HCC.

Epigenetic alterations refer to the reversible and heritable changes in gene expression that occur without alteration to the DNA sequence itself [13, 14]. The term “epigenome” is used to define a cell’s overall epigenetic state. The changed epigenome of HCC is characterized by gene-specific hypermethylation or hypomethylation, global genomic hypomethylation, abnormal expression of DNA methyltransferases and histone modifying enzymes, altered histone modification patterns, and aberrant expression of microRNAs [15, 16].

Methylation of DNA cytosine residues at the carbon 5 position (5mC) in cytosine–guanine dinucleotides (CpG sites) is a common and major epigenetic mechanism in eukaryotic DNA, playing an important role in regulation of gene activities. Aberrant DNA methylation has been detected in many cancers including HCC [17–20]. Gene-specific promoter hypermethylation and global hypomethylation have been linked with silenced TSGs, DNA repair genes, other cancer-related genes and genomic instability [18, 21]. Aberrant DNA methylation can be detected in premalignant lesions and noninvasive carcinomas, indicating that DNA methylation changes are an early event in the process of malignant transformation [22, 23].

2 Gene-Specific DNA Hypermethylation and Hepatocellular Carcinoma

DNA methylation is found in so-called CpG islands, guanine-cytosine-rich regions containing relatively high densities of CpG sites located primarily in the promoter of genes, and genes with promoter hypermethylation are usually transcriptionally silent [17]. The growing list of genes inactivated by promoter region hypermethylation provides an opportunity to investigate the profile of inactivation of those genes among different cancers [10, 17].

In HCC, among genes frequently hypermethylated are tumor suppressor genes, DNA repair genes and other cancer-related genes including *RASSF1A* [24], *p16^{INK4A}* [25, 26], *p15^{INK4B}* [27, 28], *RBI* [29], *GSTP1* [30, 31], *SOCS1* [32], *APC* [33], *E-cadherin* [33], *Hint1* [34], *SOCS3* [35], *RIZ1* [36], *MGMT* [37], *p73* [33] and many more. The number is still increasing. These genes are involved in different pathways of signal transduction and activities of biological regulation, especially in cell proliferation, cell cycle control, DNA repair and suppression of cytokine signaling which are very important in HCC development.

The most striking methylation pattern in HCC is the concurrent methylation of multiple tumor suppressor genes. Even though promoter hypermethylation of one or two TSGs could be seen in non-tumor or cirrhotic liver tissue, the majority of HCC harbor three or more TSGs with promoter hypermethylation. This phenomenon suggests that disruption of multiple signal transduction pathways is biologically required during hepatocarcinogenesis [33].

Chronic HBV and HCV infections are well-known major risk factors for HCC. The presence of HBV and HCV in host hepatocytes may result in both genetic (insertional mutation) and epigenetic events, especially aberrant promoter hypermethylation in HCC development [38].

Zhong and coworkers reported that epigenetic silencing of *RASSF1A* and *GSTP1* expression by CpG island DNA hypermethylation is common in human HBV-associated HCC [30, 39]. A high rate of *p16* gene promoter hypermethylation was also associated with HBV infection in HCC [40]. These results suggested that HBV may enhance the aberrant methylation of those genes. Abnormalities in DNA methyltransferase (DNMT) activity during chronic HBV infection may also contribute to the abnormal de novo CpG island hypermethylation changes affecting *RASSF1A*, *GSTP1*, *p16* and other genes, which accumulate during the pathogenesis of human HCC [30, 39, 40]. HBV encoded protein x (HBx) may also directly interact with DNMTs, directing their recruitment to specific genes affecting their methylation and expression. For example, it was found that *p16* hypermethylation was associated with HBV x protein expression [41].

Despite the fact that HCV is an RNA virus without a DNA intermediate, HCV viral proteins may actively participate in epigenetic regulation of hepatic cancer stem cell phenotypes and induce HCC specific epigenetic changes. Methylation of some TSGs, such as the *SOCS-1*, *GADD45b*, *STAT1*, *APC*, and *p15*, was observed at a higher prevalence in HCV-positive than in HCV-negative HCC [38]. A recent study investigated DNA methylation status of 10 genes (*APC*, *CCND2*, *CDKN2A*, *GSTP1*, *HOXA9*, *RARB*, *RASSF1A*, *RUNX*, *SFRP1*, and *TWIST1*) using the MethyLight assay. Five genes (*APC*, *CCND2*, *HOXA9*, *RASSF1A*, *RUNX*) were significantly more frequently methylated

in malignant liver tissue than normal liver tissue. Among HCC cases *HOXA9*, *RASSF1A*, and *SFRP1* were methylated more frequently in HBV positive HCC cases, while *RARB* and *CDKN2A* were methylated only in HCV positive HCC cases. *P16* was significantly more frequently methylated in HCV positive HCC cases [42].

The correlation between HBV and HCV and promoter hypermethylation is still controversial and exact mechanisms underlying virus-DNA methylation interaction are still unclear.

Alcohol consumption is a risk factor for HCC and alcohol acts as a potent carcinogen and exhibits strong synergistic effects with HBV, HCV, AFB₁ exposure, and obesity [14]. It is also associated with different epigenetic changes including gene promoter hypermethylation. One study investigated the frequency of DNA hypermethylation in HCC patients with alcohol intake and found that 67, 44, and 60 % of HCC cases have promoter hypermethylation for *RASSF1A*, *GSTP1*, and *DOK1*, respectively [43]. This result suggests that alcohol intake may have a strong effect on the methylation status of specific genes in HCC. *S*-Adenosylmethionine (SAM) plays a pivotal role as a methyl donor in biological and biochemical events. The mechanism underlying the epigenetic changes caused by alcohol intake may involve SAM.

AFB₁ is a dietary chemical carcinogen and several studies have implicated AFB₁ exposure as an etiological agent in HCC and suggested an interaction with chronic HBV infection [44]. Worldwide studies suggest that high AFB₁ exposure is associated with a specific mutation at codon 249 in the *p53* tumor suppressor gene in HCC. In the past 10 years, a series of studies found a statistically significant association between promoter hypermethylation of *RASSF1A* [24], *p16* [45], *MGMT* [37], and *GSTP1* [31] and the level of AFB₁-DNA adducts in HCC. The results suggest that AFB₁ is associated with aberrant methylation in tumor suppressor genes and cancer-related genes. AFB₁ mediates its toxicity via its biotransformation to an 8,9-epoxide that binds DNA, RNA, and proteins, forming adducts and cause damage on those macromolecules. AFB₁ may bind preferentially to methylated CpG sites and/or specific structure in chromatin including the damage of DNA and histone [14]. The precise mechanism by which AFB₁ alters methylation status needs further exploring.

Methylated DNA has been suggested as a potential biomarker for early detection of cancer [46]. It is conceivable that methylation-dependent epigenetic events occur in chronic liver disease and/or in the preneoplastic (cirrhotic) liver, in addition to HCC [47]. The possible diagnostic value of aberrant promoter hypermethylation of three tumor suppressor genes, *RASSF1A*, *p16*, and *p15* in serum DNA for early detection of HCC was explored and the investigators found hypermethylated *p16*, *p15*, and *RASSF1A* in 44, 24, and 70 % of HCC patients, respectively. Hypermethylation of these three genes could be detected 1–9 years before the clinical

diagnosis of cancer [48]. These results showed that epigenetic biomarkers applied to preclinical testing of high-risk individuals exhibit good sensitivity, small early liver neoplastic lesions may be detected and the clinical course of HCC monitored.

3 Genome-Wide DNA Methylation and Hepatocellular Carcinoma

The human genome contains approximately 29 million CpGs that exist in methylated, hydroxymethylated or unmethylated states; 7 % of all CpGs are within CpG islands (CGIs), a majority of which are unmethylated [49]. Approximately 45 % of CpGs are within repetitive elements and presumed constitutively methylated [50]. The methylation status of cytosines in CpGs and occasionally in non-CpG cytosines influences protein–DNA interaction, gene expression, and chromatin structure and stability.

Several methods have been used to detect genome-wide methylation profiles [51]. Illumina (CA, USA) methylation arrays are mid-range to large-scale platforms that quantitatively interrogate 1505 individual CpG sites in 807 genes (GoldenGate) or 27,578 individual CpG sites in approximately 14,000 genes (Infinium™ HumanMethylation 27K BeadChip) [52]. The Illumina HumanMethylation450 BeadChip includes 99 % of RefSeq genes and 96 % of CpG islands, making it ideal for epigenome-wide association studies (EWAS). Thus far, Illumina methylation assays are the mostly frequently used method to detect methylation patterns in HCC samples. Ammerpohl and colleagues first investigated patterns of methylation using HumanMethylation 27K BeadChip arrays in 12 HCC, 15 cirrhotic controls and 12 normal liver samples and found 167 hypomethylated loci and 100 hypermethylated loci in cirrhosis and HCC as compared to normal controls [53]. GoldenGate or HumanMethylation 27K BeadChip arrays were used on HCC tumor and adjacent non-tumor tissues from Japan [54, 55], Korea [56], France [57], and the USA [58], but with relatively small sample sizes of 5–38 cases. Shen and colleagues analyzed paired tumor and adjacent non-tumor tissues from 62 Taiwanese HCC cases by using Infinium HumanMethylation 27K BeadChip; a total of 2,324 CpG sites significantly differed in methylation level, with 684 CpG sites significantly hypermethylated and 1,640 hypomethylated in tumor, compared to nontumor tissues [59]. Recently, Infinium HumanMethylation 450K BeadChip arrays have been used to examine genome-wide DNA methylation profiles in 66 pairs of US HCC tumor and adjacent non-tumor tissues by the same group; a total of 130,512 CpG sites significantly differed in methylation level, with 28,017 CpG sites significantly hypermethylated and 102,495 hypomethylated in tumor compared to non-tumor tissues [60]. Most (60.1 %) significantly hypermethylated CpG sites are located in CpG islands, with 21.6 %

in CpG shores and 3.6 % in shelves. In contrast, only a small proportion (8.2 %) of significantly hypomethylated CpG sites are situated in islands, while most are found in open seas (60.2 %), shore (17.3 %) or shelf (14.3 %) regions. Aberrant DNA methylation profiles across the genome were identified in tumor tissues from US HCC cases that are predominantly related to HCV infection [60]. In a recent study, genome-wide methylation analysis was performed using HumanBeadchip27k arrays, and *SMPD3* and *NEFH* were identified as tumor suppressor genes in HCC. A reduced level of *SMPD3* was an independent prognostic factor for early recurrence of HCC [61].

A global methylation profile of single hepatocytes isolated from liver tissue of HBV-related HCC was carried out by using Infinium HumanMethylation27 BeadsChips, and combined bisulfite restriction analysis (COBRA) and bisulfite sequencing were used to validate the 20 significant hypermethylated genes identified. Many noteworthy differences in the genome-wide methylation profiles of hepatocytes from HBV-related HCC were found, including 7 novel genes (*WNK2*, *EMILIN2*, *TLX3*, *TM6SF1*, *TRIM58*, *HIST1H4F* and *GRASP*) [62].

Genome-wide DNA methylation detection provides the most comprehensive analysis to date of the methylome of HCC samples and has produced novel insights into the mechanisms of methylome alteration during hepatocarcinogenesis. A series of novel methylated genes found in these investigations have the potential to be molecular markers for HCC diagnosis, treatment and prognosis, and to aid in understanding the effects of methylation status in different pathways in the development of HCC.

4 Global DNA Hypomethylation and Hepatocellular Carcinoma

Global DNA hypomethylation, in both noncoding repetitive sequences and in genes, contributes to carcinogenesis by causing chromosome instability and increased gene expression and has been detected in different human cancer tissues, including HCC [63]. Hypomethylation of the genome mainly affects intergenic and intragenic regions of DNA, particularly repeat sequences and transposable elements [64]. More than 90 % of all 5mC lies within transposons, including short interspersed nucleotide elements (SINEs) and long interspersed nucleotide elements (LINEs), which are comparatively rich in CpG dinucleotides [63]. Satellite 2 (Sat2) DNA sequences are located as tandem repeats in the pericentromeric and juxtacentromeric heterochromatin of several chromosomes [65] and other repetitive elements include short stretches of DNA originally characterized by the action of the *Arthrobacter luteus* restriction endonuclease (Alu element). Loss of DNA methylation in these sequences is believed to mainly account for global hypomethylation [66].

Global hypomethylation of liver tumor DNA was observed in a mouse model [67]. Analysis of methylation levels of Sat2, LINE1 and Alu are frequently used as surrogate measures of genomic global methylation level. Several studies showed hypomethylation of LINE1, Alu and Sat2 in human HCC [68, 69]. One study demonstrated that global hypomethylation in HCC was associated with gene specific hypermethylation including *p16* and *ER* [68], but further studies are needed to validate the relationship between global hypomethylation and hypermethylation of specific genes.

Global hypomethylation is a marker of poor prognosis in several cancers including HCC and may be promoting progression via chromosomal instability, activation of protooncogenes, and reactivation of transposable elements. In patients with HCC, serum and tumor LINE1 hypomethylation is considered a poor prognostic marker involved in tumor progression and is associated with high tumor grade and poor survival rate [11, 70].

A previous study found that three repetitive DNA elements Sat2, Alu and LINE1, showed discordance in timing of hypomethylation along the multistep pathway in hepatocarcinogenesis from normal to HCC: comparing HCC tumor and non-neoplastic liver tissues hypomethylation occurred earlier in Sat2 than in LINE1 and Alu [71]. Hypomethylation also differed according to geographic location of the subjects and their hepatitis infection status; mean LINE1 methylation in tumor samples was lower in hepatitis-positive cases than in hepatitis negative cases [68]. These findings suggest that HBV and HCV infections can influence global DNA hypomethylation status. An *in vivo* study demonstrated that HBx represses *E-cadherin* expression via activation of DNA methyltransferase 1 [72] and induces global hypomethylation of Sat2 repeat sequences by downregulating DNA methyltransferase 3 [73]. This may be partially explained by the fact that the HBx protein can induce altered DNA methyltransferase activity, hypermethylation of specific genes and global hypomethylation [72, 73].

Recently, detection of global hypomethylation has been carried out using several different methods: the methyl acceptance assay, MethyLight and pyrosequencing to analyze Sat2 and LINE1 in paired HCC samples. Consistent hypomethylation in tumor compared to adjacent non-tumor tissue was found by all three methods; AFB₁ exposure was also associated with DNA hypomethylation [74]. These results suggest that AFB₁ exposure may cause DNA hypomethylation during the development of HCC in humans.

Methylation has also been evaluated in white blood cells (WBC). The main challenge is making inferences from studies examining the association of DNA methylation in WBC with cancer risk and/or risk factors is that the studies were retrospective in nature. There is some evidence that decreased methylation of particular repetitive elements such as Alu and LINE1 in blood collected before cancer diagnosis is associated with increased risk of cancer [75, 76]. In a recent study, decreased Sat2 methylation

of WBC DNA from peripheral blood was significantly associated with increased HCC risk later in life [77]. These results suggest that global hypomethylation may be a useful biomarker of HCC susceptibility.

5 Gene-Specific Hypomethylation and HCC

There is evidence that promoter hypomethylation of some cancer-related genes may be associated with cancer development by regulating the activity of genes. The demethylated genes are mainly involved in cell growth, cell adhesion and communication, signal transduction, mobility, and invasion functions that are essential for cancer progression and metastasis [78]. For example, CD147, a transmembrane, protein with a highly glycosylated modification exhibits the characteristics of the immunoglobulin superfamily members. Promoter hypomethylation upregulates *CD147* expression through increasing SP1 (sequence-specific transcriptional factor) binding and is associated with poor prognosis in HCC [79]. Trefoil factor 3 (*TFF3*) is a marker of columnar epithelium and is expressed in a variety of tissues. *TFF3* overexpression and promoter hypomethylation is frequent in mouse and human HCC, suggesting that *TFF3* overexpression may be a critical process in mouse and human hepatocarcinogenesis [80, 81]. Specific promoter hypomethylation may be one of the regulatory mechanisms of *TFF3* overexpression in HCC [81]. The human synuclein-gamma (*SNCG*) gene is a tissue-specific gene primarily expressed in brain and is abnormally expressed in a high percentage of liver cancers. Analyses of the methylation status of the CpG island of *SNCG* by methylation specific PCR (MSP) confirmed that all tumor samples contained the demethylated gene. There was no correlation between *SNCG* expression and HBV and HCV infection. Chemical carcinogens AFB₁ and N-nitrosodimethylamine (DMN) are strong inducer of *SNCG* expression [82].

Robust changes in the landscape of DNA methylation in HCC patients occur and show that promoter specific hypomethylation is associated with activation of cancer-driving genes [78]. The existence of two opposing hypermethylation and hypomethylation events in the same functional pathways complement or enhance each other in the disruption of cellular homeostasis favoring progression of HCC [83]. The high frequency of promoter hypomethylation was also confirmed in HCC samples in another study by genome-wide methylation analysis [84]. The mechanisms of this aberration of DNA methylation, especially, for the association between promoter specific hypomethylation and HBV and HCV infection still needs larger studies.

6 DNA Methyltransferases and Hepatocellular Carcinoma

DNA methylation is a biochemical process catalyzed by enzyme called DNA methyltransferases (DNMTs). The mammalian DNMT is encoded by three distinct families of DNMT genes: *DNMT1*, *DNMT2*, and *DNMT3*. DNMT1 is the most abundant DNMT involved in the maintenance of methylation and DNMT3 functions as a de novo methyltransferase and consists of two related genes: *DNMT3a* and *DNMT3b* [85]. *Dnmt1* is 5- to 30-fold more active on hemimethylated DNA as compared with unmethylated substrate in vitro, but it is still more active at de novo methylation than other DNMTs; DNMT3 family could methylate hemimethylated and unmethylated CpG at the same rate [86]. The DNA methyltransferase activity of Dnmt2 was found to be substantially weaker than that for other DNA methyltransferases [87, 88].

