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Molecular Diagnosis and Targeting for Thoracic and Gastrointestinal Malignancy

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Molecular Diagnosis and Targeting for Thoracic and Gastrointestinal Malignancy

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Preface

Recent advances in molecular biology have resulted in the mechanisms responsible for cancer progression being elucidated in detail, and molecular targeted drugs are now used in the treatment of various malignant diseases. Analyses of the molecular backgrounds of tumors are indispensable for the diagnosis and precise treatment of cancer. However, tumor-specific and comprehensive mechanisms are involved in these processes.

This book focuses on these issues and provides information from the viewpoint of molecular biology and its clinical applications. The molecular mechanisms of thoracic and gastrointestinal malignancies and their clinical applications are also outlined. This book is primarily for clinical oncologists but is also relevant to basic oncologists. It may also attract clinicians who have an interest in this field and are considering the initiation of molecular diagnoses and targeted therapies.

Although several good textbooks are currently available on molecular diagnoses and targeted therapies, our book places a strong emphasis on clinical applications from the viewpoint of oncologists specializing in specific organs. Furthermore, we focus on the role of molecular diagnoses and targeted therapies in the course of surgical treatment.

We hope this book will provide timely and useful information to clinicians.

Kyoto, Japan — Yutaka Shimada
Tokyo, Japan — Katsuhiko Yanaga

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Chapter 1
Molecular Diagnosis and Targeting for Lung Cancer

Kazue Yoneda and Fumihiro Tanaka

Abstract Lung cancer is the leading cause of cancer deaths associated with poor prognosis. Patients with advanced lung cancer had been "uniformly" treated with platinum-based cytotoxic chemotherapy, which had provided an only modest clinical benefit. However, recent advances in molecular diagnosis and systemic treatment targeting cancer hallmarks such as angiogenesis, oncogenic gene alteration, and evasion from cancer immunity have dramatically changed treatment strategies associated with a tremendous improvement in outcomes of lung cancer patients. Here, we review current status and future perspectives of molecular diagnosis and personalized "precision" medicine for lung cancer.

Keywords Angiogenesis • Oncogenic alteration • EGFR (epidermal growth factor receptor) • ALK (anaplastic lymphoma kinase) • Immune checkpoint inhibitor

1.1 Introduction

Lung cancer is the leading cause of cancer-related death worldwide [1, 2]. Lung cancer is classified into several histologic categories including four major subtypes as follows: small cell lung cancer (SCLC, ~15%), squamous cell carcinoma (~25%), adenocarcinoma (~50%), and large cell carcinoma (~10%). SCLC is biologically and clinically distinct from other subtypes, as characterized by early lymphatic and

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distant metastatic spread as well as by higher responsiveness to cytotoxic chemotherapeutic agents [1, 3, 4]. The other histologic subtypes have been clinically categorized as a whole into non-small cell lung cancer (NSCLC), for which cytotoxic agents provided an only modest clinical benefit [1].

The optimal treatment for the cure of cancer patients is surgical removal, but less than 20% of lung cancer patients present with localized disease for which surgery may be indicated. The majority of patients present with metastatic disease and are treated with systemic treatment. During the last few decades, systemic chemotherapy using cytotoxic agents, especially platinum-doublet chemotherapy consisting a platinum agent (cisplatin or carboplatin) plus one non-platinum agent, has been employed as the "standard care of treatment" and has provided an only modest survival benefit with the median overall survival (OS) of less than 2 years [1, 5–7]. However, recent discoveries of molecular hallmarks of cancer have changed our understanding of lung cancer, especially NSCLC, as a single disease to a disease comprising a variety of molecularly distinct subtypes, and novel agents targeting various cancer hallmarks have provided a tremendous improvement in treatment outcomes for NSCLC carrying specific targets [1, 8–13] (Table 1.1). Among them, the

Table 1.1 Overview of molecular targets and targeting agents for lung cancer

Agent			
Name	Target	Class	Approved indication[a]
Angiogenesis inhibitors			
Bevacizumab	VEGF	Humanized MoAb (IgG1)	Non-squamous NSCLC
			In combination with cytotoxic agents
Ramucirumab	VEGFR-2	Fully human MoAb (IgG1)	Previously treated NSCLC
			In combination with DOC
Nintedanib	VEGFR-1, VEGFR-2, VEGFR-3	TKI	Adenocarcinoma, after first-line chemotherapy
	FGFR-1, FGFR-2, FGFR-3		In combination with DOC
	PDGFR-α, PDGFR-β		# approved only by EMA
Inhibitors for driver oncogenic alterations			
Gefitinib	EGFR	TKI	*EGFR*-mutated NSCLC
Erlotinib	EGFR	TKI	*EGFR*-mutated NSCLC
Afatinib	EGFR	TKI	*EGFR*-mutated NSCLC
Osimertinib	EGFR	TKI	T790M-positive, *EGFR*-mutated NSCLC, after treatment with EGFR-TKI (gefitinib, erlotinib, or afatinib)
Crizotinib	ALK	TKI	*ALK*-rearranged NSCLC
Alectinib	ALK	TKI	*ALK*-rearranged NSCLC
Ceritinib	ALK	TKI	*ALK*-rearranged NSCLC, after treatment with crizotinib

Table 1.1 (continued)

Agent			
Name	Target	Class	Approved indication[a]
Crizotinib	ROS1	TKI	*ROS1*-rearranged NSCLC
			# approved only by FDA and EMA
Immune checkpoint inhibitors			
Nivolumab	PD-1	Fully human MoAb (IgG4)	Previously treated NSCLC
Pembrolizumab	PD-1	Humanized MoAb (IgG4)	NSCLC with PD-L1 expression
Atezolizumab	PD-L1	Humanized MoAb (IgG1)	Previously treated NSCLC
			# approved only by FDA

[a]Indication, in Japan as of December 2016
VEGF vascular endothelial growth factor, *EGFR* vascular endothelial growth factor receptor, *FGFR* fibroblast growth factor receptor, *PDGFR* platelet-derived growth factor receptor, *EGFR* epidermal growth factor receptor, *ALK* anaplastic lymphoma kinase, *T790M* a "threonine (T) to methionine (M)" substitution at codon 790 of EGFR, *PD-1* programmed cell death protein 1, *PD-L1* programmed death-ligand 1, *MoAb* monoclonal antibody, *TKI* tyrosine kinase inhibitor, *NSCLC* non-small cell lung cancer, *DOC* docetaxel, *EMA* European Medicines Agency, *FDA* Food and Drug Administration

discovery of activating mutations in the epidermal growth factor receptor (EGFR) gene and development of inhibitors targeting tyrosine kinase of EGFR that is constitutionally activated by these mutations have provided a paradigm shift in the treatment of NSCLC; EGFR tyrosine kinase inhibitors (TKIs) are very effective for NSCLC harboring *EGFR* mutations accounting for 30–50% of NSCLC in East Asian patients, but not for NSCLC without *EGFR* mutations [14]. Similarly, TKIs of anaplastic lymphoma kinase (ALK) are effective only for tumor with *ALK* rearrangements, which accounts for only 3–5% of NSCLC. Accordingly, precise molecular diagnosis is essential in achieving personalized, optimal treatment for each patient with lung cancer [13–16]. Future perspectives as well as current status of molecular diagnosis and targeting therapy in lung cancer are reviewed and discussed.

1.1.1 Angiogenesis and Inhibitors

Angiogenesis plays essential roles in the development and progression of a variety of malignant tumors, and blockade of tumor angiogenesis may be a promising strategy for treating cancer [8, 17–19]. Tumor angiogenesis is mediated primarily through the interaction of vascular endothelial growth factor (VEGF) and its receptor (VEGFR-2), and several targeting agents have been developed and evaluated in clinical trials for lung cancer. For SCLC, anti-angiogenic agents including bevacizumab, an anti-VEGF antibody, have failed to provide a significant clinical benefit [20–23]. For NSCLC, based on results in randomized controlled trials (RCTs) comparing anti-angiogenic agents plus cytotoxic

chemotherapy with cytotoxic chemotherapy alone, two anti-angiogenic agents (bevacizumab [24–27] and ramucirumab [28]) are currently available for clinical use, and another agent (nintedanib [29]) is approved only by the European Medicines Agency (EMA) (Table 1.2).

The most important adverse event associated with the use of anti-angiogenic agents may be fatal bleeding. In fact, in a randomized phase 2 trial to investigate the efficacy and safety of an anti-VEGF antibody, bevacizumab, in addition to chemotherapy using carboplatin plus paclitaxel (CbP) for advanced NSCLC, an increased risk of a major life-threating bleeding described as grade ≥ 3 hemoptysis or hematemesis was documented in patients with squamous cell carcinoma (30.7%, 4 of 13 patients) but not in patients with other histologic types including adenocarcinoma and large cell carcinoma (non-squamous NSCLC; 3.7%, 2 of 54 patients) [30]. Accordingly, only non-squamous NSCLC patients have been eligible in subsequent clinical trials of bevacizumab, and bevacizumab is approved and indicated only for non-squamous NSCLC patients (Table 1.1). The E4599, conducted by the Eastern Cooperative Oncology Group (ECOG), is the landmark phase 3 trial showing a significant survival advantage of the use of bevacizumab in combination with chemotherapy (median overall survival time [MST], 10.3 months with CbP-alone versus 12.3 months with CbP plus bevacizumab; hazard ratio [HR] for death, 0.79; $P = 0.003$) [24]. Additional phase 3 trials have also indicated significant clinical benefits of the use of bevacizumab in combination with platinum-based chemotherapy [25–27] (Table 1.2).

Ramucirumab is a fully human antibody against VEGFR-2 [19]. In a placebo-controlled phase 3 trial (REVEL) conducted for NSCLC with disease progression after first-line chemotherapy, ramucirumab plus docetaxel (DOC) has provided a significant survival advantage over DOC plus placebo (Table 1.2) [26] and is approved for previously treated NSCLC of all histologic subtypes including squamous cell carcinoma (Table 1.1).

Nintedanib is a small molecule TKI targeting multiple angiogenic pathways mediated by VEGFRs (VEGFR-1, VEGFR-2, VEGFR-3), fibroblast growth factor receptors (FGFR-1, FGFR-2, FGFR-3), and platelet-derived growth factor receptors (PDGFR-α, PDGFR-β) [29]. Nintedanib is approved worldwide for the treatment of idiopathic pulmonary fibrosis (IPF) based on results of placebo-controlled trials demonstrating a significant reduction in annual decline in lung function by approximately 50% [31]. For the treatment of NSCLC, however, nintedanib is approved only by EMA (Table 1.1), as only a modest clinical benefit has been achieved with the use of nintedanib [29] (Table 1.2).

A variety of biological markers associated with clinical responses or survival benefits achieved with anti-angiogenic agents have been tested. Unfortunately, no clinically useful predictive marker has been established. For example, serum levels of several angiogenesis-related molecules (VEGF, basic fibroblast growth factor [bFGF], soluble intercellular adhesion molecule [ICAM], and E-selectin) were prospectively measured and correlated with the effect of bevacizumab in the E4599 trial. Serum VEGF levels proved to be predictive of tumor response to bevacizumab, but failed to predict a survival benefit with bevacizumab [32].

Table 1.2 Key randomized clinical trials of anti-angiogenic agents for advanced non-small cell lung cancer (NSCLC)

Trial	Key eligible criteria	Arm (No. of pts)	Results		
			ORR	PFS (median)	OS (median)
Bevacizumab (anti-VEGF antibody)					
E4599 phase 3 [24]	Non-squamous NSCLC, previously untreated	CbP ($n = 433$)	15%	4.5 m	10.3 m
		CbP + Bev (15) ($n = 417$)	35% ($P < 0.001$)	6.2 m HR = 0.66 (95% CI, 0.57–0.77; $P < 0.001$)	12.3 m HR = 0.79 (95% CI, 0.67–0.92; $P = 0.003$)[a]
AVAil (BO17704) phase 3 [25, 26]	Non-squamous NSCLC, previously untreated	CdG + Pla ($n = 347$)	20.1%	6.1 m	13.1 m
		CdG + Bev (7.5) ($n = 345$)	34.1% ($P < 0.0001$)	6.7 m HR = 0.75 (95% CI, 0.62–0.91; $P = 0.003$)[a]	13.6 m HR = 0.93 (95% CI, 0.78–1.11; $P = 0.420$)
		CdG + Bev (15) ($n = 351$)	30.4% ($P = 0.0023$)	6.5 m HR = 0.82 (95% CI, 0.68–0.98; $P = 0.03$)[a]	13.4 m HR = 1.03 (95% CI, 0.86–1.23; $P = 0.761$)
BEYOND (YO25404) phase 3 [27]	Non-squamous NSCLC, previously untreated	CbP + Pla ($n = 138$)	26%	6.5 m	17.7 m
		CbP + Bev (15) ($n = 138$)	54% ($P < 0.001$)	9.2 m HR = 0.40 (95% CI, 0.29–0.54; $P < 0.001$)[a]	24.3 m HR = 0.68 (95% CI, 0.50–0.93; $P = 0.0154$)
Ramucirumab (anti-VEGFR-2 antibody)					
REVEL phase 3 [28]	NSCLC, PD after 1st-line platinum-based chemotherapy	DOC + Pla ($n = 625$)	14%	3.0 m	9.1 m
		DOC + Ram ($n = 628$)	23% ($P < 0.0001$)	4.5 m HR = 0.76 (95% CI, 0·68–0·86; $P < 0.0001$)	10.5 m HR = 0.86 (95% CI, 0·75–0·98; $P = 0.023$)[a]
Nintedanib (multi-targeting TKI)					
LUME-Lung1 phase 3 [29]	NSCLC, PD after First-line chemotherapy	DOC + Pla ($n = 659$)	3.3%	Primary 2.7 m	9.1 m
		DOC + Nin ($n = 655$)	4.4% ($P = 0.3067$)	3.4 m HR = 0.79 (95% CI, 0.68–0.92; $P = 0.0019$)[a]	10.1 m HR = 0.94 (95% CI, 0.83–1.05; $P = 0.2720$)

[a]Indicates the primary endpoint of each trial

ORR objective response rate, *PFS* progression-free survival, *OS* overall survival, *HR* hazard ratio, *CI* confidence interval, *PD* progressive disease, *VEGF* vascular endothelial growth factor, *CbP* carboplatin plus paclitaxel, *CdG* cisplatin plus gemcitabine, *Pla* placebo, *Bev (15)* bevacizumab (15 mg/kg), *Bev (7.5)* bevacizumab (7.5 mg/kg), *DOC* docetaxel, *Ram* ramucirumab, *Nin* nintedanib

1.1.2 Driver Oncogenic Gene Alterations and Targeting Agents

Since the discovery of activating mutations in the *EGFR* gene [33, 34], a number of gene alterations to "drive" oncogenic transformation have been identified in non-squamous NSCLC, mainly in adenocarcinoma (Fig. 1.1) [9, 10, 35–38]. As noted, the majority of "oncogenic" alterations are seen in genes coding receptor tyrosine kinase (RTK) such as EGFR, HER2, MET, ALK, ROS1, and RET and are caused through two different types of mechanisms as follows: (1) "small-scale" mutations including substitution, deletion, and insertion of nucleotides within a gene (*EGFR*, *HER2*, *KRAS*, *BRAF*, *MET*) and (2) "large-scale" alterations in chromosomal structure including chromosomal transformation to rearrange a gene, leading to the formation of a new "oncogenic" fusion gene with a partner gene (*ALK*, *ROS1*, *RET*).

"Driver" alterations may confer hypersensitivity to specific inhibitors. EGFR-TKIs and ALK-TKIs are currently approved worldwide for treating advanced NSCLC with EGFR mutations and ALK rearrangements, respectively. In addition, crizotinib has been recently approved by EMA and by the Food and Drug Administration (FDA) (Table 1.1). Today, molecular testing is mandatory at the time of initial diagnosis, before the initiation of systemic treatment, of advanced non-squamous NSCLC [15].

1.1.2.1 EGFR Mutations and EGFR-TKIs

EGFR is a transmembrane spanning RTK, which is composed of an extracellular ligand-binding region, a transmembrane region, a juxta-membrane region, and an intracellular region. The intracellular region contains the tyrosine kinase (TK)

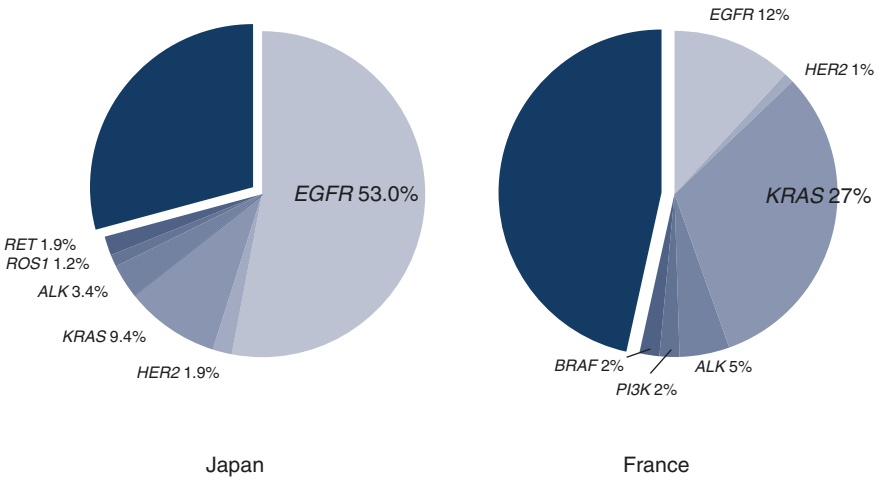


Fig. 1.1 Driver oncogenic gene alterations in lung adenocarcinoma in Japan (*left*, data from references [9, 10]) and in France (*right*, data from reference [11])

domain and the carboxyl-terminal (C-terminal) domain that contains at least five tyrosine residues that may be autophosphorylated [39–43] (Fig. 1.2a). The EGFR family members, EGFR and its three close relatives (HER-2, HER-3, and HER-4), play important roles in normal biological processes such as cell proliferation, differentiation, and migration [40, 43–45]. On binding to specific activating ligands such as EGF, transforming growth factor-α, betacellulin, heparin-binding EGF-like

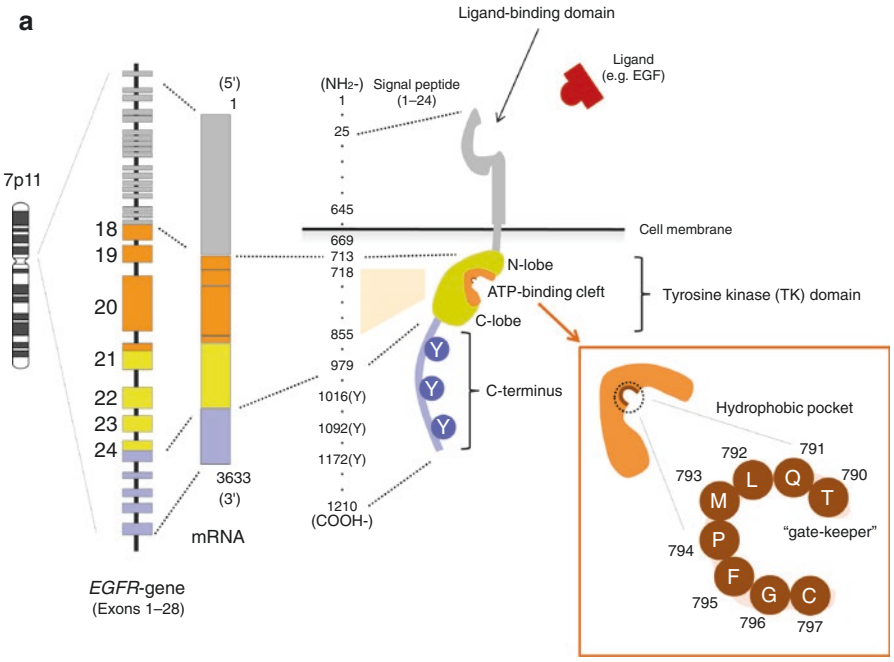
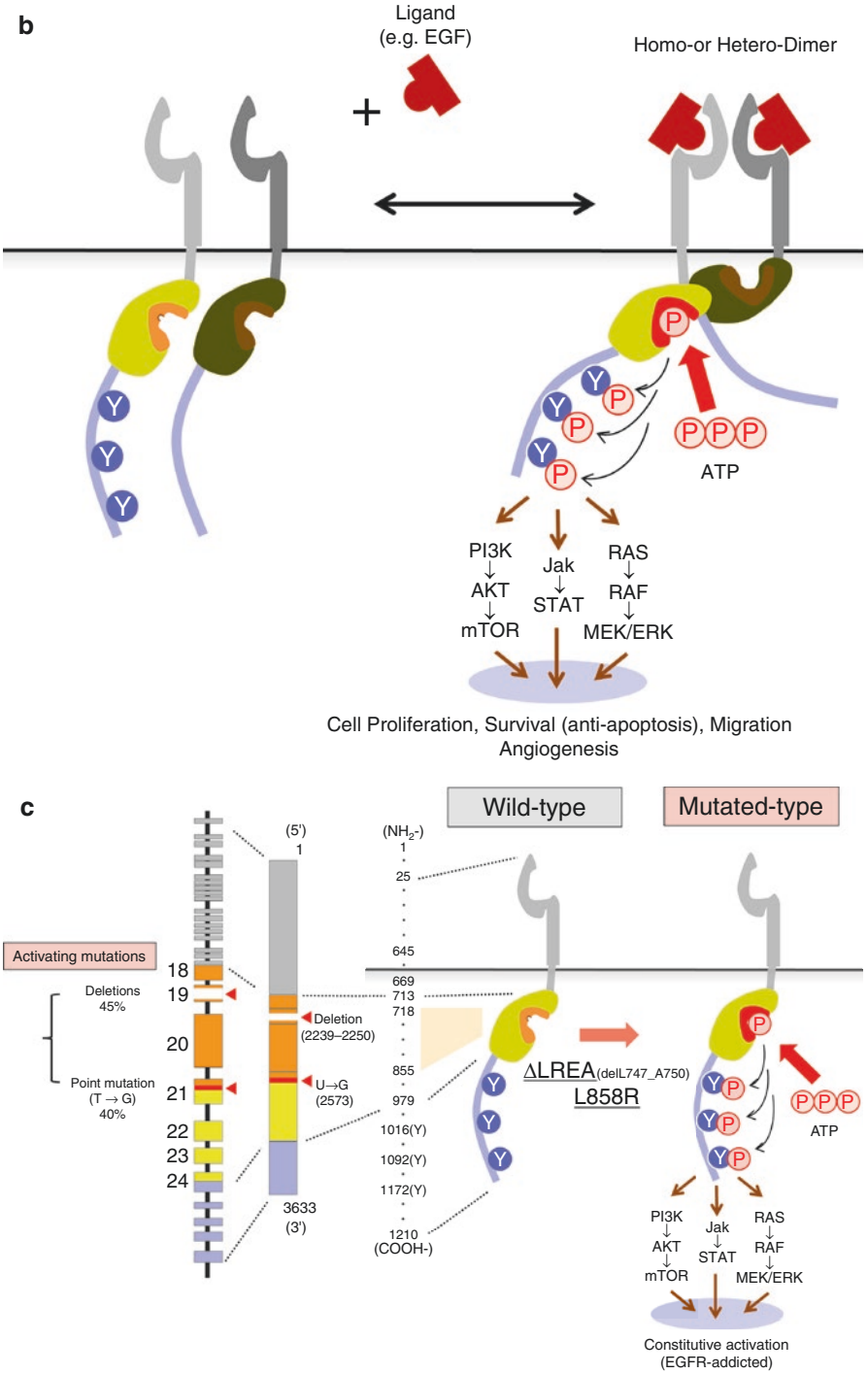


Fig. 1.2 (**a**) Structure of epidermal growth factor receptor (EGFR) and EGFR gene comprising of 28 exons. Exons 18–24 code the intracellular tyrosine kinase (TK) domain including adenosine triphosphate (ATP)-binding pocket. The hydrophorbic pocket in the back of the ATP-binding cleft comprises of eight amino acids as follows: threonine (T) at codon 790, the "gatekeeper" at the entrance of the pocket, glutamine (Q) at codon 791, leucine (L) at codon 792, methionine (M) at codon 793, proline (P) at codon 794, phenylalanine (F) at codon 795, glycine (G) at codon 796, cysteine (C) at codon 797. The carboxyl-terminus (C-terminus) contains at least five tyrosine (Y) residues for autophosphorylation. (**b**) Ligand-dependent activation of TK of EGFR. Dimerization of EGFR leads to activation of TK domain followed by autophosphorylation of tyrosine residues at the C-terminus, which results in activation of downstream signaling pathways associated with cell proliferation, cell survival, cell migration, and angiogenesis. (**c**) Deletions in exon 19 affecting elimination of amino-acid sequences of "leucine (L) at codon 747, arginine (R) at codon 748, glutamic acid (E) at codon 749, alanine (A) at codon 750" (ΔLREA) as well as a point mutation in exon 21 affecting a "leucine (L) to arginine (R)" substitution at codon 858 (L858R) are oncogenic through ligand-independent, constitutive TK activation. Dimerization partner in activated form of mutated EGFR is deleted in the figure. (**d**) Activated TK caused by oncogenic *EGFR* mutations can be effectively inhibited by tyrosine kinase inhibitors (TKIs). Reversible "first-generation" inhibitors (gefitinib and erlotinib) competitively inhibit ATP binding, and irreversible inhibitors such as afatinib and osimertinib covalently bind to C797

Fig.1.2 (continued)



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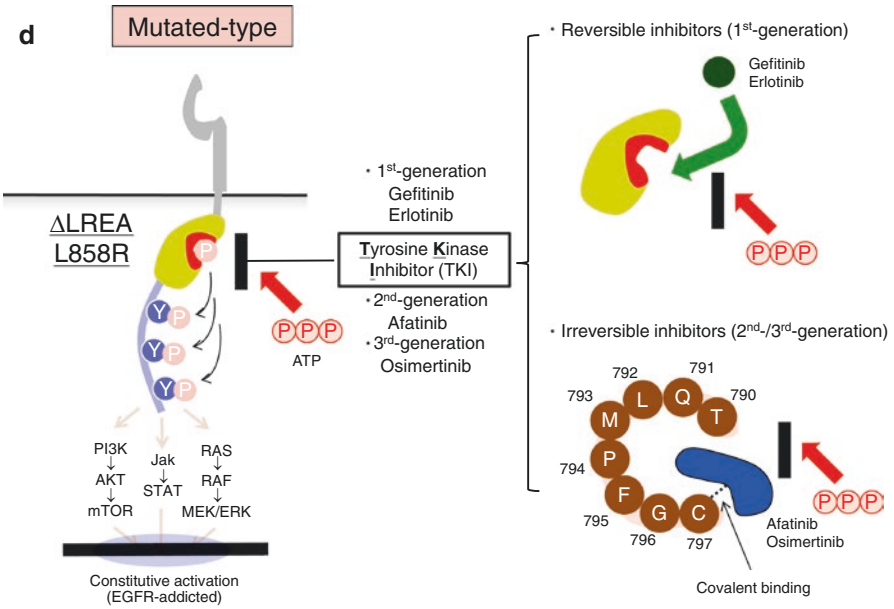

growth factor, amphiregulin, epiregulin, and epigen, EGFR forms homo- or heterodimers with other EGFR members, which leads to activation of intracellular TK domain. The activated TK domain catalyzes autophosphorylation of specific tyrosine residues within the C-terminal domain through transferring γ-phosphate of adenosine triphosphate (ATP) and subsequently activates several signaling pathways such as Ras-Raf-mitogen-activated protein kinase (MAPK) pathway, phosphatidylinositol-3 kinase (PI3K)-AKT pathway, and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway; activation of these pathways finally results in accelerated cell proliferation and migration, evasion from apoptosis, and prolonged cell survival, promoting angiogenesis [39–42] (Fig. 1.2b).

The *EGFR* gene, located on the chromosome 7p11.2 region, comprises 28 exons, and exons 18–24 encode the TK domain of EGFR [46] (Fig. 1.2a). In 2004, somatic mutations in the EGFR gene were discovered in NSCLC patients who response to an EGFR-TKI (gefitinib) [33, 34]. Since the discovery, a variety of *EGFR* mutations have been identified, which cluster in exons 18–21 that encode the ATP-binding pocket of the TK domain [14]. The most common mutations are in-frame deletions in exon19 and a point mutation in exon 21, comprising over 90% of *EGFR* mutations documented in NSCLC [14, 46]; deletions in exon 19 including codons 747–750 affect elimination of the conserved amino-acid sequences of "leucine (L) at codon 747, arginine (R) at codon 748, glutamic acid (E) at codon 749, and alanine (A) at codon 750" (ΔLREA), and the point mutation in exon 21 affects a "leucine (L) to arginine (R) substitution" at codon 858 (L858R) [33, 34, 46–50] (Fig. 1.2c). Several studies have revealed that these mutations are oncogenic through constitutive and ligand-independent activation of tyrosine kinase of EGFR by stabilizing the

active conformation as well as by promoting dimerization [42, 51–53]. As noted, these activating mutations are predominantly detected in lung adenocarcinoma, and the incidence is significantly different according to ethnicity (47.9% in East Asian patients with adenocarcinoma versus 19.2% in Western patients with adenocarcinoma) and smoking status (37.6–62.5% in never-light smokers versus 8.4–35.9% in ever-heavy smokers) (Fig. 1.1) [15, 54–56].

In *EGFR*-mutated tumor, malignant phenotype such as autonomic cell proliferation solely depends on constitutively activated tyrosine kinase of EGFR caused by activating mutations ("oncogene addiction"), which may be effectively inhibited by agents targeting tyrosine kinase of EGFR (Fig. 1.2d) [57]. Several experimental studies have shown that "first-generation" EGFR-TKIs, either gefitinib or erlotinib, competitively bind to the ATP-binding site of EGFR and effectively inhibit autophosphorylation and activation of consequent downstream signaling cascades [50, 58]. In clinical setting, a number of retrospective studies have indicated a significant correlation between the presence of *EGFR* mutations and response to EGFR-TKIs in NSCLC [15, 59]. The IRESSA Pan-Asia Study (IPASS), comparing gefitinib with chemotherapy (CbP) as a first-line treatment of advanced lung adenocarcinoma among nonsmokers or former light smokers in East Asia, is the landmark trial to confirm that the presence of *EGFR* mutations is the predictor of the response to an EGFR-TKI (objective response rate [ORR] with gefitinib group, 71.2% for *EGFR* mutation-positive patients versus 1.1% for *EGFR* mutation-negative patients) [60, 61]. All subsequent phase 3 trials comparing a first-generation EGFR-TKI (gefitinib or erlotinib) with platinum-doublet chemotherapy for previously untreated patients with advanced NSCLC harboring "sensitizing" *EGFR* mutations (i.e., ΔLREA and L858R) have met the primary endpoint of improving progression-free survival (PFS), although no significant improvement in overall survival (OS) has been indicated mainly due to impact of post-study treatment crossover (e.g., gefitinib after failure of chemotherapy) on OS [62–68] (Table 1.3).

Afatinib is a "second-generation" EGFR-TKI, which covalently binds to a cysteine residue at codon 797 (C797) at the edge of ATP-binding cleft of the TK domain of EGFR (Fig. 1.2d). Owing to covalent, irreversible nature of the binding, afatinib may achieve a greater inhibitory effect as compared with reversible "first-generation" EGFR-TKIs. Afatinib also inhibits tyrosine kinase of other EGFR family members (HER-2, HER-3, and HER-4), which may provide a more potent antitumor activity [74]. Two phase 3 trials comparing afatinib with platinum-doublet chemotherapy for untreated *EGFR*-mutated NSCLC have demonstrated a significantly prolonged PFS with afatinib [69–71] (Table 1.3). Based on these results, first-line treatment with an EGFR-TKI, either gefitinib, erlotinib, or afatinib, is recommended as the "standard care" of patients with advanced NSCLC harboring sensitizing *EGFR* mutations [16, 75–77].

Despite initial favorable responses, a vast majority of patients eventually develop progressive disease due to "acquired resistance" after 10–14 months of treatment with EGFR-TKIs. A variety of mechanisms of acquired resistance to "first-generation" EGFR-TKIs have been identified as follows: (1) target alterations by

Table 1.3 Key randomized clinical trials of EGFR-TKIs for previously untreated, advanced non-small cell lung cancer (NSCLC) harboring activating *EGFR* mutations

Trial	Key eligible criteria	Arm (No. of pts)	Results		
			ORR	PFS (median)	OS (median)
EGFR-TKI versus *chemotherapy*					
NEJ002 phase 3 [62, 63]	ΔLREA, L858R, or other *EGFR* mutations (6.1%)	CbP ($n = 114$)	30.7%	5.4 m	26.6 m
		Gefitinib ($n = 114$)	73.7% ($P < 0.001$)	10.8 m HR = 0.30 (95% CI, 0.22–0.41; $P < 0.001$)	27.7 m HR = 0.887 (95% CI, 0.634–1.241; $P = 0.483$
WJTOG3405 phase 3 [64]	ΔLREA or L858R	CdDOC ($n = 86$)	32.2%	6.3 m	37.3 m
		Gefitinib ($n = 86$)	62.1% ($P < 0.0001$)	9.2 m HR = 0.489 (95% CI, 0.336–0.710; $P < 0.0001$)	34.8 m HR = 1.252 (95% CI, 0.883–1.775; $P = 0.206$)
OPTIMAL phase 3 [65, 66]	ΔLREA or L858R	CbG ($n = 72$)	36%	4.6 m	27.2 m
		Erlotinib ($n = 82$)	83% ($P < 0.0001$)	13.7 m HR = 0.16 (95% CI, 0.10–0.26; $P < 0.0001$)	22.8 m HR = 1.19 (95% CI, 0.83–1.71; $P = 0.2663$)
EURTAC phase 3 [67]	ΔLREA or L858R	CdDOC/CdG CbDOC/CbG, allowed ($n = 87$)	14.9%	5.2 m	19.6 m
		Erlotinib ($n = 86$)	58.1%	9.7 m HR = 0.37 (95% CI, 0.25–0.54; $P < 0.0001$)	22.9 m HR = 0.92 (95% CI, 0.63–1.35; $P = 0.68$)
ENSURE phase 3 [68]	ΔLREA or L858R	CdG ($n = 107$)	33.6%	5.5 m	25.5 m
		Erlotinib ($n = 110$)	62.7%	11.0 m HR = 0.34 (95% CI, 0.22–0.51; $P < 0.0001$)	26.3 m HR = 0.91 (95% CI, 0.63–1.31; $P = 0.6073$)
LUX-lung 3 phase 3 [69, 70]	ΔLREA, L858R, or other *EGFR* mutations (10.7%)	CdPem ($n = 115$)	23%	6.90 m	28.2 m
		Afatinib ($n = 230$)	56% ($P = 0.001$)	11.14 m HR = 0.58 (95% CI, 0.43–0.78; $P = 0.001$)	28.2 m HR = 0.88 (95% CI, 0.66–1.17; $P = 0.39$)

(continued)

Table 1.3 (continued)

Trial	Key eligible criteria	Arm (No. of pts)	Results		
			ORR	PFS (median)	OS (median)
LUX-lung 6 phase 3 [70, 71]	ΔLREA, L858R, or other *EGFR* mutations (11.0%)	CdG (n = 122)	23.0%	5.6 m	23.5 m
		Afatinib (n = 242)	66.9% (P < 0.0001)	11.0 m HR = 0.28 (95% CI, 0.20–0.39; P < 0.0001)	23.1 m HR = 0.93 (95% CI, 0.72–1.22; P = 0.61)
Gefitinib versus afatinib					
LUX-lung 7 phase 2B [72, 73]	ΔLREA or L858R	Gefitinib (n = 159)	56%	10.9 m	24.5 m
		Afatinib (n = 160)	70% (P = 0.0083)	11.0 m HR = 0.73 (95% CI, 0.57–0.95; P = 0.017)	27.9 m HR = 0.86 (95% CI, 0.66–1.12; P = 0.2580)

[a]Progression-free survival (PFS) as the primary endpoint in each trial, and overall survival (OS) and time-to-treatment failure as co-primary endpoints in the LUX-Lung7 trial
EGFR-TKI tyrosine kinase inhibitor of epidermal growth factor receptor *ORR* objective response rate, *PFS* progression-free survival, *OS* overall survival, *HR* hazard ratio, *CI* confidence interval, *ΔLREA* deletion mutations in exon 19 of EGFR gene (affecting elimination of the conserved amino-acid sequences of "leucine (L) at 747, arginine (R) at 748, glutamic acid (E) at 749 alanine (A) at codon 750"), *L858R* point mutation in exon 21 of EGFR gene (affecting a "leucine (L) to arginine (R)" substitution at codon 858), *CbP* carboplatin plus paclitaxel, *CdDOC* cisplatin plus docetaxel, *CbDOC* carboplatin plus docetaxel, *CdG* cisplatin plus gemcitabine, *CbG* carboplatin plus gemcitabine, *CdPEM* cisplatin plus pemetrexed

second mutations in the EGFR gene, (2) activation of downstream signalings or bypass tracks including MET pathway, and (3) phenotype changes including epithelial-mesenchymal transition (EMT) or change to SCLC [78–81] (Fig. 1.3). A second mutation (a single base-pair change from cytosine to thymidine [C to T] at position 2368) in exon 20, affecting a "threonine (T) to methionine (M)" at codon 790 of EGFR (T790M), is most common, accounting for 50–60% of acquired resistance to "first-generation" EGFR-TKIs [78–80]. The threonine at codon 790 (T790) is so-called gatekeeper, as it is located at the entrance to a hydrophorbic pocket in the back of ATP-binding cleft in the TK domain of EGFR [42] (Fig. 1.2a). As the position is a key determinant of affinity and specificity of inhibitors, the substitution of a larger amino acid (M) for a small amino acid (T) may cause an increased affinity of ATP as well as a decreased affinity of first-generation EGFR-TKIs to the ATP-binding cleft, leading to acquired resistance [78, 82–84] (Fig. 1.3). Acquired resistance caused by T790M can be overcome by more potent inhibitors that covalently and irreversibly bind to TK domain, and experimental studies have shown that afatinib is effective for tumors carrying T790M in addition to L858R [85]. In clinical studies, however, afatinib has provided only a modest clinical benefit in

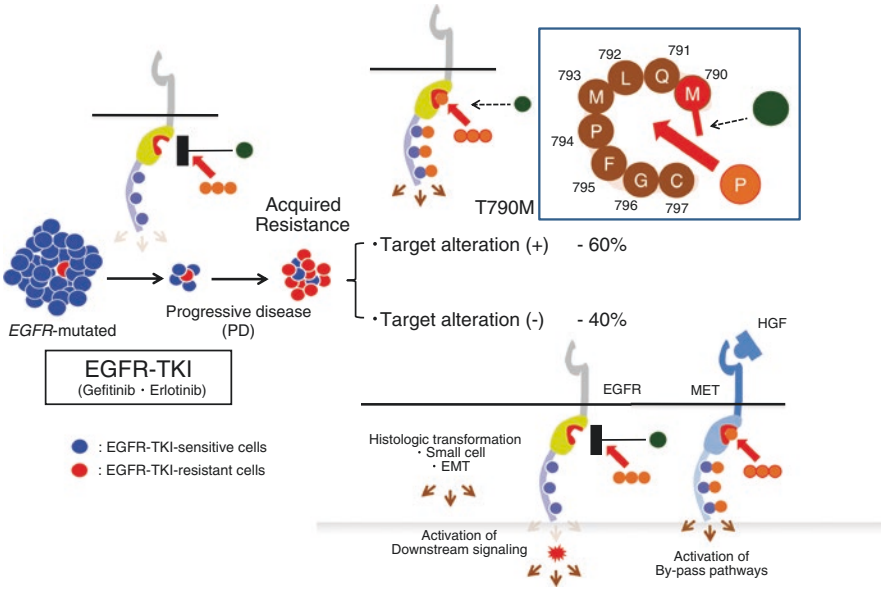

Fig. 1.3 Mechanisms of "acquired resistance" for tyrosine kinase inhibitors of epidermal growth factor (EGFR-TKIs). Target alteration by a resistant mutation in exon 20 affecting a "threonine (T) to methionine (M)" substitution at codon 790 (T790M) accounts for approximately 50–60% of acquired resistance following treatment with first-generation EGFR-TKIs

patients who progressed after treatment with either gefitinib or erlotinib (ORR, 5.9–7%) [86, 87], whereas a phase 2b "head-to-head" trial (LUX-Lung7) comparing afatinib with gefitinib as first-line treatment has shown a superior PFS with afatinib (Table 1.3) [72, 73]. Afatinib may cause increased toxicity by inhibiting both mutated and wild-type EGFRs, which limits the use of escalated afatinib doses that are enough to overcome T790M in clinical setting.

Accordingly, "third-generation" EGFR-TKIs that are designed to overcome T790M through mutant-selective inhibition have been developed [88–91]. Osimertinib (AZD9291) is the only approved agent for treatment of patients with T790M-positive NSCLC who progressed after treatment with EGFR-TKI (gefitinib, erlotinib, or afatinib) [92]. In preclinical models, osimertinib potently inhibits tyrosine kinase of mutated EGFR (ΔLREA or L858R), with or without T790M, through covalent binding to C797 (Fig. 1.2d), but shows only limited inhibitory effects for wild-type EGFR [93]. Osimertinib, as expected form its mutant-selective profile of EGFR inhibition, showed a significant clinical benefit (ORR, 61–70%; median PFS, 9.6–9.9 months) with a favorable safety profile in early clinical trials conducted for T790M-positive NSCLC [94, 95]. A phase 3 trial (AURA3) comparing osimertinib with chemotherapy (pemetrexed plus either cisplatin or carboplatin) for T790M-positive, *EGFR*-mutated NSCLC patients who progressed after first-line

EGFR-TKI therapy has confirmed a greater efficacy with osimertinib, showing a significantly longer PFS (median PFS, 10.1 months with osimertinib versus 4.4 months with chemotherapy; HR, 0.30; 95% confidence interval [CI], 0.23–0.41; $P < 0.001$), a higher ORR (71% versus 31%; odds ratio, 5.39; 95% CI, 3.47–8.48; $P < 0.001$), and a lower incidence of adverse events of grade 3 or higher (23% versus 47%) [96]. Based on these results, at the time of progression after first-line treatment with an EGFR-TKI (either gefitinib, erlotinib, or afatinib), re-biopsy to explore the mechanism responsible for acquired resistance is recommended for decision-making of subsequent treatment; osimertinib is recommended if T790M is positive, and platinum-doublet chemotherapy is recommended if T790M is negative [15, 81, 97–99].

1.1.2.2 ALK Rearrangements and ALK-TKIs

ALK is a RTK which is thought to play a role in the development of nervous system and exert its effects in specific neurons [100]. In the adult human, ALK expression is restricted to the central nervous system and is not detected in other normal tissues [101]. The ALK gene, located on the chromosome 2p23 region, encodes, may be oncogenic through generation of fusion to another gene as a result of chromosomal translocation. The first identified "oncogenic" fusion gene was *NPM1-ALK* causing anaplastic large cell lymphoma [102]. The most common fusion gene found in NSCLC is *EML4-ALK*, which is generated as a result of a small inversion between *ALK* and *EML4* located on the same chromosome (2p21). In its product (EML4-ALK) comprising the amino-terminal portion of EML4 fused to the kinase domain of ALK, tyrosine kinase is constitutively activated, leading to oncogenic transformation (Fig. 1.4) [103]. Since the discovery of *EML4-ALK*, several rearranged *ALK* fusion genes such as *KIF5B-ALK* [104] and *KCL1-ALK* [105] have been identified in NSCLC.

The diagnosis of *ALK* rearrangements is clinically important, because ALK-TKIs are effective only in *ALK*-rearranged (*ALK*+) tumor which accounts for 3–6% of NSCLC. Unlike *EGFR* mutations for which detection assays with gene amplification by polymerase chain reaction (PCR) are usually employed, *ALK* rearrangements may be diagnosed with several assays including fluorescence in situ hybridization (FISH), reverse-transcription PCR (RT-PCR), and immunohistochemistry (IHC) (Fig. 1.4) [15, 106]. The current "gold standard" assay for selection of patients for ALK-TKIs is the FISH assay to detect the chromosomal "break" within *ALK* locus on 2p23, as it can detect all types of *ALK* fusions and is validated in several clinical trials of ALK-TKIs. However, the FISH assay may not be feasible in routine clinical practice, especially in screening, due to a higher cost and its need for technical expertise in sample preparation as well as in evaluation of the results [15, 106]. Alternatively, IHC may be useful as a rapid and relatively inexpensive method for screening, which is applicable for routine formalin-fixed

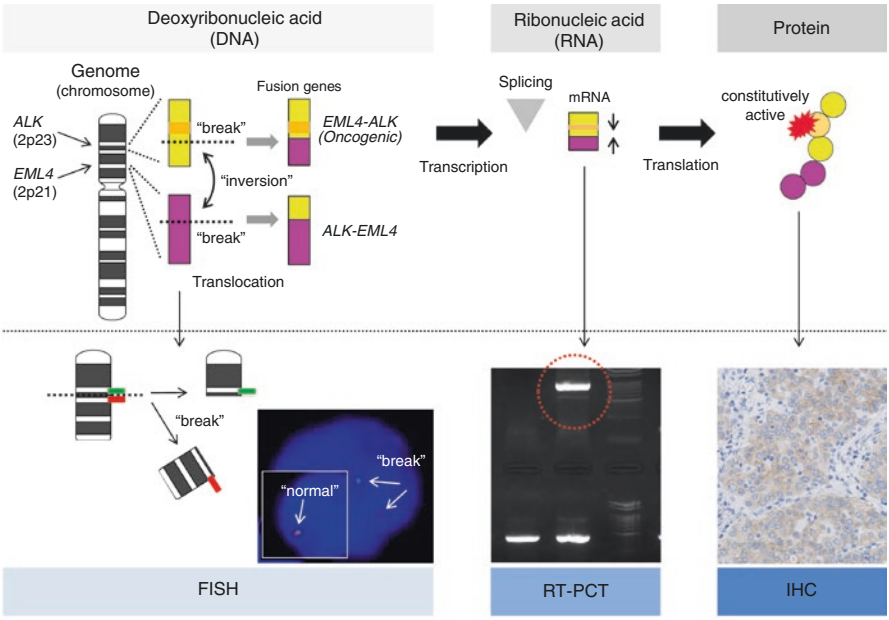

Fig. 1.4 Rearrangements of anaplastic lymphoma kinase (ALK) gene by chromosomal translocation may generate oncogenic fusion genes such as *EML4-ALK*. *ALK* rearrangement can be diagnosed with fluorescence in situ hybridization (FISH) to detect the chromosomal "break" within *ALK* locus on 2p23, reverse-transcription polymerase chain reaction (RT-PCR) to detect fusion genes, and immunohistochemistry (IHC) to detect expression of ALK that is not expressed in normal lung tissues

paraffin-embedded (FFPE) tissues [104]. A commonly employed anti-ALK antibody (D5F3 or 5A4) reacts with not only rearranged ALK but also wild-type ALK. However, as ALK is not expressed in normal lung tissues, the presence of ALK expression strongly suggests the presence of rearranged ALK. In fact, several studies have revealed high concordance rates, over 90%, between a positive IHC result and a positive FISH result. Accordingly, ALK-IHC may be recommended in screening for patients with *ALK* + NSCLC who are eligible for treatment with ALK-TKIs [15]. RT-PCR is the most sensitive method to detect *ALK* fusions [107], but the routine clinical use is challenging because of its difficulty in applying for FFPE tissues. In addition, only known *ALK* fusions, for which specific primers shall be prepared, can be amplified and detected [15, 106].

Crizotinib is the first approved ALK-TKI, which had been originally developed as a TKI of MET and subsequently was found to inhibit ALK and ROS1 [108]. Early clinical trials have indicated a promising clinical activity for *ALK* + NSCLC (ORR, approximately 60%) [109, 110]. Subsequent two phase 3 trials comparing crizotinib with chemotherapy, one (PROFLILE1014) in first-line setting and the other (PROFILE1007) in the second-line setting, have proved superior clinical

benefits of crizotinib over chemotherapy (Table 1.4) [111, 112], and crizotinib is recommend for *ALK* + NSCLC [16, 77, 114].

Despite initial dramatic response to crizotinib, most patients develop disease progression within 1 year after initiation of treatment, and a variety of mechanisms of acquired resistance including target alterations by second *ALK* mutations have been identified [80, 114–117]. Unlike acquired resistance to EGFR-TKIs, the "gatekeeper" mutation, L1196M, comprises only a small fraction (up to 10%) of mechanisms of resistance to crizotinib [80, 114–117]. Diverse and heterogeneous mechanisms including *ALK* amplification and various mutations other than L1196 may be an important issue in treating patients who progressed after treatment with crizotinib. To overcome acquired resistance to crizotinib, a number of "new-

Table 1.4 Key randomized clinical trials of ALK-TKIs for advanced non-small cell lung cancer (NSCLC) with *ALK* rearrangement (*ALK*+)

Trial	Key eligible criteria	Arm	Results		
			ORR	PFS (median)	OS (median)
Crizotinib versus chemotherapy					
PROFILE1007 phase 3 [111]	*ALK* + NSCLC, previously untreated	CdPem or CdPem (n = 171)	45%	7.0 m	NR
		Crizotinib (n = 172)	75% (P < 0.001)	10.9 m HR = 0.45 (95% CI, 0.35–0.60; P < 0.001)	NR HR = 0.82 (95% CI, 0.54–1.26; P = 0.36)
PROFILE1014 phase 3 [112]	*ALK* + NSCLC, PD after one platinum-based chemotherapy	DOC or Pem (n = 174)	20%	3.0 m	22.8 m
		Crizotinib (n = 173)	65% (P < 0.001)	7.7 m HR = 0.49 (95% CI, 0.37–0.64; P < 0.001)	20.3 m HR = 1.02 (95% CI, 0.68–1.54; P = 0.54)
Crizotinib versus *Alectinib*					
J-ALEX phase 3 [113]	*ALK* + NSCLC, previously untreated	Crizotinib (n = 104)	70.2%	10.2 m	Not reported
		Alectinib (n = 103)	85.4% (P < 0.0001)	NR HR = 0.34 (95% CI, 0.17–0.71; P < 0.0001)	Not reported

[a]Progression-free survival (PFS) as primary endpoint of each trial

ALK-TKI tyrosine kinase inhibitor of anaplastic lymphoma kinase (ALK), *ORR* objective response rate, *PFS* progression-free survival, *OS* overall survival, *HR* hazard ratio, *CI* confidence interval, *NR* not reached for estimation of median survival time, *CdPem* cisplatin plus pemetrexed, *CbPem* carboplatin plus pemetrexed, *DOC* docetaxel, *Pem* pemetrexed

generation" ALK-TKIs such as ceritinib [118–120], alectinib [121, 122], and brigatinib [123] have been developed and showed promising activities (ORR, 38–71%) in clinical trials. Based on these results, ceritinib and alectinib are approved for treatment of *ALK* + NSCLC patients who progressed after crizotinib treatment. Alectinib is also active for patients with crizotinib-naïve *ALK* + NSCLC in early clinical studies [124], and a recent phase 3 trial (J-ALEX) comparing alectinib with crizotinib as a first-line treatment showed a significantly better PFS and a more favorable toxicity profile with alectinib (Table 1.4) [113], suggesting alectinib is a new standard first-line treatment option for *ALK* + NSCLC patients.

1.1.2.3 Other Oncogenic Alterations and Targeting Agents

ROS1 rearrangement is a rare oncogenic alteration seen in NSCLC, predominantly in adenocarcinoma developing in never-to-light smokers like *ALK* rearrangement [125] (Fig. 1.1). As crizotinib inhibits tyrosine kinase of ROS1 as well as that of ALK in preclinical models, clinical studies have been conducted and have shown a robust efficacy for *ROS1*-rearranged NSCLC (ORR, 72–80%; median PFS, 9.1–19.2 months) [16, 126].

Other promising agents targeting oncogenic alterations in NSCLC are vandetanib for *RET*-rearranged tumor [127], dabrafenib for *BRAF*-mutated tumor [128, 129] and and *MET* exon 14 skipping mutated tumor [130, 131].

KRAS mutations are the most common "driver" alteration in Western NSCLC patients (Fig. 1.1) [11], which account in approximately 30% of adenocarcinoma of the lung [11–13, 132]. Unlike *EGFR* mutations, *KRAS* mutations are not frequently found in East Asian patients and are more commonly seen in smokers [13]. Unfortunately, no effective agent or treatment strategy has been established for *RAS*-mutated NSCLC [13].

1.1.3 Immune Checkpoints and Inhibitors

Cancer immunotherapy is the use of the immune system to treat cancer. Traditional immunotherapy is principally aimed to activate immune cells such as cytotoxic T-cells (CTLs), but has failed to provide a significant clinical benefit in most malignant tumors. However, novel strategies to inhibit negative regulators ("immune checkpoints") of cancer immunity have changed the "tide" after tremendous success of recent clinical trials for multiple tumors including NSCLC [133–137].

Cancer immunity comprises a series of steps ("cancer-immunity cycle") as follows [134]: (1) release of neoantigens from cancer cells and capture of neoantigens by antigen-presenting cells (APCs) such as dendritic cells (DCs), (2) presentation of processed fragments of neoantigens (peptides) on major histocompatibility complex (MHC) class I and class II molecules by APCs to T-cells, (3)

priming and activation of effector T-cells that respond against cancer-specific neoantigens (cytotoxic T-cells, CTLs), (4) trafficking CTLs to tumors through blood vessels, (5) infiltration of CTLs to tumors, (6) recognition and binding to cancer cells by CTLs through interaction between T-cell receptor (TCR) on CTLs and its cognate neoantigen on MHC class I molecules, and (7) killing of target cancer cells. The "cancer-immunity cycle" is controlled not only by positive regulators to enhance immunity but also by negative regulators ("immune checkpoints") to prevent overactivation of immunity. Cancer cells may evade from immune attack by utilizing immune checkpoints to survive and proliferate, and the inhibition has emerged a new strategy of cancer immunotherapy [133, 134]. Among various immune checkpoint molecules, major targets in today's cancer immunotherapy are cytotoxic T-lymphocyte-associated protein 4 (CTLA-4, also known as CD152) that acts primarily at the step of priming and activation of T-cells and programmed death protein 1 (PD-1, also known as CD279) that mainly acts at later step and inhibits immune attack by CTLs (Fig. 1.5) [134–137]. In lung cancer, several antibodies inhibiting PD-1 by blocking PD-1 or its ligand (PD-L1) have been evaluated in clinical trials, and some are currently available in clinical practice (Table 1.1) [136, 137].

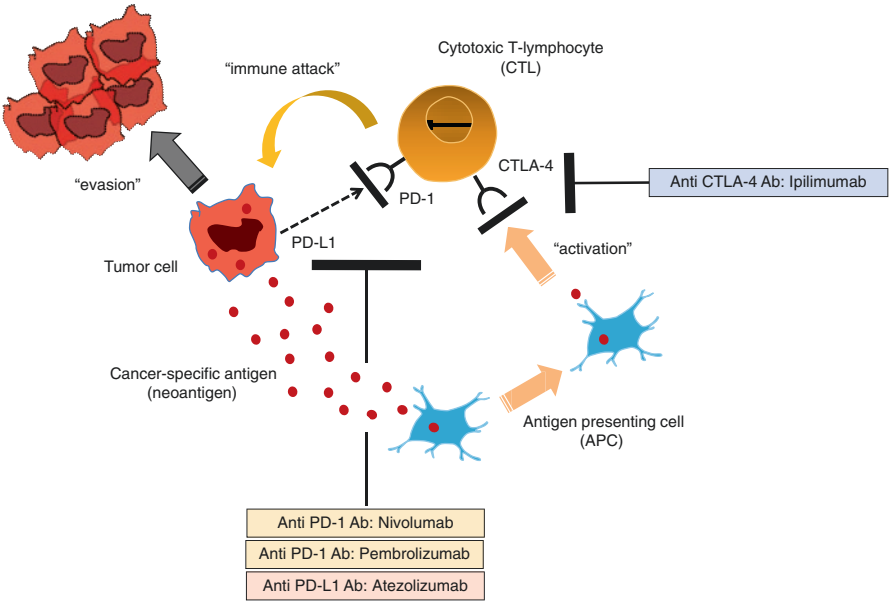

Fig. 1.5 Cancer immunity comprising several steps including activation of cytotoxic T-cells (CTLs) by antigen-presenting cells (APCs) and killing tumor cells by CTLs. Immune checkpoint molecules such as cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and programmed death protein 1 (PD-1) negatively regulate cancer immunity. Tumor cells may evade from immune attack by expressing a ligand of PD-1 (PD-L1). Blockade of immune checkpoint molecules may be a promising strategy for treating cancer

1.1.3.1 Immune Checkpoint Inhibitors for NSCLC

Nivolumab is the first antibody against PD-1 that is approved for treatment of NSCLC. In early clinical studies, nivolumab has showed durable antitumor activities for previously treated NSCLC [137–139]. Two phase 3 trials comparing nivolumab with chemotherapy (DOC) for previously treated NSCLC, one (CheckMate017) for squamous NSCLC and the other (CheckMate057) for non-squamous NSCLC, showed a significantly better overall survival (OS) with nivolumab (Table 1.5) [143, 144].

As cancer cells may evade from immune attack by CTLs through expressing PD-L1, PD-L1 expression on tumor cells is a potentially promising biomarker to predict the efficacy of antibodies against PD-1/PD-L1 [147, 148]. The CheckMate057 indicated a significant correlation between clinical benefits with nivolumab and PD-L1 expression evaluated with IHC using an anti-PD-L1 antibody (clone 28–8); ORR with nivolumab was significantly higher in tumor-expressing PD-L1 (Fig. 1.6), and a significantly longer PFS or OS with nivolumab was documented in PD-L1-positive tumor but not in PD-L1-negative tumor [144]. In contrast, in the CheckMate017, nivolumab was associated with significantly better clinical benefits regardless of PD-L1 expression status (Fig. 1.6) [143]. These results suggest that the correlation between PD-L1 expression and antitumor activity of nivolumab may differ between squamous NSCLC and non-squamous NSCLC.

