Cancer Treatment and Research

Steven T. Rosen, M.D., Series Editor

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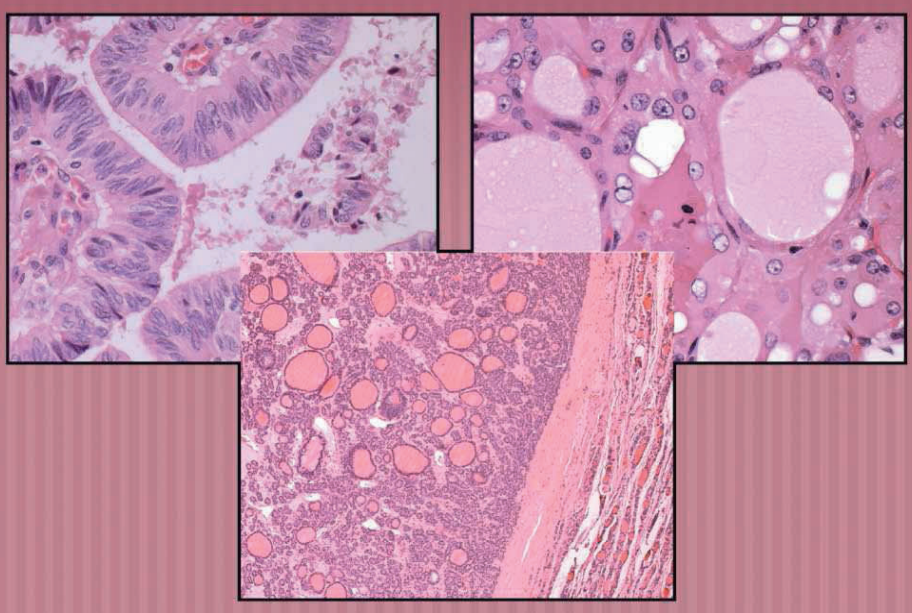
Molecular Basis of Thyroid Cancer



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MOLECULAR BASIS OF THYROID CANCER

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PREFACE

Thyroid cancer is the fastest growing cancer in the U.S., especially among women. Given the relative success we have had in its treatment, there is a need to capitalize on it by better understanding the factors that underpin this malignancy, and exploring better strategies for diagnosis, treatment, and follow-up. To do so, we must take full advantage of the revolution in modern biology.

This comprehensive volume addresses the needs of a broad readership. Against a backdrop of the complexity of the origins of cancer, pathology of thyroid tumors, including lymphomas, is discussed, as are molecular genetic lesions associated with spontaneous and radiation-related thyroid tumors, diagnostic tests available to surmise tumor subtypes (including a review of the potential of DNA micro arrays), advances in therapeutics (including recombinant hTSH superagonists, allowing for better treatment of well differentiated thyroid carcinoma and its imaging), and combinations of drugs that might influence the course of poorly differentiated and anaplastic thyroid cancer. In this book, we glimpse into the promise of gene therapy in the future management of otherwise lethal anaplastic and poorly differentiated thyroid tumors. The molecular genetics of medullary thyroid cancer is considered in depth, and malignancy is used as a showcase for genetic prediction and counseling.

The Molecular Basis of Thyroid Cancer is an indispensable companion for endocrinologists (particularly those with an interest in thyroid cancer), thyroid surgeons, nuclear medicine physicians, molecular oncologists, clinical biochemists, and those in biotechnology intent on the innovation of better diagnosis and therapy of cancer.

I am fortunate and privileged to be the editor of *The Molecular Basis of Thyroid Cancer.* I compiled a list of top scientists based on their areas of expertise in thyroid cancer whom I hoped would contribute, and received enthusiastic and positive responses. Their chapters are superb.

Throughout this project I have been supported and advised by two wonderful individuals from Kluwer: the untiring Maureen Tobin, editorial assistant and Laura Walsh, editor. They have made the task of putting this book together a pleasure.

Nadir R. Farid

1. THE ORIGIN OF CANCER

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INTRODUCTION

Cancer is a complex genetic disease. Work over the past fifty years confirms that the genetic alterations found associated with human cancers impair the function of pathways critical to controlling cell growth and differentiation. In aggregate, these genetic mutations allow a malignant cell to acquire a set of biologic attributes leading to autonomous proliferation and metastatic spread. Despite this paradigm, the precise nature and timing of each of the events that conspire to program the malignant cell remain incompletely understood.

Although familial cancer syndromes are responsible for only a minority of human cancers, the study of these kindreds has facilitated our understanding of cancer genetics. In many such syndromes, individuals inherit one defective, predisposing allele in the germline, and only later in life do they acquire a second loss of function mutation. As first described by Knudson, this "two hit" hypothesis helps explain such inherited cancer syndromes such as retinoblastoma and Wilms' tumors (1). Although the tumors in these patients express mutations in specific inherited genes, the finding that these tumors also harbor a myriad of other genetic changes indicates that further alteration by somatic mutation are required for tumor development (2).

However, the majority of human cancers lack a readily definable predisposing genetic defect and appear to be the result of a concert of acquired genetic alterations. Work from many laboratories, using both patient-derived material and experimental cancer models, have begun to define these malignant genetic mechanisms.

In spontaneously arising human cancers, we still cannot determine the exact number and nature of genetic alterations involved in the process of transformation from a normal cell to a malignant one. Since cancer encompasses more than 100 different types of malignant diseases with great heterogeneity of clinical characteristics, every tumor could hypothetically be completely unique. Thus, cancers, in general, could harbor an undecipherable number of genetic and epigenetic changes leading to their development.

Alternatively, pathogenesis of human cancers may be dependent on a distinct set of genetic and biochemical alterations that apply uniformly to most if not all human tumors. These changes may alter the functions of specific pathways involved in important biological functions and facilitate malignant transformation, endowing cells with specific changes in cell physiology, termed "acquired capabilities," ensuring their survival and continued success (3). In particular, cancer cells generate their own mitogenic signals, proliferate without limits, resist cell cycle arrest, evade apoptosis, induce angiogenesis, and eventually devise mechanisms for invasion and metastasis.

GENETIC REQUIREMENTS FOR CANCER

Epidemiologic analyses have shown that four to six rate-limiting events must occur before a tumor becomes clinically apparent (4,5). The changes that must occur are genetic and/or epigenetic in nature. Most of these events result from somatic mutations that occur infrequently or are induced by carcinogen exposure, and only in aggregate do they lead to the tumorigenic state.

The colorectal carcinoma model

In a seminal series of studies, Vogelstein and his colleagues described a stepwise genetic history of colorectal tumors (6). Since colorectal carcinoma develops intraluminally and tissue is readily available for examination, specific histopathological alterations that occur in cancer development are readily observed in different stages. By studying tissue derived from specific histopathologic stages, ranging from normal colonic epithelium to frank carcinoma, they catalogued genetic alterations specific for each stage, thereby developing a model that dissected an accumulation of separate genetic mutations that could in combination lead to malignancy (7,8).

A vast majority of early adenomatous polyps were found to exhibit an inactivated mutant form of the tumor suppressor gene, adenomatous polyposis coli *(APC)* (9). Alterations in this gene had been previously shown to be responsible for Familial Adenomatous Polyposis (FAP) (10,11). However, patients with germline mutations of *APC* have a greater risk for but do not necessarily develop colorectal cancer. In addition to the germline mutation, somatic mutation of the wild-type *APC* allele must also occur (9,12).

When they investigated intermediate size adenomas, they found that approximately half carry activating mutant *RAS* oncogenes (6,13). Interestingly, normal colonic epithelium harboring *RAS* mutations alone, do not lead to neoplasia (14), and these cells may eventually succumb to apoptosis (15), suggesting that other genetic alterations are necessary for *RAS* mutation to contribute to tumor formation. In a subset of larger

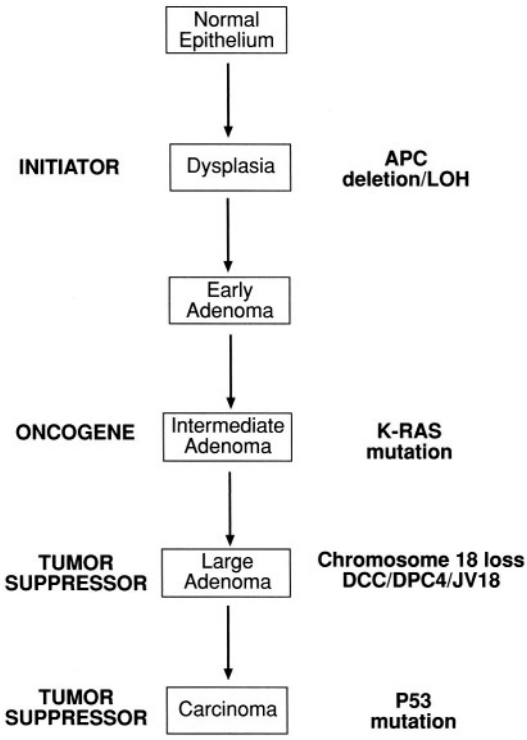

Figure 1. Genetic Model of Colorectal Carcinoma Development. Multiple genetic alterations are found at different stages of development from pre-malignant lesions on to frank carcinoma. These genetic lesions may represent necessary alterations to progress to the next developmental phase toward cancer.

adenomas, alteration of a chromosome 18-associated tumor suppressor gene such as *DCC, DPC4,* or *JV18,* were common. Finally, 80% of colorectal carcinomas show evidence of genetic alterations of the *P53* tumor suppressor gene (16). Surprisingly, however, patients with Li-Fraumeni syndrome, who have germline mutations of *P53*, do not have a higher risk of colorectal cancer development and do not even tend to develop polyposis (17). Thus, although both *P53* and *RAS* play individual roles in colon cancer pathogenesis, these observations suggest that oncogenesis cannot be accomplished by a random accumulation of mutations. The order of alteration, as well as the necessity for an initiator like *APC* deletion, may both be important determinants for formation of the resultant tumor (Figure 1).

These observations provide evidence that the history of human cancer follows a stepwise progression of genetic events. This model, however, demonstrates only one of many potential pathways to the neoplastic state.

While these observations in colorectal cancer certainly suggest that all cancers progress through a similar series of ordered events, no other human cancer has been similarly mapped, and abundant evidence indicates that specific mutations differ among

particular cancers. Understanding the combination of events required in each type of human cancer remains an important goal of future studies.

EXPERIMENTAL MODELS

Initial studies of human cancer cells were limited to samples obtained from tumor biopsy specimens. To facilitate further study, cells from these tumors were frequently adapted into cell lines that grow in culture (18). These cell lines are useful for many purposes, however, it is impossible to determine the order or even a set of defined genetic or biochemical changes that lead to neoplastic development. Complicating matters further is the high likelihood that additional genetic alterations are acquired over time through propagation in culture.

Recently, transcriptional profiling has been helpful in evaluating the simultaneous expression of thousands of genes in particular cancers or cancer cell lines (19,20). Unfortunately, while these studies have provided us with tools to better classify cancers, they have not yet yielded insight into the functionally important gene expression changes required for cancer growth. It is still impossible from these analyses to determine which genes have true functional roles in the transformation to the malignant state. Thus, a complementary approach to studying the genetic alterations necessary to form a tumor is to transform normal cells, *in vitro,* by serially introducing multiple oncogenes. An alternative method of cancer modeling is through the production of genetically altered mice harboring specific alterations associated with human cancer.

Rodent cell transformation

In rodent systems, single oncogenes fail to transform primary cells without the presence of prior predisposing mutations (21,22). In contrast, two introduced oncogenes convert embryonic rodent cells to a tumorigenic phenotype (23,24). These observations indicated that the conversion of normal cells into cancer cells requires multiple genetic changes to occur.

Collaborating oncogenes that induced transformation in these cultured rodent primary cells included *Myc/Ras* or *E1a/ ras* (23,24). Further confirmation of this collaboration through transgenic mouse experiments occurred when a *Ras* or a *Myc* transgene was placed under the control of mammary- or prostate- specific promoters (25,26). Dysplasia in promoter specific organs developed at high rates in the transgenic mice expressing single oncogenes, but frank tumors did not develop unless mice expressed both transgenes. These findings support the concept that specific oncogenes collaborate to aid in tumor development *in vivo,* as well as in cultured cells.

Barriers to human cellular immortalization

While two oncogenes appeared to suffice to transform rodent cells, the transformation of primary human cell lines proved to be more complex. This difference is in part because human cells require more genetic alteration to bypass the barriers of immortalization (Figure 2). When normal human cells are grown in culture, their proliferative potential is limited and they eventually enter an irreversible, quiescent state, termed mortality stage 1 (M1) or replicative senescence (27). Although these cells are still viable, they can no longer be stimulated to divide. The exact trigger for entry into replicative

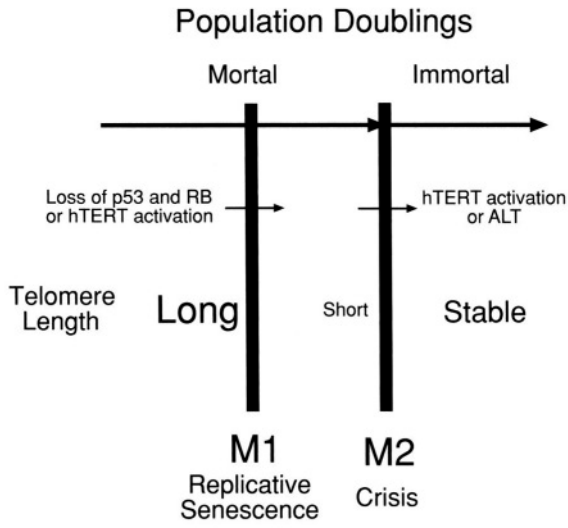

Figure 2. Barriers to Human Cellular Immortalization. Normal passage of cells is halted at MI unless this barrier is bypassed by p53 and RB inactivation or hTERT expression. These cells can then continue dividing until their telomeres become critically short at M2. hTERT expression or ALT allows telomere length stabilization and cellular immortalization.

senescence is still unclear, although there are a variety of stimuli that have a role in this process (28).

Pre-senescent cells can be experimentally manipulated to bypass replicative senescence through ectopic expression of certain genes. Expression of *hTERT,* the catalytic subunit of telomerase is capable of bestowing some but not all primary cells with immortality (29,30). Another mechanism of bypassing this first proliferative barrier is through simultaneous abrogation of the *P53* tumor suppressor and retinoblastoma *(RB)* pathways (28). Expression of viral oncoproteins, such as SV40 large T antigen (31) or human papillomavirus E6 and E7 oncoproteins (32), which bind to and inactivate p53 and RB (33), respectively, offer experimental methods of achieving this dual inactivation.

Cells that lack telomerase overexpression but that express the above mentioned viral oncoproteins, may then undergo another 10–20 population doublings before they encounter mortality stage 2 (M2) or crisis. Here the vast majority of cells have short telomeres (34), display karyotypic abnormalities (35), and die by apoptosis (36). Since in culture telomeres shorten by 50-100 base pairs during each cell replication (37), ongoing passage allows telomeres to shorten to a critical length. This results in an inability to protect the ends of chromosomes, leading to genomic instability and triggering crisis (38).

Rare variants, approximately 1 in 10 million cells, emerge from crisis, and have infinite proliferative capability (31). These cells typically exhibit stable telomere lengths and express the *hTERT* gene with preserved activity (38), which is felt to be expressed

at low levels in normal cells (39). These findings have been corroborated by observations in post-senescent, pre-crisis cells that avoid crisis and proliferate indefinitely after transduction with *hTERT* (40–42). However, a subgroup of cells may become immortal without significant *hTERT* or telomerase expression (43,44). These cells have a separate mechanism of telomere length maintenance, termed alternative lengthening of telomeres (ALT), which likely involves recombination (45).

Human cell transformation

The observations that suggested that human cell immortalization is more complex than rodent cell immortalization also complicated attempts for experimental transformation of human cells. From this set of observations, Sager and her colleagues postulated that the senescence program is a barrier to cancer development (46,47). Recent work, however, has begun to identify combinations of genetic alterations that suffice to confer human cellular transformation.