Overexpression of these methyltransferase has been associated with promoter hypermethylation of tumor suppressor genes in various human cancers, including HCC [89–91]. Significant overexpression of DNMT3a and DNMT3b was found in HCC compared with the corresponding noncancerous liver tissue. The average level of mRNA for DNMT1 and DNMT3a was significantly higher in noncancerous liver tissue showing chronic hepatitis or cirrhosis than in histologically normal liver tissues and was even higher in HCC. Significant overexpression of DNMT3b and reduced expression of DNMT2 were observed in HCC compared with the corresponding noncancerous liver tissues, suggesting that overexpression of DNMT3a and DNMT1 is an early event in hepatocarcinogenesis [92]. Similar results were reported by other groups [93, 94]. The incidence of increased DNMT1 protein expression in HCC significantly correlated with tumor poor differentiation and portal vein involvement, suggesting that DNMT1 protein expression may play a critical role in the malignant progression of HCC and could be a biological predictor of both HCC recurrence and poor prognosis in HCC patients [95]. In another clinical-pathological study an increased expression of DNMT1, DNMT3a and DNMT3b mRNA and progressive increase in the number of methylated genes from normal liver, chronic hepatitis/cirrhosis to HCC was found; an increase in the DNMT3a and DNMT3b mRNA levels in HCC relative to their non-cancerous tissues may be a predictor of poor survival [96].

The HBV has been implicated as a potential trigger of epigenetic alterations. HBx represses the expression of *E-cadherin*, a tumor suppressor gene, by promoter hypermethylation through activating expression of DNMT1 [72, 97]. Expression of HBx can increase total DNMT activity by the upregulation of DNMT1 and DNMT3 selectively facilitating the promoter hypermethylation of some tumor

suppressor genes [73]. HBx can trigger epigenetic modification at different loci via its interaction with DNMT3A to specifically modulate target gene transcription, providing an alternative epigenetic mechanism by which HBx contributes to the pathogenesis of HBV-associated hepatitis and HCC [98]. *E-cadherin* promoter hypermethylation represents the most common cause for its inactivation and has been observed in many cancers including HCC [33]. HCV core protein downregulates *E-cadherin* expression at the transcriptional level; this effect was abolished after treatment of 5'-Aza-2'-deoxycytidine, a specific inhibitor of DNMTs. This repression was strongly correlated with hypermethylation of the CpG islands of the *E-cadherin* promoter via concerted action of both DNMT1 and DNMT3b in core-expressing cells. This finding suggests that HCV core protein plays a role in hepatocarcinogenesis by favoring cell detachment from surrounding cells and migration outside of the primary tumor sites [99]. The interaction between HBV and HCV infections and DNMT and how these interactions play a critical role in HCC development remain to be further investigated.

7 Aberrant Histone Modification and Hepatocellular Carcinoma

Histones are the chief protein components of chromatin, acting as spools around which DNA winds, and play a role in gene regulation. Five major families of histones exist: H1/H5, H2A, H2B, H3, and H4. Histone H2A, H2B, H3, and H4 are known as the core histones, while histones H1 and H5 are known as the linker histones. Histone modifications, recognized as a "histone code," affect chromatin structure, function, and gene expression as well as play an important role in the establishment of gene silencing during tumorigenesis [18]. Histone modifications comprise covalent posttranslational modifications of histone proteins. The N-terminal tails of nucleosomal histones are subject to different modifications, including acetylation, butylation, methylation, phosphorylation, and ubiquitination, which appear to work together with other epigenetic mechanisms in establishing and maintaining gene activity states, and regulating a wide range of cellular processes [38]. It is now accepted that histone modifications influence chromatin-based processes, including gene transcription, DNA repair, and DNA replication [100].

Enrichment in acetylation of histone tails is associated with transcriptional activation of genes. The functional consequences of methylation depend on the number of methyl groups, the residue itself, and its location within the histone tail. So far, the most studied modifications are acetylation and methylation of specific lysine residues on histone H3 and H4. For example, histone 3 lysine 4 dimethylation and trimethylation (H3K4me2 and

H3K4me3) and histone 3 lysine 9 monomethylation (H3K9me1) are associated with open chromatin and active gene expression. Histone 3 lysine 27 dimethylation and trimethylation (H3K27me2 and H3K27me3) and histone 3 lysine 9 dimethylation and trimethylation (H3K9me2 and H3K9me3) are associated with inactive chromatin and repression of gene expression [101]. Histone 3 lysine 4 monomethylation (H3K4me1) and histone 3 lysine 27 acetylation (H3K27ac) are found in the enhancer elements of genes and can influence gene expression [102].

HCC has been reported to display altered histone modification and as a result an altered cellular epigenetic state. HCC associated aberrant histone modification events affect expression of critical cellular genes and impair normal cellular activities [38]. High expression of H3K4me3 by immunohistochemical detection is associated with poor prognosis in HCC patients, particularly those at early stage [103]. Cai and colleagues reported high expression of H3K27me3 in HCC patients correlates closely with vascular invasion and predicts worse prognosis; the frequency of high expression of H3K27me3 was significantly greater in HCC tissues than in adjacent non-tumor tissues. Further correlation analysis demonstrated that high expression of H3K27me3 in HCC was associated with large tumor size, multiplicity, poor differentiation, serum α -fetoprotein level, late clinical stage, and relapse [104]. In HCC cell lines, *P16* and *RASSF1a* genes silencing was closely related to high levels of H3K9me2 and DNA methylation. In contrast, progesterone receptor (*PGR*) and estrogen receptor (*ER*) gene silencing was dependent on a high level of H3K27me3 rather than DNA methylation [105]. Another in vitro study demonstrated that the enrichment of H3K27 trimethylation, independent of H3K9 dimethylation, trimethylation, and DNA methylation, is an early event in the silencing of *p16* during HCC development [106]. Both promoter methylation and histone H3K9 modification are found to be involved in inactivation of the tumor suppressor gene, retinoblastoma-interacting zinc finger gene (*RIZ1*) in HCC [107]. Aberrant expression of histone 3 phosphorylation was revealed to play a role in hepatocarcinogenesis [108]. A recent study identified two modified forms of histone H4, H4K20me2 and H4K16ac, as new biomarkers of microvascular invasion in HCC that could be easily assessed in clinical practice by immunohistochemistry suggesting it would be helpful in management of patients with HCC [109].

Histone modification patterns are dynamically regulated by enzymes that add and remove covalent modifications to histone proteins. Histone acetyltransferases (HATs) and histone methyltransferases (HMTs) add acetyl and methyl groups respectively, whereas histone deacetylases (HDACs) and histone demethylases (HDMs) remove acetyl and methyl groups, respectively. These histone-modifying enzymes interact with each other as well as

other DNA regulatory mechanisms to tightly link chromatin state and transcription [110].

Expression of HDACs 1, 2, 3 and Ki-67 is significantly higher in HCC cells compared to normal tissue. In HCC tissues, HDACs 1–3 expression levels were highly correlated with Ki-67 expression and tumor grade and high HDAC2 was associated with poor survival in low-grade and early-stage HCC [113]. Several studies have revealed an elevated expression of HDACs and SIRT1 (a class III HDAC) in HCC that correlates with clinical-pathological features and recurrence of HCC [104, 111–113]. Enhancer of zeste homolog 2 (EZH2) is the catalytic subunit of the Polycomb-repressive complex 2 (PRC2) that represses gene transcription through H3K27me3. Combinatorial EZH2 and HDAC inhibition dramatically reduced the levels of nuclear β -catenin, T-cell factor-dependent transcriptional activity, and downstream pro-proliferative targets *CCND1* and *EGFR*. Functional analysis revealed that downregulation of EZH2 reduced HCC cell growth, partially through the inhibition of β -catenin signaling [114]. HDAC3 is essential for efficient DNA replication and DNA damage control. Deletion of HDAC3 impaired DNA repair and greatly reduced chromatin compaction and heterochromatin content. These defects corresponded to increases in histone H3k9, H3K14ac, H4K5ac, and H4K12ac in late S phase of the cell cycle, and histone deposition marks were retained in quiescent HDAC3-null cells; liver specific deletion of HDAC3 culminated in HCC [115]. Interestingly, it was discovered that HBx can interact directly with HDAC1 and trigger epigenetic modifications [98]. Histone H3 lysing 4 methyltransferase SMYD3 was found to be over-expressed and have a pro-carcinogenesis effect in HCC. Yang and colleagues reported HBx protein upregulated expression of SMYD3 in an HCC cell line and may promote hepatoma development and progression [116]. Activation of SMYD3 may be a key factor in hepatocarcinogenesis [111]. These findings may explain how HBV could affect expression of different histone modification machineries and result in HCC initiation and progression.

8 microRNA and Hepatocellular Carcinoma

MicroRNA are a class of single-stranded, small (18–26 nucleotide), noncoding RNAs. Most miRNAs are expressed in a temporal- and tissue-specific manner and play crucial roles in cell proliferation, apoptosis and differentiation during mammalian development [117, 118]. So far more than 2,000 miRNA have been identified. Aberrant expression of miRNAs is found in variety of cancers including HCC [119]. miRNA genes are generally transcribed from transcription start sites (TSS) by RNA polymerase II to form primary transcripts (pre-miRNA). Polymerase II-transcribed pri-miRNAs

are capped with 7-methylguanosine and are polyadenylated. The nuclear RNA polymerase III enzyme Drosha and its cofactor DiGeorge syndrome critical region gene 8 (DRCG8) process pre-miRNA into approximately 60-nt precursor miRNA, which forms an imperfect stem-loop structure. Pre-miRNA is transported into the cytoplasm by exportin 5 and is subsequently cleaved by Dicer, an RNase III enzyme, into mature miRNA [118, 120]. It is now widely accepted that miRNA undergoes the same regulatory mechanisms of any other protein-coding gene (PCG), including epigenetic regulation. Intriguingly, a subgroup of these ncRNAs (epi-miRNAs) control, directly and indirectly, the expression of epigenetic effectors such as DNMTs, HDACs, and polycomb genes, casting a new light on the functions of miRNAs as both genetic and epigenetic regulators [121].

Many deregulated miRNAs have been proven to play crucial roles in the initiation and progression of cancers and have been referred to as so-called oncomirs for their functions as oncogenes and tumor suppressor genes [119]. It has been shown that miRNAs are frequently located in cancer-associated genomic regions (CAGRs), which include minimal regions of amplification, loss of heterozygosity (LOH), common breakpoint regions in or near oncogenes and tumor suppressor genes, and fragile sites [122]. Involvement of miRNAs in HCC has been demonstrated, and the development of HCC via deregulation of various molecular pathways, including *p53*, *RAS/MAPS*, *PI3K/AKT/mTOR*, *WNT/β-catenin*, *MET*, *MYC*, and transforming growth factor beta, may be influenced by the expression of aberrant miRNAs thus affecting these pivotal cancer-associated pathways [123].

miRNA-122 is unique among the deregulated miRNAs; it is specifically expressed and highly abundant in the human liver. In patients with HCC, lower levels of miR-122 were associated with a shorter time to recurrence, whereas higher cyclin G1, a target of miR-122, expression was related to lower survival rate; miR-122 is downregulated in approximately 70 % of cases, suggesting miR-122 has tumor suppressor function in HCC [124]. miR-221, one of the oncogene-like miRNAs, is upregulated in 70–80 % of HCC samples, and there is evidence in support of its tumorigenesis activity [125]. By modulating multiple gene targets, miR-221 has been shown to be involved in several cancer-associated pathways including *CDKN1B/p27*, *CDKN1C/p57* [125], and *PTEN (PI3K/AKT/mTOR)* [126]. Overexpression of miR-221 leads to increased growth, proliferation, invasion, and migration and inhibits apoptosis in hepatocarcinogenesis [127]. A number of studies have documented aberrant expression, both downregulation and upregulation, of miRNAs that have been associated with every aspect of HCC development. For example, downregulated miRNAs are: miR-26, let-7 members, miR-199a-3p [128–130]. Upregulated miRNAs are miR-21, miR-155, miR-17-5p, miR-191 [131–134].

By using candidate and genome-wide approaches, more than 100 miRNAs were identified as dysregulated in HCC tumor tissues compared with non-tumor tissues [135].

miRNAs regulate HBV infection at the transcriptional level either by targeting cellular transcription factors required for HBV gene expression, or by directly binding to HBV transcripts [136]. miR-152 and miR148a target DNMT1 that can methylate viral DNA and inhibit the replication of HBV [92]. miR-1 regulates the expression of several host genes to enhance HBV replication and reverse the cancer cell phenotype, which is apparently beneficial for HBV replication [137]. HBV-encoded proteins could also influence expression of miRNAs; HBx was found to significantly upregulate 7 miRNAs expression, but downregulate let-7 family miRNAs and other several miRNAs expression [138, 139]. Chronic HCV infection is a risk factor for the development of HCC, especially in Western countries. miR-122, a liver-specific miRNA, and miR-320, a cell-cycle associated miRNA were downregulated in HCV positive samples; miR-16 induced cell cycle arrest and was upregulated among HCV positive samples [140]. miR-196 plays a protective role in HCV-induced HCC by upregulating heme oxygenase (decycling) 1 (*HMOX1*) expression and inhibiting HCV transcription [141]. More and more studies have shown that miRNAs are important mediators of HBV and HCV infections in hepatocarcinogenesis. Further studies are needed to enable more detailed mechanistic analysis of the miRNAs identified and to evaluate the usefulness of miRNAs as diagnostic/prognostic markers and potential therapeutic target molecules for HCC.

Recently, detection of genome-wide aberrant DNA methylation of microRNA host genes in HCC has been reported. Tumor and adjacent non-tumor tissues from 62 paired HCC cases was analyzed using Infinium HumanMethylation27 Analysis BeadChips that include 254 CpG sites covering 110 miRNAs from 64 host genes. 54 CpG sites from 27 host genes significantly differed in methylation levels between tumor and adjacent non-tumor tissues. DNA methylation alteration frequently occurs in miRNAs' host gene in HCC. Expression levels of three identified miRNAs were also measured. Decreased miR-10a expression in tumor tissues was related to its host gene's hypermethylation, supporting a tumor suppressor role for miR-10a in the repression of downstream target oncogenes. Therefore, aberrant miRNAs' methylation and expression may be useful molecular biomarkers for assessing HCC risk and early diagnosis [142].

miRNAs are notably stable in blood, and their expression patterns appear to be tissue-specific. miRNAs also circulate in the blood in a cell-free form [143, 144]. These characteristics make circulating miRNAs good candidates for noninvasive testing for cancer and precancerous conditions. Serum miRNAs have been proven to be highly promising diagnostic, prognostic and metastatic markers for HCC [145, 146]. Zhou et al. analyzed plasma miRNA

to diagnose HBV-related HCC and found that seven miRNAs (miR-122, miR-192, miR-21, miR-223, miR-26a, miR-27a and miR-801) provided high diagnostic accuracy. The miRNA panel can also differentiate HCC from healthy chronic HBV infection and cirrhosis [146]. miRNA expression profiles in HBV serum were compared with that in control serum and 13 miRNAs identified that were differentially expressed in HBV serum. These 13 miRNA-based biomarkers accurately discriminated not only HBV infected subjects from controls and HCV infected subjects, but also HBV positive HCC cases from control and HBV infected subjects. 6 miRNAs were identified as significantly upregulated in HCC compared with controls. These results demonstrated that serum miRNA profiles can serve as novel and noninvasive biomarkers for HBV infection and HBV-positive HCC diagnosis [147]. In a recent study, genome-wide miRNA expression detection and quantitative RT-PCR were used; the combination of plasma miR-483-5p level, which was statistically significantly over-expressed in cases, and HCV status can significantly differentiate HCC cases from controls with an area under the curve (AUC) of 0.908 ($p < 0.0001$) [148]. The sensitivity and specificity were 75.5 and 89.8 %, respectively. Aberrant expression of miR-483-5p in a large prospective study will provide further support for its applications to HCC detection. Similar results were also reported by Zhang et al. [149]. Apart from detection of serum or plasma miRNAs, urinary miRNAs as biomarkers were investigated for the early detection of HCC among high-risk HCV-infected patients. The sensitivity of miR-650 and the specificity of the miR-618/miR-650 combination were greatly improved compared to the traditional α -fetoprotein (AFP) level-based detection method. The proposed HCC miRNA signatures may be of great value for the early diagnosis of HCC, before the onset of disease in HCV-positive patients. The significance of this approach is amplified by the use of urine as a sample source as it offers a noninvasive approach for developing screening methods that could reduce mortality [150]. The possibility of profiling miRNAs in circulating samples, such as serum, plasma, and urine, represents a noninvasive approach to detect HCC-related specific miRNAs and is a promising field in the clinical surveillance for the development and recurrence of HCC.

miRNAs are recognized as one of the major regulatory gatekeepers of protein-coding genes in the human genome with different functions [125], but miRNAs may have other as yet unknown functions including the activation of gene transcription. More novel miRNAs were discovered and investigated in hepatocarcinogenesis. Aberrant miRNAs have the potential to be used for early and noninvasive detection of HCC. Further investigation and evaluation of the candidate miRNAs, as HCC diagnosis, prognosis, metastasis, and recurrence biomarkers, are needed before translation into clinical practice.

9 Conclusions and Perspectives

Epigenetics is one of the most flourishing areas in biomedical research today. Epigenetic changes such as DNA methylation, histone modification, and changes in expression of miRNAs are associated with a broad range of diseases and could represent important pathways by which environmental factors influence disease risks, both within individuals and across generations [151]. Mounting studies have shown that aberrant epigenetic changes are involved in the development of HCC, from the precancerous condition to advanced metastatic stage. Great progress and discovery in epigenetics provide us with a deeper insight into the molecular mechanisms underlying the pathogenesis of HCC. The progressive accumulation of epigenetic alterations during development of HCC gives a unique opportunity to use these changes as biomarkers in HCC detection. For example, miRNA expression profiles altered in HCC paves the way for early detection and prediction of HCC. Chronic HBV and HCV infections are the most important risk factors for HCC. Some evidence suggests that there are correlations between epigenetic changes (gene specific hypermethylation, miRNA alterations, and others) and viral infection, but further studies should also address whether epigenetic changes induced by HBV and/or HCV infection promote HCC development by inducing direct deregulation of epigenetic states or if the changes are consequences of the activation of inflammatory pathways [38].