Pembrolizumab is another approved antibody against PD-1. In a phase 1 trial (KEYNOTE001) for NSCLC, correlations between clinical benefits with pembrolizumab and PD-L1 expression status have been evaluated; PD-L1 expression was assessed with IHC using an anti-PD-L1 antibody (clone 22C3) and is classified according to the proportion of tumor cells expressing PD-L1 (proportion score, PS). As a result, clinical benefits with pembrolizumab were enhanced along with increased PD-L1 expression (ORR, 10.7% in PS <1%, 16.5% in PS 1–49%, and 45.2% in PS ≥ 50%; median PFS, 4.0 months, 4.1 months, 6.4 months, respectively; median OS, 10.4 months, 10.6 months, not reached for evaluation, respectively) [149]. In a subsequent phase 2/3 trial (KEYNOTE010) comparing pembrolizumab at two doses (2 mg/kg or 10 mg/kg) with chemotherapy (DOC) for previously treated NSCLC (KEYNOTE010), patients are eligible only when PD-L1 expression was positive (PS ≥ 1%) [142]. Pembrolizumab at either dose has provided superior clinical benefits including a significant prolongation of OS (median OS, 8.5 months with DOC, 10.4 months with pembrolozimab [2 mg/kg], and 12.7 months with pembrolizumab [10 mg/kg]) as well as a significant increase in ORR (ORR, 9.3% with DOC, 18.0% with pembrolozimab [2 mg/kg], and 18.5% with pembrolizumab [10 mg/kg) (Table 1.5). The survival benefits are enhanced for cases with strong PD-L1 expression as defined PS ≥ 50% (median OS, 8.2 months with DOC, 14.9 months with pembrolozimab [2 mg/kg], and 17.3 months with pembrolizumab [10 mg/kg]), and ORR at each dose of pembrolizumab is higher in cases with strong PD-L1 expression (Fig. 1.6) [142].

Recently, a phase 3 trial (KEYNOTE024) comparing pembrolizumab with platinum-doublet chemotherapy for previously untreated NSCLC with strong

Table 1.5 Key randomized clinical trials comparing immune checkpoint inhibitors with chemotherapy for advanced non-small cell lung cancer (NSCLC)

Trial	Key eligible criteria	Arm	Results		
			ORR	PFS (median)	OS (median)
First-line					
KEYNOTE024 phase 3 [140]	NSCLC, PD-L1 expression on at least 50% of tumor cells, without *EGFR* mutations nor *ALK* rearrangements	Platinum-doublet regimen[a] (n = 151)	27.8%	6.0 m	NR (72.4% at 6 m)
		Pembrolizumab (n = 154)	44.8%	10.3 m HR = 0.50 (95% CI, 0.37–0.68; $P < 0.001$)[c]	NR (80.2% at 6 m) HR = 0.60 (95% CI, 0.41–0.89; $P = 0.005$)
CheckMate026 phase 3 [141]	NSCLC, PD-L1 expression on at least 1% of tumor cells (efficacy, evaluated in only NSCLC, PD-L1 expressing at least 5% of tumor cells), without *EGFR* mutations nor *ALK* rearrangements	Platinum-doublet regimen[b] (n = 212)	33.5%	5.9 m	13.2 m
		Nivolumab (n = 211)	26.1%	4.2 m HR = 1.15 (95% CI, 0.91–1.45; $P = 0.2511$)[c]	14.4 m HR = 1.02 (0.80–1.30)
Second-line or more					
KEYNOTE010 phase 2/3 [142]	NSCLC, previously treated and PD-L1 expression on at least 1% of tumor cells	DOC (n = 343)	9.3%	4.0 m	8.5 m
		Pembrolizumab (2 mg/kg) (n = 344)	18.0% ($P = 0.0005$)	3.9 m HR = 0.88 (95% CI, 0.71–1.05; $P = 0.07$)[c]	10.4 m HR = 0.71 (95% CI, 0.58–0.88; $P = 0.0008$)[c]
		Pembrolizumab (10 mg/kg) (n = 346)	18.5% ($P = 0.0002$)	4.0 m HR = 0.79 (95% CI, 0.66–0.94; $P = 0.004$)[c]	12.7 m HR = 0.61 (95% CI, 0.49–0.75; $P < 0.0001$)[c]

CheckMate017 phase 3 [143]	Squamous NSCLC, PD after one platinum-based chemotherapy	DOC (n = 137)	9%	2.8 m	6.0 m
		Nivolumab (n = 135)	20% (P = 0.008)	3.5 m HR = 0.62 (95% CI, 0.47–0.81; P < 0.001)	9.2 m HR = 0.59 (95% CI, 0.44–0.79; P < 0.001)[c]
CheckMate057 phase 3 [144]	Non-squamous NSCLC, PD after one platinum-based chemotherapy	DOC (n = 290)	12%	4.2 m	9.4 m
		Nivolumab (n = 292)	19% (P = 0.002)	2.3 m HR = 0.92 (95% CI, 0.77–1.11; P = 0.39)	12.2 m HR = 0.73 (95% CI, 0.59–0.89; P = 0.002)[c]
POPLAR phase 2 [145]	NSCLC, PD after one or two chemotherapy regimens including one platinum-based chemotherapy	DOC (n = 143)	14.7%	3.0 m	9.7 m
		Atezolizumab (n = 144)	14.6%	2.7 m HR = 0.94 (95% CI, 0.72–1.23; P = 0.645)	12.6 m HR = 0.73 (95% CI, 0·53–0·99; P = 0·040)[c]
OAK phase 3 [146]	NSCLC, PD after one or two chemotherapy regimens including one platinum-based chemotherapy	DOC (n = 425)	13.4%	4.0 m	9.6 m
		Atezolizumab (n = 425)	13.6%	2.8 m HR = 0.95 (95% CI, 0.82–1.10; P = 0.4928)	13.8 m HR = 0·73 (95% CI, 0.62–0.87; P = 0·0003)[c]

[a]Investigator's choice from one of the following five regimens: carboplatin plus pemetrexed, cisplatin plus pemetrexed, carboplatin plus gemcitabine, cisplatin plus gemcitabine, or carboplatin plus paclitaxel

[b]Investigator's choice from one of the following regimens: carboplatin plus gemcitabine, cisplatin plus gemcitabine, or carboplatin plus paclitaxel for squamous NSCLC; carboplatin plus pemetrexed or cisplatin plus pemetrexed for non-squamous NSCLC

[c]Indicates the primary endpoint of each trial; progression-free survival (PFS) and overall survival (OS) are co-primary endpoints of the KEYNOTE010 trial

ORR objective response rate, *PFS* progression-free survival, *OS* overall survival, *HR* hazard ratio, *CI* confidence interval, *NR* not reached for estimation of median survival time, *PD-L1* programmed death-ligand 1, *EGFR* epidermal growth factor receptor, *ALK* anaplastic lymphoma kinase, *DOC* docetaxel, *PD* progressive disease

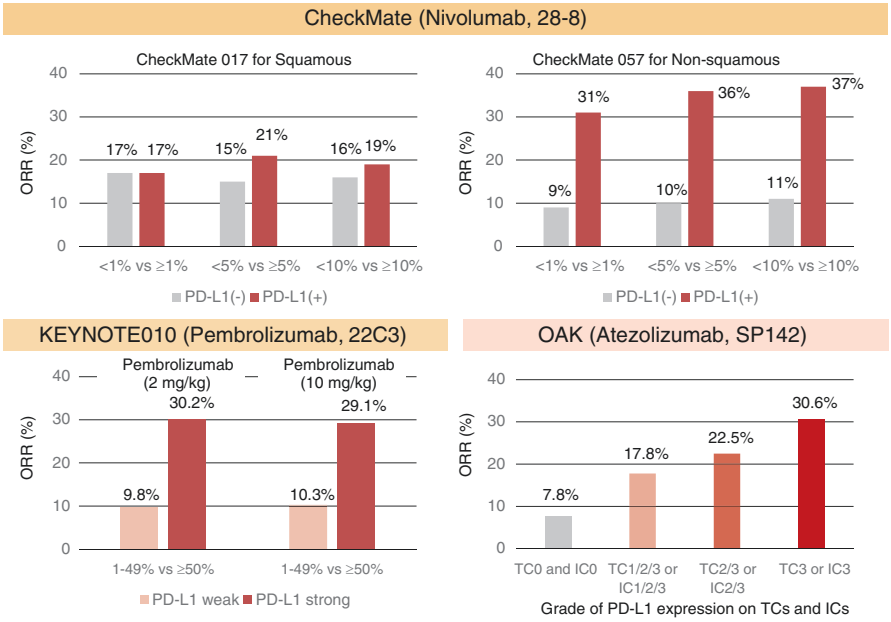

Fig. 1.6 Correlation between overall response rates (ORRs) with antibodies against PD-1/PD-L1 according to immunohistochemically evaluated PD-L1 expression status in clinical trials. Different antibodies ("28-8" in CheckMate trials, "22C3" in KEYNOTE010 trial, and "SP142" in OAK trial) and different cut-off values as percentages of tumor cells expressing PD-L1 (1/5/10% in CheckMate trials, 1/50% in KEYNOTE010 trial, 1%/5%/50%, and 1/550% in OAK trial) are employed to evaluate PD-L1 expression status. In the OAK trial, PD-L1 status on tumor-infiltrating immune cells (ICs) as well as that on tumor cells (TCs) is evaluated and scored as follows: IC0/1/2/3 for PD-L1 expression on ICs and TC0/1/2/3 for PD-L1 expression on TCs

PD-L1 expression (PS ≥ 50%) has shown a significantly longer PFS and OS and a more favorable toxicity profile with pembrolizumab (Table 1.5) [140], indicating that pembrolizumab is a new standard of care as first-line treatment for advanced NSCLC without *EGFR* mutations nor *ALK* rearrangements and with strong PD-L1 expression. In addition, a recent phase 2 study (KEYNOTE021) has shown that pembrolizumab plus platinum-doublet (carboplatin plus pemetrexed) can be a promising first-line treatment option for non-squamous NSCLC regardless of PD-L1 expression status (ORR, 57% in PS < 1% versus 54% in PS ≥ 1%) [150]. In contrast, a phase 3 trial (CheckMate026) comparing nivolumab with platinum-doublet chemotherapy for previously untreated of NSCLC with positive PD-L1 expression (PS ≥ 5%), which is designed as with KEYNOTE024, has failed to show greater survival benefits with nivolumab in patients with strong PD-L1 expression (PS ≥ 50%) as well as in all patients (Table 1.5) [141].

Atezolizumab is the only agent targeting PD-L1 approved for treatment of NSCLC (only by FDA). Early clinical studies have suggested that clinical benefits with

atezolizumab are significantly correlated not only with PD-L1 expression on tumor cells but also with that on tumor-infiltrating immune cells. In these studies, PD-L1 expression is detected with IHC and is scored according to the percentage of PD-L1-expressing cells as follows: TC1/2/3 or IC1/2/3, defined as PD-L1 expression on 1% or more of tumor cells or tumor-infiltrating immune cells; TC2/3 or IC2/3, defined as PD-L1 expression on 5% of these cells; TC3, defined as PD-L1 expression on 50% or more of tumor cells; IC3, defined as 10% or more of tumor-infiltrating immune cells; TC0, defined as PD-L1 expression on less than 1% of tumor cells; IC0, defined as PD-L1 expression on less than 1% of tumor-infiltrating immune cells [145, 151]. A randomized phase 2 trial (POPLAR) comparing atezolizumab with DOC for previously treated NSCLC has shown a significantly better OS with atezolizumab (HR, 0.73; $P = 0.04$) (Table 1.5) The survival benefit with atezolizumab is enhanced in patients with higher PD-L1 expression on either TCs or ICs, but has disappeared in patients with no PD-L1 expression on neither TCs nor ICs (HR, 0.49 in TC3 or IC3; 0.54 in TC2/3 or IC2/3; 0.59 in TC1/2/3 or IC1/2/3; 1.04 in TC0 and IC0). Similarly, an increased ORR is documented when PD-L1 expression on either TCs or ICs is stronger (ORR, 37.5% in TC3 or IC3; 22.0% in TC2/3 or IC2/3; 18.3% in TC1/2/3 or IC1/2/3; 7.8% in TC0 and IC0) [145]. A subsequent phase 3 trial (OAK) confirmed a significantly longer OS with atezolizumab for previously treated NSCLC (Table 1.5). In the phase 3 trial, a superior survival benefit is seen across all patients regardless of PD-L1 expression status (HR, 0.41 in TC3 or IC3; 0.67 in TC2/3 or IC2/3; 0.74 in TC1/2/3 or IC1/2/3; 0.75 in TC0 and IC0), whereas a higher ORR is documented in patients with stronger PD-L1 expression (Fig. 1.6) [146].

PD-L1 expression status on tumor cells is a potentially important predictive marker in selecting patients who will benefit from antibodies against PD-1 or PD-L1. In fact, PD-L1 status, as assesses with IHC using the antibody, 22C3, is approved as the companion diagnostic for the use of pembrolizumab in NSCLC. However, PD-1/PD-L1 inhibitors such as nivolumab and atezolizumab may provide superior clinical benefits over cytotoxic chemotherapy even for PD-L1-negative NSCLC patients. These controversies may indicate several issues in current IHC assays to evaluate PD-L1 expression as follows: (1) different antibodies employed for IHC (22C3 in trials with pembrolizumab, 22-8 in trials with nivolumab, SP142 in trials with atezolizumab, SP263 in trials with durvalumab that is another anti-PD-L1 antibody, evaluated in clinical trials), (2) different cut-off values, and (3) different PD-L1 expressing cells evaluated (only tumor cells in trials with nivolumab and pembrolizumab, both tumor cells and tumor-infiltrating immune cells in trials with atezolizumab) [147, 148]. The "Blueprint PD-L1 IHC Assay Comparison Project" has been conducted to compare different antibodies employed for IHC, and early results have showed that the percentage of PD-L1-positive tumor cells is comparable with 22C2, 28-8, and SP263 assays but is lower with SP142 assay [152]. In addition to PD-L1, other factors involved in "cancer immunity" should be examined in future studies to more precisely select patients who will benefit from PD-1/PD-L1 inhibitors.

1.1.3.2 Immune Checkpoint Inhibitors for SCLC

Despite recent advances in targeting agents, no novel treatment option has been added to the current standard chemotherapy of platinum-doublet regimens (platinum agent plus either etoposide or irinotecan for treatment of advanced SCLC [3, 4]. Immune checkpoint blockade may be promising strategies to improve treatment outcomes in SCLC as well as in NSCLC [153]. For example, a phase 2 study has shown that the median PFS with an anti-CTLA-4 antibody, ipilimumab, in combination with cytotoxic chemotherapy (CbP) is significantly longer than that with chemotherapy alone [154]. However, a large-scale placebo-controlled phase 3 trial has failed to show a significant improvement of OS with the addition of ipilimumab to chemotherapy (etoposide plus either cisplatin or carboplatin; median OS, 11.0 months versus 10.9 months; HR, 0.94; $P = 0.3775$) [155]. These results may suggest that rapid tumor growth with corresponding symptomatic disease and performance status decline may lead to patient drop-off as a poor drug tolerability or disease progression. Accordingly, more potent regimens such as double blockade of PD-1/PD-L1 and CTLA-4 may be necessary to achieve more durable clinical benefits. In fact, a phase 1/2 trial has indicated that dual CTLA-4 and PD-1 blockade with the combination of ipilimumab and nivolumab may be promising [156].

1.2 Conclusions and Future Perspectives

Recent advances in understanding molecular characteristics and developing agents targeting "cancer hallmarks" have changed treatment strategies for advanced lung cancer which had been uniformly treated with cytotoxic agents including platinum agents. Today, lung cancer is classified according to not only histologic subtypes but also molecular characteristics including oncogenic alterations (*EGFR* mutations and *ALK-/ROS1* rearrangement) and PD-L1 status and shall be "precisely" treated with specific agents. Accordingly, tissue biopsy and molecular diagnosis are mandatory to achieve "precision" medicine, but tissue biopsy may be associated with some limitations such as some risk and pain for patients and difficulty in accessibility, especially in case of re-biopsy. Alternatively, "liquid biopsy," detection, and analyses of either tumor cells that are shed from primary tumor and circulate in the peripheral blood (circulating tumor cells, CTCs) or deoxynucleotide (DNA) fragments derived from tumor cells (cell-free tumor DNA, ctDNA) may be promising as a noninvasive and useful assay to reveal molecular characteristics of tumor [157–159]. Based on accumulating evidence to support the use of ctDNA for detection of *EGFR* mutations, plasma testing for *EGFR* mutations (sensitizing mutations for the use of erlotinib, only by FDA; T790M for the use of osimertinib) has been recently approved for clinical use, which may provide new insights toward personalized "precision" medicine.

References

1. Herbst RS, Heymach JV, Lippman SM. Lung cancer. N Engl J Med. 2008;359:1367–80.
2. Siegel RL, Miller KD, Jemal A. Cancer statistics 2016. CA Cancer J Clin. 2016;6:7–30.
3. Rudin CM, Ismaila N, Hann CL, et al. Treatment of small-cell lung cancer: american society of clinical oncology endorsement of the American college of chest physicians guideline. J Clin Oncol. 2015;33:4106–11.
4. Bunn PA Jr, Minna JD, Augustyn A, et al. Small cell lung cancer: can recent advances in biology and molecular biology be translated into improved outcomes? J Thorac Oncol. 2016;11:453–4748.
5. Schiller JH, Harrington D, Belani CP, et al. Comparison of four chemotherapy regimens for advanced non-small-cell lung cancer. N Engl J Med. 2002;10(346):92–8.
6. Ohe Y, Ohashi Y, Kubota K, et al. Randomized phase III study of cisplatin plus irinotecan versus carboplatin plus paclitaxel, cisplatin plus gemcitabine, and cisplatin plus vinorelbine for advanced non-small-cell lung cancer: four-arm cooperative study in Japan. Ann Oncol. 2007;18:317–23.
7. Non-Small Cell Lung Cancer Collaborative Group. Chemotherapy and supportive care versus supportive care alone for advanced non-small cell lung cancer. Cochrane Database Syst Rev. 2010;12(5):CD007309.
8. Hanahan D, Weingberg RA. Hallmarks of cancer: the next generation. Cell. 2011;144:646–74.
9. Kohno T, Ichikawa H, Totoki Y, et al. KIF5B-RET fusions in lung adenocarcinoma. Nat Med. 2012;18:375–7.
10. Takeuchi K, Soda M, Togashi Y, et al. RET, ROS1 and ALK fusions in lung cancer. Nat Med. 2012;18:378–81.
11. Barlesi F, Mazieres J, Merlio JP, et al. Routine molecular profiling of patients with advanced non-small-cell lung cancer: results of a 1-year nationwide programme of the French cooperative thoracic intergroup (IFCT). Lancet. 2016;387:1415–26.
12. Li T, Kung HJ, Mack PC, Gandara DR. Genotyping and genomic profiling of non-small cell lung cancer: implications for current and future therapies. J Clin Oncol. 2013;31:1039–49.
13. Tsao AS, Scagliotti GV, Bunn PA Jr, et al. Scientific advances in lung cancer 2015. J Thorac Oncol. 2016;11:613–38.
14. Kobayashi Y, Mitsudomi T. Not all epidermal growth factor receptor mutations in lung cancer are created equal: perspectives for individualized treatment strategy. Cancer Sci. 2016;107:1179–86.
15. Lindeman NI, Cagle PT, Beasley MB, et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. J Thorac Oncol. 2013;8:823–59.
16. Masters GA, Temin S, Azzoli CG, et al. American Society of Clinical Oncology clinical practice systemic therapy for stage IV non-small-cell lung cancer: American Society of Clinical Oncology clinical practice guideline update. J Clin Oncol. 2015;33:3488–515.
17. Folkman J. Clinical applications of research on angiogenesis. N Engl J Med. 1995;333:1757–63.
18. Hanahan D, Folkman J. Patterns and emerging mechanism of the angiogenic switch during tumorigenesis. Cell. 1996;86:353–64.
19. Bremnes RM, Camps C, Sirera R. Angiogenesis in non-small cell lung cancer: the prognostic impact of neoangiogenesis and the cytokines and bFGF in tumors and blood. Lung Cancer. 2006;51:143–58.
20. Horn L, Dahlberg SE, Sandler AB, et al. Phase II study of cisplatin plus etoposide and bevacizumab for previously untreated, extensive-stage small-cell lung cancer: eastern cooperative oncology group study E3501. J Clin Oncol. 2009;27:6006–11.
21. Spigel DR, Townley PM, Waterhouse DM, et al. Randomized phase II study of bevacizumab in combination with chemotherapy in previously untreated extensive-stage small-cell lung cancer: results from the SALUTE trial. J Clin Oncol. 2011;29:2215–22.

22. Ready NE, Dudek AZ, Pang HH, et al. Cisplatin, irinotecan, and bevacizumab for untreated extensive-stage small-cell lung cancer: CALGB 30306, a phase II study. J Clin Oncol. 2011;29:4436–41.
23. Pujol JL, Lavole A, Quoix E, et al. Randomized phase II-III study of bevacizumab in combination with chemotherapy in previously untreated extensive small-cell lung cancer: results from the IFCT-0802 trial. Ann Oncol. 2015;26:908–14.
24. Sandler A, Gray R, Perry MC, et al. Pacritaxel-carboplatin alone or with bevacizumab for non-small-cell lung cancer. N Engl J Med. 2006;355:2542–50.
25. Reck M, von Pawel J, Zatloukal P, et al. Phase III trial of cisplatin plus gemcitabine with either placebo or bevacizumab as first-line therapy for nonsquamous non-small-cell lung cancer: AVAil. J Clin Oncol. 2009;27:1227–34.
26. Reck M, von Pawel J, Zatloukal P, et al. Overall survival with cisplatin-gemcitabine and bevacizumab or placebo as first-line therapy for nonsquamous non-small-cell lung cancer: results from a randomised phase III trial (AVAiL). Ann Oncol. 2010;21:1804–9.
27. Zhou C, Wu YL, Chen G, et al. BEYOND: a randomized, double-blind, placebo-controlled, multicenter, phase III study of first-line carboplatin/paclitaxel plus bevacizumab or placebo in Chinese patients with advanced or recurrent nonsquamous non-small-cell lung cancer. J Clin Oncol. 2015;33:2197–204.
28. Gorbunova V, Kowalyszyn RD, Pikiel J, et al. Ramucirumab plus docetaxel versus placebo plus docetaxel for second-line treatment of stage IV non-small-cell lung cancer after disease progression on platinum-based therapy (REVEL): a multicentre, double-blind, randomised phase 3 trial. Lancet. 2014;384:665–73.
29. Reck M, Kaiser R, Mellemgaard A, et al. LUME-lung 1 study group. Docetaxel plus nintedanib versus docetaxel plus placebo in patients with previously treated non-small-cell lung cancer (LUME-lung 1): a phase 3, double-blind, randomised controlled trial. Lancet Oncol. 2014;15:143–55.
30. Johnson DH, Fehrenbacher L, Novotny WF, et al. Randomized phase II trial comparing bevacizumab plus carboplatin and paclitaxel with carboplatin and paclitaxel alone in previously untreated locally advanced or metastatic non-small-cell lung cancer. J Clin Oncol. 2004;22:2184–91.
31. Richeldi L, du Bois RM, Raghu G, et al. Efficacy and safety of nintedanib in idiopathic pulmonary fibrosis. N Engl J Med. 2014;370:2071–82.
32. Dowlati A, Gray R, Sandler AB, Schiller JH, Johnson DH. Cell adhesion molecules, vascular endothelial growth factor, and basic fibroblast growth factor in patients with non-small cell lung cancer treated with chemotherapy with or without bevacizumab - an eastern cooperative oncology group study. Clin Cancer Res. 2008;14:1407–12.
33. Lynch TJ, Bell DW, Sordella R, et al. Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. N Engl J Med. 2004;350:2129–39.
34. Paez JG, Jänne PA, Lee JC, et al. EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. Science. 2004;304:1497–500.
35. Saito M, Shiraishi K, Kunitoh H, Takenoshita S, Yokota J, Kohno T. Gene aberrations for precision medicine against lung adenocarcinoma. Cancer Sci. 2016;107:713–20.
36. Sonobe M, Manabe T, Wada H, Tanaka F. Mutations in the epidermal growth factor receptor gene are linked to smoking-independent, lung adenocarcinoma. Br J Cancer. 2005;93:355–63.
37. Sonobe M, Manabe T, Wada H, Tanaka F. Lung adenocarcinoma harboring mutations in the ERBB2 kinase domain. J Mol Diagn. 2006;8:351–6.
38. Rahman S, Kondo N, Yoneda K. Frequency of epidermal growth factor receptor mutations in Bangladeshi patients with adenocarcinoma of the lung. Int J Clin Oncol. 2014;19:45–9.
39. Schlessinger J. Ligand-induced, receptor-mediated dimerization and activation of EGF receptor. Cell. 2002;110:669–72.
40. Lemmon MA, Schlessinger J. Cell signaling by receptor tyrosine kinases. Cell. 2010;141:1117–34.

41. Lemmon MA, Schlessinger J, Ferguson KM. The EGFR family: not so prototypical receptor tyrosine kinases. Cold Spring Harb Perspect Biol. 2014;6:a020768. https://doi.org/10.1101/cshperspect.a020768.
42. Kovacs E, Zorn JA, Huang Y, Barros T, Kuriyan J. A structural perspective on the regulation of the epidermal growth factor receptor. Annu Rev Biochem. 2015;84:739–64.
43. Roskoski R Jr. ErbB/HER protein-kinases: structures and small molecule inhibitors. Pharmacol Res. 2014;87:42–59.
44. Blume-Jensen P, Hunter T. Oncogenic kinase signaling. Nature. 2001;411:355–65.
45. Yarden Y, Sliwkowski MX. Untangling the ErbB signalling network. Nat Rev Mol Cell Biol. 2001;2:127–37.
46. Kumar A, Perti ET, Halmos B, Boggon T. Structure and clinical relevance of the epidermal growth factor receptor in human cancer. J Clin Oncol. 2008;26:1742–51.
47. Pao W, Miller V, Zakowski M, et al. EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. Proc Natl Acad Sci U S A. 2004;101:13306–11.
48. Shigematsu H, Lin L, Takahashi T, et al. Clinical and biological features associated with epidermal growth factor receptor gene mutations in lung cancers. J Natl Cancer Inst. 2005;97:339–46.
49. Kosaka T, Yatabe Y, Endoh H, Kuwano H, Takahashi T, Mitsudomi T. Mutations of the epidermal growth factor receptor gene in lung cancer: biological and clinical implications. Cancer Res. 2004;64:8919–23.
50. Yun CH, Boggon TJ, Li Y, Woo MS, Greulich H, Meyerson M, et al. Structures of lung cancer-derived EGFR mutants and inhibitor complexes: mechanism of activation and insights into differential inhibitor sensitivity. Cancer Cell. 2007;11:217–27.
51. Ji H, Li D, Chen L, Shimamura T. The impact of human EGFR kinase domain mutations on lung tumorigenesis and in vivo sensitivity to EGFR-targeted therapies. Cancer Cell. 2006;9:485–95.
52. Sordella R, Bell DW, Haber DA, Settleman J. Gefitinib-sensitizing EGFR mutations in lung cancer activate anti-apoptotic pathways. Science. 2004;305:1163–7.
53. Shan Y, Eastwood MP, Zhang X. Oncogenic mutations counteract intrinsic disorder in the EGFR kinase and promote receptor dimerization. Cell. 2012;149:860–70.
54. Dearden S, Stevens J, Wu YL, Blowers D. Mutation incidence and coincidence in non small-cell lung cancer: meta-analyses by ethnicity and histology (mutMap). Ann Oncol. 2013;24:2371–6.
55. Midha A, Dearden S, McCormack R. EGFR mutation incidence in non-small-cell lung cancer of adenocarcinoma histology: a systematic review and global map by ethnicity (mutMapII). Am J Cancer Res. 2015;5:2892–911.
56. Chapman AM, Sun KY, Reustow P, Cowan DM, Madl AK. Lung cancer mutation profile of EGFR, ALK, and KRAS: meta-analysis and comparison of never and ever smokers. Lung Cancer. 2016;102:122–34.
57. Weinstein IB, Joe A. Oncogene addiction. Cancer Res. 2008;68:3077–80.
58. Carey KD, Garton AJ, Romero MS. Kinetic analysis of epidermal growth factor receptor somatic mutant proteins shows increased sensitivity to the epidermal growth factor receptor tyrosine kinase inhibitor, erlotinib. Cancer Res. 2006;66:8163–71.
59. Carlson JJ, Garrison LP, Ramsey SD, Veenstra DL. Epidermal growth factor receptor genomic variation in NSCLC patients receiving tyrosine kinase inhibitor therapy: a systematic review and meta-analysis. J Cancer Res Clin Oncol. 2009;135:1483–93.
60. Mok TS, Wu YL, Thongprasert S, et al. Gefitinib or carboplatin-paclitaxel in pulmonary adenocarcinoma. N Engl J Med. 2009;361:947–57.
61. Fukuoka M, Wu YL, Thongprasert S, et al. Biomarker analyses and final overall survival results from a phase III, randomized, open-label, first-line study of gefitinib versus carboplatin/paclitaxel in clinically selected patients with advanced non-small-cell lung cancer in Asia (IPASS). J Clin Oncol. 2010;29:2866–74.

62. Maemondo M, Inoue A, Kobayashi K. Gefitinib or chemotherapy for non-small-cell lung cancer with mutated EGFR. N Engl J Med. 2010;362:2380–8.
63. Inoue A, Kobayashi K, Maemondo M. Updated overall survival results from a randomized phase III trial comparing gefitinib with carboplatin-paclitaxel for chemo-naïve non-small cell lung cancer with sensitive EGFR gene mutations (NEJ002). Ann Oncol. 2013;24:54–9.
64. Mitsudomi T, Morita S, Yatabe Y, et al. Gefitinib versus cisplatin plus docetaxel in patients with non-small-cell lung cancer harbouring mutations of the epidermal growth factor receptor (WJTOG3405): an open label, randomised phase 3 trial. Lancet Oncol. 2010;11:121–8.
65. Zhou C, Wu YL, Chen G, et al. Erlotinib versus chemotherapy as first-line treatment for patients with advanced EGFR mutation-positive non-small-cell lung cancer (OPTIMAL, CTONG-0802): a multicentre, open-label, randomised, phase 3 study. Lancet Oncol. 2011;12:735–42.
66. Zhou C, Wu YL, Chen G. Final overall survival results from a randomised, phase III study of erlotinib versus chemotherapy as first-line treatment of EGFR mutation-positive advanced non-small-cell lung cancer (OPTIMAL, CTONG-0802). Ann Oncol. 2015;26:1877–83.
67. Rosell R, Carcereny E, Gervais R, et al. Erlotinib versus standard chemotherapy as first-line treatment for European patients with advanced EGFR mutation-positive non-small-cell lung cancer (EURTAC): a multicentre, open-label, randomised phase 3 trial. Lancet Oncol. 2012;13:239–46.
68. Wu YL, Zhou C, Liam CK, et al. First-line erlotinib versus gemcitabine/cisplatin in patients with advanced EGFR mutation-positive non-small-cell lung cancer: analyses from the phase III, randomized, open-label, ENSURE study. Ann Oncol. 2015;26:1883–9.
69. Sequist LV, Yang JC, Yamamoto N, et al. Phase III study of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations. J Clin Oncol. 2013;31:3327–34.
70. Yang JC, Wu YL, Schuler M, et al. Afatinib versus cisplatin-based chemotherapy for EGFR mutation-positive lung adenocarcinoma (LUX-lung 3 and LUX-lung 6): analysis of overall survival data from two randomised, phase 3 trials. Lancet Oncol. 2015;16:141–51.
71. Wu YL, Zhou C, Hu CP, et al. Afatinib versus cisplatin plus gemcitabine for first-line treatment of Asian patients with advanced non-small-cell lung cancer harbouring EGFR mutations (LUX-lung 6): an open-label, randomised phase 3 trial. Lancet Oncol. 2014;15:213–22.
72. Park K, Tan EH, O'Byrne K, et al. Afatinib versus gefitinib as first-line treatment of patients with EGFR mutation-positive non-small-cell lung cancer (LUX-lung 7): a phase 2B, open-label, randomised controlled trial. Lancet Oncol. 2016;17:577–89.
73. Paz-Ares L, Tan EH, Zhang L, et al. Afatinib (a) vs gefitinib (G) in patients (pts) with EGFR mutation-positive (EGFRm+) non-small-cell lung cancer (NSCLC): overall survival (OS) data from the phase IIb trial LUX-lung 7(LL7). Ann Oncol. 2016;27(suppl 6):vi589. (abstract LBA43 PR)
74. Keating GM. Afatinib: a review in advanced non-small cell lung cancer. Target Oncol. 2016;11:825–35.
75. Sebastian M, Schmittel A, Reck M. First-line treatment of EGFR-mutated nonsmall cell lung cancer: critical review on study methodology. Eur Respir Rev. 2014;23:92–105.
76. Socinski MA, Evans T, Gettinger S, et al. Treatment of stage IV non-small cell lung cancer: diagnosis and management of lung cancer, 3rd ed: American College of Chest Physicians evidence-based clinical practice guidelines. Chest. 2013;143(5 Suppl):e341S–68S.
77. Novello S, Barlesi F, Califano R, et al. ESMO guidelines committee. Metastatic non-small-cell lung cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol. 2016;27(suppl 5):v1–v27.
78. Kobayashi S, Boggon TJ, Dayaram T. EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med. 2005;352:786–92.
79. Yu HA, Arcila ME, Rekhtman N, et al. Analysis of tumor specimens at the time of acquired resistance to EGFR-TKI therapy in 155 patients with EGFR-mutant lung cancers. Clin Cancer Res. 2013;19:2240–7.

80. Camidge DR, Pao W, Sequist LV. Acquired resistance to TKIs in solid tumours: learning from lung cancer. Nat Rev Clin Oncol. 2014;11:473–81.
81. Zhou C, Yao LD. Strategies to improve outcomes of patients with EGRF-mutant non-small cell lung cancer: review of the literature. J Thorac Oncol. 2016;11:174–86.
82. Pao W, Miller VA, Politi KA, et al. Acquired resistance of lung adenocarcinomas to gefitinib or erlotinib is associated with a second mutation in the EGFR kinase domain. PLoS Med. 2005;2(3):e73.
83. Kwak EL, Sordella R, Bell DW, et al. Irreversible inhibitors of the EGF receptor may circumvent acquired resistance to gefitinib. Proc Natl Acad Sci U S A. 2005;102:7665–70.
84. Yun CH, Mengwasser KE, Toms AV, et al. The T790M mutation in EGFR kinase causes drug resistance by increasing the affinity for ATP. Proc Natl Acad Sci U S A. 2008;105:2070–5.
85. Li D, Ambrogio L, Shimamura T, et al. BIBW2992, an irreversible EGFR/HER2 inhibitor highly effective in preclinical lung cancer models. Oncogene. 2008;27:4702–11.
86. Miller VA, Hirsh V, Cadranel J, et al. Afatinib versus placebo for patients with advanced, metastatic non-small-cell lung cancer after failure of erlotinib, gefitinib, or both, and one or two lines of chemotherapy (LUX-lung 1): a phase 2b/3 randomised trial. Lancet Oncol. 2012;13:528–38.
87. Katakami N, Atagi S, Goto K. LUX-lung 4: a phase II trial of afatinib in patients with advanced non-small-cell lung cancer who progressed during prior treatment with erlotinib, gefitinib, or both. J Clin Oncol. 2013;31:3335–41.
88. Zhou W, Ercan D, Chen L, et al. Novel mutant-selective EGFR kinase inhibitors against EGFR T790M. Nature. 2009;462:1070–4.
89. Ohashi K, Maruvka YE, Michor F, Pao W. Epidermal growth factor receptor tyrosine kinase inhibitor-resistant disease. J Clin Oncol. 2013;31:1070–80.
90. Ercan D, Choi HG, Yun CH, et al. EGFR mutations and resistance to irreversible pyrimidine-based EGFR inhibitors. Clin Cancer Res. 2015;21:3913–23.
91. Hossam M, Lasheen DS, Abouzid KA. Covalent EGFR inhibitors: binding mechanisms, synthetic approaches, and clinical profiles. Arch Pharm (Weinheim). 2016;349:573–93.
92. Yver A. Osimertinib (AZD9291)–a science-driven, collaborative approach to rapid drug design and development. Ann Oncol. 2016;27:1165–70.
93. Cross DA, Ashton SE, Ghiorghiu S. AZD9291, an irreversible EGFR TKI, overcomes T790M-mediated resistance to EGFR inhibitors in lung cancer. Cancer Discov. 2014;4:1046–61.
94. Jänne PA, Yang JC, Kim DW, et al. AZD9291 in EGFR inhibitor-resistant non-small-cell lung cancer. N Engl J Med. 2015;372:1689–99.
95. Goss G, Tsai CM, Shepherd FA, et al. Osimertinib for pretreated EGFR Thr790Met-positive advanced non-small-cell lung cancer (AURA2): a multicentre, open-label, single-arm, phase 2 study. Lancet Oncol. 2016;17:1643–52.
96. Mok TS, Wu YL, Ahn MJ, et al. Osimertinib or platinum-pemetrexed in EGFR T790M-positive lung cancer. N Engl J Med. 2016;376(7):629–40.
97. Socinski MA, Villaruz LC, Ross J. Understanding mechanisms of resistance in the epithelial growth factor receptor in non-small cell lung cancer and the role of biopsy at progression. Oncologist. 2016;22:3–11.
98. Tan DS, Yom SS, Tsao MS, et al. The International Association for the Study of Lung Cancer consensus statement on optimizing management of EGFR mutation-positive non-small cell lung cancer: status in 2016. J Thorac Oncol. 2016;11:946–63.
99. Mayor S. Osimertinib effective in EGFR T790M-positive lung cancer. Lancet Oncol. 2016;18(1):e9.
100. Mano H. ALKoma: a cancer subtype with a shared target. Cancer Discov. 2012;2:495–502.
101. Pulford K, Lamant L, Morris SW, et al. Detection of anaplastic lymphoma kinase (ALK) and nucleolar protein nucleophosmin (NPM)-ALK proteins in normal and neoplastic cells with the monoclonal antibody ALK1. Blood. 1997;89:1394–404.
102. Morris SW, Kirstein MN, Valentine MB, et al. Fusion of a kinase gene, ALK, to a nucleolar protein gene, NPM, in non-Hodgkin's lymphoma. Science. 1994;263:1281–4.

103. Soda M, Choi YL, Enomoto M, et al. Identification of the transforming EML4-ALK fusion gene in non-small-cell lung cancer. Nature. 2007;448:561–6.
104. Takeuchi K, Choi YL, Togashi Y, et al. KIF5B-ALK, a novel fusion oncokinase identified by an immunohistochemistry-based diagnostic system for ALK-positive lung cancer. Clin Cancer Res. 2009;15:3143–9.
105. Togashi Y, Soda M, Sakata S, et al. KLC1-ALK: a novel fusion in lung cancer identified using a formalin-fixed paraffin-embedded tissue only. PLoS One. 2012;7(2):e31323.
106. Takeuchi K, Choi YL, Soda M, et al. Multiplex reverse transcription-PCR screening for EML4-ALK fusion transcripts. Clin Cancer Res. 2008;14:6618–24.
107. Kerr KM, López-Ríos F. Precision medicine in NSCLC and pathology: how does ALK fit in the pathway? Ann Oncol. 2016;27(Suppl 3):iii16–24.
108. Blackhall F, Cappuzzo F. Crizotinib: from discovery to accelerated development to front-line treatment. Ann Oncol. 2016;27(Suppl 3):iii35–41.
109. Kwak EL, Bang YJ, Camidge DR, et al. Anaplastic lymphoma kinase inhibition in non-small-cell lung cancer. N Engl J Med. 2010;363:1693–703.
110. Camidge DR, Bang YJ, Kwak EL, et al. Activity and safety of crizotinib in patients with ALK-positive non-small-cell lung cancer: updated results from a phase 1 study. Lancet Oncol. 2012;13:1011–9.
111. Solomon BJ, Mok T, Kim DW, et al. First-line crizotinib versus chemotherapy in ALK-positive lung cancer. N Engl J Med. 2014;371:2167–77.
112. Shaw AT, Kim DW, Nakagawa K, et al. Crizotinib versus chemotherapy in advanced ALK-positive lung cancer. N Engl J Med. 2013;368:2385–94.
113. Nokihara H, Hida T, Kondo M, et al. Alectinib (ALC) versus crizotinib (CRZ) in ALK-inhibitor naive ALK-positive nonsmall-cell lung cancer (ALKþ NSCLC): primary results from the J-ALEX study. J Clin Oncol. 2016;34(suppl):abstract 9008.
114. Dagogo-Jack I, Shaw AT. Crizotinib resistance: implications for therapeutic strategies. Ann Oncol. 2016;27(Suppl 3):iii42–50.
115. Katayama R, Shaw AT, Khan TM, et al. Mechanisms of acquired crizotinib resistance in ALK-rearranged lung cancers. Sci Transl Med. 2012;4(120):120ra17.
116. Doebele RC, Pilling AB, Aisner DL, et al. Mechanisms of resistance to crizotinib in patients with ALK gene rearranged non-small cell lung cancer. Clin Cancer Res. 2012;18:1472–82.
117. Gainor JF, Dardaei L, Yoda S, et al. Molecular mechanisms of resistance to first- and second-generation ALK inhibitors in ALK-rearranged lung cancer. Cancer Discov. 2016;6:1118–33.
118. Shaw AT, Kim DW, Mehra R, et al. Ceritinib in ALK-rearranged non-small-cell lung cancer. N Engl J Med. 2014;370:1189–97.
119. Kim DW, Mehra R, Tan DS, et al. Activity and safety of ceritinib in patients with ALK-rearranged non-small-cell lung cancer (ASCEND-1): updated results from the multicentre, open-label, phase 1 trial. Lancet Oncol. 2016;17:452–63.
120. Crinò L, Ahn MJ, De Marinis F, et al. Multicenter phase II study of whole-body and intracranial activity with ceritinib in patients with ALK-rearranged non-small-cell lung cancer previously treated with chemotherapy and crizotinib: results from ASCEND-2. J Clin Oncol. 2016;34:2866–73.
121. Gadgeel SM, Gandhi L, Riely GJ, et al. Safety and activity of alectinib against systemic disease and brain metastases in patients with crizotinib-resistant ALK-rearranged non-small-cell lung cancer (AF-002JG): results from the dose-finding portion of a phase 1/2 study. Lancet Oncol. 2014;15:1119–28.
122. Shaw AT, Gandhi L, Gadgeel S, et al. Alectinib in ALK-positive, crizotinib-resistant, non-small-cell lung cancer: a single-group, multicentre, phase 2 trial. Lancet Oncol. 2016;17:234–42.
123. Gettinger SN, Bazhenova LA, Langer CJ, et al. Activity and safety of brigatinib in ALK-rearranged non-small-cell lung cancer and other malignancies: a single-arm, open-label, phase 1/2 trial. Lancet Oncol. 2016;17:1683–96.
124. Seto T, Kiura K, Nishio M, et al. CH5424802 (RO5424802) for patients with ALK-rearranged advanced non-small-cell lung cancer (AF-001JP study): a single-arm, open-label, phase 1-2 study. Lancet Oncol. 2013;14:590–8.

125. Zhu Q, Zhan P, Zhang X, Lv T, Song Y. Clinicopathologic characteristics of patients with ROS1 fusion gene in non-small cell lung cancer: a meta-analysis. Transl Lung Cancer Res. 2015;4:300–9.
126. Shaw AT, Ou SH, Bang YJ, et al. Crizotinib in ROS1-rearranged non-small-cell lung cancer. N Engl J Med. 2014;371:1963–71.
127. Yoh K, Seto T, Satouchi M, et al. Vandetanib in patients with previously treated RET-rearranged advanced non-small-cell lung cancer (LURET): an open-label, multicentre phase 2 trial. Lancet Respir Med. 2017;5:42–50.
128. Planchard D, Kim TM, Mazieres J, et al. Dabrafenib in patients with BRAF(V600E)-positive advanced non-small-cell lung cancer: a single-arm, multicentre, open-label, phase 2 trial. Lancet Oncol. 2016;17:642–50.
129. Planchard D, Besse B, Groen HJ, et al. Dabrafenib plus trametinib in patients with previously treated BRAF(V600E)-mutant metastatic non-small cell lung cancer: an open-label, multicentre phase 2 trial. Dabrafenib plus trametinib in patients with previously treated BRAFV600E-mutant metastatic non-small cell lung cancer: an open-label, multicentre phase 2 trial. Lancet Oncol. 2016;17:984–93.
130. Frampton GM, Ali SM, Rosenzweig M, et al. Activation of MET via diverse exon 14 splicing alterations occurs in multiple tumor types and confers clinical sensitivity to MET inhibitors. Cancer Discov. 2015;5:850–9.
131. Awad MM, Oxnard GR, Jackman DM, et al. MET exon 14 mutations in non-small-cell lung cancer are associated with advanced age and stage-dependent MET genomic amplification and c-met overexpression. J Clin Oncol. 2016;34:721–30.
132. Wood K, Hensing T, Malik R, Salgia R. Prognostic and predictive value in KRAS in non-small-cell lung cancer: a review. JAMA. 2016;2:805–12.
133. Mellman I, Coukos G, Dranoff G. Cancer immunotherapy comes of age. Nature. 2011;480:480–9.
134. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. Immunity. 2013;39:1–10.
135. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med. 2010;363:711–23.
136. Brahmer JR, Tykodi SS, Chow LQ, et al. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med. 2012;366:2455–65.
137. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012;366:2443–54.
138. Gettinger SN, Horn L, Gandhi L, et al. Overall survival and long-term safety of nivolumab (anti-programmed death 1 antibody, BMS-936558, ONO-4538) in patients with previously treated advanced non-small-cell lung cancer. J Clin Oncol. 2015;33:2004–12.
139. Rizvi NA, Mazières J, Planchard D, et al. Activity and safety of nivolumab, an anti-PD-1 immune checkpoint inhibitor, for patients with advanced, refractory squamous non-small-cell lung cancer (CheckMate 063): a phase 2, single-arm trial. Lancet Oncol. 2015;16:257–65.
140. Reck M, Rodríguez-Abreu D, Robinson AG, et al. Pembrolizumab versus chemotherapy for PD-L1-positive non-small-cell lung cancer. N Engl J Med. 2016;375:1823–33.
141. Socinski M, Creelan B, Horn L, et al. CheckMate 026: a phase 3 trial of nivolumab vs investigator's choice (IC) of platinum-based doublet chemotherapy (PT-DC) as first-line therapy for stage IV/recurrent programmed death ligand 1 (PD-L1)-positive NSCLC. Ann Oncol. 2016;27(suppl 6):vi588. (abstract LBA7 PR)
142. Herbst RS, Baas P, Kim DW, et al. Pembrolizumab versus docetaxel for previously treated, PD-L1-positive, advanced non-small-cell lung cancer (KEYNOTE-010): a randomised controlled trial. Lancet. 2016;387:1540–50.
143. Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. N Engl J Med. 2015;373:123–35.
144. Borghaei H, Paz-Ares L, Horn L, et al. Nivolumab versus docetaxel in advanced nonsquamous non-small-cell lung cancer. N Engl J Med. 2015;373:1627–239.

145. Fehrenbacher L, Spira A, Ballinger M, et al. Atezolizumab versus docetaxel for patients with previously treated non-small-cell lung cancer (POPLAR): a multicentre, open-label, phase 2 randomised controlled trial. Lancet. 2016;387:1837–46.
146. Rittmeyer A, Barlesi F, Waterkamp D, et al. Atezolizumab versus docetaxel in patients with previously treated non-small-cell lung cancer (OAK): a phase 3, open-label, multicentre randomised controlled trial. Lancet. 2016;389(10066):255–65.
147. Yu H, Boyle TA, Zhou C, Rimm DL, Hirsch FR. PD-L1 expression in lung cancer. J Thorac Oncol. 2016;11:964–75.
148. Shien K, Papadimitrakopoulou VA, Wistuba II. Predictive biomarkers of response to PD-1/PD-L1 immune checkpoint inhibitors in non-small cell lung cancer. Lung Cancer. 2016;99:79–87.
149. Garon EB, Rizvi NA, Hui R, et al. Pembrolizumab for the treatment of non-small-cell lung cancer. N Engl J Med. 2015;372:2018–28.
150. Langer CJ, Gadgeel SM, Borghaei H, et al. Carboplatin and pemetrexed with or without pembrolizumab for advanced, non-squamous non-small-cell lung cancer: a randomised, phase 2 cohort of the open-label KEYNOTE-021 study. Lancet Oncol. 2016;17:1497–508.
151. Herbst RS, Soria JC, Kowanetz M, et al. Predictive correlates of response to the anti-PD-L1 antibody MPDL3280A in cancer patients. Nature. 2014;515:563–7.
152. Hirsch FR, McElhinny A, Stanforth D, et al. PD-L1 immunohistochemistry assays for lung cancer: results from phase 1 of the "blueprint PD-L1 IHC assay comparison project". J Thorac Oncol. 2016;12(2):208–22. https://doi.org/10.1016/j.jtho.2016.11.2228.
153. Facchinetti F, Marabelle A, Rossi G, Soria JC, Besse B, Tiseo M. Moving immune checkpoint blockade in thoracic tumors beyond NSCLC. J Thorac Oncol. 2016;11:1819–36.
154. Reck M, Bondarenko I, Luft A, et al. Ipilimumab in combination with paclitaxel and carboplatin as first-line therapy in extensive-disease-small-cell lung cancer. Results from a randomized, double-blind, multicenter phase 2 trial. Ann Oncol. 2013;24:75–83.
155. Reck M, Luft A, Szczesna A, et al. Phase III randomized trial of ipilimumab plus etoposide and platinum versus placebo plus etoposide and platinum in extensive-stage small-cell lung cancer. J Clin Oncol. 2016;34:3740–8.
156. Antonia SJ, López-Martin JA, Bendell J, et al. Nivolumab alone and nivolumab plus ipilimumab in recurrent small-cell lung cancer (CheckMate 032): a multicenter, open-label, phase 1/2 trial. Lancet Oncol. 2016;17:883–95.
157. Tanaka F, Yoneda K, Hasegawa S. Circulating tumor cells (CTCs) in lung cancer: current status and future perspectives. Lung Cancer Targets Ther. 2010;1:77–84.
158. Sacher AG, Paweletz C, Dahlberg SE, et al. Prospective validation of rapid plasma genotyping for the detection of EGFR and KRAS mutations in advanced lung cancer. JAMA Oncol. 2016;2:1014–22.
159. Oxnard GR, Thress KS, Alden RS, et al. Association between plasma genotyping and outcomes of treatment with osimertinib (AZD9291) in advanced non-small-cell lung cancer. J Clin Oncol. 2016;34:3375–82.

Chapter 2
Molecular Diagnosis and Targeting Therapy for Breast Cancer

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Abstract Breast cancer (BC) is a representative cancer for which molecular targeting therapy is most popular, because systemic therapy is selected according to tumor biological subtypes, luminal A, luminal B, HER2-enriched, and basal-like; those are decided by gene expression pattern or estrogen receptor (ER), HER2, and tumor proliferation measured by Ki67 expression in immunohistochemistry. Approximately 70–80% of BC is ER positive. Adjuvant therapy is selected according to the guideline based on the large-scale randomized control trials. Selective estrogen receptor modulators (SERM), like tamoxifen or toremifene, and gonadotropin-releasing hormone agonist (GnRH) are used in combination or alone for premenopausal metastatic BC (MBC) and in adjuvant setting. Aromatase inhibitor (AI) targeting the enzyme aromatase is recommended for postmenopausal BC in adjuvant and MBC both in pre- and postmenopausal.

A multigene assay predicts the prognosis of luminal-type BC and selects the candidates for chemotherapy (CT). Selective ER downregulator (SERD), fulvestrant, is used for MBC. Overexpression of the human epidermal growth factor 2 (HER2) worsens the prognosis of BC, but the monoclonal antibody, trastuzumab, has drastically improved the prognosis of HER2-overexpressing BC. Anti-HER2 blockade (trastuzumab/lapatinib or trastuzumab/pertuzumab) is associated with chemotherapy (CT), or Trastuzumab-Emtansine (T-DM1) can be used for trastuzumab-resistant MBC. mTOR inhibitor everolimus or cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with AI can be used for ER-positive MBC.

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New therapeutic approach like apoptosis induction, inhibition of anti-apoptosis, cell cycle progression, and signal transduction are now under developing.

Keywords Breast cancer • Molecular targeting therapy • ER • PgR • Human epidermal growth factor 2 (HER2) • VEGF • Cyclin-dependent kinase 4/6 inhibitor

Breast cancer (BC) is the most common cancer worldwide, being also the leading cause of cancer death among women [1]. It is also the most common cancer in female with increasing morbidity year after year in Japan, and the most common affected generation is 40th (http://ganjoho.jp/reg_stat/statistics/stat/annual.html). The number of the patients is increasing in the world, but the mortality from BC in North America and the European Union (EU) has decreased from the end of twentieth century. In 2016, mortality from BC in the EU is expected to drop by 8% [2]. Increased survival is due to the drastic success in early diagnosis by screening and breakthrough of the treatment, with targeting the molecules. The first epoch-making target of BC is estrogen receptor (ER) and the next is CerbB2.

2.1 Surgical Anti-hormonal Treatment

BC has the longest history of treatment among all cancers [3].

In 1896, George Thomas Beatson published a paper entitled "On Treatment of Inoperable Cases of Carcinoma of the Mamma: Suggestions for a New Method of Treatment," with detailed treatment of three patients with advanced breast cancer through bilateral oophorectomy. Oophorectomy became the standard treatment for advanced BC over the following years. He is considered the father of anti-hormonal treatment of BC [4]. Oophorectomy and bilateral adrenalectomy have been introduced in treatment for advanced BC for long time [5].

2.2 Estrogen Receptor (ER) and Anti-Estrogen Receptor Targeting Therapy

ER, binding sites for 17β-estradiol, was found in rat uterus, and it was also recognized existing in BC tissue [6]. Drugs targeting for ER have been developed and became the major arms for BC. The first drug targeting ER is tamoxifen, a selective estrogen receptor modulator (SERM). Gonadotropin-releasing hormone (GnRH) agonist and aromatase inhibitor (AI) have been developed and became the gold standard therapeutic agents after several large-scale randomized controlled clinical trials (RCT).

2.3 Human Epidermal Growth Factor Receptor 2 (HER2) and Targeting Therapy

HER2/neu or ErbB-2 is a member of the epidermal growth factor receptor (EGFR) family, along with HER1 (EGFR), HER3, and HER4 [7]. These receptors, functioning as homo- or heterodimers, activate multiple cellular pathways, such as the p44/42 mitogen-activated protein kinase (MAPK) and the phosphatidylinositol-3-kinase (PI3K) pathways, and stimulate cell growth, survival, and differentiation [8, 9]. Unlike other members of the family, HER2 is not activated by a specific ligand and is always in an active conformational state, ready to interact with other ligand-activated EGF receptors [10], particularly HER3 [11]. Overexpression of the membrane HER2 in BC cells is known as a major negative prognostic factor [12]. Overexpression occurs in 20–25% of cases and is detected either as gene amplification (fluorescence in situ hybridization: FISH) or as protein expression with immunohistochemistry (IHC) [13]. The anti-HER2 humanized monoclonal antibody, trastuzumab (Herceptin®), has been introduced in 1998, and a tyrosine kinase inhibitor of EGFR and HER2, lapatinib (Tykerb®), in 2007; they showed significant improvement in the outcome of patients with HER2-overexpressing BC.

2.4 Subtype of BC as a Decision Making for Targeting Treatment

Since the groundbreaking works at the beginning of this millennium [14, 15], BC is considered to consist of at least four different clinically relevant molecular subtypes: luminal A, luminal B, HER2-enriched, and basal-like. Yet, scientifically, up to ten different molecular subtypes have been identified using gene copy number and expression analyses [16]. The four original subtypes can either be directly determined with a multigene assay such as Prosigna (NanoString Technologies) or BluePrint (Agendia) or indirectly reconstructed with immunohistochemistry (IHC) with formalin-fixed paraffin-embedded tumor tissue [17]. Subtypes according to ER, progesterone receptor (PgR), and HER2, as well as tumor proliferation measured by Ki67 status are as follows: luminal A-like (ER or PgR positive, or both, HER2 negative, low proliferation), luminal B-like (ER or PgR positive, or both, HER2 negative, high proliferation), HER2, non-luminal (HER2 positive and ER and PgR negative) or luminal (HER2 positive and ER or PgR positive, or both), and basal-like (HER2 negative and ER and PgR negative; triple-negative breast cancer) (Table 2.1) [18].

In accordance with the St Gallen consensus, systemic therapy for early BC is guided by these molecular subtypes (Fig. 2.1) [19, 20].

Table 2.1 Molecular Subtypes of Breast Cancer

a. Luminal A: ER positive, HER2 negative, Ki-67 protein low, and PR high
b. Luminal B: ER positive, HER2 negative, and either Ki-67 protein high or PR low [23]
c. Basal-like breast cancer: typically lacks expression of the molecular targets that confer responsiveness to highly effective targeted therapies such as tamoxifen and aromatase inhibitors (AIs) or trastuzumab (HER2 amplification) [24]
d. Triple-negative breast cancer (TNBC): ER-, PR-, and HER2-negative tumors [24]. Most BRCA1 breast cancers are basal-like TNBC. Triple negative also includes some special histological types such as (typical) medullary and adenoid cystic carcinoma with low risks of distant recurrence [25]
e. HER2+: ERBB2+ has amplified HER2/neu. HER-2/neu status can be analyzed by fluorescence in situ hybridization (FISH) assays. HER2-positive cancer is diagnosed in 10–20% of breast cancer patients. This cancer is particularly aggressive and more likely to spread rapidly than other types of breast cancer [17]
f. Claudin low: a more recently described class; often triple negative, but distinct in that there is low expression of cell–cell junction proteins including E-cadherin. Infiltration with lymphocytes is common

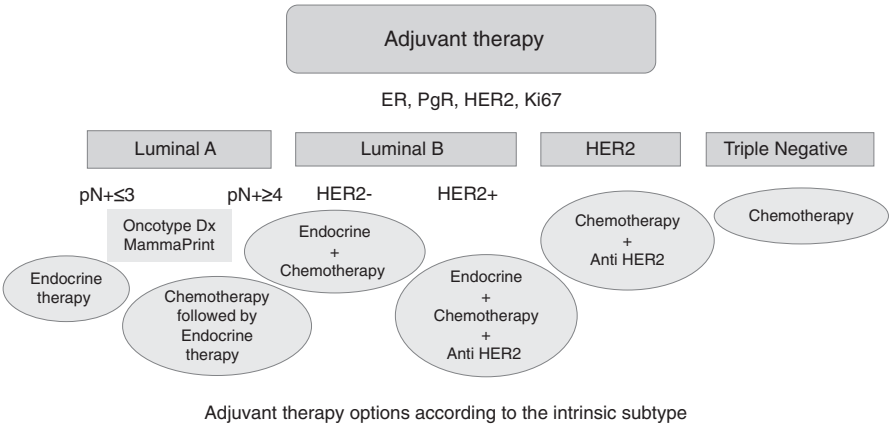

Fig. 2.1 In patients with luminal tumors, several multigene assays like MammaPrint and Oncotype DX assess long-term relapse risk, duration of adjuvant ET, and adoption of CT

In daily clinical practice, the difficulty is distinguishing between luminal A and luminal B on the basis of proliferation assessed by non-standardized Ki67 values. Values of 10% or less are generally considered low risk, and values between 20% and 29% are considered as a minimum criterion for high proliferation. Yet, because of the lack of a prospectively validated study for cutoff value, intermediate values between 10% and about 30% should not be used as the sole criterion for indicating adjuvant chemotherapy. International standardization for Ki67 is still missing, and the measured inter laboratory variability is rather high [21].