Thus, specifically targeting each of the barriers of immortalization by introduction of the SV40 Early Region, which encodes the large T oncoprotein, in combination with the *hTERT* gene into normal human fibroblasts and kidney cells suffice for immortalization (48,49). Since the SV40 Early Region also encodes for small t oncoprotein, subsequent transduction with oncogenic *RAS* results in the ability to develop tumors in immunocompromised mice, hence transformation. Additional studies have revealed this combination of genetic alterations to be sufficient to transform multiple cell types, including cells of mammary (50), lung (51), prostate, ovarian, mesothelial (52), endothelial, and neuroectodermal (53) origin. Thus, it is necessary to understand the roles of these basic genetic elements involved in transformation in regards to the critical pathways that they effect. For example, the large T oncoprotein may functionally inactivate the p53 and RB pathways, but the inactivation of these two pathways may in sum not equal the effects of the oncoprotein alone, as there may be additional functions gained with large T. Thus, a myriad of other genetic mutations that lead down similar or parallel paths may also bestow specific "acquired capabilities," leading to similar functional endpoints or the neoplastic phenotype.

MOLECULAR CHANGES

Experimental evidence has allowed the delineation of a few crucial pathways in human primary cell transformation. Although there are many important cellular capabilities, allowing a normal cell to bypass cell cycle arrest checkpoints, escape apoptosis, guard against crisis, and provide its own mitogenic signals, may be sufficient to allow for transformation to the oncogenic phenotype. These basic genetic elements may be generalized to most human cancers, however, specific alterations that contribute to oncogenesis are found in some cancers, such as thyroid cancer. These well-defined specific molecular alterations involved both in thyroid-specific and general malignant transformation are described below.

The *P53* tumor suppressor gene

Perhaps one of the most common alterations in human cancers is mutation of the *P53* pathway, found altered in most, if not all, human cancers (54). Loss of wild-type

p53 protein expression, in conjunction with gain-of-function from mutant proteins (55), contribute to acquisition of specialized cell properties, such as proliferative and survival advantages. p53 performs these tasks by acting as a transcription factor induced in response to DNA damage, hypoxia, or oncogene activation (54,56). This, in turn, initiates a program of gene regulation leading down at least two major separate pathways, one for cell cycle arrest to allow time to repair damaged DNA and another for apoptosis to trigger the cell to euthanize (54,57).

Wild-type p53 protein may act as a cellular defense mechanism through its effects on cell cycle arrest and apoptosis, both major obstacles to tumor formation. Cells that are unable to arrest and correct DNA damage have increased potential to develop genetic instability with ongoing replication. At the same time, survival of a neoplastic cell, also includes evasion of apoptosis, preventing the cell-suicide program from taking an antitumor effect. Thus, abrogation of wild-type p53 function, may be sufficient in some tumor types to dismantle the apoptotic machinery (58). However, in other tumors, specific components of the apoptotic cascade, such as bcl-2 (59), Akt (60), or caspases (61), must also be inactivated.

p53 regulates a number of genes involved in the cell cycle. One of these proteins, $p21^{CIP1}$, is upregulated by p53 and inhibits the cyclin dependent kinases, resulting in G1 cell cycle checkpoint arrest. Another is Hdm2, a negative regulator of p53, which is also positively regulated by p53 protein itself (Figure 3). Hdm2 physically binds p53

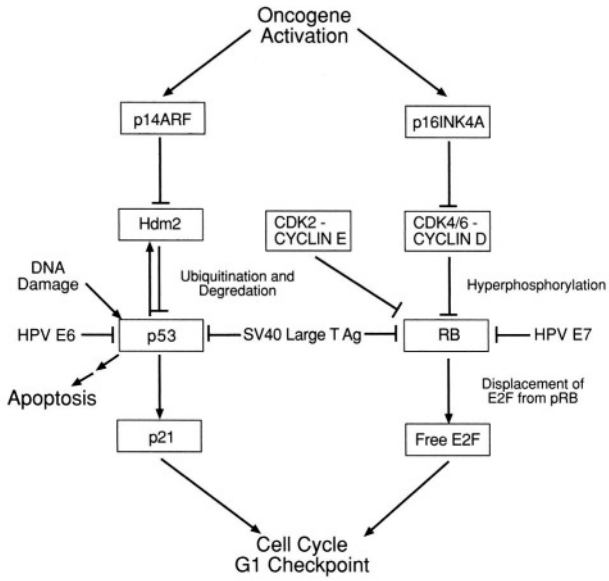

Figure 3. The P53 and RB tumor suppressor pathways. These are both central molecular pathways that are often dysregulated in cancer. Each of these tumor suppressors are regulated by multiple proteins, and disruption can occur at any of these points in human cancer. The role of p53 in apoptosis entails a complex pathway that is not shown on this diagram. Arrows signify activation of the target while blunt lines act in an inhibitory fashion.

protein, inhibiting its activity as a transcriptional factor, meanwhile catalyzing p53 ubiquitination which marks it for proteasomal degredation (62) Hdm2 is itselfregulated by $P14^{ARF}$, another tumor suppressor whose protein product binds to and inactivates Hdm2 (63).

While *P53* may be directly mutated in over half of all human cancers, in some tumors no *P53* mutation is observed, yet other genes in the pathway are altered. For example, Hdm2 can be overexpressed and antagonize p53 protein function in a variety of cancers, including B-cell lymphomas (64), melanomas (65), and breast cancers (66). Other tumors harbor $P14^{ARF}$ deletions or suppression by methylation, permitting Hdm2 to remain active and drive the degredation of p53 (63,67). Thus, a various array of genetic and biochemical alterations can converge to enforce a common resultant phenotype, aiding in tumor development and progression.

As will be described in greater detail elsewhere, in thyroid carcinoma, *P53* alterations have been found more frequently in both poorly differentiated and undifferentiated thyroid carcinomas (68). Thus, p53 may have a role in the dedifferentiation process. A combination of mutation (69), loss of heterozygosity (70), and overexpression (71,72), presumably from decreased degredation, have all been found in thyroid cancer, again declaring the importance of this critical pathway. (See Chapter 8).

The retinoblastoma (RB) protein

Regulation of passage through the G_1 checkpoint of the cell cycle is one of the most important roles of the retinoblastoma protein (73). In its hypophosphorylated form, this protein inhibits cellular commitment to mitosis by blocking cell cycle entry into S-phase. In that state, it is bound to various members of the E2F family of proteins (74). These RB-E2F complexes can inhibit gene transcription by multiple methods: (1) Interfering the ability of free E2Fs' to act as transcriptional factors for cyclin E, cyclin A, and multiple other genes necessary for DNA replication (75) (2) Actively recruiting histone deacetylases (HDACs) (76) and other chromatin remodeling factors to E2F responsive promoters (77).

RB inactivation is a crucial step in allowing a cell to pass the G_1 checkpoint and continue through the cell cycle (Figure 3). Normally, one of the cyclin D subtypes (D1, D2, or D3) assembles with one of the cyclin-dependent kinases, CDK4 or CDK6, and cyclin E binds to CDK2. These active holoenzymes phosphorylate RB proteins. Once in a hyperphosphorylated state, RB is unable to bind E2F or HDACs, and releases the repression on genes required for S-phase entry.

Several other tumor suppressor genes also contribute to the phosphorylation status of pRB. For instance, $p16^{INK4A}$ inhibits the activity of cyclin D-dependent kinases to prevent RB phosphorylation and halt cell division (78). The cyclin E-CDK2 complex is inhibited by both $p21^{CIP1}$ (79) and $p27^{KIP1}$ (80). However, when a strong mitogenic stimulus is present, increased cyclin D1 tends to complex with CDK4, and this combination sequesters $p27^{KIP1}$. This leaves cyclin E-CDK2 free from $p27^{KIP1}$ inhibition to phosphorylate and inactivate RB. E2F, as a result, dissociates from hyperphosphorylated RB and acts as a transcription factor for a number of responder genes,

including cyclin E. The transcription of these responder genes are required for cell cycle progression through the G1 restriction checkpoint, facilitating cellular division.

Like *P53,* mutations in *RB* or its associated tumor suppressor genes occur frequently, and disabling this pathway may be required for the formation of human cancer cells (81,82). For example, loss of function mutations of *RB* also can be found in osteosarcomas and lung cancers, particularly small cell tumors (81). Although *RB* mutations do occur in non-small cell lung carcinomas, they appear to be present in approximately 20–30% of cases as compared to 80% of the small cell subtype (75). However, $p16^{INK4A}$ loss is evident in over half of all non-small cell lung cancers. Inactivation of $P16^{INK4A}$, by genetic lesions or by methylation, disrupts the RB pathway in a large array of other cancers, including pancreatic, breast, glioblastoma multiforme, and T cell ALL (67,75). Cyclin D1 overexpression drives the cell cycle forward and can also substitute for RB inactivation, as noted in breast cancers (83) and mantle cell lymphomas, where there is juxtaposition of the cyclin D1 gene with the immunoglobulin heavy chain promoter enhancer via a t(11:14) translocation (75). Cyclin E overexpression in breast cancers have also been noted and may help drive past the *RB* inhibition checkpoint in G1 (84). Finally, in many cervical cancers, human papillomaviruses (HPV) E7 oncoprotein sequesters and tags RB for degredation (85). Even in those cervical carcinomas that do not express HPV E7, *RB* somatic mutation is detectable. Alterations in the *RB* pathway seem to be mutually exclusive, as usually only one component of the pathway is mutated or lost; nonetheless, convergence on the loss of growth suppression by RB does seem to exist in the majority of human cancers (81).

However, the role of the *RB* in human thyroid cancer remains unclear. Although there are several human immunohistochemical studies (86–88) that remain inconclusive as well as studies evaluating *E2f* and *Rb* in rodents (89–91), definitive molecular evidence for the role of *RB* in human thyroid cancers is lacking. (See Chapter 8).

Mitogenic stimuli and oncogenic *RAS*

Normal and cancer cells differ in their innate ability to proliferate in the absence of mitogenic stimulation. The presence of surrounding growth factors are crucial for the continued proliferation of normal human cells. Cancer cells, in contrast, have reduced their dependence on external stimuli due to the activation of oncogenic mutations that generate constitutively active mitogenic signals (92). For example, alterations in growth-factor receptors, such as *HER2/NEU* amplification in breast cancer (93,94) or epidermal growth factor receptor mutation in most carcinomas (95), function as autonomous growth stimuli.

In human thyroid cancer, multiple activating receptors have been implicated in disease pathogenesis. Characteristic chromosomal rearrangements linking the promoter and amino-terminus domains of unrelated gene(s) to the carboxy-terminus of the *RET* gene result in a constitutively active chimeric receptor, termed (RET/PTC). This event may initiate papillary thyroid cancers (96). Constitutive activation of this mutant kinase promotes interaction with SHC adaptor proteins, intermediates in the *RAS* signaling pathway (97). Although rare, another early event in papillary thyroid cancers, may involve rearrangements of specific TRK tyrosine kinase receptors (98).

Both epidermal growth factor receptor (EGFR) and its ligands, epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α), are also widely expressed in both normal thyroid and thyroid neoplastic tissue (99,100); however, EGF has a higher binding affinity for neoplastic thyroid tissue when compared to normal tissue (101). EGF and its receptor stimulate proliferation of thyroid cancer cells and enhance invasion (102), suggesting their potential role in malignant progression.

Multiple intracellular protein networks exist downstream of growth factor receptors that can become constitutively active in a mutated state, conferring a growth-inducing effect. As discussed above, introduction of one of these aberrant signals, *H-RAS,* turns an activating switch on and facilitates malignant transformation to previously immortalized human and rodent primary cells. (See Chapter 7).

Various RAS proteins, members of a large superfamily of low-molecular-weight GTP-binding proteins, control several crucial signaling pathways that regulate cell proliferation. Their ability to effect downstream intracellular signaling proteins first rely on post-translational farnesylation to localize the *RAS* protein to the cell membrane. Then the ratio of biologically active RAS-GTP to inactive RAS-GDP depends upon the presence and activity of various guanine nucleotide exchange factors (GEFs) and their antagonists, GTPase activating proteins (GAPs) (103).

Multiple effector pathways lay immediately downstream of *RAS* (Figure 4). The RAF family of proteins, which can trigger a cascade of phosphorylating events through the mitogen-activated protein kinase (MAPK) pathway, leads to cell cycle progression. There is resultant ERK-mediated transcriptional upregulation of angiogenic factors, and increased capability for invasiveness through expression of matrix metalloproteinases. Through RAS stimulation of phosphatidylinositol 3-kinases (PI3Ks), RAC, which is a Rho family protein, can also increase invasiveness through its effects on the actin cytoskeleton. PI3K also triggers a strong anti-apoptotic survival signal through Akt/protein kinase B (PKB). Much like Akt, RALGDS, which is activated by RAS, inhibits the Forkhead transcription factors of the FoxO family which have a role in cell cycle arrest through induction of $p27^{KIP1}$ and apoptosis through the expression of BIM and FAS ligand (104). Finally, phospholipase C (PLC) is another RAS effector which promotes activation of protein kinase C and calcium mobilization (105). Alterations in the RAS proteins or their downstream effectors can therefore have the potential to lead to constitutively active signals, aiding the oncogenic phenotype. (See Chapter 7).

Activating point mutations of *RAS* occur in approximately 20% of human tumors, most frequently in pancreatic, thyroid, colorectal, and lung carcinomas, obviating the requirement for the neoplastic cells to encounter external growth stimuli (106,107). In general human cancer and thyroid cancer cells, somatic *RAS* mutations seem to be an early event. These activating mutations are frequently found in follicular thyroid carcinomas and occasionally papillary thyroid carcinoma (108).

Three members of the *RAS* family, *K-RAS* (around 85% of total), which is ubiquitously expressed, *N-RAS* (about 15%), and *H-RAS* (less than 1%), are commonly found to be activated by mutation in human tumors (109). These point mutations all prevent GAP induced GTPase activity, leaving RAS in its active, GTP-bound form. GAP deletion also leads to a similar resultant RAS activation; *NF1* or neurofibromin

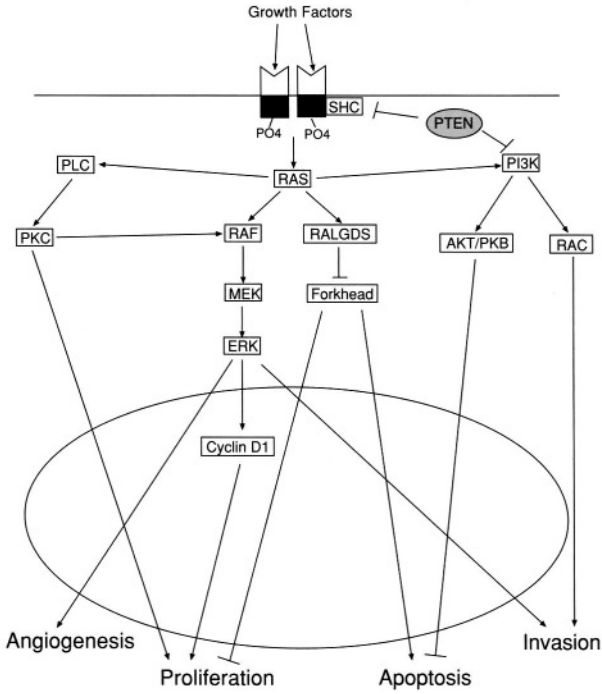

Figure 4. Downstream Mediators of *RAS.* The RAS family of proteins lead down multiple signal transduction networks to not only effect a mitogenic stimulus, but also to provide other important cellular capabilities important for cancer cells. These signaling pathways can lead to cell survival, angiogenic potential, and invasion.

loss is an example of this phenomenon and leads to benign and occasionally malignant tumors of neural crest origin (110). These single point mutations in *RAS* contribute to many of the "acquired capabilities" of cancer cells, including dysregulated growth, inappropriate survival, invasiveness, and angiogenesis (111).

In many cancers that lack *RAS* mutations, downstream effectors of RAS signaling are frequently altered, leading to acquisition of a similar set of neoplastic attributes (105). Mutations of the *BRAF* gene were initially found to be present in around 66% of melanomas and also approximately 12% of colon cancers (112). Recently, two unique somatic mutations of the *BRAF* gene have been identified in papillary thyroid carcinoma (113,114), and they offer genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway (113). Amplification of the *P110α* gene results in *PI3K* activation in 40% of ovarian tumors, while one of its downstream targets, AKT2, can also be amplified in breast and ovarian carcinomas (115). Finally, the *PTEN* tumor suppressor gene, acts as a phosphatase on specific downstream targets of PI3K, such as AKT, inactivating that pathway; *PTEN* deletions occurs in 30–40% of human cancers (116). Altogether, in human cancers the RAS proteins are not only

central mediators of both upstream growth factor receptors, but also their downstream targets play critical cellular roles, bestowing constitutively active mitogenic signals as well as multiple other important functions for oncogenesis.