More and more studies are finding that serum methylated DNA and serum miRNAs could be potential biomarkers for early detection and prediction of HCC. Since these methods are relatively noninvasive and appear to have good sensitivity and specificity, they have a great advantage for HCC screening and prospective investigations in epigenetic epidemiology studies. The discovery of more novel circulating epigenetic biomarkers will further enhance mechanism-based approaches to early diagnosis and prediction of recurrence and metastasis in HCC.

Several methods have been used to detect DNA methylation: bisulfite genomic sequencing, methylation-specific PCR (MSP), real-time PCR-based assays (including MethyLight, a sodium-bisulfite-dependent, quantitative, fluorescence-based, real-time PCR method), and restriction enzyme digest. Traditional MSP was widely used to detect gene-specific hypermethylation in HCC. A deeper understanding of the cellular epigenome will require the development of reliable techniques that allow measurement of the DNA methylation status of specific CpGs at the single cell level [152]. High-throughput techniques for characterization of epigenetic changes are being developed. A large number of genome-wide methods and novel Next-Generation Sequencing for detection of epigenetic alterations are making studies feasible now and in the future.

Significant progress in cancer epigenetics has increased our understanding of the molecular mechanisms in different cellular processes and in abnormal events involved in tumorigenesis. But there are many questions that still need to be answered; a critical one in the field of cancer epigenetics regards the factors that trigger primary epigenetic alterations. Further investigation should provide insight to understand the epigenetic mechanisms for hepatocarcinogenesis and to develop novel strategies for prevention, early detection, and treatment of hepatocellular carcinoma.

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Specific Type Epigenetic Changes in Cervical Cancers

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Abstract

Cancer is a genetic and epigenetic disease. Multiple genetic and epigenetic changes have been studied in cervical cancer; however, such changes are selected for during tumorigenesis and tumor aggression is not yet clear. Cervical cancer is a multistep process with accumulation of genetic and epigenetic alterations in regulatory genes, leading to activation of oncogenes and inactivation or loss of tumor suppressor genes. In cervical cancer, epigenetic alterations can affect the expression of papillomaviral as well as host genes in relation to stages representing the multistep process of carcinogenesis.

Key words Cervical cancer, Epigenetics, Papilloma virus, Tumor suppressor genes

1 Introduction

Cervical cancer is the third most common tumor in women worldwide with more than 85 % of the cases occurring in developing countries [1]. And cervical cancer is seriously harmful to the health of women, remains a leading cause of cancer-related death for women in the world. Now, the principal treatment for cervical cancer is the radical hysterectomy, patients with advanced stages are mainly treated with combined radiotherapy and chemotherapy or radiotherapy alone [2]. However, survival has not improved with current treatment for patients with recurrent or distant metastatic cervical cancer [3]. It is difficult to foresee a dramatic increase in cure rate even with the most optimal combination of cytotoxic drugs, surgery, and radiation. Therefore, it's necessary for the development of more effective and novel treatment. The diagnosis and treatment of this disease can have a profound impact on the quality of patients' lives.

Cancer is a genetic and epigenetic disease. In contrast to genetic mutations, epigenetic events lead to changes in gene expression other than by means of DNA sequence alterations including DNA methylation, histone modifications, and miRNA dysregulation. Aberrations epigenetic regulation in cell proliferation, apoptosis,

autophagy and changes in cell adhesion motility is involved even in the early and precancerous stages of human carcinogenesis [4, 5]. From what is presently known, epigenetics are more and more involved in the development and progression of cervical cancers. Here we discuss specific type epigenetic changes in cervical cancers.

2 Specific Type Epigenetic Changes

2.1 HPV Genome Methylation

Cervical cancer is a multistep process with accumulation of genetic and epigenetic alterations in regulatory genes, leading to activation of oncogenes and inactivation or loss of tumor suppressor genes. According to several epidemiological and biological studies, Infection with oncogenic HPV has been demonstrated to be the most significant risk factor in the etiology of cervical cancer [6–8] and its precursor lesion, cervical intraepithelial neoplasia [9–11]. However, the mechanism responsible for HPV initiated cervical carcinogenesis remains elusive [12].

Human papillomaviruses (HPV) is a member of the Papovaviridae family of virus. They consist of a 72-capsomere capsid containing the viral genome. Each capsomer is composed of two viral proteins: L1 and L2. Epidemiological and laboratory data suggest that the presence of specific HPV subtypes and the integration of their HPV DNA into the genome are important factors in the development of cervical neoplasia. Genital HPVs are classified according to their potential to induce malignant transformation as follows: high-risk types (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 68, 73, and 82); probable high-risk types (26, 53, and 66), and low-risk types (6, 11, 40, 42, 43, 44, 54, 61, 70, 72, 81, and CP6108) [13]. Among high-risk strains, HPV 16 and 18 are those most closely associated with cervical carcinoma and are found in >50 and 20 % of squamous cell carcinomas, respectively [7]. The association of the HPV genome and the cellular DNA replication machinery is mediated by E1 viral protein. The carcinogenic role of human papillomavirus (HPV) lies mainly on the E6 and E7 oncoproteins which initiate dysregulation of cellular proliferation and apoptotic mechanisms at well-defined targets, the primary targets are p53 and retinoblastoma (Rb) tumor suppressor proteins, respectively. Expression of E6 and E7 genes are highly related to the progression of cervical neoplasia. The role of E6 protein is to switch off apoptotic defense activated by genomic instability arising in consequence of E7 induced cell cycle activation. The early papillomaviral protein E2 mediates a negative feedback on E6/E7 expression through the E2 binding elements of the LCR. During HPV DNA integration, the viral genome usually breaks in the E1/E2 region. The break generally leads to loss of the E1 and E2 regions. Loss of E2 function results in over-expression of E6/E7. Over-expression of E6/E7, meanwhile, has been observed to lead to malignant transformation of host cells

and to tumor formation [14]. HPV viral integration into host genome DNA is associated with progression from polyclonal to monoclonal status in cervical intraepithelial neoplasia (CIN), and these events play a fundamental role in the progression from low-grade to high-grade cervical neoplasia [15].

Infection with high risk type human papilloma viruses is the main risk factor for cervical cancer [16]. High risk type human papilloma viral infections are extremely widespread throughout the world, but malignant transformation is a rare consequence of the infection. The progression from precursor intraepithelial lesions to cervical carcinomas is accompanied by additional genetic and epigenetic changes that have not been fully characterized. Genetic changes with resultant genomic instability have long been recognized as an important mechanism for cervical carcinogenesis [17, 18]. Increasing reports of DNA methylation findings in cervical cancer and precancerous lesions support a role for this phenomenon in cervical cancer development [19–21]. The role of HPV genome DNA hypermethylation has of late been the subject of study. Sushma et al. performed a study in two HPV-18 cervical cancer cell lines, Hela and C4-1, and clinical samples. They found that there was also clonal heterogeneity in the methylation status of the different regions analyzed. There was complete or partial methylation, respectively, of the HPV enhancer in three of six the cancers, and lack of methylation in the eight samples from asymptomatic patients. The promoter sequences were methylated in three of the six cancers and four of the six smears [22]. Turan et al. performed a study to assess the status of the HPV-18 genome; they found that HPV-18L1 was mostly unmethylated in asymptomatic infections and infections with precancerous lesions. In contrast, L1 sequences were strongly methylated in all cervical carcinomas [23]. Kalantari et al. had studied L1 and LCR regions by bisulfite modification in 115 clinical samples. They found that high heterogeneity on methylation status in different patients' sample and even in different samples of the same patient. The methylation frequency of L1 was 30 % and lower in other positions, particularly at a CPG site located in the linker between two nucleosomes positioned over HPV-16 enhancer and promoter. However, methylation at most sites was higher in carcinomas than in dysplasia, maybe it's related to the tandem repetition and chromosomal integration that occurs in invasive lesions [24].

HPV DNA methylation patterns appear to influence viral transcription *in vitro*. Highly methylated regions of the HPV viral upstream regulatory region of HPV lead to inactive transcription *in vitro*, which can decrease viral proliferation. DNA methylation status plays an important effect in diagnosing, treating and evaluating the prognosis of cervical cancers. It will be of major interest to analyze human papillomavirus oncogene expression in cervical tumors before and after treatment of patients with DNA methylation.

2.2 Genes' Promoter Hypermethylation

Epigenetic mechanisms that result in aberrant gene expression are prominent features of many cancer types, and promoter hypermethylation is one of the main epigenetic mechanisms of gene silencing. The silencing of specific genes by the DNA promoter hypermethylation has been suggested to modify the biological characteristics of human cancers. Also, it is believed that unique promoter hypermethylation profiles exist for the various human cancer types, in which some gene changes are shared while others are cancer-type specific [25]. Many studies have indicated that aberrant DNA methylation may play roles in carcinogenesis as a result of (a) increased gene mutagenicity due to deamination of 5-methylcytosine to thymine; (b) possible association of aberrant DNA methylation with genetic instability; and (c) silencing of tumor-related genes through DNA methylation on CpG islands in cooperation with histone modification.

Cervical cancer is the leading cause of cancer-related mortality in women worldwide. In addition to human papillomavirus infection—the well-known critical event in the malignant transformation and immortalization of cervical epithelial cells—other factors, such as inactivation of tumor suppressors, are involved in its the multistage pathogenesis of cervical cancer [26]. A growing number of cancer-related genes are being recognized that harbor dense methylation of cytosine in normally unmethylated CpG-rich sequences, called CpG islands, located within the 5' gene promoter regions [27]. Several studies have indicated that methylation is essential for normal development [28], X chromosome inactivation [29], and imprinting [30, 31]. Aberrant promoter methylation is an important mechanism for loss of gene function in tumors and may be more frequent than mutations in coding regions [32–35].

There are numerous reports demonstrating that abnormal promoter hypermethylation lead to silencing or diminished expression of tumor suppressor genes in cervical carcinoma.

P53 is the first tumor suppressor gene linked to apoptosis. As a transcription factor, P53 can detect DNA damage and subsequently arrest cells in the G1 phase of the cell cycle and allow repair them. The p53 pathway responds to stresses can disrupt the fidelity of cell division and DNA replication. It becomes evident that the mutation of p53 can disrupt the apoptosis way in the majority of human tumors. However, the p53 pathway can be aberrant methylated in some of their components. P73, a member of the p53 family, was found that represents a critical alternative mechanism for inactivation of TAP73 gene via CpG-island hypermethylation in cervical cancer. TAP73 has been reported as involved in cellular response to DNA damage induced by radiation and chemotherapeutic agents and when it is overexpressed in cells, it can trigger p53-independent apoptosis by activating transcription of p53-responsive genes such as p21, Bax, Mdm2, and GADD45 and inhibits cell growth in a p53-like manner by inducing apoptosis [36, 37].

P16 was first reported as a novel tumor suppress gene in 1994, located in 9p21 which is important to the negative regulation of cell cycle. Mutation of p16 will lead to loss and confusion of regulation to cell division, and induce abnormal multiplication of cell and form tumor. P16 can be repeated and specific competition with cyclinD1 control CDK4, inhibiting the kinase activity of both, so that cells cannot be fast through the G1 / S conversion. P16 gene has been one of the most studied in cervical cancer. Loss of expression of p16 gene, often associated with aberrant methylation, is a frequent alteration in many types of human neoplasia [38, 39]. The methylation alteration of p16 is the earliest step of cervical cancer. The tumor suppressor gene p16^{INK4A} was almost overexpressed in high-risk HPV cervical cancer and precancerous lesions specimens [40]. Another study was performed to the pattern of aberrant methylation during the multistage pathogenesis of cervical cancers, they found that p16 methylation rate was over 20 % and there was no association of aberrant methylation with HPV, smoking, or hormone use.

The tumor suppressor gene adenomatous polyposis coli (APC) located at human chromosome 5p21-22 which acts as an antagonist of the Wnt signaling pathway leading to proteolytic degradation of β -catenin. The Wnt signaling pathway is known to play key roles during development and in maintaining homeostasis in many adult tissues. Its aberrant activation is associated with cancers in many tissues such as breast, colon, pancreas, skin, and liver. In the absence of Wnt stimulation, β -catenin enters the nucleus and associates with LEF/TCF transcription factors, leading to the transcription of Wnt target genes such as cyclin D1, Myc, and TCF-1. It is established that mutational or epigenetic inactivation of APC contributes to uncontrolled proliferation in many tumor types. APC can be inactivated by promoter hypermethylation in cervical, ovarian and endometrial cancers [41]. And further study found that APC promoter hypermethylation is frequent in adenocarcinoma and seems incidental in squamous cell carcinoma in cervical cancers [42–44].

The spleen tyrosine kinase (Syk) gene was originally cloned from porcine cDNA by Japanese scholars Taniguchi et al. in 1991 [45]. Its coding protein is a kind of non-receptor tyrosine kinase. Syk is one of the two members of the Syk family (Syk and ZAP-70), which is widely expressed in multiple cell types. The Syk protein which contains 629 amino acid residues is comprised of (1) a kinase domain and (2) two SH2 domains: SH2 (N) and SH2 (C), in tandem. Loss of Syk expression has been reported with malignant phenotypes such as increased motility and invasion [46]. Emerging evidence indicates that wild-type Syk suppresses cell growth, and it may thus act as a tumor suppressor [47, 48]. In cervical cancer, it has been reported that Syk mRNA expression decreases and is frequently lost throughout the progression of cervical carcinoma.

Hypermethylation in the Syk promoter correlates with loss of Syk mRNA expression in human cervical carcinoma and is likely the mechanism underlying this loss. These data suggest that epigenetic inactivation of Syk may contribute to the development or/and progression of cervical carcinoma [49].

The fragile histidine triad gene (FHIT) is a putative tumor suppressor and belongs to the histidine triad superfamily of nucleotide-binding proteins. It is located at the fragile site locus FRA3B on chromosome 3p14.2, a region of the genome which shows LOH in a variety of cancers. The abnormality in structure and function of the suppressor gene is obviously associated with the pathogenesis of some tumors, such as esophageal, head and neck, lung, gastric, breast, cervical carcinomas, and precancerous and cancerous lesions. In cervical cancer, FHIT promoter hypermethylation correlates inversely with gene expression and FHIT has been detected in up to 75 % of cervical carcinomas [50–52]. These findings support that FHIT promoter hypermethylation has a significant oncogenic effect in cervical carcinogenesis. Further study suggesting that hypermethylation of this gene has been associated with loss of expression and advanced stages of cervical carcinoma, suggesting its participation in disease progression [50].

The expression pattern of human telomerase reverse transcriptase (hTERT), the telomerase catalytic subunit gene, is correlated with telomerase activity. The promoter region of the hTERT gene has been located in a CpG island and may therefore be regulated by DNA methylation. Data on hTERT expression in cervical cancer has revealed that 0–33 % of normal cervixes exhibited hTERT mRNA expression, whereas 80–100 % of cervical cancers showed hTERT expression [53–56]. Despite it is expected that hypermethylation decreases gene expression, a study has found a correlation between reduced expression and catalytic subunit activity with demethylation [57]. This may explain what was found with regard to better prognosis of patients with cervical cancer whose tumors lack hTERT methylation and hTERT methylation may be of prognostic significance in ovarian and cervical cancer [58].

Death-associated protein kinase (DAPK) was first found by Kimchi in 1995. DAPK gene is located in 9q34.1 which is an actin-associated, calcium/calmodulin-dependent enzyme with serine/threonine kinase activity [59, 60]. DAPK suppresses tumor growth and metastasis by increasing the occurrence of apoptosis *in vivo*, and therefore has been characterized as a tumor suppressor gene [61]. DAPK is a pro-apoptotic gene and participates in various apoptotic systems. DAPK acts as a tumor suppressor, and its inactivation by promoter hypermethylation has been frequently observed in various human cancers. A large number of studies demonstrated consistently the hypermethylated state of this gene in cervical cancer; therefore, it may serve as a marker for detection of this malignancy. Moreover, DAPK promoter methylation is more prominent in SCC than in AC of the uterine cervix [42–44, 62–65].

The Ras Association Domain family 1 (RASSF1A) gene consists of two main variants (RASSF1A and RASSF1C), which are transcribed from distinct CpG-island promoters. Aberrant promoter hypermethylation and subsequent decreasing of expression is a frequently detected epigenetic alteration in several malignancies including cervical cancer. RASSF1A protein is actively involved in microtubule regulation, genomic stability maintenance, cell-cycle regulation, apoptosis modulation, cell motility, and invasion control, and may act as a negative Ras effector inhibiting cell growth and inducing cell death [66–74]. Studies in patients with cervical cancer have demonstrated its silencing by methylation in up to 30 % of tumors [75–78]. RASSF1A promoter hypermethylation was found in HPV-negative but not in HPV-positive cervical cancer cell lines [76]. In agreement with the cell lines, the primary cervical tumors harboring HPV had a low frequency RASSF1A promoter hypermethylation. This low frequency can be refined by the histological type; it is more frequent in AC than in SCC [75, 79, 80]. These suggest that HPV and RASSF1A may be involved in the same tumorigenic pathway.

E-cadherin is one of the major constituents of cell-adhesion complexes in epithelial cells [81, 82]. It is a 97-kDa trans-membrane glycoprotein encoded by the E-cadherin gene (CDH1) located on chromosome 16q22.1. It plays important roles in the establishment of adherent-type junctions by mediating calcium-dependent cellular interactions, and is thought to be a tumor suppressor protein. Partial or total loss of E-cadherin expression occurs in the majority of human carcinomas including cervical cancer. Besides its role in physical cell–cell adhesions, E-cadherin is also thought to be involved in intracellular signaling in normal epithelial cells, since down regulation of this molecule in epithelial cells is frequently associated with tumor formation and differentiation [83]. Aberrant hypermethylation of CpG islands in CDH1 promoter regions is associated with suppressed transcriptional activity and it is frequently found in invasive cervical cancer, particularly in SCC histologic type [43, 62, 63]. The CDH1 gene can be detected in serum samples of invasive cervical cancer patients and promoter hypermethylation can indicate an increased risk for relapse [84, 85].