2.5 Adjuvant Therapy According to Subtype of BC

2.5.1 Endocrine Therapy (ET)

In all luminal—i.e., hormone receptor (HR) positive (ER or PgR positive or both)—early BC, adjuvant endocrine therapy over the course of 5–10 years is considered standard. Current guidelines consider any ER or PgR staining (i.e., ≥1%) as being positive; endocrine sensitivity is directly correlated to the degree of HR positivity [22]. In premenopausal patients, 20 mg tamoxifen per day is the standard endocrine therapy. The Early Breast Cancer Trialists' Collaborative Group (EBCTCG) meta-analysis showed that 5 years of tamoxifen treatment reduced the recurrence not just in the first 4 years in patients with ER-positive disease. This effect was independent of PgR status, age, nodal status, and chemotherapy use. BC mortality was reduced by about a third throughout the first 15 years of follow-up [23].

2.6 Chemotherapy (CT)

The benefit of CT is more pronounced in ER-negative BC. CT is recommended in the majority of TNBC, in HER2-positive BC, and in high-risk luminal tumors. The current CT standards in early BC are anthracyclines and taxanes, given as a combination or in sequence over a period of 18–24 weeks. Generally, recommended regimens do not differ between neoadjuvant and adjuvant settings. The EBCTCG meta-analysis suggested that anthracycline and taxane-containing CT reduced 10-year BC mortality by about one-third [24]. Anthracycline and taxane sequence is as effective as their combination [25]. Four times anthracycline followed by four times docetaxel is equally effective as the combination of the same drugs (six times TAC [docetaxel, doxorubicin, and cyclophosphamide]) but has a different toxicity pattern [26]. A population-based analysis showed that delays beyond 91 days between surgery and start of adjuvant CT are associated with an impaired outcome, particularly in triple-negative breast cancer [27].

For patients with triple-negative BC, standard regimens containing anthracycline and taxane should be used, preferably as neoadjuvant therapy. Trials have indicated that adding platinum to a neoadjuvant anthracycline-taxane combination or sequence improves pathological complete response (pCR) [28].

2.7 Multigene Assay for ER+ HER2-BC

In patients with luminal tumors, several multigene assays assess long-term relapse risk, duration of adjuvant ET, and adopt of CT. EndoPredict [Myriad Genetics] [29], MammaPrint [Agendia] [30], Oncotype DX [Genomic Health] [31], and Prosigna [32] have been validated for risk assessment and prediction of CT response. Most of these assays give information not only about risk of early recurrence (first 5 years) but also about risk of late recurrence (>5 years). Prospective trial results for test validation only exist for Oncotype DX and MammaPrint. For Oncotype DX, the TAILORx trial for pN0 [33] and the WSG PlanB trial for pN0–1 prospectively confirmed its prognostic effect [34]. MammaPrint showed that patient outcome is not compromised if adjuvant CT is omitted in clinically high-risk and genomically low-risk early BC. All other multigene assays have been retrospectively validated. Prospective outcome data are still missing from the randomized comparisons of the large international trials that used Oncotype DX for risk group assessment (i.e., TAILORx [pN0], RxPONDER [pN1]). The protein-based ELISA assay for uPA/PAI-1 (Femtelle [American Diagnostica/Sekisui Diagnostics]) has also been validated at the highest level of evidence for its prognostic and predictive effect by a prospective clinical trial [35] and European Organisation for Research and Treatment of Cancer pooled analysis [36]. By contrast with multigene assays, this test requires fresh-frozen tumor tissue; it can be an alternative option for risk assessment because of its low overall costs [37].

2.8 Targeting Agents for ER

2.8.1 Selective Estrogen Receptor Modulator (SERM)

Tamoxifen (TAM), ICI 46,474, and orphan drug have been described as antifertility agent in rats, and its modest activity has been investigated looking for a therapeutic application for MBC. Initial clinical studies demonstrated that it was safe and effective for the treatment of MBC in postmenopausal women [38–40]. Targeting study for aromatase enzyme was started also in 1977.

2.8.2 Gonadotropin-Releasing Hormone (GnRH) Agonist

The hormone secretions of the ovary are controlled by the secretions from the pituitary, which in turn is controlled by the hypothalamus through its own secretions. With the elucidation of the last-mentioned secretions in the form of the structure of gonadotropin-releasing hormone (GnRH) by Guillemin and Schally in 1967, it became possible to synthesize thousands of different analogues of the primary

decapeptide, GnRH [41]. Leuprorelin and goserelin and the other synthetic models were made and tested for clinical use [42]. They were introduced in prostate and BC treatment. Those agonists induce reversible hypogonadism (decrease the level of LH) by achieving through receptor downregulation by internalization of receptors.

2.9 Aromatase Inhibitor

Aromatase is the enzyme which synthesizes estrogen from androgen. Aromatase inhibitor (AI) inhibits aromatization and decreases estrogen level. AI has been also proposed as the drug for female infertility by its ovarian stimulation. There are two types of AIs approved to treat BC. Irreversible steroidal inhibitors, such as exemestane (Aromasin®), form a permanent and deactivating bond with the aromatase enzyme. Nonsteroidal inhibitors, such as anastrozole (Arimidex®) and letrozole (Femara®), inhibit the synthesis of estrogen via reversible competition for the aromatase [43].

In postmenopausal BC patients, TAM and AI are both valid therapeutic options, as each monotherapy for 5 years. Sequential AIs followed by 2–3 years TAM also significantly reduce recurrences by 30%, but not mortality, compared with TAM. 5 years of AI significantly reduce BC mortality by 15% compared with 5 years of TAM treatment [44]. In postmenopausal patients, upfront AI therapy is preferred [45].

SOFT and TEXT trial results showed additional treatment of GnRH to TAM or even administering GnRH together with an AI enhances efficacy in premenopausal patients with a high-risk recurrence (i.e., after chemotherapy or age $\leq$ 35 years) [46, 47].

2.10 Selective ER Downregulator (SERD)

The SERD fulvestrant, selective ER downregulator, blocks ERα dimerization and promotes ERα protein degradation, resulting in the inhibition of ERα function. It has a steroidal structure and high affinity with the cellular membrane and matrix, as well as high chemical stability, resulting in long-lasting effects [48].

2.11 Anti-HER2 Therapy

Two drugs are currently approved for HER2-positive BC: trastuzumab (Herceptin®), introduced in 1998, and lapatinib (Tykerb®), in 2007.

The introduction of the anti-HER2 humanized monoclonal antibody, "trastuzumab," was associated with a significant improvement in the outcome of patients with HER2-overexpressing BC. Clinical studies have shown that, in the HER2-positive BC, the addition of trastuzumab to chemotherapy (CT) significantly improves both recurrence-free survival (RFS) and overall survival (OS) in the adjuvant setting [49, 50].

It also increases the rate of pathologic complete response (pCR) in the primary systemic therapy (PST) setting for HER2-positive BC [51] and also improves OS in the metastatic setting [52]. HER2 positive was decided as >30% in IHC or FISH/CEP17 ratio > 2.2 according to American Society of Clinical Oncology/College of American Pathologists clinical practice guideline [53]. Trastuzumab should not be administered routinely concomitantly with anthracyclines because of its cardiotoxicity [54]. Combination with taxanes is safe and has been demonstrated to be more effective than sequential treatment. Trastuzumab may also be safely combined with radiotherapy (RT) and endocrine therapy (ET) [55]. Anti-HER2 therapy is recommended as early as possible in patients with HER2-positive MBC. Even though efficacy of trastuzumab or lapatinib together with AI was shown in several phase 2–3 trials for postmenopausal patients [56] and led to registration of these combinations, combination with chemotherapy is currently recommended in early lines of therapy because of the overall survival advantage. In the neoadjuvant setting, dual anti-HER2 blockade associated with CT (trastuzumab/lapatinib or trastuzumab/pertuzumab) has led to improvements in the outcomes when compared with CT associated with one anti-HER2 agent [57]. However, long-term outcomes are not known, and such a treatment cannot be recommended outside of clinical trials.

Trastuzumab still leads the market but with biosimilar and new-generation agents now on the horizon. Pertuzumab plus trastuzumab plus docetaxel regimen now is a first-line therapy for patients with HER2-positive MBC [58]. Dual HER-2 blockade has been shown to be more effective than single blockade in the metastatic setting. FDA approved the first antibody–drug conjugate for the treatment of HER2-positive metastatic BC, T-DM1 (Kadcyla®) on February 2013. T-DM1 is trastuzumab-maytansinoid (microtubule-depolymerizing agents) conjugates through a non-reducible linker showed greater activity compared with nonconjugated trastuzumab while maintaining selectivity for HER2-overexpressing tumor cells. It offers improved efficacy and pharmacokinetics and reduced toxicity [59].

2.12 Cross Talk of ER and HER2

In vitro and in vivo models suggested the existence of a cross talk between the two downstream pathways. Estrogens act via a nuclear/genomic and a nonnuclear/non-genomic activity. Nonnuclear ER interacts directly or indirectly (e.g., via G proteins) with HER 2/HER1–4 dimers activating their downstream kinase

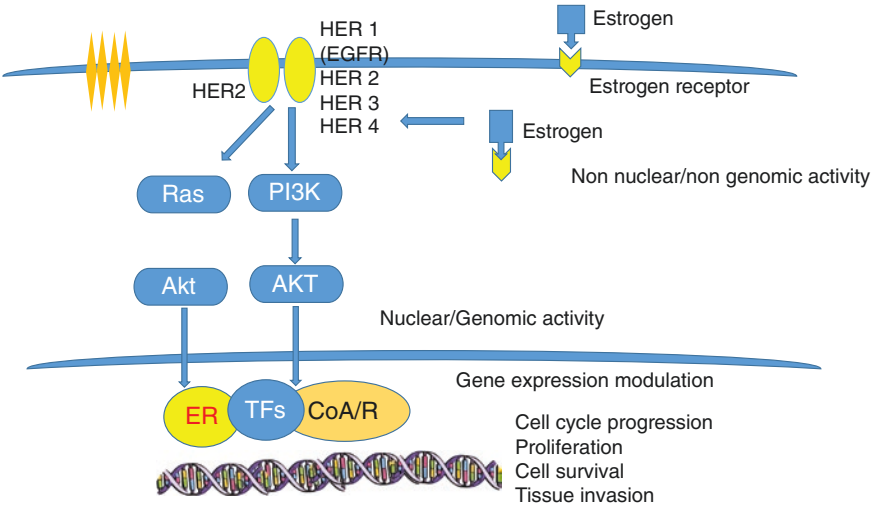

Fig. 2.2 Cross talk of ER and HER2 estrogens act via a nuclear and nonnuclear activity. Nonnuclear ER interacts directly or indirectly (via G proteins) with HER 2/HER1–4 dimers activating their downstream kinase pathways (Ras-MAPK and PI3K-Akt pathways), which in turn phosphorylate ER and other transcription factors (TFs) and coactivators/corepressors (CoA/R), modulating gene expression. HER2 signaling pathways also reduce ER expression at both mRNA and protein levels. ER also promotes HER2, other tyrosine kinase receptors (TKR), and TKR ligands' gene expression. This bidirectional cross talk leads to cancer cell cycle progression, proliferation, survival, and invasiveness [60]

pathways (e.g., Ras-MAPK and PI3K-Akt pathways), which in turn phosphorylate ER and other transcription factors (TFs) and coactivators/corepressors (CoA/R), modulating gene expression. HER2 signaling pathways also reduce ER expression at both mRNA and protein levels. ER also promotes HER2, other tyrosine kinase receptors (TKR) and TKR ligands' gene expression. This bidirectional cross talk leads to cancer cell cycle progression, proliferation, survival, and invasiveness (Fig. 2.2) [60].

2.13 Anti-Vascular Endothelial Growth Factor Targeting Agents

Anti-vascular endothelial growth factor (VEGF) antibody bevacizumab (Avastin®) is a specific drug for HER2-negative metastatic BC. Bevacizumab improved PFS 9.2 vs. 6.7 months but not overall survival when given together with first-line chemotherapy such as paclitaxel or capecitabine [61]. Bevacizumab (Avastin®) was approved by the European Committee for Medicinal Products for Human Use (CHMP) but not by FDA and thus constitutes a therapy option only in individual countries.

2.14 New Molecular Targeting Agents

2.14.1 Phosphatidylinositol 3-Kinase/Mammalian Target of Rapamycin (PI3K/mTOR) Pathway

Phosphatidylinositol 3-kinase/mammalian target of rapamycin (PI3K/mTOR) pathway is commonly dysregulated in BC [62]. mTOR inhibitor has demonstrated antitumor activity in a variety of cancer types, including ER positive [63]. mTOR inhibitor, everolimus can be used for postmenopausal patients after failure of AI treatment [64]. Afinitor® (everolimus) is the only FDA-approved inhibitor of mTOR to be used in combination with exemestane to treat postmenopausal women with advanced HR+, HER2-BC from 2012.

PI3K pathway activation occurs frequently in TNBC and confers susceptibility to mTOR inhibitors [65]. Gonzalez-Angulo et al. investigated the addition of everolimus to paclitaxel in the neoadjuvant setting for the treatment of TNBC and showed that downregulation of mTOR was achieved after 48 h [66].

2.15 Cyclin-Dependent Kinase (CDK) Targeting Agents

CDK targeting treatment has been emerging in the last few years. The cyclin-dependent kinase 4/6 (CDK 4/6) inhibitor palbociclib (Ibrance®) together with letrozole also improved median PFS in postmenopausal patients without previous systemic treatment for MBC [67]. Palbociclib received approval in the USA and Europe from November 2016. PALOMA 3 study also showed efficacy of palbociclib together with fulvestrant for fulvestrant alone in progressive disease and the relapse cases after previous endocrine therapy. The efficacy of palbociclib was similar in premenopausal patients received additional goserelin and postmenopausal patients [68]. An OS advantage versus standard therapy has not been reported for any CDK 4/6 inhibitor. For the PALOMA studies, final OS analyses are still pending. Ribociclib and abemaciclib are two additional CDK 4/6 inhibitors that are being assessed in clinical trials. Data from the ribociclib first-line registration trial (MONALEESA 2; NCT01958021) showed a substantial progression-free survival benefit for letrozole plus ribociclib versus letrozole alone [69]. On the same aspect, abemaciclib, a potent inhibitor of CDK4/6, is under investigation by Lilly Inc.

The aforementioned protein, enzyme, and molecular targeting agents are listed on Table 2.2. New therapeutic approach for apoptosis induction, inhibition of antiapoptosis, cell cycle progression, and signal transduction are now under developing.

Table 2.2 List of currently approved targeted drug by FDA for breast cancer

Agents	Target molecules or protein	Indications
SERM		
Tamoxifen	ER	Premenopausal and postmenopausal
Toremifene	ER	
GnRH agonist		
Goserelin		Premenopausal
Leuprorelin		Premenopausal
Aromatase inhibitor		
Anastrozole (Arimidex®)	Aromatase	Postmenopausal
Letrozole (Femara®)	Aromatase	Postmenopausal
Exemestane (Aromasin®)	Aromatase	Postmenopausal
Trastuzumab (Herceptin®)	HER2 (ERBB2/neu)	(HER2+) cancer
Pertuzumab (Perjeta®)	HER2 (ERBB2/neu)	(HER2+)
Ado-trastuzumab emtansine (Kadcyla®)	HER2 (ERBB2/neu)	(HER2+)
Lapatinib (Tykerb®)	HER2 (ERBB2/neu), EGFR (HER1/ERBB1) (HER2+)	
Bevacizumab (Avastin®)	VEGF ligand	
Everolimus (Afinitor®)	mTOR	(HR+, HER2-)
Palbociclib (Ibrance®)	CDK4, CDK6	(ER+, HER2-)

References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65(2):87–108.
2. Malvezzi M, Carioli G, Bertuccio P, Rosso T, Boffetta P, Levi F, La Vecchia C, Negri E. European cancer mortality predictions for the year 2016 with focus on leukemias. Ann Oncol. 2016;27:725–31.
3. Akram M, Siddiqui SA. Breast cancer management: past, present and evolving. Indian J Cancer. 2012;49(3):277–82.
4. Raven RW. Cancer of the breast treated by oophorectomy. Br Med J. 1950;1(4666):1343–5.
5. Huggins C, Dao TL. Adrenalectomy and oophorectomy in treatment of advanced carcinoma of the breast. J Am Med Assoc. 1953;151(16):1388–94.
6. Johansson H, Terenius L, Thorén L. The binding of estradiol-17beta to human breast cancers and other tissues in vitro. Cancer Res. 1970;30(3):692–8.
7. Stem DF. Tyrosine kinase signaling in breast cancer. Breast Cancer Res. 2000;2(3):176–83.
8. Rubin I, Yarden Y. The basic biology of HER2. Ann Oncol. 2001;12(Suppl 1):S3–8.
9. Citri A, Yarden Y. EGF-ERBB signalling: towards the systems level. Nat Rev Mol Cell Biol. 2006;7(7):505–16.
10. Graus-Porta D, Beerli RR, Daly JM, Hynes NE. ErbB-2, the preferred heterodimerization partner of all ErbB receptors, is a mediator of lateral signaling. EMBO. 1997;16(7):1647–55.
11. Lee-Hoeflich ST, Crocker L, Yao E, Pham T, Munroe X, Hoeflich KP, et al. A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. Cancer Res. 2008;68(14):5878–87.

12. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. Science. 1987;235:177–82.
13. Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, Hortobagyi GN. The HER-2 receptor and breast cancer: ten years of targetedanti-HER-2 therapy and personalized medicine. Oncologist. 2009;14:320–68.
14. Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, et al. Molecular portraits of human breast tumours. Nature. 2000;406:747–52.
15. Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, et al. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A. 2001;98:10869–74.
16. Curtis C, Shah SP, Chin SF, Turashvili G, Rueda OM, Dunning MJ, et al. The genomic and transcriptomic architecture of 2000 breast tumours reveals novel subgroups. Nature. 2012;486:346–52.
17. Harbeck N, Salem M, Nitz U, Gluz O, Liedtke C. Personalized treatment of early-stage breast cancer: present concepts and future directions. Cancer Treat Rev. 2010;36:584–94.
18. Harbeck N, Gnant M. Breast cancer. Lancet. 2016;6736(16):31891–8.
19. Goldhirsch A, Winer EP, Coates AS, Gelber RD, Piccart-Gebhart M, Thürlimann B, Senn HJ, Panel Members. Personalizing the treatment of women with early breast cancer: highlights of the St Gallen international expert consensus on the primary therapy of early breast cancer 2013. Ann Oncol. 2013;24:2206–23.
20. Coates AS, Winer EP, Goldhirsch A, Gelber RD, Gnant M, Piccart-Gebhart M, Thürlimann B, Senn HJ, Panel Members. Tailoring therapies-improving the management of early breast cancer: St Gallen international expert consensus on the primary therapy of early breast cancer 2015. Ann Oncol. 2015;26:1533–46.
21. Polley MY, Leung SC, McShane LM, Gao D, Hugh JC, Mastropasqua MG, et al. International Ki67 in breast cancer working Group of the Breast International Group and North American Breast Cancer Group. An international Ki67 reproducibility study. J Natl Cancer Inst. 2013;105:1897–906.
22. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Relevance of breast cancer hormone receptors and other factors to the efficacy of adjuvant tamoxifen: patient-level meta-analysis of randomised trials. Lancet. 2011;378:771–84.
23. Fleming GF, Colleoni M, Láng I, Gomez HL, Tondini C, Burstein HJ, et al. TEXT and SOFT investigators; international breast cancer study group. Adjuvant exemestane with ovarian suppression in premenopausal breast cancer. N Engl J Med. 2014;371:107–18.
24. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Comparisons between different polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100,000 women in 123 randomised trials. Lancet. 2012;379:432–44.
25. Citron ML, Berry DA, Cirrincione C, Hudis C, Winer EP, Gradishar WJ, et al. Randomized trial of dose-dense versus conventionally scheduled and sequential versus concurrent combination chemotherapy as postoperative adjuvant treatment of node-positive primary breast cancer: first report of intergroup trial C9741/cancer and Leukemia group B trial 9741. J Clin Oncol. 2003;21:1431–9.
26. Eiermann W, Pienkowski T, Crown J, Sadeghi S, Martin M, Chan A, et al. Phase III study of doxorubicin/cyclophosphamide with concomitant versus sequential docetaxel as adjuvant treatment in patients with human epidermal growth factor receptor 2-normal, node-positive breast cancer: BCIRG-005 trial. J Clin Oncol. 2011;29:3877–84.
27. Chavez-MacGregor M, Clarke CA, Lichtensztajn DY, Giordano SH. Delayed initiation of adjuvant chemotherapy among patients with breast cancer. JAMA Oncol. 2016;2:322–9.
28. Sikov WM, Berry DA, Perou CM, Singh B, Cirrincione CT, Tolaney SM, et al. Impact of the addition of carboplatin and/or bevacizumab to neoadjuvant once-per-week paclitaxel followed by dose-dense doxorubicin and cyclophosphamide on pathologic complete response rates in stage II to III triple-negative breast cancer: CALGB 40603 (alliance). J Clin Oncol. 2015;33:13–21.

29. Filipits M, Rudas M, Jakesz R, Dubsky P, Fitzal F, Singer CF, For the EP Investigators, et al. A new molecular predictor of distant recurrence in ER-positive, HER2-negative breast cancer adds independent information to conventional clinical risk factors. Clin Cancer Res. 2011;17:6012–20.
30. van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, et al. A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med. 2002;347:1999–2009.
31. Paik S, Shak S, Tang G, Kim C, Baker J, Kim W, et al. A multigene assay to predict recurrence of tamoxifen-treated, node-negative breast cancer. N Engl J Med. 2004;351:2817–26.
32. Dowsett M, Nielsen TO, A'Hern R, Bartlett J, Coombes RC, Cuzick J, For the International Ki-67 in Breast Cancer Working Group, et al. Assessment of Ki67 in breast cancer: recommendations from the international Ki67 in breast cancer working group. J Natl Cancer Inst. 2011;103:1656–64.
33. Sparano JA, Gray RJ, Makower DF, Pritchard KI, Albain KS, Hayes DF, et al. Prospective validation of a 21-gene expression assay in breast cancer. N Engl J Med. 2015;373:2005–14.
34. Gluz O, Nitz U, Christgen M, Kates RE, Shak S, Clemens M, et al. West German study group phase III PlanB trial: first prospective outcome data for the 21-gene recurrence score assay and concordance of prognostic markers by central and local pathology assessment. J Clin Oncol. 2016;34:2341–9.
35. Harbeck N, Schmitt M, Meisner C, Friedel C, Untch M, Schmidt M, for the Chemo-N 0 Study Group, et al. Ten-year analysis of the prospective multicentre chemo-N0 trial validates American Society of Clinical Oncology (ASCO)-recommended biomarkers uPA and PAI-1 for therapy decision making in node negative breast cancer patients. Eur J Cancer. 2013;49:1825–35.
36. Look MP, van Putten WL, Duffy MJ, Harbeck N, Christensen IJ, Thomssen C, et al. Pooled analysis of prognostic impact of urokinase-type plasminogen activator and its inhibitor PAI-1 in 8377 breast cancer patients. J Natl Cancer Inst. 2002;94:116–28.
37. Jacobs VR, Kates RE, Kantelhardt E, Vetter M, Wuerstlein R, Fischer T, et al. Health economic impact of risk group selection according to ASCO-recommended biomarkers uPA/PAI-1 in node-negative primary breast cancer. Breast Cancer Res Treat. 2013;138:839–50.
38. Cole MP, Jones CT, Todd ID. A new anti-oestrogenic agent in late breast cancer. An early clinical appraisal of ICI46474. Br J Cancer. 1971;25:270–5.
39. Maximov PY, Lee TM, Jordan VC. The discovery and development of selective estrogen receptor modulators (SERMs) for clinical practice. Curr Clin Pharmacol. 2013;8(2):135–55.
40. Ward HW. Anti-oestrogen therapy for breast cancer: a trial of tamoxifen at two dose levels. Br Med J. 1973;1:13–4.
41. Magon N. Gonadotropin releasing hormone agonists: expanding vistas. Indian J Endocrinol Metabol. 2011;15(4):261–7.
42. Nicholson RI, Maynard PV. Anti-tumour activity of ICI 118630, a new potent luteinizing hormone-releasing hormone agonist. Br J Cancer. 1979;39(3):268–73.
43. Legro RS, Brzyski RG, Diamond MP, Coutifari C, Schlaff WD, Casson P, et al. Letrozole versus clomiphene for infertility in the polycystic ovary syndrome. N Engl J Med. 2014;371:119–29.
44. Early Breast Cancer Trialists' Collaborative Group (EBCTCG). Aromatase inhibitors versus tamoxifen in early breast cancer: patient-level meta-analysis of the randomised trials. Lancet. 2015;386:1341–52.
45. Dowsett M, Cuzick J, Ingle J, Coates A, Forbes J, Bliss J, et al. Meta-analysis of breast cancer outcomes in adjuvant trials of aromatase inhibitors versus tamoxifen. J Clin Oncol. 2010;28:509–18.
46. Pagani O, Regan MM, Walley BA, Fleming GF, Colleoni M, Láng I, For the TEXT and SOFT Investigators, International Breast Cancer Study Group, et al. Adjuvant exemestane with ovarian suppression in premenopausal breast cancer. N Engl J Med. 2014;371:107–18.
47. Francis PA, Regan MM, Fleming GF, Láng I, Ciruelos E, Bellet M, For the SOFT Investigators, International Breast Cancer Study Group, et al. Adjuvant ovarian suppression in premenopausal breast cancer. N Engl J Med. 2015;372:436–46.

48. Flemming J, Madarnas Y, Franek JA. Fulvestrant for systemic therapy of locally advanced or metastatic breast cancer in postmenopausal women: a systematic review. Breast Cancer Res Treat. 2009;115(2):255–68.
49. Slamon D, Eiermann W, Robert N, Pienkowski T, Martin M, Press M, Breast Cancer International Research Group, et al. Adjuvant trastuzumab in HER2-positive breast cancer. N Engl J Med. 2011;365:1273–83.
50. Gianni L, Dafni U, Gelber RD, Azambuja E, Muehlbauer S, Goldhirsch A, Herceptin Adjuvant (HERA) Trial Study Team, et al. Treatment with trastuzumab for 1 year after adjuvant chemotherapy in patients with HER2-positive early breast cancer: a 4-year follow-up of a randomized controlled trial. Lancet Oncol. 2011;12:236–44.
51. Buzdar AU, Ibrahim NK, Francis D, Booser DJ, Thomas ES, Theriault RL, et al. Significantly higher pathologic complete remission rate after neoadjuvant therapy with trastuzumab, paclitaxel, and epirubicin chemotherapy: results of a randomized trial in human epidermal growth factor receptor 2-positive operable breast cancer. J Clin Oncol. 2005;23(16):3676–85.
52. Marty M, Cognetti F, Maraninchi D, Snyder R, Mauriac L, Tubiana-Hulin M, et al. Randomized phase II trial of the efficacy and safety of trastuzumab combined with docetaxel in patients with human epidermal growth factor receptor 2-positive metastatic breast cancer administered as first line treatment: the M77001 study group. J Clin Oncol. 2005;23:4265–74.
53. Wolff AC, Hammond ME, Hicks DG, Dowsett M, McShane LM, Allison KH, et al. Recommendations for human epidermal growth factor receptor 2 testing in breast cancer: American Society of Clinical Oncology/College of American Pathologists clinical practice guideline update. J Clin Oncol. 2013;31:3997–4013.
54. Ewer SM, Ewer MS. Cardiotoxicity profile of trastuzumab. Drug Saf. 2008;31(6):459–67.
55. Turner N, Biganzoli L, Di Leo A. Continued value of adjuvant anthracyclines as treatment for early breast cancer. Lancet Oncol. 2015;16(7):e362–e9.
56. Huober J, Fasching PA, Barsoum M, Petruzelka L, Wallwiener D, Thomssen C, et al. Higher efficacy of letrozole in combination with trastuzumab compared to letrozole monotherapy as first-line treatment in patients with HER2-positive, hormone-receptor-positive metastatic breast cancer—results of the eLEcTRA trial. Breast. 2012;21:27–33.
57. Sang RY, Finn RS. Beyond trastuzumab: novel therapeutic strategies in HER2-positive metastatic breast cancer. Br J Cancer. 2012;106(1):6–13.
58. Swain SM, Im YH, Im SA, Chan V, Miles D, Knott A, et al. Safety profile of pertuzumab with trastuzumab and docetaxel in patients from Asia with human epidermal growth factor receptor 2-positive metastatic breast cancer: results from the phase III trial CLEOPATRA. Oncologist. 2014;19(7):693–701.
59. Lewis Phillips GD, Li G, Dugger DL, Crocker LM, Parsons KL, Mai E, Blättler WA, Lambert JM, et al. Targeting HER2-positive breast cancer with trastuzumab-DM1, an antibody-cytotoxic drug conjugate. Cancer Res. 2008;68(22):9280–90.
60. Schettini F, Buono G, Cardalesi C, Desideri I, De Placido S, Del Mastro L. Hormone receptor/human epidermal growth factor receptor 2-positive breast cancer: where we are now and where we are going. Cancer Treat Rev. 2016;46:20–6.
61. Miles DW, Dieras V, Cortes J, Duenne AA, Yi J, O'Shaughnessy J. First-line bevacizumab in combination with chemotherapy for HER2-negative metastatic breast cancer: pooled and subgroup analyses of data from 2447 patients. Ann Oncol. 2013;24:2773–80.
62. Vinayak S, Carlson RW. mTOR inhibitors in the treatment of breast cancer. Oncology. 2013;27(1):38–44.
63. Yardley DA. Combining mTOR inhibitors with chemotherapy and other targeted therapies in advanced breast cancer: rationale, clinical experience, and future directions. Breast Cancer (Auckl). 2013;7:7–22.
64. Piccart M, Hortobagyi GN, Campone M, Pritchard KI, Lebrun F, Ito Y, et al. Everolimus plus exemestane for hormone-receptor-positive, human epidermal growth factor receptor-2-negative advanced breast cancer: overall survival results from BOLERO-2. Ann Oncol. 2014;25:2357–62.

65. Mancini P, Angeloni A, Risi E, Orsi E, Mezi S. Standard of care and promising new agents for triple negative metastatic breast cancer. Cancer. 2014;6(4):2187–223.
66. Gonzalez-Angulo AM, Akcakanat A, Liu S, Green MC, Murray JL, Chen H, et al. Open-label randomized clinical trial of standard neoadjuvant chemotherapy with paclitaxel followed by FEC versus the combination of paclitaxel and everolimus followed by FEC in women with triple receptor-negative breast cancerdagger. Ann Oncol. 2014;25(6):1122–7.
67. Finn RS, Crown JP, Lang I, Boer K, Bondarenko IM, Kulyk SO, et al. The cyclin-dependent kinase 4/6 inhibitor palbociclib in combination with letrozole versus letrozole alone as first-line treatment of oestrogen receptor-positive, HER2-negative, advanced breast cancer (PALOMA-1/TRIO-18): a randomised phase 2 study. Lancet Oncol. 2015;16:25–35.
68. Turner NC, Ro J, Andre F, Loi S, Verma S, Iwata H, et al. Palbociclib in hormone-receptor-positive advanced breast cancer. N Engl J Med. 2015;373:209–19.
69. Hortobagyi GN, Stemmer SM, Burris HA, Yap YS, Sonke GS, Paluch-Shimon S, et al. Ribociclib as first-line therapy for hr-positive, advanced breast cancer. N Engl J Med. 2016;375:1738–48.

Chapter 3
Clinical Application of Stem Cell Biology in Esophageal Cancer

Tomoyuki Okumura, Hirohumi Kojima, Tetsuji Yamaguchi, and Yutaka Shimada

Abstract Despite recent progress in its diagnosis and multimodal therapies, prognosis in patients with esophageal squamous cell carcinoma (ESCC) remains poor, because of high incidence of metastasis and therapeutic resistance.

Cancer stem cells (CSCs) represent a small number of cells which possess stem cell properties, such as self-renewal ability, tumorigenicity, metastasis, and chemoresistance. Recently, mitotically quiescent CSCs have been demonstrated in tumors, which enhance malignant potential. In ESCC, several candidate CSC markers have been reported, such as p75 neurotrophin receptor (p75NTR), CD44, and CD90. Reports using ESCC cell lines demonstrated that majority of p75NTR-positive/CD44-negative/CD90-negative cells were in a mitotically quiescent state, along with significantly higher stem cell properties, indicating that the cells represent a candidate quiescent CSC population. Stem cell-related molecules, such as Hedgehog, Bmi-1, p63, and Nanog, as well as chemoresistant molecules, such as ABCG2 and ERCC1, were strongly expressed in the p75NTR-positive fraction, suggesting potential use of these molecules for therapeutic application. Interactions between p75NTR-positive candidate CSCs and tumor microenvironment, such as tumor matrix, cytokines, various types of stromal cells, and immune checkpoints, are now under investigation. For diagnostic application, our two-color flow

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cytometric investigation using peripheral blood samples of ESCC patients revealed that p75NTR-positive, but not p75NTR-negative, circulating tumor stem cell counts correlated with clinically diagnosed distant metastasis and pathological venous invasion in ESCC tumors.

Further investigation targeting CSCs which are specifically characterized by multiple markers may provide us with novel therapeutic and diagnostic strategies for patients with ESCC.

Keywords Esophageal cancer • Cancer stem cells • p75NTR • Circulating tumor cells

3.1 Esophageal Squamous Cell Carcinoma (ESCC)

Despite recent progress in its diagnosis and multimodal therapies, such as preoperative chemo- or chemoradiotherapy followed by radical surgery [1, 2], many of the patients with locally advanced esophageal squamous cell carcinoma (ESCC) still exhibit postoperative tumor recurrence with a 5-year progression-free survival rate of about 40% [3, 4], indicating the presence of chemoresistant micrometastasis [5].

3.2 Cancer Stem Cells

Cancer stem cells (CSCs) represent a small number of cells which possess stem cell properties, such as self-renewal ability, tumorigenicity, and chemo- and/or radioresistance [6, 7]. CSCs are also known to play a key role in metastasis through epithelial-mesenchymal transition (EMT), invasion into vessels, systemic circulation, and tumor initiation in the metastatic sites [8–10]. Recently, the existence of mitotically quiescent CSCs has been demonstrated in tumors, such as melanoma [11], ovarian [12], breast [13], and pancreatic cancer [14]. These "dormant CSCs" even enhance invasiveness, metastasis, and chemoresistance, consequently leading a late relapse after surgery [13–18].

3.3 Proposed Cancer Stem Cell Markers in ESCC

Several cell surface antigens were reported to be expressed in candidate CSCs in ESCC, such as p75 neurotrophin receptor (p75NTR, CD271), CD44, CD90, ABC subfamily G member 2 (ABCG2), and aldehyde dehydrogenase (ALDH)-1 [19–24]. In addition, functional assays using flow cytometry, such as dye-effluxing side population, which is based on ABCG2 expression [25], and Aldefluor, which was based on ALDH-1 expression [26, 27], have been reported to isolate candidate CSCs in ESCC.

All these markers were reported to identify certain subpopulations with stem cell properties in vitro. However, the expression of these molecules was heterogeneous among tumors, even within a tumor, which required a combination of multiple markers for more specific identification of CSCs [28–30].

3.4 p75NTR as a Candidate Cancer Stem Cell Marker in ESCC

p75NTR is a 75-kDa cell surface receptor glycoprotein, which is a member of the tumor necrosis factor receptor superfamily [31] and involved in regulation of malignant phenotypes in various types of cancer.

p75NTR is expressed in mitotically quiescent basal cells in normal esophageal epithelium [32, 33], while it is expressed in the infiltrating margin of ESCC tumor [20, 29].

Reports using ESCC cell lines demonstrated that p75NTR-positive cells have higher ability of sphere/colony formation, higher tumorigenicity in mouse xenograft model, and resistance to chemotherapy [19, 20, 29, 34].

Detailed observation using combined expression of p75NTR, CD44, CD90, and a proliferation marker ki67 revealed that majority of the p75NTR-positive/CD44-negative/CD90-negative cells were in the resting phase of cell cycle in surgically resected ESCC tumors ([29, 33], Fig. 3.1). In addition, investigation using ESCC cell lines demonstrated that majority of p75NTR-positive/CD44-negative/CD90-negative cells isolated by flow cytometry were in a mitotically quiescent state, along with significantly higher colony formation (Fig. 3.2a), enhanced tumor formation in mice (Fig. 3.2b), and greater chemoresistance (Fig. 3.2c), indicating that p75NTR-positive/CD44-negative/CD90-negative cells were candidate quiescent CSCs in ESCC [29].

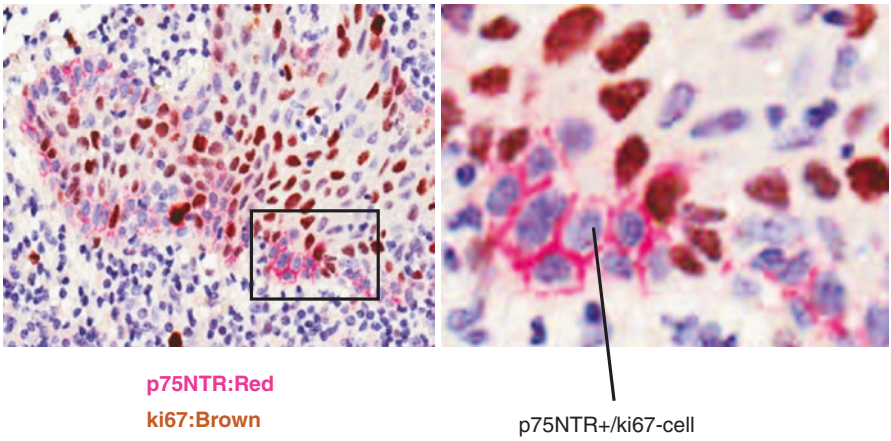
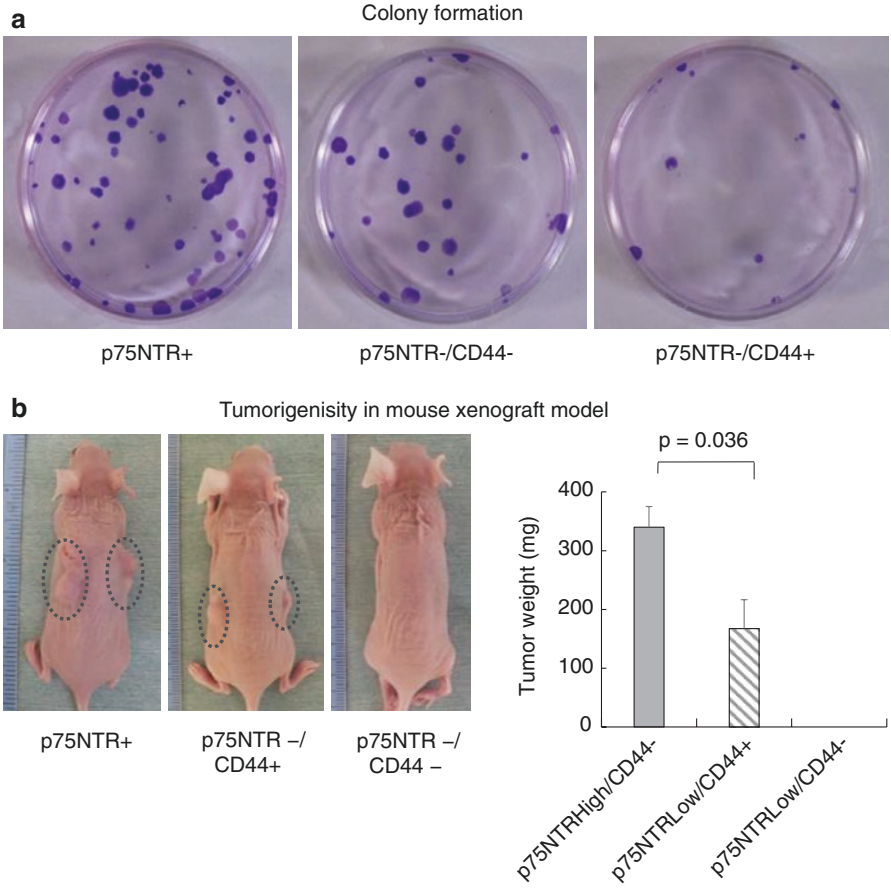


Fig. 3.1 A representative photograph of immunohistochemical staining for p75NTR in ESCC specimens (Reproduced from [29])


a
Colony formation
p75NTR+
p75NTR-/CD44-
p75NTR-/CD44+
b
Tumorigenisity in mouse xenograft model
p75NTR+
p75NTR –/ CD44+
p75NTR –/ CD44 –
p = 0.036
Tumor weight (mg)
400
300
200
100
0
p75NTRHigh/CD44-
p75NTRLow/CD44+
p75NTRLow/CD44-
c
Resistance to chemotherapy
MTT assay
Cell viability (%)
90
75
60
45
30
15
0
p75NTR+/CD44-
p75NTR-/CD44+
p75NTR-/CD44-
25
50
100
200
Cisplatin (μM)

3.5 Molecular Mechanisms Which Regulate Cancer Stem Cells in ESCC

3.5.1 CSC-Specific Signal Transduction Pathways

Signal transduction pathways which regulate embryonic and normal tissue stem cells, such as Wnt/*β*-catenin, Notch, Hedgehog (Hh), and Bmi-1, have been reported to play a crucial role in stem cell self-renewal in various types of cancer [35, 36]. In ESCC, activation of the Wnt/β-catenin pathway promotes tumor sphere formation and in vivo tumorigenesis under control of microRNAs [37, 38]. Glioma-associated oncogene homolog 1 (Gli1), a key mediator of the Hh pathway, is involved in malignant potential of ESCC [39, 40]. Bmi-1 has been also reported to play an important role in controlling cell proliferation and self-renewal of esophageal cancer stemlike cells [41]. The oncogene p63 has been reported to be expressed in normal stem cells and regulates the proliferation and differentiation of keratinocytes in the epidermis and esophagus [42, 43]. Reports demonstrated p63-mediated regulation of growth and invasion in ESCC cell lines [44, 45]. Growth inhibition and epithelial differentiation along with downregulation of p63 by induced expression of mir-203 have also been reported [46].

Embryonic pluripotency markers, such as Oct-4, Sox2, and Nanog transcription factors, have been linked to the stemlike phenotype of CSCs [47]. In ESCC, Nanog expression was found to be an independent prognostic factor [48]. In addition, induction of Nanog expression in ESCC cell lines resulted in promotion of proliferation, invasiveness, and drug resistance against CDDP [49].

In p75NTR-positive candidate CSCs, stem cell-related genes, such as Nanog, Bmi-1, and p63, were strongly and exclusively expressed [19, 29, 30, 34], suggesting that these molecules have a key role in regulating stem cell properties in p75NTR-positive candidate CSCs.

3.5.2 Drug Resistance

Most of cancer stem cells express ATP-binding cassette (ABC) transporter proteins, such as ABCB1, ABCC1, ABCC2, and ABCG2, which can transport and excrete substances including metabolites, toxic substances, and anticancer drugs, and thus they have been associated with multidrug resistance [50, 51]. The overexpression of

Fig. 3.2 Stem cell phenotype of isolated p75NTR-positive/CD44-negative/CD90-negative cells. (**a**) Colony formation, (**b**) Tumor formation in mice, (**c**) Chemoresistance against cisplatin. (Reproduced from [29])

ABCG2 has been reported to be associated with response to chemotherapy and prognosis in ESCC [52].

In addition, p75NTR-positive cell fraction of ESCC cell lines have chemoresistance against CDDP, along with strong expression of ABCG2 and ERCC1 [19, 29, 30, 34], suggesting the involvement of these molecules in chemoresistance of CSCs in ESCC.

3.5.3 Other CSC-Related Molecules

Tumor suppressor p53; retinoblastoma protein (RB); cyclin-dependent protein kinase inhibitors, such as p21, p27, and p57; Notch-related molecules; and various microRNAs have been reported to be involved in the regulation of stem cell quiescence [53]. Gene mutation and altered expression of these molecules in development and progression of ESCC have been reported [33, 54–58]. An immunohistochemical investigation demonstrated that the altered expression of hTERT and p53 first occurred in p75NTR-positive quiescent basal layer of the esophageal epithelium during progression to dysplasia, followed by phenotypic changes from quiescent to active proliferation in the basal layer [33], suggesting that oncogenic events that disrupt mitotic regulation in the p75NTR-positive quiescent basal layer may play a crucial role in malignant transformation.

The NGF/proNGF/p75NTR axis was demonstrated to play a critical role in regulating self-renewal of quiescent CSCs, as well as promoting EMT, in breast cancer [59]. In addition to the expression of p75NTR in the candidate CSCs, overexpression of NGF and its autocrine loop was also shown to enhance cell proliferation and migration in ESCC cell lines [52], suggesting p75NTR signaling plays a role in regulation of quiescent CSC in ESCC as well.

3.5.4 Interaction Between CSCs and Tumor Microenvironment

Tumor microenvironment, such as tumor matrix, cytokines, and various types of stromal cells, were shown to play important roles in regulating self-renewal and differentiation of CSCs [60]. In stromal cells, myeloid-derived suppressor cells (MDSCs), as well as tumor-associated monocytes and macrophages (TAMs), have been demonstrated to create a stem cell niche and control CSC phenotype in pancreatic, ovarian, and breast cancer [61–63]. Inflammatory cytokines, such as IL-1, IL-6, and IL-8, have shown to regulate CSC self-renewal, tumor growth, and EMT, through modulating MDSCs, monocytes, and/or macrophages [64–66]. Involvement of these cytokines and tumor-infiltrating stromal cells in malignant potential of ESCC has also been demonstrated [67–74]. Immune checkpoints, such as the programmed cell death-1 (PD-1)/PD-L1 axis and the cytotoxic T lymphocyte antigen 4

(CTLA-4)/B7 axis, have been shown to suppress T cell-mediated immune response to protect CSCs in various types of cancer, including head and neck squamous cell carcinoma [75, 76]. The expression of PD-L1 is associated with poor prognosis in patients with ESCC [67, 69, 77], suggesting a role of immune checkpoints in survival and progression of CSCs in ESCC, as well.

Further investigations focusing on the interaction between tumor microenvironment and candidate CSCs in ESCC may provide us with a better understanding of CSC regulation in ESCC.

3.6 Clinical Application of Stem Cell Biology in Esophageal Cancer

3.6.1 Therapeutic Application

Therapeutic application of stem cell biology includes directory targeting the CSCs to eliminate them taking advantages of specific marker expression, the stem cell-specific signaling pathways, and CSC niche in tumor microenvironment.

Inhibition of Wnt-β-catenin pathway with small molecules, such as nonsteroidal anti-inflammatory drugs and retinoic acid, has been shown to have anti-tumorigenic effects in ESCC [39, 40, 78–80], as well as colon cancer [81–83]. Trastuzumab has been shown to target tumor-initiating cells via disrupting notch pathway in breast cancer [84]. In ESCC, targeting EGFR and HER-2 with cetuximab and trastuzumab has been shown to have potential therapeutic significance [85, 86].

Hedgehog signaling pathway inhibitors, such as cyclopamine and vismodegib, have shown therapeutic effect in various solid tumors, such as basal cell and pancreatic cancer [87, 88]. In ESCC, cyclopamine has been shown to inhibit proliferation and migration in vitro [89].

Targeting the tumor microenvironment, inhibitors against inflammatory cytokines, such as IL-1, IL-6, and IL-8, and their receptors have been shown to disrupt cancer stem cell phenotype in prostate cancer and breast cancer cells [90–92]. Immune checkpoint inhibitors, such as PD-1 and PD-L1 antibodies, have been shown to overcome immune resistance against CSCs in non-small-cell lung cancer, melanoma, renal cell cancer, and Hodgkin's lymphoma [93, 94]. Several strategies in immune targeting against CSCs have been reported, such as adoptive transfer of CSC-primed T cells in solid tumors [95] and CSC-based dendritic cell vaccines [96–98].

Reports have demonstrated strategies to target CSCs using nanoparticles, such as conventional chemotherapeutic drugs, against the CSC surface markers in solid tumors including breast and pancreatic cancer [91, 92, 99–102].

Because fundamental biological processes which regulate CSCs are commonly crucial in various types of cancer, these therapeutic strategies are promising for development of novel therapies targeting CSCs in ESCC, as well.

3.6.2 *Diagnostic Application*

Diagnostic application of stem cell biology includes detection of CSCs in various samples, such as tumor specimens and peripheral blood, as biomarker for early detection and for personalized therapies. In recent years, reports have demonstrated that circulating tumor cells (CTCs) in peripheral blood sample of the patients were detected based on the expression of epithelial-specific markers, such as EpCAM [103]. Quantification of CTCs has been reported to be an early detection marker for metastasis and prognostic factor in patients with various types of cancer [104–106]. More recent reports suggested that circulating tumor stem cells (CTSCs), rather than CTCs, were more accurate diagnostic marker for metastasis ([6, 7], Grover et al. 2014), because CSCs are responsible for metastasis through processes such as epithelial-mesenchymal transition (EMT), circulation, and tumor initiation in the metastatic sites [8–10].

Our two-color flow cytometric investigation using peripheral blood samples of ESCC patients (Fig. 3.3) revealed that EpCAM+p75NTR+cell count was significantly higher in ESCC patients than healthy controls [30]. More importantly, EpCAM+p75NTR+, but not EpCAM+p75NTR- CTC counts, correlated with clinically diagnosed distant metastasis ($p = 0.003$, Table 3.1) and pathological venous invasion in surgically resected primary ESCC tumors ($p = 0.016$, Table 3.2), suggesting that detection of CTSCs based on the expression of p75NTR was a more accurate diagnostic marker than CTC detection using EpCAM alone. Investigations based on large-scale prospective studies with long-term follow-up are now ongoing to provide evidences for its clinical use.

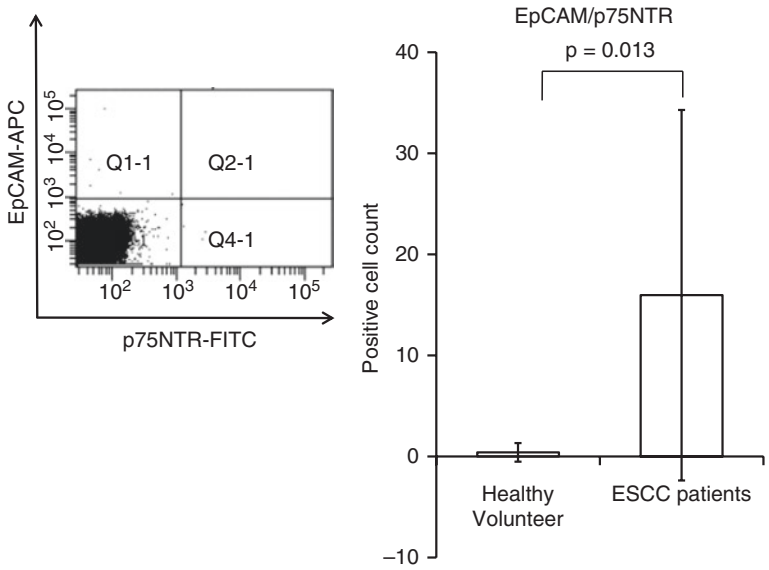


Fig. 3.3 Mononuclear cells from 3 mL peripheral blood of an ESCC patient were co-stained with anti-EpCAM-APC and anti-p75NTR-FITC, and analyzed by two-color flow cytometry (left panel). Quadrant markers were set according to isotype-matched controls. EpCAM+p75NTR+cell count was significantly higher in ESCC patients than healthy controls (right panel). (Reproduced from [30] with permission from BioMed Central)

Table 3.1 Relationship between the clinicopathological features and mean EpCAM + p75NTR + or EpCAM + p75NTR- CTC counts in patients who underwent surgery (Reproduced from [30] with permission from BioMed Central)

Characteristics	*n*	EpCAM + p75NTR + (average ± SD)	*p*-value	EpCAM + p75NTR- (average±SD)	*p*-value
T 0–2/3–4	1/12	7.0/16.4 ± 13.0	0.501	0/23.9 ± 30.6	0.468
N 0/1–3	1/12	7.0/16.4 ± 13.0	0.501	0/23.9 ± 30.6	0.468
M 0/1	8/5	7.1 ± 5.9/29.4 ± 6.4	*0.003	19.5 ± 20.8/26.2 ± 43.7	0.713
Stage1–2/3–4	1/12	7.0/16.4 ± 13.0	0.501	0/23.9 ± 30.6	0.468

CTC Circulating tumor cell, *SD* standard deviation
$^{*}p < 0.05$

Table 3.2 Relationship between the clinical features and mean EpCAM + p75NTR + or EpCAM + p75NTR- CTC counts in patients who received chemotherapy or chemoradiotherapy (Reproduced from [30] with permission from BioMed Central)

Characteristics	*n*	EpCAM + p75NTR + (average ± SD)	*p*-value	EpCAM + p75NTR- (average ± SD)	*p*-value
pT 0–2/3–4	4/6	2.0 ± 2.8/26.2 ± 29.3	0.146	27.0 ± 50.1/ 2.0 ± 4.9	0.246
pN 0/1–3	7/3	14.9 ± 27.7/20.3 ± 23.1	0.773	0.8 ± 2.3/ 38.0 ± 55.7	0.090
Ly 0/1–2	7/3	14.9 ± 27.7/20.3 ± 23.1	0.773	0.8 ± 2.3/ 38.0 ± 55.7	0.090
v 0/1–2	7/3	5.0 ± 4.2/43.3 ± 35.6	*0.016	0.8 ± 2.3/ 38.0 ± 55.7	0.090
pStage 1–2/3–4	7/3	15.7 ± 27.2/18.3 ± 25.1	0.891	15.4 ± 38.2/4.0 ± 6.9	0.632

CTC Circulating tumor cell, *ly* lymphatic invasion, *v* venous invasion, *SD* standard deviation
$^{*}p < 0.05$

3.7 Summary

Identification and characterization of candidate CSCs using various markers have been reported in ESCC, enabling us to investigate biological processes in more specific cell subsets based on combined expression of multiple markers. Our investigation using ESCC cell lines revealed that majority of p75NTR-positive/CD44-negative/CD90-negative cells were in a mitotically quiescent state, along with higher colony formation, enhanced tumorigenicity, and greater chemoresistance, indicating that the cells represent a quiescent CSC fraction. Investigation in molecular mechanisms which regulate stem cell properties in our candidate CSC fraction will now be underway. With recent progress in clinical application of CSC research in various types of cancer, further investigation targeting candidate CSCs in ESCC may lead to development of novel therapeutic and diagnostic strategies in the near future.

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References

1. Thallinger CM, Raderer M, Hejna M. Esophageal cancer: a critical evaluation of systemic second-line therapy. J Clin Oncol. 2011;29:4709–14.
2. van Hagen P, Hulshof MC, van Lanschot JJ, et al. Preoperative chemoradiotherapy for esophageal or junctional cancer. N Engl J Med. 2012;366(22):2074–84.
3. Ando N, Kato H, Igaki H, et al. A randomized trial comparing postoperative adjuvant chemotherapy with cisplatin and 5-fluorouracil versus preoperative chemotherapy for localized advanced squamous cell carcinoma of the thoracic esophagus (JCOG9907). Ann Surg Oncol. 2012;19(1):68–74.
4. Hara H, Tahara M, Daiko H, et al. Phase II feasibility study of preoperative chemotherapy with docetaxel, cisplatin, and fluorouracil for esophageal squamous cell carcinoma. Cancer Sci. 2013;104(11):1455–60.
5. Kell MR, Winter DC, O'Sullivan GC, et al. Biological behaviour and clinical implications of micrometastases. Br J Surg. 2000;87(12):1629–39.
6. Clarke MF, Dick JE, Dirks PB, et al. Cancer stem cells-perspectives on current status and future directions: AACR workshop on cancer stem cells. Cancer Res. 2006;66(19):9339–44.
7. Reya T, Morrison SJ, Clarke MF, et al. Stem cells, cancer, and cancer stem cells. Nature. 2001;414(6859):105–11.
8. Dean M, Fojo T, Bates S. Tumour stem cells and drug resistance. Nat Rev Cancer. 2005;5(4):275–84.
9. Schatton T, Frank MH. Cancer stem cells and human malignant melanoma. Pigment Cell Melanoma Res. 2008;21(1):39–55.
10. Wicha MS, Hayes DF. Circulating tumor cells: not all detected cells are bad and not all bad cells are detected. J Clin Oncol. 2011;29(15):1508–11.
11. Roesch A, Fukunaga-Kalabis M, Schmidt EC, et al. A temporarily distinct subpopulation of slow-cycling melanoma cells is required for continuous tumor growth. Cell. 2010;141:583–94.
12. Gao MQ, Choi YP, Kang S, et al. CD24+ cells from hierarchically organized ovarian cancer are enriched in cancer stem cells. Oncogene. 2010;29(18):2672–80.
13. Pece S, Tosoni D, Confalonieri S, et al. Biological and molecular heterogeneity of breast cancers correlates with their cancer stem cell content. Cell. 2010;140(1):62–73.
14. Dembinski JL, Krauss S. Characterization and functional analysis of a slow cycling stem cell-like subpopulation in pancreas adenocarcinoma. Clin Exp Metastasis. 2009;26(7):611–23.
15. Dick JE. Stem cell concepts renew cancer research. Blood. 2008;112(13):4793–807.
16. Li L, Bhatia R. Stem cell quiescence. Clin Cancer Res. 2011;17(15):4936–41.
17. Mani SA, Guo W, Liao MJ, et al. The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell. 2008;133(4):704–15.
18. Sosa MS, Bragado P, Aguirre-Ghiso JA. Mechanisms of disseminated cancer cell dormancy: an awakening field. Nat Rev. Cancer. 2014;9:611–22.
19. Huang SD, Yuan Y, Liu XH, et al. Self-renewal and chemotherapy resistance of p75NTR positive cells in esophageal squamous cell carcinomas. BMC Cancer. 2009;9:9.
20. Okumura T, Tsunoda S, Mori Y, et al. The biological role of the low-affinity p75 neurotrophin receptor in esophageal squamous cell carcinoma. Clin Cancer Res. 2006;12(17):5096–103.
21. Tang KH, Dai YD, Tong M, et al. A CD90(+) tumor-initiating cell population with an aggressive signature and metastatic capacity in esophageal cancer. Cancer Res. 2013;73(7):2322–32.
22. Zhao JS, Li WJ, Ge D, Zhang PJ, et al. Tumor initiating cells in esophageal squamous cell carcinomas express high levels of CD44. PLoS One. 2011;6(6):e21419.
23. Zhang G, Ma L, Xie YK, Miao XB, Jin C. Esophageal cancer tumor spheres involve cancer stem-like populations with elevated aldehyde dehydrogenase enzymatic activity. Mol Med Rep. 2012;6(3):519–24.
24. Zhang M, Mathur A, Zhang Y, et al. Mithramycin represses basal and cigarette smoke-induced expression of ABCG2 and inhibits stem cell signaling in lung and esophageal cancer cells. Cancer Res. 2012;72(16):4178–92.