Telomeres and telomerase

Telomeres are terminal structures at the ends of each eukaryotic chromosome and are composed of guanine rich, DNA 5′-TTAGGG-3′ repeats, as well as multiple DNA-binding proteins (117,118). At the end of each telomere is a single stranded 3′ overhang (119–121) that forms a large secondary loop structure, termed a T-loop (122). Telomeric DNA is maintained by telomerase, a RNA-dependent, DNA polymerase (123). Telomerase is composed of multiple subunits, two of which are crucial for enzymatic function, the RNA component (hTERC) and catalytic component (hTERT) (124) hTERT is the rate limiting component of the holoenzyme, as hTERT expression is restricted solely to cells that demonstrate telomerase enzymatic activity (125).

One of the main functions of telomeres are to protect the ends of chromosomes from forming illegitimate fusions, which would lead to genetic instability (126–128). Many DNA damage-associated proteins, such as the MRE11 complex (129) and Ku 70/80 (130,131) bind to telomere associated proteins. Thus, it has also been hypothesized that the telomere may serve as a cap, guarding the chromosome end from recognition as damaged DNA (132,133).

Both telomere length and maintenance are associated with human cell lifespan, genetic instability, senescence, immortalization, and transformation. In approximately 90% of human tumors, telomere maintenance and replicative immortality may be achieved through activation of telomerase; the remaining tumors may be maintained through "alternative lengthening of telomeres" (ALT), a telomerase-independent mechanism (134). Interestingly, studies examining malignant transformation in ALT cells lacking *P53* and *RB* function, but expressing oncogenic RAS, confirm that malignant transformation is impossible even with stable telomere lengths unless hTERT is ectopically introduced (135). Thus, 3′ overhang and T-loop maintenance by hTERT may have a role in the mechanism of transformation (136). Additionally, hTERT itself may serve some physical capping function that may be important for malignant transformation. Finally, it remains possible that hTERT has some either direct or indirect role in regulation of other important gene(s) that are critical for transformation.

In thyroid cancer, the correlation of telomere length to telomerase activity is poor, implying that there are other mechanisms that regulate telomere dynamics (137). However, most thyroid cancer cells do have sustained telomere length and have assayable telomerase activity while telomerase negative cell telomeres are likely maintained through ALT (138). Thus, similar to other types of cancers, telomere length is also important for thyroid cancer; although hTERT function and telomerase activity in thyroid cancer require further delineation of their mechanism(s) in cancer pathogenesis.

GENETIC INSTABILITY

Although the above discussed molecular alterations and their regulatory pathways are crucial to the development of a neoplastic cell, one additional hallmark may be

necessary to achieve a malignant state. This cardinal feature is genetic instability, which likely allows a cell to more rapidly acquire additional neoplastic attributes through the stepwise accumulation of mutations.

When the homeostatic mechanisms that guard the integrity of chromosomes are disrupted, additional genetic alterations may accumulate that lead to further deleterious effects. Though the various components of DNA damage detection, signaling, and repair mechanisms (139) are poorly understood, the operation of this repair machinery is incompletely understood, the adequate operation of this repair machinery is integral in preventing the survival of aberrant cells with neoplastic potential. An abnormal level of genetic instability is consistently found in many tumors (140). This instability, however, can be at either a DNA sequence level or at the level of the chromosome, in the form of aneuploidy.

Cytogenetic deformities are not an absolute finding in human cancers, as subtle DNA sequence changes can occasionally suffice to predispose to tumor formation. For instance, mutations in DNA mismatch repair genes, such as *MSH2* or *MLH1* (141), give rise to instability at the nucleotide sequence level since common replication errors can no longer be properly repaired. These tumors demonstrate microsatellite instability, which is detectable as short DNA sequence repeats seen scattered throughout the genome (142,143). These markers identify tumors that typically have two to three times as many single nucleotide mutations as compared to normal cells or cancers of the same histology but are mismatch repair proficient (144,145)

Different sets of proteins recognize and repair various types of physical DNA lesions. For instance, ultraviolet light induces adjacent pyrimidine dimerization, affecting DNA transcription and replication. Such events are repaired by the nucleotide excision repair proteins (NER) (146). In contrast, double-strand breaks (DSBs) develop in response to ionizing radiation, oxidative stress, or the stalling of replication forks at sites of DNA damage (147). These DSBs can only be repaired by homologous recombination or non-homologous end-joining (148). Thus, the cell must have unique mechanisms to recognize low levels of DNA damage at any location in the genome and shuttle the specific repair proteins required for that type of lesion. Examples include the *Xeroderma pigmentosum* group C protein involved in NER (146), MUTS proteins which bind to mismatched bases (149), and the Ku heterodimer which binds to DSBs (150). If these repair mechanisms are not in proper order, a dividing cell could improperly segregate, and depending on the type of lesion, possibly result in aneuploidy.

One important feature for a DNA damage response is the slowing or arrest of the defective cell at specific DNA damage checkpoint (151,152). This serves to delay important cell cycle transitions until repair has occurred. In human cells, the *ATR/ATM* signaling network, which can together detect a wide variety of DNA lesions through genomic surveillance during DNA replication, has a large role in this action. *ATR* disruption is lethal; *ATM* defects are not, although they are responsible for ataxia telangiectasia, which causes hypersensitivity to agents causing DSBs and increases cancer risk (152) *ATR,* when necessary, is likely to be the initiator of a global DNA damage response by activating downstream proteins like CHK1, CHK2, and RAD53. This leads to cell-cycle arrest, chromatin modulation, and further upregulation of other repair pathway proteins (139).

Alterations in DNA damage pathways, conferring genetic instability, may be early events in tumorigenesis, as is the case in microsatellite instability tumors. A heterogeneous population of cells will undergo a selective process in regards to instability. Cells with excess instability accumulate increasing amounts of DNA damage with continued proliferation eventually surpassing the threshold of viability, succumbing to apoptosis. Other cells with either too little or no genetic instability, halt at the natural barriers to immortalization. Certain cells with the appropriate amount of instability develop a survival and proliferative advantage by selecting out the right set of mutations, typically an accumulation of oncogenes and tumor suppressor genes that are now no longer able to be repaired (153). This eventually leads to clonal outgrowth and tumor formation.

ANGIOGENESIS, INVASION, AND METASTASIS

Although the initiating event in oncogenesis is not reliant upon angiogenesis, the continued success and maintainence of a tumor depends upon the utilization of this system for sustenance and eventual dissemination. In general, a solid tumor cannot grow successfully beyond 2 mm in diameter without neovascularization through the switch to the angiogenic phenotype (154,155). Thus, a tumor must acquire its own blood supply by developing new vascular structures that connect directly with existing host vasculature.

Angiogenesis is achieved by the secretion of proangiogenic factors, namely vascular endothelial growth factor (VEGF), angiopoetins, and basic fibroblast growth factors (bFGF); alternatively, the down-regulation of antiangiogenic proteins, such as endostatin, angiostatin, and thrombospondin-1 (TSP-1), also have a similar effect (155,156). These proteins signal to a complex array of downstream signaling proteins that cooperate to facilitate the overall regulation of angiogenesis. Unfortunately, this molecular circuitry remains poorly understood at this time.

An example of how tightly tumor angiogenesis may be tied to the other crucial pathways involved in tumorigenesis, is the intricate involvement of the p53 protein. Not only is p53 involved in G1 cell cycle regulation and apoptosis, but it also has a role in the regulation of angiogenesis, mainly through its interactions with TSP-1. The wild-type p53 protein can act on *TSP-1* promoter sequences and stimulate endogenous TSP-1 production (157). Since TSP-1 is a potent inhibitor of angiogenesis, wild-type *P53* gene expression loss, coincides with the switch to the angiogenic phenotype. Additionally, mutant p53 cells have been shown to upregulate VEGF (158), perhaps the most potent proangiogenic agent.

Invasion and metastasis are the final steps in tumor progression, but a unified set of responsible genetic elements has yet to be identified. Studies have described target genes that may be intimately involved in tumor migration, such as matrix metalloproteinases (159), however, the discovery of integral pathways to invasion and metastasis require further study. Just as studies have led to the discovery of common pathways for cell cycle progression, apoptosis, and autonomous growth stimulation, the discovery of a "metastasis pathway" could have utility for better understanding and treatment of cancers.

BEYOND CANCER GENOMES

To date, genetic alterations are still the easiest changes in cancer cells to detect and study experimentally. In the future, successful sequencing of an entire human cancer cell genome will certainly yield more important information for the further study of cancer. However, even with this data, many important alterations will be missed, since not all changes occur at the DNA sequence level and are instead occurring at a non-genetic level. For example, both epigenetic phenomena and post-translational modifications have critical roles in the regulation of important cellular capabilities that contribute to the neoplastic phenotype.

Epigenetic alterations

Alterations in gene expression that do not involve mutations of DNA sequences are epigenetic events. These arise during cell development and proliferation and serve as an additional method of adaptation to environmental and selective pressures. It has become clear in recent years, that epigenetic changes have an impact to the development of human cancers through silencing of tumor suppressors and DNA damage elements, chromosomal instability, and even activation of oncogenes (160,161).

Hypermethylation-mediated silencing of tumor suppressor genes may be important for tumor development since, among other advantages, it tends to lead to a selective cellular growth advantage (160,161). Methylation of cytosine residues at CpG dinucleotides occurs, and 70–80% of these dinucleotides are heavily methylated in human cells (162). Long GC-rich stretches of DNA in the human genome, termed CpG islands, are often uniquely associated with flanking genes and are protected from modification (163). These normally unmethylated CpG islands may become methylated in cancer cells, resulting in loss of expression of the flanking genes (160). This form of methylation-induced silencing affects tumor suppressors genes such as *CDH1* (164) and *P16*(165), both implicated in cancer development. Epigenetic alterations found in familial and non-hereditary forms of breast and colon cancer offer further supportive evidence for the role of methylation in neoplastic formation (166).

Although the exact mechanism of abnormal epigenetic changes leading to neoplasia is unknown, likely candidates include changes in expression of the key enzymes that regulate DNA methylation, such as the DNA methyltransferases (DNMTs). Overexpression of *DNMT* mRNA levels have been found in malignancies of various histological origin, including lung (167), colon (168,169), and ovarian (170) cancer cells. Further evidence is the fact that overexpression of DNMT1 leads to de novo methylation of CpG islands (171), and can facilitate cellular transformation (172,173).

CONCLUSIONS

The development of human cancer is a complex process that entails the alteration of multiple cell physiologic functions. Although possibilities for genetic and/or epigenetic alterations are innumerable, common principles have recently been delineated that ensure the success of any cell exhibiting a malignant phenotype. The specific pathways

and principles discussed above are known to contribute in an intimate manner to this process, but the foundation is just being set.

At this time, the study of cancer is frequently performed through experimentation with artificial cell lines or genetically altered and transformed primary cells. Although useful, it is still impossible to know the exact conditions and order of events that occur *in vivo* during metamorphosis to a neoplastic cell. Perhaps, in the future, when better cancer cell models are developed, we will define cancer by a set of distinct pathways intersecting with common principles.

Thyroid carcinomas, in particular, offer excellent models for studying cancer in general, as they offer a broad spectrum of tumor subtypes. For instance, both papillary and follicular tumors tend to be well-differentiated and may have utility in studying early genetic lesions involved in neoplastic formation. Anaplastic tumors offer the other end of the differentiation spectrum. Medullary thyroid carcinomas are associated with the MEN2 familial syndrome and supply another model to study genetic predisposition to cancer.

In the following chapters, the specific molecular defects involved in thyroid cancers will be discussed in detail. These defects may be specific for different subsets of thyroid carcinomas, but they typically lead into unifying pathways that phenotypically result in specific "acquired capabilities" for the cells. It is important to recognize these molecular changes, not only to gain greater understanding of the origin of thyroid cancers, but also to use this information for superior drug development and treatment.

Early stage thyroid cancer is standardly treated and often cured by surgical resection. However, advanced stage disease is still incurable, and current treatment measures with chemotherapy have not been shown to significantly improve morbidity or mortality. Thus, identifying these important molecular changes may one day lead to the development of new targeted therapies that can be readily tested in the metastatic setting.

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2. THE PATHOLOGY OF THYROID CANCER

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Thyroid nodules are extremely common in the general population; it has been estimated that about 20% of the population has a palpable thyroid nodule and approximately 70% has a nodule that can be detected by ultrasound (1). The prevalence of thyroid nodules is greater in women than in men, and multiple nodules are more common than solitary nodules.

The differential diagnosis of the thyroid nodule includes numerous entities, non-neoplastic and neoplastic, benign and malignant (2–5). The pathologist has an important role to play in their evaluation. The use of fine needle aspiration biopsy has significantly improved our ability to identify specific high-risk disorders and to facilitate their management in an expeditious and cost-effective manner. Patients who require surgery for further confirmation of the disease process rely upon the pathologist to correctly characterise their nodule and pathologists are actively involved in research to clarify the pathogenesis of thyroid disease.

While some of these entities are readily diagnosed based on specific features seen in a routine slide stained with conventional dyes, the morphologic evaluation of many of these lesions is fraught with controversy and diagnostic criteria are highly variable from Pathologist to Pathologist (6). Nevertheless, histology remains the gold standard against which we measure outcomes of cytology, intraoperative consultations, molecular and other studies, and it represents the basis on which we determine patient management and the efficacy of various therapies. Unfortunately, no current morphologic criteria provide adequate information to predict outcome for many follicular nodules of thyroid.

Advances in our understanding of the molecular basis of thyroid cancer will allow more accurate characterisation of specific subtypes of neoplasia and malignancy even on single cells obtained at fine needle aspiration biopsy. This should further enhance the usefulness of this technique and better guide the management of patients with a thyroid nodule.

THYROID FOLLICULAR HYPERPLASIA AND NEOPLASIA

Follicular nodules are the most commonly encountered problems in the surgical pathology of the thyroid. These lesions can be classified along the full spectrum of thyroid pathology from hyperplastic nodules to benign follicular adenomas and malignant follicular carcinomas.

Nodular goitre

Sporadic nodular goitre is characterised by numerous follicular nodules with heterogeneous architecture and cytology, features that have suggested a hyperplastic rather than neoplastic pathogenesis (7–10). The gland may be distorted by multiple bilateral nodules and can achieve weights of several hundred to a thousand grams, but this disorder is often identified as a dominant nodule in what clinically appears to be an otherwise normal gland. Histologically, the nodules are irregular; some are poorly circumscribed while others are surrounded by scarring and condensation of thyroid stroma, creating the appearance of complete encapsulation. They are composed of follicles of variable size and shape. Some follicles are large, with abundant colloid surrounded by flattened, cuboidal or columnar epithelial cells, often with cellular areas composed of small follicles lined by crowded epithelium with scant colloid in a small lumen, alone or pushing into large colloid-filled follicles as "Sanderson's polsters" (Figure 1). There may be focal necrosis, haemorrhage with haemosiderin deposition and cholesterol clefts, fibrosis, and granulation tissue; these degenerative changes are usually found in the centre of large nodules, creating stellate scars.