Retinoic acid receptor beta (RAR- β 2) binds retinoic acid, the biologically active form of vitamin A, which mediates cellular signaling in embryonic morphogenesis, cell growth and differentiation. RAR- β 2 limits growth of many cell types by regulating gene expression. Reduction or lack of RAR- β 2 gene expression has been detected in various malignancies. Loss of its expression due to promoter hypermethylation was first detected in colon and breast cancers. In cervical cancer, the RAR- β 2 gene is of particular interest because retinoic acid inhibits transformation of human keratinocytes by HPV-16 and leads to regression of moderate cervical dysplasia [86, 87]. CpG methylation of the 5' region of RAR- β 2 gene contributes to gene silencing also in cervical carcinogenesis.

Rate of RAR- β 2 methylation progressively increases from 11 % in low grade to 29 % in high grade lesions and from 33 to 63 % in invasive cancers [19, 62, 88, 89], suggesting that this abnormality is an early event in multistage cervical carcinogenesis.

HIC1 is a zinc finger transcription factor that is transcriptionally silenced by promoter methylation in several types of human cancer. Homozygous disruption of Hic1 impairs development and results in embryonic and perinatal lethality in mice, while heterozygous mice develop many different spontaneous malignant tumors including a predominance of epithelial cancers in males and lymphomas and sarcomas in females, suggesting that this gene acts as a tumor suppressor gene. The complete loss of Hic1 function in heterozygous mice appears to involve dense methylation of the remaining wild-type allele promoter [90]. It has been found that the HIC1 gene is down regulated in many cervical cancer cell lines and re-expressed upon treatment with a demethylating drug [19]. In primary cervical tumors, its methylation rate varies between 18 and 45 % [42, 62]. It has recently been shown that its loss of function accentuates the tumorigenic effect of loss of p53 [91]. These results suggest that the tumor suppressor role of HIC1 and its inactivation by promoter methylation in cervical cancer may be a critical epigenetic change in tumor development.

The tumor suppressor gene in lung cancer-1 (TSLC1) at chromosomal region 11q23.2 is abrogated or markedly reduced in a number of human cancer tissues and cell lines, including lung, prostate, breast, cervical, esophageal, and pancreatic cancers [20, 92–96]. The IGSF4/TSLC1 gene codes for an immunoglobulin-like intercellular adhesion molecule which mediate homophilic or heterophilic interactions in a calcium-independent way. It was identified as a potential tumor suppressor in small cell lung cancer. This gene was first identified as a tumor suppressor gene in lung cancer and silencing can derive from loss of heterozygosity or promoter hypermethylation [97]. It was demonstrated also in cervical cancer that its silencing can be a result of LOH due to promoter hypermethylation. It has been shown that normal epithelium and CIN-I lesions are free of methylation at IGSF4, whereas methylation rate in CIN-III is 35 %, which increases to 58 and 65 % in invasive tumors [98, 99]. These data demonstrate that IGSF4 may play an important role in cervical cancer development.

2.3 Global Methylation Changes

In human cancer, global DNA hypomethylation leads to genomic instability, affects repeated DNA sequences, tissue-specific genes and proto-oncogenes or causes loss of imprinting with a biallelic expression. Further, the level of DNA hypomethylation increases with tumor progression [100, 101]. The main mechanisms set forward in attempting to explain cancer causation by hypomethylation are chromosome instability and reactivation of transposable elements and/or inappropriate gene activation. Also for cervical

cancer, there are data on global genomic and gene-specific epigenetic changes. The first evidence for global DNA hypomethylation in cervical neoplastic tissues was provided by [³H]-S-adenosylmethionine incorporation assay. As expected, the extent of ³H-methyl group incorporation was increased threefold and sevenfold in DNA from cervical dysplasia and cancer as compared with DNA of normal cervixes and within dysplasias as long as they progress from normal to low- and high-grade [101]. These findings were confirmed in a study using a computer-assisted assay based on quantitative analysis of DNA methylation in individual interphase nuclei by immunolabelling with anti-5-methylcytosine antibodies, progressive hypomethylation was observed in dysplastic and cancer cells as compared to normal controls [102]. These data, along with observations of gene promoter hypermethylation of a number of genes during pre-invasive to invasive stages of cervical cancer, suggest that both phenomena are coincident during carcinogenesis of the cervical cancer.

2.4 Histone Modifications

Histone modifications are strongly associated with formation of the nucleosome structure and are closely linked to CpG island methylation by interacting with Methyl-CpG-binding proteins (MBD's) and DNA methyltransferases (DNMT's). Modifications including methylation, acetylation or phosphorylation of certain position of the histone tails. Whereas histone methylation is associated either to activation or to repression, histone hypoacetylation mediated by histone deacetylases leads mostly to DNA relaxation and subsequent accessibility for transcriptional factors with repression of transcription. It was reported that during the tumorigenic process, cancer cells had a loss of monoacetylated and trimethylated forms of histone H4, predominantly at acetylated Lys16 and trimethylated Lys20 residues of histone H4, which were associated with hypomethylation of DNA repetitive sequences, a hallmark of cancer cells [103]. In cervical cancer, it has been reported that phosphorylated and acetylated forms of histone H3 in cytologic smears shows a marked association of histone H3 modifications with progression of the disease from CIN I to CIN II and CIN III [104].

2.5 MicroRNA and Epigenetics

MicroRNA (miRNA) is a novel class of short noncoding RNA molecules regulating a wide range of cellular functions through translational repression of their target genes. DNA methylation is one of the heritable epigenetic modifications, repressing gene expressions and consequent phenotypic alterations without changing the DNA sequence. Recent researches have proved that the abnormal interaction miRNAs and epigenetics definitively linked to HPV associated cervical cancer. Aberrant epigenetics are involved in silencing of tumor-suppressor miRNAs in carcinogenesis. On the other hand, miRNAs can improve the repair of viral DNA and silence tumor-suppressor genes by regulating DNA

methylation or histone modifications, suggesting that a distinct connection between aberrant expression of miRNAs and the development of cancer and miRNAs can be potential therapeutic targets. Many studies have indicated that a variety of miRNA expression was significantly upregulated [105–109] or downregulated [105, 107, 110–112] in cervical cancer.

Some miRNAs are controlled by epigenetic alterations such as DNA methylation and histone modification in human cancer cells. Activation of tumor suppressor miRNAs by chromatin modifying drugs may cause downregulation of target oncogenes and could be a novel strategy for the prevention and treatment of human cancer [113]. DNA methylation and histone modification play critical roles in chromatin remodeling and general regulation of gene expression in mammalian development and in human diseases, such as cancer [114]. The epigenetic changes occupy an important position in the MiRNA regulation process, such as the expression of DNA methyltransferase enzyme, the maintenance of cell DNA methylation or histone change. miRNA 203 (miR-203) is expressed specifically in suprabasal layers of stratified epithelia, as well as in psoriatic plaques, implicating it as a regulator of epithelial maturation. When miR-203 is highly expressed in HPV-positive cervical cells, they can promote the repair of genomic methylation to inhibit the viral DNA amplification. The high levels of miR-203 are inhibitory to HPV amplification and that HPV proteins act to suppress expression of this microRNA to allow productive replication in differentiating cells. HPV E7 protein downregulates miR-203 expression upon differentiation which may occur through the mitogen-activated protein (MAP) kinase/protein kinase C (PKC) pathway. This can make the abnormal proliferation of differentiated cells to maintain and achieve a large number of viral DNA amplification. miR-203 is also inversely correlated with amounts of oncogenes p63, Bax [115].

In recent years, miRNA and epigenetic changes for cervical cancer diagnosis, treatment, prognosis has opened up a new field. Many studies have indicated that several miRNAs have the function of oncogenes or tumor suppressor genes.

3 Conclusion

Cervical cancer is one of the major causes of death in women worldwide [116]. Infection with oncogenic human papillomavirus (HPV), which can be detected in virtually all cases of cervical cancers, is the most significant risk factor in the etiology of this type of cancer [7]. However, HPV infection is necessary but not sufficient to cause cervical cancer. The molecular mechanisms underlying such an inefficient HPV-initiated cervical carcinogenesis remain elusive. In cervical cancer, a number of epigenetic alterations occurring during all stages of cervical carcinogenesis in both human

papillomavirus and host cellular genomes have been identified. Epigenetic changes have been reported in many cancers and are now recognized to be at least as common as genetic changes. These include global DNA hypomethylation, hypermethylation of key tumor suppressor genes, histone modifications, and miRNA. Moreover, epigenetic abnormalities occur very early in the carcinogenic process they can potentially serve as molecular markers for early detection. Aberrant hypermethylation of CpG islands, which are CpG dinucleotide-rich areas located mainly in the promoter regions of many genes, serves as an alternative mechanism for inactivation of tumor suppressor genes (TSGs) in cancers [33, 117–119]. Such hypermethylation of gene promoters has been increasingly pinpointed as an early event in cervical carcinogenesis [117, 120, 121]. The realization that genetic and epigenetic alterations are present at the earliest steps of the malignant progression of cervix uteri has led to testing the presence of these abnormalities, such as p16 expression [122, 123].

A large number of studies looking at the methylation status of tumor suppressor genes have uncovered that some genes are found hypermethylated in preinvasive lesions, raising the possibility that developing a panel of methylation markers may therefore have value in early detection of cancer precursors, provide added reassurances of safety for women who are candidates for less frequent screens, and predict outcomes of women infected with carcinogenic human papillomavirus infections [124]. In the therapeutic field, transcriptional therapy is a very promising form of cancer treatment that is being extensively evaluated. Unlike genetic changes, the aberrant DNA methylation and histone modifications of neoplastic cells are reversible, suggesting strategies to revert these epigenetic alterations via pharmacological manipulation. Therefore, the reversible nature of epigenetic changes constitutes a target for transcriptional therapies, namely, DNA methylation and histone deacetylase inhibitors which can reactivate expression of tumor suppressor genes and induce histone hyperacetylation in the tumors of patients with cervical cancer after treatment with these agents. But further investigations in clinical trials are needed to prove and integrate epigenetic pathway modulating agents. Furthermore, epigenetic inhibitors have potential in cancer treatment.

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Epigenetics in Head and Neck Cancer

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Abstract

Epigenetics refers to the study of heritable changes in gene expression that occur without a change in DNA sequence. Research has shown that epigenetic mechanisms provide an “extra” layer of transcriptional control that regulates how genes are expressed. These mechanisms are critical components in the normal development and growth of cells. Epigenetic abnormalities have been found to be causative factors in cancer, genetic disorders, and pediatric syndromes. Head and neck cancers are a group of malignancies with diverse biological behaviors and a strong, well-established association with environmental effects. Although the hunt for genetic alterations in head and neck cancer has continued in the past two decades, with unequivocal proof of a genetic role in multistage head and neck carcinogenesis, epigenetic alteration in association with promoter CpG islands hypermethylation has emerged in the past few years as one of the most active areas of cancer research. Silencing of the genes by hypermethylation or induction of oncogenes by promoter hypomethylation is a frequent mechanism in head and neck cancer and achieves increasing diagnostic and therapeutic importance. In this context it is important for clinicians to understand the principles of epigenetic mechanisms and how these principles relate to human health and disease. It is important to address the use of epigenetic pathways in new approaches to molecular diagnosis and novel targeted treatments across the clinical spectrum.

Key words Epigenetics, Head and neck cancer, Promoter methylation, Epigenetic silencing, CpG islands, Hypermethylation, Hypomethylation

1 Introduction: Epigenetic Abnormalities in Head and Neck Cancer

In principle “epigenetics” refers to the somatically heritable differences in gene expression not attributable to intrinsic alterations in the primary sequence of DNA. In a single individual, cells of all different types have same genome, but alternatively multiple, complex, and different “epigenomes” which define their respective phenotypes. Epigenetic control occurs throughout the genome, as well as in regulatory elements, far distant from the genes they control, and can mediate interactions between chromosomes. Core elements of the epigenetic regulation of gene expression include how DNA is packaged or wrapped around nucleosomes, and how those nucleosomes are positioned throughout the genome [1].

Similarly key regulatory features of epigenetics include how chromatin and nucleosomes are modified by a complex series of enzymes and their subsequent interactions with proteins which recognize these modifications.

Cancer cells have diverse biological capabilities which are conferred by numerous genetic and epigenetics changes. Increasingly, it has become evident that epigenetic changes in cancer constitute driver events in tumorigenesis, and exome sequencing studies have revealed recurrent mutations in key chromatin modifiers in multiple tumor types. Cancers of all types, and even different cell populations within a single patient's cancer, have different genomes resulting from the many mutations which alter the DNA. In addition, extensive and biologically significant epigenetic changes are increasingly recognized; these changes constitute the "cancer epigenome." Currently, the most studied and recognized cancer specific epigenetic changes are alterations in DNA methylation and histone acetylation, which have been extensively reviewed in literature [2]. Briefly, these modifications include global hypomethylation, regional hypermethylation and chromatin events that are closely tied to DNA methylation changes.

The epigenetic alterations involved in cancer, occur within a larger context of extensive alterations to chromatin in neoplastic cells in comparison with the normal cells from which they are derived. These involve both losses and gains of DNA methylation as well as altered patterns of histone modifications. Although the molecular determinants which that underlie these types of chromatin changes in tumor are only beginning to be elucidated, the best understood component is the transcriptional repression of a growing list of tumor-suppressors and candidate tumor suppressor genes. This suppression is associated with abnormal methylation of DNA at certain CpG islands that often lie in the promoter regions of these genes. By this mechanism of "silencing" the expression of these tumor-suppressor genes in the cancer cells can be reduced or eliminated as an alternative mechanism to genetic mutation [3]. Random searches of the cancer-cell genome are now being carried out to detect changes in methylation and chromatin status, either overall or in specifically hypermethylated (which results in gene silencing) or hypomethylated (which results in increased transcription) regions. This might lead to the discovery of new factors important for tumor initiation and progression.

Genome methylation patterns are being developed as biomarker for tumor type, and also as informative markers for risk assessment, early detection, and monitoring of prognosis, as well as indicators of susceptibility or response to therapy [4] (Table 1). The ramification of global hypomethylation for tumor progression are less understood, but might contribute to genomic instability, structural changes in chromosomes and increases in gene expression. Local changes in promoter methylation, and concomitant

Table 1
Epigenetic alterations in cancer cells and their consequences

Epigenetic change	Consequence and mechanism
DNA hypomethylation	Activation of cellular oncogenes and transposable elements resulting in increased proliferation, growth advantage, genomic instability, and transcriptional noise
DNA hypermethylation	De novo hypermethylation of CpG islands within gene promoters leading to silencing of tumor suppressor genes resulting in genomic and chromosomal instability, increased proliferation, and growth advantage
Loss of imprinting (LOI)	Reactivation of silent alleles, biallelic expression of imprinted gene resulting in expansion of precursor cell population
Relaxation of X-chromosome inactivation	Apparently age related but results in altered gene dosage and growth advantage
Histone acetylation	Could be gain of function or loss of function but results in activation of tumor promoting genes and defects in DNA repair and checkpoints
Histone deacetylation	Silence tumor suppressor genes causing genomic instability and increased proliferation
Histone methylation	Loss of heritable patterns of gene expression (“cellular memory”)
MicroRNAs amplification in cancer	Function as oncogenes and result in neoplastic transformation
MicroRNAs deletion in cancer	Function as tumor suppressors and cause neoplastic transformations

loss of gene expression, have been the recent focus of investigation, and the effect of these changes on tumor biology is becoming increasingly apparent. As investigators have continued to search for promoter hypermethylation in candidate tumor-suppressor genes, it has become apparent that many genes, which are located across all chromosomal locations, are epigenetically silenced in cancer cells. Importantly, many epigenetically silenced genes have not been found to contain any genetic mutations at all, even though they are transcriptionally repressed in many different cancer-cell types [5].

It is now well established that one mechanism contributing to tumor-suppressor gene inactivation involves the abnormal methylation of their promoters. Although a global hypomethylation characterizes the greater length of the DNA stretch, the majority of CpG islands become hypermethylated, causing transcriptional repression of their associated genes, and this has particular interest in tumor diagnostics and therapy [6]. Delving deeper into biology frequently uncovers a complexity greater than predicted. For nearly two decades research into the cancer epigenome has been blinkered by

experimental approaches and the concept of “one methylated CpG island equals one silent gene.” However, recent data from epigenomic and transcription profiling studies has challenged this underlying assumption and demonstrated that epigenetic changes in cancer are not always focal, but can be global encompassing large chromosomal regions, resulting in Long Range Epigenetic Silencing [7].

Identification of methylated genes or sets of methylated genes provides the promise of novel biomarkers for cancer detection or prognosis. In the candidate or global studies, the location of the methylated genes or the potential influence of CpG island methylation on the neighboring gene was not addressed in past. However recent studies have found DNA hypermethylation in cancer is not always restricted to discrete CpG islands or single genes but can encompass multiple adjacent CpG rich regions resulting in concordant gene silencing across large chromosomal domains. A concordant methylation of adjacent CpG island gene promoters, but on a restricted scale, has been reported for a number of other coding regions including the three human leukocyte antigens (HLA) class I genes located on chromosome 6p21.3 in esophageal cancer [8]. Both global hypomethylation and region-specific hypermethylation of CpG islands in specific regions of genetic promoters are common epigenetic events which take place in the mammalian genome, leading to oncogenesis [9]. It is also indicated that global hypomethylation can lead to activation of proto-oncogenes and chromosomal instability, whereas regional hypermethylation leads to the suppression of housekeeping and cell cycle control genes as well as tumor suppressor and DNA repair genes.