25. Zhao Y, Bao Q, Schwarz B, et al. Stem cell-like side populations in esophageal cancer: a source of chemotherapy resistance and metastases. Stem Cells Dev. 2013;23(2):180–92.
26. Almanaa TN, Geusz ME, Jamasbi RJ. A new method for identifying stem-like cells in esophageal cancer cell lines. J Cancer. 2013;4(7):536–48.
27. Fujiwara D, Kato K, Nohara S, et al. The usefulness of three-dimensional cell culture in induction of cancer stem cells from esophageal squamous cell carcinoma cell lines. Biochem Biophys Res Commun. 2013;434(4):773–8.
28. Medema JP. Cancer stem cells: the challenges ahead. Nat Cell Biol. 2013;15(4):338–44.
29. Yamaguchi T, Okumura T, Hirano K, et al. p75 neurotrophin receptor expression is a characteristic of the mitotically quiescent cancer stem cell population present in esophageal squamous cell carcinoma. Int J Oncol. 2016;48(5):1943–54.
30. Yamaguchi T, Okumura T, Hirano K, et al. Detection of circulating tumor cells by p75NTR expression in patients with esophageal cancer. World J Surg Oncol. 2016;14(1):40.
31. Rodriguez-Tebar A, Dechant G, Gotz R, et al. Binding of neurotrophin-3 to its neuronal receptors and interactions with nerve growth factor and brain-derived neurotrophic factor. EMBOJ. 1992;11(3):917–22.
32. Okumura T, Shimada Y, Imamura M, et al. Neurotrophin receptor p75(NTR) characterizes human esophageal keratinocyte stem cells in vitro. Oncogene. 2003;22(26):4017–26.
33. Okumura T, Shimada Y, Sakurai T, et al. Abnormal cell proliferation in the p75NTR-positive basal cell compartment of the esophageal epithelium during squamous carcinogenesis. Dis Esophagus. 2015;28(7):634–43.
34. Li S, Yue D, Chen X, et al. Epigenetic regulation of CD271, a potential cancer stem cell marker associated with chemoresistance and metastatic capacity. Oncol Rep. 2015;33(1):425–32.
35. Borah A, Raveendran S, Rochani A, et al. Targeting self-renewal pathways in cancer stem cells: clinical implications for cancer therapy. Oncogene. 2015;4:e177.
36. Oren O, Smith BD. Eliminating cancer stem cells by targeting embryonic signaling pathways. Stem Cell Rev. 2016;13(1):17–23.
37. Ge C, Wu S, Wang W, et al. miR-942 promotes cancer stem cell-like traits in esophageal squamous cell carcinoma through activation of Wnt/β-catenin signalling pathway. Oncotarget. 2015;6(13):10964–71097.
38. He J, Zhou M, Chen X, et al. Inhibition of SALL4 reduces tumorigenicity involving epithelial-mesenchymal transition via Wnt/β-catenin pathway in esophageal squamous cell carcinoma. J Exp Clin Cancer Res. 2016;35(1):98. https://doi.org/10.1186/s13046-016-0378-z.
39. Yang Q, Wang R, Xiao W, et al. Cellular retinoic acid binding protein 2 is strikingly downregulated in human esophageal squamous cell carcinoma and functions as a tumor suppressor. PLoS One. 2016;11(2):e0148381. https://doi.org/10.1371/journal.pone.0148381.
40. Yang Z, Cui Y, Ni W, et al. Gli1, a potential regulator of esophageal cancer stem cell, is identified as an independent adverse prognostic factor in esophageal squamous cell carcinoma. J Cancer Res Clin Oncol. 2016;143(2):243–54.
41. Yu X, Jiang X, Li H, et al. miR-203 inhibits the proliferation and self-renewal of esophageal cancer stem-like cells by suppressing stem renewal factor Bmi-1. Stem Cells Dev. 2014;23(6):576–85. https://doi.org/10.1089/scd.2013.0308.
42. Daniely Y, Liao G, Dixon D, et al. Critical role of p63 in the development of a normal esophageal and tracheobronchial epithelium. Am J Phys Cell Phys. 2004;287:C171–81.
43. Truong AB, Kretz M, Ridky TW, et al. p63 regulates proliferation and differentiation of developmentally mature keratinocytes. Genes Dev. 2006;20:3185–97.
44. Lee KB, Ye S, Park MH, et al. p63-mediated activation of the β-catenin/c-Myc signaling pathway stimulates esophageal squamous carcinoma cell invasion and metastasis. Cancer Lett. 2014;353(1):124–32. https://doi.org/10.1016/j.canlet.2014.07.016.
45. Ye S, Lee KB, Park MH, et al. p63 regulates growth of esophageal squamous carcinoma cells via the Akt signaling pathway. Int J Oncol. 2014;44(6):2153–9. https://doi.org/10.3892/ijo.2014.2374.
46. Okumura T, Shimada Y, Moriyama M, et al. MicroRNA-203 inhibits the progression of esophageal squamous cell carcinoma with restored epithelial tissue architecture in vivo. Int J Oncol. 2014;44(6):1923–32. https://doi.org/10.3892/ijo.2014.2365.

47. Hadjimichael C, Chanoumidou K, Papadopoulou N, et al. Common stemness regulators of embryonic and cancer stem cells. World J Stem Cells. 2015;7(9):1150–84. https://doi.org/10.4252/wjsc.v7.i9.1150. Review
48. Shimada Y, Okumura T, Sekine S, et al. Expression analysis of iPS cell - inductive genes in esophageal squamous cell carcinoma by tissue microarray. Anticancer Res. 2012;32(12):5507–14.
49. Yang L, Zhang X, Zhang M, et al. Increased nanog expression promotes tumor development and cisplatin resistance in human esophageal cancer cells. Cell Physiol Biochem. 2012;30(4):943–52. https://doi.org/10.1159/000341471.
50. Di C, Zhao Y. Multiple drug resistance due to resistance to stem cells and stem cell treatment progress in cancer. Exp Ther Med. 2015;9(2):289–93.
51. Wang JL, Yu JP, Sun ZQ, Sun SP. Radiobiological characteristics of cancer stem cells from esophageal cancer cell lines. World J Gastroenterol. 2014;20(48):18296–305. https://doi.org/10.3748/wjg.v20.i48.18296.
52. Tsunoda S, Okumura T, Ito T, et al. ABCG2 expression is an independent unfavorable prognostic factor in esophageal squamous cell carcinoma. Oncology. 2006;71(3–4):251–8.
53. Cheung TH, Rando TA. Molecular regulation of stem cell quiescence. Nat Rev. Mol Cell Biol. 2013;14(6):329–40. https://doi.org/10.1038/nrm3591.
54. Dong S, Zhang P, Liang S, et al. The role of the retinoblastoma protein-interacting zinc finger gene 1 tumor suppressor gene in human esophageal squamous cell carcinoma cells. Oncol Lett. 2013;6(6):1656–62.
55. Kawamura T, Goseki N, Koike M, et al. Acceleration of proliferative activity of esophageal squamous cell carcinoma with invasion beyond the mucosa: immunohistochemical analysis of Ki-67 and p53 antigen in relation to histopathologic findings. Cancer. 1996;77:843–9.
56. Nakajima Y, Miyake S, Tanaka K, et al. The expressions of p21 and pRB may be good indicators for the sensitivity of esophageal squamous cell cancers to CPT-11: cell proliferation activity correlates with the effect of CPT-11. Cancer Sci. 2004;95(5):464–8.
57. Ohbu M, Kobayashi N, Okayasu I. Expression of cell cycle regulatory proteins in the multistep process of oesophageal carcinogenesis: stepwise over-expression of cyclin E and p53, reduction of p21(WAF1/CIP1) and dysregulation of cyclin D1 and p27(KIP1). Histopathology. 2001;39(6):589–96.
58. Zhang LY, Wu JL, Qiu HB, et al. PSCA acts as a tumor suppressor by facilitating the nuclear translocation of RB1CC1 in esophageal squamous cell carcinoma. Carcinogenesis. 2016;37(3):320–32. https://doi.org/10.1093/carcin/bgw010.
59. Tomellini E, Touil Y, Lagadec C, et al. Nerve growth factor and proNGF simultaneously promote symmetric self-renewal, quiescence, and epithelial to mesenchymal transition to enlarge the breast cancer stem cell compartment. Stem Cells. 2015;33(2):342–53. https://doi.org/10.1002/stem.1849.
60. Borovski T, De Sousa EMF, Vermeulen L, et al. Cancer stem cell niche: the place to be. Cancer Res. 2011;71:634–9.
61. Cui TX, Kryczek I, Zhao L, et al. Myeloid-derived suppressor cells enhance stemness of cancer cells by inducing microRNA101 and suppressing the corepressor CtBP2. Immunity. 2013;39(3):611–21.
62. Lu H, Clauser KR, Tam WL, et al. A breast cancer stem cell niche supported by juxtacrine signalling from monocytes and macrophages. Nat Cell Biol. 2014;16(12):1105–17.
63. Panni RZ, Sanford DE, Belt BA, et al. Tumor-induced STAT3 activation in monocytic myeloid-derived suppressor cells enhances stemness and mesenchymal properties in human pancreatic cancer. Cancer Immunol Immunother. 2014;63(5):513–28.
64. Korkaya H, Liu S, Wicha MS. Regulation of cancer stem cells by cytokine networks: attacking cancer's inflammatory roots. Clin Cancer Res. 2011;17(19):6125–9.
65. Marigo I, Bosio E, Solito S, et al. Tumor-induced tolerance and immune suppression depend on the C/EBPbeta transcription factor. Immunity. 2010;32:790–802.
66. Wang L, Yi T, Kortylewski M, et al. IL-17 can promote tumor growth through an IL-6-Stat3 signaling pathway. J Exp Med. 2009;206:1457–64.

67. Chen K, Cheng G, Zhang F, et al. Prognostic significance of programmed death-1 and programmed death-ligand 1 expression in patients with esophageal squamous cell carcinoma. Oncotarget. 2016;7(21):30772–80. 10.18632/oncotarget.8956.
68. Chen MF, Kuan FC, Yen TC, et al. IL-6-stimulated CD11b + CD14+ HLA-DR- myeloid-derived suppressor cells, are associated with progression and poor prognosis in squamous cell carcinoma of the esophagus. Oncotarget. 2014;5(18):8716–28.
69. Chen X, Wang L, Wang W, et al. B7-H4 facilitates proliferation of esophageal squamous cell carcinoma cells through promoting interleukin-6/signal transducer and activator of transcription 3 pathway activation. Cancer Sci. 2016;107(7):944–54. https://doi.org/10.1111/cas.12949.
70. Karakasheva TA, Waldron TJ, Eruslanov E, et al. CD38-expressing myeloid-derived suppressor cells promote tumor growth in a murine model of esophageal cancer. Cancer Res. 2015;75(19):4074–85. https://doi.org/10.1158/0008-5472.CAN-14-3639.
71. Okamura S, Fujiwara H, Yoneda M, et al. Overexpression of IL-6 by gene transfer stimulates IL-8-mediated invasiveness of KYSE170 esophageal carcinoma cells. Anticancer Res. 2013;33(4):1483–9.
72. Sugimura K, Miyata H, Tanaka K, et al. High infiltration of tumor-associated macrophages is associated with a poor response to chemotherapy and poor prognosis of patients undergoing neoadjuvant chemotherapy for esophageal cancer. J Surg Oncol. 2015;111(6):752–9. https://doi.org/10.1002/jso.23881.
73. Takase N, Koma Y, Urakawa N, et al. NCAM- and FGF-2-mediated FGFR1 signaling in the tumor microenvironment of esophageal cancer regulates the survival and migration of tumor-associated macrophages and cancer cells. Cancer Lett. 2016;380(1):47–58. https://doi.org/10.1016/j.canlet.2016.06.009.
74. Zhao ZF, Li JX, Ye R, et al. Interleukin-6 as a potential molecular target in esophageal squamous cell carcinoma. Oncol Lett. 2016;11(2):925–32.
75. Lyford-Pike S, Peng S, Young GD, et al. Evidence for a role of the PD-1:PD-L1 pathway in immune resistance of HPV-associated head and neck squamous cell carcinoma. Cancer Res. 2013;73(6):1733–41.
76. Nirschl CJ, Drake CG. Molecular pathways: coexpression of immune checkpoint molecules: signaling pathways and implications for cancer immunotherapy. Clin Cancer Res. 2013;19(18):4917–24.
77. Ohigashi Y, Sho M, Yamada Y, et al. Clinical significance of programmed death-1 ligand-1 and programmed death-1 ligand-2 expression in human esophageal cancer. Clin Cancer Res. 2005;11(8):2947–53.
78. Najafzadeh N, Mazani M, Abbasi A, et al. Low-dose all-trans retinoic acid enhances cytotoxicity of cisplatin and 5-fluorouracil on CD44(+) cancer stem cells. Biomed Pharmacother. 2015;74:243–51. https://doi.org/10.1016/j.biopha.2015.08.019.
79. Yusup G, Akutsu Y, Mutallip M, et al. A COX-2 inhibitor enhances the antitumor effects of chemotherapy and radiotherapy for esophageal squamous cell carcinoma. Int J Oncol. 2014;44(4):1146–52. https://doi.org/10.3892/ijo.2014.2300.
80. Zhang L, Wu YD, Li P, et al. Effects of cyclooxygenase-2 on human esophageal squamous cell carcinoma. World J Gastroenterol. 2011;17(41):4572–80. https://doi.org/10.3748/wjg.v17.i41.4572.
81. Chan TA. Nonsteroidal anti-inflammatory drugs, apoptosis, and colon-cancer chemoprevention. Lancet Oncol. 2002;3(3):166–74. Review
82. Fujii N, You L, Xu Z, et al. An antagonist of dishevelled protein-protein interaction suppresses β-catenin-dependent tumor cell growth. Cancer Res. 2007;67:573–9.
83. Thun MJ, Henley SJ, Patrono C. Nonsteroidal antiinflammatory drugs as anticancer agents: mechanistic, pharmacologic, and clinical issues. J Natl Cancer Inst. 2002;94:252–66.
84. Magnifico A, Albano L, Campaner S, et al. Tumor-initiating cells of HER2-positive carcinoma cell lines express the highest oncoprotein levels and are trastuzumab sensitive. Clin Cancer Res. 2009;15(6):2010–21.
85. Kawaguchi Y, Kono K, Mimura K, et al. Targeting EGFR and HER-2 with cetuximab- and trastuzumab-mediated immunotherapy in oesophageal squamous cell carcinoma. Br J Cancer. 2007;97(4):494–501.

86. Kono K, Mimura K, Fujii H, et al. Potential therapeutic significance of HER-family in esophageal squamous cell carcinoma. Ann Thorac Cardiovasc Surg. 2012;18(6):506–13. Review
87. LoRusso PM, Rudin CM, Reddy JC, et al. Phase I trial of hedgehog pathway inhibitor vismodegib (GDC-0449) in patients with refractory, locally advanced or metastatic solid tumors. Clin Cancer Res. 2011;17:2502–11.
88. McMillan R, Matsui W. Molecular pathways: the hedgehog signaling pathway in cancer. Clin Cancer Res. 2012;18(18):4883–8.
89. Mori Y, Okumura T, Tsunoda S, et al. Gli-1 expression is associated with lymph node metastasis and tumor progression in esophageal squamous cell carcinoma. Oncology. 2006;70(5):378–89.
90. Kim SY, Kang JW, Song X, et al. Role of the IL-6-JAK1-STAT3-Oct-4 pathway in the conversion of non-stem cancer cells into cancer stem-like cells. Cell Signal. 2013;25(4):961–9. https://doi.org/10.1016/j.cellsig.2013.01.007.
91. Yang C, Xiong F, Wang J, et al. Anti-ABCG2 monoclonal antibody in combination with paclitaxel nanoparticles against cancer stem-like cell activity in multiple myeloma. Nanomedicine. 2013;9:45–60.
92. Yang DR, Ding XF, Luo J, et al. Increased chemosensitivity via targeting testicular nuclear receptor 4 (TR4)-Oct4-interleukin 1 receptor antagonist (IL1Ra) axis in prostate cancer CD133+ stem/progenitor cells to battle prostate cancer. J Biol Chem. 2013;288(23):16476–83.
93. Ansell SM, Lesokhin AM, Borrello I, et al. PD-1 blockade with nivolumab in relapsed or refractory Hodgkin's lymphoma. N Engl J Med. 2015;372(4):311–9. https://doi.org/10.1056/NEJMoa1411087.
94. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med. 2012;366(26):2443–54.
95. Monjazeb AM, Hsiao HH, Sckisel GD, et al. The role of antigen-specific and non-specific immunotherapy in the treatment of cancer. J Immunotoxicol. 2012;9:248–58.
96. Huang J, Li C, Wang Y, et al. Cytokine-induced killer (CIK) cells bound with anti-CD3/anti-CD133 bispecific antibodies target CD133(high) cancer stem cells in vitro and in vivo. Clin Immunol. 2013;149(1):156–68.
97. Luo H, Zeng C, Fang C, et al. A new strategy using ALDHhigh-CD8 + T cells to inhibit tumorigenesis. PLoS One. 2014;9:e103193.
98. Zhu X, Prasad S, Gaedicke S, et al. Patient-derived glioblastoma stem cells are killed by CD133-specific CAR T cells but induce the T cell aging marker CD57. Oncotarget. 2015;6:171–84.
99. Bostad M, Berg K, Høgset A, et al. Photochemical internalization (PCI) of immunotoxins targeting CD133 is specific and highly potent at femtomolar levels in cells with cancer stem cell properties. J Control Release. 2013;168:317–26.
100. Swaminathan SK, Roger E, Toti U, et al. CD133-targeted paclitaxel delivery inhibits local tumor recurrence in a mouse model of breast cancer. J Control Release. 2013;171:280–7.
101. Wei X, Senanayake TH, Warren G, et al. Hyaluronic acid-based nanogel-drug conjugates with enhanced anticancer activity designed for the targeting of cd44-positive and drug-resistant tumors. Bioconjug Chem. 2013;24:658–68.
102. Yu Z, Ni M, Xiong M, et al. Poly(lactic-co-glycolic acid) nanoparticles conjugated with CD133 aptamers for targeted salinomycin delivery to CD133+ osteosarcoma cancer stem cells. Int J Nanomedicine. 2015;10:2537–54.
103. King MR. Rolling in the deep: therapeutic targeting of circulating tumor cells. Front Oncol. 2012;2:184. https://doi.org/10.3389/fonc.2012.00184.
104. Cohen SJ, Punt CJ, Iannotti N, et al. Relationship of circulating tumor cells to tumor response, progression-free survival, and overall survival in patients with metastatic colorectal cancer. J Clin Oncol. 2008;26(19):3213–21.
105. Cristofanilli M, Budd GT, Ellis MJ, et al. Circulating tumor cells, disease progression, and survival in metastatic breast cancer. N Engl J Med. 2004;351(8):781–91.
106. de Bono JS, Scher HI, Montgomery RB, et al. Circulating tumor cells predict survival benefit from treatment in metastatic castration-resistant prostate cancer. Clin Cancer Res. 2008;14(19):6302–9.

Chapter 4
Molecular Diagnosis and Targeted Therapy for Gastric Cancer

Nobuhisa Matsuhashi, Kazuhiro Yoshida, Kazuya Yamaguchi, and Toshiyuki Tanahashi

Abstract Gastric cancer (GC) is a heterogeneous cancer with widely varied outcome and similar clinical and pathological features in Caucasians and Asians. The treatment results from Asian countries seem to be better than those of Western countries. There is an urgent need to clarify the differences in results between Eastern and Western countries and to determine the best management of patients with GC. Now, the molecular biology of cancer is gradually becoming clear. Molecular-targeted drugs are on the rise, and classifications corresponding to the biomarkers of these drugs are beginning to be used in clinical settings. In addition, huge quantities of genome information are gradually being analyzed in a short time with the appearance of next-generation sequencers that can identify gene variation and copy number abnormalities. Now, because a classification based on the characteristics of the genome level of GC has been reported, we review the latest information on GC.

Keywords Molecular diagnosis • Targeted therapy • Gastric cancer

4.1 Introduction

Estimates of the worldwide incidence, mortality, and prevalence of 26 cancers in the year 2012 are now available in the GLOBOCAN series of the International Agency for Research on Cancer. The most commonly diagnosed cancers are lung (1.35 million), breast (1.15 million), and colorectal (1 million); the most

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common cause of cancer death is lung cancer (1.18 million deaths), and the second most common cause is gastric cancer (GC) (700,000 deaths) [1]. However, GC is the most common cancer in several areas of the world, most notably in Japan, Korea, and China. In Japan, the incidence of GC is almost ten times higher than that in the United States. In most areas in Japan, the incidence of GC in men is almost twice that of women [2]. GC, one of the most common human cancers, is a heterogeneous disease with different phenotypes and varying prognoses and responses to treatment. Among the various viewpoints, the newly developed concept of oncogene addiction provides a rationale for the use of targeted therapies.

4.1.1 Genetic and Molecular Alterations During Gastric Carcinogenesis Helicobacter pylori

Recent epidemiological studies have indicated that *Helicobacter pylori* plays a key role in the development of both intestinal-type and diffuse-type gastric carcinomas [3–5]. *H. pylori* is a gram-negative, spiral-shaped bacterium that infects the stomach of about half of the world's population. The acidic environment in the stomach usually prevents the survival of viruses, bacteria, and other microorganisms, but *H. pylori* has evolved to uniquely overcome this harsh environment. *H. pylori* secretes urease, a special enzyme that converts urea to ammonia to neutralize the acidity of the stomach, making the stomach a more hospitable place for *H. pylori*. With *H. pylori*'s ability to survive this harsh environment, the stomach provides it with a special living niche. Host inflammatory/immune cells that would normally recognize and attack invading bacteria are unable to cross from blood vessels through the stomach epithelial mucosa. Instead, the ineffective host cells continue to respond to the site of infection, where they die and release nutrients that feed the gastric pathogen. *H. pylori* infection is primarily acquired during childhood, and the transmission occurs through a fecal–oral or oral–oral mode, primarily within families. In the majority of cases, *H. pylori* infection is lifelong in the absence of eradication with antibiotics [6–8]. It is now well established that *H. pylori* infection predisposes individuals to gastric adenocarcinoma later in life [9, 10]. *H. pylori* has been classified by the International Agency for Research on Cancer (IARC) as a definite carcinogen to humans (group 1) [11]. *H. pylori* infection induces a chronic inflammation of the gastric mucosa that is intensified by the host inflammatory immune response with high levels of several cytokines. This chronic process leads, in a minority of infected individuals, to the development of GC through a series of intermediate progressive stages including mild and severe chronic gastritis; atrophic gastritis; gastric atrophy, characterized by hypochlorhydria; and intestinal metaplasia [4, 12]. Recent meta-analyses have estimated that *H. pylori* infection increases the risk of GC by two–threefold, with higher estimates for noncardiac GC [13–15].

4.1.2 *Oncogenic Mechanisms of* H. pylori *CagA Protein*

The CagA gene, which encodes a 120–135 KDa immunodominant protein, CagA, is localized at one end of the Cag pathogenicity island (*cag* PAI), a 40 kb DNA segment that is thought to be horizontally transferred into the *H. pylori* genome [16, 17]. *H. pylori* CagA is the first bacterial oncoprotein to be identified in relation to human cancer [18]. CagA is delivered into gastric epithelial cells through a bacterial type IV secretion system and localizes to the plasma membrane, where it undergoes tyrosine phosphorylation by host cell kinases. Infection with *cagA*-positive *H. pylori* strains has been associated with higher grades of gastric mucosal inflammation and severe atrophic gastritis and has been thought to play an important role in the development of gastric carcinoma. This link is further supported by the results of a combined analysis of 16 studies showing a twofold increase in the risk of GC associated with *cagA*-positive *H. pylori* compared to the risk of GC associated with *CagA*-negative *H. pylori* [19, 20].

It is well documented that chronic infection with *CagA*-positive *H. pylori* induces progressive histopathological changes in gastric mucosa that lead to intestinal-type gastric adenocarcinoma: superficial gastritis, atrophic gastritis, intestinal metaplasia, dysplasia, and adenocarcinoma [21–23] (Fig. 4.1). In addition, in terms of CagA polymorphisms, the vast majority of *H. pylori* isolates in East Asian countries bear East Asian CagA. In contrast, most if not all of the *H. pylori* cagA-positive strains isolated in non-East Asian countries carry Western CagA. A clear exception is Southeast Asia, where *H. pylori* strains carrying East Asian CagA and Western CagA coexist at various ratios in different areas and countries [23–25]. Chronic infection with cagA-positive strains of *H. pylori* hyperstimulates gastric epithelial turnover by constitutively exposing cells to oncogenic stress [26]. Long-term sustenance of such a situation substantially increases the chance of epithelial cells acquiring genetic and epigenetic defects in signaling pathways including those involved in senescence and apoptosis, the malfunctioning of which is an important hallmark of cancer [27] (Fig. 4.2).

4.1.3 *Claudin-18 Loss*

Claudin-18 has two alternative splicing forms, the lung and stomach types, which use a different first exon and the same exons 2–4; the two isoforms are regulated by different tissue-specific promotors. Because stomach-type claudin-18 is the predominant claudin expressed in stomach, it is expected to regulate the stomach-specific properties of the paracellular barrier, including resistance to H^+ leakage and/or pepsin, as implied by its overexpression in MDCK II cells [28, 29]. Epithelial cells adhere to each other to form cell sheets, and when the intercellular spaces between epithelial cells are sealed by tight junctions, the paracellular barrier function is established [30, 31, 32]. Sanada et al. [33] reported the downregulation of claudin-18

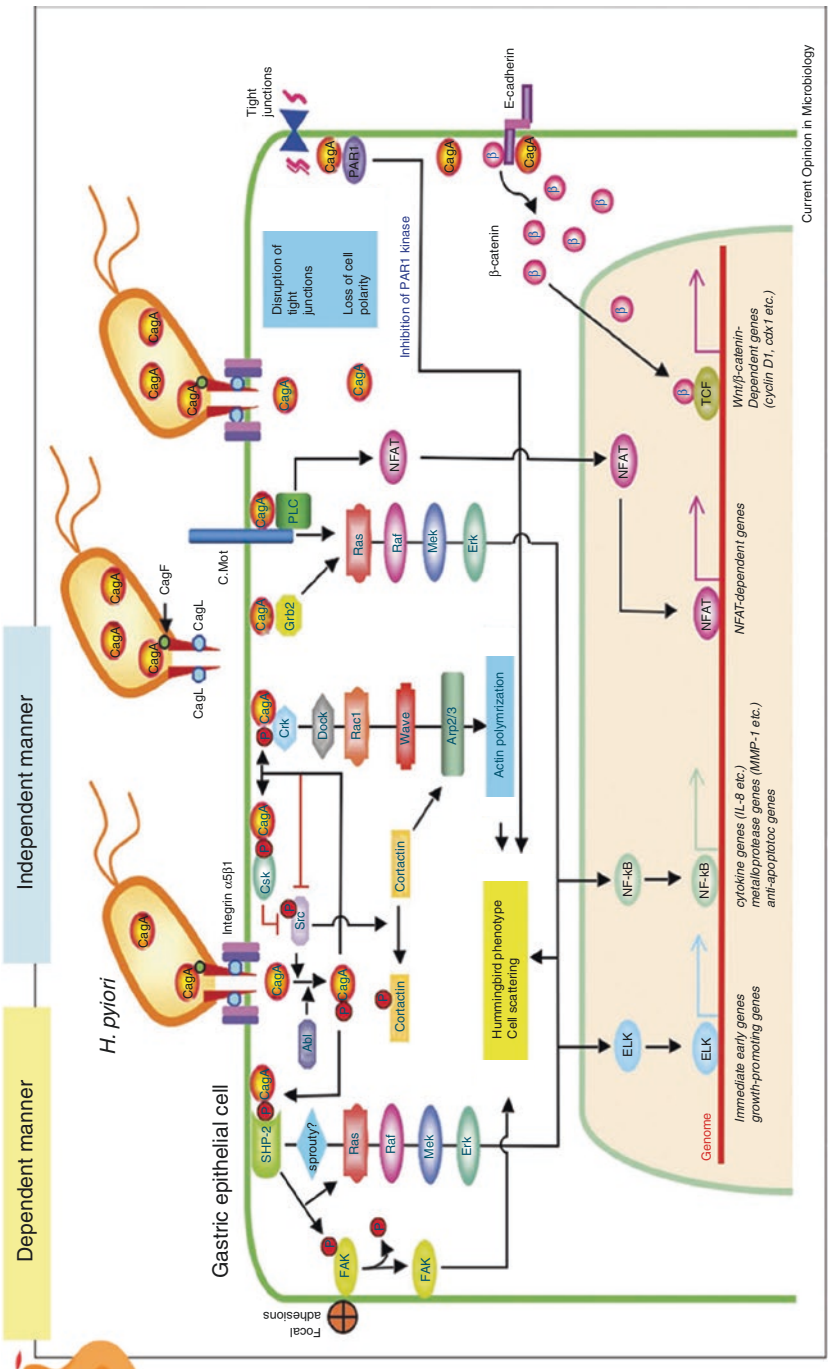

Fig. 4.1 Oncogenic mechanisms of *Helicobacter pylori* CagA protein. Source: Refs. [22, 23]

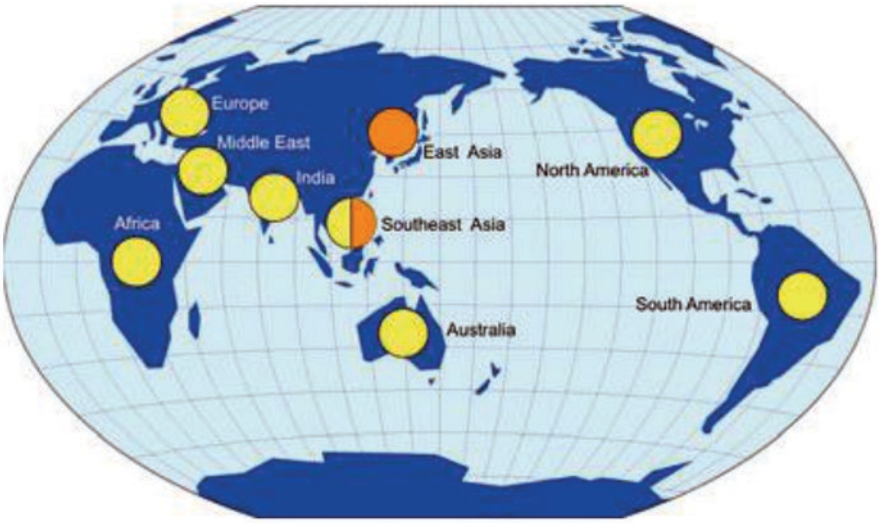

Fig. 4.2 Global distribution of *Helicobacter pylori* Western CagA (*yellow*) and East Asian CagA (*orange*). Source: Ref. [23]

expression in human GC. Hayashi et al. [34] reported that that claudin-18-dependent formation of the paracellular barrier against H^+ diffusion is likely to play a specific role in the prevention of gastritis. Because claudin-18 is the major tight junction (TJ) component of stomach epithelial cells, barrier dysfunction of the tight junctions is reduced in *H. pylori*-induced atrophic gastritis and intestinal metaplasia. According to this result, claudin-18 can lead to a precancerous condition [33, [34].

4.1.4 Overexpression of HER2, EGFR, and c-MET

Recently, significant achievements have been made in the discovery and advancement of treatments for lung cancer due to the recognition of distinct molecular subtypes generally headlined by an actionable "driver mutation" such as rearrangement in anaplastic lymphoma kinase (ALK), c-ROS kinase (ROS1), or rearrangement during transformation (RET kinase) or amplification of *HER2* or *MET*, or mutations in epidermal growth factor receptor (EGFR), BRAF (V600E), and KRAS. Many of these same mutations have been described in GC [35] (Fig. 4.3).

HER2 overexpression has been observed in 9–38% of GC patients and occurs more frequently in gastroesophageal junction (GEJ) and intestinal-type tumors [36]. Treatment with the anti-HER2 monoclonal antibody trastuzumab has been proven to achieve improved survival in patients with HER2-positive advanced GC [37–39].

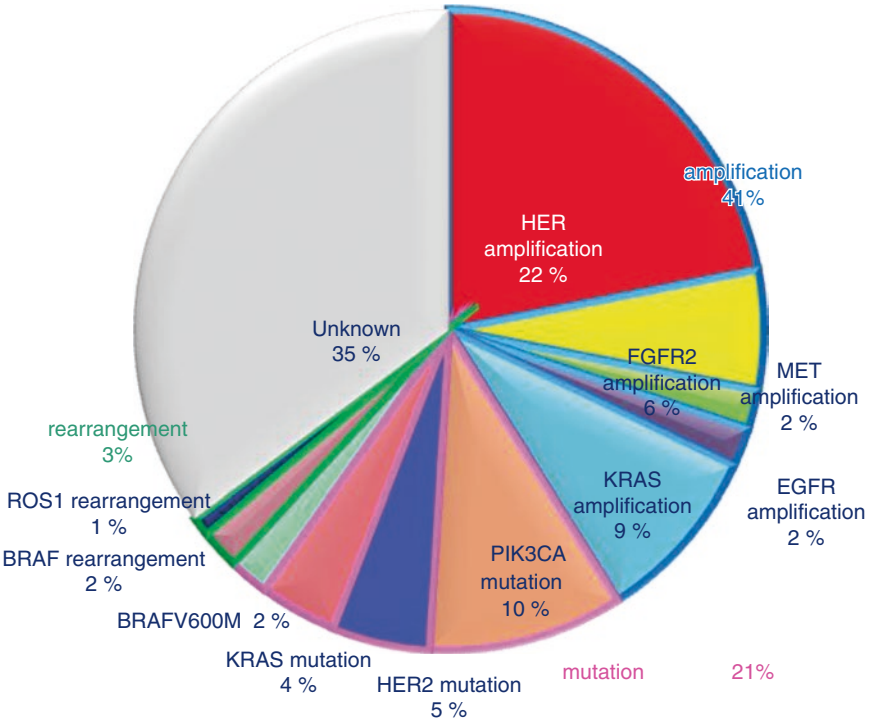

Fig. 4.3 Proportion of potential driver mutations identified in gastric carcinoma. Source: Ref. (Lee J, Ou SH. Discov Med, 2013, 16:7–14)

The Trastuzumab for Gastric Cancer (ToGA) trial investigated the addition of trastuzumab to standard cisplatin and 5-fluorouracil-based chemotherapy to determine whether it would significantly improve the rate of overall survival (OS). Among 3665 patients with advanced GC or GEJ carcinoma who were successfully screened for HER2 overexpression, 810 (22.1%) were positive for HER2 overexpression. The rates of HER2 positivity were similar in Europe (23.6%) and Asia (23.5%) [40] and were higher in GEJ than GC (33.2% vs. 20.9%; $p < 0.001$) and in intestinal than diffuse/mixed cancer (32.2% vs. 6.1%/20.4%; $p < 0.001$) [40]. Of the patients enrolled in ToGA, 81.8% (478/584) had GC, whereas the rest had GEJ carcinoma. Most of the ToGA patients had intestinal histology (75.5%), with diffuse histology seen in 8.8% of the patients and mixed histology seen in the remaining 15.7% of patients. Slightly more than half of the enrolled patients (52.9%, 309/584) were Asians. Stratification was by performance status, stage, primary cancer site, and measurability of disease. The addition of trastuzumab to the chemotherapy regimen significantly improved the objective response rate to 47% as compared with 35% for chemotherapy alone (odds ratio [OR], 1.70; 95% confidence interval [CI], 1.22–2.38; $p = 0.0018$). Trastuzumab also significantly improved the rate of progression-free survival (PFS) from 5.5 months to 6.7 months (hazard ratio [HR] = 0.71; 95% CI, 0.59–0.85, $p = 0.0002$), and of most importance, the addition of trastuzumab

significantly improved OS from 11.1 months to 13.8 months (HR = 0.74; 95% CI, 0.60–0.91, p = 0.0045). Post hoc subgroup analysis showed that OS had improved significantly in patients from Europe (n = 190, HR = 0.63; 95% CI, 0.44–0.89) and Central/South America (n = 52; HR = 0.44; 95% CI, 0.21–0.90) but that in Asian patients had not significantly improved (n = 319; HR = 0.82; 95% CI, 0.61–1.11) [40]. To date, the only successful and approved targeted therapy for GC is trastuzumab in combination with chemotherapy in GC with HER2 overexpression [41].

The prognostic effect and clinicopathological features of EGFR and c-MET have also been studied. EGFR overexpression, which was observed in 27–44% of GC patients, has generally been reported to be a poor prognostic factor [42]. The correlation between EGFR status and clinicopathological characteristics has not been clearly elucidated. EGFR-positive status was reported to be frequently associated with the following factors: non-curatively treated GC [43], older age, moderately to poorly differentiated histological appearance, higher-stage disease [44], and recurrence after curative surgery and higher disease stages [45]. On the whole, c-MET overexpression, which was observed in 22–82% of GC patients, has also been reported to be associated with poor prognosis [42, 46, 47]; however, findings from a few other studies were contradictory. Recently, Fuse et al. [48] investigated co-overexpression of HER2, EGFR, and c-MET in patients with advanced GC who received standard chemotherapy and found that only c-MET was a significant and independent prognostic factor, which suggests that c-MET would be a good candidate for molecular-targeted agents [49] (Fig. 4.4). In the future, a new treatment strategy for patients simultaneously positive for EGFR or c-MET and HER2 is required.

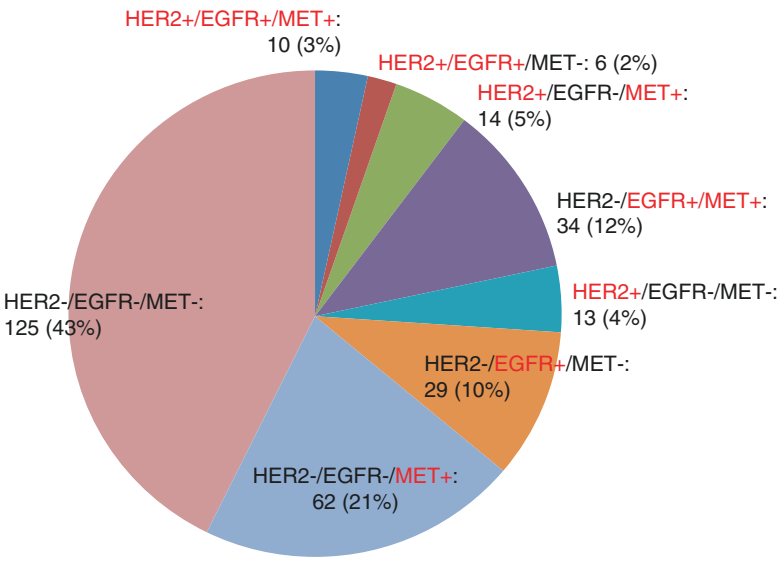

Fig. 4.4 Co-overexpression status of human epidermal growth factor receptor 2 (HER2), epidermal growth factor receptor (EGFR), and c-MET. Source: Ref. [48]

4.1.5 VEGF-Directed Therapies

The VEGF family consists of five ligands [VEGFA, VEGFB, VEGFC, VEGFD, and placental growth factor (PIGF)] and three receptor tyrosine kinases [VEGF-R1, R2, and R3]. Of the VEGF receptors, VEGF-R2 expression is restricted to the vasculature and appears to play a key role in angiogenesis [49]. The failure of the AVAGAST trial was a setback for anti-angiogenic therapy for this disease [50]. Ramucirumab is a monoclonal antibody that binds to and prevents the activation of VEGF-R2. The recent REGARD trial, a randomized phase III trial of ramucirumab vs. placebo in patients with advanced, pretreated GC, met its primary endpoint of increased OS [51]. The subsequent RAINBOW trial pitting paclitaxel + ramucirumab against paclitaxel + placebo for advanced pretreated GC confirmed the survival advantage of this anti-angiogenic agent in GC [52]. Most (60%) of the patients were North American or European, and the remainder were Asian. Those patients treated with ramucirumab + paclitaxel experienced statistically significant and clinically meaningful improvement in OS compared with those treated with paclitaxel alone (9.63 m vs. 7.26 m, HR = 0.807; 95% CI, 0.678–0.962; $p = 0.0169$). Improvements of the response rate and PFS were also comparable in the study's experimental arm. A subgroup analysis showed that Asian patients did not obtain the same benefit of OS as the non-Asian patients did. Although the reasons for the discrepant outcome between Asian and Caucasian patients are unclear, one possibility is that Asian GC patients have a relatively less aggressive disease biology and often undergo third- and fourth-line therapies. The rates for these additional lines of therapy were 75% in Japanese patients but <40% in non-Asian patients. Compared with the Asian patients in this study, those from Europe or North America clearly derived more benefit from anti-angiogenic therapy [53].

4.1.6 Definition of Gastric and Intestinal Phenotypes of GC

GC, one of the most common human cancers, is a heterogeneous disease with different phenotypes and varying prognoses and responses to treatment. Therefore, subtype classification of GC is necessary to predict prognosis and decide on effective treatments. Histologically, GC demonstrates marked heterogeneity at both the architectural and cytologic levels, often with the coexistence of several histologic elements [54]. In Eastern and Western countries, the histologic classification of GC has largely been based on Lauren's criteria, in which intestinal-type and diffuse-type adenocarcinoma are the two major histologic subtypes, plus mixed type as an uncommon variant [55]. The relative frequencies are approximately 54% for intestinal type, 32% for diffuse type, and 15% for mixed type [55–57]. There are indications that the intestinal-type adenocarcinoma is more often associated with

intestinal metaplasia and *H. pylori* infection, whereas the diffuse-type gastric carcinoma is more often seen in families, females, and young individuals. Although the Lauren classification provides important information in clinical practice, it is not critical for predicting prognosis or determining treatment. Oue et al. [58] reported that GC can also be classified into a gastric or intestinal phenotype according to mucin expression. Accumulating evidence has indicated that gastric and intestinal phenotypes of GC have distinct clinical characteristics and exhibit specific genetic and epigenetic changes. Oue et al. [59] also focused on the clinical and molecular characteristics of the gastric and intestinal phenotypes of GC and reported that theTP53 mutation and allelic deletion of the APC gene are detected more frequently in the intestinal phenotype of GC. In contrast, CDH1 gene mutation is detected in differentiated-type GC showing a gastric phenotype [60]. Microsatellite instability (MSI) is detected more frequently in the gastric phenotype of GC [61]. Yasui et al. [56, 57] reported that GC cases showing CK7−/CK20+ were frequently found in the intestinal phenotype of GC, whereas GC cases showing CK7+/CK20- were commonly found in the gastric phenotype of GC. Nuclear β-catenin staining was frequently found in the intestinal phenotype of GC. However, expression of MMP7, laminin γ2, or HER2 was not correlated with gastric or intestinal phenotypes of GC [62, 63].

Whole genome or exon sequencing in GC has been performed, and mutation of the *RHOA* gene in undifferentiated-type GC has been reported [64] (Fig. 4.5). According to the COSMIC website (http://cancer.sanger.ac.uk), the most frequently mutated gene is *TP53* (32%), and the second most frequently mutated gene is *ARID1A* (14%). Frequencies of other gene mutations are approximately 10% or less [65]. Although the associations between mutation of these genes and gastric and intestinal phenotypes are unclear, driver gene mutation is a rare event, and it is difficult to plan an effective treatment according to such gene mutations. In contrast,

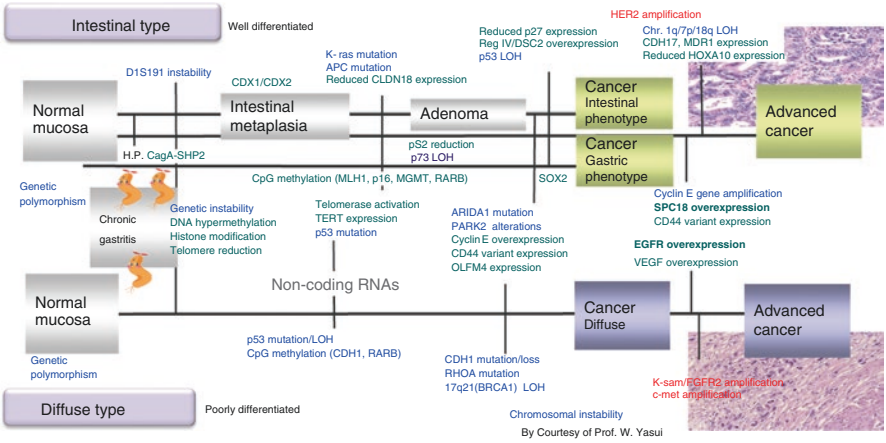

Fig. 4.5 Definition of gastric and intestinal phenotypes of gastric cancer. Source: Ref. [60]

the Cancer Genome Atlas Network has reported that GC can be classified into four distinct molecular subtypes: GC positive for Epstein-Barr (EB) virus, microsatellite unstable GC, genomically stable GC, and GC with chromosomal instability [65]. As described above, MSI is detected more frequently in the gastric phenotype of GC. GC positive for EB virus is also frequently found in the gastric phenotype of GC [58]. However, the mucin phenotypes of genomically stable GC and GC with chromosomal instability remain unclear. In the future, classification of these subtypes may be used to provide personalized medicine.

4.1.7 Whole-Exome/Genome Sequencing Analyses of GC in Asia

GC is a leading cause of global cancer mortality, with high incidence rates in Asia and parts of Latin America [66]. Survival outcomes differ across geographical regions, with rates of 5-year OS being 10–15% in North America and 45–50% in East Asia [67–69]. These differences cannot be explained simply by improved early diagnosis in Asian countries as they persist even after stratifying for disease stage [70]. It has been suggested that these differences may reflect geographic variability in clinical practice. However, Asian patients treated in Western countries still exhibit superior outcomes compared with Caucasians, albeit worse outcomes than patients from Asian registries in their home countries [71, 72]. Lin et al. [1] assembled nine independent GC microarray cohorts comprising 1016 tumor gene expression profiles, six from Asian localities (n = 890) and three from outside Asia (n = 126). Except for tumor location, most of the clinicopathologic parameters, such as age, sex, and stage, were not significantly different between the Asian and non-Asian cohorts. However, there were significantly more cases of tumors in the upper third of the stomach in the non-Asian vs. Asian cohorts ($p = 0.04$). This study showed that for major cancer oncogenes such as KRAS, HER2, and FGFR2, somatic gene mutations and gene amplification rates are basically similar between Asian and non-Asian GCs. However, the association of GC with enrichment of tumor-infiltrating T cells and T-cell gene expression signatures, including CTLA-4 signaling, was stronger in non-Asian GCs [1]. In the future, differences in tumor immunity may contribute to geographical differences in clinical outcome and the design of future trials particularly in immuno-oncology.

4.1.8 Immune Checkpoint Blockade of PD-1/PD-L1

PD-1/PD-L1 blockade has recently been shown to be a promising treatment in a variety of tumor types [74–76]. Pembrolizumab is a humanized monoclonal anti-PD-1 antibody of the IgG4-kappa isotype that blocks the interaction between PD-1 and its ligands, PD-L1 and PD-L2. Pembrolizumab is FDA approved for the

treatment of unresectable or metastatic melanoma and for PD-L1-positive metastatic non-small cell lung cancer [76–80]. Le et al. [81] reported that mismatch repair status predicted the clinical benefit of immune checkpoint blockade with pembrolizumab. In their cohort of patients with mismatch repair-deficient colorectal cancer (cohort A), median PFS and median OS were not reached. Contrastingly, in those with mismatch repair-proficient cancers (cohort B), median PFS was only 2.2 months (95% CI, 1.4–2.8), and median OS was 5.0 months (95% CI, 3.0 to not estimable). The median PFS in their cohort C (patients with mismatch repair-deficient noncolorectal cancer) was 5.4 months (95% CI, 3 to not estimable), and median OS was not reached. KEYNOTE-012 was a multicenter, open-label, phase 1b trial in which patients with advanced GC, urothelial cancer, triple-negative breast cancer, and head and neck cancer were treated. In their report, Seiwert et al. [80] described the results of the cohort with advanced GC, which comprised 39 patients (19 from East Asia and 20 from other areas in the world). Specimens from 24 patients with microsatellite instability were also analyzed. Four (17%) of these 24 patients had tumors with high microsatellite instability (two [8%] Asian patients and two [8%] from elsewhere) and the remaining 20 (83%) had tumors with microsatellite stability. Among all 32 patients with at least one post-baseline tumor assessment, 17 (53%) experienced a decrease in their target lesion size from baseline. A central review showed the median time to response to be 8 weeks. At the final analysis, four of the eight responders were alive, had no disease progression, and required no additional anticancer therapy. The median duration of response was 40 weeks, and decreased tumor burden was maintained over several assessments. One patient experienced 100% reduction in the target region but was not judged to have a complete response because of the subsequent development of new lesions. In the four patients with GC with high microsatellite instability, two experienced a partial response, but the disease progressed in the other two. From these results, mismatch repair status was unable to predict the clinical benefit of immune checkpoint blockade with pembrolizumab in GC [81]. Several ongoing studies are continuing to investigate the efficacy and safety of pembrolizumab in patients with advanced gastric or GEJ cancer. In view of the mechanism of action of pembrolizumab, the known expression of PD-L1 in GC and data from patients with non-small cell lung cancer suggest an improved response in patients whose tumors express PD-L1 [82] (Fig. 4.6a–c).

4.1.9 Conversion Therapy for Stage IV GC

4.1.9.1 Proposal of New Biological Categories of Classification

The strategy for treating stage IV GC remains controversial. Due to poor prognosis, the variance in physical status, and severe symptoms, it is important to determine the optimal strategy for treating each individual patient with stage IV disease. The survival efficacy of palliative gastrectomy by reductive gastrectomy for advanced

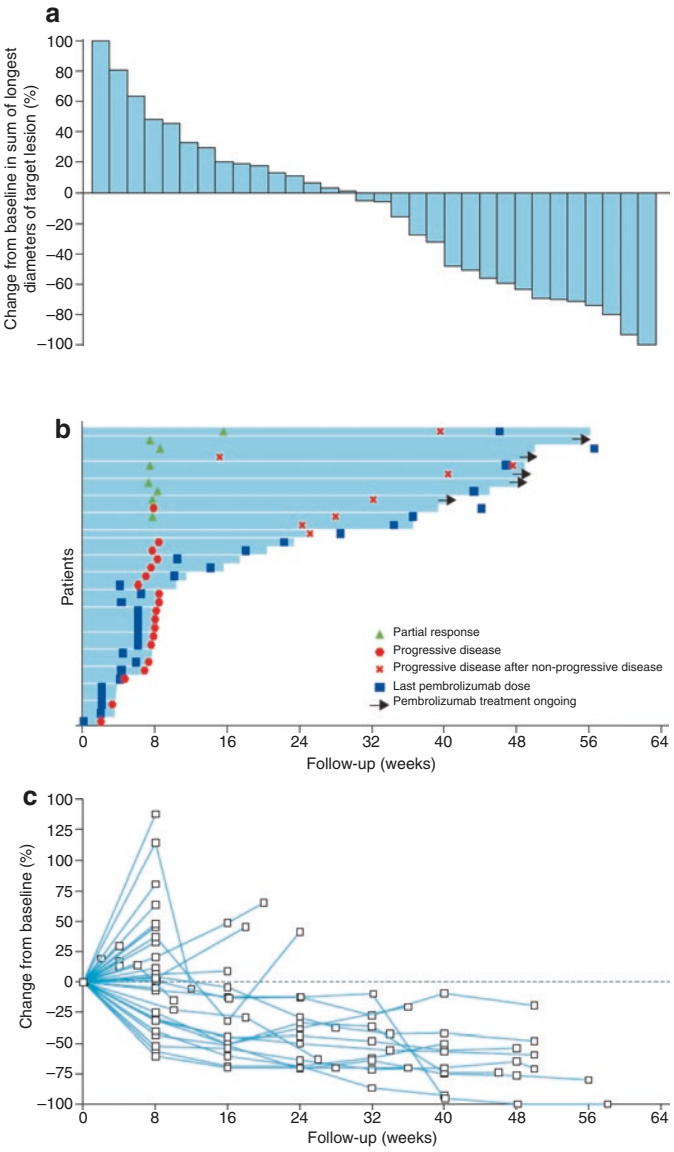

Fig. 4.6 (a–c) Assessment of the effect of immune factors on geographic locality-based and chemotherapy-based survival. Source: Ref. [82]

tumors has been denied in three Asian countries in the REGATTA trial [83]. However, the development of molecular technology and targeted therapies has drawn attention due to their potentially greater anticancer activity and fewer side effects than traditional chemotherapeutic agents. This suggests that the development of new cancer treatment strategies will require the discovery of more

candidates to target. For this reason, we reviewed the current status of GC to better understand the biology and indications of curative surgery as conversion therapy. We have proposed new categories for the classification of stage IV GC, taking into account the heterogeneous situation and treatment trends in general practice. In the new categories of classification, we have divided stage IV GC into two entities of macroscopic-positive and macroscopic-negative patients, who are further classified into four categories [84]. Patients without macroscopic peritoneal dissemination are classified into category 1 and category 2. The patients with potentially resectable metastasis are classified into category 1, whereas those with marginally resectable metastasis are classified into category 2. Patients with macroscopic peritoneal dissemination are classified into category 3 and category 4. The patients in category 3 are considered to be incurable and have unresectable metastases; however, resection may be performed to achieve local palliation. The patients in category 4 have non-curable metastases. It is essentially impossible to achieve a cure in any patient with peritoneal carcinomatosis from GC, irrespective of the extent of pretreatment or the ability to achieve an R0 resection. However, the survival outcomes differ according to the degree of disease progression and the extent of the disease, in addition to the response to therapy. Longer survival can be expected in patients in categories 1 and 2 who are treated with curative intent, whereas treatment of the patients in the other categories focuses on "care." The concept of conversion therapy or adjuvant surgery principally includes patients in category 2, some patients in category 3, and rarely patients in category 4 when operations are performed with the goal of achieving an R0 resection or a surgical cure [85] (Fig. 4.7). This suggests that the development of new cancer treatment strategies will require the discovery of more candidates to target. A retrospective cohort study is now being conducted in Asia through the

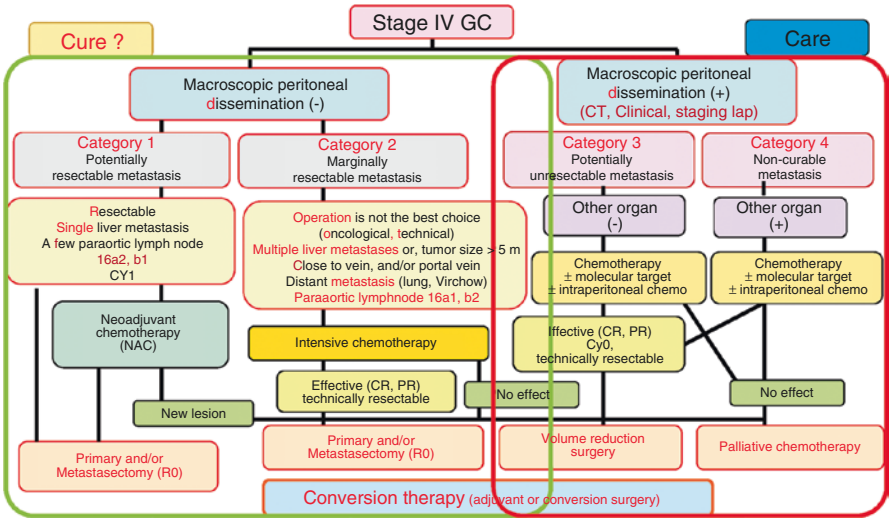

Fig. 4.7 The new biological categories for the classification of stage IV gastric cancer. Source: Ref. [85]

Federation of Asian Clinical Oncology (FACO), which consists of the Japanese Society of Clinical Oncology (JSCO), the Korean Association of Clinical Oncology (KACO), and the Chinese Society of Clinical Oncology (CSCO), with the support of the Japanese Gastric Cancer Association (JGCA), the Korean Gastric Cancer Association (KGCA), and the Gastric Cancer Association of the Chinese Anti-cancer Association. Further analysis will prove to clarify the benefits of conversion therapy in the new strategic approach for stage IV GC.

In conclusion, the development of new DNA sequencing technologies, such as next-generation sequencing techniques, may dramatically increase the speed and reduce the cost of DNA sequencing, thus enabling more rapid and detailed analysis of gene amplifications and genetic alterations in GC. In turn, the development of more potent molecular diagnosis and targeted therapy for the treatment of GC will be expected.

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References

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin. 2015;65(2):87–108.
2. Ito Y, Ioka A, Tanaka M, Nakayama T, Tsukuma H. Trends in cancer incidence and mortality in Osaka, Japan: evaluation of cancer control activities. Cancer Sci. 2009;100:2390–5.
3. Nomura A, Stemmermann GN, Chyou PH, Kato I, Perez GI, Blaser MJ. Helicobacter pylori infection and gastric carcinoma among Japanese Americans in Hawaii. N Engl J Med. 1991;325:1132–6.
4. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, et al. Helicobacter pylori and the risk of gastric carcinoma. N Engl J Med. 1991;325:1127–31.
5. Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, et al. Helicobacter pylori infection and the development of gastric cancer. N Engl J Med. 2001;345:784–9.
6. Kudo M, Asaka M, Kato M, Katagiri M, Kagaya H, Nishikawa K, et al. Role of helicobacter pylori in chronic gastritis: a prospective study. J Clin Gastroenterol. 1995;21(Suppl 1):S174–8.
7. Asaka M, Kudo M, Kato M, Kimura T, Meguro T, Mitani S, et al. The role of helicobacter pylori infection in the pathogenesis of gastritis. J Gastroenterol. 1994;29(Suppl 7):100–4.
8. Haruma K, Komoto K, Kawaguchi H, Okamoto S, Yoshihara M, Sumii K, et al. Pernicious anemia and helicobacter pylori infection in Japan: evaluation in a country with a high prevalence of infection. Am J Gastroenterol. 1995;90(7):1107–10.
9. Kuwahara Y, Kono S, Eguchi H, Hamada H, Shinchi K, Imanishi K. Relationship between serologically diagnosed chronic atrophic gastritis, helicobacter pylori, and environmental factors in Japanese men. Scand J Gastroenterol. 2000;35(5):476–81.