The morphologic classification of cellular follicular nodules in nodular glands can be extremely difficult. Hyperplasia may be extremely difficult to distinguish from neoplasia. Classical guidelines that allow distinction of a hyperplastic nodule from a follicular adenoma include the following: (i) multiple lesions suggest hyperplasia whereas a solitary lesion is likely to be neoplastic, (ii) a poorly encapsulated nodule is likely hyperplastic; a well developed capsule suggests a neoplastic growth, (iii) variable architecture reflects a polyclonal proliferation whereas uniform architecture suggests a monoclonal neoplastic growth, (iv) cytologic heterogeneity suggests hyperplasia; monotonous cytology is characteristic of neoplasia, (v) the presence of multiple lesions in hyperplasia means that areas similar to the lesion in question will be present in the adjacent gland; in contrast, neoplasms have a distinct morphology compared with the surrounding parenchyma, (vi) classically hyperplastic nodules are said not to compress the surrounding gland whereas neoplasms result in compression of the adjacent parenchyma. For the most part, large nodules in multinodular glands tend to be incompletely encapsulated and poorly demarcated from the internodular tissue. However, in some glands, large encapsulated lesions with relatively monotonous architecture

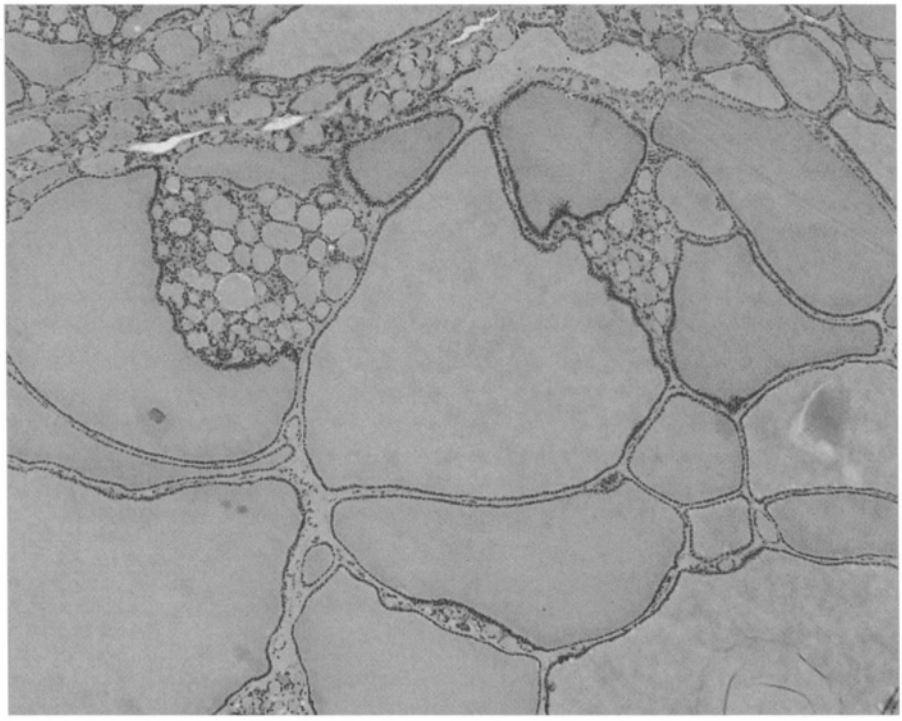

Figure 1. A hyperplastic nodule of thyroid is characterized by architectural and cytologic heterogeneity, usually with abundant colloid and often with subfollicle formation within larger follicles.

and cytology make distinction of hyperplasia from adenoma difficult. Many pathologists have applied nonspecific terms such as "adenomatoid nodules" to describe such lesions.

The pathophysiology of nodule formation remains poorly understood. The aetiology of this disorder has long remained elusive, since the goitres do not appear to be TSH-dependent (9). The work of Stüder suggests that the initial proliferation is a polyclonal one involving cells that are intrinsically more rapidly growing than their neighbours (7,10,11). While the stimulus for growth is not certain, high levels of circulating thyroid growth-stimulating immunoglobulins (TGI) and defects in T suppressor cell function have been documented in patients with sporadic nodular goitre (12,13), implicating autoimmunity in the pathogenesis of this disease. Drexhage and colleagues (12) compared immunoglobulin preparations of patients who have goitrous Graves' disease with those of patients who have sporadic nodular goitre and have found that the former are approximately 10-fold more potent in inducing growth than the latter. It has been postulated that the weaker stimuli result in proliferation of only the most sensitive of the heterogeneous follicular epithelial cell population, hence the nodularity, and that "toxic" nodular goitre results from preferential replication of cells which

are highly responsive to TSH stimulation (14,15). These data implicating an autoimmune pathogenesis explain the presence of chronic inflammation that is usually focally associated with nodular hyperplasia.

In contrast, molecular studies have indicated that the dominant nodules of multinodular goitres are monoclonal proliferations, and therefore represent benign neoplasms (8,16,17). It may be that these represent true adenomas arising in the background of a hyperplastic process that is mediated by growth stimulating immunoglobulins. Moreover, most hyperfunctioning nodules are also now thought to represent clonal benign neoplasms with activating mutations of the TSH receptor or Gsα (18–22). The evidence of clonal proliferation in sporadic nodular goitre and the identification of ras mutations as early events in morphologically classified hyperplastic nodules in this disorder (23) indicates that the thyroid is a site for the hyperplasia-neoplasia sequence. Nevertheless, clinical experience has shown us that the vast majority of these lesions remain entirely benign.

Follicular adenoma

Solitary follicular nodules have been unequivocally shown to be monoclonal (24,25,26) and in the absence of invasive behaviour or of markers of papillary carcinoma, these lesions are considered to be benign. Follicular adenomas are described as solitary encapsulated follicular lesions that exhibit a uniform architectural and cytologic pattern. However, the inclusion of nodules in sporadic nodular goitre in this category alters these criteria.

On aspiration cytology, the diagnosis of "follicular lesion" covers both follicular adenoma and follicular carcinoma, which are difficult if not impossible to distinguish because the diagnostic criteria do not rest on cytologic characteristics. The aspirate of a follicular lesion is usually cellular with follicular cells in sheets or microfollicular arrangements. The follicular cells are monotonous with elongated, bland nuclei and micronucleoli. Worrisome features include nuclear crowding, altered polarity, pleomorphism, macronucleoli and coarse chromatin. The main practical role of cytology is to distinguish a colloid nodule or papillary carcinoma from a follicular neoplasm.

Follicular adenomas are well delineated and usually thickly encapsulated neoplasms that can be classified histologically according the size or presence of follicles and degree of cellularity, each adenoma tending to have a consistent microscopic pattern (Figure 2). The subclassification of follicular adenomas into simple, microfollicular, trabecular, oxyphil, atypical, papillary and signet ring cell types has no prognostic significance.

Atypical adenomas are highly cellular tumours with unusual gross and/or histologic appearances that suggest the possibility of malignancy but these tumours lack evidence of invasion. They may have necrosis, infarction, numerous mitoses or unusual cellularity. Many so-called "atypical adenomas" are indeed papillary carcinomas. The distinction of an encapsulated follicular variant papillary carcinoma from follicular adenoma relies on cytologic characteristics. The presence of the cytologic features of papillary carcinoma described below should indicate that diagnosis, despite lack of invasion. Whether some follicular nodules classified histologically as adenomas have the biologic potential to become carcinoma is not clear; aneuploid cell populations

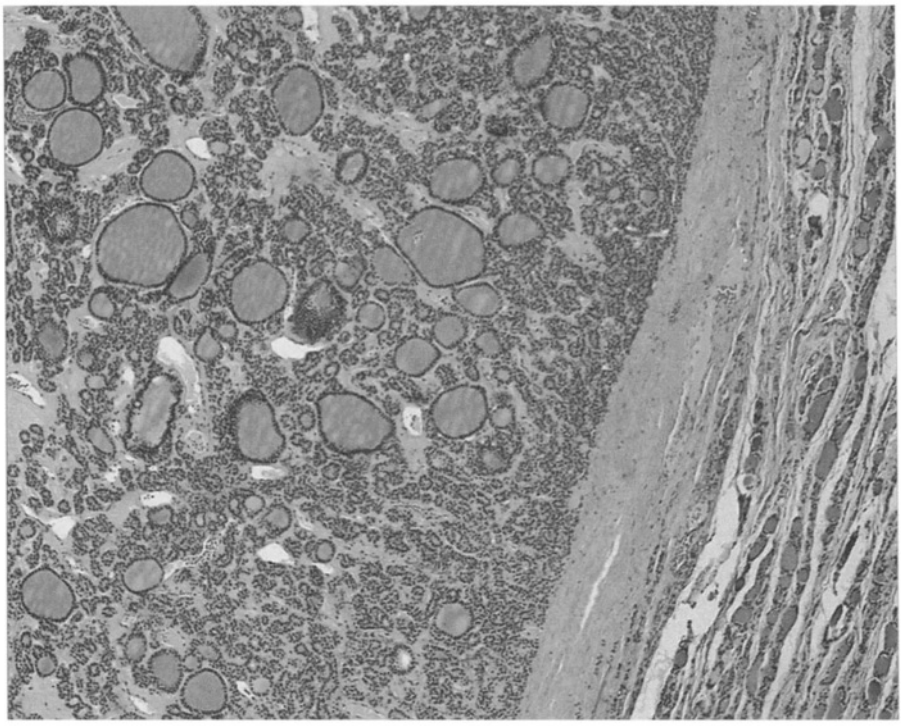

Figure 2. A follicular adenoma is usually well delineated and often surrounded by a thick fibrous capsule. The lesion is generally characterized by uniformity of architecture and cytology.

have been described in a significant percentage of these lesions, suggesting that some of these may represent carcinoma in situ.

Follicular carcinoma

Follicular adenoma and most follicular carcinomas are indistinct with respect to their clinical presentation, radiographic appearance, cytologic findings and microscopic features. In most cases, the parenchymal component of both tumour types is essentially the same histomorphologically. The distinction between these two conditions has been considered possible only by recognition of invasion or metastasis. As indicated above, some encapsulated follicular adenomas exhibit evidence of aneuploidy and may in fact represent in situ follicular carcinomas.

Nuclear and cellular atypia and mitotic figures may be present in adenomas as well as in carcinomas and therefore cytologic characteristics are not helpful. Most follicular tumours are composed of cells with nuclei that are round to oval with uniformly speckled chromatin; the nuclei are evenly spaced and lack the crowded, overlapping appearance found in papillary carcinoma. As stated previously, these lesions cannot be

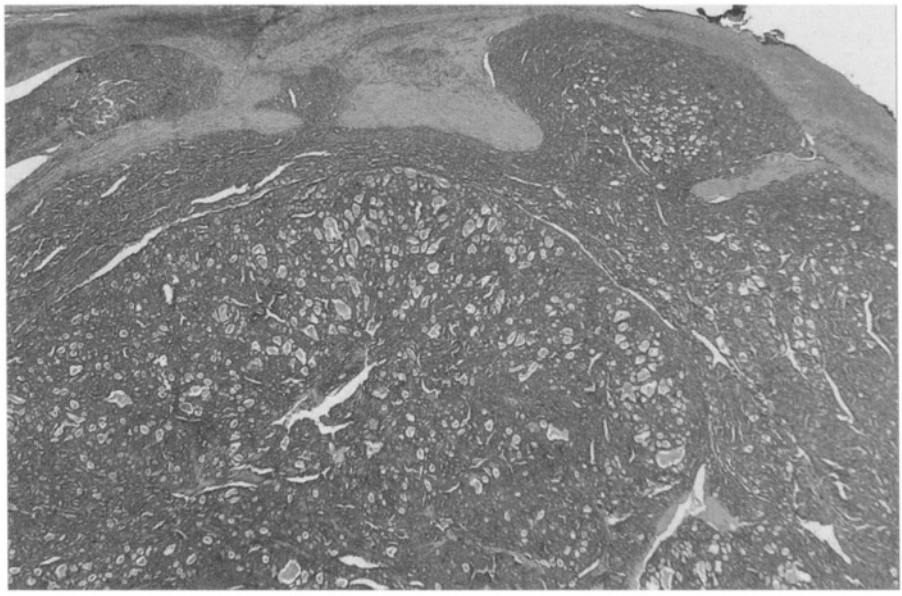

Figure 3. The distinction of follicular carcinoma from follicular adenoma relies on unequivocal evidence of invasive behaviour, as in the multifocal mushrooming capsular penetration exhibited by this lesion.

diagnosed as benign or malignant by fine needle aspiration; the diagnosis should be restricted to "follicular lesion".

Follicular carcinoma can only be diagnosed by the pathologist on high quality sections of well-fixed tissues that demonstrate capsular and/or vascular invasion (Figure 3). At the time of intraoperative consultation, frozen section will reveal only a very small number of these lesions, since the likelihood of identifying microinvasive foci on a single frozen section are low. The use of multiple frozen sections is not cost effective in the evaluation of these lesions (27).

Follicular carcinomas are divided into groups that reflect the biology of tumour growth and metastasis. *Widely invasive follicular carcinomas,* which are usually identifiable as invasive grossly, and certainly are not difficult to recognise as invasive microscopically, carry a poor prognosis with a 25–45% ten year survival (28,29). However, such lesions tend to be insular carcinomas (see below). In contrast, the more common scenario is that of minimal capsular invasion and patients with these tumors have an excellent prognosis. The diagnosis of follicular neoplasms requires very careful and thorough examination of the entire capsule of the follicular neoplasm by the pathologist (30). *Minimally invasive follicular carcinoma* is identified by invasion through but not widely beyond the capsule. Borderline lesions include those with invasion into the capsule beyond the bulk of the lesion but not through the full thickness of the capsule or situations in which islands of tumor are trapped within a capsule, associated with perpendicular rupture of collagen. The finding of nests, cords, or individual tumour

cells within a tumour capsule leads some pathologists to the diagnosis of minimally invasive follicular carcinoma, however, this may represent an artefact in a patient who has undergone fine needle aspiration biopsy, with trapping by fibrosis or displacement of tumour cells into the capsule. The pathologist is therefore advised to carefully search for evidence of fine needle aspiration biopsy in the adjacent tissue. This would include finding focal haemorrhage, deposition of haemosiderin-laden macrophages, the presence of granulation tissue and/or fibrosis, all of which would indicate a needle biopsy site and the possibility of artifactual invasion rather than genuine invasion.

The concept of unencapsulated follicular carcinoma was raised by the identification of tumours that lack a capsule. In one report of four such cases, one patient developed metastases, and this gave rise to citations of a 25% metastatic rate by such lesions (31). However, this has not been substantiated in larger series and this concept has largely been abandoned.

Patients with minimally invasive follicular carcinomas are on average about 10 years younger than those with widely infiltrative carcinomas and since traces of capsule are found in about 24% of widely invasive lesions, it is possible that encapsulated follicular carcinoma is a precursor of the widely invasive lesion (32). Minimally invasive carcinomas have ten year survival rates of 70–100% (33) and therefore some argue that this disease does not warrant the painstaking search for microscopic invasion that distinguishes it from follicular adenoma. Nevertheless, the investigators that have reported these promising data have treated their patients for carcinoma rather than for benign disease (34).

Vasculoinvasive follicular carcinomas are aggressive and require management accordingly. While vascular invasion is more reliable for the diagnosis of malignancy, again the criteria are vague. Vascular invasion cannot be evaluated within the tumour and therefore again the circumference of the lesion is the site that warrants careful examination. Bulging of tumour under endothelium does not qualify as vascular invasion if the endothelium is intact. Nests of tumour cells within an endothelial lumen generally are accepted as representing invasion, however, it is recognised that artefactual implantation of tumour cells into blood vessels can occur during the surgical procedure or sectioning. Therefore, invasive tumour cells infiltrating the wall of an endothelial-lined space and thrombus adherent to intravascular tumour are required to distinguish true invasion from artefact.

Elastin stains are of little value in assessing vascular invasion, since the involved vessels are usually thin-walled veins with little if any elastic tissue. Immunohistochemical markers such as factor-8 related antigen, type IV collagen, CD31 and CD34 can be used to improve the recognition of vascular invasion in follicular carcinoma.

It is obvious that the diagnosis of malignancy in well-differentiated encapsulated follicular tumours rests on subjective criteria. The search for objective markers of malignancy has yielded only one candidate thus far; HBME-1, a marker of mesothelial cells, is immunohistochemically detected in 40% of thyroid follicular malignancies of papillary or follicular differentiation (35–37) and has been used successfully in cytology studies as well as histopathologic evaluation (Figure 4) of thyroid nodules (36,37). Recent studies have advocated the use of galectin-3 as another marker of malignancy

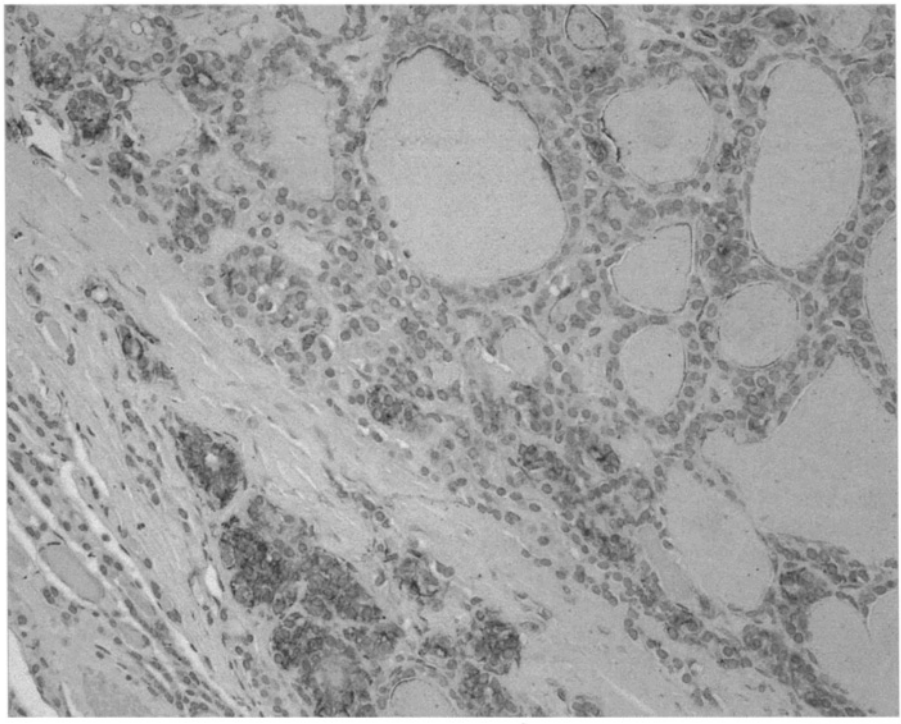

Figure 4. Immunoreactivity for HBME-1 is a feature of thyroid malignancies of epithelial cell derivation, such as this follicular carcinoma with superficial capsular invasion.