The mechanism which involves in Long Range Epigenetic Silencing of contiguous genes is open for debate. Long Range Epigenetic Silencing predicts that even if some CpG island genes are targeted for methylation, neighboring genes may also be affected by default. One reason could be the density of CpG islands in the region that results in Long Range Epigenetic Silencing. It is intriguing to speculate that if “seeds” of CpG methylation trigger hypermethylation of neighboring sites, then may be methylated CpG islands “seeds” or promote the spread of hypermethylation to the neighboring CpG islands within the enriched CpG island zone. Similarly, it could be the density of subsequent DNA hypermethylation that recruits the histone methylation machinery and results in a global heterochromatization spreading between the CpG rich zones. It is possible that the genes which are initially methylated in cancers are those genes which expressed only at the basal levels in normal cells and the genes resistant to methylation are expressed at higher levels in the normal cells, supporting the hypothesis that the level of active transcription is a key determinant in protecting CpG islands from hypermethylation in cancer [10]. In contrast, to the “silencing & seeding” model for promoting DNA

hypermethylation, it has been suggested that the propensity for methylation is based on a “sequence signature” which is associated with protection from or susceptibility to aberrant methylation in cancer [11].

Epigenetic changes, including alterations in histone modifications and DNA methylation, commonly occur in cancer and are associated with aberrant gene expression. However, most studies have focused on epigenetic gene silencing events, and thus, the mechanism that promotes gene activation in carcinogenesis is still poorly appreciated. Studies examining the underlying mechanism of epigenetic deregulation in cancer have primarily concentrated on DNA hypermethylation and gene silencing, rather than DNA demethylation and epigenetic gene activation. Genome-wide hypomethylation [3] is one of the primary epigenetic aberrations found in tumors; similarly long range hypomethylation domains cover nearly half the cancer genome [12] and commonly occur at partially methylated domains in somatic cells. Even though epigenetic activation of specific genes has been documented in cancer, as yet no genome-wide studies have specifically addressed the extent and genomic context of epigenetic activation in cancer [13].

The widespread and focal shifts in chromatin and DNA methylation in Partially Methylated Domains suggest that in cancers there is a loss of boundaries that regulate chromatin domains. NBL2, a tandem array of 1.4-kb repeat unit is located mostly on short arm of the acrocentric chromosomes. Overall hypomethylation in some cancer specimens and hypermethylation in others was found in NBL2. Similarly, the epigenetics of D4Z4 is of great interest because of the linkage of a short array of these tandem 3.3-Kb repeats at 4q35 to facioscapulohumeral muscular dystrophy (FSHD), a severely debilitating, dominantly inherited disease. Satellite 2 and other human satellite DNAs are very frequently hypomethylated in diverse cancers compared with a wide variety of control tissues. There is clearly much plasticity in the epigenetics of these tandem repeats arrays [14].

2 Epigenetics and Clinical Management of Head and Neck Cancer: Prognostic and Therapeutic Implications

Head and neck cancers constitute the sixth most common malignant tumors worldwide [15]. Head and neck cancers are a heterogeneous group of malignant neoplasms with a wide range of biological behaviors. These malignancies encompass tumors arising from the epithelium of the nasal and oral cavity, paranasal sinus, pharynx, and larynx. Oral cancer is the most frequent cancer of the head and neck district, with squamous cell carcinoma being by far the commonest single entity, accounting alone for about 90 % of all malignancies of the oral cavity [16]. Oral carcinogenesis is a

multistep process modulated by endogenous environmental factors. Among the latter, a major role is played by regular intake of tobacco and alcohol [17], as well as by Human Papillomavirus (HPV) persistent infection [18].

Although a clear correlation between the epigenetic-driven deregulation of gene expression and the cancer (head and neck) progression is at present not fully demonstrated, hypermethylation and consequent silencing of several tumor suppressor genes, has been identified in head and neck cancer. The genes found hypermethylated (Table 2) in head and neck cancers cover a wide range of cellular processes, including cell cycle control, apoptosis, Wnt signaling, cell–cell adhesion, and DNA repair.

Aberrant DNA methylation events have been hypothesized to accumulate initially in a stochastic fashion and, through positive selection, result in clones that have a growth advantage leading to the genesis of a rapidly dividing tumor. It is also observed that

Table 2
Genes causing cancer due to promoter methylation

Gene	Locus	Function
ABO	9q34	Blood group antigen
APC	5q21	Tumor suppressor
ATM	11q22	Tumor suppressor
BLU	3p21.3	Tumor suppressor
CCNA1	13q12.3	Cell cycle
C/EBP α	19q13	Tumor suppressor
CDH1	16q22.1	Cell adhesion
CDKN2A	9p21	Cell cycle
CHFR	12q24.33	Early cell cycle checkpoint
CRABP2	1q21	Nuclear transcriptional regulator
DAPK1	9q	Apoptosis
DCC	18q21	Tumor suppressor
DLC1	8p22	Tumor suppressor
DLEC1	3p21.3	Tumor suppressor
DKK3	11p	Transcriptional regulator
E-cadherin	16q22	Signal transduction
EDNRB	13q22	Signal transduction
GSTP1	11q13	Detoxification of carcinogens

(continued)

Table 2
(continued)

Gene	Locus	Function
H3K4	1q21.2	Histone
HIN-1	12p13	Tumor suppressor
h MLH1	3p21	DNA repair
h MSH 2	2p21	DNA repair
KIF1A	10q22.1	Axonal transport of synaptic vesicles and cell division
LHX6	9q33	Transcriptional regulator
MGMT	10q26	DNA repair
MINT1	9q13-q21.1	Synaptic vesicle exocytosis
MINT31	Not clear	Tumor suppression
miR137	1p21.3	Tumor suppressor
miR193a	17q11.2	Tumor suppressor
MX1	21q22	Not known
p14	9p21	Tumor suppressor
p15	9p21	Tumor suppressor
p16	9p21	Tumor suppressor
p53	17p13	Tumor suppressor
p73	1p36	Apoptosis
PTEN	10q23	Tumor suppressor
PGP9.5 (UCHL1)	4p14	Cell signaling
RAR β	17q21	Transcriptional regulator
RASSF1A	3p21	Tumor suppressor
RASSF2	3p21	Tumor suppressor
Rb	13q14	Tumor suppressor
RUNX3	1p36	Transcriptional regulator
SFRP1	8p11.21	Transcriptional regulator
TCF21	6q23-q24	Epithelial–mesenchymal interactions
THBS1	15q15	Cell–cell and cell–matrix interactions
TIG1	3q25.32	Response to retinoic acid receptors
TIMP3	22q12	Inhibition of angiogenesis
WIF1	12q14	Transcriptional regulators
14-3-3 sigma	1p36	Signal transduction

highly significant association between copy number and DNA methylation profiles, definitively showing that these modes of gene regulation are linked in head and neck cancer [19]. These factors can directly or indirectly cause damage to DNA molecules, resulting in irreversible genetic aberrations. For the past two decades, unparalleled emphasis has been placed on the characterization of genetic abnormalities that are traditionally thought to be the predominant or sole molecular basis of tumor development in human malignancies, including head and neck cancer. LOH (Loss of heterozygosity) analysis is a technique commonly used to identify tumor-suppressor genes. Head and neck cancer also frequently show expansion or deletion of some intronic simple repeated (CACACA)_n or microsatellite sequence. Increased frequency of this change is referred to as MSI (microsatellite instability), which is caused by a defective DNA mismatch repair system, and the elucidation of these genetic alterations in head and neck cancer provides support for genetic basis for human malignancy and is confirmed in many cases [20].

One fundamental question regarding the gene inactivation in cancer in association with promoter CpG island hypermethylation is how the epigenetic DNA methylation silences transcription of the involved gene. The mechanisms whereby CpG island methylation suppresses gene transcription are now better understood and multiple steps, starting from initiation and maintenance of methylation to protein acetylation and chromatin organization, appear to work as layers in cancer cells to achieve transcription silencing [21]. It has become apparent that different tumor types possess a different spectrum, profile, or clustering of genes hypermethylation, referred to as “methylotype” as opposed to genetic term “genotype.” The rapid advances in detection techniques and expansion of our knowledge in tumor epigenetics have raised expectations for transferring these techniques from the basic research laboratory to the clinic.

Unlike genetic alterations, epigenetic changes are potentially reversible, and this feature makes them attractive targets for therapeutic intervention. Recent progresses in the knowledge of epigenetics of cancer have allowed the development of several inhibitors of DNA methyltransferase, such as 5-azacitidine and decitabine, and histone deacetylase, successfully used in the treatment of several malignancies of the hematopoietic system. The NIH repository for clinical trials reports some trials involving epigenetic-based drugs in head and neck cancer treatment: Azacitidine and Cisplatin have been tested in combined chemotherapy in advanced, recurrent and metastatic squamous cell carcinoma of head and neck but no data is available to date about the outcome of study. The use of epigenetic inhibitors in association with traditional anticancer therapeutic agents looks very promising as a tool to improve the chemosensitivity of non-responsive cancers [22].

3 Detection of Aberrant DNA Methylation in Body Fluids as an Early Detection Tool

The frequency with which DNA methylation patterns are altered in early stage tumors has fueled efforts to develop diagnostic biomarkers, utilizing cancer-specific alterations such as hypermethylation of promoter region for early detection of potentially fatal tumors. To be suitable for population screening, diagnostic biomarkers are usually based on readily available body fluids. The rationale behind blood tests for detecting solid tumors is that tumor cells may shed epigenetically altered DNA into the blood stream or that blood cells may have undergone epigenetic changes representative of those present in the tumor; for example, in response to specific environmental influences.

DNA methylation is a well-known epigenetic phenomenon, and growing evidence during the past decade supports promoter CpG island hypermethylation as a bona fide mechanism for gene inactivation [23]. Loss of gene function due to promoter hypermethylation has several characteristics that bear striking similarity to loss of tumor suppressor gene function by somatic mutation: first, promoter hypermethylation in one allele is frequently accompanied by deletion of the opposite allele, resulting in loss of heterozygosity of the gene. Second, gene inactivation in association with promoter hypermethylation is fully heritable. Finally, loss of gene function due to epigenetic alterations leads to selective growth advantage in a manner identical to loss of tumor suppressor function due to somatic mutations [21].

The appropriate approach for analysis of DNA methylation depends upon the goals of the study. A major advancement in DNA methylation analysis was the development of a methods for sodium bisulfite modification of DNA to convert unmethylated cytosines to uracil, leaving methylated cytosines unchanged. This allows one to distinguish methylated from unmethylated DNA via PCR amplification and analysis of the PCR products. During PCR amplification, unmethylated cytosines amplify as thymine and methylated cytosines amplify as cytosine. Most methods for analyzing DNA methylation at specific loci are based on this approach.

High-performance liquid chromatography is a classical method to quantify global DNA methylation and is highly quantitative and reproducible. This method requires large amounts of high-quality genomic DNA, however, is not suitable for high-throughput analyses. To circumvent these problems, several bisulfite-based PCR methods have been developed to approximate global DNA methylation by assessing repetitive DNA elements such as Alu elements and long interspersed nucleotide elements [24]. However, as global methylation analyses provide no information on the genomic position at which methylation is altered, it is difficult to link such changes to functional outcomes.

Gene specific methylation analysis methods can be characterized as either “candidate gene” or “genome wide” approaches. Candidate gene approach could be “sensitive” if methylated or unmethylated alleles are detected by designing primers overlapping multiple CpG dinucleotides. In quantitative approach of candidate gene method, primers are designed to amplify both methylated and unmethylated alleles with equal efficiency, and the methylation level is analyzed by various approaches. Methylation sensitive PCR (MSP), a sensitive method of candidate gene approach, is a rapid and very sensitive technique to screen for methylation. Following bisulfite modification, PCR is performed using two sets of primers designed to amplify either methylated or unmethylated alleles. MSP has the advantage of being highly sensitive and can be used on DNA samples of limited quantity and quality, although MSP is not quantitative. Variations of MSP called MethylLight [25] or quantitative analysis of methylated alleles (QAMA) [26] were developed using real-time PCR for methylation detection.

Except for methylation-sensitive restriction analysis by Southern blotting, all quantitative methods for measuring DNA methylation at specific loci are based on modification of DNA by sodium bisulfite. Allele-specific bisulfite sequencing is the gold standard for mapping methylation across CpG sites within post-bisulfite PCR products [27]. In this method, a region of interest is amplified from bisulfite-modified DNA using PCR primers not overlapping CpG sites, to amplify both methylated and unmethylated alleles. PCR products are ligated to a cloning vector and transfected to competent cells. Antibiotic resistant colonies are grown on agar plates, selected individually, and expanded by growing in LB medium. The plasmid DNA is isolated and sequenced, if a sufficient number of clones are sequenced, this method can be quantitative. As each clone represents a single allele, the data provides information on allele specific methylation.

An alternative approach employing direct radioactive sequencing of post-bisulfite PCR products and quantitation by phosphorimaging is also validated [28]. Bisulfite PCR followed by restriction analysis (COBRA) has been used as a quantitative technique for methylation detection [29]. After bisulfite modification and PCR amplification, the PCR product is digested with restriction enzyme and quantitated using gel electrophoresis and densitometry. This method provides data only for specific restriction enzyme cutting sites and is relatively time-consuming compared with MSP. Bisulfite pyrosequencing, on the other hand, relies on bisulfite conversion and PCR amplification. To facilitate the conversion of PCR product to single-stranded DNA for later pyrosequencing, the PCR reaction is performed with either one primer biotinylated or using a tailed primer in combination with a biotin-labeled universal primer in the same reaction. This method has the advantages of

introducing an internal control and allowing accurate quantitation of multiple CpG methylation sites in the same reaction. The significant drawback is that only 25–30 base pairs can be sequenced in each reaction, limiting the number of CpG sites that can be assessed.

Another high-throughput method utilizes base-specific cleavage of nucleic acids and analyzes samples by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) [30]. In this method, regions of interest are PCR-amplified from bisulfite-modified DNA using primers with a T7RNA polymerase tag. The PCR products are *in vitro* translated into a single-stranded RNA using T7 polymerase and subsequently cleaved by an endoribonuclease such as RNase A.

Just as gene expression microarrays accelerated and revolutionized the study of transcriptional regulation, rapidly improving technologies are increasingly enabling researchers to access locus-specific DNA methylation on a genome-wide scale. Several genome-wide DNA methylation analysis methods do not require microarrays. A classical method is Restriction Landmark Genome scanning (RLGS)—a two dimensional DNA gel electrophoresis technique. In combination with methylation-sensitive restriction enzymes, this technique provides methylation profiles of thousands of loci at once [31]. Another non-array method, methylation specific digital karyotyping (MSDK), is developed. MSDK is conceptually similar to serial analysis of gene expression (SAGE) and relies on the cleavage of genomic DNA with a methylation-sensitive enzyme.

Three main classes of microarray based methods have been developed to map ^{5m}C patterns in genome: methods enriching for highly methylated regions using an antibody specific for ^{5m}C or methyl-binding proteins, methods based upon bisulfite modification, and methods utilizing methylation-sensitive restriction enzymes. Methylated DNA immunoprecipitation (MeDIP) immunoprecipitates DNA using an anti-methylcytosine antibody, and then hybridizes the immunoprecipitated DNA to microarrays. This technique is independent of the specific methylation-sensitive restriction sites within the target sequence, but requires large amount of genomic DNA and antibody. Ligation-mediated PCR (LM-PCR) has been used to perform MeDIP with limited quantities of DNA [32]. Methylated CpG island recovery assay (MIRA) has been used for genome-wide methylation analysis in cancers [33]. Differential methylation hybridization (DMH) is one of the first described methods. Genomic DNA is digested, ligated with linkers, and then digested to remove unmethylated fragments. The digested DNA is amplified by primers complementary to the linker sequence and the products are labeled and hybridized to array (Table 3).

Table 3
Techniques used to detect DNA methylation in genes involved
in head and neck cancer

Technique	Genes analyzed
MSP Methylation-specific PCR	14-3-3 WTF1 TIMP3 TIG1 SFRP1 MGMT h MSH2 h MLH1 CDH1
QMSP Quantitative methylation-specific PCR	p16 KIF1A EDNRB DLEC1 DLC1 DCC BLU
MSRE Methylation-specific restriction enzyme analysis	p14 MINT31 MINT1
SRM-PCR Sensitive restriction multiplex PCR	p15
MS-MLPA Methylation-specific multiplex ligation-dependent probe amplification	RASSF1A RAR β DAPK1 CHFR APC
COBRA Combined bisulfite restriction analysis	RASSF2
PS Pyro-sequencing	CCNA1
BS Bisulfite sequencing	PGP9.5
PCR-DHPLC PCR-denaturing high-performance liquid chromatography	HIN-1

4 Strategies for Epigenetic Biomarkers Development in Head and Neck Cancer

The goal of clinical biomarkers is to provide physicians with relevant information about the presence or absence of disease (diagnostic biomarkers) as well as about patients and disease characteristics that influence treatment decisions (prognostic and therapy optimization biomarkers). Various genetic markers, such as point mutation, LOH, and MSI, have been used extensively in head and neck cancer for early tumor detection, prognostic prediction, and elucidation of the genetic progression model [34]. However, promoter hypermethylation is an alternative, attractive epigenetic marker that has recently gained popularity. Conceptually, epigenetic biomarkers consists of two complementary building blocks: an experimental assay that provides accurate measurements of epigenetic alterations in a given patient sample, either at a single locus or at multiple genomic region and a sample classifier that translates the experimental read out into a biomarker outcome, e.g., the predicted disease subtype or tumor grading.

Epigenetic biomarker development has so far focused mainly on DNA methylation, both because of practical considerations (as DNA methylation is relatively stable and easy to measure with current technologies) and because of the well-established role of DNA methylation in cancer. Complementary to early diagnosis of developing tumors, a second class of epigenetic biomarkers aims to support clinical decision-making once a tumor has been identified. Such prognostic and therapy-optimization biomarkers help address the following questions by measuring relevant aspects of tumor biology:

- Is the pathological diagnosis confirmed at the molecular level?
- Does the tumor fall into a known subclass of epigenetically characterized tumor?
- Can and should the tumor be treated?
- Which type of therapy is appropriate?
- Which should be used for radiation and chemotherapy?
- How strong will the side effects be and how can they be minimized?

A more systematic approach to biomarker development has the potential to overcome many of these challenges. The procedure is usually based on three key concepts:

- To maximize genomic coverage in the early stages of the search
- To employ computational methods for identifying and optimizing a small number of highly promising candidate biomarkers
- To validate biomarkers performances in large cohorts using highly targeted assays.