10. Fukuda S, Tanaka M, Soma Y, Shimoyama T, Mikami T, Crabtree JE, et al. Histological analysis of gastritis and helicobacter pylori infection in patients with early gastric cancer: a case-control study. J Gastroenterol Hepatol. 2000;15(12):1370–6.
11. Schistosomes, liver flukes and helicobacter pylori. IARC working group on the evaluation of carcinogenic risks to humans. Lyon, 7-14 June 1994. IARC Monogr Eval Carcinog Risks Hum. 1994;61:1–241.
12. Kuipers EJ, Uyterlinde AM, Pena AS, Roosendaal R, Pals G, Nelis GF, et al. Long-term sequelae of helicobacter pylori gastritis. Lancet. 1995;345:1525–8.
13. Helicobacter and Cancer Collaborative Group. Gastric cancer and helicobacter pylori: a combined analysis of 12 case-control studies nested within prospective cohorts. Gut. 2001;49:347–53.
14. Eslick GD, Lim LL, Byles JE, Xia HH, Talley NJ. Association of Helicobacter pylori infection with gastric carcinoma: a meta-analysis. Am J Gastroenterol. 1999;94:2373–9.
15. Huang JQ, Sridhar S, Chen Y, Hunt RH. Meta-analysis of the relationship between helicobacter pylori seropositivity and gastric cancer. Gastroenterology. 1998;114:1169–79.
16. Hatakeyama M. Helicobacter pylori CagA and gastric cancer: a paradigm for hit-and-run carcinogenesis. Cell Host Microbe. 2014;15(3):306–16.
17. Covacci A, Censini S, Bugnoli M, Petracca R, Burroni D, Macchia G, et al. Figura N molecular characterization of the 128-kDa immunodominant antigen of helicobacter pylori associated with cytotoxicity and duodenal ulcer. Proc Natl Acad Sci U S A. 1993;90(12):5791–5.
18. Hatakeyama M. Oncogenic mechanisms of the helicobacter pylori CagA protein. Nat Rev. Cancer. 2004;4(9):688–94. Review
19. Blaser MJ, Perez-Perez GI, Kleanthous H, Cover TL, Peek RM, Chyou PH, et al. Infection with helicobacter pylori strains possessing cagA is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res. 1995;55:2111–5.
20. Parsonnet J, Friedmann GD, Orentreich N, Vogelman H. Risk for gastric cancer in people with CagA positive or CagA negative helicobacter pylori infection. Gut. 1997;40:279–301.
21. Enroth H, Kraaz W, Engstrand L, Nyren O, Rohan T. Helicobacter pylori strain types and risk of gastric cancer: a case-control study. Cancer Epidemiol Biomark Prev. 2000;9:981–4.
22. Hatakeyama M. Helicobacter pylori and gastric carcinogenesis. J Gastroenterol. 2009;44(4):239–48.
23. Hatakeyama M. Anthropological and clinical implications for the structural diversity of the helicobacter pylori CagA oncoprotein. Cancer Sci. 2011;102(1):36–43.
24. Vilaichone RK, Mahachai V, Tumwasorn S, Wu JY, Graham DY, Yamaoka Y. Molecular epidemiology and outcome of helicobacter pylori infection in Thailand: a cultural cross roads. Helicobacter. 2004;5:453–9.
25. Truong BX, Mai VT, Tanaka H, et al. Diverse characterization of the cagA gene of helicobacter pylori strains in gastric cancer and peptic ulcer patients from southern Vietnam. J Clin Microbiol. 2009;47:4021–8.
26. Schmidt HM, Goh KL, Fock KM, et al. Distinct cagA EPIYA motifs are associated with ethnic diversity in Malaysia and Singapore. Helicobacter. 2009;14:256–63.
27. Saito Y, Murata-Kamiya N, Hirayama T, Ohba Y, Hatakeyama M. Conversion of helicobacter pylori CagA from senescence inducer to oncogenic driver through polarity-dependent regulation of p21. J Exp Med. 2010;201:2154–74.
28. Hanahan D, Weinberg RA. The hallmark of cancer. Cell. 2000;100:57–70.
29. Muto S, Hata M, Taniguchi J, et al. Claudin-2-deficient mice are defective in the leaky and cation-selective paracellular permeability properties of renal proximal tubules. Proc Natl Acad Sci U S A. 2010;107:8011–6.
30. Tamura A, Hayashi H, Imasato M, et al. Loss of claudin-15, but not claudin-2, causes Na_ deficiency and glucose malabsorption in mouse small intestine. Gastroenterology. 2011;140:913–23.
31. Madara JL. Regulation of the movement of solutes across tight junctions. Annu Rev. Physiol. 1998;60:143–59.
32. Umeda K, Ikenouchi J, Katahira-Tayama S, et al. ZO-1 and ZO-2 independently determine where claudins are polymerized in tight junction strand formation. Cell. 2006;126:741–54.

33. Sanada Y, Oue N, Mitani Y, et al. Down-regulation of the claudin-18 gene, identified through serial analysis of gene expression data analysis, in gastric cancer with an intestinal phenotype. J Pathol. 2006;208:633–42.
34. Hayashi D, Tamura A, Tanaka H, Yamazaki Y, Watanabe S, Suzuki K, et al. Deficiency of claudin-18 causes paracellular H+ leakage, up-regulation of interleukin-1β, and atrophic gastritis in mice. Gastroenterology. 2012;142(2):292–304.
35. Lee J, Ou SH. Towards the goal of personalized medicine in gastric cancer-time to move beyond HER2 inhibition. Part II: Targeting gene mutations and gene amplifications and the angiogenesis pathway. Discov Med. 2013:16(86):7–14.
36. Lee JW, Soung YH, Kim SY, Park WS, Nam SW, Kim SH, et al. ERBB2 kinase domain mutation in a gastric cancer metastasis. APMIS. 2005;113(10):683–7.
37. Gravalos C, Jimeno A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. Ann Oncol. 2008;19:1523–9.
38. Yonemura Y, Ninomiya I, Yamaguchi A, Fushida S, Kimura H, Ohoyama S, et al. Evaluation of immunoreactivity for erbB-2 protein as a marker of poor short term prognosis in gastric cancer. Cancer Res. 1991;51:1034–8.
39. Allgayer H, Babic R, Gruetzner KU, Tarabichi A, Schildberg FW, Heiss MM. C-erbB-2 is of independent prognostic relevance in gastric cancer and is associated with the expression of tumor associated protease systems. J Clin Oncol. 2000;18:2201–9.
40. Bang Y, Chung H, Xu J, Lordick F, Sawaki A, Lipatov O, et al. Pathological features of advanced gastric cancer (GC): relationship to human epidermal growth factor receptor 2 (HER2) positivity in the global screening programme of the ToGA trial. J Clin Oncol. 2009;27(suppl):abstr#4556.
41. Bang YJ, Van Cutsem E, Feyereislova A, Chung HC, Shen L, Sawaki A, Lordick F, Ohtsu A, Omuro Y, Satoh T, Aprile G, Kulikov E, Hill J, Lehle M, Rüschoff J, Kang YK; ToGA Trial Investigators. Trastuzumab in combination with chemotherapy versus chemotherapy alone for treatment of HER2-positive advanced gastric or gastro-esophageal junction cancer (ToGA): a phase 3 open-label randomized controlled trial. Lancet 2010;376(9742):687–697.
42. Galizia G, Lieto E, Orditura M, Castellano P, Mura AL, Imperatore V, et al. Epidermal growth factor receptor (EGFR) expression is associated with a worse prognosis in gastric cancer patients undergoing curative surgery. World J Surg. 2007;31:1458–68.
43. Kim MA, Lee HS, Lee HE, Jeon YK, Yang HK, Kim WH. EGFR in gastric carcinomas: prognostic significance of protein overexpression and high gene copy number. Histopathology. 2008;52:738–46.
44. Hayashi M, Inokuchi M, Takagi Y, Yamada H, Kojima K, Kumagai J, et al. High expression of HER3 is associated with a decreased survival in gastric cancer. Clin Cancer Res. 2008;14:7843–9.
45. Terashima M, Kitada K, Ochiai A, Ichikawa W, Kurahashi I, Sakuramoto S, et al. Impact of expression of human epidermal growth factor receptors EGFR and ERBB2 on survival in stage II/III gastric cancer. Clin Cancer Res. 2012;18:5992–6000.
46. Lee HE, Kim MA, Lee HS, Jung EJ, Yang HK, Lee BL, et al. MET in gastric carcinomas: comparison between protein expression and gene copy number and impact on clinical outcome. Br J Cancer. 2012;107:325–33.
47. Ha SY, Lee J, Kang SY, Do IG, Ahn S, Park JO, et al. MET overexpression assessed by new interpretation method predicts gene amplification and poor survival in advanced gastric carcinomas. Mod Pathol. 2013;26:1632–41.
48. Fuse N, Kuboki Y, Kuwata T, Nishina T, Kadowaki S, Shinozaki E, et al. Prognostic impact of HER2, EGFR, and c-MET status on overall survival of advanced gastric cancer patients. Gastric Cancer. 2016;19(1):183–91.
49. Lu D, Jimenez X, Zhang H, Bohlen P, Witte L, Zhu Z. Selection of high affinity human neutralizing antibodies to VEGFR2 from a large antibody phage display library for antiangiogenesis therapy. Int J Cancer. 2002;97:393–9.

50. Van Cutsem E, de Haas S, Kang YK, Ohtsu A, Tebbutt NC, Ming Xu J, et al. Bevacizumab in combination with chemotherapy as first-line therapy in advanced gastric cancer: a biomarker evaluation from the AVAGAST randomized phase III trial. J Clin Oncol. 2012;30:2119–27.
51. Fuchs CS, Tomasek J, Yong CJ, Dumitru F, Passalacqua R, Goswami C, et al. Ramucirumab monotherapy for previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (REGARD): an international, randomised, multicentre, placebo-controlled, phase 3 trial. Lancet. 2014;383:31–9.
52. Wilke H, Muro K, Van Cutsem E, Oh SC, Bodoky G, Shimada Y, Hironaka S, Sugimoto N, Lipatov O, Kim TY, Cunningham D, Rougier P, Komatsu Y, Ajani J, Emig M, Carlesi R, Ferry D, Chandrawansa K, Schwartz JD, Ohtsu A; RAINBOW Study Group. Ramucirumab plus paclitaxel versus placebo plus paclitaxel in patients with previously treated advanced gastric or gastro-oesophageal junction adenocarcinoma (RAINBOW): a double-blind, randomised phase 3 trial. Lancet Oncol 2014;15(11):1224–1235.
53. Javle M, Smyth EC, Chau I. Ramucirumab: successfully targeting angiogenesis in gastric cancer. Clin Cancer Res. 2014;20(23):5875–81.
54. Nakamura K, Sugano H, Takagi K. Carcinoma of the stomach in incipient phase: its histogenesis and histological appearances. Gan. 1968;59:251–8.
55. Lauren P. The two histological main types of gastric carcinoma. Diffuse and so-called intestinal type carcinoma: an attempt at histological classification. Acta Pathol Microbiol Scand. 1965;64:31–49.
56. Vauhkonen M, Vauhkonen H, Sipponen P. Pathology and molecular biology of gastric cancer. Best Pract Res Clin Gastroenterol. 2006;20:651–74.
57. Yasui W, Oue N, Ito R, Kuraoka K, Nakayama H. Search for new biomarkers of gastric cancer through serial analysis of gene expression and its clinical implications. Cancer Sci. 2004;95:385–92.
58. Yasui W, Sentani K, Sakamoto N, Anami K, Naito Y, Oue N. Molecular pathology of gastric cancer: research and practice. Pathol Res Pract. 2011;207:608–12.
59. Oue N, Oshimo Y, Nakayama H, et al. DNA methylation of multiple genes in gastric carcinoma: association with histological type and CpG island methylator phenotype. Cancer Sci. 2003;94:901–5.
60. Oue N, Sentani K, Sakamoto N, Yasui W. Clinicopathologic and molecular characteristics of gastric cancer showing gastric and intestinal mucin phenotype. Cancer Sci. 2015;106(8):951–8.
61. Endoh Y, Tamura G, Watanabe H, Ajioka Y, Motoyama T. The common 18-base pair deletion at codons 418–423 of the E-cadherin gene in differentiated-type adenocarcinomas and intramucosal precancerous lesions of the stomach with the features of gastric foveolar epithelium. J Pathol. 1999;189:201–6.
62. Shibata N, Watari J, Fujiya M, Tanno S, Saitoh Y, Kohgo Y. Cell kinetics and genetic instabilities in differentiated type early gastric cancers with different mucin phenotype. Hum Pathol. 2003;34:32–40.
63. Sentani K, Matsuda M, Oue N, et al. Clinicopathological significance of MMP-7, laminin gamma2 and EGFR expression at the invasive front of gastric carcinoma. Gastric Cancer. 2014;17:412–22.
64. Kakiuchi M, Nishizawa T, Ueda H, et al. Recurrent gain-of-function mutations of RHOA in diffuse-type gastric carcinoma. Nat Genet. 2014;46:583–7.
65. Forbes SA, Beare D, Gunasekaran P, et al. COSMIC: exploring the world's knowledge of somatic mutations in human cancer. Nucleic Acids Res. 2015;43:D805–11.
66. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of gastric adenocarcinoma. Nature. 2014;513:202–9.
67. Ferlay J, Soerjomataram I, Ervik M, et al. Cancer incidence and mortality worldwide: IARC CancerBase No. 11; 2013.
68. Greenlee RT, Hill-Harmon MB, Murray T, et al. Cancer statistics, 2001. CA Cancer J Clin. 2001;51:15–36.

69. Lee WJ, Lee WC, Houng SJ, et al. Survival after resection of gastric cancer and prognostic relevance of systematic lymph node dissection: twenty years experience in Taiwan. World J Surg. 1995;19:707–13.
70. Mok YJ, Koo BW, Whang CW, et al. Cancer of the stomach: a review of two hospitals in Korea and Japan. World J Surg. 1993;17:777–82.
71. Fuchs CS, Mayer RJ. Gastric carcinoma. N Engl J Med. 1995;333:32–41.
72. Hundahl SA, Phillips JL, Menck HR. The National Cancer Data Base Report on poor survival of U.S. gastric carcinoma patients treated with gastrectomy: fifth edition American joint committee on cancer staging, proximal disease, and the "different disease" hypothesis. Cancer. 2000;88:921–32.
73. Lin SJ, Gagnon-Bartsch JA, Tan IB, Earle S, Ruff L, Pettinger K, et al. Signatures of tumour immunity distinguish Asian and non-Asian gastric adenocarcinomas. Gut. 2015;64(11):1721–31.
74. Robert C, Ribas A, Wolchok JD, et al. Anti-programmed death- receptor-1 treatment with pembrolizumab in ipilimumab-refractory advanced melanoma: a randomized dose-comparison cohort of a phase 1 trial. Lancet. 2014;384:1109–17.
75. Topalian SL, Sznol M, McDermott DF, et al. Survival, durable tumor remission, and long-term safety in patients with advanced melanoma receiving nivolumab. J Clin Oncol. 2014;32:1020–30.
76. Brahmer J, Reckamp KL, Baas P, et al. Nivolumab versus docetaxel in advanced squamous-cell non-small-cell lung cancer. N Engl J Med. 2015;373:123–35.
77. Motzer RJ, Escudier B, McDermott DF, et al. Nivolumab versus everolimus in advanced renal-cell carcinoma. N Engl J Med. 2015;373:1803–13.
78. FDA; 2016. Available online: http://www.accessdata.fda.gov/scripts/cder/drugsatfda/index.cfm?fuseaction=Search.Label_ApprovalHistory
79. Larkin J, Hodi FS, Wolchok JD. Combined nivolumab and ipilimumab or monotherapy in untreated melanoma. N Engl J Med. 2015;373:1270–1.
80. Seiwert TY, Burtness B, Mehra R, Weiss J, Berger R, Eder JP, et al. Safety and clinical activity of pembrolizumab for treatment of recurrent or metastatic squamous cell carcinoma of the head and neck (KEYNOTE-012): an open-label, multicentre, phase 1b trial. Lancet Oncol. 2016;17(7):956–65.
81. Le DT, Uram JN, Wang H, et al. PD-1 blockade in tumors with mismatch-repair deficiency. N Engl J Med. 2015;372:2509–20.
82. Muro K, Chung HC, Shankaran V, Geva R, Catenacci D, Gupta S, Eder JP, Golan T, Le DT, Burtness B, McRee AJ, Lin CC, Pathiraja K, Lunceford J, Emancipator K, Juco J, Koshiji M, Bang YJ. Pembrolizumab for patients with PD-L1-positive advanced gastric cancer (KEYNOTE-012): a multicentre, open-label, phase 1b trial. Lancet Oncol. 2016;17(6):717–26.
83. Fujitani K, Yang HK, Mizusawa J, Kim YW, Terashima M, Han SU, Iwasaki Y, Hyung WJ, Takagane A, do Park J, Yoshikawa T, Hahn S, Nakamura K, Park CH, Kurokawa Y, Bang YJ, Park BJ, Sasako M, Tsujinaka T; REGATTA study investigators. Gastrectomy plus chemotherapy versus chemotherapy alone for advanced gastric cancer with a single non-curable factor (REGATTA): a phase 3, randomised controlled trial. Lancet Oncol 2016;17(3):309–318.
84. Yoshida K, Yamaguchi K, Okumura N, Tanahashi T, Kodera Y. Is conversion therapy possible in stage IV gastric cancer: the proposal of new biological categories of classification. Gastric Cancer. 2016;19(2):329–38.
85. Yamaguchi K, Yoshida K, Tanaka Y, Matsuhashi N, Toshiyuki T, Takahashi T. Conversion therapy for stage IV gastric cancer-the present and future. Transl Gastroenterol Hepatol. 2016;1:50.

Chapter 5
Colorectal Cancers Developed from Proximal and Distal Tumor Location Belong to the Distinct Genetic Entity and Show Different Oncologic Behavior

Nagahide Matsubara

Abstract Colorectal cancer is now understood as a genetic disease.

Because of the importance of this highly prevalent disease, intense research efforts during the past two decades have focused on molecular processes to gain a better understanding of carcinogenesis. Since then, colorectal cancer has become a leading research model for the genetic basis of cancer. Attempt of molecular classification of colorectal cancer was made in order to offer precision medicine.

Colorectal cancer located either proximal or distal to the splenic flexure has been considered as belonging to different clinicopathological or physiological categories. Now, tumor location in colorectum is becoming an important surrogate marker to estimate prognosis and to determine the treatment decision including selection of chemotherapy agents for CRC.

Keywords Colorectal cancer • Tumor location • Chemotherapy • Carcinogenesis • Molecular classification

5.1 Introduction

Colorectal cancer (CRC) is the third most common cancers in developed countries [1]. CRC is a significant cause of morbidity and mortality in Western population. Majority of CRC develops from distal part of the colon (descending, sigmoid colon and rectum), but recently the number of CRC develops from proximal part of the colon (cecum, ascending and transverse colon) is gradually increasing especially in elderly female population. Interestingly, majority of CRCs of Lynch syndrome, one of the common

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hereditary CRC syndromes, develop from proximal part of the colon. The idea that CRCs located either proximal or distal to the splenic flexure of the colon belong to the different clinicopathological or physiological categories was not new. Nearly three decades ago, Bufill proposed that colon cancer located to the proximal and distal part of colorectum may rise from different biological pathways [2]. Differences in the embryologic origin of epithelium of proximal and distal segments may determine the differences in the susceptibility to the environmental carcinogenesis.

Recent advancement in the molecular biology supports an idea that differently accumulated genetic alterations on each side of the colon may underlie the pathologically different colorectal cancer. More recently, primary tumor location of CRCs has been considered as a prognostic factor: patients with proximal-sided tumors have worse prognosis than those with distal-sided tumors. Treatment effect of certain anticancer medicine may be different depending on the tumor location.

In this chapter, tumor location and CRCs are widely discussed especially hereditary, and acquired alterations on proximal or distal part of CRCs are discussed.

5.2 Biology of Normal Colon

Embryologic origins are different between proximal and distal segment of colorectum [3]. Distal part of the colorectum (those originating in the splenic flexure, descending colon, sigmoid colon, rectum) is derived from the embryonic hindgut [3]. In contrast, proximal part of the colorectum (those originated in the cecum, ascending colon, hepatic flexure, or transverse colon) is derived from the embryonic midgut, just as part of the duodenum and small intestine [3]. Vascular supply to the proximal and distal colon is also totally different, as midgut-originated proximal colon is served by the superior mesenteric artery, whereas hindgut-originated distal part of the large intestine is served by the inferior mesenteric artery [3].

Endocrine component is also different in tumor location. Accumulation of chromogranin immunoreactive cells are observed in the distal large intestine and few of those cells are observed in the proximal part of colon [4]. Ornithine decarboxylase (ODC) is a key enzyme in polyamine synthesis, and its functional activity closely parallels cellular proliferative activity in normal colonic mucosa. GTP-activated isoform of ODC is predominantly distributed in proximal colon [5].

5.3 Colorectal Cancer

In general proximal CRCs were more frequently diagnosed in elderly woman, and distal CRCs were more frequently diagnosed in men [6]. Patients with proximal CRC complain less symptoms, and comorbidities were more common in patients with proximal CRC [6]. Histologically, mucinous, undifferentiated, and signet ring cell carcinomas were more frequently diagnosed in patients with proximal CRC.

Consistent with these differences in embryological origin, distal-sided and proximal-sided CRC possess different karyotypic and enzymatic profiles. For example, expression of ODC is frequently elevated in many human neoplasms including CRC. High levels of ODC expression and the presence of a GTP-activated isoform for proximal CRC predict a favorable prognosis in CRC [5]. It is known that cancers developed in the distal part of colon have more unstable in karyotype and show frequent loss of heterozygosity of chrosomose compared to those developed in the proximal colon.

5.3.1 Hereditary Colorectal Cancer

Hereditary CRCs account for approximately 5–10% of the total CRC burden. Genetic germline mutations are the basis of inherited colon cancer syndromes. Two forms of hereditary colorectal cancer syndromes—one with and without associated polyposis of the colon—are known as familial adenomatous polyposis (FAP) and Lynch syndrome (LS). Interestingly CRCs based on FAP often develop in the distal part colon, and those on LS more often develop in proximal part of the colon.

5.3.2 Lynch Syndrome (LS)

LS is the most frequently observed hereditary syndrome developing CRC. It accounts for approximately 3–6% of the total CRC burden. The Lynch syndrome is an autosomal dominant syndrome with 30–74% penetrance. The syndrome is characterized by an onset of CRC at an early age, right-sided predominance, excess of synchronous and metachronous CRCs, and extracolonic tumors of the endometrium, renal pelvis, ureter, and other locations. Pathologic characteristics of CRCs in Lynch syndrome include poor differentiation, mucin production, peritumoral lymphocytic infiltrate, and Crohn's-like reaction. Causing genes for LS are mismatch repair genes including *MLH1*, *MSH2*, *MSH6*, and *PMS2*.

5.3.3 Familial Adenomatous Polyposis (FAP)

FAP is an autosomal-dominant inherited disease characterized by the development of multiple adenomas throughout the colorectum. It represents about 0.5–1% of all CRC cases and is the most common gastrointestinal polyposis syndrome. Germline mutations in the *APC* gene are responsible for most cases of FAP. Classic FAP is characterized by the presence of hundreds to thousands adenomatous polyps throughout the colon and rectum. At the time of adolescence, the polyps are usually identified in the rectosigmoid as small polyps and, thereafter, increase in size and

number. About half of FAP patients develop adenomas by 15 years of age and 95% by age 35 years. CRC inevitably occurs throughout the colorectum at an earlier age than sporadic CRC (average age of 35 years) but mainly occurs in distal part of the colon. Attenuated FAP (AFAP) is a variant of FAP with a mild disease course, characterized by a reduced number of polyps (10–100), later age of onset, frequently proximal-sided distribution of polyps, and lower CRC risk (up to 70%). Clinical definition of AFAP is controversial and should be considered in any patient with 10–99 adenomas, although a precise diagnosis is often difficult in a single patient. In many FAP patients, extracolonic manifestations are present, including gastric and duodenal polyps, desmoid tumors, thyroidal and brain tumors, osteomas, congenital hypertrophy of the retinal pigment epithelium, supernumerary teeth, and epidermoid cysts.

5.4 Molecular Carcinogenesis of Sporadic Colorectal Cancer

Recent advances have contributed to the understanding of the molecular basis of these various patterns of sporadic CRC. CRC develops as a result of the pathologic transformation of normal colonic epithelium to an adenomatous polyp and ultimately an invasive cancer. Mutations in two classes of genes, tumor-suppressor genes and proto-oncogenes, are thought to impact a proliferative advantage to cells and contribute to development of the malignant phenotype. The multistep progression requires years and possibly decades and is accompanied by a number of recently characterized genetic alterations.

Two molecular pathways for colorectal carcinogenesis are well known [7]. Genomic instability is critical for carcinogenesis. It accelerates the neoplastic evolutionary process, and without this, acquisition of new genetic alteration would occur too slowly for cancer development. One common genomic instability is chromosomal instability (CIN) [8]. The molecular model of the adenoma-carcinoma sequence (traditional pathway) is attributed to the CIN, which is characterized by stepwise mutation or deletion of *KRAS*, *APC*, *DCC*, and *TP53* [8, 9] (Fig. 5.1). As a gatekeeper gene, *APC* is an important regulator of the CIN pathway [10]. This pathway is involved in the formation of dysplastic aberrant crypt foci (ACF) with *KRAS* mutations [11]. A minority of dysplastic ACF develops into simple and then advanced adenomatous polyps and finally produces an invasive cancer [12, 13]. Sporadic CRC resulted in CIN pathway mainly develops in distal part of the colon.

The second pathway responsible to the genomic instability is the mutator pathway—microsatellite instability (MIN) pathway. In this pathway, dysfunction of a mismatch repair (MMR) genes (e.g., *MLH1*, *MSH2*, *MSH6*, or *PMS2*) results in genetic instability characterized by the accumulation of numerous mutations specifically target of repetitive DNA sequences called microsatellite. Thus, this phenomenon is termed microsatellite instability (MSI). The high frequency of MSI detected throughout the genome after inactivation of a MMR gene is termed high-level MSI (MSI-H) [14]. A subset (10–15%) of sporadic CRC exhibits MSI-H, and

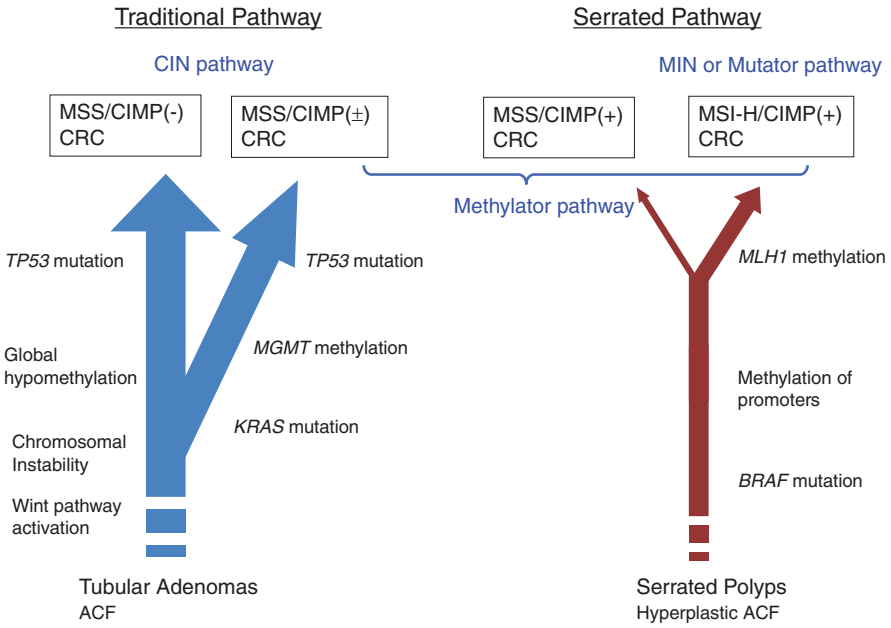

Fig. 5.1 Traditional and serrated pathway of carcinogenesis in colorectal cancer. *CIN* chromosomal instability, *MSS* microsatellite stable, *CIMP* CpG island methylator phenotype, *MIN* microsatellite instability pathway, *MSI*, microsatellite instability, *ACF* aberrant crypt foci (Matsubara [7])

most of those are caused by silencing of *MLH1* due to promoter hypermethylation, one of the epigenetic events that may lead to multiple genetic changes in tumor cells. Sporadic CRC following MIN pathway mainly develops in the proximal portion of the colon.

5.4.1 Methylator Pathway in Colorectal Cancer

Currently a subset of CRC can be distinguished by the status of methylation at several promoter loci. This panel (marker promoters) takes advantage to classify cancers as CpG island methylator phenotype (CIMP+) or not (CIMP-), just like NIH microsatellite panel does to distinguish MSI status [15, 16]. Depending on the marker used, 24–51% of CRCs belong to CIMP+ subtype. The first proposed CIMP panel includes promoter regions of *MLH1*, *p16*, *MINT1*, *2*, and *31* [15]. CIMP+ CRCs are often developed in older women, with a predominance of proximal colon, high grade, and mucinous type. CIMP+ CRCs are associated with hypermethylation of many promoters other than original five markers. Since CIMP CRCs frequently show promoter methylation at *MLH1*, it is obvious that CIMP cancers share a similar phenotype with sporadic MSI-H. It is interesting that those CIMP+/MSS CRCs are associated with a worse prognosis, while MSI-H CRCs show better prognosis [17] (Fig. 5.1).

The additional events regulating both prognosis and *MLH1* methylation and, thus, MSI status are unclear. Not all researchers in this field have accepted the concept of CIMP. Over the past few years, there has been debate as to whether the CIMP tumors represent a biologically distinct subgroup of CRCs or an artificially selected group from a continuum of tumors showing different degrees of methylation at particular loci. Since original CIMP panel was inadequate to classify CRCs into well-defined subsets, an alternative panel of markers (*CACNA1G, IGF2, NEUROG1, RUNX3, SOCS1*) has been proposed [18]. By this new panel, CRCs distribute bimodal into new CIMP+ and new CIMP- cases, with an even close correlation between CRCs with new CIMP+ and CRCs with *BRAF* mutation. In other words, CRSs with new CIMP+ is almost the same as the sporadic CRC with MSI-H. We have shown that the degrees of promoter methylation at multiple loci in CRC are closely related to the mutational status of *BRAF* and *KRAS*. Since *BRAF* and *KRAS* mutations occur in a mutually exclusive manner, a pathway common to both is critical in developing cancers [19]. The *RAS-RAF-MEK-ERK* signaling pathway is important in apoptosis and particular in anoikis, the process of apoptosis following loss of the epithelial connection to the basement membrane. Failure of anoikis has an important role in developing hyperplastic polyps and serrated adenomas, which are the postulated precursors of CIMP+ colorectal cancers. *BRAF* and *KRAS* mutations interrupt the *RAS-RAF-MEK-ERK* signaling pathway at different levels, impairing normal anoikis [20].

5.4.2 Serrated Pathway

The hypothesis of the "serrated neoplasia pathway," in which serrated polyps (sessile serrated adenomas, hyperplastic polyps, serrated adenomas, and admixed polyps) are the precursors of the sporadic MSI-H CRCs, is supported by the recent finding that 78% of sessile serrated adenomas exhibit *BRAF* mutation. Cancers from this pathway may begin as hyperplastic aberrant crypt foci (ACF), becoming right-sided sessile serrated adenomas, and ultimately develop to MSI-H CRCs (Fig. 5.1). *BRAF* mutation and associated failure of anoikis may be important at least in the early stage of this pathway to form a serrated architecture. The methylator pathway is usually associated with *BRAF* mutation with or without promoter methylation of *MLH1,* resulting in MSI-H or MSS CRC, respectively [21]. It is interesting that there is an association between *MGMT* methylation and *KRAS* mutation in a subset of MSS/CIMP+ cancers. There may be an alternate methylator pathway, without *BRAF* mutation, but rather with the acquisition or maintenance of *KRAS* (G to A) mutation following and the result of the promoter methylation of *MGMT* (Fig. 5.1). It is possible that mutation of *BRAF* with or without promoter methylation of *MLH1* may define one methylator pathway, while the methylation of *MGMT* and *KRAS* mutation could characterize an "alternate methylator" subtype. The precursor lesions for these ultimately "*KRAS* mutant/*MGMT*-methylated" cancers may be adenoma partly being serrated polyp, but this is an area requiring for further

research. Accordingly we proposed the four molecular carcinogenesis pathway of CRC (Fig. 5.1).

5.5 Recent Molecular Classification After Next-Generation Sequencing Era

Gene expression-based subtyping is widely accepted as a relevant source of disease stratification [22]. After emergence of powerful next-generation sequencing, more comprehensive and precise genetic cross-nation analysis was made. Cancer Genome Atlas (TCGA) Research Network has reported integrated genome-wide studies of ten distinct malignancies including colon (COAD) and rectal (READ) adenocarcinomas (Cancer Genome Atlas Research Network, 2012), lung squamous cell carcinoma (LUSC) (Cancer Genome Atlas Research Network, 2012), breast cancer (BRCA) (Cancer Genome Atlas Research Network, 2012), acute myelogenous leukemia (AML) (Cancer Genome Atlas Research Network, 2013), endometrial cancer (UCEC) (Kandoth et al., 2013), renal cell carcinoma (KIRC) (Cancer Genome Atlas Research Network, 2013), and bladder urothelial adenocarcinoma (Cancer Genome Atlas Research Network, 2014) [22]. The subclassification is based on recurrent genetic and epigenetic alterations that converge on common pathways (e.g., *p53* and/or *Rb* checkpoint loss; *RTK/RAS/MEK* or *RTK/PI3K/AKT* activation). Meaningful differences in clinical behavior are often correlated with the single-tissue tumor types, and, in a few case, single-tissue subtype identification has led to therapies that target the driving subtype-specific molecular alteration(s). *EGFR* mutant lung adenocarcinomas and *ERBB2*-amplified breast cancer are two well-established examples [22]. Despite the widespread use, its translational and clinical utility is hampered by discrepant results, likely related to differences in data processing and algorithms applied to diverse patient cohorts, sample preparation methods, and gene expression platforms. Attempt to elucidate intrinsic subtypes of CRC was made elsewhere [23]. Inspection of the published gene expression-based CRC classifications revealed only superficial similarities [24]. For example, all groups identified one tumor subtype enriched for microsatellite instability (MSI) and one subtype characterized by high expression of mesenchymal genes, but failed to achieve full consistency among the other subtypes [23].

5.6 Chemotherapy and Treatment Response

Predictive and prognostic meaning of tumor location is not well understood. Such knowledge may shed light on interactions linking tumor location and treatment response and outcome that may guide personalized therapy. Notably, proximal-sided tumors are more frequently characterized by a host of adverse prognostic

factors, including *BRAF* mutation positivity, MSI (prognostic in stage IV disease), hypermutation, serrated pathway signature positivity, and mucinous histology; conversely, distal-sided tumors more frequently possess gene expression profiles characteristic of EFGR inhibitor-sensitive phenotype (i.e., EGFR/ERBB2 amplified, epiregulin high, and possessing classic chromosomal instability) [25]. The existence of six subtypes of CRC based on the combined analysis of gene expression profiles are suggested and differential response to cetuximab. These subtypes are phenotypically distinct in their DFS and vary in degree of response to cetuximab and standard-of-care chemotherapy. These CRC subtypes are associated with distinctive anatomical regions of the colon phenotype and with location-dependent differentiation states and Wnt signaling activity. Candidate biomarkers that might be developed into clinical qRT-PCR or immunohistochemical assays were identified to classify CRC tumors into one of six subtypes as a guide to assignment of subtype-specific therapeutic agents. With regard to first-line chemotherapy, particular subtypes might show beneficial responses to FOLFIRI in either adjuvant or metastatic settings, whereas in unselected CRC, this treatment did not improve survival in the adjuvant setting. Stemlike-subtype tumors, both in the adjuvant and metastatic settings, as well as inflammatory-subtype tumors in the adjuvant setting, may best be treated with FOLFIRI. Additionally, the transit-amplifying sub-subtypes and the goblet-like subtype will probably not respond to FOLFIRI in the adjuvant setting. Watchful surveillance might spare patients with these forms of disease from the harmful side effects of debilitating and ineffective FOLFIRI treatment. Moreover, and in contrast to the adjuvant setting, the CS-TA or CR-TA subtype might be effectively treated with cetuximab or a cMET inhibitor, respectively, in the metastatic setting [26]. These molecular differences manifest as differential clinical behavior, with right-sided tumors typically displaying worse prognosis. Nevertheless, primary tumor location has not traditionally been included as a stratification criterion in clinical trials, and the influence of tumor location on responsiveness to particular therapies remains incompletely understood. However, primary tumor location could be an important prognostic factor in previously untreated metastatic CRC. Given the consistency across an exploratory set and two confirmatory phase III studies, side of tumor origin should be considered for stratification in randomized trials [27]. Primary tumor location and *KRAS* codon 12/13 mutational status interact on the outcome of patients with metastatic CRC receiving cetuximab- based first-line therapy. Distal-sided primary tumor location might be a predictor of cetuximab efficacy [28]. Also, retrospective analysis of the NCIC CTG CO.17 trial recently reported that tumor location was predictive of treatment benefit. In this population of chemotherapy-refractory patients with *KRAS* wild-type metastatic CRC, adding cetuximab to best supportive care significantly benefitted patients with distal-sided tumors, but has limited benefit in patients with proximal-sided tumors. Furthermore, a significant interaction was observed between tumor location and treatment for progression-free survival. Patients with a proximal-sided primary have more negative prognostic factors and indeed have inferior outcomes compared with those with a distal-sided primary [29]. In the *RAS* wild-type

population of CRYSTAL and FIRE-3, patients with distal-sided tumors had a markedly better prognosis than those with proximal-sided tumors. First-line FOLFIRI plus cetuximab clearly benefitted patients with distal-sided tumors (vs FOLFIRI or FOLFIRI plus bevacizumab, respectively), whereas patients with proximal-sided tumors derived limited benefit from standard treatments [25]. Nibolumab (nivo) showed durable responses and disease control in heavily pretreated patients with dMMR/MSI-H metastatic CRC. Treatment was well tolerated, with no new safety signals (ASCO abstracts).

5.7 Conclusions

At present CRC is understood as a genetic disease, and the attempt of advanced molecular classification is applied to the patients to accomplish personalized medicine. In order to identify molecular classification, examination of several surrogate markers instead of going through precise genetic alterations is desired. CRCs located either proximal or distal to the splenic flexure of the colon have been considered as belonging to the different clinicopathological or physiological categories. Now tumor location in colorectum can possibly become an important surrogate marker to estimate prognosis and [6] to determine the treatment decision including selection of chemotherapy agents for CRC.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global cancer statistics. CA Cancer J Clin. 2011;61:69–90.
2. Bufill JA. Colorectal cancer: evidence for distinct genetic categories based on proximal or distal tumor location. Ann Intern Med. 1990;113:779–88.
3. Langman J. Medical embryology. 4th ed. Baltimore: Williams & Wilkins; 1981.
4. Facer P, Bishop AE, Cole GA, Aitchison M, Kendall CH, van Aswegen G, Penketh RJ, Rodek CH, McKeever P, Polak JM. Developmental profile of chromogranin, hormonal peptides, and 5-hydroxytryptamine in gastrointestinal endocrine cells. Gastroenterology. 1989;97:48–57.
5. Matsubara N, Hietala OA, Gilmour SK, Yum KY, Litwin S, Watts P, Brennan O'B. Association between high levels of ornithine decarboxylase activity and favorable prognosis in human colorectal carcinoma. Clin Cancer Res. 1995;1:665–71.
6. Benedix F, Schmidt U, Mroczkowski P, Gastinger I, Lippert H, Kube R, Study Group Colon/Rectum C. Colon Carcinoma--classification into right and left sided cancer or according to colonic subsite?--analysis of 29,568 patients. Eur J Surg Oncol. 2011;37:134–9.
7. Matsubara N. Epigenetic regulation and colorectal cancer. Dis Colon Rectum. 2012;55:96–104.
8. Iino H, Simms L, Young J, Arnold J, Winship IM, Webb SI, Furlong KL, Leggett B, Jass JR. DNA microsatellite instability and mismatch repair protein loss in adenomas presenting in hereditary non-polyposis colorectal cancer. Gut. 2000;47:37–42.
9. Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal-tumor development. N Engl J Med. 1988;319:525–32.

10. Kane MF, Loda M, Gaida GM, Lipman J, Mishra R, Goldman H, Jessup JM, Kolodner R. Methylation of the hMLH1 promoter correlates with lack of expression of hMLH1 in sporadic colon tumors and mismatch repair-defective human tumor cell lines. Cancer Res. 1997;57:808–11.
11. Herman JG, Umar A, Polyak K, Graff JR, Ahuja N, Issa JP, Markowitz S, Willson JK, Hamilton SR, Kinzler KW, Kane MF, Kolodner RD, Vogelstein B, Kunkel TA, Baylin SB. Incidence and functional consequences of hMLH1 promoter hypermethylation in colorectal carcinoma. Proc Natl Acad Sci U S A. 1998;95:6870–5.
12. Nephew KP, Huang TH. Epigenetic gene silencing in cancer initiation and progression. Cancer Lett. 2003;190:125–33.
13. Feinberg AP, Tycko B. The history of cancer epigenetics. Nat Rev Cancer. 2004;4:143–53.
14. Jass JR. Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology. 2007;50:113–30.
15. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci U S A. 1999;96:8681–6.
16. Rashid A, Issa JP. CpG island methylation in gastroenterologic neoplasia: a maturing field. Gastroenterology. 2004;127:1578–88.
17. Ward RL, Cheong K, Ku SL, Meagher A, O'Connor T, Hawkins NJ. Adverse prognostic effect of methylation in colorectal cancer is reversed by microsatellite instability. J Clin Oncol. 2003;21:3729–36.
18. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW. CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet. 2006;38:787–93.
19. Nagasaka T, Sasamoto H, Notohara K, Cullings HM, Takeda M, Kimura K, Kambara T, MacPhee DG, Young J, Leggett BA, Jass JR, Tanaka N, Matsubara N. Colorectal cancer with mutation in BRAF, KRAS, and wild-type with respect to both oncogenes showing different patterns of DNA methylation. J Clin Oncol. 2004;22:4584–94.
20. Oberst MD, Beberman SJ, Zhao L, Yin JJ, Ward Y, Kelly K. TDAG51 is an ERK signaling target that opposes ERK-mediated HME16C mammary epithelial cell transformation. BMC Cancer. 2008;8:189.
21. Nagasaka T, Koi M, Kloor M, Gebert J, Vilkin A, Nishida N, Shin SK, Sasamoto H, Tanaka N, Matsubara N, Boland CR, Goel A. Mutations in both KRAS and BRAF may contribute to the methylator phenotype in colon cancer. Gastroenterology. 2008;134:1950–60.
22. Hoadley KA, Yau C, Wolf DM, Cherniack AD, Tamborero D, Ng S, Leiserson MD, Niu B, MD ML, Uzunangelov V, Zhang J, Kandoth C, Akbani R, Shen H, Omberg L, Chu A, Margolin AA, Van't Veer LJ, Lopez-Bigas N, Laird PW, Raphael BJ, Ding L, Robertson AG, Byers LA, Mills GB, Weinstein JN, Van Waes C, Chen Z, Collisson EA, Cancer Genome Atlas Research N, Benz CC, Perou CM, Stuart JM. Multiplatform analysis of 12 cancer types reveals molecular classification within and across tissues of origin. Cell. 2014;158:929–44.
23. Guinney J, Dienstmann R, Wang X, de Reynies A, Schlicker A, Soneson C, Marisa L, Roepman P, Nyamundanda G, Angelino P, Bot BM, Morris JS, Simon IM, Gerster S, Fessler E, EMF DS, Missiaglia E, Ramay H, Barras D, Homicsko K, Maru D, Manyam GC, Broom B, Boige V, Perez-Villamil B, Laderas T, Salazar R, Gray JW, Hanahan D, Tabernero J, Bernards R, Friend SH, Laurent-Puig P, Medema JP, Sadanandam A, Wessels L, Delorenzi M, Kopetz S, Vermeulen L, Tejpar S. The consensus molecular subtypes of colorectal cancer. Nat Med. 2015;21:1350–6.
24. Cancer Genome Atlas N. Comprehensive molecular characterization of human colon and rectal cancer. Nature. 2012;487:330–7.
25. Tejpar S, Stintzing S, Ciardiello F, Tabernero J, Van Cutsem E, Beier F, Esser R, Lenz HJ, Heinemann V. Prognostic and predictive relevance of primary tumor location in patients with

RAS wild-type metastatic colorectal cancer: retrospective analyses of the CRYSTAL and FIRE-3 trials. JAMA Oncol. 2016;3(2):194–201.
26. Sadanandam A, Lyssiotis CA, Homicsko K, Collisson EA, Gibb WJ, Wullschleger S, Ostos LC, Lannon WA, Grotzinger C, Del Rio M, Lhermitte B, Olshen AB, Wiedenmann B, Cantley LC, Gray JW, Hanahan D. A colorectal cancer classification system that associates cellular phenotype and responses to therapy. Nat Med. 2013;19:619–25.
27. Loupakis F, Yang D, Yau L, Feng S, Cremolini C, Zhang W, Maus MK, Antoniotti C, Langer C, Scherer SJ, Muller T, Hurwitz HI, Saltz L, Falcone A, Lenz HJ. Primary tumor location as a prognostic factor in metastatic colorectal cancer. J Natl Cancer Inst. 2015;107:dju427.
28. von Einem JC, Heinemann V, von Weikersthal LF, Vehling-Kaiser U, Stauch M, Hass HG, Decker T, Klein S, Held S, Jung A, Kirchner T, Haas M, Holch J, Michl M, Aubele P, Boeck S, Schulz C, Giessen C, Stintzing S, Modest DP. Left-sided primary tumors are associated with favorable prognosis in patients with KRAS codon 12/13 wild-type metastatic colorectal cancer treated with cetuximab plus chemotherapy: an analysis of the AIO KRK-0104 trial. J Cancer Res Clin Oncol. 2014;140:1607–14.
29. Price TJ, Beeke C, Ullah S, Padbury R, Maddern G, Roder D, Townsend AR, Moore J, Roy A, Tomita Y, Karapetis C. Does the primary site of colorectal cancer impact outcomes for patients with metastatic disease? Cancer. 2015;121:830–5.

Chapter 6
Evolving Immunotherapy Approaches for Hepatocellular Carcinoma

Ken Takahashi and Hiroyuki Marusawa

Abstract Hepatocellular carcinoma (HCC) is a serious therapeutic challenge, with poor prognosis. Therapeutic options for HCC are limited, particularly for the patients at advanced stage who are not eligible for curative therapies such as radio-frequency ablation (RFA), hepatectomy, or hepatic transplantation. Thus, novel approaches are urgently needed for the treatment of this prevalent malignancy. Recent advance in cancer immunotherapy such as immune checkpoint blockade has revolutionized the landscape of cancer therapy, and the efficacy of several classes of immunotherapy has been tested in clinical trials. The current issue reviewed the current status of immunotherapy for HCC as well as the unique tolerogenic character of liver immune system and the immune evasion mechanisms of HCC. Taking into the account of the immunosuppressive forces operating in the hepatic tumor microenvironment—combination therapies of different strategies might be encouraged for achieving optimal clinical outcome.

Keywords liver cancer • cancer immunotherapy • cytotoxic T cell • PD-1 • CTLA-4

Abbreviations

ACT	Adoptive cell transfer
AE	Adverse event

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AFP	α-Fetoprotein
ALT	Alanine aminotransferase
APC	Antigen-presenting cell
AST	Aminotransferase
CAR	Chimeric antigen receptor
CIK	Cytokine-induced killer cell
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte protein-4
DC	Dendritic cell
DCR	Disease control rate
DFS	Disease-free survival
GITR	Glucocorticoid-induced TNFR-related protein
GPC3	Glypican-3
HBV	Hepatitis B virus
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
KC	Kupffer cell
KIR	Killer inhibitory receptor
LAG3	Lymphocyte-activation gene 3
LSEC	Liver sinusoidal endothelial cell
MART-1	Melanoma-associated antigen recognized by T cells-1
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
OS	Overall survival
PD-1	Programmed cell death protein-1
PR	Partial response rate
RECIST	Response evaluation criteria in solid tumors
RFA	Radio-frequency ablation
RFS	Recurrence-free survival
TAA	Tumor-associated antigens
TACE	Transarterial chemoembolization
TAM	Tumor-associated macrophage
TCR	T cell receptor
TERT	Telomerase reverse transcriptase
TGF-β	Transforming growth factor-β
TIL	Tumor-infiltrating lymphocyte
TIM-3	T cell immunoglobulin and mucin-domain containing-3
Treg	Regulatory T cell
TTP	Time to progression

6.1 Introduction

HCC is the most common type of hepatic malignancies, accounting for approximately 85% of primary liver cancer and the leading cause of cancer-related death worldwide [1]. The main risk factor for HCC is hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, which induces a chronic inflammation within the infected liver [2]. Alcohol consumption, obesity, and aflatoxin contact are also known as etiologic factors of HCC [3]. HCC patients at early stage benefit from curative therapies including percutaneous tumor ablation, surgical resection, and liver transplantation. For advanced stage patients, however, only limited options are available such as chemoembolization, radioembolization, or sorafenib, none of which is curative. Furthermore, the recurrence even after curative treatment is problematic. Therefore, there is an urgent need for developing new effective therapies for HCC.

The field of cancer immunotherapy is rapidly evolving. In particular, immune checkpoint blockade therapy targeting immunosuppressive molecules that negatively regulate T cell response has undoubtedly been one of the most impressive advancements in recent years. Cytotoxic T lymphocyte protein-4 (CTLA-4) and programmed cell death protein-1 (PD-1) are the most studied checkpoint molecules that induce T cell tolerance at priming phase and effector phase, respectively [4]. Great clinical achievements of antagonizing monoclonal antibodies to CTLA-4 and PD-1 in melanoma have clearly proved the potential of the immune system to eradicate tumor cells, validating the concept of harnessing patient's own immune system for controlling cancer [5]. In preclinical and clinical investigations, checkpoint blockade therapies are now intensively studied across a range of tumor types. In addition, there are other classes of cancer immunotherapy that include administration of agonistic antibody targeting co-stimulating molecule, cancer vaccine, adoptive cell transfer (ACT), and oncolytic virus [6]. Combinations of immunotherapies among themselves or with standards of care are expected to be the key approaches for the future cancer treatment.

For HCC that is characterized by metachronous multicentric occurrence, immunotherapy could be an appealing option, since it is capable of inducing systemic and durable anticancer response. However, most importantly to note is that the liver is an organ in which immune response is biased toward tolerance. This tolerogenic property of the liver is attributed to non-parenchymal liver cells such as resident dendritic cells (DCs), liver sinusoidal endothelial cells (LSECs), and Kupffer cells (KCs) as well as parenchymal hepatocytes [7]. Furthermore, HCC develops several mechanisms for escaping from host immune surveillance. Such additive immunosuppressive force in the HCC patients may represent a major impediment to effective antitumor immune response and thus must be counterbalanced when immunotherapeutic approaches are designed for HCC. In this review, we will discuss the unique immune characteristics of the liver, the interaction of HCC with immune system, and the current strategies for HCC immunotherapy, emphasizing on the importance of combination therapy.

6.2 The Liver as an Immunotolerant Organ

Because the liver is continuously exposed to abundant antigens contained in food and microbes from the intestine and produces metabolite-derived antigens, the liver has a unique immune environment that induces tolerance for preventing overactivation of immune response [7].

Different types of non-parenchymal cells are resident in the liver that promote tolerance through several mechanisms. LSECs function as cellular barrier between sinusoidal blood and hepatocytes. LSECs express pattern recognition receptors such as Toll-like receptors and class I and II major histocompatibility complex (MHC) molecules [8], functioning as antigen-presenting cells (APCs). However, low levels of CD80 and CD86 and high levels of PD-L1 expressed by LSECs limit their ability to effectively activate T cells [9]. Furthermore, IL-10 and TGF-β secreted by LSECs [10] also contribute to impairing CD8+ T cell activation. Thus, their main role in the liver seems to be the induction of T cell tolerance to maintain the immune homeostasis against constant supply of food- or bacteria-derived molecules. KCs are resident macrophages in the liver that represent the largest population of tissue-resident macrophages in the body [11]. While KCs play an important role in pathogen clearance [12], KCs induce immune tolerance under physiological conditions [13]. Exposure to lipopolysaccharide inhibits KCs to activate lymphocytes and stimulates KCs to secrete immunosuppressive cytokine IL-10 [14]. KCs also produce TGF-β [15] and prostaglandin E2, both of which can contribute to abrogating T cell activation [16] and preferentially expand regulatory T cells (Tregs), thereby inducing systemic T cell tolerance [17]. Resident hepatic DCs capture antigens and function as APCs but appear to be less potent activator of T cells compared with their counterparts from other organs [18]. A unique cytokine environment where IL-10 and TGF-β are secreted by KCs and LSECs may contribute to the immature phenotypes of resident DCs [19]. IL-10 produced by hepatic DCs promotes a shift from Th1- to Th2-type response, thereby inhibiting cellular immunity that is required for tumor elimination and promoting the development of Tregs [20].

Besides these non-parenchymal cells that serve as APCs in the liver, hepatocytes and parenchymal cells accounting for about 80% of total liver cells also play a potential immunological role. Hepatocytes can directly interact with naïve T cells and present antigens to the T cell receptor (TCR) in the context of MHC molecule. Due to the lack of expression of co-stimulatory molecules such as CD80 and CD86, however, they induce an anergic phenotype in T cells [21]. All these features of the liver as an immunotolerant organ represent a barrier for the development of immunity against HCC.

6.3 Immune Evasion Mechanisms of HCC

Multiple mechanisms of immune evasion have been identified in HCC. HCC tumor cells escape from the host's immunosurveillance by silencing of tumor antigens or defect in expression of genes involved in antigen processing and presentation, both of which allow escape from cytotoxic T cell (CTL) killing [22, 23]. Furthermore,

HCC cells also escape from immunity by producing various immunosuppressive factors such as IL-10, TGF-β, indoleamine 2,3-dioxygenase (IDO), arginase, adenosine, and immunoinhibitory checkpoint molecules [24].

In addition to tumor cells, several immune inhibitory stromal cells abundant in the tumor microenvironment also suppress effective antitumor T cell response. Tregs, characterized by the expression of CD25, CD4, and transcription factor FOXP3, physiologically play an essential role in inducing immunological self-tolerance by suppressing self-reactive T cells [25]. Due to their immunosuppressive functions, Tregs impede immunosurveillance against cancers. Indeed, in HCC patients, the increased numbers of Tregs in peripheral blood and their marked infiltration in the tumor microenvironment have been reported, and importantly the number of tumor-infiltrating Tregs positively correlates with the poor prognosis [26]. Myeloid-derived suppressor cells (MDSCs) are immunosuppressive myeloid cells, with a variety of tumor-supportive effects. Increased numbers of MDSCs in the tumor specimen and peripheral blood from HCC patients have reportedly been associated with tumor progression [27, 28]. MDSCs suppress antitumor T cell responses by the increased arginase activity and the production of reactive oxygen and nitrogen species [29]. MDSCs promote induction and expansion of Tregs by IL-10 and TGF-β secretion. Tumor-supportive M2 macrophages characterized by low IL-12 and high IL-10 production are distinguished from M1 macrophages that produce high IL-12 and low IL-10 levels. HCC microenvironment biases toward M2 phenotype, the characteristic of tumor-associated macrophages (TAMs). In addition to their immunosuppressive functions, TAMs promote tumor progression by angiogenesis, tumor invasion, and metastasis [30]. Regarding immune checkpoint molecules whose physiological role is controlling hyperactive immune response to pathogens or self, their expressions in the tumor microenvironment of HCC are dysregulated and result in the inhibition of antitumor effect by CTLs [31]. Among them, PD-1/PD-L1 axis is notably implicated in HCC and discussed in the separate section below.

6.4 HCC Immunogenicity

As described above, the intrinsic tolerogenic nature of hepatic immunity and the immune evasion mechanisms operating in the tumor microenvironment appear to suppress the induction of effective antitumor immunity. Nonetheless, significant number of HCC shows spontaneous regression presumably by immune-mediated mechanisms [32]. There are several evidences that HCC is potentially immunogenic. HCC immunogenicity is suggested by the presence of tumor-infiltrating lymphocytes (TILs) [33]. The ratio of CTLs to Tregs in TILs is reportedly an independent prognostic factor of disease-free survival (DFS) and OS after surgical resection of HCC [34]. Another study demonstrated that tumor antigen-specific CD8+ T cells were detected in the peripheral blood of more than 50% of HCC patients and that the frequency and magnitude of their responses were associated with patient survival [35].

All these observations suggest that HCC might be an attractive target for immunotherapy, if immune system is optimally stimulated or enhanced. In the following sections, I will discuss the current strategies for HCC immunotherapy that include checkpoint blockade, ACT, cancer vaccines, cytokine therapies, and combination therapies.

6.5 Strategies for HCC Immunotherapy

6.5.1 Immune Checkpoint Blockade

The intensity of an immune response is determined by the balance between stimulatory and inhibitory signals, known as immune checkpoints. The checkpoint molecules can be activated by cancers, resulting in promoting tumor evasion from immunity. Among many investigated immune checkpoints, CTLA-4 and PD-1 are the most studied ones, and the dramatic success of checkpoint blockade therapy with antibodies against CTLA-4 and PD-1 in melanoma has revolutionized the field of cancer therapy [5].

6.5.1.1 PD-1/PD-L1 Blockade

PD-1 is a negative co-stimulatory molecule that belongs to the CD28 superfamily. PD-1 is primarily expressed on T cells as well as B cells and monocytes. PD-L1 and PD-L2 are PD-1 ligands that belong to B7 co-stimulatory molecule family. PD-L1 is expressed in APCs and non-lymphoid cells, including parenchymal cells, while PD-L2 is exclusively expressed in DCs and macrophages [4]. Upon ligand ligation, PD-1 transduces inhibitory signals for TCR, leading to the inhibition of T cell proliferation and cytokine release. Although physiological role of PD-1 is suppressing hyperactive immune response and inducing peripheral tolerance, cancer cells utilize PD-1/PD-L1 system by expressing PD-L1 to activate PD-1 in TILs, thereby evading immunosurveillance [36]. PD-L1 is highly expressed in both HCC tumor cells and non-parenchymal APCs such as LSECs, KCs, and tumor-associated monocytes [37–39]. PD-L1 expression in clinical HCC samples reportedly ranges from 45% to 100% [39–42]. Importantly, in the analysis of surgically resected HCC, the expression levels of PD-L1 were associated with shorter DFS [41], suggesting that PD-1/PD-L1 axis critically participates in the immune evasion mechanisms of HCC.

Nivolumab is a human IgG4 monoclonal antibody targeting PD-1. A phase I/II clinical study for assessing safety and efficacy of nivolumab was conducted for either uninfected, HBV-infected, or HCV-infected HCC patients at advanced stage with excellent hepatic function [43]. The patients had history of pretreatment including sorafenib. Treatment was tolerable. Grade 4 adverse event (AE) was only an elevated level of lipase. Grade 3 AEs of elevated aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were observed in 11% and 9%, respectively,

none of which leads to serious hepatic failure or autoimmune hepatitis. The efficacy was promising. CR and an overall objective response rate were 5% and 19%, respectively. OS rate at 12 months was 62%. Importantly, in some patients, durable responses more than 12 months were observed. Nivolumab showed an antiviral activity in some HCV-infected cases, while antitumor effect was observed regardless of the etiology (i.e., no infection, HBV and HCV infection). In the latter part of this clinical study, the safety and efficacy of the combination therapy of nivolumab and anti-CTLA-4 antibody (ipilimumab) will be investigated for the advanced HCC patients (NCT01658878).