(38–40). While this marker also stains normal, hyperplastic and inflamed thyroid tissue, positivity in malignancies is more diffuse and strong. These data should limit the application of this technique for cytology but this has not been widely recognized (41).

Another molecular marker with application to follicular carcinoma is a gene rearrangement that involves the thyroid transcription factor Pax 8 and the peroxisome proliferator-activated receptor γ (PPARγ) gene (42). Normal thyroid follicular cells express Pax 8 at high levels; this transcription factor is essential for thyroid development, involved in regulating expression of the endogenous genes encoding thyroglobulin, thyroperoxidase, and the sodium/iodide symporter. PPARγ, a transcription factor that is implicated in the inhibition of cell growth and promotion of cell differentiation, is also expressed by normal thyroid follicular epithelium. However, this in-frame rearrangement results in a fusion protein that likely interferes with the normal function of both differentiating factors, thereby explaining its potential role in thyroid tumorigenesis. The rearrangement is most reliably detected using fluorescence in situ hybridization (FISH) technology to identify the translocation of the two genes that are normally localized on chromosomes 2q13 (Pax 8) and 3p25 (PPARγ). The presence of overexpressed

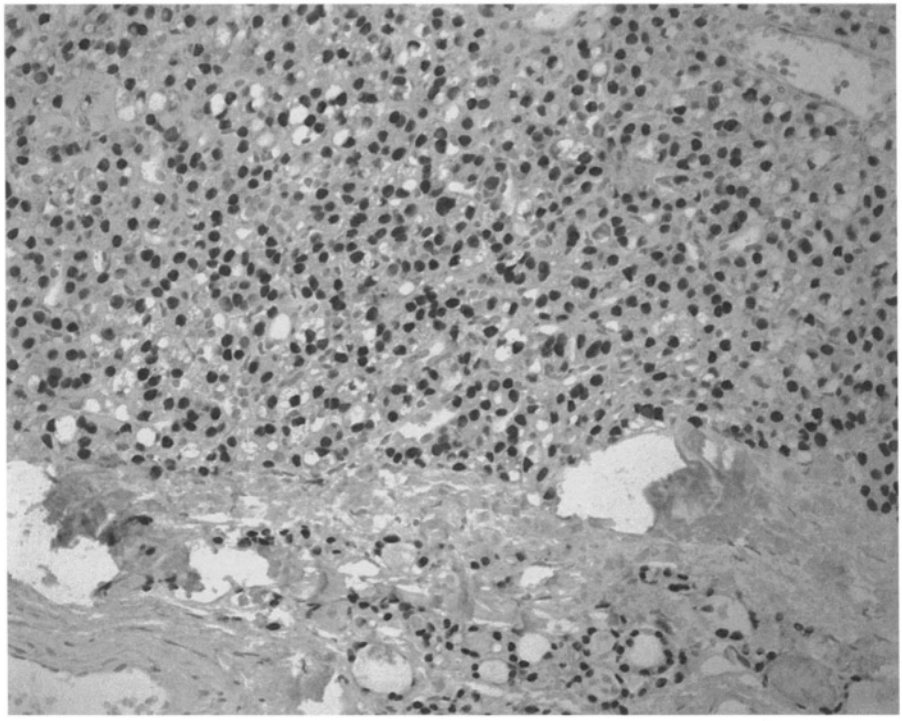

Figure 5. Overexpression of PPAR-γ due to a Pax 8-PPAR-γ gene rearrangement is detectable by immunohistochemistry. This finding has been correlated with aggressive behaviour, usually with vascular invasion.

protein can also be identified using immunostains for PPARγ where strong nuclear staining identifies tumours harboring a translocation (Figure 5). Although follicular carcinomas of thyroid are rare (43), and the numbers of cases studied has been small, it appears to be a useful tool for the diagnosis of malignancy in thyroid follicular lesions, particularly to predict vascular spread and aggressive behaviour (44).

DNA aneuploidy is a well-recognised feature of human malignant tumors and it was initially hoped that ploidy analyses could help to distinguish adenomas from carcinomas of the thyroid. However, it has now been recognised that about 27% of follicular adenomas are aneuploid and about 40% of follicular carcinomas are diploid (45). Therefore such measurements are of limited diagnostic value for the individual patient. In contrast, however, ploidy may be a useful adjunct in determining prognosis.

The significance of this diagnosis must be interpreted in light of clinical data that assess the behaviour of this disorder. The dominant determinant of cause-specific mortality in patients with follicular carcinoma is the presence of distant metastases (46–48). Most studies have indicated that morbidity and mortality for patients with non-metastatic encapsulated follicular carcinoma is very low and correlates better with

patient age than with any other parameter. Some have suggested that capsular invasion alone does not alter the incidence of distant metastases or cancer-related death (33).

Since the incidence of follicular carcinoma is low (43), most investigators still advocate total thyroidectomy and radioactive iodine therapy (34,49,50). The rationale for total thyroidectomy is not bilateral carcinoma; multifocal disease in follicular carcinoma is exceedingly rare and the identification of occult papillary carcinoma in the contralateral lobe is not an indication for further surgery (51). The only logical rationale for completion thyroidectomy is to allow selective uptake of radioactive iodine by metastatic tumour deposits rather than by residual thyroid gland. Uptake of radioactive iodine by distant metastases is a favourable prognostic factor and is improved by pretherapeutic total thyroidectomy, resulting in improved survival (52–54). In contrast, external beam radiotherapy is not thought to be of use in patients with differentiated thyroid carcinoma, apart from those with locally advanced tumours such as widely invasive follicular carcinomas that involve extrathyroidal soft tissues of the neck and cannot be completely resected (54).

The last few decades have seen a decrease in the incidence of follicular thyroid carcinoma, probably due to dietary iodine supplementation (43). However, misdiagnosis of this tumour continues. Benign lesions, such as partly encapsulated hyperplastic nodules or nodules exhibiting pseudoinvasion after fine needle aspiration (55), are often overdiagnosed as malignant; papillary carcinomas with follicular architecture are often misinterpreted as follicular carcinoma. The clinical features, pathophysiology and biological behaviour of follicular cancer differ significantly from those of the entities with which it is often confused. Only careful histopathologic classification will allow correct evaluation of treatment options and prognosis.

PAPILLARY LESIONS OF THYROID

Hyperplastic nodules and adenomas with papillary architecture

The "papillary hyperplastic nodule" of the thyroid is usually identified in girls, usually teenagers in and around the age of menarche. These present as solitary nodules and it is unusual for them to be associated with clinical hyperfunction, although that might occur. These lesion are distinguished from papillary carcinoma in that they are totally encapsulated, often show central cystic change, have subfollicle formation in the centres of broad oedematous papillae, and do not show nuclear features of papillary carcinoma (Figure 6). Although one analysis of clonality has suggested that these are polyclonal hyperplasias (56), the detection of Gsα or TSH receptor activating mutations in such nodules suggests that they are neoplasms (18–22). Their behaviour is almost always benign. Some have advocated the name "papillary adenoma" for these tumours; while scientifically appropriate, this term carries historical connotations that some feel are unacceptable (5).

In adults, one can have a similar histologic appearance in a "hot" nodule, that is, a thyroid nodule that is associated with clinical toxicity or subclinical hyperthyroidism and iodine uptake on scan. These lesions may be solitary but are often seen in the setting of sporadic nodular goitre (see above).

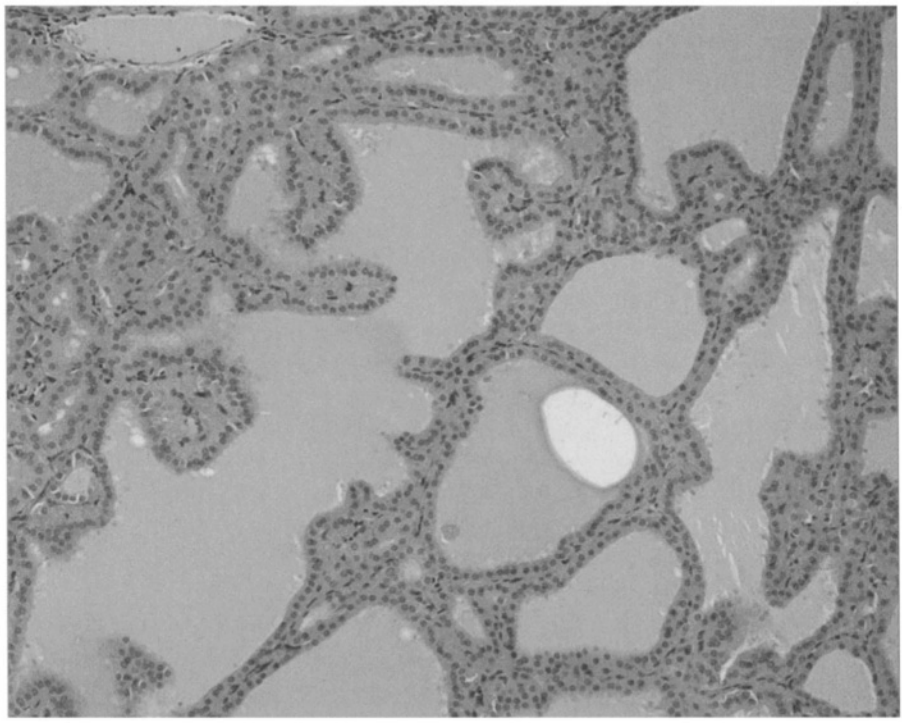

Figure 6. A benign adenoma with true papillary architecture has an organized centripedal orientation of the papillae that are lined by cells with bland nuclei that lack the atypia of papillary carcinoma.

On fine needle aspiration and on histologic evaluation, particularly at frozen section, papillary hyperplastic nodules or adenomas can be very alarming and lead to a false positive diagnosis of papillary carcinoma. Indeed, these entities give rise to well formed papillae but on higher magnification, the cytologic criteria for the diagnosis of papillary carcinoma, including powdery nuclear chromatin, multiple micro- and/or macronucleoli, intranuclear cytoplasmic inclusions, and linear chromatin grooves (57), are lacking.

Papillary carcinoma

Papillary carcinoma comprises at least 80% of thyroid epithelial malignancies diagnosed in regions of the world where goitres are not endemic. The terminology is misleading; papillary carcinomas can exhibit papillary architecture (Figure 7) but they may also have follicular (Figure 8) or mixed papillary and follicular patterns (58–62). It is now recognised that the diagnosis of papillary carcinoma is based on what the WHO has described as "a distinctive set of nuclear characteristics" (63). In contrast to true follicular carcinomas, these lesions are usually more indolent and most have an excellent prognosis with a 20 year survival rate of 90% or better (64,65).

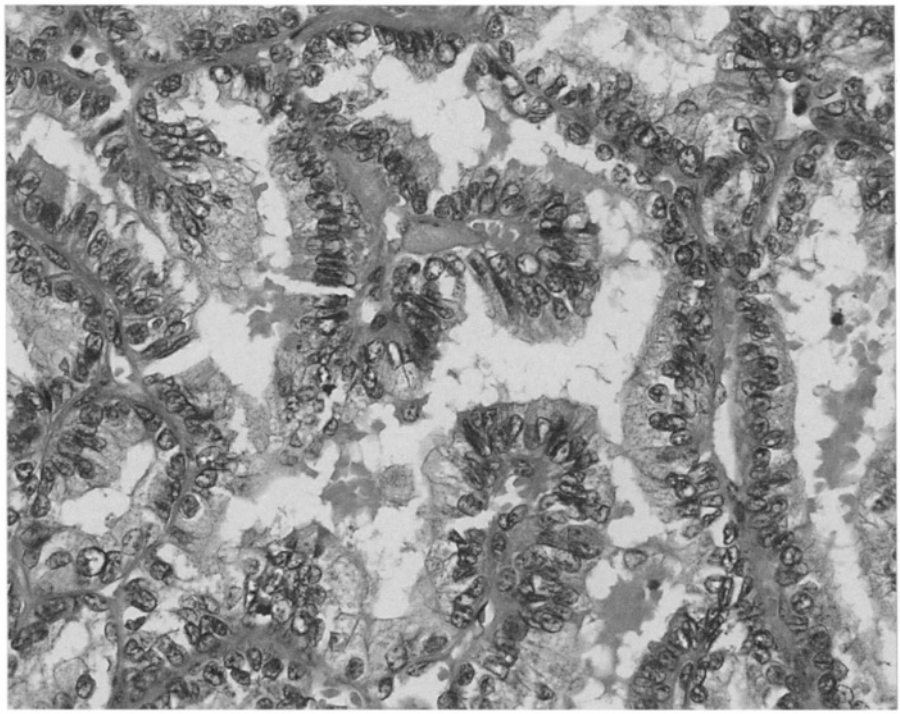

Figure 7. Papillary carcinoma was named as such because many of these lesions have complex papillary architecture. The papillae are lined by crowded cells with nuclear atypia.

The defining nuclear features are readily seen on cytology of fine needle aspirates as well as on histologic sections (Figure 9). They include an alteration of the size and the roundness of the normal follicular cell nucleus to one that is large and oval. Due to peripheral margination of chromatin, the centre of the nucleus has an empty appearance, which when pronounced has been termed "ground glass" (66). The chromatin and nucleolus are pushed to the edge of the nucleus. The nuclear contour is strikingly irregular, resulting in a "crumpled paper" appearance, intranuclear cytoplasmic pseudoinclusions and nuclear grooves (67,68). No one specific feature is absolutely diagnostic of papillary carcinoma; a constellation or combination of nuclear features is required for the diagnosis.

Papillary carcinomas may be multifocal; this has been interpreted as reflective of intraglandular lymphatic dissemination, but the identification of such microcarcinomas in up to 24% of the population (69) and the detection of different clonal rearrangements in multifocal lesions (70) support the interpretation of multifocal primary lesions in most patients. Nevertheless, when these lesions do invade, they show preference for lymphatic involvement with a high percentage of regional lymph node metastases.

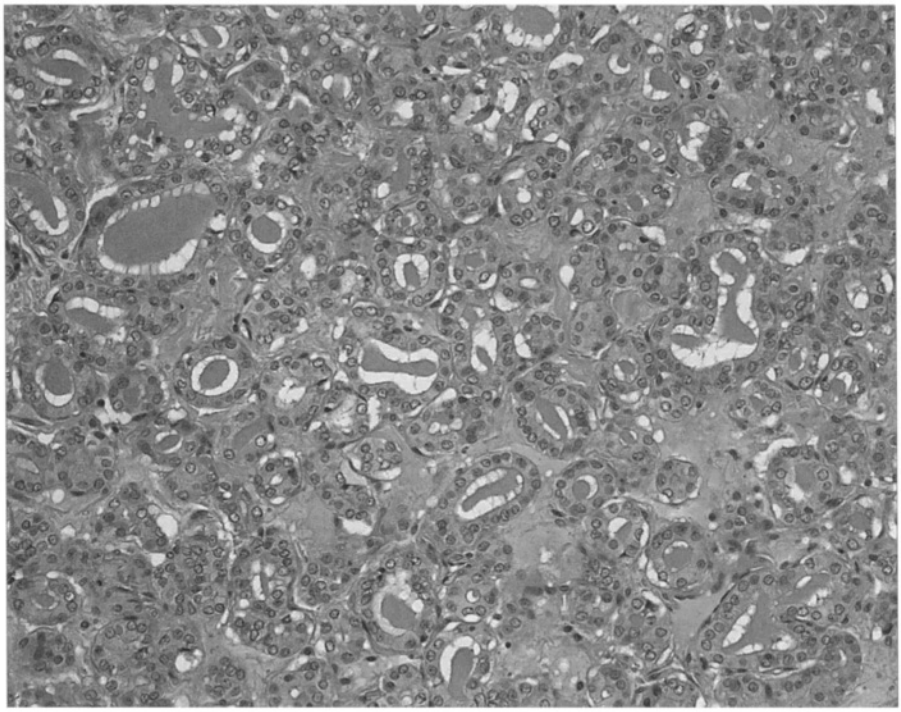

Figure 8. Papillary carcinoma may have partial or complete follicular architecture. The follicles usually harbour hypereosinophilic colloid that has peripheral scalloping. The nuclei exhibit characteristic atypia.