As genome scale analysis of DNA methylation is still a costly exercise, large scale epigenetic biomarkers development becomes more feasible when several experimental methods with very different trade-offs between genomic coverage and per-sample costs are combined. Several experimental methods enable robust DNA methylation measurements of a small number of CpGs at low cost, addressing key requirements of biomarker validation and subsequent clinical use. Bisulfite pyrosequencing, methylation-sensitive single nucleotide primer extension (Ms-SNuPe), combined bisulfite restriction analysis (COBRA) and mass spectrometry provide quantitative DNA methylation information for individual CpGs, conferring increased robustness against random fluctuations. In contrast, MethyLight and methylation specific PCR (MSP) query the DNA methylation status of several CpGs simultaneously, enabling highly sensitive detection of specific methylation patterns. Finally, clonal bisulfite sequencing (regarded as gold standard in DNA methylation analysis) is useful for assay quality control and for identifying the most representative CpGs within a given region.

Selecting the most promising biomarkers for validation is arguably the most crucial and challenging step. Clearly, the main selection criterion is the predictive power for the disease condition of interest, which can be estimated from the experimental data accumulated. However, to maximize the chances of selecting biomarker candidates that validate well and that may also provide new insights into disease mechanisms, further data should be taken into account. To enable validation in large cohorts and subsequent clinical use, a targeted assay has to be developed specifically for each candidate region, in such a way that it maintains the predictive power for the disease condition, but is significantly more cost-efficient, robust, and easy to handle.

Using statistical learning software packages and cross-validation on the training data, the most suitable classification model is selected and a preliminary performance assessment is derived. Based on these results, the most promising biomarker candidates are selected for confirmation in an independent validation cohort that is clinically comparable with the primary case-control cohort [35]. As validation studies are highly dependent on biostatistical methods, they are best performed using statistical packages such as R, SAS/STAT, or SPSS PASW. Furthermore, tools supporting data integration and clinical trials management can facilitate the complete logistics of large scale biomarker validation.

5 Future Prospects

The epigenome records a variety of dietary, lifestyle, behavioral, and social cues, providing an interface between the environment and the genome. Epigenetic variation, whether genetically or

environmentally determined, contributes to interindividual variation in gene expression and thus to variation in common complex disease risk. Interventions based upon epigenetic agents, including DNA methyltransferase inhibitors and histone deacetylase inhibitors, have been in clinical use for many years, but their role outside treatment of specific cancers is not yet established. Epigenetic therapies can only be fruitful if epigenetic mechanisms are casually related to the disease being treated. Evidences linking epigenetic variation to specific disease phenotypes to date are lacking [36]. Understanding the molecular details behind “epigenetic cancer diseases” holds potentially important prospects for medical treatment, allowing to develop novel strategies for drug discovery.

From a larger perspective, two phenomena demonstrate the significance of epigenetic in cancer development. First, the influence of epigenetics on tumor development is reflected in the importance of extracellular matrix signaling and tumor cell-stromal cell interactions. Notwithstanding the importance of genetic alterations enabling tumor cells to escape defense mechanisms such as the p53 and pRB pathways and the “telomere clock” malignant transformation is highly dependent on the surroundings for successful outgrowth. Second, directly proving the importance of epigenetics in tumorigenic processes are the emerging successful treatments of cancers with inhibitors of epigenetic regulators. The fundamental principle behind this type of epigenetic therapy is that reversal of epigenetic silencing will reinstate cellular cancer defense mechanisms, for instance via induced expression of the cyclin-dependent kinase inhibitors p16 and p21.

Epigenetic research as a rapidly evolving field offers exciting new opportunities for the diagnosis and treatment of complex clinical disorders. In this context it is important for clinicians to understand the principles of epigenetic mechanisms and how these principles relate to human health and disease. It is important to address the use of epigenetic pathways in new approaches to molecular diagnosis and novel targeted treatments across the clinical spectrum. Epigenetic mechanisms regulate DNA accessibility throughout a Person’s lifetime. Immediately following fertilization, the paternal genome undergoes rapid DNA demethylation and histone modifications [37]. Genome imprinting allows genes to “remember” whether they were inherited from the mother or the father so that only the maternally or paternally inherited allele is expressed. Imprinting is regulated by DNA methylation and histone modifications and is important in the context of a variety of developmental and pediatric disorders [38].

Besides gene-specific imprinting effects, global imprinting changes can occurs in embryos that completely lack one parental genome. Evidences suggest the manipulation of embryos for the purpose of assisted reproduction or cloning may impose inherent risks to normal development. Similarly, in vitro culture in animal

models has been found to lead to reduced viability and growth, developmental abnormalities, behavioral changes, and loss of imprinting [39]. In addition, epigenetic abnormalities, including imprinting defects, are likely responsible for the substantial embryonic losses (up to 98 %), placental hyperplasia and respiratory abnormalities observed in animal cloning models [40]. To date, epigenetic therapies are few in number, but several are currently being studied in clinical trials or have been approved for specific cancer types [41]. Nucleoside analogues such as azacitidine incorporated into replicating DNA inhibit methylation and reactivate previously silenced genes [42]. Both increases and decreases in DNA methylation are associated with the aging process, and evidence is accumulating that age-dependent methylation changes are involved in the development of neurologic disorders, autoimmunity, and cancer in elderly people [43].

Our increased knowledge of epigenetic mechanisms over the last 10 years is beginning to be translated into new approaches to molecular diagnosis and targeted treatments across the clinical spectrum. With the Human Genome Project completed, the Human epigenome Project has been proposed and will generate genome-wide methylation maps [44]. By examining both healthy and diseased tissues, specific genomic regions will be identified that are involved in development, tissue-specific expression, environmental susceptibility, and pathogenesis. Use of these epigenetic maps will lead to epigenetic therapies for complex disorders across the clinical spectrum.

In many cancers, several tumor-suppressor pathways have become inactivated, and epigenetic therapy offers the potential of targeting several genes with one drug. The other side of that coin is many “innocent bystander genes” are likely to be affected and, as yet, relatively little is known about potential side effects in patients [45]. The significance of epigenetic mechanisms for gene regulation in cancer is now evident with cancer related mechanisms acting at all epigenetic layers. The study of epigenetic imprints is still in its infancy, as not all epigenetic marks are known, and only few of the known ones are understood in any detail. Hence, the near future is likely to bring important new insights into epigenetic gene regulation in both normal and neoplastic development as high-quality tools such as methyl-specific histone antibodies and new chromatin exploration methodologies emerge.

Through examining the role of environmental factors in causing variation in epigenetic patterns (exposure/epigenotype) and ultimately exploring the causal impact of epigenotype on disease outcomes (epigenotype/disease) using genetically epigenomics and other methods, progress towards epigenetic interventions can be made. As genome-wide association studies and other approaches identify robust associations between genetic variants and epigenetics patterns, possibilities for elucidating causal pathways

and predicting the effect of manipulation—through environmental (including lifestyle) modification or pharmacotherapeutic means—is considerable. In this way, epigenetic markers may become targets for modification as well as biomarkers for exposure and disease risk. The International Human Epigenome Consortium is poised to invest millions of dollars to map 1,000 reference epigenomes in a range of normal tissues and define the level of variation that exists between individuals [46].

Our understanding of the role of epigenetic abnormalities in disease processes is still in its infancy. The primary goals over the next decade will include improving our understanding of the interplay between epigenetic mechanisms, gene expression, and the environment, and moving from animal models to clinical trials of novel epigenetic therapies directed at a wide variety of diseases. By decreasing the risk of epigenetic instability that leads to disease and by correcting epigenetic abnormalities that predispose to diseases later in life, the promise for epigenetic therapies as an essential treatment option will be fulfilled. The field of epigenetic in relation to common complex disease will undoubtedly continue to be the focus of much attention, and its progress, now that it has passed the starting line, will be followed with considerable interest.

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Epigenetic Therapy for Colorectal Cancer

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Abstract

Aberrations in epigenome that include alterations in DNA methylation, histone acetylation, and miRNA (microRNA) expression may govern the progression of colorectal cancer (CRC). These epigenetic changes affect every phase of tumor development from initiation to metastasis. Since epigenetic alterations can be reversed by DNA demethylating and histone acetylating agents, current status of the implication of epigenetic therapy in CRC is discussed in this article. Interestingly, DNA methyltransferase inhibitors (DNMTi) and histone deacetylase inhibitors (HDACi) have shown promising results in controlling cancer progression. The information provided here might be useful in developing personalized medicine approaches.

Key words Colon cancer, Epigenetic therapy, Methylation, Acetylation

1 Introduction

Colorectal cancer (CRC) is the third leading cause of cancer related deaths in the USA. The process of tumorigenesis has been reportedly linked with gene mutations, deletions or translocations. Other than genetic lesions which are heritable as such, the epigenetic changes do play a crucial role in CRC progression [1]. Epigenetic changes are defined as those heritable alterations which occur in the genetic material due to chemical modifications in a way that do not involve changes in the primary gene nucleotide sequence [2, 3]. These changes include DNA methylation, histone alterations, chromatin remodelers and variations in noncoding RNAs including miRNAs (microRNAs) that are crucial to ensure the stable propagation of gene activity in cell lineages [4, 5]. Any abrupt deviations in these distinct and mutually reinforcing epigenetic mechanisms lead to inappropriate gene expression, causing cancer development and other epigenetic diseases [6, 7]. In recent years, epigenetics has represented an important tool for advanced understanding of disease pathogenesis, in particularly tumorigenesis [8, 9] and has played an interesting role in developing strategies for cancer treatment and prevention. Targeting epigenetic modifications offers a

new therapeutic approach in CRC and may dictate the future of personalized medicine. Resistance to conventional drugs such as 5-fluorouracil, oxaliplatin, and irinotecan also involve epigenetic mechanisms. Thus, combined therapies with agents that modify epigenetic systems may reverse drug resistance, sensitize refractory tumors and enhanced synergistic anticancer efficacy. In this regard DNA methyltransferase inhibitors (DNMTi) and Histone deacetylase inhibitors (HDACi) are extensively investigated. Though outcomes and responses are not very convincing in CRC, future research is needed in this area. In this review article we have briefly described the intriguing current epigenetic cancer therapeutic modalities modulating DNA methylation and histone acetylation patterns.

2 DNA Methylation

2.1 An Overview

DNA methylation, a natural covalent modification of DNA, is a natural event in eukaryotic species such as vertebrates and plants. On the contrary, yeast and drosophila have shown little methylation of their DNA. DNA methylation is the addition of a methyl group to the 5' position of the cytosine ring, converting it to 5-methyl cytosine (5-mC) and typically occurs in CpG (cytosine–guanine) islands (CpG-rich 0.3–2 kb stretches of DNA) within the gene promoter regions. The key areas of a normal cell genome which harbor extensively methylated CpG islands are DNA repeat sequences, telomeres, centromeres, and inactive X-chromosomes. Generally, DNA hypermethylation in gene promoters can lead to the silencing of the respective gene expression by modulating the accessibility of DNA to transcription factors and also by recruiting various silencing-associated proteins [10]. Cytosine methylation has also been observed outside of CpG sequences (e.g., CpA or CpT) but such non-CpG methylation is said to be a representative feature of stem cells and occurs relatively rarely in differentiated cells [11, 12].

DNA methyltransferases (DNMTs) are responsible for the establishment and maintenance of DNA methylation patterns, which recognize palindrome dinucleotides CpG and catalyze the transfer of a methyl group from the donor S-adenosyl-L-methionine (SAM) to the C5 cytosine carbon atom. There are four known DNA methyltransferase isoenzymes to date: DNMT1, DNMT2, DNMT3A, and DNMT3B [13]. DNMT3A and DNMT3B perform de novo DNA methylation during early embryogenesis [14–16]. However, patterns of DNA methylation is maintained in daughter cells by ubiquitin-like, PHD and RING finger domains 1 (UHRF1) which tends to recognize the hemi-methylated DNA regions and redirects DNMT1 to complete the methylation at the appropriate cytosine during successive replications [17, 18].

Moreover, it has now been proposed that in somatic cells also DNMT3A/B is required for the maintenance of DNA methylation pattern particularly at the repeat regions and imprinted genes [19]. DNMT2 enzyme is characterized to have a weak in vitro DNMT activity but can effectively catalyze tRNA methylation [20].

Previous reports via the estimation of global content of 5-mC of tumors suggested that hypomethylation was a common feature of tumorigenesis [21–23]. However, malignant cells show an opposite pattern with more gene promoter hypermethylation as compared to decreased global methylation. Subsequent epigenetic studies revealed that various tumor suppressor genes and miRNAs are silenced due to promoter DNA methylation [24–26]. Currently, genome-wide studies have reported some distinct patterns of methylome in cancerous tissues compared with their normal counterparts [27, 28] and approximately 600 regions rich in CpG islands were found to have altered DNA methylation patterns in cancers when compared to normal [29].

Variations in DNA methylation patterns could be a better diagnostic tool for early detection of different tumor types because these changes often occur in early stages of tumorigenesis [30, 31]. Utilizing DNA methylome analysis the most recently proposed prognostic biomarkers are the methylation suppressed expression of prolyl 3-hydroxylase P3H2 associated with estrogen-receptor-positive breast cancers [32] and the homeobox transcription factor EN1 (analyzed in stool DNA), common in most CRC [33]. Plenty of advantages are offered by DNA methylation analysis in comparison to gene expression microarrays or proteomic approaches because DNA is a very stable molecule and the assays for individual markers are universal, i.e., independent of tumor type [34]. DNA methylation patterns are not only quantifiable but also differ qualitatively between cancer and respective normal cells. A diagnostic tool was launched by Epigenomics AG under the trade mark Epi proColon, which can detect CRC based on the aberrantly methylated DNA of the SEPT9 gene in blood plasma with more than 70 % accuracy [34].

2.2 Clinical Significance of Inhibition of DNMTs

The epigenome can serve as a potential anticancer therapeutic target. Like any other reversible chemical reaction, DNA methylation is also a reversible process in which genes get demethylated and restored to their original expression and function. For this purpose, DNA methyltransferase inhibitors or DNMT inhibitors (DNMTi) have been investigated as anticancer agents as they may restore the expression of tumor suppressor genes by blocking DNMTs activity [35–37].

2.2.1 Nucleoside Analogs

These drugs form covalent bonds with DNMTs and block their enzymatic activities [38]. Two nucleoside analogs have been developed and tested in numerous clinical trials, 5-aza-2-deoxycytidine

(5-azaCdR or decitabine) and 5-azacytidine (azacitidine or 5-azaCR or Vidaza) [39, 40]. Vidaza and Decitabine were approved by US Food and Drug Association (FDA) in 2004 and 2006, respectively, for the treatment of myelodysplastic syndrome (MDS) [41]. Additionally, these drugs are also undergoing phase II clinical trial for various solid tumors and leukemia [42]. Zebularine which is another nucleoside analog has come up with better results as it has shown to remove 25–60 % 5-mC from the genes in a panel of human cancer cell lines [43]. It is more stable than Vidaza and Decitabine and displayed a half-life of ~21 days at pH 10 helping its oral administration [44]. Moreover, it was found that Zebularine can reactivate various silenced genes in tumor cells, e.g., p16 and E-cadherin [45].

2.2.2 Small Molecules

Hydralazine and Procainamide are FDA approved drugs for hypertension and cardiac arrhythmia, respectively, have demonstrated DNMT inhibitor like activities [46, 47]. However, no clinical trial has yet been conducted for their DNMT inhibitor role. Another DNMT1 specific inhibitor RG108 was developed using molecular modeling approach using DNMT1 catalytic pocket and it showed significant in vivo and in vitro demethylating activities [48–50]. RG108 was found to restore tumor suppressor genes SRFP1 and TIMP-3 in colon cancer cells and displayed less toxicity to normal human cells [51]. However, this molecule is still in preclinical phase of analysis.

2.2.3 Antisense Oligonucleotide Inhibitors

MG98 is a 20 bp antisense deoxyoligonucleotide that binds with the 5'UTR (5' Untranslated region) of human DNMT1 mRNA to inhibit its translation [52]. During phase I/II trials many tumor types were screened which could be sensitive for MG98. In patients MG98 has been shown to downregulate DNMT1 but this suppression was not consistent and dose dependent [53, 54]. Moreover, it was documented that a continuous dosing of MG98 for 7-days was well tolerated by patients suffering from advanced solid tumors but the end effect of the study is not concluded [55].

3 Histone Acetylation

3.1 An Overview

Chromatin contains repeated units of nucleosomes, where each nucleosomal core contains DNA wrapping around a histone octamer that has duplicate copies of each of the four core histones H2A, H2B, H3, and H4. The fifth or the linker histone, histone H1, binds to the DNA linking two adjacent nucleosomal cores. In comparison to histone H1, each core histone comprises a C-terminal globular domain and an unstructured N-terminal tail. The globular domain (C-terminal) is responsible for forming the histone octamer [56], whereas the unstructured tails (N-terminal) contain many

residues for covalent modifications, including acetylation [20]. The N-terminal tails possess multiple lysine residues for specific acetylation although the globular domains of H3 and H4 have also demonstrated some sites of acetylation in them. Functionally, histone acetylation does not act alone but actively crosstalk with other histone modifications such as methylation, phosphorylation, ubiquitination, and DNA methylation. Histone acetylation controls transcription via enzymes acetyltransferases, and deacetylases by modifying the acetylation state of histones or other promoter-bound transcription factors. Due to histone acetylation the positive charge of histones get significantly reduced which in turn disrupts electrostatic interactions between histones and DNA and makes complex chromatin easily accessible to the molecular machineries involved in transcriptional control. However, histone deacetylation antagonistically favors transcriptional repression by inducing chromatin compaction [57].