Pembrolizumab (MK3475) is a humanized monoclonal IgG4 antibody against PD-1. A single-arm phase II trial of pembrolizumab for advanced, unresectable HCC is now planned. The primary objective is to assess the therapeutic efficacy and toxicity profile of pembrolizumab. In addition, the expression levels of PD-L1 in tumor tissue will also be evaluated in this study for elucidating which cases may benefit from anti-PD-1/PD-L1 therapy (NCT02658019). Another anti-PD-1 antibody pidilizumab (CT-011) was also evaluated in a phase I clinical trial but unfortunately terminated due to slow accrual (NCT00966251).

PD-L1 blockade for HCC is also under investigation. Durvalumab (MEDI4736) is a human IgG1 monoclonal antibody to PD-L1. A phase I/II clinical study with durvalumab is recruiting patients and now ongoing for assessing the effectiveness and safety of durvalumab for advanced HCC. The preliminary results demonstrated that disease control rate (DCR) was 21% at 12 weeks, although there were no responders according to RECIST criteria. Durvalumab is tolerable with lower incidence of hepatotoxicity than that observed in anti-CTLA-4 therapy for HCC patients [44].

6.5.1.2 CTLA-4 Blockade

CTLA-4, also known as CD152, is the first negative co-stimulatory molecule identified that belongs to the CD28 superfamily [45]. CTLA-4 is rapidly induced in T cells upon T cell activation but not expressed on naive T cells. The ligands of CTLA-4 are co-stimulatory molecules CD80 and CD86 on APCs. CTLA-4 suppresses T cell activation by binding to CD80 and CD86 more avidly than CD28 that is required for T cell activation [46]. CTLA-4 is also constitutively expressed on Tregs. CTLA-4 on Tregs downregulates CD80 and CD86 on APCs and thus reduces the potency of APCs to prime naïve T cells, thereby controlling immunosuppressive function of Tregs [47]. Despite the encouraging results from clinical trial on HCC, only limited data are available regarding the mechanistic insights into anti-HCC effect induced by CTLA-4 blockade.

Tremelimumab is a fully human IgG2 monoclonal antibody against CTLA-4. A multicenter phase II noncontrolled clinical trial was conducted in 21 patients with advanced HCC and chronic HCV infection, and the promising results were recently reported [48]. Tremelimumab was administered every 90 days until tumor progression or severe toxicity. The antitumor effect was particularly encouraging. PR and

DCR were 17.6% and 76.4%, respectively. Median OS was 8.2 months, and median time to progression (TTP) was 6.5 months, which was favorable compared with historical controls. Overall good safety profile was recorded, although grade 3 and 4 transaminase toxicity was observed in 45% of patients. This AE was reversible and did not progress to liver failure. Importantly, tremelimumab was demonstrated to have an antiviral activity, inducing a significant decrease in viral load, with 15% of the patients achieving sustained virological response. The antitumor and antiviral effect of tremelimumab might be advantageous for treating viral hepatitis-induced HCC. Another phase I/II clinical study with tremelimumab is currently ongoing to determine the effectiveness and safety of tremelimumab in combination with transarterial chemoembolization (TACE) or RFA in advanced HCC. The preliminary results were promising, demonstrating that the combination therapy was acceptably safe and effective, with TTP increasing up to 7.4 months [49].

6.5.1.3 Other Checkpoint Targets

Although CTLA-4 and PD-1 are best characterized to date, other immune checkpoint molecules such as LAG3 [50], TIM-3 [51], and KIR [52] can be targeted for enhancing T cell-mediated tumor killing. Although no data on HCC patients is as yet available, preclinical studies have demonstrated antitumor activity of LAG3 and TIM-3 [51, 53]. Alternatively, immunostimulatory molecules such as CD40, CD137, OX40, and GITR can also be targeted [54, 55], some of which have already shown antitumor activity in HCC animal models [56, 57]. The monoclonal antibodies targeting these molecules are under development for clinical application in solid tumors ([58], NCT01471210, NCT01862900, NCT01239134), but the efficacy and safety in HCC remain to be elucidated.

6.5.2 Immunotherapy Other Than Checkpoint Blockade

In addition to checkpoint blockade, there are several classes of immunotherapy strategies for HCC, including ACT, cancer vaccines, and cytokine therapy.

6.5.2.1 ACT

ACT includes three types of cellular immunotherapy: cytokine-induced killer cells (CIKs), TILs, and genetically engineered T cells. CIKs are generated from peripheral blood mononuclear cells treated ex vivo with IFN-γ, IL-2, and anti-CD3 antibodies. CIK cells are characterized by the expression of both CD3 T cell biomarker and CD56 NK cell biomarker [59]. CIKs exhibit potent cytolytic activity against a broad spectrum of tumor cells, independently of MHC restriction [60]. Among ACTs, CIK therapy has been most studied in HCC patients. Recent two

meta-analyses of CIK immunotherapy for HCC revealed a significant superiority in prolonged OS, PFS, or RFS [61, 62]. TILs obtained from surgically resected tumor tissues are ex vivo expanded and transferred back into the patients. Impressive complete and durable response by TIL infusion therapy has been demonstrated in metastatic melanoma [63, 64]. In HCC, the intratumoral density of TILs was reportedly correlated with tumor progression [34]. Preliminary results from a phase I clinical trial of autologous TIL therapy in HCC have shown the transient increase in the frequency of T cells without serious AEs after ACT and no evidence of disease in 80% of enrolled patients after a median follow-up of 14 months [65]. The concept of genetically engineered T cell therapies is generating tumor antigen-specific T cells by the genetic transfer of antigen-specific TCRs. For this aim, either cloned physiological "MHC-restricted" TCR or "non-MHC-restricted" chimeric antigen receptor (CAR) is utilized. The use of these engineered T cells circumvents technical difficulties in isolation and expansion of TILs. In metastatic melanoma, the infusion of T cells genetically modified to express TCR against tumor-specific antigen MART-1 resulted in the objective tumor regression in a substantial number of patients [66]. Despite no data on TCR-transgenic T cells directed against HCC-specific antigen, Gehring et al. introduced HLA-A2-restricted HBV-specific TCRs into T cells of HBV-related HCC patients and demonstrated that TCR-redirected HBV-specific T cells recognized HCC cells with natural HBV DNA integration [67]. CAR essentially consists of ecto- and endo-domain. The former is a single-chain variable fragment constructed from monoclonal antibody against defined tumor antigen, while the latter contains adaptor signaling protein CD3ζ and one or more co-stimulatory modules such as CD28, CD137, or OX40, thereby transmitting activation signals triggered by antigen recognition into the cells [68]. MHC restriction-independent direct antigen recognition by CAR-T cells circumvents not only the problem of MHC restriction inherent to TCR-transgenic T cells but also the tumor immune evasion mechanism of low expression of MHC antigens. In contrast to hematological malignancies, however, the efficacy and safety of CAR-T cells have not been confirmed in solid tumors [69–71]. Improvement of insufficient T cell migration into the tumor lesions and defining of the tumor-specific target would be crucial to elicit an effective immune response with minimal toxicities against solid tumors like HCC.

6.5.2.2 Cancer Vaccines

Peptide-based vaccine and DC vaccine are the two main strategies of cancer vaccine. To develop effective peptide-based cancer vaccines, the choice of optimal antigens is crucial. Cancer antigens are categorized into two classes: tumor-associated antigens (TAAs) and neo-antigens. While TAAs are shared antigens, neo-antigens are authentic "private" cancer-specific antigens originated from non-synonymous mutations in the tumors. All cancer vaccines for HCC tested so far have targeted TAAs such as α-fetoprotein (AFP), glypican-3 (GPC3), and telomerase reverse transcriptase (TERT). High-level expression of AFP in HCC makes it an ideal

candidate for peptide vaccine. Increased frequencies of circulating AFP-specific T cells have been reported [72]. In an early clinical trial, vaccination with cocktail of four AFP-derived peptides with HLA-A2 restriction was tested in six HCC patients. The results showed that AFP-specific T cell response was observed in all patients [73]. GPC3 is another highly expressed antigen in HCC. A phase I trial showed that vaccination with GPC3 peptides restricted to HLA-A24 and HLA-A2 was found safe and induced specific CTLs in advanced HCC patients. OS was correlated with the frequency of peptide-specific CTL response, although objective tumor response was observed in only 1 patient among 33 treated patients [74]. Upregulation of telomerase expression and activity was reported in HCC [75, 76]. However, a phase II trial of TERT-derived peptide vaccine showed no clinical activity without peptide-specific CTL response [77]. The design of neo-antigen vaccine is based on the whole exome data obtained by next-generation sequencing, combined with the following computational prediction of antigen presentation on individual HLA allelic variants. Neo-antigen-based vaccine enables fully personalized cancer immunotherapy [78]. Although it is at its infancy, it is beyond doubt that this strategy holds promise as the future cancer vaccine for HCC. DCs, the most potent APCs, play a central role in inducing antitumor CTL response. Indeed, the prognosis of HCC patients after surgical resection was associated with the frequency of infiltrated DCs in HCC nodules [79]. The therapeutic concept of DC vaccine is eliciting antitumor immune response by activating DCs ex vivo with tumor antigens and infusing them back into the patient. DC vaccines have been reported to be capable of not only inducing antigen-specific CTLs but also activating NK cells and inhibiting Tregs in HCC patients [80, 81]. Several DC vaccine clinical trials utilizing DCs pulsed with either autologous or tumor cell line-derived lysate have been conducted in advanced HCC patients. DC vaccines have proven to be safe. Unfortunately, however, most studies demonstrated only marginal clinical response [82–85]. Improvements such as combination with other therapeutic options might be needed for increasing the efficacy of DC vaccines.

6.5.2.3 Oncolytic Virotherapy

Oncolytic virotherapy is an emerging therapeutic strategy [86]. JX-594 is a genetic engineered vaccinia virus that is designed to replicate selectively in the tumor cells. JX-594 causes virus replication-dependent oncolysis that results in the antigen release from dead cells and the subsequent induction of tumor-specific immunity. This immunostimulation can be further enhanced by the expression of GM-CSF transgene integrated in JX-594 [87]. A randomized phase II trial on JX-594 for advanced HCC has shown promising results [88]. Low- or high-dose JX-594 was intratumorally administered for two different groups. Both doses showed equivalent tumor regression in injected and distant non-injected tumors, while survival duration was significantly correlated with dose. Median survival was 14.1 months and 6.7 months at high and low dose, respectively [88]. All patients experienced manageable AE of flu-like symptoms. Further phase II or III clinical trials for evaluating

the efficacy of JX-594 on advanced HCC are currently underway (NCT01636284, NCT01171651, NCT02562755).

6.5.2.4 Cytokine Therapy

Cytokine therapy could exhibit direct antitumor effect on HCC. Accordingly, interferon (IFN) has been extensively studied in HCC, based on its immunomodulatory effect and experience in the treatment of viral hepatitis. However, the conflicting results from several randomized studies, together with its serious side effect, might not encourage the use of IFN for HCC immunotherapy at least currently.

6.6 Combination Therapies

As described above, besides the intrinsic immunosuppressive microenvironment of the liver, HCC evades immune response by multiple mechanisms. To overcome these impediments, combination immunotherapy strategies would be an appealing approach for HCC.

Combination therapy using anti-PD-1 and anti-CTLA-4 antibodies [89] has been found to improve the objective clinical outcome in melanoma, albeit at the expense of high rates of immune-mediated AEs. As indicated before, clinical study for assessing the safety and efficacy of the combination therapy of nivolumab (anti-PD-1 antibody) and ipilimumab (anti-CTLA-4 antibody) is underway for the advanced HCC patients (NCT01658878).

Alternative combination strategy includes the blockade of PD-1 (or CTLA-4) plus other immune checkpoint molecules. In animal model, the additive antitumor effect was observed by combinational inhibition of PD-1 and LAG-3 co-expressed on tumor-specific CD8+ T cells in the suppressive tumor microenvironment [90]. In a clinical setting, a phase I/II study of anti-KIR (lirilumab) in combination with nivolumab is now ongoing for advanced refractory solid tumors (NCT01714739). Combination of immune checkpoint blockade and activation of immunostimulatory molecules such as OX40 and CD137 would be theoretically another option. Indeed, administration of anti-PD-L1, anti-CD137, and anti-OX40 antibodies in a transgenic HCC mouse model was reported to show extended survival of treated mice in a CD8+ T cell-dependent manner [57]. A phase I/II clinical study of nivolumab in combination with urelumab (agonistic anti-CD137 antibody) is currently conducted for solid tumors and B-cell non-Hodgkin's lymphoma (NCT02253992).

Combination of checkpoint inhibitors and cancer vaccination is also a reasonable approach. Sawada et al. reported that PD-1/PD-L1 blockade enhanced the antitumor effects of GPC3 peptide vaccine by increasing the immune response of vaccine-induced CTLs. This study gives a rationale for the development of combination therapy of GPC3 vaccination and PD-1 blockade [91]. Another report demonstrated that the treatment of breast cancer-bearing mice with anti-PD-1 antibody and mul-

tipeptide vaccine prolonged progression-free survival, associated with enhanced antigen reactivity of tumor-infiltrating CD8+ T cells [92]. Combination therapy of GM-CSF-based vaccines (GVAX) with ipilimumab (anti-CTLA-4 antibody) for advanced pancreatic cancer patients showed favorable antitumor effect compared with ipilimumab monotherapy [93]. Taken together, these results support the concept of synergy between cancer vaccines and immune checkpoint blockade for tumor treatment.

Locoregional therapies such as TACE, RFA, and radiation that are widely applied as a standard care in HCC patients might be an ideal combination partner for immunotherapy, because they cause a release of tumor antigens, thereby triggering innate immune response leading to the activation of systemic antitumor immunity. A foregoing study demonstrated the significant expansion and activation of AFP-specific CD4+ T cell against three immunodominant epitopes after embolization for HCC [94]. Importantly, higher frequencies of AFP-specific CD4+ T cells after embolization were associated with higher degree of tumor necrosis and improved clinical outcome. Despite no available data on HCC, tumor ablation combined with immunotherapies has shown promising results in several preclinical studies [95]. CTLA-4 blockade combined with cryoablation of a primary tumor was demonstrated to induce systemic antitumor immunity, thereby inhibiting the growth of secondary tumors at a distant site in a prostate cancer model [96]. Recently, Victor et al. demonstrated that optimal response in melanoma and other cancer types requires radiation and dual blockade of CTLA-4 and PD-L1/PD-1. In their mouse models, radiation enhances the diversity of the TCR repertoire, while anti-CTLA-4 promotes expansion of T cells and anti-PD-1 reverses T cell exhaustion [97]. These results encourage the development of combination therapy with radiation and checkpoint inhibitors for HCC patients. As described in Sect. 5.1.2, a clinical study for assessing the efficacy and safety of tremelimumab in combination with locoregional therapies including TACE and RFA is underway for advanced HCC. Importantly, all evaluable patients showed immune cell infiltration in the outside lesions of TACE- or RFA-treated tumors, and in some cases partial objective response was achieved [49].

Systemic chemotherapy might also be combined with immunotherapy. The main effect of cancer chemotherapeutic agents is killing of tumor cells as well as other highly replicating cells such as immune cells. Therefore, they are generally considered as immunosuppressive agents. Contradictorily, however, they also potentially induce effective antitumor immunity: one mechanism is that they induce immunogenic cell death that causes tumor antigen release, resulting in the maturation of Th1-polarizing DCs that is required for the development of antitumor immunity [98]. In addition, some chemotherapeutic agents selectively kill immunosuppressive cells such as MDSCs and Tregs in the tumor microenvironment [99]. Accordingly, the synergistic effect of peptide-based cancer vaccines combined with cisplatin and gemcitabine has been reported [100, 101]. In another study, adoptive transfer of CIK cells combined with gemcitabine has shown enhanced antitumor effect for the metastatic solid tumors [102]. As for HCC, the combination therapy of low-dose cyclophosphamide and telomerase vaccine was tested in a single clinical

study [77]. Although neither effective antitumor response nor prolonged TTP was unfortunately observed in this study, the combination of chemotherapeutic agents with other immunotherapy such as checkpoint inhibitors would be warranted in the future.

Finally, the combination of immunotherapy with sorafenib, a tyrosine kinase inhibitor that is approved in clinical use, might be an option, because sorafenib reportedly acts as a positive immunomodulator by enhancing the functions of tumor-specific effector T cells and NK cells [103, 104]. However, there are also conflicting data showing that sorafenib negatively impacts immune response by impairing DC function, upregulating intratumoral PD-1 expression, and recruiting Tregs [105, 106]. Thus, further careful evaluation is required to determine whether sorafenib can be an optimal partner for immunotherapy.

6.7 Conclusions

HCC is a common cancer with a high mortality. The current standard treatment is unsatisfactory, and there is an urgent need for the development of novel therapeutic approaches. Cancer immunotherapy has a bright future, and different immunotherapeutic modalities are rapidly under development, among which the use of monoclonal antibodies targeting immune checkpoint molecules holds a good promise. In case of HCC, however, it must be taken into account that the liver has an intrinsic tolerogenic immune environment and that HCC subverts antitumor immunity by multiple mechanisms. Therefore, the combination of checkpoint blockade with either other immunotherapeutic approaches or standard therapies of proven value would be a future trend in this field. Further studies including basic and clinical research are warranted to achieve successful immunotherapy for HCC.

References

1. McGlynn KA, Petrick JL, London WT. Global epidemiology of hepatocellular carcinoma: an emphasis on demographic and regional variability. Clin Liver Dis. 2015;19:223–38.
2. Bosetti C, Turati F, La Vecchia C. Hepatocellular carcinoma epidemiology. Best Pract Res Clin Gastroenterol. 2014;28:753–70.
3. Welzel TM, Graubard BI, Quraishi S, et al. Population-attributable fractions of risk factors for hepatocellular carcinoma in the United States. Am J Gastroenterol. 2013;108:1314–21.
4. Okazaki T, Chikuma S, Iwai Y, et al. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. Nat Immunol. 2013;14:1212–8.
5. Postow MA, Callahan MK, Wolchok JD. Immune checkpoint blockade in cancer therapy. J Clin Oncol. 2015;33:1974–82.
6. Melero I, Berman DM, Aznar MA, et al. Evolving synergistic combinations of targeted immunotherapies to combat cancer. Nat Rev Cancer. 2015;15(8):457–72.
7. Crispe IN. The liver as a lymphoid organ. Annu Rev Immunol. 2009;27:147–63.

8. Limmer A, Ohl J, Kurts C, et al. Efficient presentation of exogenous antigen by liver endothelial cells to CD8+T cells results in antigen specific T-cell tolerance. Nat Med. 2000;6:1348–54.
9. Diehl L, Schurich A, Grochtmann R, et al. Tolerogenic maturation of liver sinusoidal endothelial cells promotes B7-homolog 1-dependent CD8+T cell tolerance. Hepatology. 2008;47:296–305.
10. von Oppen N, Schurich A, Hegenbarth S, et al. Systemic antigen cross-presented by liver sinusoidal endothelial cells induces liver-specific CD8 T-cell retention and tolerization. Hepatology. 2009;49:1664–72.
11. Mackay IR. Hepatoimmunology: a perspective. Immunol Cell Biol. 2002;80:36–44.
12. Bilzer M, Roggel F, Gerbes AL. Role of Kupffer cells in host defense and liver disease. Liver Int. 2006;26:1175–86.
13. You Q, Cheng L, Kedl RM, et al. Mechanism of T cell tolerance induction by murine hepatic Kupffer cells. Hepatology.
14. Knolle P, Schlaak J, Uhrig A. Human Kupffer cells secrete IL-10 in response to lipopolysaccharide (LPS) challenge. J Hepatol. 1995;22:226–9.
15. Zhang M, Xu S, Han Y, et al. Apoptotic cells attenuate fulminant hepatitis by priming Kupffer cells to produce interleukin-10 through membrane-bound TGF-β. Hepatology. 2011;53:306–16.
16. Knolle PA, Gerken G. Local control of the immune response in the liver. Immunol Rev. 2000;174:21–34.
17. Breous E, Somanathan S, Vandenberghe LH, et al. Hepatic regulatory T cells and Kupffer cells are crucial mediators of systemic T cell tolerance to antigens targeting murine liver. Hepatology. 2009;50:612–21.
18. Pillarisetty VG, Shah AB, Miller G, et al. Liver dendritic cells are less immunogenic than spleen dendritic cells because of differences in subtype composition. J Immunol. 2004;172:1009–17.
19. Lau AH, Thomson AW. Dendritic cells and immune regulation in the liver. Gut. 2003;52:307–14.
20. Bamboat ZM, Stableford JA, Plitas G, et al. Human liver dendritic cells promote T cell hyporesponsiveness. J Immunol. 2009;182:1901–11.
21. Holz LE, Benseler V, Bowen DG, et al. Intrahepatic murine CD8 T-cell activation associates with a distinct phenotype leading to Bim-dependent death. Gastroenterology. 2008;135:989–97.
22. Dunn GP, Bruce AT, Ikeda H, et al. Cancer immunoediting: from immunosurveillance to tumor escape. Nat Immunol. 2002;3:991–8.
23. Matsui M, Machida S, Itani-Yohda T, et al. Downregulation of the proteasome subunits, transporter, and antigen presentation in hepatocellular carcinoma, and their restoration by interferon-gamma. J Gastroenterol Hepatol. 2002;17:897–907.
24. Prieto J, Melero I, Sangro B. Immunological landscape and immunotherapy of hepatocellular carcinoma. Nat Rev Gastroenterol Hepatol. 2015;12:681–700.
25. Wing K, Sakaguchi S. Regulatory T cells exert checks and balances on self tolerance and autoimmunity. Nat Immunol. 2010;11:7–13.
26. Chen KJ, Lin SZ, Zhou L, et al. Selective recruitment of regulatory T cell through CCR6–CCL20 in hepatocellular carcinoma fosters tumor progression and predicts poor prognosis. PLoS One. 2011;6:e24671.
27. Arihara F, Mizukoshi E, Kitahara M, et al. Increase in CD14+HLA-DR −/low myeloid-derived suppressor cells in hepatocellular carcinoma patients and its impact on prognosis. Cancer Immunol Immunother. 2013;62:1421–30.
28. Hoechst B, Ormandy LA, Ballmaier M, et al. A new population of myeloid-derived suppressor cells in hepatocellular carcinoma patients induces CD4+CD25+Foxp3+ T cells. Gastroenterology. 2008;135:234–43.
29. Nagaraj S, Gupta K, Pisarev V, et al. Altered recognition of antigen is a mechanism of CD8+ T cell tolerance in cancer. Nat Med. 2007;13:828–35.
30. Qian B, Deng Y, Im JH, et al. A distinct macrophage population mediates metastatic breast cancer cell extravasation, establishment and growth. PLoS One. 2009;4:e6562.

31. Zou W, Chen L. Inhibitory B7-family molecules in the tumour microenvironment. Nat Rev Immunol. 2008;8:467–77.
32. Epstein RJ, Leung TW. Reversing hepatocellular carcinoma progression by using networked biological therapies. Clin Cancer Res. 2007;13:11–7.
33. Yoong KF, McNab G, Hubscher SG, et al. Vascular adhesion protein-1 and ICAM-1 support the adhesion of tumor-infiltrating lymphocytes to tumor endothelium in human hepatocellular carcinoma. J Immunol. 1998;160:3978–88.
34. Gao Q, Qiu SJ, Fan J, et al. Intratumoral balance of regulatory and cytotoxic T cells is associated with prognosis of hepatocellular carcinoma after resection. J Clin Oncol. 2007;2007(25):2586–93.
35. Flecken T, Schmidt N, Hild S, et al. Immunodominance and functional alterations of tumor-associated antigen-specific CD8+ T-cell responses in hepatocellular carcinoma. Hepatology. 2014;59:1415–26.
36. Okazaki T, Iwai Y, Honjo T. New regulatory co-receptors: inducible co-stimulator and PD-1. Curr Opin Immunol. 2002;14:779–82.
37. Kuang DM, Zhao Q, Peng C, et al. Activated monocytes in peritumoral stroma of hepatocellular carcinoma foster immune privilege and disease progression through PD-L1. J Exp Med. 2009;206:1327–37.
38. Wu K, Kryczek I, Chen L, et al. Kupffer cell suppression of CD8+T cells in human hepatocellular carcinoma is mediated by B7-H1/programmed death-1 interactions. Cancer Res. 2009;69:8067–75.
39. Zeng Z, Shi F, Zhou L, et al. Upregulation of circulating PD-L1/PD-1 is associated with poor post-cryoablation prognosis in patients with HBV-related hepatocellular carcinoma. PLoS One. 2011;6:e23621.
40. Cariani E, Pilli M, Zerbini A, et al. Immunological and molecular correlates of disease recurrence after liver resection for hepatocellular carcinoma. PLoS One. 2012;7:e32493.
41. Gao Q, Wang XY, Qiu SJ, et al. Overexpression of PD-L1 significantly associates with tumor aggressiveness and postoperative recurrence in human hepatocellular carcinoma. Clin Cancer Res. 2009;15:971–9.
42. Wang BJ, Bao JJ, Wang JZ, et al. Immunostaining of PD-1/PD-Ls in liver tissues of patients with hepatitis and hepatocellular carcinoma. World J Gastroenterol. 2011;17:3322–9.
43. El-Khoueiry AB, Melero I, Crocenzi TS, et al. Phase I/II safety and antitumor activity of nivolumab in patients with advanced hepatocellular carcinoma (HCC): CA209-040. J Clin Oncol. 2015;33(suppl):Abstract LBA101.
44. Segal N, Hamid O, Hwu W, et al. A phase I multi-arm dose-expansion study of the anti-programmed cell death-ligand-1 (PD-L1) antibody MEDI4736: preliminary data. Ann Oncol. 2014;25:iv361–72.
45. Brunet JF, Denizot F, Luciani MF, et al. A new member of the immunoglobulin superfamily—CTLA-4. Nature. 1987;328:267–70.
46. Walker LS, Sansom DM. Confusing signals: recent progress in CTLA-4 biology. Trends Immunol. 2015;36:63–70.
47. Wing K, Onishi Y, Prieto-Martin P, et al. CTLA-4 control over Foxp3+ regulatory T cell function. Science. 2008;322:271–5.
48. Sangro B, Gomez-Martin C, de la Mata M, et al. A clinical trial of CTLA-4 blockade with tremelimumab in patients with hepatocellular carcinoma and chronic hepatitis C. J Hepatol. 2013;59:81–8.
49. Duffy AG, Makarova-Rusher OV, Kerkar SP, et al. A pilot study of tremelimumab–a monoclonal antibody against CTLA-4–in combination with either trans catheter arterial chemoembolization (TACE) or radiofrequency ablation (RFA) in patients with hepatocellular carcinoma (HCC). J Clin Oncol. 2015;33(suppl):abstr 4081.
50. Nguyen LT, Ohashi PS. Clinical blockade of PD1 and LAG3--potential mechanisms of action. Nat Rev Immunol. 2015;151:45–56.
51. Li FJ, Zhang Y, Jin GX, et al. Expression of LAG-3 is coincident with the impaired effector function of HBV-specific CD8(+) T cell in HCC patients. Immunol Lett. 2013;150:116–22.

52. Cariani E, Missale G. KIR/HLA immunogenetic background influences the evolution of hepatocellular carcinoma. Oncoimmunology. 2013;2:e26622.
53. Li H, Wu K, Tao K, et al. Tim-3/galectin-9 signaling pathway mediates T-cell dysfunction and predicts poor prognosis in patients with hepatitis B virus-associated hepatocellular carcinoma. Hepatology. 2012;56:1342–51.
54. Grewal IS, Flavell RA. CD40 and CD154 in cell-mediated immunity. Annu Rev Immunol. 1998;16:111–35.
55. Melero I, Hirschhorn-Cymerman D, Morales-Kastresana A, et al. Agonist antibodies to TNFR molecules that costimulate T and NK cells. Clin Cancer Res. 2013;19:1044–53.
56. Gauttier V, Judor JP, Le Guen V, et al. Agonistic anti-CD137 antibody treatment leads to antitumor response in mice with liver cancer. Int J Cancer. 2014;135:2857–67.
57. Morales-Kastresana A, Sanmamed MF, Rodriguez I, et al. Combined immunostimulatory monoclonal antibodies extend survival in an aggressive transgenic hepatocellular carcinoma mouse model. Clin Cancer Res. 2013;19:6151–62.
58. Beatty GL, Torigian DA, Chiorean EG, et al. A phase I study of an agonist CD40 monoclonal antibody (CP-870,893) in combination with gemcitabine in patients with advanced pancreatic ductal adenocarcinoma. Clin Cancer Res. 2013;19:6286–95.
59. Zoll B, Lefterova P, Csipai M, et al. Generation of cytokine-induced killer cells using exogenous interleukin-2, −7 or −12. Cancer Immunol Immunother. 1998;47:221–6.
60. Introna M, Golay J, Rambaldi A. Cytokine induced killer (CIK) cells for the treatment of haematological neoplasms. Immunol Lett. 2013;155:27–30.
61. Li X, Dai D, Song X, et al. A meta-analysis of cytokine-induced killer cells therapy in combination with minimally invasive treatment for hepatocellular carcinoma. Clin Res Hepatol Gastroenterol. 2014;38:583–91.
62. Ma Y, Xu YC, Tang L, et al. Cytokine- induced killer (CIK) cell therapy for patients with hepatocellular carcinoma: efficacy and safety. Exp Hematol Oncol. 2012;1:11.
63. Ellebaek E, Iversen TZ, Junker N, et al. Adoptive cell therapy with autologous tumor infiltrating lymphocytes and low-dose interleukin-2 in metastatic melanoma patients. J Transl Med. 2012;10:169.
64. Rosenberg SA, Yang JC, Sherry RM, et al. Durable complete responses in heavily pretreated patients with metastatic melanoma using T-cell transfer immunotherapy. Clin Cancer Res. 2011;17:4550–7.
65. Jiang SS, Tang Y, Zhang YJ, et al. A phase I clinical trial utilizing autologous tumor-infiltrating lymphocytes in patients with primary hepatocellular carcinoma. Oncotarget. 2015;6:41339–49.
66. Johnson LA, Morgan RA, Dudley ME, et al. Gene therapy with human and mouse T-cell receptors mediates cancer regression and targets normal tissues expressing cognate antigen. Blood. 2009;114:535–46.
67. Gehring AJ, Xue SA, Ho ZZ, et al. Engineering virus-specific T cells that target HBV infected hepatocytes and hepatocellular carcinoma cell lines. J Hepatol. 2011;55:103–10.
68. Jensen MC, Riddell SR. Design and implementation of adoptive therapy with chimeric antigen receptor-modified T cells. Immunol Rev. 2014;257:127–44.
69. Kershaw MH, Westwood JA, Parker LL, et al. A phase I study on adoptive immunotherapy using gene-modified T cells for ovarian cancer. Clin Cancer Res. 2006;12:6106–15.
70. Lamers CH, Sleijfer S, van Steenbergen S, et al. Treatment of metastatic renal cell carcinoma with CAIX CAR-engineered T cells: clinical evaluation and management of on-target toxicity. Mol Ther. 2013;21:904–12.
71. Morgan RA, Yang JC, Kitano M, et al. Case report of a serious adverse event following the administration of T cells transduced with a chimeric antigen receptor recognizing ERBB2. Mol Ther. 2010;18:843–51.
72. Greten TF, Duffy AG, Korangy F. Hepatocellular carcinoma from an immunologic perspective. Clin Cancer Res. 2013;19:6678–85.

73. Butterfield LH, Ribas A, Meng WS, et al. T-cell responses to HLA-A*0201 immunodominant peptides derived from alpha-fetoprotein in patients with hepatocellular cancer. Clin Cancer Res. 2003;9:5902–8.
74. Sawada Y, Yoshikawa T, Shimomura M, et al. Phase I trial of a glypican-3-derived peptide vaccine for advanced hepatocellular carcinoma: immunologic evidence and potential for improving overall survival. Clin Cancer Res. 2012;18:3686–96.
75. Saini N, Srinivasan R, Chawla Y, et al. Telomerase activity, telomere length and human telomerase reverse transcriptase expression in hepatocellular carcinoma is independent of hepatitis virus status. Liver Int. 2009;29:1162–70.
76. Shimada M, Hasegawa H, Gion T, et al. The role of telomerase activity in hepatocellular carcinoma. Am J Gastroenterol. 2000;95:748–52.
77. Greten TF, Forner A, Korangy F, et al. A phase II open label trial evaluating safety and efficacy of a telomerase peptide vaccination in patients with advanced hepatocellular carcinoma. BMC Cancer. 2010;10:209.
78. Schumacher TN, Schreiber RD. Neoantigens in cancer immunotherapy. Science. 2015;348:69–74.
79. Cai XY, Gao Q, Qiu SJ, et al. Dendritic cell infiltration and prognosis of human hepatocellular carcinoma. J Cancer Res Clin Oncol. 2006;132:293–301.
80. Bray SM, Vujanovic L, Butterfield LH. Dendritic cell-based vaccines positively impact natural killer and regulatory T cells in hepatocellular carcinoma patients. Clin Dev Immunol. 2011;2011:249281. https://doi.org/10.1155/2011/249281.
81. Sun JC, Pan K, Chen MS, et al. Dendritic cells-mediated CTLs targeting hepatocellular carcinoma stem cells. Cancer Biol Ther. 2010;10:368–75.
82. El Ansary M, Mogawer S, Elhamid SA, et al. Immunotherapy by autologous dendritic cell vaccine in patients with advanced HCC. J Cancer Res Clin Oncol. 2013;139:39–48.
83. Lee WC, Wang HC, Hung CF, et al. Vaccination of advanced hepatocellular carcinoma patients with tumor lysate-pulsed dendritic cells: a clinical trial. J Immunother. 2005;28:496–504.
84. Palmer DH, Midgley RS, Mirza N, et al. A phase II study of adoptive immunotherapy using dendritic cells pulsed with tumor lysate in patients with hepatocellular carcinoma. Hepatology. 2009;49:124–32.
85. Tada F, Abe M, Hirooka M, et al. Phase I/II study of immunotherapy using tumor antigen-pulsed dendritic cells in patients with hepatocellular carcinoma. Int J Oncol. 2012;41:1601–9.
86. Hernández-Alcoceba R. Recent advances in oncolytic virus design. Clin Transl Oncol. 2011;13:229–39.
87. Kim JH, Oh JY, Park BH, et al. Systemic armed oncolytic and immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF. Mol Ther. 2006;14:361–70.
88. Heo J, Reid T, Ruo L, et al. Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia JX-594 in liver cancer. Nat Med. 2013;19:329–36.
89. Wolchok JD, Kluger H, Callahan MK, et al. Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med. 2013;369:122–33.
90. Gros A, Robbins PF, Yao X, et al. PD-1 identifies the patient-specific CD8+ tumor-reactive repertoire infiltrating human tumors. J Clin Invest. 2014;124:2246–59.
91. Sawada Y, Yoshikawa T, Shimomura M, et al. Programmed death-1 blockade enhances the antitumor effects of peptide vaccine-induced peptide-specific cytotoxic T lymphocytes. Int J Oncol. 2015;46:28–36.
92. Karyampudi L, Lamichhane P, Scheid AD, et al. Accumulation of memory precursor CD8 T cells in regressing tumors following combination therapy with vaccine and anti-PD-1 antibody. Cancer Res. 2014;74:2974–85.
93. Le DT, Lutz E, Uram JN, et al. Evaluation of ipilimumab in combination with allogeneic pancreatic tumor cells transfected with a GM-CSF gene in previously treated pancreatic cancer. J Immunother. 2013;36:382–9.

94. Ayaru L, Pereira SP, Alisa A, et al. Unmasking of alpha-fetoprotein-specific CD4(+) T cell responses in hepatocellular carcinoma patients undergoing embolization. J Immunol. 2007;178:1914–22.
95. Chuand KF, Dupuy DE. Thermal ablation of tumours: biological mechanisms and advances in therapy. Nat Rev Cancer. 2014;14:199–208.
96. Waitz R, Solomon SB, Petre EN, et al. Potent induction of tumor immunity by combining tumor cryoablation with anti-CTLA-4 therapy. Cancer Res. 2012;72:430–9.
97. Victor CT, Rech AJ, Maity A, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. Nature. 2015;16:373–7.
98. Zitvogel L, Apetoh L, Ghiringhelli F, et al. The anticancer immune response: indispensable for therapeutic success? J Clin Invest. 2008;118:1991–2001.
99. Duffy AG, Greten TF. Immunological off-target effects of standard treatments in gastrointestinal cancers. Ann Oncol. 2014;25:24–32.
100. Dijkgraaf EM, Santegoets SJ, Reyners AK, et al. A phase 1/2 study combining gemcitabine, Pegintron and p53 SLP vaccine in patients with platinum-resistant ovarian cancer. Oncotarget. 2015;6:32228–43.
101. van der Sluis TC, van Duikeren S, Huppelschoten S, et al. Vaccine-induced tumor necrosis factor-producing T cells synergize with cisplatin to promote tumor cell death. Clin Cancer Res. 2015;21:781–94.
102. Morisaki T, Hirano T, Koya N, et al. NKG2D-directed cytokine-activated killer lymphocyte therapy combined with gemcitabine for patients with chemoresistant metastatic solid tumors. Anticancer Res. 2014;34:4529–38.
103. Chen ML, Yan BS, Lu WC, et al. Sorafenib relieves cell-intrinsic and cell-extrinsic inhibitions of effector T cells in tumor microenvironment to augment antitumor immunity. Int J Cancer. 2014;134:319–31.
104. Sprinzl MF, Reisinger F, Puschnik A, et al. Sorafenib perpetuates cellular anticancer effector functions by modulating the crosstalk between macrophages and natural killer cells. Hepatology. 2013;57:2358–68.
105. Chen Y, Ramjiawan RR, Reiberger T, et al. CXCR4 inhibition in tumor microenvironment facilitates anti-programmed death receptor-1 immunotherapy in sorafenib-treated hepatocellular carcinoma in mice. Hepatology. 2015;61:1591–602.
106. Hipp MM, Hilf N, Walter S, et al. Sorafenib, but not sunitinib, affects function of dendritic cells and induction of primary immune responses. Blood. 2008;111:5610–20.

Chapter 7
Molecular Diagnosis and Targeting of Biliary Tract Cancer

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Abstract Biliary tract cancer (BTC) is a highly aggressive malignant tumor arising from epithelial cells lining the bile duct and occurring at distinct anatomical locations: intrahepatic, extrahepatic, and the gallbladder. The incidence of BTC has increased globally; however, surgery is the only curative treatment, and no other effective therapies are available. Patients with BTC have a poor prognosis because most tumors are only detected at an advanced stage, making early diagnosis and treatment essential.

Recent developments in diagnosis and therapy for BTC, especially in the field of molecular biology, have resulted in better long-term survival rates, especially in patients undergoing curative resection at an early stage. This trend needs to continue to further improve prognosis in BTC patients. In addition, more effective molecular diagnosis and targeting therapies are needed to target essential biochemical and signaling pathways or mutant proteins that are required for tumor cell growth and survival. To this end, the field requires better molecular classification of BTC and more precise categorization of tumors with respect to prognosis such that clinicians can make more informed choices about surgical or nonsurgical therapies for individual patients.

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This review focuses on the molecular diagnosis and targeting therapy for BTC in surgical and nonsurgical patients, as well as the special relationship between tumor behavior and anatomical specificity in this disease.

Keywords Biliary tract cancer • Bile duct cancer • Gallbladder cancer • Molecular diagnosis • Molecular targeting therapy

7.1 Introduction

Biliary tract cancer (BTC) comprises a group of highly aggressive malignant tumors with a poor prognosis because most patients are diagnosed at an advanced stage. Therefore, better approaches for the early diagnosis and treatment of BTC are essential for improving patient prognosis. Somatic mutations of KRAS and BRAF are the most common genetic alterations associated with BTC [1, 2]; however, no effective molecular targeted therapies have been approved. While surgery is the only curative treatment for BTC, more than half of all surgical patients have non-curative resection with minimally invasive metastasis and positive resected margin due to unexpectedly advanced disease. In addition, no standard chemotherapy regimens have been established for inoperative cases or those showing recurrence after surgical resection [2]; thus, 5-year overall survival rates for BTC after resection remain at 20–40% [3–5].

Recent developments in the diagnosis and treatment of BTC have improved long-term survival, especially in patients undergoing curative resection at an early stage [3, 4]; however, further research is warranted into better diagnostic and/or targeted therapeutic strategies, especially specific molecular approaches.

This review focuses on the molecular diagnosis and targeting therapy of BTC in surgical and nonsurgical patients.

7.2 Classifications of Biliary Tract Cancer

Biliary tract cancer (BTC) comprises bile duct cancer (BDC) and gallbladder cancer (GBC) and is classified by the local site of malignancy. Twenty years ago, Nakeeb et al. [6] proposed that BDC could be classified as intrahepatic, perihilar, or distal (Table 7.1). More recently, a liver cancer study in Japan [7] found that intrahepatic BDC could be classified further macroscopically into mass-forming (MF), periductal-infiltrating (PI), and intraductal growth (IG) types.

In 2013, the Japanese Society of Hepato-Biliary-Pancreatic Surgery [8, 9] demonstrated that the extrahepatic bile duct is divided into a perihilar bile duct (Bp) and distal bile duct (Bd) by a line dividing the duct equally between the upper margin of the common hepatic duct and the point where the common bile duct enters the wall

Table 7.1 Classifications of biliary tract cancer

Classification	Subclassification
Intrahepatic bile duct cancer	Mass forming (MF)
	Periductal infiltrating (PI)
	Intraductal growth (IG)
Extrahepatic bile duct cancer	Perihilar bile duct (Bp)
	Distal bile duct (Bd)
Gallbladder cancer	Cystic duct (C)
	Neck of gallbladder (Gn)
	Body of gallbladder (Gb)
	Fundus of gallbladder (Gf)

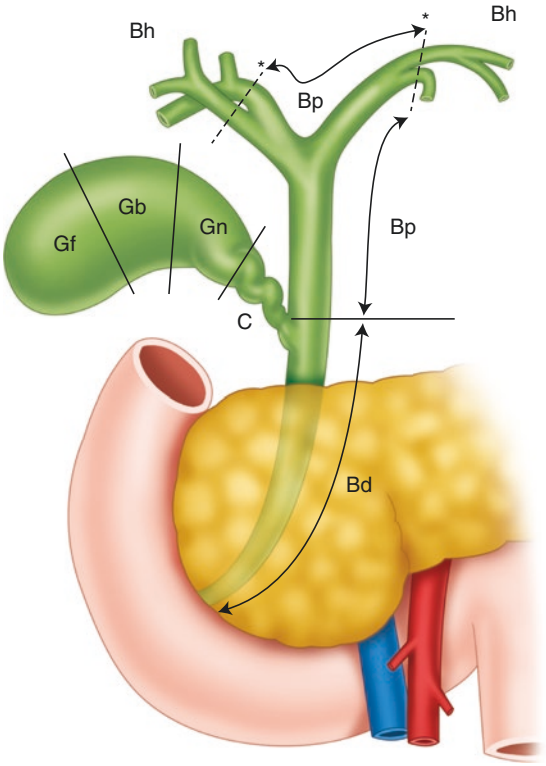

Fig. 7.1 Portion of the extrahepatic bile ducts and gallbladder. *Bd* distal bile duct, *Bh* intrahepatic bile duct, *Bp* perihilar bile duct, *C* cystic duct, *Gb* body of gallbladder, *Gf* fundus of the gallbladder, *Gn* neck of the gallbladder (quoted from Reference No. ,[9])

of the duodenum. This line is principally positioned at the origin of the cystic duct as a guide (Fig. 7.1) [3, 4, 9], and the proximal borders of the Bp, between the intrahepatic (Bh) and extrahepatic bile ducts, are defined using the portal system as a reference [8, 9]. The ducts of the left and right side borders are located topologically at the right side of the umbilical portion of the left portal vein and the left side of the origin of the right posterior portal vein, respectively (Fig. 7.2a) [3, 4, 9], as judged principally by computed tomography (CT). In cases with an independent right

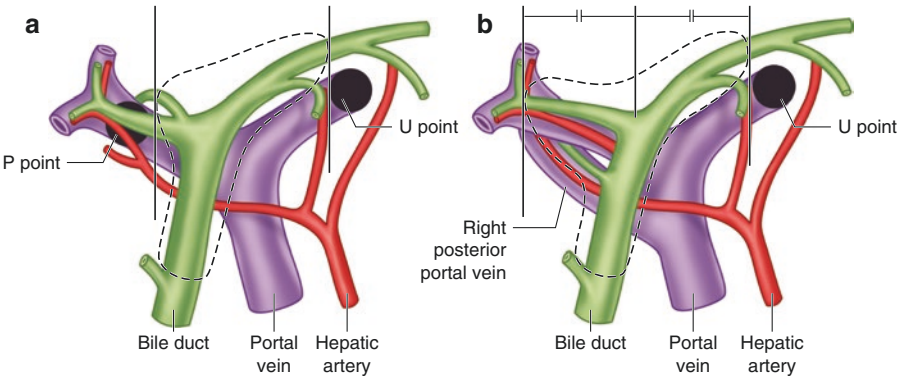

Fig. 7.2 Definition of the perihilar bile duct (*dotted lines*). (**a**) A case with standard anatomy. (**b**) A case with an independent right posterior portal vein branching from the main portal vein. P point, origin of the right posterior portal vein; U point, umbilical portion of the left portal vein (quoted from Reference No. [9])

posterior portal vein branching from the main portal vein, the right side border is determined based on the length between the confluence of the left and right anterior portal veins and the right side of the umbilical portion of the left portal vein to the right side (Fig. 7.2b) [3, 4, 9]. Table 7.1 summarizes these classifications of BDC.

On the other hand, GBC is classified simply based on the following tumor locations: cystic duct (C), neck of gallbladder (Gn), body of gallbladder (Gb), and fundus of gallbladder (Gf) (Fig. 7.1) [3, 4, 9].

7.3 Molecular Diagnosis

Currently, the diagnosis of BTC relies mainly on imaging and intraoperative exploration due to brush cytology having low sensitivity and the standard blood markers, such as carcinoembryonic antigen (CEA) and carbohydrate 19–9 (CA 19–9), having neither sufficient sensitivity nor specificity to be used for differential diagnosis and early-stage detection. Thus, better noninvasive methods are needed to distinguish between normal and pathological tissue in diagnosing BTC.

MicroRNAs (miRNAs) are small, single-stranded, noncoding RNA molecules ranging from 20 to 22 nucleotides that regulate physiological mechanisms and that have been implicated in carcinogenesis [10, 11]. Recent studies detected miRNAs in multiple body fluids, where they showed great stability, either as free entities or trapped in circulating microvesicles such as exosomes. miRNAs are ideal biomarkers for screening and predicting prognosis in BTC and for contributing to the clinical decisions at different stages of cancer treatment. Consequently, progress in analyzing circulating miRNAs in serum, plasma, and bile could yield potential diagnostic and prognostic markers of BTC [12].

7.3.1 Bile Duct Cancer

Intrahepatic BDC has been associated with various gene activities, in particular KRAS mutations and overexpression of TP53. The type of mutation present seems related to different risk factors, such as hepatolithiasis and fluke infection associated with intrahepatic BDC. Moreover, macroscopic and histopathological characteristics of intrahepatic BDC are associated with specific genetic changes, including KRAS mutation being significantly more frequent with PI-type compared with MF-type tumors. Recent studies also identified that different genes (BAP1, ARID1A, PBMR1, IDH1, and IDH2) are significantly downregulated in intrahepatic BDC compared to normal tissue, and mutations of these genes are related to survival in patients undergoing surgical resection [13].

A recent meta-analysis of 37 studies and 2371 patients identified a number of prognostic biomarkers for resected extrahepatic BDC, with the following nine biomarkers predictive of overall survival (HR, 95% CI): VEGF (2.32, 1.57–3.44), COX-2 (1.94, 1.01–3.71), GLUT-1 (2.09, 1.52–2.89), cyclin D1 (1.96, 1.02–3.76), p16 (0.68, 0.47–0.98), p27 (0.48, 0.3–0.78), E-cadherin (0.47, 0.35–0.63), fascin (2.19, 1.35–3.55), and Ki-67 (1.69, 1.02–2.79) [14].

It was also recently suggested that distinct differences in genetic mutation patterns exist between intrahepatic BDC and hepatocellular carcinoma (HCC) [15]. Whole-genome sequencing analysis revealed that the genome-wide substitution patterns of intrahepatic BDC developed in chronic hepatitis livers overlapped with those developed with HCC, whereas those of hepatitis-negative intrahepatic BDC diverged. In addition, hepatitis-positive patients with intrahepatic BDC showed more frequent TERT promoter and PBRM1 mutations compared with hepatitis-negative patients, and the frequencies of KRAS and IDHs mutations, which are associated with poor disease-free survival, were significantly higher in hepatitis-negative intrahepatic BDC [15]. In 2009, Lee et al. [16] reported that viral hepatitis-associated intrahepatic BDC shares common disease processes with HCC, possibly because both arose from the same hepatic progenitor cells.

Taken together, these findings indicate that viral hepatitis-positive intrahepatic BDC shares similar biological behaviors with HCC and confirm the importance of therapeutic strategies in such patients including the selection of targeted drugs.

Interestingly, Churi et al. [17] found significant genetic differences between intra- and extrahepatic BDC. Namely, intra- and extrahepatic BDC differed in regard to the nature and frequency of specific genetic aberrations (GAs), with IDH1 and DNA repair gene alterations occurring more frequently in intrahepatic BDC, while ERBB2 GAs occurred in the extrahepatic BDC. Commonly occurring GAs in intrahepatic BDC were TP53 (35%), KRAS (24%), ARID1A (20%), IDH1 (18%), MCL1 (16%), and PBRM1 (11%), and the most frequent GAs in extrahepatic BDC were TP53 (45%), KRAS (40%), ERBB2 (25%), SMAD4 (25%), FBXW7 (15%), and CDKN2A (15%). In intrahepatic BDC patients, KRAS, TP53, or MAPK/mTOR GAs were significantly associated with a worse prognosis, while FGFR GAs correlated with a relatively indolent disease course. IDH1 GAs did not

have any prognostic significance, while those in chromatin-modulating genes, BAP1 and PBRM1, were associated with bone metastases and worse survival in extrahepatic BDC. Radiological responses and clinical benefit were noted in patients with BTC showing EGFR, FGFR, C-met, BRAF, and MEK inhibitors. Next-generation sequencing can potentially identify such disease subsets with distinct prognostic and therapeutic implications [17]. Another recent report [18] suggested that IDH1/2 and BAP1 mutations were characteristic of intrahepatic BDC, while KRAS and TP53 were more frequent in extrahepatic BDC and gallbladder cancer (GBC).

In 2016, Ruzzenente et al. [19] used mutational gene profiling to identify different gene mutations in distal BDC, perihilar BDC, and intrahepatic BDC. The most frequently mutated genes in distal BDC were KRAS (48%), TP53 (24%), and ARID1A (16%); in perihilar BDC, they were KRAS (22%), PBRM1 (17%), and PIK3CA (17%) and in intrahepatic BDC, IDH1 (17%), NRAS (17%), and BAP1 (14%). The presence of mutations in ALK, IDH1, and TP53 genes was significantly associated with poor prognosis in patients with distal BDC, with mutations in TP53 also significantly associated with poor prognosis in patients with perihilar BDC. The presence of mutations in ARID1A, PIK3C2G, STK11, TGFBR2, and TP53 genes was significantly associated with poor prognosis in patients with intrahepatic BDC. Such prognostic specificity in gene alteration could be used to identify patients with poor prognosis after curative surgery that might benefit from traditional or targeted adjuvant treatments.

Mutations in genes involved in the phosphatidylinositide 3-kinase (PI3K) cell signaling pathway, including PI3CA, PTEN, and AKT1, have also been reported in BDC, as have mutations of previously identified hotspots in IDH1 and IDH2. Jiao et al. [20] also identified frequent mutations in previously reported hotspots of the IDH1 and IDH2 genes that encode metabolic enzymes in intrahepatic BDC.

Checkpoint blockades turn on a new paradigm shift in immunotherapy for cancer. Remarkable clinical efficacy, durable responses, and low toxicity of program death 1 (PD-1) and its ligand (PD-L1) have been observed in various malignancies [21]. The overexpression of PD-L1 is an important and widely explored predictive biomarker for a patient's response to PD-1/PD-L1 antibodies; however PD-L1 staining cannot be used to accurately select patients for PD-1/PD-L1 pathway blockade due to the low prediction accuracy and prevalent dynamic changes. Tumor-infiltrating immune cells and molecules in the tumor microenvironment, along with PD-L1 expression, may also be important in predicting clinical benefits of PD-1/PD-L1 checkpoint blockades, and challenges must be overcome in accurately identifying patients who will benefit from this exciting therapeutic approach [21]. Recently PD-1 and PD-L1 have been identified as potential therapeutic targets for BDC, when expression of PD-L1 was noted among a majority of patients with intrahepatic BDC and PD-L1 expression within the tumor front was associated with a 60% decreased survival. Future clinical trials are necessary to assess the safety and efficacy of anti-PD-L1 therapies among patients with intrahepatic BDC and other BTCs [22].

Judging from the abovementioned findings, the prognosis and carcinogenesis of BDC might depend on both the anatomical classification sites and the prevailing genetic mutations.

7.3.2 Gallbladder Cancer

Gallbladder cancer (GBC) is associated with very high lethality, mainly due to the lack of symptoms until the tumor is in an advanced stage. As many as 80% of patients are diagnosed at very late stages of disease, allowing only palliative therapy. As a result, most patients with GBC will die within 6 months of diagnosis, and the average 5-year survival does not exceed 5% [23].

Clinical data show that groups of patients with long-standing gallstone disease are at increased risk of developing gallbladder cancer [24]. Since excessive preventative cholecystectomy is not a viable financial option for the healthcare system, a combination of risk assessment by genetic screening and analysis of biomarkers seems a feasible strategy to tackle this unmet need in the future. In light of the ever-decreasing cost of DNA sequence analysis, genetic screening is a stratification option with strong promise [23].

Recent studies indicate that alterations in miR-138 expression are associated with the development and progression of human tumors such as leukemia, nasopharyngeal carcinoma, neuroblastoma, and lung cancer [25–27]. MiR-138 is frequently downregulated in different cancer types and has been implicated in the progression of tumorigenesis. In a GBC study, Ma et al. [28] reported MiR-138 downregulation in cancer cells compared to normal cells. This group also studied the biological effects of miR-138 and Bag-1 (Bcl-2-associated athanogene-1) on cell proliferation using 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide and apoptosis assays, finding that overexpression of miR-138 inhibited cell proliferation by directly suppressing the expression of Bag-1. These results provide the first evidence that miR-138 plays an important role in inhibiting the growth of GBC [28].

There is scant data about genetic changes in GBC. KRAS gene mutations have been found in 39–59% of GBC cases, and 50–83% of KRAS gene mutations were identified in patients with an anomalous junction of the pancreatico-biliary duct, suggesting that pancreatic juice reflux might contribute to the carcinogenic process.

TP53 gene mutations have also been reported in 35–92% of GBC patients, with two-thirds of such studies showing a frequency greater than 50% [29]. Analyses of exon 5–8 of TP53 demonstrated point mutations in 31–70% of GBC cases, but no particular “hotspot” has been identified [29, 30].

Shukla et al. [31] reported that telomerase activity was mainly concentrated in poorly differentiated adenocarcinomas (83%) and increased expression was present in the advanced stages. Telomerase activity is also a reliable marker for detecting tumor cells in postsurgical resection margins; thus, telomerase may serve as a molecular marker for the diagnosis of GBC and may have prognostic and therapeutic implications for treatment strategies. Future studies may help to find a correla-

tion between telomerase activity and tumor size, lymph node involvement, and stage.

Various studies suggested that genetic alterations in PIK3CA, KRAS, p53, p63, and p16 play a significant part in gallbladder carcinogenesis; thus, identifying such mutations in GBC patients might help in formulating a more effective and targeted approach for disease management [32]. In addition, activating mutations in PIK3CA were identified exclusively in GBC (12.5%) [33], which has clinical implications for both the diagnosis of GBC and targeted therapies such as PI3K inhibitors. GBC also has a high frequency of KRAS codon 12 mutation with poorer outcomes in resected stage II and III disease [34]. Kim et al. [35] demonstrated that p16, p63, and p53 can be used as prognostic markers in GBC, with p53 and p63 predicting a poor prognosis and p16 a favorable prognostic marker. TP53 and beta-catenin expression in GBC were also significantly higher compared to normal cells at 56% and 75%, respectively. TP53 expression correlates with increasing tumor grade, while beta-catenin nuclear expression correlates with tumor grade and depth of invasion, thus suggesting a role for these genes in tumor progression [36]. p16 is a cyclin-dependent kinase 4 inhibitor and is linked to the regulation of cell cycle in mammalian cells, with the genes encoding these inhibitors located at 9p21 [37]. The frequency of p16 gene mutations was observed in 31% of patients with GBC [37].

Inactivation of tumor suppressor genes involves different genetic mutational events that favor one allele and lead to allelic loss of the other alleles. This allelic loss is known as loss of heterozygosity (LOH) and can be detected by using microsatellite markers. Recent studies found a high incidence of LOH at several chromosomes in GBC, particularly on chromosomes 1p, 3p, 5q, 8p, 9p, 9q, 13q, 16q, and 17p, which has been highlighted. LOH on 3p, 9p, 13q, 16q, and 17p was also detected in preneoplastic lesions and in the early phase of GBC during multistep carcinogenesis. Many chromosomal regions of LOH in GBC have important associated genes such as the following: 3p (incidence, 76–100%), 5q21 (66%), 8p21–23 (100%), 13q14 (20–56%), 16q24 (61%), and 17p13 (42–91%) [38].

Retinoblastoma (Rb) gene was the first tumor suppressor protein to be discovered in human Rb, and it is mutated in various types of cancer [39]. Xuan et al. [39] noted a reduced expression of the Rb and p27 genes and increased expression of p53, cyclin D1, Ki-67, and MSH2 in GBC, while expression of p53, Ki67, p27, and MSH2 correlated with clinical stage. Another novel finding is that Ki-67 and MSH2 overexpression was more frequent in elderly patients with GBC (>65 years) and that expression of p27 was greater in males. Adenomas also showed reduced expression of Rb but increased expression of cyclin D1 [39].

The precise molecular abnormality and signaling mechanisms underlying neoplastic transformation in the gallbladder epithelium remain unclear. A better understanding of molecular events is therefore needed for improved early detection of GBC [40].

A systematic review and meta-analysis of 80 candidate gene variants and 173 polymorphisms among 1046 cases of GBC and 2310 controls revealed that most studies regarding OGG1, TP53, CYP1A1, and GSTM1 were underpowered and they could not confirm previous association results upon meta-analysis [23, 41].