Metastases beyond the neck are unusual in common papillary carcinoma and probably only occur in about 5 to 7% of cases.

The most useful prognostic markers in papillary carcinoma are patient variables, tumour size and extent of disease (28,29,53,71). Patients under the age of 45 usually have an excellent prognosis; in contrast those over 45 years of age generally have a poorer outlook. Sex has also been said in the past to be an important determinant of tumour biology but more recent studies have suggested that there is no major difference in the behaviour of papillary carcinoma in men compared to women. Tumour size is exceedingly important (72). Tumours less than 1 cm are common and appear to be different biologically than larger tumours (73–75); a recent study has shown that occult papillary carcinomas are identified in up to 24% of the population in thyroids that are removed for non-malignant or unrelated disease (69). In contrast, tumours greater than 1 cm are thought to be of clinical significance and those larger than 3 cm generally have a poorer prognosis than do smaller tumours. The presence of cervical lymph node metastasis, whether microscopic or identified clinically, is thought to increase the risk of recurrence of disease but has been shown to have no impact on mortality.

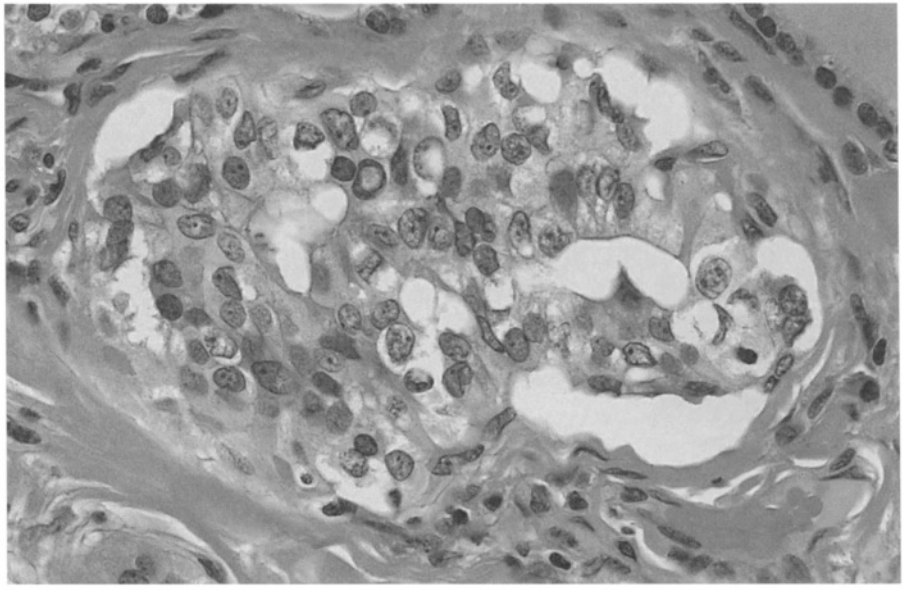

Figure 9. The nuclear features of papillary carcinoma encompass clearing of nucleoplasm and peripheral margination of chromatin, prominent and often multiple nucleoli, and irregular nuclear contours that result in formation of linear grooves and cytoplasmic pseudoinclusions.

Extrathyroidal extension, in contrast, predicts a worse prognosis and the presence of distant metastases is the hallmark of an aggressive tumour that will bear the potential for high mortality.

Grossly, papillary carcinomas vary in size from microcarcinomas (also called small, tiny, occult and minute), which are defined as lesions measuring less than 1 cm (usually 4 to 7 mm) to large neoplasms that extend extrathyroidally beyond the thyroid capsule into surrounding soft tissue. The bulk of clinical papillary carcinomas are intrathyroidal tumours confined within the capsule of the thyroid and may have an encapsulated appearance (this is usual for the follicular variant) or an irregularly infiltrative appearance. One can see gross cystic change but usually papillary carcinoma is a firm tumor and some are calcified or even ossified.

Microscopically, papillary carcinomas classically are composed of papillae but virtually all contain follicular elements. Ghosts of dead papillae or infarcted papillae calcify with a concentric whorled pattern that is characteristic of psammoma bodies (Figure 10); these are found in 40 to 50% of classical papillary carcinomas, either in the tumour stroma or in the surrounding non-tumourous thyroid, but they are distinctively uncommon in lesions with follicular architecture.

Inflammatory infiltrates within papillary carcinomas and in the surrounding thyroid parenchyma have been noted by several authors, although the prognostic significance of this is not clear (76,77). Some people have postulated that this inflammatory infiltrate

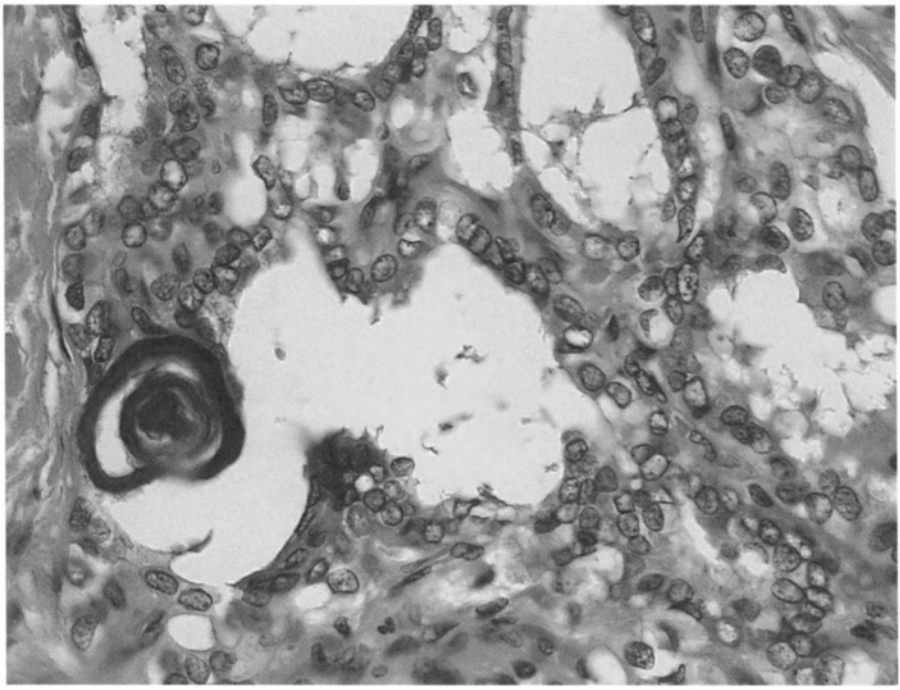

Figure 10. A minority of papillary carcinomas form psammoma bodies, concentric calcifications.

may indicate host-tumour immune interactions that are responsible for the general indolence of this type of thyroid carcinoma (76).

Variants

Although there are reports to the contrary, the exact histological variant of papillary carcinoma usually cannot be predicted from the appearance of the fine needle aspirate (78). Nevertheless, the histologic distinctions, which are characteristic (3,5,63,79–81), are of prognostic value.

Papillary microcarcinoma (75), *cystic and encapsulated variants of papillary carcinoma* (82) have an apparently better prognosis than usual papillary carcinoma.

The follicular variant has been recognized more frequently in the past 20 years (5,63,83,84). It has either been misdiagnosed as follicular carcinoma or underdiagnosed as follicular adenoma or atypical adenoma. Any lesion with follicular architecture and characteristic nuclear features of papillary carcinoma should be classified as this tumor. Infiltrating areas and metastases may exhibit a more striking papillary appearance and may even have psammoma bodies. It is unclear what the ultimate biological and clinical behaviour of follicular variant is, since some of these may be underdiagnosed as atypical adenomas and it is likely that the initial reports of this tumour included the aggressive biological spectrum of this variant.

The presence of cytologic atypia may raise the possibility of papillary carcinoma without being sufficiently convincing for unequivocal diagnosis. In some cases the changes may be induced by previous needle biopsy. The presence of haemorrhage, granulation tissue and hemosiderin laden-macrophages, inflammation and foreign body giant cells and even foreign material should point to this possibility. There may be calcification that can be mistaken for psammoma bodies. Various metaplastic changes occur. These changes have been described with the acronym WHAFFT which stands for *"Worrisome Histological Alterations Following FNA of Thyroid"* (55). The diagnosis of papillary carcinoma should not be made in this situation unless the lesion is entirely unequivocal.

In cases where the features are suggestive of papillary carcinoma but not entirely diagnostic, specific markers of this tumour as well as other markers if malignancy may be useful. A proportion of malignancies of thyroid follicular epithelium stain for HBME-1 (35–37)and some investigators have advocated the use of galectin-3 as a marker of thyroid carcinoma (38–41). Stains for high molecular weight cytokeratins may be useful. This technique, also considered controversial in the past, has recently been shown to be useful when applied to paraffin sections with microwave antigen retrieval (85). The results of these studies indicate that moderate to strong diffuse staining is confined to papillary carcinoma (Figure 11) whereas follicular neoplasms and hyperplastic nodules are negative or show only focal staining in areas of reaction to degeneration or previous fine needle aspiration biopsy. Nevertheless, only approximately 60% of papillary carcinomas are positive; a positive stain is therefore helpful, but negative stains are unable to assist in the diagnostic process.

The diagnosis of this entity has been further advanced by the recognition of a family of gene rearrangements that are specific to papillary carcinoma (86). The *ret*/PTC oncogenes (1 through 15, depending on the site of rearrangement, reviewed in (87)) are the result of DNA damage with rearrangements that transpose various cellular genes adjacent to the gene encoding the intracellular tyrosine kinase domain of the *ret* protooncogene (88–92). The rearrangements result in constitutive tyrosine kinase activation and translocation of the fusion protein to the cytoplasm (93). Animal models have shown the tumorigenicity of these fusion proteins (94–96); the rearrangements are common in radiation-induced tumors (97–101) but are also found in sporadic papillary carcinomas (102–105) and appear to be an early event in tumour development (106). Immunohistochemical staining with antisera directed against the carboxy terminus of *ret* allows rapid and clinically useful detection of this marker of papillary carcinoma which is present in almost 80% of occult papillary microcarcinomas and approximately 50% of clinically detected lesions (70). Again, a negative stain is not useful, however, the combination of high molecular weight cytokeratins and *ret* provides a set of immunohistochemical markers that aids in the diagnosis of papillary carcinoma in equivocal cases (107). At the moment, antisera or antibodies to *ret* offer inconsistent detection of these rearrangements and molecular diagnostics using RT-PCR remain the gold standard of this diagnostic tool. This methodology has been applied to FNA specimens when collected in suspension (108) and application of this technique enhances the cytological diagnosis of papillary carcinoma.

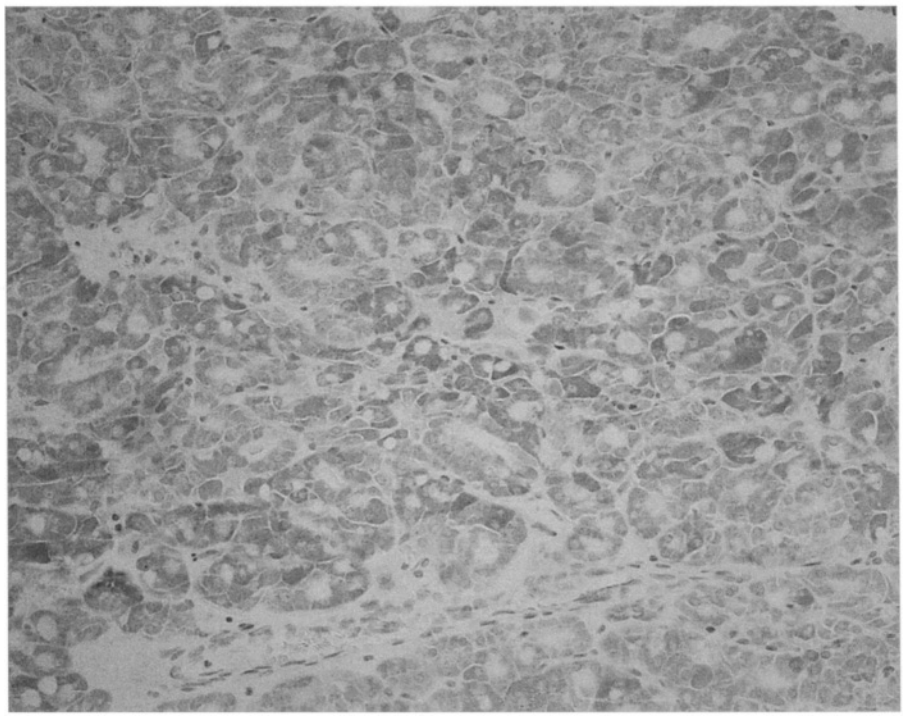

Figure 11. A diffuse cytoplasmic staining pattern for high molecular weight cytokeratins and cytokeratin-19 are the hallmark of papillary carcinomas of all types.

An unusual variant of papillary carcinoma is the *hyalinizing trabecular tumour.* This tumour was originally described by pioneers such as Zipkin in 1905 (109), Masson in 1922 (110), and Ward et al. in 1982 (111). The terminology "hyalinizing trabecular adenoma" (HTA) was defined by Carney et al. in 1987 (112). This lesion has also been designated "paraganglioma-like adenoma of thyroid" (PLAT) by Bronner et al (113) because of its unusual histologic pattern (Figure 12). Since the original descriptions, a malignant counterpart, hyalinizing trabecular carcinoma (HTC), has been described (114–116) and both HTA and HTC are now incorporated under the umbrella of hyalinizing trabecular tumors (HTT). Their main importance lies in the fact that they are sometimes mistaken for other entities such as paraganglioma or medullary carcinoma (112). Immunohistochemical stains for neuroendocrine markers will easily discriminate between HTT and paraganglioma or medullary carcinoma. However, it was noted that many features of HTT were also seen in papillary carcinoma; both lesions are of thyroid follicular epithelial origin and therefore both express thyroglobulin; several cases of HTT have been reported in patients with Hashimoto's thyroiditis or who have had a history of neck irradiation (117); HTT can co-exist with papillary carcinoma (5); HTT can often exhibit papillary carcinoma-like histologic features such as

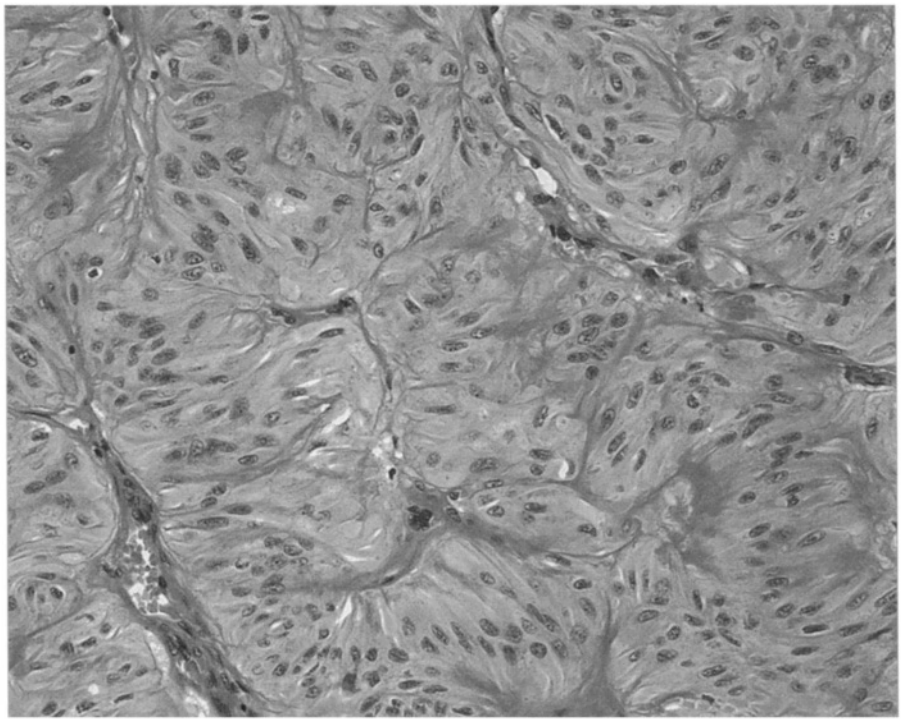

Figure 12. The hyalinizing trabecular tumour of thyroid is characterized by elongated spindle-shaped cells with hyaline cytoplasm, as well as stromal hyaline fibrosis. The tumour cells exhibit the nuclear atypia of papillary carcinoma.

psammoma-body formation, and characteristic nuclear changes including elongation, hypochromasia, grooves and pseudoinclusions (112). Based on these observations, a number of authors have hypothesized that these two entities are related and may in fact share a similar pathogenesis (118). These lesions are generally well delineated tumors characterised architecturally by trabecular and nesting architecture and elongated tumor cells which can have abundant pale eosinophilic cytoplasm and scattered "yellow bodies" (112,113,117,119). There is perivascular hyaline fibrosis and the cytoplasmic hyaline is usually identified as cytoplasmic filaments of cytokeratin. Occasional cases are immunoreactive for S100 protein. Most importantly, the tumour cells harbour large clear nuclei with irregular and elongated contours, grooves and inclusions as well as micronucleoli, features of papillary carcinoma. Application of *ret*/PTC analysis identified rearrangements in these lesions at a rate identical to that found in other papillary carcinomas (120,121) and many pathologists now consider this to be a variant of papillary carcinoma. However, some continue to maintain that these are distinct lesions (122,123) .