Specific recruitment of histone acetyltransferases (HAT) and histone deacetylases (HDAC) containing complexes to selected gene promoter elements generates localized domains of modified histones that influence transcriptional activity [58]. There are three major groups of HATs: Gcn5 (general control non-derepressible 5)-related *N*-acetyltransferases (GNATs), E1A-associated protein of 300 kDa (p300)/CREB (cAMP-responsive element binding protein)-binding protein (CBP), and MYST proteins. HDACs oppose the activities of HATs by removing acetyl groups from lysine residues of histone tails. Major four classes of HDACs include: Class I (HDAC 1, 2, 3, and 8, localized in nucleus), Class II (HDAC 4, 5, 6, 7, 9, and 10, present in both the nucleus and cytoplasm), Class III (seven sirtuins [Sir2(tu) like proteins], SIRT1-7, mainly nuclear but may be found in the cytoplasm too), Class IV (only one member, HDAC11, mainly nuclear; its sequence displays characteristic features of class I and II members). It is well established that HDACs function as transcriptional repressors and thus diminished histone acetylation at promoter regions generally correlates with gene silencing. Contrary to this there are also evidences that HDACs activate expression of some genes for example, in yeast, the Hos2 (Hda One Similar 2) deacetylase is required for gene activation, and deletion of the Rpd3 (reduced potassium dependency 3) deacetylase leads to repression of transcription at telomeric loci [59, 60].

3.2 Clinical Significance of HDAC Inhibitors

Administrations of HDAC inhibitors (HDACi) have shown to increase in histone acetylation and upregulation of gene expression. However, many proteins get acetylated for intracellular signaling and majority of those proteins are targets for HATs and HDACs [61]. HDAC superfamily consists of two types of enzymes, which are either classical or zinc²⁺ dependent [class I, II, and IV] and sirtuin or NAD⁺ dependent [class III] [62]. HDACi, which

target classical family enzymes, are often referred as classical HDACi [63–65]. Higher sensitivity to classical HDACi has been shown by tumor cells as compared to normal cells [66]. Therefore, these inhibitors have constituted an exciting addition to the field of cancer therapy. Different clinical trials are either completed or still on going to test the efficacy of classical HDACi [63–65]. HDACi have been classified into five major groups: short-chain fatty acids, hydroxamic acids, cyclic peptides, benzamides, and hybrid molecules [67].

3.2.1 Short-Chain Fatty Acids

Butyrate or phenyl-butyrate and valproic acid, the short-chain fatty acids that are produced by enteric bacterial fermentation protect against colon cancer [68]. Butyrate serves as an important energy source for intestinal epithelial cells and plays a role in the maintenance of colonic homeostasis by exerting potent effects on various colonic mucosal functions [69]. Butyrate downregulates oxidative stress in the healthy colonic mucosa [70], modulates the response of oxidative and metabolic stress by altering gene expression in primary human colon cells [71], and thereby may enhance the sensitivity of colon cancer cells to trans-retinoic acid [72]. Moreover, human colon cancer tissue specimens have demonstrated lower expression of butyrate transporter protein essential for butyrate uptake [73, 74]. In phase I trial of patients having colon cancer and rectal hemangiopericytoma it was observed that phenyl-butyrate can inhibit HDACs activity [75, 76]. Valproic acid, an anticonvulsant and mood-stabilizing agent has also been studied in phase I trial in patients with advanced colorectal and esophageal cancers [77].

3.2.2 Hydroxamic Acids

Various hydroxamic acids, e.g., trichostatin A, suberoyl bishydroxamic acid, suberoylanilide hydroxamic acid or Vorinostat, LBH589, and PXD101 display high efficacy against class I and II HDACs [78, 79]. Especially, Vorinostat has demonstrated significant activity in a wide range of cancers [80]. Vorinostat can reactivate expression of a subset of genes silenced in colon and breast cancer cells which ultimately resulted in cell growth arrest, differentiation, and apoptosis [81]. Disease stabilization was observed in half of the patients in a multicenter phase II single-agent study with breast, colon, and lung cancers [82]. Vorinostat was combined with radiotherapy and the treatment was found safe in phase I study with gastrointestinal cancers [83, 84]. A novel HDACi, Scriptaid, was found to be effective in cell-cycle arrest and growth suppression and in reversal of repressive chromatin marks at the promoter region of a hyper-methylated p16 gene in CRC [85]. The therapeutic potential of combining lapatinib (tyrosine kinase inhibitor) with panobinostat (HDAC inhibitor) in colorectal cancer cell lines showed greater antitumor activity than either agent alone [86]. Thus, the combinations of HDACi with established anticancer therapies (chemotherapy or

other targeted agents) can be expected to increase the therapeutic efficacy of HDAC inhibitors.

3.2.3 *Cyclin Tetrapeptides*

Depsipeptide (FK228 or FR901228), a potent bicyclic depsipeptide, was studied in combination with gemcitabine (nucleoside analog) in a phase I trial for patients suffering with advanced solid tumor [87, 88]. Apicidin which is another novel cyclic tetrapeptide, displayed marked anti-proliferative effects via upregulation of p21^{CIP1/WAF1} and gelsolin in a wide variety of human cancer cell lines such as osteosarcoma, breast and gastric origin, as well as in v-Ras-transformed NIH3T3 cells [89].

3.2.4 *Benzamides*

These compounds contain a benzamide moiety and were postulated to bind zinc at the catalytic site of classical HDACs [90, 91]. MS-275 and CI-994 are the two members of this group. MS-275 is structurally and functionally different from many other HDACi that demonstrated an increased expression of p21^{CIP1/WAF1} and accumulation of tumor cells at G1 phase [92, 93]. MS-275 has also displayed prominent anti-proliferative activity in various human cancer cell lines, including breast, colorectal, leukemia, lung, ovary, and pancreas [92].

3.2.5 *Hybrid Molecules*

MGCD0103, a hybrid compound was evaluated in a phase I/II trial in combination with gemcitabine in patients with solid tumors [94]. Isothiocyanate, as a member of hybrid molecules, derived from glucosinolates in cruciferous vegetables, such as watercress, cabbage, and broccoli, has antioxidative properties and chemopreventive effects on the development of lung and colon cancers [95, 96]. Isothiocyanates have protected a cohort of Chinese men at high risk for gastric cancer where glutathione S-transferases was found to be a modulator of the chemopreventive effect of isothiocyanates [97].

4 Nonconventional Therapeutic Approaches

Statins, cholesterol reducing drugs, which reduce CRC risk by interacting with the epigenetic mechanisms, inhibit EZH2. Growth arrest of CRC cells was further augmented when co-administered with HDACi [98]. Another report demonstrated that statins chemo-sensitize CRC cells through demethylation of the promoter of bone morphogenetic protein-2 (BMP-2) gene [99]. Natural dietary compounds including epigallocatechin 3-gallate (EGCG) from green tea, polyphenols and flavonoids from coffee, genistein from soybean, curcumin from turmeric, folate and vitamins influence the epigenetic mechanisms in CRC and thus could be utilized as epigenetic chemopreventive drugs [100]. Radiation affects epigenetic pattern and thus promotes apoptosis in cancer cells through inhibition of DNMTs and histone modifications [101].

5 Conclusion

DNMTi, have shown remarkable effects in suppressing the tumor progression by activating the methylated genes. Clinical trials are in progress to characterize DNMTi according to their mode of actions with fewer side effects. Along with the DNA hypermethylation, the DNA wrapping histone proteins do play a significant role in gene expression profiles. Two chemically distinct stages of histone molecules either acetylated or deacetylated dictate the nature of chromatin being active or inactive, respectively. Various histone deacetylase enzymes or HDACs are abruptly expressed in CRC. HDACi have shown significant effects in gastrointestinal tumor regression and hence serve as novel therapeutic drugs. Several DNMTi and HDACi are approved by FDA for treatment of various malignancies after qualifying clinical trials.

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Chapter 41

Epigenetics of Gastric Cancer

Mingzhou Guo and Wenji Yan

Abstract

Epigenetic changes frequently occur in human gastric cancer. Gene promoter region hypermethylation, genomic global hypomethylation, histone modifications, and alterations of noncoding RNAs are major epigenetic changes in gastric cancer. As a key risk factor of gastric cancer, *H. pylori* infection is an independent predictive indicator of gene methylation. A growing number of epigenetic studies in gastric cancer have provided lots of potential diagnostic and prognostic markers and therapeutic targets.

Key words Gastric cancer, Epigenetic, Hypermethylation, Hypomethylation, Histone modification, MicroRNA, Long noncoding RNA, *H. pylori*

Abbreviations

5-aza-CR	5-azacytidine
5-aza-CdR	5-aza-2'-deoxycytidine
GC	Gastric cancer
HATs	Histone acetyltransferases
HDACs	Histone deacetylases
HDMs	Histone demethylases
HMTs	Histone methyltransferases
LncRNA	Long noncoding RNA
TSA	Trichostatin A

1 Introduction

Gastric cancer (GC) is one of the most common malignancies, ranking the fourth in males and the fifth in females worldwide. It is a leading cause of cancer-related death [1]. Because of the lack of early detection markers and effective therapeutic strategies, gastric cancer is usually diagnosed at late stage and the patients often die of metastasis and recurrence, with low 5-year survival rate [2, 3]. Gastric cancer is mainly divided into two histological types: the better differentiated intestinal-type carcinomas, with cohesive,

glandular-like cell groups and the poorly differentiated, diffuse-type carcinomas, with infiltrating, non-cohesive tumor cells according to the Lauren classification [4]. The intestinal type of GC is developed following multiple steps from normal gastric mucosa, acute and chronic gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and finally to gastric cancer [5]. By contrast, the diffuse-type gastric cancer is not characterized by preceding steps other than the chronic gastritis associated with the *Helicobacter pylori* infection [4, 6, 7]. It indicates that gastric cancer was developed through distinct molecular pathways [8]. Each histological type of gastric cancer is attributed to a progressive accumulation of genetic and epigenetic alterations.

The term epigenetics was first introduced by C.H. Waddington in 1939 to name “the causal interactions between genes and their products, which bring the phenotype into being” [9]. It was later defined as heritable changes in gene expression that are not due to alterations in gene sequence. In addition to genetic alterations, epigenetic changes are recognized as a common molecular alteration in human cancers [10]. DNA methylation, histone modification, and noncoding RNA are three important epigenetic regulation factors, which were involved in different biological behaviors, including cell cycle, apoptosis, proliferation, metastasis, and DNA repair.

In this chapter, we overviewed the major epigenetic changes and the applications in gastric cancer.

2 DNA Methylation

The procession of DNA methylation is addition of a methyl moiety at the fifth carbon position of cytosine residue within CpG dinucleotides that are usually located in CpG-rich regions or CpG islands and around the gene promoter [11, 12]. DNA methylation in gene promoter region represses gene transcription by recruiting proteins that bind methylated CpG sequences (methyl-CpG-binding domain [MBD] proteins) complexed with histone deacetylases (HDACs) and HMTs promoting coordinated epigenetic modifications of surrounding chromatin [13–15]. In contrast, methylation in gene bodies does not block transcription and is sometimes associated with active transcription [16]. DNA methylation is mediated by a family of enzymes known as DNA methyltransferases (DNMT) [17]. DNMT1 is responsible for maintenance of pre-existent methylation patterns during the DNA replication. DNMT3A and DNMT3B are responsible for de novo methylation. It was reported overexpression of these DNMTs was involved in gastric cancer [18, 19]. Another DNA methyltransferase family member, DNMT3L, is required for the methylation of imprinted genes in germ cells, and interacts with DNMT3a and 3b in de

novo methyltransferase activity [20]. And the function of DNMT2 remains unclear; the character of strong binding to DNA suggests that it may mark specific sequences in the genome.

As the best-known epigenetic modification, DNA methylation was begun to be studied from the initial finding of global hypomethylation in human tumors [21], and it was soon followed by the identification of promoter region hypermethylation in tumor-suppressor genes [22–26]. Transcriptional silencing of tumor-suppressor gene by promoter region hypermethylation was the most studied epigenetic alteration in human cancer. The role of DNA methylation on carcinogenesis has become a hot topic in the field of oncology [27].

2.1 The Role of DNA Hypermethylation in Gastric Cancer

Regional hypermethylation and global hypomethylation are supposed to be the hallmarks of cancer cells [21, 24, 28]. A number of tumor suppressor genes, such as *hMLM1*, *p14*, *p15*, *p16*, *GSTP1*, *RASSF1*, *COX-2*, *APC*, *CDH1*, *CDH4*, *DAP-K*, *THBS1*, *TIMP-3*, *RAR β* , *MGMT*, *CHFR*, *DCC*, *RUNX3*, *TSLC1*, *BCL2L10*, *IRX1*, *CMDM*, and *UCHL1*, were frequently silenced by hypermethylation in gastric cancer [29–31]. These genes are key components in different signaling pathways, which involves in proliferation, apoptosis, cell cycle, metastasis, and DNA damage repair. Accumulation of aberrant methylations of these genes is thought to promote carcinogenesis through disrupting normal signaling (Table 1).

2.1.1 DNA Methylation Is Involved in Gastric Carcinogenesis

RUNX protein was involved in transforming growth factor (TGF)- β signaling and inhibited TGF- β -induced epithelial-mesenchymal transition (EMT) [32, 33]. And RUNX3 cooperates with FoxO3a to induce apoptosis in gastric cancer cells [34]. Re-expression of RUNX3 increases p27 and caspase3 expression and induces cell apoptosis in vitro [35]. Stepwise accumulation of *RUNX3* promoter methylation was observed during gastric carcinogenesis; the ratio of methylation was 16 % in chronic atrophic gastritis, 37 % in intestinal metaplasia, 42 % in gastric adenoma, 55 % in dysplasia, and 75 % in advanced gastric cancer.

2.1.2 DNA Methylation Is Related to Invasion, Metastasis, and Prognosis in Gastric Cancer

Homeobox D10 (*HoxD10*) gene plays a critical role in cell differentiation and morphogenesis during development. HoxD10 induced cell apoptosis and suppressed cell migration and invasion both in vitro and in vivo [36]. HoxD10 expression was downregulated by promoter region hypermethylation in gastric cancer.

Death-associated protein kinase (DAPK) is frequently methylated in gastric cancer [37] and methylation is related to poor differentiation and lymph node metastasis and poor survival in gastric cancer significantly [38]. BNIP3 is a pro-apoptotic member of the Bcl-2 family [39, 40]. Promoter methylation-mediated *BNIP3* inactivation has been reported in gastric cancer [38, 41] and *BNIP3* methylation is related to poor prognosis in gastric cancer. Both *BNIP3* and *DAPK* methylation reduced sensitivity of fluoropyrimidine-based chemotherapy to gastric cancer patients [38].

Table 1
Methylation status of some tumor-suppressor genes in gastric cancer

Hypermethylated genes	Association with <i>H. pylori</i> infection	Main functions	Potential value	References
<i>UCHL1</i>	Unknown	Upregulated p53 protein expression	Detection biomarker	[125]
<i>ADAMTS9</i>	Unknown	Inhibits tumor growth by blocking AKT/mTOR signaling pathway	Detection biomarker	[126]
<i>FOXD3</i>	Yes	A tumor suppressor that regulates CYFIP2 and RARB	Detection biomarker	[62]
<i>MGMT</i>	Yes	DNA repair	Detection biomarker	[127]
<i>MLH1</i>	Yes	DNA mismatch repair	Detection biomarker	[127]
<i>GATA4</i> and <i>GATA5</i>	Yes	Transcription regulatory proteins	Detection and prognostic biomarker	[128, 129]
<i>PCDH10</i>	Unknown	Inhibits cell proliferation and induces cell apoptosis	Prognostic biomarker	[130]
<i>BCL6B</i>	Unknown	Inhibits cell proliferation and induces cell apoptosis	Prognostic biomarker	[131]
<i>RASSF1A</i>	Unknown	Cell cycle control, microtubule stabilization, cellular adhesion, motility, and apoptosis	Prognostic biomarker	[132, 133]
<i>Galectin-7</i>	Unknown	Suppressed proliferation, migration, and invasion	Prognostic biomarker	[134]
<i>BMP4</i>	Unknown	Enhances multiple pro-oncogenic traits and induces epithelial–mesenchymal transition	Inversely correlated with cisplatin resistance	[135]
<i>CHFR</i>	Unknown	Mitotic checkpoint gene	Predict the sensitivity of gastric cancers to microtubule inhibitors	[45]
<i>VEZT</i>	Yes	Targets GPR56, CDC42, and TCF19 and inhibits growth and invasion	A potential target for therapeutic	[136]

CDH1 (E-cadherin) is a transmembrane glycoprotein of epithelial cells [42]. Downregulation of CDH1 contributes to tumor progression through increasing proliferation, invasion, and metastasis [42]. Frequently methylation of CDH1 is found in primary gastric cancer and related to poor prognosis, particularly in the poorly differentiated and diffuse type of gastric cancer [43, 44].

Other genes related to cancer cell proliferation, invasion, metastasis, and prognosis were found frequently methylated in gastric cancer, including *p16* and *PRDM5*, *bMLH1*, *MGMT* [12], and *CHFR* [45].

2.2 Hypomethylation in Gastric Cancer

In contrast to the studies on promoter region hypermethylation, only a few studies have investigated genomic hypomethylation in gastric cancer. Genomic hypomethylation mainly affects repetitive transposable DNA elements that are normally heavily methylated and causes the elevated transcription [46, 47]. Generalized genomic hypomethylation contributes to genomic or chromosomal instability, resulting in cancer development [48, 49]. In addition, hypomethylation of normally methylated promoter CpG islands can lead to elevated expression of possible oncogenes [50]. Following are cases of hypomethylated genes.

LINE-1 hypomethylation has been demonstrated to be a prognostic marker in several types of human cancer, including ovary, colon, liver, and lung cancers. Bae et al. reported the similar role of *LINE-1* in gastric cancer, suggesting that *LINE-1* and *ALU* hypomethylation are early events in multistep gastric carcinogenesis. The study also revealed that *LINE-1* methylation status may be a tumor biomarker for advanced gastric cancer and a prognostic indicator independent of cancer stage to help identify a subgroup of GC patients with poor prognosis.