Since gallstones represent a significant risk factor for GBC, the Lith genes predisposing to gallstones represent important genetic risk factors. Several genes have been associated with the development of human gallstone disease, including APOE and APOB, cholesterol 7 α-hydroxylase, CCKAR, the low-density LRPAP1, and CETP [23]. Genome-wide association analyses and functional studies also identified the p.D19H variant of the hepatic cholesterol hemitransporter (ABCG8) as associated with susceptibility for human gallstones, and a similar Japanese study of GBC revealed a strong positive signal at rs7504990 within the DCC gene on chromosome 18q21.3 [23].

Various association studies in different populations also confirmed a potential role for CYP1A1, which metabolizes estrogen and xenobiotics [23]. A meta-analysis of 268 studies examining the role of CYP1A1 polymorphisms in cancer susceptibility indicated the need for further investigating this polymorphism in GBC [23, 42]. The usefulness of stratification by presence or absence of gallstones was shown by Sharma et al. [43] when 410 GBC cases and 230 healthy controls were analyzed for an association of genetic variants in MMP-2, MMP-7, and MMP-9 and TIMP-2 with gallbladder disease. Different associations were found in patients with or without accompanying gallstones, indicating differences in the genetic etiology between these entities. Unfortunately, no gallstone-bearing but cancer-free controls were available [23].

7.4 Molecular Targeting Therapy

The incidence of BTC has been increased globally; however, no effective targeted molecular therapies are currently available. Nakamura et al. [44] characterized 260 BTCs and uncovered spectra of genomic alterations that included new potential therapeutic targets. Gradient spectra of mutational signatures with a higher burden of the APOBEC-associated mutation signature were observed in GBC and extrahepatic BDC and were associated with APOBEC3B expression and higher mutation number, preferentially contributed to GBC rather than BDC and contributed to extrahepatic BDC more than to intrahepatic BDC. Although APOBEC3B gene expression is broadly involved in the genesis of cancer genomes, intrinsic cellular and microenvironmental differences undoubtedly also contribute to mutational processes in the biliary tract epithelium [45]. Thirty-two significantly altered genes, including ELF3, were identified, and nearly 40% of cases harbored targetable genetic alterations. Gene fusions involving FGFR2 and RKACA or PRKACB preferentially occurred in intrahepatic and extrahepatic BDC, respectively, and the subtype-associated prevalence of actionable growth factor-mediated signals was noteworthy. The subgroup with the poorest prognosis had significant enrichment of hyper-mutated tumors and a characteristic elevation in the expression of immune checkpoint molecules. Accordingly, immune-modulating therapies might also be potentially promising options for these patients [44]. The organ-specific prevalence of targetable growth factor-mediated signaling pathways (FGFR in intrahepatic

BDC, PKA in extrahepatic and intrahepatic BDC, and EGFR and ERBB3 in GBC) is therefore clinically important [44].

Huch et al. [46] showed that primary human bile duct cells can readily be expanded in vitro as bipotent stem cells into 3D organoids and that these cells differentiate into functional hepatocytes upon transplantation. Extensive analysis of the genetic stability of cultured organoids in vitro demonstrated that the expanded cells preserve their genetic integrity over months in culture [46]. This report also suggested that activation of cAMP-dependent signaling is required for the immortalization of biliary tract epithelial cells and thus could present an encouraging new drug target.

Cancer development largely depends on the ability of mutant cells to hijack and exploit the normal physiological processes of the host. Each stage of cancer development is exquisitely susceptible to regulation by immune cells (BOX 1). On the other hand, full activation of adaptive immune cells in response to the tumor might result in eradiation of malignant cells, and chronic activation of various types of innate immune cells in or around premalignant tissues might actually promote tumor development. Namely, there is a paradoxical relationship between innate and adaptive immune cells. Chronic inflammation by the host immune system, a key epidemiological basis for BTC, demonstrates two paradoxical roles during carcinogenesis: antitumor activity and tumor promotion [47]. Cancer chemotherapies were initially identified through screens for compounds that kill rapidly dividing cells. These drugs remain the backbone of current treatment, but they are limited by a narrow therapeutic index, significant toxicities, and frequently acquired resistance. Gene expression profiling demonstrated that patients with the poorest prognosis (cluster 4) presented elevated expression of components of the host immune system but also counteracting ICM and antiapoptotic signatures, suggesting an immune environment "tailored" by the tumor cells. Targeting ICMs could be complementary to genotype-based molecular therapy against cancer, and immune-modulating therapies could also be promising for BTC, depending on future proof of concept in the clinic [48].

Recent noteworthy topics regarding molecular targeting therapy of each BDC and GBC are herein described.

7.4.1 Bile Duct Cancer

Somatic mutations of KRAS and BRAF are the most common genetic alterations in BDC. Surgical resection is the only curative treatment for BDC, and no standard chemotherapy regimens have been established for inoperative cases or those showing recurrence after surgical resection.

Fibroblast growth factor receptor (FGFR) genes are involved in multiple biological processes, ranging from cell transformation, angiogenesis, and tissue repair to embryonic development. Treatment with the FGFR kinase inhibitors BGJ398 and

PD173074 effectively suppressed transformation [2]. FGFR2 fusions occur in 13.6% of intrahepatic BDC [2]. The expression pattern of these fusions in association with sensitivity to FGFR inhibitors warrants a new molecular classification of BDC and suggests a new therapeutic approach [2].

Based on the specific relevant genomic alterations, tyrosine kinase inhibitors (TKIs) have been developed into effective therapies [49, 50]. Gu et al. [51] demonstrated ROS tyrosine kinase as a promising therapeutic target and diagnostic molecular marker in BDC. By integrating genetic, epigenetic, proteomic, and phosphoproteomic information, we can begin to understand the pathogenesis of BDC and identify novel therapeutic targets [49]. It was shown that small-molecule FGFR inhibitors, BGJ398 and PD173074, efficiently blocked the downstream signaling and oncogenic activity of intrahepatic BDC-specific FGFR2 fusions [2]. High-throughput cell line profiling has also revealed amplifications or mutations of FGFR genes in cancer cell lines able to predict sensitivity to the selective pan-FGFR inhibitor BGJ398 [52]. This drug is currently in a phase I study in patients with advanced solid tumors and FGFR1/2 amplification or FGFR3 mutation (Novartis, Basel, Switzerland; Clinical Trials. Gov identifier: NCT01004224). Cells harboring FGFR fusions showed enhanced sensitivity to the FGFR inhibitors PD173074 and pazopanib, suggesting that cancers with FGFR fusions might respond to targeted FGFR kinase inhibition [53]. These findings justify the clinical development of FGFR inhibitors in selected patients with cancer-harboring tumors and the identified predictors of sensitivity [52]. Clinical investigations akin to those conducted in other solid tumors with oncogenic fusion kinases are also warranted to examine the efficacy of FGFR inhibitors in treating a defined subset of BDC cases harboring FGFR2 fusions [2].

7.4.2 Gallbladder Cancer

GBC is the most aggressive malignancy of the biliary tract and has the worst prognosis. Improvement of GBC patient care requires better understanding of the biological signaling pathways and application of newly discovered drugs for cancer therapy.

Standard care for advanced GBC cases involves combination chemotherapy with gemcitabine and cisplatin, but this strategy has no significant impact on the median overall survival, which is less than 6 months after diagnosis. Long-term survival in the small proportion of cases is primarily seen in those detected incidentally during routine cholecystectomy for gallstones. A poor understanding of the molecular pathogenesis and aberrant signaling pathways involved and the effect of targeted therapeutic agents on this tumor type have hampered our ability to devise effective strategies to deal with this disease [54].

Li et al. [55] identified somatic mutations for GBC through a combination of exome sequencing and ultra-deep sequencing of cancer-related genes, revealing

those with a significant frequency of non-silent mutations including TP53 (47%), KRAS (8%), and ERBB3 (12%). Moreover, ErbB signaling, including EGFR, ERBB2, ERBB3, ERBB4, and their downstream genes, is the most extensively mutated pathway, detected in 37% of the GBC samples, and multivariate analysis showed that these cases with ErbB pathway mutations had a worse outcome. These findings provide insight into the somatic mutational landscape in GBC and highlight both the key role of ErbB signaling in GBC pathogenesis and the consequent potential for an effective therapeutic approach against GBC in future.

Somatic mutation analysis has become a useful tool in selecting personalized therapy for many solid tumors and yielded other new, broad-based molecular techniques for molecularly profiling tumors to identify potential therapeutic targets. Voss et al. [1] identified BTC as expressing BRAF, EGFR, MET, and PIK3CA. Although EGFR-targeted therapy on 42 BTC patients in a phase II study showed a partial response in only three patients, the benefits of vemurafenib, a next-generation BRAF inhibitor, and multiple inhibitors targeting the MET pathway are expected to be revealed by future clinical trials [56]. A recent study identified PIK3CA-activating mutations in 13% of GBC, but no mutations in BDC [33]. Similarly, Reiner et al. [57] identified only one PIK3CA mutation in 11 intrahepatic BDC cases, although Xu et al. [58] identified PIK3CA mutations in 32% patients with BDC within the Chinese population. Therefore, it appears that PIK3CA mutations are relatively rare yet present in a subset of BTC including GBC that could be responsive to novel PIK3CA-tageted therapies [1].

7.5 Summary

At present, KRAS, BRAF, and circulating miRNA may be the most promising biomarkers for the early detection of BTC. Table 7.2 summarizes the anatomically specific biomarkers used for molecular diagnosis and targeting therapy in BTC. Such anatomical specificity, such as intrahepatic and extrahepatic BDC and GBC, is highly important because markers with such specificities are already available as shown in Table 7.2.

Clinically, finding a precise differential diagnosis between perihilar BTC and intrahepatic BTC based on PI type might be solved in the future with the further development of genetic targets akin to the anatomically specific markers. Based on current genomic data, further preclinical studies and clinical evaluations are expected to effectively determine the validity of these therapeutic targets in BTC.

The discovery of crucial molecular pathways that promote BTC growth and maintenance together with the development of drugs that specifically inhibit these pathways has ushered in a new era of BTC therapy.

In future, the molecular classification of BTC and the precise definition of categories based on prognosis will help clinicians select patients for surgical or nonsurgical therapies.

Table 7.2 Anatomically specific biomarkers in the molecular diagnosis and targeting therapy of biliary tract cancer

Classification	Molecular diagnosis	Targeting therapy	Reference no.
1) Intrahepatic bile ductal cancer	KRAS, TP53, BAP1, ARID1A, PBMR1, IDH1, IDH2, DNA repair gene alterations, NRAS, PIK3C2G, STK11, TGFBR2, PD-L1, FGFR2, EPHA2, SMAD4, ARID1A, GNAS	Anti-PD-L1 therapy	[2, 13, 17, 19, 22, 44, 51, 53]
		FGFR kinase inhibitor	
		Tyrosine kinase inhibitor (TKI)	
2) Extrahepatic bile ductal cancer	KRAS, TP53, VEGF, COX-2, GLUT-1,Cyclin D1, p16, p27, E-cadherin, fascin, Ki-67, ERBB2, SMAD4, FBXW7, CDKN2A, ARID1A, GNAS		[14, 17, 44]
Perihilar	KRAS, TP53, PBRM1, PIK3CA	PIK3CA-targeted therapy	[19]
Distal	KRAS, TP53,ARID1A, ALK, IDH1		[19]
3) Gallbladder cancer	KRAS, TP53, miR-138, PIK3CA, p63, p16,p27, MSH2, CYP1A1, EGFR, ERBB2, ERBB3,ERBB4, MET, PTEN, ARID2, MLL2, MLL3, APOBEC	PI3 kinase inhibitor	[1, 23, 28, 32, 38, 39, 44, 55]
		Inhibitor of ErbB pathway	
		Inhibitor of MET pathway	
		PIK3CA-targeted therapy	

References

1. Voss JS, et al. Molecular profiling of cholangiocarcinoma shows potential for targeted therapy treatment decisions. Hum Pathol. 2013;44:1216–22.
2. Arai Y, et al. Fibroblast growth receptor 2 tyrosine kinase fusions define a unique molecular subtype of cholangiocarcinoma. Hepatology. 2014;59:1427–14134.
3. Nagino M. Perihilar cholangiocarcinoma: a surgeon's system viewpoint on current topics. J Gastroenterol. 2012;47:1165–76.
4. Ebata T, et al. Proposal to modify the International Union against Cancer staging system for perihilar cholangiocarcinoma. Br J Surg. 2014;101:79–88.
5. Endo I, et al. Intrahepatic cholangiocarcinoma: rising frequency, improved survival, and determinants of outcome after resection. Ann Surg. 2008;248:84–96.
6. Nakeeb A, et al. Cholangiocarcinoma. A spectrum of intrahepatic, perihilar, and distal tumors. Ann Surg. 1996;224:463–73. (discussion)
7. Liver Cancer Study Group in Japan. The general rules for the clinical and pathological study of primary liver cancer. 6th ed. Tokyo: Kanehara & Co., Ltd.; 2015. (in Japanese)
8. Japanese Society of Hepato-Biliary-Pancreatic Surgery. General rules for clinical and pathological studies on cancer of the biliary tract. 6th ed. Tokyo: Kanehara & Co., Ltd.; 2013. (in Japanese)
9. Miyazaki M, et al. Classification of biliary tract cancers established by the Japanese Society of Hepato-Biliary-Pancreatic Surgery: 3rd English edition. J Hepato Biliary Pancreatic Sci. 2015;22:181–96.

10. Lu J, et al. Microrna expression profiles classify human cancers. Nature. 2005;435:834–8.
11. Reinhart BJ, et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis Elegans. Nature. 2000;403:901–6.
12. Latelier P, et al. Circulating microRNAs as biomarkers in biliary tract cancers. Int J Mol Sci. 2016;17:791–814.
13. Ruzzenente A, et al. Role of surgery in the treatment of intrahepatic cholangiocarcinoma. Eur Rev Med Pharmacol Sci. 2015;19:2892–900.
14. Jones RP, et al. Prognostic molecular markers in resected extrahepatic biliary tract cancers; a systemic review and meta-analysis of immunohistochemically detected biomarkers. Biomark Med. 2015;9:763–75.
15. Fujimoto A, et al. Whole-genome mutational landscape of liver cancers displaying biliary phenotype reveals hepatitis impact and molecular diversity. Nat Commun. 2015;6:6120–7.
16. Lee CH, et al. Viral hepatitis-associated with intrahepatic cholangiocarcinoma shares common disease processes with hepatocellular carcinoma. Br J Cancer. 2009;100:1765–70.
17. Churi CR, et al. Mutation profiling in cholangiocarcinoma: prognostic and therapeutic implications. PLoS One. 2014;9:e115383–405.
18. Simbolo M, et al. Multigene mutational profiling of cholangiocarcinomas identifies actionable molecular subgroups. Oncotarget. 2014;5:2839–52.
19. Ruzzenente A, et al. Cholangiocarcinoma heterogeneity revealed by multigene mutational profiling: clinical and prognostic relevance in surgically resected patients. Ann Surg Oncol. 2016;23:1699–707.
20. Jiao Y, et al. Exome sequencing identifies frequent inactivating mutations in BAP1, ARID1A and PBRM1 in intrahepatic cholangiocarcinoma. Nat Genet. 2013;45:1470–3.
21. Meng X, et al. Predictive biomarkers in PD-1/PD-L1 checkpoint blockade. Cancer Treat Rev. 2015;41:868–76.
22. Gani F, et al. Program death 1 immune checkpoint and tumor microenvironment: implications for patients with intrahepatic cholangiocarcinoma. Ann Surg Oncol. 2016;23:2610–7.
23. Liebe R, et al. Modifiable factors and genetic predisposition associated with gallbladder cancer. A concise review. J Gastrointestin Liver Dis. 2015;24:339–48.
24. Zatonski WA, et al. Epidemiologic aspects of gallbladder cancer: a case-control study of the SEARCH program of the International Agency for Research on Cancer. J Natl Cancer Inst. 1997;89:1132–8.
25. Liu X, et al. MiR-138 suppressed nasopharyngeal carcinoma growth and tumorigenesis by targeting the CCND1 oncogene. Cell Cycle. 2012;11:2495–506.
26. Zhao X, et al. miR-138 might reverse multidrug resistance of leukemia cells. Leuk Res. 2010;34:1078–82.
27. Gao Y, et al. miR-138-5p reverses gefitinib resistance in non-small cell lung cancer cells via negatively regulating G protein-coupled receptor 124. Biochem Biophys Res Commun. 2014;446:179–86.
28. Ma F, et al. MiR-138 suppresses cell proliferation by targeting bag-1 in gallbladder carcinoma. PLoS One. 2015;10:e126499–510.
29. Lazcano-Ponce EC, et al. Epidemiology and molecular pathology of gallbladder cancer. CA Cancer J Clin. 2001;51:349–64.
30. Fujii K, et al. High frequency of p53 gene mutation in adenocarcinomas of the gallbladder. Cancer Epidemiol Biomark Prev. 1996;5:461–6.
31. Shukla VK, et al. Telomerase activation-one step on the road to carcinoma of the gall bladder. Anticancer Res. 2006;26:4761–6.
32. Kumari N, et al. Mutation profiling in gallbladder cancer in Indian population. Indian J Pathol Microbiol. 2014;57:9–12.
33. Deshpande V, et al. Mutational profiling reveals PIK3CA mutations in gallbladder carcinoma. BMC Cancer. 2011;11:60–6.
34. Kazmi HR, et al. Prognostic significance of K-ras codon 12 mutation in patients with resected gallbladder cancer. Dig Surg. 2013;30:233–9.

35. Kim K, et al. Expression of cell cycle-related proteins, p16, p53 and p63 as important prognostic markers in gallbladder adenocarcinoma. Pathol Oncol Res. 2014;20:409–15.
36. Ghosh M, et al. p53 and beta-catenin expression in gallbladder tissues and correlation with tumor progression in gallbladder cancer. Saudi J Gastroenterol. 2013;19:34–9.
37. Dixit R, et al. Molecular alterations in gallbladder cancer. World J Pathol. 2012;1:31–4.
38. Kuroki T, et al. Genetic alterations in gallbladder carcinoma. Surg Today. 2005;35:101–5.
39. Xuan YH, et al. An immunohistochemical study of the expression of cell-cycle-regulated proteins p53, cyclin D1, RB, p27, Ki67 and MSH2 in gallbladder carcinoma and its precursor lesions. Histol Histopathol. 2005;20:59–66.
40. Dwivedi AN, et al. Gall bladder carcinoma: aggressive malignancy with protean loco-regional and distant spread. World J Gastroenterol. 2015;16:231–44.
41. Srivastava K, et al. Candidate gene studies in gallbladder cancer: a systemic review and meta-analysis. Mutat Res. 2011;728:67–79.
42. He XF, et al. Association between the CYP1A1 T3801C polymorphism and risk of cancer. Evidence from 268 case-control studies. Gene. 2013;534(2):324–44.
43. Sharma KL, et al. Higher risk of matrix metalloproteinase (MMP-2, 7,9) and tissue inhibitor of metalloproteinase (TIMP-2) genetic variants to gallbladder cancer. Liver Int. 2012;32:1278–86.
44. Nakamura H, et al. Genomic spectra of biliary tract cancer. Nat Genet. 2015;47:1003–10.
45. Burns MB, et al. Evidence for APOBEC3B mutagenesis in multiple human cancers. Nat Genet. 2013;45:977–83.
46. Huch M, et al. Long-term culture of genome-stable bipotent stem cells from adult human liver. Cell. 2015;160:299–312.
47. de Visser KE, et al. Paradoxical roles of the immune system during cancer development. Nat Rev Cancer. 2006;6:24–37.
48. Vanneman M, Dranoff G. Combining immunotherapy and targeted therapies in cancer treatment. Nat Rev Cancer. 2012;12:237–51.
49. Mitelman F, et al. The impact of translocations and gene fusions on cancer causation. Nat Rev Cancer. 2007;7:233–45.
50. Ablain J, et al. The drug-induced degradation of oncoproteins: an unexpected Achilles' heel of cancer cells? Cancer Discov. 2011;1:117–27.
51. Gu TL, et al. Survey of tyrosine kinase signaling reveals ROS kinase fusions in human cholangiocarcinoma. PLoS One. 2011;6:e15640–8.
52. Guagnano V, et al. FGFR genetic alterations predict for sensitivity to NVP-BGJ398, a selective pan-FGFR inhibitor. Cancer Discov. 2012;2:1118–33.
53. Wu YM, et al. Identification of targetable FGFR gene fusions in diverse cancers. Cancer Discov. 2013;3:636–47.
54. Mittal B, Yadav S. Targeting the hedgehog pathway for gallbladder cancer therapy? Chin Clin Oncol. 2016;5:2–4.
55. Li M, et al. Whole-exome and targeted gene sequencing of gallbladder carcinoma identifies recurrent mutations in the ErbB pathway. Nat Genet. 2014;46:872–6.
56. Phlip PA, et al. Phase II study of erlotinib in patients with advanced biliary cancer. J Clin Oncol. 2006;24:3069–74.
57. Riener MO, et al. Rare PIK3CA hotspot mutations in carcinomas of biliary tract. Genes Chromosom Cancer. 2008;47:363–7.
58. Xu RF, et al. KRAS and PIK3CA but not BRAF genes are frequently mutated in Chinese cholangiocarcinoma patients. Biomed Pharmacother. 2011;65:22–6.

Chapter 8
Molecular Analysis for Therapeutic Targets of Pancreatic Cancer

Shinji Tanaka

Abstract Pancreatic cancer is one of the most lethal malignancies in humans and still remains a challenging problem in targeted therapy compared to other malignancies. Accumulation of multiple molecular abnormalities is required for generation and progression of cancers, and one of the malignant hallmarks is the dependency on the abnormalities in cancer and host cells. Disruption of such addiction should cause specifically the cancer cell death, indicating a feasible rationale for molecular targeted therapy. Recent whole genomic, epigenomic, and transcriptomic studies identified four molecular subtypes of pancreatic cancer in respect to the cell lineages and signatures: (1) aberrantly differentiated endocrine exocrine subtype with KRAS signal network, (2) squamous trans-differentiation subtype with TP53 and KDM6A mutations, (3) immunogenic subtype with T-/B-cell signatures, and (4) pancreatic progenitor subtype with stemness identity. Based on the subtype classification, this review summarizes the molecular pathogenesis and therapeutic targets of pancreatic cancer. The subtype-directed precision medicine might be a promising strategy to treat the pancreatic cancer in the perspective of oncogenic and epigenetic pathways as well as immune regulatory checkpoints and stem cell fates.

Keywords Molecular subtype • KRAS • KDM6A • Treg • PD-1 • Cancer stem cells

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8.1 Introduction

Mature pancreas is mainly comprised of three distinct tissue populations: exocrine, endocrine, and ductal cell types [1]. In pancreatic development, the specific bud within the posterior foregut region of the definitive endoderm is first indicated by expression of PDX1 transcription factor that marks the progenitors of all cell lineages of the pancreas including exocrine acinar, endocrine islet, and ductal types (Fig. 8.1). Under oncogenic stress in acinar cells, upregulation of transcription

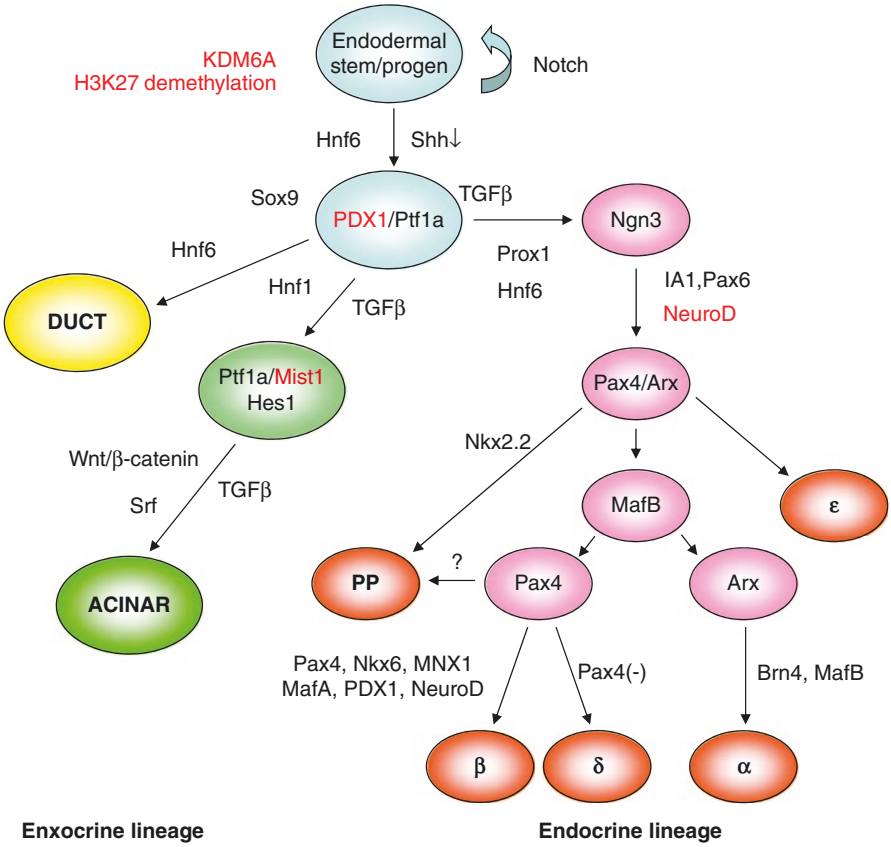

Fig. 8.1 Cell lineages of the pancreatic organ. Pancreas specification within the foregut endoderm is first indicated by expression of PDX1 transcription factor, marking the progenitors of all exocrine, endocrine, and ductal cell types [1]. Correct reprogramming and specific differentiation during pancreatic ectodermal development require H3K27 demethylase KDM6A. As the embryonic organ grows, multipotent progenitor cells also express Ptf1a transcription factor. These progenitors are later segregated into specific sub-lineages, prior to differentiation. Exocrine acinar cells arise from precursors that express high levels of Ptf1a and Mist1 transcription factors, while endocrine islet cells arise from precursors that transiently express NeuroD transcription factor. Function of Hnf6 transcription factor might be restricted to the distinct ductal lineage. Acinar-to-ductal metaplasia with reprogramming is promoted by PDX1 and Sox9 but inhibited by Mist1 transcription factor that maintains stable acinar differentiation [2]. Ductal dedifferentiation/retrogression is associated with downregulation of Sox9 transcription factor that stabilizes the mature ductal cell identity [3]

factors PDX1 and Sox9 promotes ductal reprogramming, namely, acinar-to-ductal metaplasia (ADM) [2]. The ductal reprogramming of acinar cells is inhibited by Mist1 transcription factor that maintains stable acinar differentiation. In contrast, dedifferentiation/retrogression of ductal cells is associated with downregulation of Sox9 transcription factor. The ductal retrogression might be inhibited by Sox9 expression that stabilizes the mature ductal cell identity [3]. Either ADM or ductal retrogression initiates the distinct pancreatic precancerous lesions causing ductal adenocarcinomas [2].

Pancreatic ductal adenocarcinoma is the most common malignancy of the pancreas (approximately 90%) and usually referred to as "pancreatic cancer" in general [4]. Pancreatic cancer is one of the leading causes of cancer deaths, and the incidence is still increasing in the United States, European countries, and Japan. Five-year survival is approximately 5% in the patients diagnosed with pancreatic ductal adenocarcinoma, and such poor prognosis has remained virtually unaltered in the past decades. Recent studies on cancer genomics and transcriptomics are revolutionizing the understanding of pancreatic carcinogenesis [3], and subtype-specific therapy which specifically targets the molecular abnormalities emerged to be a novel approach for the innovative and effective medical treatment [5]. In order to fulfill this promise, there is an urgent need to cluster the molecular subtypes and identify the individual optimal targets utilized for radical treatment of the pancreatic cancer. The sharpshooting strategy for the addictive molecule(s) in each subtype has led to understating pancreatic carcinogenesis and progression, leading to a precision medicine approach for treating pancreatic cancer.

8.2 Molecular Subtypes of Pancreatic Cancer

Pancreatic ductal adenocarcinoma arises usually from a spectrum of preneoplastic lesions with ductal morphology, so-called pancreatic intraepithelial neoplasias (PanINs) that might be derived from ADMs [5]. Accumulation of genetic alterations is associated with the histological progression from PanIN-1 (hyperplasia) through PanIN-2 (atypia) and PanIN-3 (carcinoma in situ) to invasive ductal adenocarcinoma (Fig. 8.2). Genetic sequencing revealed mutations in *KRAS* (95%), *CDKN2A/P16* (>90%), *TP53* (75%), and *SMAD4* (55%) are commonly detected in pancreatic ductal adenocarcinoma [6]. *KRAS* mutations occur as the earliest genetic abnormalities, even in PanIN-1 lesions, whereas inactivation of *TP53* and *SMAD4* is detected in PanIN-3 lesions and invasive carcinomas. Similar PanIN-mimic lesions are developed in genetically engineered mouse models carrying a mutant *KRAS* gene expressed under the pancreas-specific promoter of *PDX1* gene (Fig. 8.1) [7]. In combination with *KRAS* mutation, either *CDKN2A* or *TP53* mutation leads to the frequent development of pancreatic ductal adenocarcinoma in the mouse models [7]. Whole genome/exome sequencing analysis recently revealed a SWI/SNF chromatin-remodeling complex as another candidate of the major driver mutations in pancreatic cancer [8] (Fig. 8.3). Approximately 34% of pancreatic ductal adenocarcinoma carries genomic aberrations in the SWI/SNF subunits such as ARID1A, ARID1B, ARID2, PBRM1, and BRG1 [8, 9]. BRG1 is one of the core SWI/SNF

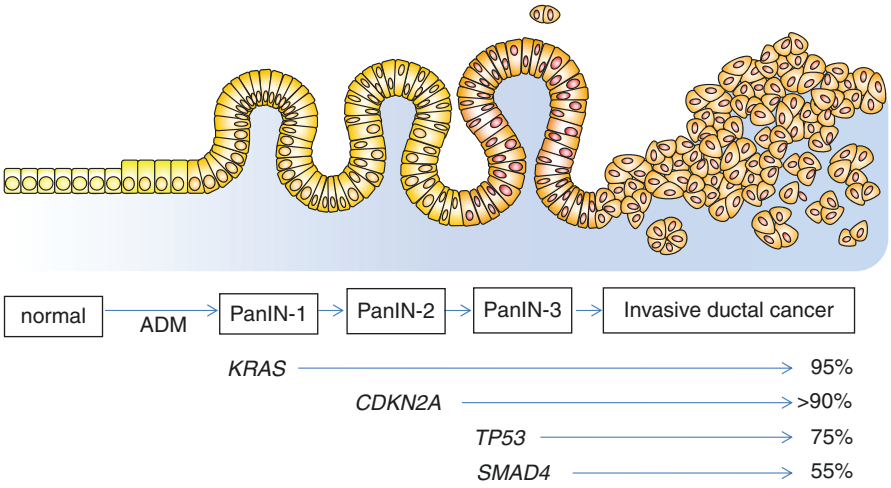

Fig. 8.2 Schematic of common pancreatic carcinogenesis and genetic alterations. *ADM* acinar-to-ductal metaplasia, *PanIN* pancreatic intraepithelial neoplasm, *PanIN-1* hyperplasia, *PanIN-2* atypia, *PanIN-3,* carcinoma in situ. Pancreatic cancer develops and progresses stepwise through a particular sequence of genetic alterations: KRAS, followed by CDKN2A, then TP53, and SMAD4

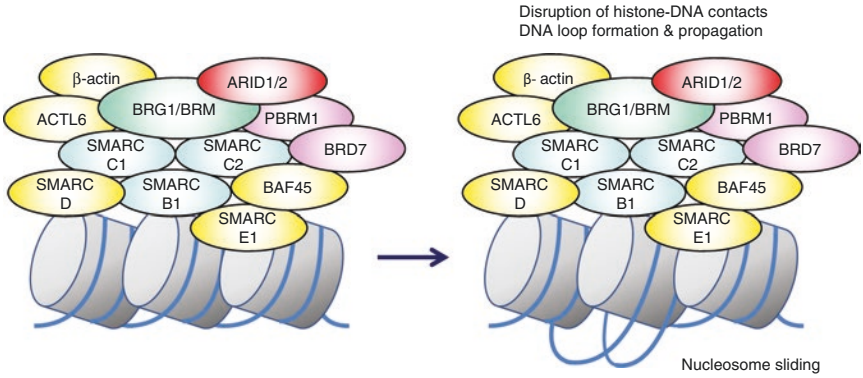

Fig. 8.3 Chromatin remodeling of SWI/SNF complexes. ARID1A or ARID1B-containing BAF and ARID2-containing PBAF complexes constitute the major subclasses. PBRM1 and BRD7 are unique to PBAF complexes, but not to BAF complexes. The steps of remodeling include SWI/SNF binding, disruption of histone-DNA contacts, the creation of a loop of DNA that propagates around the nucleosome in a wavelike manner, and the repositioning of DNA with respect to the nucleosome sliding

enzymatic subunits that function as ATP-dependent helicase to disrupt histone-DNA contacts to create a loop of DNA. Loss of *BRG1* cooperated with oncogenic *KRAS* formed intraductal papillary mucinous neoplasm (IPMN), another type of preneoplastic lesions possibly derived from the ductal retrogression and then progressed to pancreatic ductal adenocarcinoma in the conditional knockout mouse model, indicating the tumor suppressor function of BRG1 [10].

More recently, integrated genomic, epigenomic, and transcriptomic analyses identified that four molecular subtypes exist in human pancreatic adenocarcinoma [11], using bulk tumor tissues for clustering to better understand the transcriptional networks and molecular mechanisms that underpin the tumor microenvironment. They named these subtypes as (1) aberrantly differentiated endocrine exocrine (ADEX), (2) squamous trans-differentiation, (3) immunogenic, and (4) pancreatic progenitor subtypes on the basis of the differential expression of transcription factors and downstream targets important in lineage specification and differentiation during pancreas development and regeneration (Fig. 8.1). Noteworthy, squamous subtype is an independent poor prognostic factor. These four subtypes are histologically associated with specific characteristics: (1) ADEX with rare acinar cell carcinomas, (2) squamous trans-differentiation with adenosquamous carcinomas, (3) immunogenic, and (4) pancreatic progenitor with mucinous non-cystic adenocarcinomas and carcinomas arising from IPMN.

8.3 ADEX Subtype: KRAS Signal Network

The ADEX subtype is defined by transcriptional networks that are important in later stages of pancreatic development and differentiation [11]. Transcriptional networks that characterize both exocrine and endocrine lineages at later stages are upregulated. The key networks identified include upregulation of (1) Mist1 transcription factor and the downstream targets that are important in acinar cell differentiation and (2) NeuroD/Nkx transcription factors associated with endocrine differentiation (Fig. 8.1). The methylation pattern of ADEX tumors was distinct from the normal pancreas and clustered with other subtypes of pancreatic cancer. More important, ADEX tumors displayed upregulation of genes that regulate networks involved in KRAS activation.

KRAS signaling is activated from the downstream of receptor-type tyrosine kinases such as EGFR (Fig. 8.5). It is of interest that EGFR overexpression is frequently detected in KRAS-mutant pancreatic ductal adenocarcinoma in clinical human samples as well as mouse models [12]. Paradoxically, EGFR is revealed to be required for mutated KRAS-driven pancreatic tumorigenesis in the genetically engineered mice [12, 13]. Erlotinib (Tarceva, OSI774) is an oral tyrosine kinase inhibitor and selectively suppresses EGFR tyrosine kinase. A phase III clinical trial for erlotinib-gemcitabine demonstrated a significant synergistic effect of the combination with an HR of 0.82 (95%CI 0.69–0.99; $p = 0.038$) [14]. Although the erlotinib-gemcitabine arm showed a minimal 2 weeks increase in MST (6.24 months) compared with the gemcitabine-single arm (5.91 months), erlotinib is the first and only molecular-targeting agent with a statistical effect for pancreatic ductal adenocarcinoma. The activated EGFR tyrosine kinase stimulated Grb2-SOS complex, leading to activation of Ras that is farnesylated and then localized under the cellular membrane. Tipifarnib (R115777), a farnesyl transferase inhibitor, was applied for clinical trials of pancreatic ductal adenocarcinoma, resulting in no statistical

significance ($p = 0.75$) [15]. Activated form of Ras stimulates Raf serine-threonine kinase and then phosphorylates MEK kinases which finally activate Erk 1/2 of MAPK family activating intranuclear transcription factors. To date, sorafenib (Nexavar) targeting Raf kinase as well as MEK kinase inhibitors, selumetinib (AZD6244), and trametinib (Mekinis, GSK1120212) were evaluated for pancreatic duct adenocarcinoma as gemcitabine combination therapy, but no clinical benefit has been reported in the clinical trials [5, 16].

Along with Ras pathways, other downstream signals also play a critical role in pancreatic carcinogenesis, such as PI3K, STAT, and Grb7 (Fig. 8.5). We have cloned human Grb7 as a cell migration gene in cancer cells and reported a preclinical study of Grb7 peptide inhibitor targeting pancreatic cancer metastasis [17]. PI3K phosphorylates PIP2 to generate PIP3 transducing PDK which in turn activates Akt and then mTOR. A serine-threonine kinase mTOR stimulates the phosphorylation of p70 ribosomal protein S6 K and 4E-BP1/eIF4E pathway activating the ribosomal translation to amino acids. In addition, mTOR regulates lysosomal autophagy pathways, derived from amino acid deprivation. Everolimus (Afinitor, RAD001), an inhibitor of mTOR, was applied for phase II clinical trials of pancreatic ductal adenocarcinoma but neither CR nor PR obtained [18]. Since abnormalities of signaling pathways should play essential roles in pancreatic cancer progression, further studies including other signals should be enforced.

8.4 Squamous Trans-Differentiation Subtype: TP53 and KDM6A

The pancreatic squamous trans-differentiation subtype is associated with gene programs highly expressed in squamous-like class of tumors of breast, bladder, lung, and head and neck cancer defined in the TCGA pan-cancer studies (Fig. 8.4), including N-terminally truncated variant TP63 (TP63ΔN) and its target genes frequently upregulated in other squamous cell carcinomas [11]. Interestingly, the squamous trans-differentiation subtype shows hypermethylation and concordant downregulation of genes that govern pancreatic endodermal cell-fate determination (PDX1, HNF1B, MNX1, GATA6) leading to a complete loss of endodermal identity (Fig. 8.1). As in these other cancer types, the pancreatic squamous trans-differentiation subtype was associated with mutations in TP53 ($P = 0.01$) and KDM6A ($P = 0.02$). In the presence of TP53 mutations, TP63ΔN is known to regulate epithelial cell plasticity, tumorigenicity, and epithelial to mesenchymal transition in a variety of solid tumors [19]. Transcriptional network analysis identified additional key factors involved in metastasis that were upregulated in the squamous trans-differentiation subtype. Indeed, the squamous trans-differentiation subtype was an independent poor prognostic factor. TP63ΔN network cooperating with TP53 mutations is one of the therapeutic targets in this subtype of pancreatic cancer.

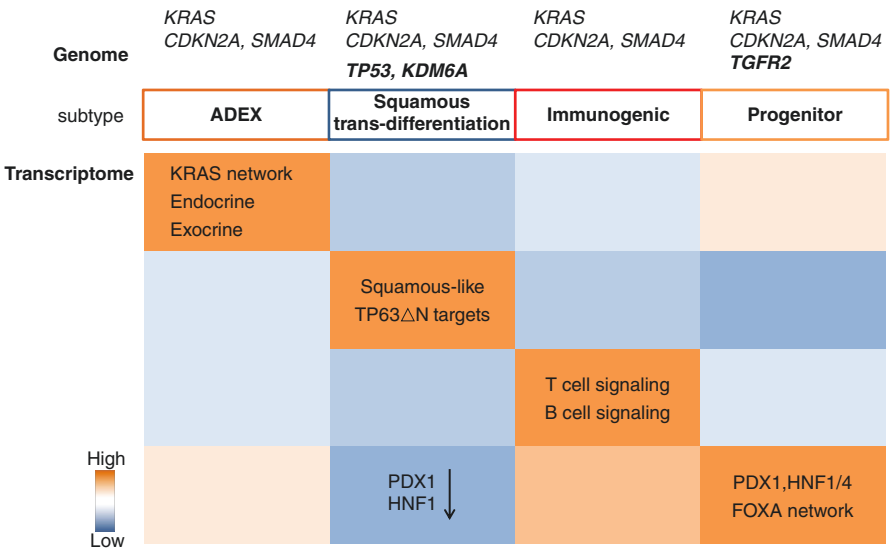

Fig. 8.4 Molecular subtypes of human pancreatic cancer proposed by Bailey et al. [10]. Four groups are classified by analyses of transcriptional networks and genome mutations. These subtypes are associated with specific histological characteristics: (1) ADEX with rare acinar cell carcinomas, (2) squamous trans-differentiation with adenosquamous carcinomas, (3) immunogenic, and (4) pancreatic progenitor with mucinous non-cystic adenocarcinomas and carcinomas arising from IPMN

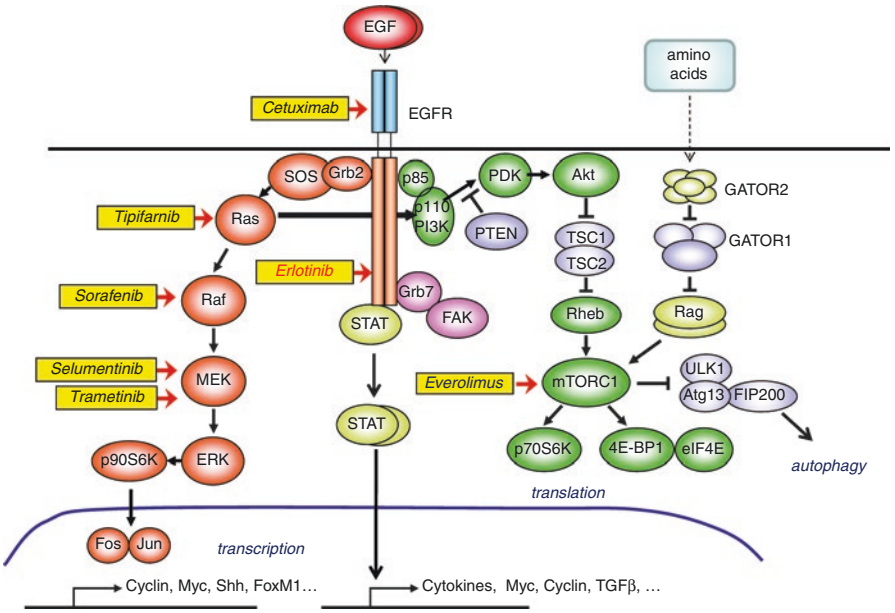

Fig. 8.5 EGFR/RAS signaling pathway. Paradoxically, EGFR is required for mutated KRAS-driven pancreatic tumorigenesis [12, 13]. Targeted agents for pancreatic cancer are boxed in *italics*

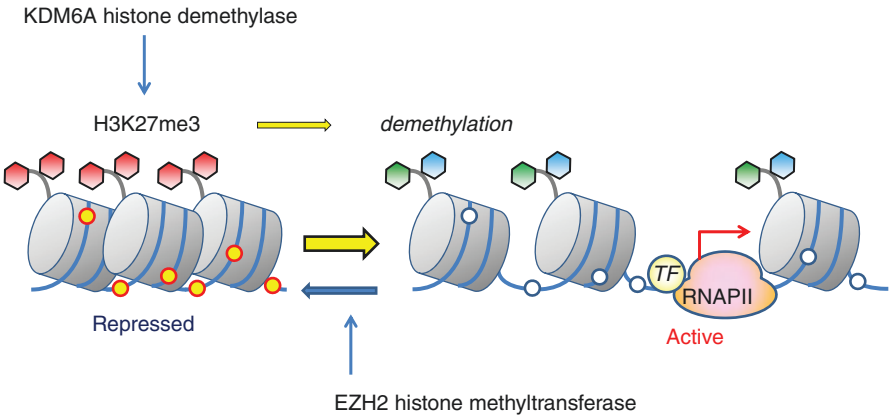

Fig. 8.6 Histone modifications in transcriptional regulation. KDM6A histone demethylase acts as an eraser of the inactive mark H3K27me3 (histone 3 lysine 27 tri-methylation). In contrast, EZH2 histone methyltransferase acts as a writer of the H3K27me3. *RNAPII* RNA polymerase II, *TF* transcription factor

KDM6A (UTX) is a histone demethylase that specifically targets di- and tri-methyl groups on lysine 27 of histone H3 (H3K27me2/3) (Fig. 8.6). KDM6A has been proven essential during pancreatic ectodermal development (Fig. 8.1), as demethylation status of H3K27 is critically required for correct reprogramming and specific differentiation [20]. Its catalytic activity has been linked to regulation of endodermal transcriptional networks, and loss-of-function mutations in KDM6A might deprive the endodermal identity. In contrast, the deletion of EZH2 H3K27me3 methyltransferase at the pancreas progenitor stage enhanced the production of endocrine progenitors [21]. These studies reveal distinct dynamics in H3K27me3 targets in vivo and a means to modulate pancreatic cell development from stem cells. Since there are several EZH2-specific inhibitors developed for clinical trials in other malignancies (tazemetostat; NCT01897571), the regulation of H3K27me3 to correct the cell fate might be a promising tool for treatment of the squamous trans-differentiation subtype in pancreatic cancer. In addition, an H3K27me2/3 demethylase-independent function for KDM6A was uncovered in promoting general chromatin remodeling in concert with the SWI/SNF complex [22]. Further studies are required on the histone and chromatin regulators as the therapeutic targets for pancreatic cancer from the perspective of synthetic lethality [23].

8.5 Immunogenic Subtype: Treg and Checkpoint

The immunogenic subtype of pancreatic cancer is associated with gene expression programs including B- and T-cell signatures, with both cytotoxic (CD8 + CTL) and regulatory T cells (CD4 + CD25 + FOXP3 + Treg) [11]. It showed evidence of a significant immune infiltrate, and the pathway analysis demonstrated enrichment

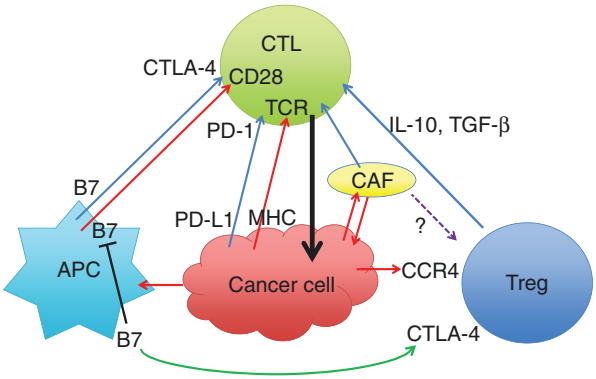

Fig. 8.7 Immunological landscape of the tumor microenvironment in pancreatic cancer. *CTL* cytotoxic CD8+ T lymphocyte, *APC* antigen-presenting cell, *Treg* regulatory CD4+CD25+FOXP3+ T cell, CAF cancer-associated fibroblast, *TCR* T-cell receptor. *Red and blue arrows* indicate stimulatory and inhibitory signals, respectively. B7/CTLA-4 signaling is also required for the maintenance of functional Treg, and the dominant captures can down-modulate B7/CD28 costimulatory signals for effector CTL

for mediators of immunosuppressive tumor microenvironment including tumor-associated M2 macrophages, stellate cells, and cancer-associated fibroblasts (CAFs) that contribute to synthesize dense desmoplastic stroma. In addition, intra-tumor immunity could be attenuated by Treg cells (Fig. 8.7). Treg is an immunosuppressive T-cell subpopulation which suppresses induction and proliferation of effector CTL by secreting IL-10 and TGF-β [24]. Studies in clinical samples have implicated that high numbers of Tregs in the tumor microenvironment are indicative of a poor prognosis [24]. Treg cells in the tumor microenvironment predominantly express CCR4, and the pancreatic cancer cells produce high levels of the CCR4 ligand, CCL22 [25]. In vivo or in vitro anti-CCR4 antibody treatment selectively depleted effector Treg cells and efficiently induced tumor-antigen-specific CD4+ and CD8+ T cells [25]. Anti-CCR4 antibody therapy is promising for evoking and enhancing tumor immunity by selectively depleting effector-type Treg cells.

The immunogenic subtype shows evidence of a significant immune infiltrate, and the predominant gene expression profiles are related not only to antigen processing and presentation but also to upregulation of CTLA-4 and PD-1 immune checkpoints essential for tumor immunosuppression pathways (Fig. 8.7). A new era is coming up by blockading the acquired immune checkpoint axis via CTLA-4 and PD-1/PD-L1 as an emerging target in the cancer treatment [26, 27]. Unfortunately, the novel checkpoint immunotherapy has achieved limited clinical benefit to date in patients with pancreatic ductal adenocarcinoma [28, 29]. Critical obstacles to immunotherapy in pancreatic cancer include the presence of a uniquely immunosuppressive tumor microenvironment. Feig et al. reported that chemokine CXCL12 secreted by CAFs functions as a barrier to CTL infiltration in pancreatic cancer [30]. Administering the CXCL12 receptor inhibitor induced rapid CTL accumulation among cancer cells and acted synergistically with α-PD-L1 to greatly diminish

cancer cells. Another recent study revealed the CXCL12-secreting CAFs are positively controlled by hyperactivated focal adhesion kinase (FAK) activity in pancreatic cancer cells [31]. A selective FAK inhibitor markedly reduced stromal fibrosis and substantially limited tumor progression. FAK inhibition rendered the previously unresponsive pancreatic cancer responsive to T-cell immunotherapy and PD-1 antagonists in the mouse model. These investigations suggest that inhibition of stromal CAFs increases immune surveillance by overcoming the fibrotic and immunosuppressive microenvironment and renders tumors responsive to immunotherapy [30, 31]. On the contrary, the other studies demonstrated that targeting the stroma resulted in undifferentiated, aggressive pancreatic cancer that responds to Treg blockade, uncovering another side of protective role by tumor stoma [32, 33]. More recent report by Honjo's group elucidated a distinct role of checkpoint PD-1 and Treg-FoxP3 in maintaining the pancreatic immunosuppressive microenvironment [34]. Given the complexity of the stroma and associated immunocytes, further investigations are required to more clearly define detrimental and beneficial aspects of niche biology in pancreatic cancer stroma and immune systems.

8.6 Pancreatic Progenitor Subtype: Cancer Stemness Identity

The pancreatic progenitor subtype is defined primarily by the transcriptional networks containing transcription factors PDX1, HES1, HNF1, HNF4, and MNX1 [11]. These transcription factors are pivotal for pancreatic endoderm cell-fate determination toward a pancreatic lineage. PDX1, in particular, is critical for pancreas development with ductal, exocrine, and endocrine cells all derived from embryonic progenitor/stem cells (Fig. 8.1) [35]. Progenitor/stemlike cancer cells have the ability for self-renewal and multi-potency to hierarchically organize the bulk with respect to tumor initiation and maintenance [5]. The so-called cancer stem cells (CSCs) lying at the apex of the hierarchy are intrinsically resistant to chemotherapeutic agents and function as a source to metastasizing and relapsing. Since CSCs might play a fundamental role in these awful malignant behaviors, investigations of the molecular targets of CSCs may reveal particularly effective therapeutic approaches [36].

"Self-renewal" is theoretically based on asymmetric divisions of stem cells that give rise to one cell of the stem cell potency and another stimulated to differentiate further into non-stem cell types [37]. In essence, stemness identity is maintained by a quiescent dormancy that is critical for protecting the stem cell components from various types of biological stress in vivo [38]. As of pancreatic CSCs, the subpopulations of $CD44^{+}CD24^{+}EpCAM^{+}$ [39], c-$Met^{high}CD44^{+}$ [40], $CD133^{+}CXCR4^{+}$ [41], and $ALDH^{high}CD44^{+}CD24^{+}$ [42] have been reported. By use of these multiple stemness markers, however, even highly purified CSCs might divide in an asymmetric manner and rapidly reconstruct mixed populations with their differentiated progeny

in the hierarchical fashion. The majority of bulk cancer cells become eventually occupied by non-CSCs dividing rapidly in a symmetric manner. Therefore, the validity of current studies remains unsatisfying in terms of evaluating molecular targets to pinpoint CSCs themselves [43].

One sophisticated solution to such a dilemma might be a monitoring system based on CSC-specific functions [44]. Dormant CSCs as well as normal stem cells are generally quiescent with low protein turnover [45], reduced metabolism [46], and downregulation of proteasome activity [47, 48]. In our recent studies, the proteasome-independent character of the dormant stem cell fate was utilized for fluorescent visualization of CSC subpopulations in human pancreatic cancer [49] as well as liver [50] and colorectal cancer cells [51]. Endogenous proteasome activity can be monitored in real time by green fluorescence-labeled degron motif that is known to be directly recognized by 26S proteasome [52], which leads to the immediate destruction of the involved protein [47]. Strikingly, this system to distinguish CSCs from non-CSCs demonstrated asymmetric cell division (Fig. 8.8a) and "self-renewal" sphere formation in a real-time manner (Fig. 8.8b), as well as an over 1000-fold increase in tumorigenicity with heterogenic expansion in vivo [49–51]. These results suggested that the novel CSC imaging system was useful to isolate populations extremely enriched with self-renewal and tumor-initiating cells.

Accumulating evidence has indicated CSCs are distinguished as the metastatic abilities [54]. Our CSC imaging system has the advantage of in vivo studies, and

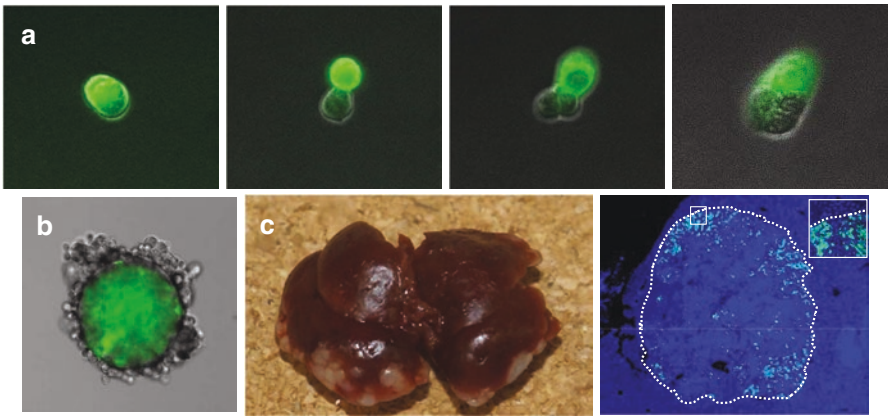

Fig. 8.8 Real-time visualization of human pancreatic CSCs [49, 53]. (**a**) Asymmetric cell division of CSC^{high} cells into CSC^{low} and CSC^{high} cells was clearly recognized, and CSC^{low} cells never divided into CSC^{high} cells, as demonstrated by time-lapse microscopy. (**b**) Sphere formation of CSC^{high} cells that attached to each other with the exclusion of CSC^{low} cells, whereas the CSC^{low} cells could not form such spheres. (**c**) Liver metastatic model of pancreatic CSC^{high} cells in mice. *Left*, liver metastatic tumors are shown in mice 8 weeks after injection of CSC^{high} but not CSC^{low} cells. *Right*, fluorescent microscopic images of the main tumor are shown. CSC^{high} cells are localized preferentially at the invading tumor margins

we visualized that pancreatic CSCs were highly metastatic and dominantly located at the invading margins of the metastatic liver tumors (Fig. 8.8c) [53]. Gene expression profiling with siRNA screening assays revealed that doublecortin-like kinase 1 (DCLK1) is essential for the invasive and metastatic properties of CSCs. Knockdown of DCLK1 completely inhibited liver metastasis. In addition, these gene expression profiles are regulated by histone modifications for open-bivalent-closed chromatin statuses. Chromatin dynamics play an essential role in cell-fate determination [55]. Recently, the chromatin dynamics are closely associated with a class of regulatory elements termed "super-enhancers," which act as switches to determine the cell type-specific gene expression as well as the essential step of cancer initiation with reprograming (Fig. 8.9a) [56]. The subgroup-specific therapeutics must be actively engaged in developing chemical inhibitors of BRD4 (JQ1, I-BET, OTX-015, TEN-010) at histone H3K27ac-enriched super-enhancer regions (Fig. 8.9b) [57].