The *diffuse sclerosis variant* occurs in young individuals and often presents as goitre without a specific mass lesion (124–127). This tumour microscopically involves thyroid

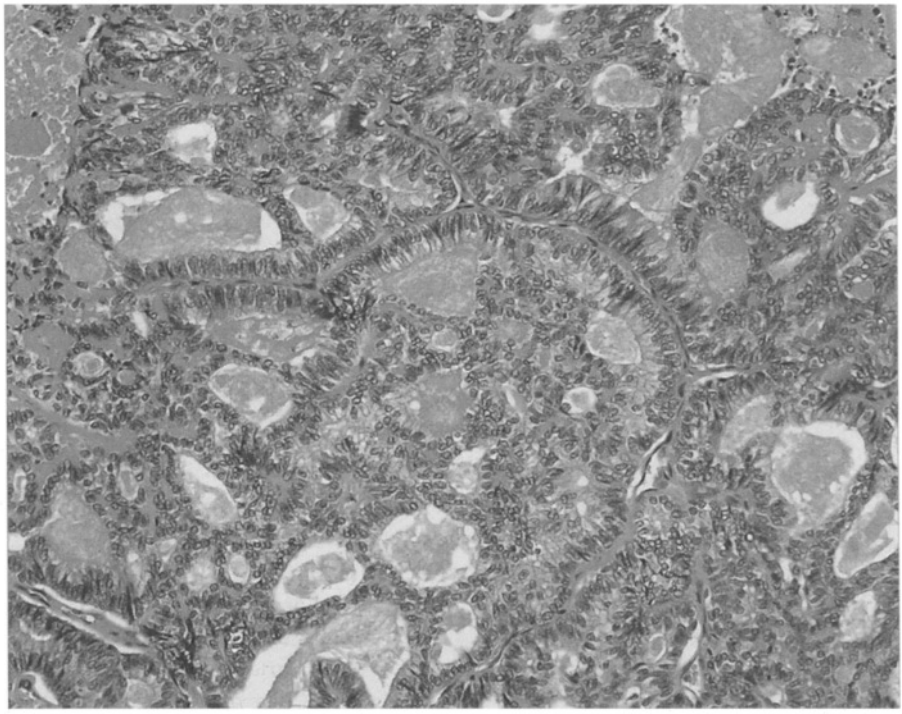

Figure 13. Patients with a family history of familial adenomatous polyposis and a germline mutation of the APC gene develop a type of papillary thyroid carcinoma that is characterized by a prominent cribriform and/or morular architecture.

lymphatics, exhibits squamous metaplasia and forms numerous psammoma bodies, giving it a very gritty appearance when examined grossly. These tumours almost always have lymph node metastases at presentation and 25% have lung metastases as well. It is interesting that about 10% of the paediatric thyroid cancers that occurred following the Chernobyl nuclear accident in 1986 were of the diffuse sclerosis type (128).

An unusual variant of papillary thyroid carcinoma known as the *cribriform-morular variant* has been identified in patients who harbour mutations of the APC gene that is responsible for familial adenomatous polyposis (FAP) syndrome (25,62,129). These lesions have unusual architecture as their name implies; they exhibit intricate admixtures of cribriform, follicular, papillary, trabecular, and solid patterns of growth (Figure 13), with morular or squamoid areas. Cribriform structures are prominent. The tumor cells are generally cuboidal or tall, with nuclear pseudostratification. Vascular and capsular invasion are common in these lesions, and while they may exhibit lymph node metastasis, there are no data to suggest that they have worse outcomes than other conventional forms of papillary carcinoma. They harbour *ret*/PTC gene rearrangements and do not exhibit loss of heterozygosity of the normal allele of the APC gene to explain an independent mechanism of tumorigenesis. Alterations in the APC

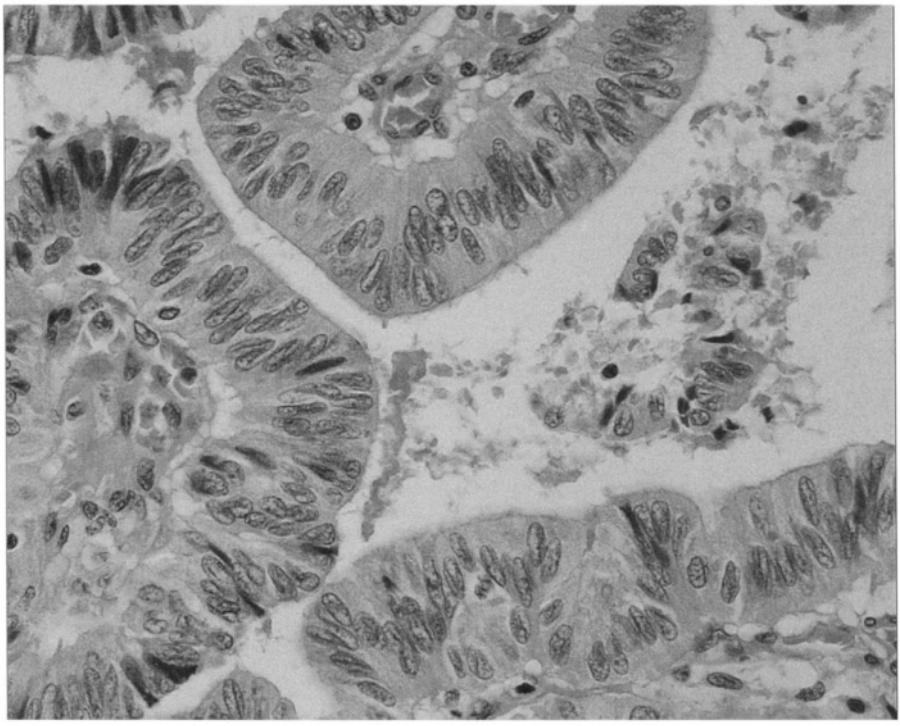

Figure 14. Tall cell papillary carcinoma is composed of a majority of tumour cells that have a height-to-width ratio that exceeds 3:1. These lesions are usually more aggressive than conventional papillary carcinomas.

gene are not thought to underlie the more common sporadic thyroid carcinomas (130,131).

Aggressive variants of papillary carcinoma include *the tall cell variant* and probably related lesions, the *trabecular and columnar cell variant* (132–137). The tall cell variant is defined as a tumor composed of cells that have a height to width ratio that exceeds 3:1 (Figure 14). They usually have complex papillary architecture and may show focal tumor cell necrosis. Tall cells generally have abundant eosinophilic cytoplasm. Columnar cells are similar to tall cells but generally are more crowded with pseudostratification and resemble endometrial lining. The two cell types tend to be found in the same tumours. Tumors that exhibit this feature in more than 30% of the tumor mass generally tend to occur in older individuals with a median age at diagnosis of 20 years older than usual papillary carcinoma, are often large lesions greater than 5 cm and often extend extrathyroidally (134). In addition to lymphatic invasion, vascular invasion is not uncommonly found in these lesions. Tumor mortality rates vary up to 25% for tall cell tumors and 90% for columnar cell carcinoma (136,138).

The management of the less aggressive forms of papillary thyroid carcinoma is controversial. Most experts advocate total thyroidectomy and radioactive iodine therapy (34,50). The rationale for total thyroidectomy is twofold, based on the frequency of bilateral carcinoma and on the need for enhancement of uptake of radioactive iodine by metastatic tumor deposits rather than residual thyroid tissue. However, as shown by the studies of Sugg et al (70), the identification of occult papillary carcinoma in the contralateral lobe is usually not attributable to intrathyroidal dissemination, which would justify further surgery for local disease. Therefore, the major indications for total thyroidectomy are the enhancement of uptake of radioactive iodine and the more sensitive use of thyroglobulin to detect persistent disease (52–54). The controversy involves the management of patients with low risk clinical and pathological parameters; some have advocated less aggressive management with unilateral thyroidectomy and no radioiodine therapy in this setting (49). Recent studies have identified potential markers of those more aggressive tumors that will metastasise to local lymph nodes, including loss of nuclear p27 and upregulation of cyclin D1 (139–141) and these may prove valuable to stratify patients for completion thyroidectomy and radioiodine therapy, but more studies are needed to validate these data. Since there are no controlled clinical trials that address this issue, the answer remains an empirical one. As for follicular carcinoma, external beam radiotherapy is not used in patients with papillary thyroid carcinoma, apart from those with locally advanced tumors that involve extrathyroidal soft tissues of the neck and cannot be completely resected (54,71).

HÜRTHLE CELL LESIONS

Hürthle cells in the thyroid represent a misnomer in that Dr. Hürthle originally described the parafollicular cell. The first description of oxyphilic cells in the thyroid is actually attributed to Askenazy. However, the term Hürthle cell is ingrained in the literature and it is unlikely that the historical error will even be corrected.

The Hürthle cell is derived from the follicular epithelium by metaplasia and possesses the capacity to produce thyroglobulin (142). Morphologically, Hürthle cells are characterised by large size, polygonal to square shape, distinct cell borders, voluminous granular and eosinophilic cytoplasm, prominent nucleus with “cherry-pink” macronucleoli. With the Papanicolau stain, the cytoplasm may be orange, green or blue. By electron microscopy, the cytoplasmic granularity is produced by large mitochondria filling the cell, consistent with oncocytic transformation (143,144). Hürthle cells have been studied by enzyme histochemistry and have been shown to contain a high level of oxidative enzymes (145,146). Somatic mutations and sequence variants of mitochondrial DNA (mtDNA) have been identified in oncocytic thyroid carcinomas (147,148). Similar changes have been found in the nontumorous thyroid tissue of patients with oncocytic neoplasms (148), suggesting that certain polymorphisms predispose to this cytologic alteration.

Hürthle cells are sometimes considered to be a cause of concern in needle biopsies (57). When they are not the major component in a thyroid aspirate, they are not diagnostic of any given lesion. Hürthle cells are found in patients with thyroiditis as

well as in several forms of thyroid neoplasia. Confusion and concern also arises with the histologic diagnosis of Hürthle cell nodules in the thyroid. Hürthle cell nodules found in the setting of thyroiditis or nodular goitre may be hyperplastic. Those lesions that arise in otherwise normal glands are usually encapsulated and are considered to be neoplastic. They can have microfollicular, macrofollicular, trabecular or solid architecture. On occasion, especially with the solid pattern and since these lesions can be extremely vascular, they may resemble medullary thyroid carcinomas and it may be necessary to resort to immunoperoxidase stains for thyroglobulin and calcitonin to obtain the correct diagnosis.

Hürthle cell adenomas and Hürthle cell follicular carcinomas are diagnosed when more than 75% of a lesion is composed of this cell type; the criteria for the diagnosis of lesions that are composed predominantly of Hürthle cells are the same as those applied to follicular lesions that do not contain Hürthle cells (149). The diagnosis of Hürthle cell papillary carcinoma (see below) is possible when the minimal cytologic criteria for papillary carcinoma are present (150).

FNA of Hürthle cell tumors may cause them to partially or totally infarct (151). This probably occurs because of the high metabolic activity of these cells and the delicate blood supply of these lesions that may readily become inadequate after direct trauma. A solitary tumor of the thyroid which occurs in a patient without thyroiditis and which is purely or predominantly composed of Hürthle cells on FNA should be excised, since Hürthle cell tumors show an average of 30% malignancy rate based on histology (149).

Hürthle cell hyperplasia

Hürthle cells are found in the thyroid in a variety of conditions and therefore are not specific for any particular disease. Individual cells, follicles or groups of follicles may show Hürthle cell features in irradiated thyroids, in ageing thyroids, in nodular goitre and in thyroiditis as well as in long-standing autoimmune hyperthyroidism (142). One can see these cells in chronic lymphocytic thyroiditis, in Graves' disease and in nodular goitre, where one can often find an entire nodule composed of oncocytes.

Hürthle cell adenoma and carcinoma

For many years it was felt that all Hürthle cell neoplasms of the thyroid (Figure 15) should be considered malignant since it was felt that the histology could not predict clinical behaviour. However, numerous studies have indicated that the criteria that apply to all follicular neoplasms of the thyroid also distinguish malignant from benign Hürthle cell lesions (149,152–158) . The larger the Hürthle cell lesion, however, the more likely it is to show invasive characteristics; a Hürthle cell tumour which is 4 cm or greater has an 80% chance of showing histologic evidence of malignancy (149). Nuclear atypia, which is the hallmark of the Hürthle cell, multinucleation, and mitotic activity are not useful to predict prognosis and therefore should not be used as diagnostic criteria for malignancy.

A subgroup of Hürthle cell neoplasm has been described which show some atypical features including marked nuclear anaplasia, mitoses, spontaneous infarction and

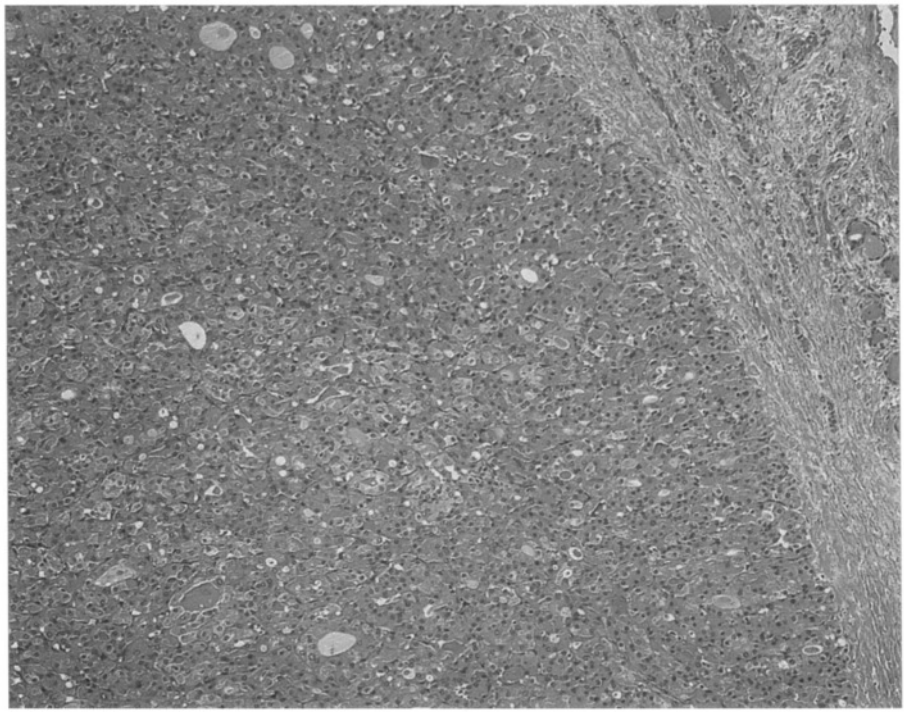

Figure 15. Hürthle cell tumours of thyroid are usually well delineated or encapsulated lesions in which more than 75% of the tumor cells have abundant eosinophilic granular cytoplasm due to the accumulation of spherulated and dilated mitochondria. These cells are derived from follicular epithelium and the criteria used to classify them should be identical to those used for non-oncocytic lesions.

trapping of tumor cells within the capsule in the absence of a preoperative FNA. Some authors have called these "atypical Hürthle cell adenoma" or "tumour of indeterminate malignancy". The great majority of these behave in a clinically benign fashion.