The *sulfatases* (or *SULFs*), *SULF1* and *SULF2*, play a critical role in the pathogenesis of human cancers. High expression of *SULFs* promoted tumor growth in vivo and correlated with higher recurrence rates and worse overall survival in gastric cancer patients. Multivariate analysis revealed that *SULF1* is an independent prognostic and lymph node metastasis indicator in patients with gastric cancer. Hypomethylation of *SULFs* is related to gastric carcinogenesis [51]. *SULF2* methylation may serve as a prognostic biomarker for gastric cancer patients treated with platinum-based chemotherapy [52].

2.3 Helicobacter pylori Infection and DNA Methylation

Helicobacter pylori (*H. pylori*) is a class I carcinogen in gastric cancer [53]. *Helicobacter pylori* infection causes gastric cancer through stepwise chronic gastritis, atrophy, intestinal metaplasia (IM), and dysplasia [54]. The risk of patients with *H. pylori* infection developing gastric cancer is increased twofold according to a lot of retrospective case control and prospective epidemiologic studies [55]. Once IM has developed, more than sixfold of risk was increased

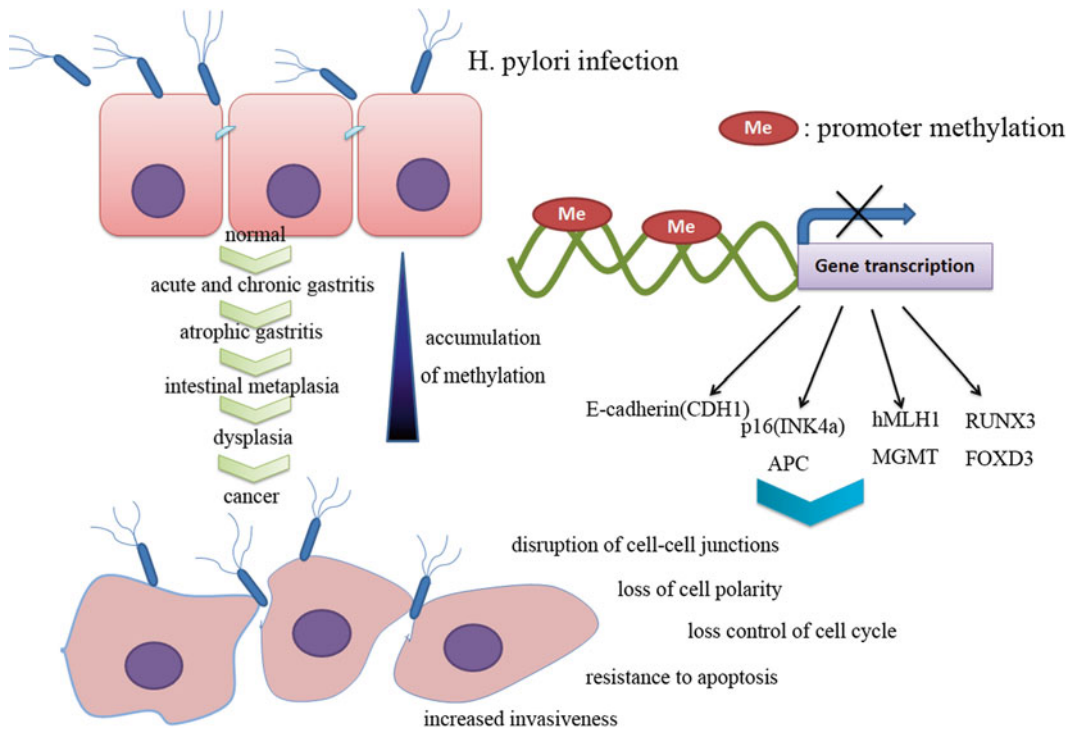


Fig. 1 The role of *H. pylori* infection-related DNA methylation in the gastric carcinogenesis through stepwise chronic gastritis, atrophy, and intestinal metaplasia

[56]. *H. pylori* infection is an independent predictive indicator of gene methylation [57, 58]. *H. pylori* infection-related DNA methylation was found in *CDH1*, *p16 (INK4a)*, *APC*, *hMLH1*, *BRCA1*, *MGMT*, *CDKN2A*, *MLH1*, and *RUNX3* [59–61]. The direct evidence of *H. pylori* infection induction of DNA methylation was found in detecting *FOXD3* methylation using *H. pylori* infected mouse model [62]. The *H. pylori* induced gene methylations participate in the gastric carcinogenesis (Fig. 1).

Accumulation of genetic and epigenetic alterations in normal appearing tissues has been considered as molecular basis for field defect. Epigenetic field defect in gastric cancer is mostly induced by *H. pylori* infection, with a higher gastric cancer risk [60]. In addition to inducing aberrant methylation of protein-coding genes, *H. pylori* infection was also reported associated with aberrant methylation of CpG islands of microRNA genes, such as miR-124a-1, miR-124a-2, miR-124a-3, and microRNA-34b/c [63, 64]. Other studies have shown that decreased DNA methylation level was found in individuals who received eradication therapy for *H. pylori* [65, 66].

3 Histone Modification

Chromatin is composed of an octameric protein core, two copies each of histone H2A, H2B, H3, and H4, wrapped around by 146 bp of DNA. The interaction between DNA and histones influences the accessibility of DNA transcription sites to RNA polymerase II and other transcription factors [67, 68]. Histone modification refers to acetylation, methylation, phosphorylation, ubiquitylation, SUMOylation, citrullination, and ADP-ribosylation of histone tails. These modifications constructing a “histone code,” caused by the action of specific proteins and plays a critical role in many biological processes including heterochromatin formation, X chromosome inactivation, and transcriptional regulation [69, 70]. In recent years, many studies have demonstrated the importance of histone modification in gastric cancer.

3.1 Histone Methylation

Histone methylation can lead to chromatin remodeling and affect DNA transcription close to the histone complex [71]. The methylation of histone tails is regulated by two kinds of enzymes: histone methyltransferases (HMTs) and histone demethylases (HDMs). Histone arginine methylation is found on residues 2, 8, 17, and 26 of histone H3 and residue 3 of histone H4 in mammals. Histone lysine methylation occurs on histones H3 and H4 and can be mono-, di-, or tri-methylated. Similar to histone lysine methylation, arginine methylation occurs in mono-methyl, symmetrical di-methyl, or asymmetrical di-methyl state [69]. The chromatin might be condensed with transcriptional inactivation or opened with activation of transcription, depending on the residue and the level of methylation. For example, the main sites of lysine methylation that have been associated with gene activity include K4, K36, and K79 of histone H3. Trimethylation of lysine 27 on histone H3 (H3K27me3), trimethylation at H3K9 and H4K20, and dimethylation at H3K9 are silencing epigenetic markers [67, 72]. The levels of H3K9me3 were shown to be associated with higher tumor stage, lymphovascular invasion, and recurrence rate in gastric cancer. Gastric cancer patients with higher H3K9me3 levels presented worse prognosis, suggesting that methylation level in H3K9 may serve as an independent prognostic factor in gastric cancer, which may due to inactivation of some tumor-suppressor genes by H3K9me3 [73].

A lot of studies have analyzed the machinery of histone methylation and the investigation was mainly focused on HMTs and HDMs. EZH2, a kind of HMT, plays a role in the trimethylation of H3K27 and is overexpressed in a variety of cancers, including gastric cancer, leading to silence of important genes during carcinogenesis [74]. It was demonstrated that knocking down EZH2 in gastric cancer cells resulted in lower H3K27me3 protein levels and

higher levels of E-cadherin expression. It was found at the same time that E-cadherin expression was associated with histone alterations but not with DNA methylation [75].

Silencing gene expression was found related to both histone modifications and DNA methylation in a variety of tumors [76–78]. For example, NGX6 is a novel tumor suppressor candidate whose inactivation is involved in the development of many cancers. Expression of NGX6 was downregulated by promoter methylation and H3-K9 methylation in gastric cancer cells, and related to tumor invasion and lymph node metastasis [79].

3.2 Histone Acetylation

Histone acetylation plays a major role in epigenetic regulation of gene expression [80]. The status of histones acetylation is controlled by two kinds of enzymes: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs add an acetyl group to lysine residues on the histone tails and promote DNA interaction in the nucleosome, leading to chromatin relaxation and subsequent increase in accessibility for transcription factors and transactivation of specific genes; in contrast, HDACs contribute to removing the acetyl group of lysine, resulting in transcriptional inactivation and compacted chromatin structure [81].

HATs have three family members, including the MOZ/YBF2/SAS2/TIP60 (MYST) family, the GCN5 *N*-acetyltransferase (GNAT) family, and the CREB binding protein (CBP)/p300 family [82]. The MYST family mainly targets histone H4, GNAT mainly targets histone H3, and the CBP/p300 family targets both H3 and H4 [83]. HATs are recruited as co-activators of transcription by transcription factors, usually in the context of large chromatin remodeling complexes [84]. In addition, PCAF, p300, and CBP acetylate multiple nonhistone proteins which play prominent roles in oncogenesis. Altered HAT activity has been reported in solid tumors, including gastric cancer. For example, PCAF expression was found reduced in gastric cancer, which was correlated to tumor invasion, tumor size, lymph node metastasis, and mutant type p53 protein expression. Patients with high-PCAF/wild-type p53 expression have a significantly better overall survival. Inactivation of HAT expression induced by gene mutation or deregulated by viral oncoproteins has been reported. Binding of these viral oncoproteins to p300 and CBP resulted in global hypoacetylation of histone H3 lysine 18(H3K18) and re-localization of these HATs to the promoter regions of a limited number of genes that promote cell growth and division, and caused gene-specific transcriptional activation [85, 86].

The role of HDACs is to balance the activity of HATs and regulate transcription through removing acetyl groups from histone tails and through deacetylation of nonhistone substrates. HDAC enzymes were divided into four classes according to their structures and functions, including class I (HDAC 1–3 and 8), II

(HDAC 4–7, 9, and 10), III (Sir-2 related—SIRT1–7), and class IV (HDAC 11). Class I, II, and IV HDACs share homolog in sequence and structure whereas the class III HDAC is of great difference in sequence or structure and requires nicotinamide adenine dinucleotide (NAD⁺) for catalytic activity [67, 87].

Increased expressions of HDAC1 and HDAC2 have been found in gastric cancer tissues [88–90]. HDAC1 and HDAC2 expressions were related to advanced gastric cancer, poor prognosis, and positive lymph node metastasis [88, 89]. It was demonstrated that inactivation of HDAC2 significantly reduced gastric cancer cell motility, invasion, colony formation, and tumor growth. Knocking down HDAC2 induced G1–S cell cycle arrest in gastric cancer cells [91]. Low level of histone acetylation has been reported to associate with the development and progression of gastric carcinoma. A lot of tumor suppressor genes was regulated by histone deacetylation, such as *GATA4* [92], *p21* (*WAF/CIP1*) [93], and *RUNX3* [94].

Action of HDAC inhibitors shifts the deacetylating activity of HDACs to the acetylating activity of HATs, and increases histone acetylation and gene expression. This is based on the assumption that histone acetylation promotes gene activation and histones are the major substrates [95]. Being the selective inhibitor for the class I and II HDACs, trichostatin A (TSA) was widely used to reactivate the expression of tumor suppressor genes (TSGs) in cancer cells.

3.3 Other Histone Modifications

Like histone acetylation, the phosphorylation of histones is highly dynamic. It takes place on serines, threonines, and tyrosines, predominantly, but not exclusively [96]. The majority of histone phosphorylation sites are within the N-terminal tails. It's modified by kinases and phosphatases [97]. High level histone H3 phosphorylation is associated with lymph node metastasis, poorer prognosis, and blood vessel invasion of intestinal-type gastric cancer [98].

Like histone methylation, the effect of histone sumoylation and ubiquitylation on gene transcription can be repression or activation, depending on the specific sites. Ubiquitin itself is a 76-amino acid polypeptide which is attached to histone lysines via the sequential action of three enzymes, E1-activating, E2-conjugating, and E3-ligating enzymes [99]. H2AK119ub1 and H2BK123ub1 are two well-characterized sites in H2A and H2B, playing the role of gene silencing or transcriptional initiation and elongation respectively [100, 101]. Sumoylation is a modification related to ubiquitylation, and involves the covalent attachment of small ubiquitin-like modifier molecules to histone lysines via the action of E1, E2, and E3 enzymes [96, 102]. It has been mainly associated with repressive function by blocking lysine substrate sites or recruiting HDACs to chromatin [67].

4 Noncoding RNA

Previous studies about human genome mainly focused on protein-coding genes, while the coding exons of these genes account for only 1.5 % of the genome [103]. In recent years, investigators began to realize the importance of nonprotein-coding portion of the genome in the occurrence of human diseases [104]. The noncoding RNAs (ncRNAs) consist of three types, long ncRNAs, mid-size ncRNAs, and short ncRNAs [103].

4.1 *MicroRNA* (*miRNA*)

MiRNAs are small single-stranded noncoding RNAs of about 18–25 nucleotides to degrade mRNA or block translation by targeting 3'-untranslated regions (UTRs) of mRNA [105]. MicroRNAs are estimated to regulate 30–60 % of human genes [106, 107]. Each miRNA may target different amount of mRNAs, and each mRNA may be targeted by different miRNAs. The expression profile is different for different tumors. The character of miRNA signature may help to distinguish different type of cancer [108, 109]. MiRNAs play an important role in several biological processes, including cell differentiation, proliferation, and apoptosis. MiRNAs can be classified as tumor-suppressive miRNA and oncogenic miRNA by their function.

The expression of miRNAs may be increased (miR-21, miR-17, and miR-20a) or reduced (miR-375 and miR-378) in gastric cancer. These increased miRNAs usually are related to cancer recurrence or progression and were known as oncogenic miRNAs. MiRNAs, which were reduced in gastric cancer, are usually related to good prognosis and are regarded as tumor-suppressive miRNAs. Gastric cancer-related miRNAs are listed in Table 2.

Increased expressions of miR-17-5p, 21, 106a, miR-378, and 106b were found in both gastric cancer tissue and patient serum. These miRNAs were regarded as serum detection marker in gastric cancer [110, 111]. More miRNAs were reported as serum markers for gastric cancer, but further validation is necessary [112].

4.2 *Long* *Noncoding RNA*

The Human Genome Project and high-throughput transcriptome analysis have revealed that human genome contains 20,000–30,000 protein-coding genes, representing about 2 % of the total genome sequence, while a substantial fraction of the human genome can be transcribed into many short or long noncoding RNAs [113–116]. Long noncoding RNA (lncRNAs) is a class of noncoding RNAs that the length is more than 200 nucleotides. More than 3,000 human lncRNAs were isolated [117, 118] and the number of new lncRNAs was growing very fast. The function of lncRNAs was associated with a spectrum of biological processes, including alternative splicing, modulation of protein activity, alternation of protein localization, and epigenetic regulation. lncRNAs can be also precursors of small RNAs and even tools for miRNAs silencing [119].

Table 2
MicroRNA expression profile in gastric cancer

MicroRNAs	Sample	Expression level	Target genes	Effect on gastric cancer	References
miR-124a	Gastric cancer cell line	Down	<i>CDK6</i>	Induces cell cycle arrest and inhibits proliferation	[63]
miR-125a-5p	Tissues and cells	Down	<i>ERBB2</i>	Suppresses cancer cells proliferation	[137]
miR-101	Cancer tissues and cells	Down	<i>EZH2, Cox-2, Mcl-1</i> and <i>Fos</i>	Suppressed tumor growth in vivo	[138]
miR-148a	Gastric cancer tissues	Down	<i>ROCK1, DNMT1</i>	Suppressed migration and invasion in vitro	[139, 140]
miR148b	Tissues and cells	Down	<i>CCKBR</i>	Inhibited proliferation and tumorigenicity	[141]
miR429	Tissues and cells	Down	<i>Myc</i>	High expression correlated with lymph node metastasis	[142]
MicroRNA-130b	Tissues and cells	Down	<i>RUNX3</i>	Promotes tumor growth	[143]
miR-200b and miR-200c	Tissues and cells	Down	<i>DNMT3A, DNMT3B</i>	Impaired cell growth and invasion	[144]
miR-21	Tissues and cells	Up	<i>PTEN</i>	Promotes tumor proliferation and invasion	[145]
miR-192 and miR-215	Tissues and cells	Up	<i>ALCAM</i>	Promotes cell growth and migration in vitro	[146]
miR-451	Tissues	Up	<i>MDR-1</i>	Correlates with poor prognosis	[147]
miR-146a	Tissues	Up	<i>SMAD4</i>	Targets SMAD4 and modulates cell proliferation and apoptosis	[148]

One of their primary tasks is regulation of protein-coding gene expression [120]. lncRNAs act mainly through four different mechanisms, which were supposed as signals, decoys, guides, and scaffolds [121]. Dysregulation of these lncRNAs is involved in different human cancers, including gastric cancer [122].

HOTAIR is an lncRNA that was identified from a custom tiling array of the HOXC locus. HOTAIR was shown to trimethylate histone H3 lysin-27 of HOXD locus with the polycomb repressive complex 2 (PRC2) and inhibit HOXD gene expression. The expression of HOTAIR was increased significantly in gastric cancer and increased expression was associated with venous invasion, lymph node metastases, and short time survival [123]. Overexpression of lncRNA HULC promotes cell proliferation, epithelial-to-mesenchymal transition (EMT), and invasion and inhibits apoptosis in gastric cancer [124]. Maternally expressed gene 3 (MEG3) is an imprinted gene located at 14q32. Down-regulated long noncoding RNA MEG3 is associated with poor prognosis in gastric cancer [122].

5 Concluding Remarks

Although there are a growing number of publications about epigenetics studies, complete understanding of cancer epigenetics is a long way to go. Discovering the epigenetic signatures might lead to developing epigenetic detection markers for diagnosis, prognosis, and chemotherapy in gastric cancer. Two kinds of epigenetic-related reagents, the HDAC inhibitor (trichostatin A [TSA]) and DNMT inhibitor (5-azacytidine [5-aza-CR] or 5-aza-2'-deoxycytidine [5-aza-CdR]), were frequently reported in experimental study, but the application in clinic has no conclusion. The application of noncoding RNA, especially lncRNA, is a new field. The promising results demonstrate that the study of lncRNA may improve the understanding of gastric cancer in the near future.

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