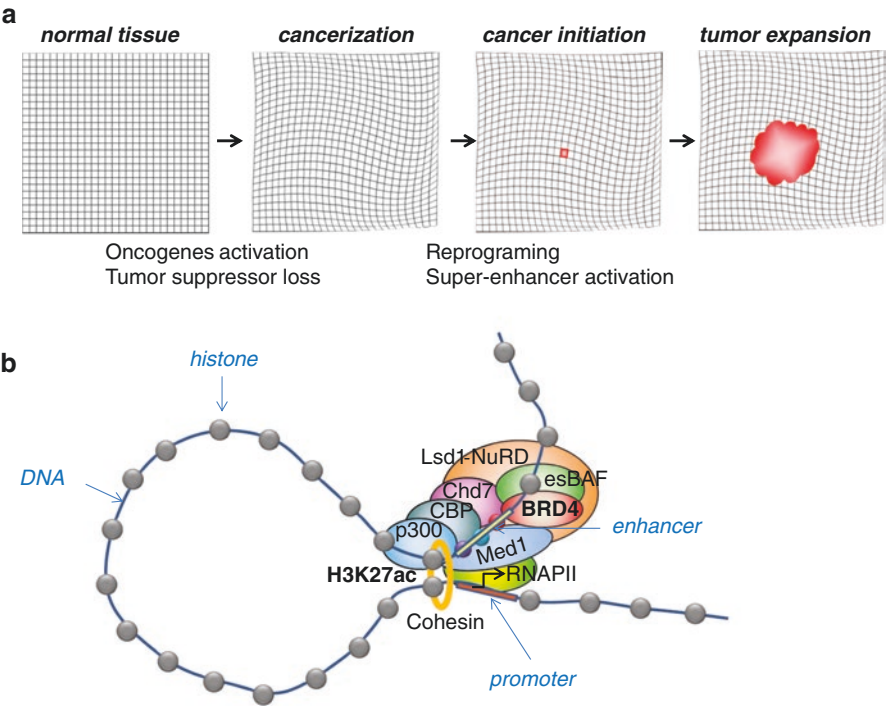

Fig. 8.9 Significance of super-enhancer activation [56]. (**a**) Model for the reemergent state of stemness reprograming through super-enhancer activation as an essential step in cancer initiation. The acquisition of genetic lesions in normal tissue leads to oncogene activation and tumor suppressor loss. It represents an initial barrier that generates a cancerized field from which rare clones overcome the additional barrier of achieving a stemness state to initiate cancer formation and then tumor expansion. (**b**) Model for the super-enhancer with RNA polymerase II (RNAPII), transcription factors, and chromatin regulators. The complexes are responsible for diverse enhancer-related functions, such as enhancer looping, gene activation, nucleosome remodeling, and histone modification

8.7 Closing Remarks

Achilles' heel of each pancreatic cancer subtype should be fatefully determined by its addiction to the genetic, epigenetic, and transcriptomic alterations [58]. As shown in Fig. 8.2, pancreatic cancer is believed to develop and progress stepwise through a particular sequence of genetic alterations: KRAS, followed by CDKN2A, then TP53, and SMAD4. Such evolutionary trajectory of pancreatic cancer progression is gradual because each alteration is acquired independently. However, Murphy et al. demonstrated that pancreatic neoplasia acquires an extensive mutation burden but remains noninvasive or non-metastatic in the mouse models [59]. Novel genome informatics on clinical samples revealed recently that the pancreatic cancer tumorigenesis is neither gradual nor follows the accepted mutation order [60]. Surprisingly, more than 60% of human pancreatic cancers exhibit complex rearrangement (chromothripsis) associated with mitotic errors, indicating "punctuated equilibrium," rather than "gradualism," as the principal evolutionary trajectory in a subset of cases (Fig. 8.10). Since the consequence of mitotic errors is not sequential but simultaneous, knockout of canonical preneoplastic genetic drivers is likely to set off the

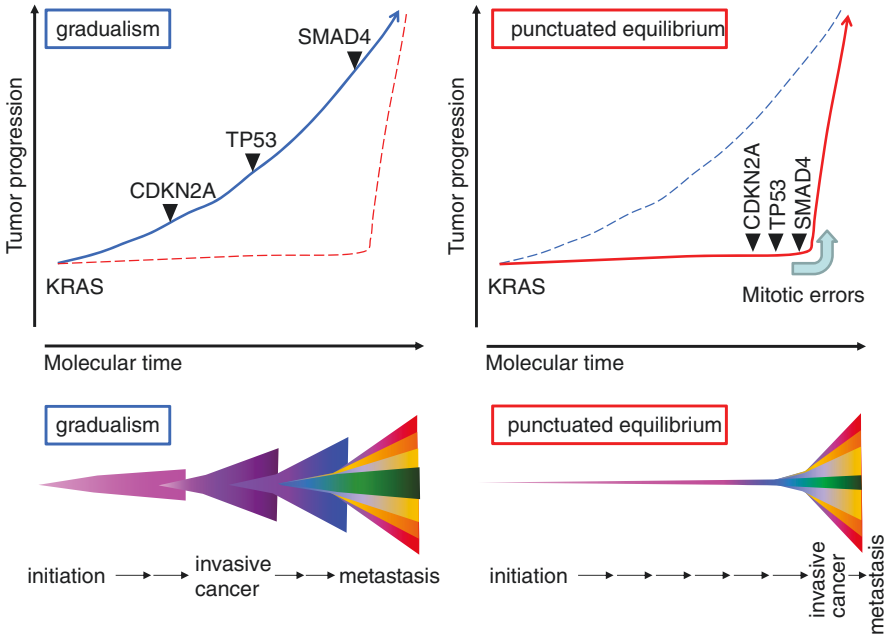

Fig. 8.10 Pancreatic cancer evolution models for classical gradualism (*left*) and alternatively punctuated equilibrium (*right*) [60]. In the classical model, there is a period of latency between the driver mutations that lead to tumor development, and the multiple transforming events are independently required for tumor development. In the punctuated equilibrium model, tumor development can be divided into two major events: the cancer-initiating event and revolutionary-transforming event. The latter event "chromothripsis" is triggered catastrophically by mitotic errors

invasive cancer growth. According to our recent studies, activity of proteasome and autophagy might be required for the punctuated initiation of pancreatic cancer and mitotic segregation, respectively [61, 62]. These findings provide new insights into the crucial processes that break through the initial trigger and metastatic chromothripsis step (Fig. 8.9). The innovative investigations of malignant evolution will be essential to guide therapeutic strategies for lethal pancreatic cancer.

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Conflicts of Interest The author discloses no conflicts.

References

1. Reichert M, Rustgi AK. Pancreatic ductal cells in development, regeneration, and neoplasia. J Clin Invest. 2011;121(12):4572–8.
2. Makohon-Moore A, Iacobuzio-Donahue CA. Pancreatic cancer biology and genetics from an evolutionary perspective. Nat Rev Cancer. 2016;16(9):553–65.
3. Kawaguchi Y. Sox9 and programming of liver and pancreatic progenitors. J Clin Invest. 2013;123(5):1881–6.
4. Vincent A, Herman J, Schulick R, Hruban RH, Goggins M. Pancreatic cancer. Lancet. 2011;378(9791):607–20.
5. Tanaka S. Molecular pathogenesis and targeted therapy of pancreatic cancer. Ann Surg Oncol. 2016;23(Suppl 2):S197–205.
6. Jones S, Zhang X, Parsons DW, Lin JC, Leary RJ, Angenendt P, Mankoo P, Carter H, Kamiyama H, Jimeno A, Hong SM, Fu B, Lin MT, Calhoun ES, Kamiyama M, Walter K, Nikolskaya T, Nikolsky Y, Hartigan J, Smith DR, Hidalgo M, Leach SD, Klein AP, Jaffee EM, Goggins M, Maitra A, Iacobuzio-Donahue C, Eshleman JR, Kern SE, Hruban RH, Karchin R, Papadopoulos N, Parmigiani G, Vogelstein B, Velculescu VE, Kinzler KW. Core signaling pathways in human pancreatic cancers revealed by global genomic analyses. Science. 2008;321(5897):1801–6.
7. Kong B, Michalski CW, Erkan M, Friess H, Kleeff J. From tissue turnover to the cell of origin for pancreatic cancer. Nat Rev Gastroenterol Hepatol. 2011;8(8):467–72.
8. Shain AH, Giacomini CP, Matsukuma K, Karikari CA, Bashyam MD, Hidalgo M, Maitra A, Pollack JR. Convergent structural alterations define SWItch/sucrose NonFermentable (SWI/SNF) chromatin remodeler as a central tumor suppressive complex in pancreatic cancer. Proc Natl Acad Sci U S A. 2012;109(5):E252–9.
9. Biankin AV, Waddell N, Kassahn KS, Gingras MC, Muthuswamy LB, Johns AL, Miller DK, Wilson PJ, Patch AM, Wu J, Chang DK, Cowley MJ, Gardiner BB, Song S, Harliwong I, Idrisoglu S, Nourse C, Nourbakhsh E, Manning S, Wani S, Gongora M, Pajic M, Scarlett CJ, Gill AJ, Pinho AV, Rooman I, Anderson M, Holmes O, Leonard C, Taylor D, Wood S, Xu Q, Nones K, Fink JL, Christ A, Bruxner T, Cloonan N, Kolle G, Newell F, Pinese M, Mead RS, Humphris JL, Kaplan W, Jones MD, Colvin EK, Nagrial AM, Humphrey ES, Chou A, Chin VT, Chantrill LA, Mawson A, Samra JS, Kench JG, Lovell JA, Daly

RJ, Merrett ND, Toon C, Epari K, Nguyen NQ, Barbour A, Zeps N, Australian Pancreatic Cancer Genome Initiative, Kakkar N, Zhao F, Wu YQ, Wang M, Muzny DM, Fisher WE, Brunicardi FC, Hodges SE, Reid JG, Drummond J, Chang K, Han Y, Lewis LR, Dinh H, Buhay CJ, Beck T, Timms L, Sam M, Begley K, Brown A, Pai D, Panchal A, Buchner N, De Borja R, Denroche RE, Yung CK, Serra S, Onetto N, Mukhopadhyay D, Tsao MS, Shaw PA, Petersen GM, Gallinger S, Hruban RH, Maitra A, Iacobuzio-Donahue CA, Schulick RD, Wolfgang CL, Morgan RA, Lawlor RT, Capelli P, Corbo V, Scardoni M, Tortora G, Tempero MA, Mann KM, Jenkins NA, Perez-Mancera PA, Adams DJ, Largaespada DA, Wessels LF, Rust AG, Stein LD, Tuveson DA, Copeland NG, Musgrove EA, Scarpa A, Eshleman JR, Hudson TJ, Sutherland RL, Wheeler DA, Pearson JV, JD MP, Gibbs RA, Grimmond SM. Pancreatic cancer genomes reveal aberrations in axon guidance pathway genes. Nature. 2012;491(7424):399–405.

10. von Figura G, Fukuda A, Roy N, Liku ME, Morris Iv JP, Kim GE, Russ HA, Firpo MA, Mulvihill SJ, Dawson DW, Ferrer J, Mueller WF, Busch A, Hertel KJ, Hebrok M. The chromatin regulator Brg1 suppresses formation of intraductal papillary mucinous neoplasm and pancreatic ductal adenocarcinoma. Nat Cell Biol. 2014;16(3):255–67.
11. Bailey P, Chang DK, Nones K, Johns AL, Patch AM, Gingras MC, Miller DK, Christ AN, Bruxner TJ, Quinn MC, Nourse C, Murtaugh LC, Harliwong I, Idrisoglu S, Manning S, Nourbakhsh E, Wani S, Fink L, Holmes O, Chin V, Anderson MJ, Kazakoff S, Leonard C, Newell F, Waddell N, Wood S, Xu Q, Wilson PJ, Cloonan N, Kassahn KS, Taylor D, Quek K, Robertson A, Pantano L, Mincarelli L, Sanchez LN, Evers L, Wu J, Pinese M, Cowley MJ, Jones MD, Colvin EK, Nagrial AM, Humphrey ES, Chantrill LA, Mawson A, Humphris J, Chou A, Pajic M, Scarlett CJ, Pinho AV, Giry-Laterriere M, Rooman I, Samra JS, Kench JG, Lovell JA, Merrett ND, Toon CW, Epari K, Nguyen NQ, Barbour A, Zeps N, Moran-Jones K, Jamieson NB, Graham JS, Duthie F, Oien K, Hair J, Grützmann R, Maitra A, Iacobuzio-Donahue CA, Wolfgang CL, Morgan RA, Lawlor RT, Corbo V, Bassi C, Rusev B, Capelli P, Salvia R, Tortora G, Mukhopadhyay D, Petersen GM, Initiative APCG, Munzy DM, Fisher WE, Karim SA, Eshleman JR, Hruban RH, Pilarsky C, Morton JP, Sansom OJ, Scarpa A, Musgrove EA, Bailey UM, Hofmann O, Sutherland RL, Wheeler DA, Gill AJ, Gibbs RA, Pearson JV, Waddell N, Biankin AV, Grimmond SM. Genomic analyses identify molecular subtypes of pancreatic cancer. Nature. 2016;531(7592):47–52.
12. Navas C, Hernández-Porras I, Schuhmacher AJ, Sibilia M, Guerra C, Barbacid M. EGF receptor signaling is essential for k-ras oncogene-driven pancreatic ductal adenocarcinoma. Cancer Cell. 2012;22(3):318–30.
13. Ardito CM, Grüner BM, Takeuchi KK, Lubeseder-Martellato C, Teichmann N, Mazur PK, Delgiorno KE, Carpenter ES, Halbrook CJ, Hall JC, Pal D, Briel T, Herner A, Trajkovic-Arsic M, Sipos B, Liou GY, Storz P, Murray NR, Threadgill DW, Sibilia M, Washington MK, Wilson CL, Schmid RM, Raines EW, Crawford HC, Siveke JT. EGF receptor is required for KRAS-induced pancreatic tumorigenesis. Cancer Cell. 2012;22(3):304–17.
14. Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada clinical trials group. J Clin Oncol. 2007;25(15):1960–6.
15. Van Cutsem E, van de Velde H, Karasek P, Oettle H, Vervenne WL, Szawlowski A, Schoffski P, Post S, Verslype C, Neumann H, Safran H, Humblet Y, Perez Ruixo J, Ma Y, Von Hoff D. Phase III trial of gemcitabine plus tipifarnib compared with gemcitabine plus placebo in advanced pancreatic cancer. J Clin Oncol. 2004;22(8):1430–8.
16. Gonçalves A, Gilabert M, François E, Dahan L, Perrier H, Lamy R, Re D, Largillier R, Gasmi M, Tchiknavorian X, Esterni B, Genre D, Moureau-Zabotto L, Giovannini M, Seitz JF, Delpero JR, Turrini O, Viens P, Raoul JL. BAYPAN study: a double-blind phase III randomized trial comparing gemcitabine plus sorafenib and gemcitabine plus placebo in patients with advanced pancreatic cancer. Ann Oncol. 2012;23(11):2799–805.

17. Tanaka S, Pero SC, Taguchi K, Shimada M, Mori M, Krag DN, Arii S. Specific peptide ligand for Grb7 signal transduction protein and pancreatic cancer metastasis. J Natl Cancer Inst. 2006;98(7):491–8.
18. Wolpin BM, Hezel AF, Abrams T, Blaszkowsky LS, Meyerhardt JA, Chan JA, Enzinger PC, Allen B, Clark JW, Ryan DP, Fuchs CS. Oral mTOR inhibitor everolimus in patients with gemcitabine-refractory metastatic pancreatic cancer. J Clin Oncol. 2009;27(2):193–8.
19. Engelmann D, Pützer BM. Emerging from the shade of p53 mutants: N-terminally truncated variants of the p53 family in EMT signaling and cancer progression. Sci Signal. 2014;7(345):re9.
20. Jiang W, Wang J, Zhang Y. Histone H3K27me3 demethylases KDM6A and KDM6B modulate definitive endoderm differentiation from human ESCs by regulating WNT signaling pathway. Cell Res. 2013;23:122–30.
21. Xu CR, Li LC, Donahue G, Ying L, Zhang YW, Gadue P, Zaret KS. Dynamics of genomic H3K27me3 domains and role of EZH2 during pancreatic endocrine specification. EMBO J. 2014;33(19):2157–70.
22. Miller SA, Mohn SE, Weinmann AS. Jmjd3 and UTX play a demethylase-independent role in chromatin remodeling to regulate T-box family member-dependent gene expression. Mol Cell. 2010;40(4):594–605.
23. McLornan DP, List A, Mufti GJ. Applying synthetic lethality for the selective targeting of cancer. N Engl J Med. 2014;371(18):1725–35.
24. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. Regulatory T cells and immune tolerance. Cell. 2008;133(5):775–87.
25. Sugiyama D, Nishikawa H, Maeda Y, Nishioka M, Tanemura A, Katayama I, Ezoe S, Kanakura Y, Sato E, Fukumori Y, Karbach J, Jäger E, Sakaguchi S. Anti-CCR4 mAb selectively depletes effector-type FoxP3+CD4+ regulatory T cells, evoking antitumor immune responses in humans. Proc Natl Acad Sci U S A. 2013;110(44):17945–50.
26. Okazaki T, Chikuma S, Iwai Y, Fagarasan S, Honjo T. A rheostat for immune responses: the unique properties of PD-1 and their advantages for clinical application. Nat Immunol. 2013;14(12):1212–8.
27. Topalian SL, Drake CG, Pardoll DM. Immune checkpoint blockade: a common denominator approach to cancer therapy. Cancer Cell. 2015;27(4):450–61.
28. Royal RE, Levy C, Turner K, Mathur A, Hughes M, Kammula US, Sherry RM, Topalian SL, Yang JC, Lowy I, Rosenberg SA. Phase 2 trial of single agent Ipilimumab (anti-CTLA-4) for locally advanced or metastatic pancreatic adenocarcinoma. J Immunother. 2010;33(8):828–33.
29. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, Pitot HC, Hamid O, Bhatia S, Martins R, Eaton K, Chen S, Salay TM, Alaparthy S, Grosso JF, Korman AJ, Parker SM, Agrawal S, Goldberg SM, Pardoll DM, Gupta A, Wigginton JM. Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med. 2012;366(26):2455–65.
30. Feig C, Jones JO, Kraman M, Wells RJ, Deonarine A, Chan DS, Connell CM, Roberts EW, Zhao Q, Caballero OL, Teichmann SA, Janowitz T, Jodrell DI, Tuveson DA, Fearon DT. Targeting CXCL12 from FAP-expressing carcinoma-associated fibroblasts synergizes with anti-PD-L1 immunotherapy in pancreatic cancer. Proc Natl Acad Sci U S A. 2013;110(50):20212–7.
31. Jiang H, Hegde S, Knolhoff BL, Zhu Y, Herndon JM, Meyer MA, Nywening TM, Hawkins WG, Shapiro IM, Weaver DT, Pachter JA, Wang-Gillam A, DeNardo DG. Targeting focal adhesion kinase renders pancreatic cancers responsive to checkpoint immunotherapy. Nat Med. 2016;22(8):851–60.
32. Özdemir BC, Pentcheva-Hoang T, Carstens JL, Zheng X, Wu CC, Simpson TR, Laklai H, Sugimoto H, Kahlert C, Novitskiy SV, De Jesus-Acosta A, Sharma P, Heidari P, Mahmood U, Chin L, Moses HL, Weaver VM, Maitra A, Allison JP, LeBleu VS, Kalluri R. Depletion of carcinoma-associated fibroblasts and fibrosis induces immunosuppression and accelerates pancreas cancer with reduced survival. Cancer Cell. 2014;25(6):719–34.
33. Rhim AD, Oberstein PE, Thomas DH, Mirek ET, Palermo CF, Sastra SA, Dekleva EN, Saunders T, Becerra CP, Tattersall IW, Westphalen CB, Kitajewski J, Fernandez-Barrena

MG, Fernandez-Zapico ME, Iacobuzio-Donahue C, Olive KP, Stanger BZ. Stromal elements act to restrain, rather than support, pancreatic ductal adenocarcinoma. Cancer Cell. 2014;25(6):735–47.
34. Zhang B, Chikuma S, Hori S, Fagarasan S, Honjo T. Nonoverlapping roles of PD-1 and FoxP3 in maintaining immune tolerance in a novel autoimmune pancreatitis mouse model. Proc Natl Acad Sci U S A. 2016;113(30):8490–5.
35. Hale MA, et al. The homeodomain protein PDX1 is required at mid-pancreatic development for the formation of the exocrine pancreas. Dev Biol. 2005;286:225–37.
36. Tanaka S. Cancer stem cells as therapeutic targets. In: Sherley JL, editor. Human stem cell toxicity. London: Royal Society of Chemistry; 2016. p. 280–94.
37. Inaba M, Yamashita YM. Asymmetric stem cell division: precision for robustness. Cell Stem Cell. 2012;11(4):461–9.
38. Cheung TH, Rando TA. Molecular regulation of stem cell quiescence. Nat Rev Mol Cell Biol. 2013;14(6):329–40.
39. Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. Identification of pancreatic cancer stem cells. Cancer Res. 2007;67(3):1030–7.
40. Li C, Wu JJ, Hynes M, Dosch J, Sarkar B, Welling TH, Pasca di Magliano M, Simeone DM. C-met is a marker of pancreatic cancer stem cells and therapeutic target. Gastroenterology. 2011;141(6):2218–27.
41. Hermann PC, Huber SL, Herrler T, Aicher A, Ellwart JW, Guba M, Bruns CJ, Heeschen C. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell. 2007;1(3):313–23.
42. Rasheed ZA, Yang J, Wang Q, Kowalski J, Freed I, Murter C, Hong SM, Koorstra JB, Rajeshkumar NV, He X, Goggins M, Iacobuzio-Donahue C, Berman DM, Laheru D, Jimeno A, Hidalgo M, Maitra A, Matsui W. Prognostic significance of tumorigenic cells with mesenchymal features in pancreatic adenocarcinoma. J Natl Cancer Inst. 2010;102(5):340–51.
43. Gupta PB, Chaffer CL, Weinberg RA. Cancer stem cells: mirage or reality? Nat Med. 2009;15(9):1010–2.
44. Li L, Bhatia R. Stem cell quiescence. Clin Cancer Res. 2011;17(15):4936–41.
45. Hernebring M, Brolén G, Aguilaniu H, Semb H, Nyström T. Elimination of damaged proteins during differentiation of embryonic stem cells. Proc Natl Acad Sci U S A. 2006;103(20):7700–5.
46. Achuthan S, Santhoshkumar TR, Prabhakar J, Nair SA, Pillai MR. Drug-induced senescence generates chemoresistant stemlike cells with low reactive oxygen species. J Biol Chem. 2011;286(43):37813–29.
47. Vlashi E, Kim K, Lagadec C, Donna LD, McDonald JT, Eghbali M, Sayre JW, Stefani E, McBride W, Pajonk F. In vivo imaging, tracking, and targeting of cancer stem cells. J Natl Cancer Inst. 2009;101(5):350–9.
48. Pan J, Zhang Q, Wang Y, You M. 26S proteasome activity is down-regulated in lung cancer stem-like cells propagated in vitro. PLoS One. 2010;5(10):e13298.
49. Adikrisna R, Tanaka S, Muramatsu S, Aihara A, Ban D, Ochiai T, Irie T, Kudo A, Nakamura N, Yamaoka S, Arii S. Identification of pancreatic cancer stem cells and selective toxicity of chemotherapeutic agents. Gastroenterology. 2012;143(1):234–45.
50. Muramatsu S, Tanaka S, Mogushi K, Adikrisna R, Aihara A, Ban D, Ochiai T, Irie T, Kudo A, Nakamura N, Tanaka H, Nakayama K, Tanaka H, Yamaoka S, Arii S. Visualization of stem cell features in human hepatocellular carcinoma enlightened in vivo significance of tumor-host interaction and clinical implication. Hepatology. 2013;58(1):218–28.
51. Munakata K, Uemura M, Tanaka S, Kawai K, Kitahara T, Miyo M, Kano Y, Nishikawa S, Fukusumi T, Takahashi Y, Hata T, Nishimura J, Takemasa I, Mizushima T, Ikenaga M, Kato T, Murata K, Carethers JM, Yamamoto H, Doki Y, Mori M. Cancer stem-like properties in colorectal cancer cells with low proteasome activity. Clin Cancer Res. 2016;22(21):5277–86.
52. Murakami Y, Matsufuji S, Kameji T, Hayashi S, Igarashi K, Tamura T, Tanaka K, Ichihara A. Ornithine decarboxylase is degraded by the 26S proteasome without ubiquitination. Nature. 1992;360(6404):597–9.

53. Ito H, Tanaka S, Akiyama Y, Shimada S, Adikrisna R, Matsumura S, Aihara A, Mitsunori Y, Ban D, Ochiai T, Kudo A, Arii S, Yamaoka S, Tanabe M. Dominant expression of DCLK1 in human pancreatic cancer stem cells accelerates tumor invasion and metastasis. PLoS One. 2016;11(1):e0146564.
54. Ischenko I, Petrenko O, Hayman MJ. Analysis of the tumor-initiating and metastatic capacity of PDX1-positive cells from the adult pancreas. Proc Natl Acad Sci U S A. 2014;111(9):3466–71.
55. Dixon JR, Jung I, Selvaraj S, Shen Y, Antosiewicz-Bourget JE, Lee AY, Ye Z, Kim A, Rajagopal N, Xie W, Diao Y, Liang J, Zhao H, Lobanenkov VV, Ecker JR, Thomson JA, Ren B. Chromatin architecture reorganization during stem cell differentiation. Nature. 2015;518(7539):331–6.
56. Kaufman CK, Mosimann C, Fan ZP, Yang S, Thomas AJ, Ablain J, Tan JL, Fogley RD, van Rooijen E, Hagedorn EJ, Ciarlo C, White RM, Matos DA, Puller AC, Santoriello C, Liao EC, Young RA, Zon LI. A zebrafish melanoma model reveals emergence of neural crest identity during melanoma initiation. Science. 2016;351(6272):aad2197.
57. Lin CY, Erkek S, Tong Y, Yin L, Federation AJ, Zapatka M, Haldipur P, Kawauchi D, Risch T, Warnatz HJ, Worst BC, Ju B, Orr BA, Zeid R, Polaski DR, Segura-Wang M, Waszak SM, Jones DT, Kool M, Hovestadt V, Buchhalter I, Sieber L, Johann P, Chavez L, Gröschel S, Ryzhova M, Korshunov A, Chen W, Chizhikov VV, Millen KJ, Amstislavskiy V, Lehrach H, Yaspo ML, Eils R, Lichter P, Korbel JO, Pfister SM, Bradner JE, Northcott PA. Active medulloblastoma enhancers reveal subgroup-specific cellular origins. Nature. 2016;530(7588):57–62.
58. Luo J, Solimini NL, Elledge SJ. Principles of cancer therapy: oncogene and non-oncogene addiction. Cell. 2009;136(5):823–37.
59. Murphy SJ, Hart SN, Lima JF, Kipp BR, Klebig M, Winters JL, Szabo C, Zhang L, Eckloff BW, Petersen GM, Scherer SE, Gibbs RA, McWilliams RR, Vasmatzis G, Couch FJ. Genetic alterations associated with progression from pancreatic intraepithelial neoplasia to invasive pancreatic tumor. Gastroenterology. 2013;145(5):1098–109.e1.
60. Notta F, Chan-Seng-Yue M, Lemire M, Li Y, Wilson GW, Connor AA, Denroche RE, Liang SB, Brown AM, Kim JC, Wang T, Simpson JT, Beck T, Borgida A, Buchner N, Chadwick D, Hafezi-Bakhtiari S, Dick JE, Heisler L, Hollingsworth MA, Ibrahimov E, Jang GH, Johns J, Jorgensen LG, Law C, Ludkovski O, Lungu I, Ng K, Pasternack D, Petersen GM, Shlush LI, Timms L, Tsao MS, Wilson JM, Yung CK, Zogopoulos G, Bartlett JM, Alexandrov LB, Real FX, Cleary SP, Roehrl MH, JD MP, Stein LD, Hudson TJ, Campbell PJ, Gallinger S. A renewed model of pancreatic cancer evolution based on genomic rearrangement patterns. Nature. 2016;538(7625):378–82.
61. Furuyama T, Tanaka S, Shimada S, Akiyama Y, Matsumura S, Mitsunori Y, Aihara A, Ban D, Ochiai T, Kudo A, Fukamachi H, Arii S, Kawaguchi Y, Tanabe M. Proteasome activity is required for the initiation of precancerous pancreatic lesions. Sci Rep. 2016;6:27044.
62. Watanabe Y, Honda S, Konishi A, Satoko Arakawa S, Murohashi M, Yamaguchi H, Torii S, Tanabe M, Tanaka S, Warabi E, Shimizu S. Autophagy controls centrosome number by degrading Cep63. Nat Commun. 2016;7:13508.

Chapter 9
Molecular Targeted Therapy for Gastroenteropancreatic Neuroendocrine Tumors

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Abstract Gastroenteropancreatic neuroendocrine neoplasms (GEP-NENs) are generally considered rare tumor. According to the recent studies, the number of patients had been increasing and frequently diagnosed as advanced stages. Surgery is the only possible way to cure GEP-NENs. However, the indication of surgical treatment for the patients with advanced GEP-NENs is limited, and for those patients other therapies such as radiofrequency ablation, transarterial chemoembolization, and/or systemic medical treatment are selected. Molecular targeted therapy is one of the promising treatments for low-grade or well-differentiated GEP-NENs. Phase III randomized studies of molecular targeted agents, such as somatostatin analogues, mTOR inhibitor, and tyrosine kinase inhibitor, had been conducted, and those studies demonstrated the antiproliferative effect in patients with GEP-NENs. Octreotide long-acting release, somatostatin analogue, was approved for gastrointestinal NENs. Lanreotide Autogel, another somatostatin analogue, was approved for GEP-NEN. Everolimus, mTOR inhibitor, was approved for GEP-NENs. Sunitinib, tyrosine kinase inhibitor, was approved for pancreatic NENs. Despite these advances, some tumors show intrinsic resistance to these targeting therapies. The arrival of novel treatment, which gives more options for the patient with GEP-NENs, is desired.

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9.1 Epidemiology of Gastroenteropancreatic Neuroendocrine Neoplasms (GEP-NENs)

Neuroendocrine neoplasms (NENs) are generally considered rare tumors [1]. According to the Surveillance, Epidemiology, and End Results (SEER) study, a US epidemiological database, the number of patients has been increasing; the incidence rate of the disease increased fivefold from 1.09 per 100,000 people in 1973 to 5.25 per 100,000 people in 2004 [2]. Ito et al. reported the result of Japanese epidemiological study: a 1.2-fold increase in the number of patients, who received treatment for pancreatic NENs (P-NENs), from 2005 to 2010 and a 1.8-fold increase in the number of patients with gastrointestinal NENs (GI-NENs) [3]. (Table 9.1).

NENs are generally considered more indolent than other gastrointestinal malignancies. However, GEP-NENs are frequently diagnosed at advanced stages. According to the SEER study, 28% of patients with NENs had distant metastasis at diagnosis, 15% of patients with gastric NENs, 30% of patients with jejunal/ileal NENs, 5% of patients with rectal NENs, and 64% of patients with P-NENs [2]. According to the Japanese epidemiological study, 6.0% of patients with GI-NENs exhibited distant metastasis at initial diagnosis and 19.9% of patients with P-NENs [3]. (Table 9.2).

Table 9.1 The trends epidemiology of NENs in SEER data and Japanese epidemiological study

	1973	1999	2004	2005	2010
SEER data[a]					
Incidence rate of all-NENs (per 100,000 population)	1.09	4.73	5.25		
Japanese epidemiological study[b]					
Total number of patients with treated for p-NEN				2845	3379
Overall prevalence of p-NENs (per 100,000 population)				2.23	2.69
Incidence rate of p-NENs (per 100,000 population)				1.01	1.27
Total number of patients with treated for GI-NEN				4406	8088
Overall prevalence of GI-NENs (per 100,000 population)				2.45	6.42
Incidence rate of GI-NENs (per 100,000 population)				2.10	3.51

[a]Yao [2]
[b]Ito [3]

Table 9.2 Distant metastasis of NENs in SEER data and Japanese epidemiological study

	Percentages of distant metastasis (%)	
SEER data (1973–2004)[a]		
Pancreas	64	
Gastric	15	
Duodenum	9	
Jejunum/ileum	30	
Rectum	5	
Japanese epidemiological study (2010)[b]		
Pancreas		19.9
Foregut (expt. pancreas)		6.0
Midgut		9.8
Hind gut		3.5

[a]Yao [2]
[b]Ito [3]

Table 9.3 WHO 2010 classification of GEP-NENs

Classification	Mitotic Count (per 10 HPF)	Ki-67 index (%)
NET G1	<2	<3
NET G2	2–20	3–20
NEC	>20	>20

WHO World Health Organization, *GEP-NENs* gastroenteropancreatic neuroendocrine neoplasms, *NET* neuroendocrine tumor, *NEC* neuroendocrine carcinoma

9.2 Pathological Classification

The 2010 World Health Organization (WHO) classification classified GEP-NENs into three categories (neuroendocrine tumor grade 1(NET G1), neuroendocrine tumor grade 2 (NET G2), and neuroendocrine carcinoma (NEC)) on the basis of Ki-67 proliferation index and/or mitotic count. A mitotic count of <2 per 10 high-power fields (hpf) and/or a Ki-67 index <3% corresponds to NET G1, a mitotic count of 2–20/10 hpf and/or a Ki-67 index of 3–20% to NET G2, and a mitotic count of >20/10 hpf and/or a Ki-67 index >20% (grade3) to NEC (Table 9.3). NET G1 and NET G2 are generally more indolent, less aggressive course than NEC [4, 5]. According to this 2010 WHO classification, both poorly differentiated small cell carcinoma and large cell neuroendocrine carcinoma (LCNEC) correspond to NEC. This classification system is pathologically simple and very useful to standardize diagnosis and treatment procedures. However, mitotic count and Ki-67 index are higher in small cell carcinoma or LACNEC than well-differentiated tumors. Recent data also suggests that it may not be correct to consider all NEC as a single entity, and some researchers have proposed that well-differentiated subtype

of NEC should be designated as NET G3 (neuroendocrine tumor grade 3) to distinguish from small cell carcinoma or LCNEC [6–9]. Some P-NENs show discordance between Ki-67 index and mitotic count; well-differentiated P-NEN that is grade 3 by Ki-67 is significantly less aggressive than poorly differentiated NECs [6]. In other study, grade 3 GI-NEN with a Ki-67 index <55% were less responsive to first-line platinum-based chemotherapy [9].

9.3 Treatments for GEP-NENs

9.3.1 Indication of Medical Treatment

Surgical treatment is only the possible way to cure the GEP-NENs and the indication of surgical treatment should be considered for all patients with GEP-NENs. Liver resection is often performed in the well-differentiated (G1 or G2) GEP-NEN patients with hepatic metastasis, depending on the tumor number, size, and location of the metastatic lesions and the extent of primary tumor [10]. The rationale of liver resection is provided by studies showing longer survival after resection of liver metastases, and the clinical effectiveness of liver resection can be partly explained by intrinsic slow progression of well-differentiated GEP-NENs [11–13]. Surgical treatment is the preferred method whenever possible; however, the patients with unresectable advanced GEP-NENs need radiofrequency ablation (RFA), transarterial chemoembolization (TACE), and/or systemic medical treatment. The goals of treatments in the patients with unresectable disease are to palliate tumor-related symptoms and prolong life span. Figure 9.1 shows treatment options in the advanced locoregional or metastatic disease. There are multiple systemic treatment options available including somatostatin analogues, molecular targeted agents, cytotoxic chemotherapy, and peptide receptor radiation therapy (PRRT). However, the rarity of this disease and the number of prospective randomized trials are limited, and the most therapeutic recommendations are based on the expert opinions.

9.3.2 Cytotoxic Chemotherapy

Cytotoxic chemotherapy has been used to treat the patients with unresectable progressive GEP-NEN for more than 50 years. Streptozocin (STZ) was approved in the USA as a cytotoxic antitumor drug for symptomatic or advanced P-NEN in 1982. STZ combined with doxorubicin (DOX) or fluorouracil (5-FU) has been used as a first-line chemotherapy for GEP-NENs based on several clinical trials including

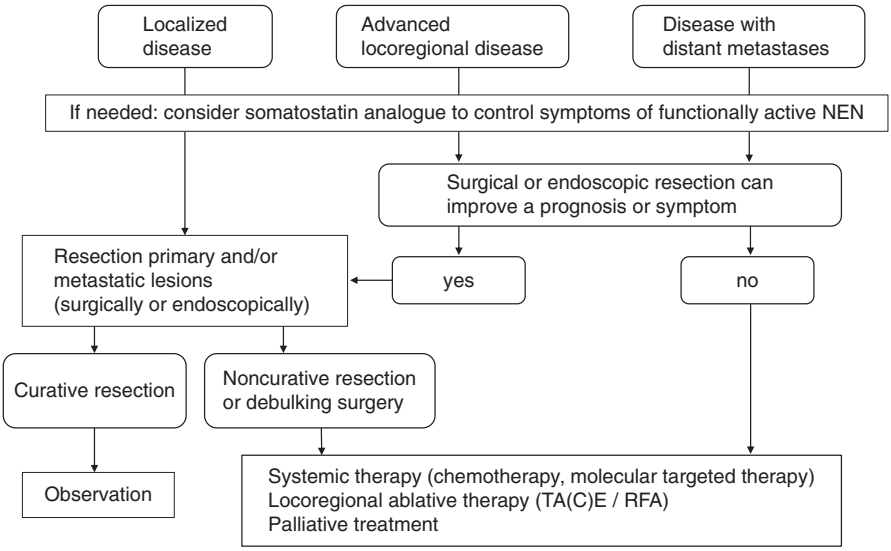

Fig. 9.1 A treatment flowchart of gastroenteropancreatic neuroendocrine neoplasms

randomized clinical trials [14–19]. However, this result was not reproduced in similar studies conducted later [20]. The combination of another alkylating agent temozolomide and capecitabine showed high response in metastatic P-NEN. Response rate of 70% was achieved, and median progressive-free survival was 18 months [21]. In the treatment of P-NENs, chemotherapy has been demonstrated to have both palliative and antitumor effects, though evidence regarding survival is still conflicting. The positioning of cytotoxic chemotherapies is still under discussion.

9.3.3 Molecular Targeted Therapies

In the recent basic and clinical research, somatostatin analogues, mammalian target of rapamycin (mTOR) inhibitors and tyrosine kinase inhibitors appear to have great potential for the treatment of advanced GEP-NENs [22–29]. Octreotide, lanreotide, sunitinib, and everolimus are the drugs evaluated within placebo-controlled studies in GEP-NENs and had evidence for the treatment. Molecular targeted treatments for advanced NENs have been approved on the basis of their antiproliferative effects, and some clinical trial data of molecular targeted therapies show the prolongation effects of progression-free survival among the patients with advanced, metastatic GEP-NENs (Table 9.4).

Table 9.4 Phase III randomized trials of molecular targeted therapy for well- to moderately differentiated advanced or metastatic GEP-NENs

Trial	Agent	Primary tumor sites functionality	Number of patients actual/placebo	Median PFS (months) actual/placebo	HR *p*-value
PROMID (2009)	Octreotide LAR 30 mg i.m., q4w	Midgut functional/nonfunctional	42/43	14.3/6.0 (TTP)	0.34 (0.20–0.59) $p = 0.000072$
CLARINET (2014)	Lanreotide autogel 120 mg s.c., q4w	Gastrointestinal/pancreas nonfunctional	101/103	Nr/18.0	0.47 (0.30–0.73) $p < 0.001$
RADIANT 3 (2011)	Everolimus 10 mg/day p.o.	Pancreas nonfunctional	207/203	11.4/5.4[a]	0.34 (0.26–0.44) $p < 0.001$
RADIANT 4 (2016)	Everolimus 10 mg/day p.o.	Lung/gastrointestinal nonfunctional	205/97	11.0/3.9	0.48 (0.35–0.67) $p < 0.00001$
Sunitinib (2011)	Sunitinib malate 37.5 mg/day p.o.	Pancreas functional/nonfunctional	86/85	11.4/5.5	0.42 (0.26–0.66) $p < 0.001$

[a]Review by central adjudication committee

GEP-NENs Gastroenteropancreatic neuroendocrine neoplasms, *LAR* long-acting-release, *i.m.* intramuscular injection, *s.c.* subcutaneous injection, *p.o.* oral administration, *PFS* progression free survival, *TTP* time to tumor progression, *HR* hazard ratio

9.4 Molecular Targeted Therapy for Advanced GEP-NENs

9.4.1 Somatostatin Receptor and Somatostatin Analogues

Somatostatin and its synthetic analogues bind to G-protein couple receptors and inhibit both secretion and growth of NENs. Somatostatin analogues have been used both for the diagnosis and therapy for NENs. Five distinct somatostatin receptor subtype genes (SSTR1–5) were cloned [30]. GEP-NENs, except insulinoma, express SSTR2 in 80–100% cases, whereas insulinomas have a lower incidence of SSTR2 expression [31]. Well-differentiated GEP-NENs usually express higher frequency of SSTRs than poorly or undifferentiated GEP-NENs [32].

The mechanisms of somatostatin receptor signaling and regulation have been elucidated. The well-known somatostatin action is inhibitory effect on secretion. This inhibitory effect is mediated by coupling of SSTR to Gi/Go proteins, and

subsequently G-protein activation leads to reduction of second messengers, cyclic AMP, and cytosolic calcium. The reduction of second messengers by somatostatin leads to inhibitory effect on hormone release [33, 34]. Another important somatostatin action is inhibition of NEN cell proliferation, and this effect can be mediated by two general signaling pathways. One pathway is activation of protein tyrosine phosphatases. The dephosphorylation of specific substrates is proposed to counteract growth factor stimulated tyrosine kinase activity and then to inhibit mitogenic signaling pathways [35–38]. The second pathway is SSTR inhibition of adenylyl cyclase. The inhibition of adenylyl cyclase leads to a reduction in cyclic AMP levels and thus to changes in cAMP response element-binding protein (CREB) and extracellular receptor kinase (ERK) signaling [39–41], although the role of this pathway is still under discussion.

As of now, three somatostatin analogues, octreotide, lanreotide, and pasireotide, are available. However, pasireotide, a novel universal somatostatin ligand, is not approved for the treatment of GEP-NENs. Focused on the antiproliferative role of somatostatin analogues, there are two phase III randomized studies, the PROMID study and the CLARINET study, which was published. Both study are placebo-controlled, double-blind, prospective, randomized study. In the PROMID study, the antitumor effect of octreotide long-acting release (LAR) on the well-differentiated metastatic NENs was examined [22]. This study included only midgut NENs. In this study, 85 patients were randomly assigned to either placebo or octreotide LAR 30 mg intramuscularly monthly until tumor progression or death. The primary end point of this study was time to tumor progression (TTP), and secondary end points were survival time and tumor response. The significant difference of TTP was observed between the octreotide LAR group and placebo group (14.3 months and 6 months, $P = 0.000072$), and antiproliferative efficacy was demonstrated [22]. Subgroup analyses of this study suggested that the antiproliferative effect was influenced by hepatic tumor burden and resection of the primary tumor [22]. In the CLARINET study, the antitumor effect of extended-release aqueous-gel formulation of lanreotide (Autogel) on the SSTR-positive, well, or moderately differentiated (Ki-67 index of <10%) metastatic GEP-NENs were examined [23]. Primary tumors were located in the pancreas, midgut, or hindgut or were of unknown origin. Two hundred four patients were randomly assigned to either placebo or lanreotide Autogel 120 mg deep subcutaneously once every 28 days for 96 weeks. The primary end point of this study was progression-free survival (PFS), and secondary end points were overall survival, quality of life, and safety [23]. The significant difference of PFS was observed between the lanreotide Autogel group and placebo group (median not reached and median of 18.0 months, $P < 0.001$). The estimated progression-free survival at 24 months was 65.1% in the lanreotide Autogel group and 33.0% in the placebo group. No significant difference in quality of life or overall survival was observed [23]. These two studies demonstrate the antiproliferative effect with long-acting somatostatin analogues in patients with NENs.

9.4.2 *mTOR Pathway and mTOR Inhibitor*

In most cases, upregulation of mTOR pathway is prevalent in P-NENs. mTOR is a serine/threonine protein kinase, belongs to the family of the phosphatidylinositol (PI) 3-kinase (PI3K)-related protein kinases, and plays a critical role in cell growth, proliferation, and migration [42]. mTOR is associated in two distinct complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Modulation of downstream mTORC1 effectors promotes protein synthesis and cell proliferation and inhibits autophagy. mTORC2 is a main modulator of cell growth. mTORC2 is directly upstream of AKT and activation of AKT stimulates downstream of mTORC1. mTORC2 also activates protein kinase c (PKC), a member of the MAPK/ERK signaling pathway. Upregulation of the PI3K/mTOR/AKT pathway is a common feature of proliferative disorder [43–45] (Fig. 9.2).

Rapamycin, a kind of mTOR inhibitor, was found as an antibiotic produced by the bacterium *Streptomyces hygroscopicus*. There are three rapamycin analogues (rapalogs) that are synthesized, CCI779, AP23573, and RAD001 (everolimus). Based on the phase II studies of everolimus, which demonstrated promising antitumor effect of everolimus in GEP-NENs [46, 47], two randomized double-blind placebo-controlled phase III trials, RADIANT 3 and RADIANT 4, was conducted. In RADIANT 3 trial, 410 patients, who had advanced low-grade or intermediate-grade P-NENs with radiologic progression within the previous 12 months, were randomly assigned to receive everolimus (207 patients) or placebo (203 patients) [26]. Everolimus significantly prolonged the progression-free survival (PFS)

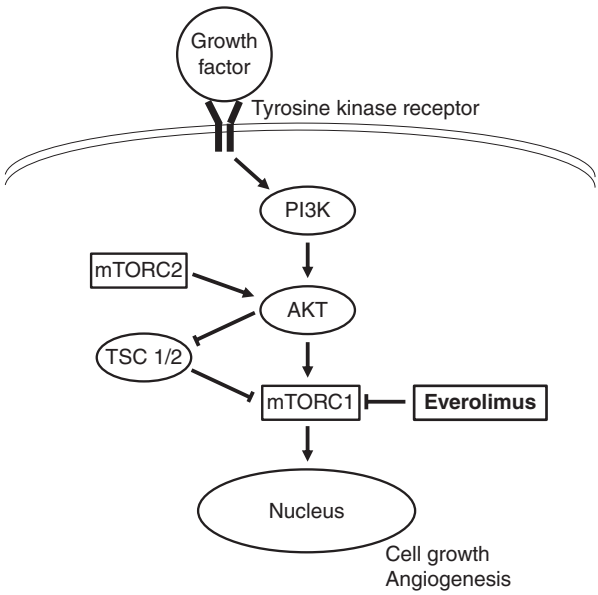

Fig. 9.2 mTOR signaling pathway. Everolimus suppresses mTOR complex 1 (mTORC1), which leads to cell growth and angiogenesis

compared with placebo (median PFS, 11.0 months vs. 4.6 months; hazard ratio for disease progression or death with everolimus, 0.35; 95% CI, 0.27–0.45; $p < 0.001$) [26]. In RADIANT 4 trial, 302 patients, who had advanced well-differentiated non-functional NEN of the lung, gastrointestinal tract origin, were randomly assigned in a 2:1 ratio to receive everolimus (205 patients) or placebo (97 patients) [27]. Everolimus significantly prolonged the PFS compared with placebo (median PFS, 11.0 months vs. 3.9 months; hazard ratio for disease progression or death with everolimus, 0.48; 95% CI, 0.40–0.67; $p < 0.00001$) [27]. The result of RADIANT 3 trial and RADIANT 4 trail indicates that the treatment with everolimus markedly extended the PFS in patients with advanced NEN of the lung, gastrointestinal tract, or pancreas origin. Based on the above two randomized trials, everolimus was approved in the USA, European countries, and other countries.

9.4.3 Angiogenesis and Tyrosine Kinase Inhibitor

Well-differentiated NENs are characterized as high vascular tumors and express high level of vascular endothelial growth factor (VEGF) [48]. Malignant P-NENs widely express platelet-derived growth factor receptors (PDGFRs) α and β, stem-cell factor receptor (c-kit), and VEGF receptor (VEGFR)-2 and VEGFR-3 [49, 50]. Sunitinib is a multi-target anti-angiogenetic tyrosine kinase inhibitor and it blocks VEGFR, PDGFR β, c-KIT, FlT-3, and RET [26] (Fig. 9.3). A phase II trial investigated the efficacy of sunitinib in both carcinoid tumors (41 patients, originated in the lung, stomach, small bowel, appendix, colon, or rectum) and P-NENs (66 patients) [51]. Patients were treated with sunitinib 50 mg/day for 4 weeks, followed by a 2 weeks off treatment. This trial suggested that sunitinib had antitumor activity in P-NENs; however, definitive effective could not be seen in carcinoid tumors [51]. Based on this phase II trial, one randomized double-blind placebo-controlled phase III trial with sunitinib was conducted. In this phase III trial, 171 patients who had advanced well-differentiated P-NEN were randomly assigned to receive sunitinib (86 patients) or placebo (85 patients) [28]. In sunitinib group, patients received best supportive care with once-daily sunitinib at a dose of 37.5 ng/day. The dose reduction (37.5 mg instead of 50 mg) was due to the increased rate of grade 3 fatigue in

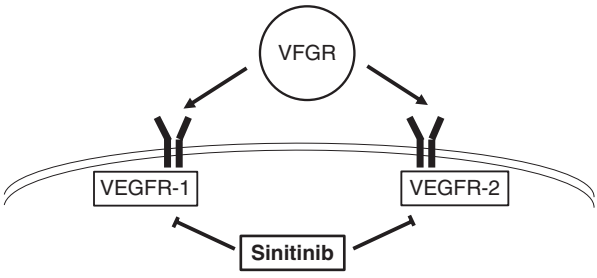

Fig. 9.3 Sunitinib suppresses VGEFR-1 and VEFGR-2

the phase II study [28, 51]. Sunitinib significantly prolonged the PFS compared with placebo (median PFS, 11.4 months vs. 5.5 months; hazard ratio for disease progression or death with everolimus, 0.42; 95% CI, 0.26–0.66; $p < 0.001$) [28]. Based on the above phase III trial, sunitinib was approved for P-NENs.

9.5 Conclusions

For the treatment of well-differentiated (NET G1/G2) GEP-NENs, promising targeted agents, such as octreotide, lanreotide, everolimus, and sunitinib, have emerged on the bases of randomized phase III trials. Despite these advances, some tumors show intrinsic resistance to these targeting therapies. Various other clinical trials of GEP-NENs are being conducted. The arrival of novel treatment, which gives more options for the patient with GEP-NENs, is desired.

References

1. Metz DC, Jensen RT. Gastrointestinal neuroendocrine tumors. Gastroenterology. 2008;135:1469–92.
2. Yao JC, Hassan M, Phan A, et al. One hundred years after "carcinoid": epidemiology of and prognostic factors for neuroendocrine tumors in 35,825 cases in the United States. J Clin Oncol. 2008;26:3063–72.
3. Ito T, Igarashi H, Nakamura K, et al. Epidemiological trends of pancreatic and gastrointestinal neuroendocrine tumors in Japan: a nationwide survey analysis. J Gastroenterol. 2015;50:58–64.
4. Rindi G, Kloppel G, Alhman H, et al. TNM staging of foregut (neuro)endocrine tumors: a consensus proposal including a grading system. Virchows Arch. 2006;449:395–401.
5. Strosberg JR, Cheema A, Weber J, et al. Prognostic validity of a novel American joint committee on cancer staging classification for pancreatic neuroendocrine tumors. J Clin Oncol. 2011;29(22):3044–9.
6. Basturk O, Yang Z, Tang LH, et al. The high-grade (WHO G3) pancreatic neuroendocrine tumor category is morphologically and biologically heterogenous and includes both well differentiated and poorly differentiated neoplasms. Am J Surg Pathol. 2015;39(5):683–90.
7. Heetfeld M, Chougnet CN, Olsen IH, et al. Characteristics and treatment of patients with G3 gastroenteropancreatic neuroendocrine neoplasms. Endocr Relat Cancer. 2015;22:657–64.
8. Coriat R, Walter T, Terris B, et al. Gastroenteropancreatic well-differentiated grade 3 neuroendocrine tumors: review and position statement. Oncologist. 2016;21:1191–9.
9. Sorbye H, Welin S, Langer SW, et al. Predictive and prognostic factors for treatment and survival in 305 patients with advanced gastrointestinal neuroendocrine carcinoma (WHO G3): the NORDIC NEC study. Ann Oncol. 2013;24(1):152–60.
10. Hill JS, McPhee JT, McDade TP, et al. Pancreatic neuroendocrine tumors: the impact of surgical resection on survival. Cancer. 2009;115:741–51.
11. Touzios JG, Kiely JM, Pitt SC, et al. Neuroendocrine hepatic metastases: does aggressive management improve survival? Ann Surg. 2005;241:776–83.
12. Fendrich V, Langer P, Celik I, et al. An aggressive surgical approach leads to long-term survival in patients with pancreatic endocrine tumors. Ann Surg. 2006;244:845–51.

13. Sarmiento JM, Heywood G, Rubin J, et al. Surgical treatment of neuroendocrine metastases to the liver: a plea for resection to increase survival. J Am Coll Surg. 2003;197:29–37.
14. Moertel CG, Hanley JA, Johnson LA. Streptozocin alone compared with streptozocin plus fluorouracil in the treatment of advanced islet-cell carcinoma. N Engl J Med. 1980;303:1189–94.
15. Moertel CG, Lefkopoulo M, Lipsitz S, et al. Streptozocin-doxorubicin, streptozocin-fluorouracil or chlorozotocin in the treatment of advanced islet-cell carcinoma. N Engl J Med. 1992;326:519–23.
16. von Schrenck T, Howard JM, Doppman JL, et al. Prospective study of chemotherapy in patients with metstatic gastrinoma. Gastroenterology. 1988;94:1326–34.
17. Eriksson B, Skogseid B, Lundqvist G, et al. Medical treatment and long-term survival in a prospective study of 84 patients with endocrine pancreatic tumors. Cancer. 1990;65:1883–90.
18. Rivera E, Ajani JA. Doxorubicin, streptozocin, and 5-fluorouracil chemotherapy for patients with metastatic islet-cell carcinoma. Am J Clin Oncol. 1998;21:36–8.
19. Kouvaraki MA, Ajani JA, Hoff P, et al. Fluorouracil, doxorubicin, and streptozocin in the treatment of patients with locally advanced and metastatic pancreatic endocrine carcinomas. J Clin Oncol. 2004;22:4762–71.
20. Cheng PN, Saitz LB. Failure to confirm major objective antitumor activity for streptozocin and doxorubicin in the treatment of patients with advanced islet cell carcinoma. Cancer. 1999;86:944–8.
21. Strosberg JR, Fine RL, Choi J, et al. First-line chemotherapy with capecitabine and temozolomide in patients with metastatic pancreatic endocrine carcinomas. Cancer. 2011;117:268–75.
22. Rinke A, Muller HH, Schade-Brittinger C, et al. Placebo-controlled, double-blind, prospective, randomized study on the effect of octreotide lar in the control of tumor growth in patients with metastatic neuroendocrine midgut tumors: a report from the promid study group. J Clin Oncol. 2009;27(28):4656–63.
23. Caplin ME, Pavel M, Ćwikła JB, et al. Lanreotide in metastatic enteropancreatic neuroendocrine tumors. N Engl J Med. 2014;371(3):224–33.
24. Jiao Y, Shi C, Edil BH, et al. DAXX/ATRX, MEN1, and mTOR pathway genes are frequently altered in pancreatic neuroendocrine tumors. Science. 2011;331:1199–203.
25. DeWilde RF, Edil BH, Hruban RH, et al. Well-differentiated pancreatic neuroendocrine tumors: from genetics to therapy. Nat Rev Gastroenterol Hepatol. 2012;9:199–208.
26. Yao JC, Shah MH, Ito T, et al. Everolimus for advanced pancreatic neuroendocrine tumors. N Engl J Med. 2011;364:514–23.
27. Yao JC, Fazio N, Singh S, et al. Everolimus for the treatment of advanced, non-functional neuroendocrine tumours of the lung or gastrointestinal tract (RADIANT-4): a randomised, placebo-controlled, phase 3 study. Lancet. 2016;387:968–77.
28. Raymond E, Dahan L, Raoul JL, et al. Sunitinib malate for the treatment of pancreatic neuroendocrine tumors. N Engl J Med. 2011;364:501–13.
29. Yao JC, Phan A. Overcoming antiangiogenic resistance. Clin Cancer Res. 2011;17:5217–9.
30. Hoyer D, Bell GI, Berelowitz M, et al. Classification and nomenclature of somatostatin receptors. Trends Pharmacol Sci. 1995;16:86–8.
31. Reubi JC, Waser B. Concomitant expression of several peptide receptors in neuroendocrine tumors as molecular basis for in vivo multireceptor tumor targeting. Eur J Nucl Med. 2003;30(5):781–93.
32. Reubi JC, Kvols LK, Waser B, et al. Detection of somatostatin receptors in surgical and percutaneous needle biopsy samples of carcinoids and islet cell carcinomas. Cancer Res. 1990;50(18):5969–77.
33. Schonbrunn A, Koch BD. Mechanism by which somatostatin inhibits pituitary hormone release. In: Reichlin S, editor. Somatostatin: basic and clinical status. New York: Plenum Press; 1987. p. 121–35.
34. Koch BD, Schonbrunn A. Characterization of the cyclic AMP-independent actions of somatostatin in GH cells. II. An increase in potassium conductance initiates somatostatin-induced inhibition of prolactin secretion. J Biol Chem. 1988;263(1):226–34.

35. Pyronnet S, Bousquet C, Najib S, et al. Antitumor effects of somatostatin. Mol Cell Endocrinol. 2008;286(1–2):230–7.
36. Dent P, Wang Y, Gu YZ, et al. S49 cells endogenously express subtype 2 somatostatin receptors which couple to increase protein tyrosine phosphatase activity in membranes and downregulate Raf-1 activity in situ. Cell Signal. 1997;9(7):539–49.
37. Reardon DB, Dent P, Wood SL, et al. Activation in vitro of somatostatin receptor subtypes 2, 3, or 4 stimulates protein tyrosine phosphatase activity in membranes from transfected Ras-transformed NIH 3T3 cells: coexpression with catalytically inactive SHP-2 blocks responsiveness. Mol Endocrinol. 1997;11(8):1062–9.
38. Florio T. Molecular mechanisms of the antiproliferative activity of somatostatin receptors (SSTRs) in neuroendocrine tumors. Front Biosci. 2008;13:822–40.
39. Tentler JJ, Hadcock JR, Gutierrez-Hartmann A. Somatostatin acts by inhibiting the cyclic 3′,5′-adenosine monophosphate (cAMP)/protein kinase a pathway, cAMP response element-binding protein (CREB) phosphorylation, and CREB transcription potency. Mol Endocrinol. 1997;11(7):859–66.
40. Drozdov I, Svejda B, Gustafsson BI, et al. Gene network inference and biochemical assessment delineates GPCR pathways and CREB targets in small intestinal neuroendocrine neoplasia. PLoS One. 2011;6(8):e22457.
41. Emery AC, Eiden MV, Mustafa T, et al. Rapgef2 connects GPCR-mediated cAMP signals to ERK activation in neuronal and endocrine cells. Sci Signal. 2013;6(281):ra51.
42. Murakami M, Ichisaka T, Maeda M, et al. mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. Mol Cell Biol. 2004;24(15):6710–8.
43. Shah T, Hochhauser D, Frow R, et al. Epidermal growth factor receptor expression and activation in neuroendocrine tumours. J Neuroendocrinol. 2006;18(5):355–60.
44. Catena L, Bajetta E, Milione M, et al. Mammalian target of rapamycin expression in poorly differentiated endocrine carcinoma: clinical and therapeutic future challenges. Target Oncol. 2011;6(2):65–8.
45. Kasajima A, Pavel M, Darb-Esfahani S, et al. mTOR expression and activity patterns in gastroenteropancreatic neuroendocrine tumours. Endocr Relat Cancer. 2011;18(1):181–92.
46. Yao JC, Phan AT, Chang DZ, et al. Efficacy of RAD001 (everolimus) and octreotide LAR in advanced low- to intermediate-grade neuroendocrine tumors: results of a phase II study. J Clin Oncol. 2008;26(26):4311–8.
47. Yao JC, Lombard-Bohas C, Baudin E, et al. Daily oral everolimus activity in patients with metastatic pancreatic neuroendocrine tumors after failure of cytotoxic chemotherapy: a phase II trial. J Clin Oncol. 2010;28(1):69–76.
48. Terris B, Scoazec JY, Rubbia L, et al. Expression of vascular endothelial growth factor in digestive neuroendocrine tumours. Histopathology. 1998;32(2):133–8.
49. Fjallskog ML, Hessman O, Eriksson B, et al. Upregulated expression of PDGF receptor Beta in endocrine pancreatic tumors and metastases compared to normal endocrine pancreas. Acta Oncol. 2007;46(6):741–6.
50. Hansel DE, Rahman A, Hermans J, et al. Liver metastases arising from welldifferentiated pancreatic endocrine neoplasms demonstrate increased VEGF-C expression. Mod Pathol. 2003;16(7):652–9.
51. Kulke MH, Lenz HJ, Meropol NJ, et al. Activity of sunitinib in patients with advanced neuroendocrine tumors. J Clin Oncol. 2008;26(20):3403–10.