Flow cytometric analyses document aneuploid cell populations in 10 to 25% of Hürthle cell neoplasms that are clinically and histologically classified as adenomas (159–161). Virtually all of these tumours behave in a benign fashion after excision. Among histologically confirmed carcinomas, patients with thyroid tumors that have diploid DNA content tend to have a better prognosis than those with aneuploid values (159,161,162). Oncocytic neoplasms show frequent chromosomal DNA imbalance, with numerical chromosomal alterations being the dominant feature (163). Activating ras mutations are infrequent in oncocytic tumors (163).

The management of Hürthle cell carcinoma is controversial (155,156,164–167). In most institutions patients undergo total thyroidectomy followed by radioactive iodine. Iodine uptake by these lesions tends to be poor. External beam radiotherapy is advocated only for locally invasive disease.

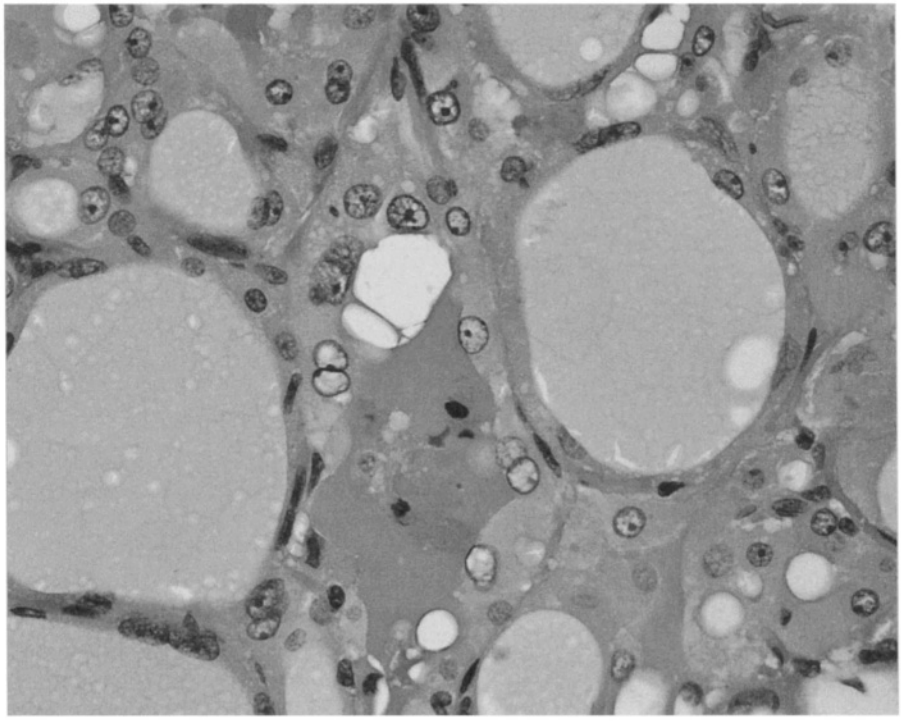

Figure 16. Oncocytic tumours with or without papillae that exhibit the nuclear features of papillary carcinoma represent Hürthle cell or oncocytic papillary carcinomas. This is an example of a follicular lesion that was not invasive, mimicking adenoma, but that harboured a ret/PTC gene rearrangement and metastasized to a local lymph node.

Hürthle cell papillary carcinoma

Many Hürthle cell tumors, whether benign or malignant, show papillary change which is really a pseudopapillary phenomenon, since Hürthle cell neoplasms have only scant stroma and may fall apart during manipulation, fixation and processing.

True oxyphilic or Hürthle cell variant of papillary carcinoma has been reported to comprise from 1 to 11% of all papillary carcinomas (144,168–173). These tumors have papillary architecture, but are composed predominantly or entirely of Hürthle cells (144,174). The nuclei may exhibit the characteristics of usual papillary carcinoma (169,175) (Figure 16), or they may instead resemble the pleomorphic nuclei of Hürthle cells, being large, hyperchromatic and pleomorphic (63,170). The clinical behaviour of this rare subtype is controversial; some authors have reported that they behave like typical papillary carcinomas (63,150,172,174,175), while others maintain that the Hürthle cell morphology confers a more aggressive behaviour (176,177) with higher rates of 10 year tumor recurrence and cause-specific mortality (170). This suggestion

of aggressive behaviour may be attributed to inclusion of tall cell variant papillary carcinoma in the group of Hürthle cell carcinomas.

One morphologic subtype of Hürthle cell papillary carcinoma which, because of a characteristic cystic change and extensive lymphocytic infiltration into the cores of the papillae of the tumour, has a striking histological resemblance to papillary cystadenoma lymphomatosum of the salivary gland and has been called "Warthin-like tumour of the thyroid" (178). This lesion occurs in the setting of chronic lymphocytic thyroiditis, predominantly in women, and is associated with a similar prognosis to usual papillary carcinoma.

The diagnosis of Hürthle cell follicular variant papillary carcinoma remains controversial. Many of these lesions have been diagnosed in the past as Hürthle cell adenoma, however, reports of aggressive behaviour suggested that this diagnosis could not be trusted (156,179). The application of *ret*/PTC analysis by RT-PCR allowed recognition of a follicular variant of Hürthle cell papillary carcinoma as a group of lesions with no invasive behaviour at the time of diagnosis but that harboured a *ret*/PTC gene rearrangement (180,181). Many of these lesions exhibit irregularity of architecture with hypereosinophilic colloid and nuclear features of papillary carcinoma, but these can be obscured by the hyperchromasia and prominent nucleoli of oncocytic change. Nevertheless, they can be recognised when there is a high index of suspicion and with the addition of immunohistochemistry for HBME-1, galectin-3, CK19 and ret or by RT-PCR studies of ret rearrangements. These tumours have the potential to metastasise (182), explaining the occurrence of malignancy in patients with a histopathological diagnosis of adenoma.

Nodules associated with hashimoto's thyroiditis

In 1912, Hashimoto described a well-defined clinicopathologic syndrome consisting of goitre, hypothyroidism, and lymphocytic thyroiditis. It is now generally accepted that the form of lymphocytic thyroiditis known as Hashimoto's thyroiditis is of autoimmune aetiology (183,184). Patients have antibodies to thyroglobulin and to thyroid peroxidase (also know as "microsomal antigen") (185). Some patients also have antibodies to a colloid component other than thyroglobulin "second colloid antigen") and, occasionally, to thyroid hormones. Patients with this disorder are most often women (female-male ratio is 10:1) between 30 and 50 years of age. They typically develop a diffuse, lobulated, asymmetrical, nontender goitre. Most patients with long-standing disease are hypothyroid. Occasionally there is a transient episode of hyperthyroidism known as "Hashitoxicosis" early in the course of the disease; this has been attributed to release of stored hormone during tissue destruction or to stimulation by antibodies to the TSH receptor (185).

The presence of thyroid growth-stimulating immunoglobulins (TGI) in these patients and/or compensatory TSH excess due to tissue destruction and hypothyroidism have been implicated in the development of hyperplastic nodules that present as discrete masses in patients with this disorder. Aspiration of these lesions yields an admixture of epithelial cells and inflammatory cells (57). The hallmark is the Hürthle

cell, a follicular epithelial cell that is characterised by abundant granular cytoplasm and a nucleus often with prominent "cherry pink" nucleolus. The background is composed of small and large lymphocytes, plasma cells, germinal centre fragments and macrophages with or without tangible bodies. Follicular cells and colloid are usually scant but may show nuclear atypia with irregular nuclear contours and prominent grooves.

The appearance of the thyroid involved by Hashimoto's thyroiditis is variable. The gland is usually enlarged and can weigh more than 200 g. It is composed of firm, lobulated, rubbery tissue with a homogeneous, pale grey, fleshy cut surface that lacks colloid translucence and resembles lymphoid tissue. Microscopically, the gland is diffusely infiltrated by mononuclear inflammatory cells, including lymphocytes, plasma cells, immunoblasts, and macrophages. Lymphoid follicles contain well-formed germinal centres. The glandular epithelium exhibits variable degrees of damage. Residual follicles are either atrophic, with sparse colloid and flattened epithelium or exhibit oxyphilic metaplasia, the accumulation of abundant eosinophilic granular cytoplasm characteristic of Hürthle cells (142). Follicular epithelial cells may also exhibit marked cytologic atypia that can be characterised by irregular nuclear membranes, grooves and even clearing of nucleoplasm. These features which in the face of inflammation are considered reactive, mimic papillary carcinoma (3,5). Areas of squamous metaplasia may be found (186). As the disease evolves, fibrosis becomes more conspicuous and in some patients, there is progression to the "fibrous variant" with less prominent lymphocytic infiltration, more prominent squamous metaplasia, and intense fibrosis that almost totally replaces thyroid tissue (187).

The nodules that usually precipitate surgical intervention are cellular areas composed of follicles with variable colloid storage. It is not uncommon for them to be composed predominantly of Hürthle cells and they may be difficult to distinguish from adenomas. The cytologic atypia that resembles that of papillary carcinoma and the fibrosis that can trap follicular epithelium create difficult diagnostic problems. The distinction of thyroid cancer from a reactive process or hyperplasia can be extremely difficult. Application of special techniques is particularly important in this setting. Stains such as HBME-1, galectin-3, CK 19 and ret can be of assistance.

Recent data indicate that glands with Hashimoto's disease express *ret*/PTC gene rearrangements (188). In the author's experience, this is the case when there are nodules of Hürthle cells or micropapillary carcinomas in the tissue submitted for examination, but not if these lesions are carefully excluded from the inflamed tissue examined (70). In general, *ret*/PTC expression in Hürthle cell nodules in this setting identifies gene rearrangements that correlate with other features of papillary carcinoma.

Sudden and rapid enlargement of a nodule in a patients with Hashimoto's thyroiditis may indicate the development of primary thyroid lymphoma which occurs usually in this setting.

POORLY DIFFERENTIATED (INSULAR) CARCINOMA

Poorly differentiated or insular carcinoma is a tumour of follicular cell origin which mimics the architecture of medullary thyroid carcinoma (189–191). The tumour may

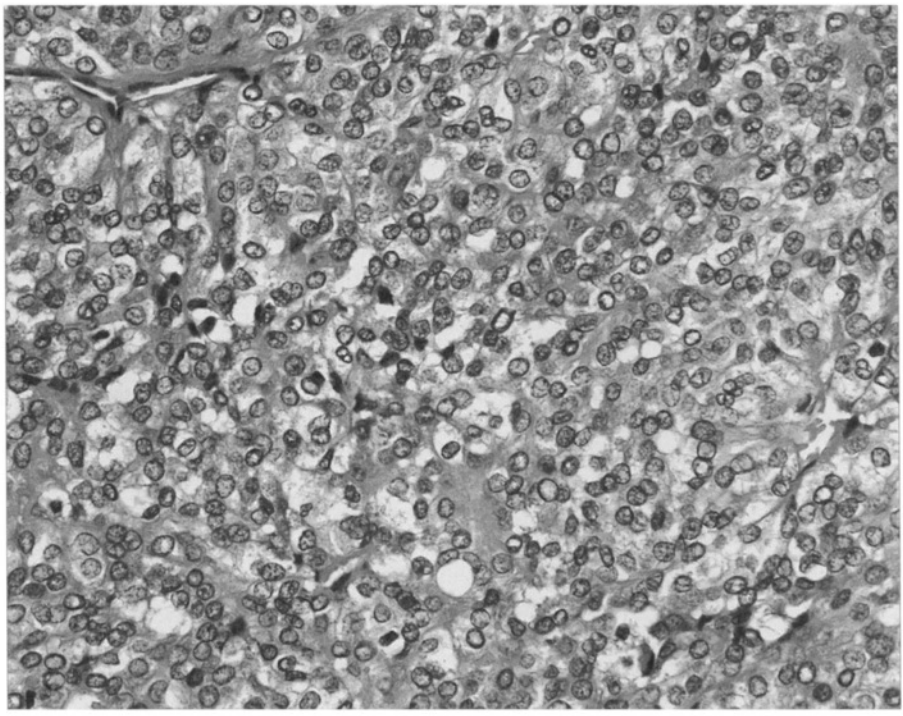

Figure 17. Insular or poorly differentiated carcinoma derived from follicular epithelium can mimick medullary carcinoma since it has a solid nesting architecture. Individual tumour cell necrosis is usually present.

have a central nidus that is encapsulated but usually the lesion exhibits frank capsular invasion and forms satellite nodules in the surrounding thyroid. The tumour architecture is characterised by large well-defined solid nests; it is largely devoid of follicular architecture and devoid of colloid (Figure 17). The tumor cells are usually small and uniform in size and there is a variable degree of mitotic activity. Sclerosis can mimic amyloid, however, congo red stains are negative and immunohistochemical stains for calcitonin, chromogranin and CEA are negative. In contrast the tumors are uniformly positive for thyroglobulin, confirming the follicular cell differentiation of this neoplasm. In contrast to anaplastic carcinomas, there is little pleomorphism and no bizarre, giant, or multinucleated cells are found, however, mitotic activity is identified. Single cell necrosis is a defining feature, but geographic necrosis is unusual.

Insular carcinoma behaves in an aggressive fashion and is often lethal. This is the lesion that most often is identified in cases that have been diagnosed as “widely invasive follicular carcinoma”. Most aggressive Hürthle cell lesions show insular growth and focal tumor cell necrosis. Vascular invasion and or metastases are frequent at the time of diagnosis. Insular carcinoma therefore occupies a position both morphologically

and biologically between differentiated papillary or follicular carcinoma and anaplastic thyroid carcinoma. These tumors are not uncommonly found associated with well differentiated carcinoma (either papillary or follicular) and the insular growth is thought to represent a dedifferentiation phenomenon. Since this entity has only been recognised relatively recently and the clinical literature does not include studies of this tumor type as a separate entity, appropriate clinical management remains to be established.

Clear cell carcinoma is a rare finding in the thyroid and raises important differential diagnoses. The identification of any clear cell lesion should alert the pathologist to the possibility of metastasis, particularly from renal or adrenal tumors (5). However, primary clear cell tumors of thyroid follicular cells occur and are thought to be due to accumulation of glycogen, lipid or even mucin (5). Proof that these represent follicular cells is obtained from thyroglobulin and TTF-1 staining. The term "clear cell tumor" should be restricted to lesions in which more than 75% of the tumor cells show this change.

ANAPLASTIC CARCINOMA

Anaplastic or undifferentiated carcinoma accounts for 5% to 10% of all primary malignant tumors of the thyroid (192) but in many centres this is decreasing with earlier detection of disease. These tumors are rapidly growing, with massive local invasion that usually overshadows the early metastases, most frequently to lung, adrenals and bone (4,5). They are highly lethal with a 5 year survival rate of 7.1% (193) and a mean survival period of 6.2 to 7.2 months (193,194).

Microscopically, anaplastic carcinomas exhibit wide variation. Three general patterns are recognised but most tumours manifest mixed morphology:

The most common type is the *giant cell variant*; as the name suggests, these tumors are composed predominantly of large cells with abundant amphophilic or eosinophilic, often granular cytoplasm and bizarre, often multiple, hyperchromatic nuclei (Figure 18). Some have round, densely acidophilic intracytoplasmic hyaline globules. These tumors grow in solid sheets; artefactual tissue fragmentation may simulate an alveolar pattern.

The squamoid variant is composed of large, moderately pleomorphic epithelial cells that form nests, resembling squamous carcinoma (Figure 19). They may even form keratin pearls.

Spindle cell anaplastic carcinomas have a fascicular architecture and dense stromal collagen with spindle-shaped tumor cells. They may resemble fibrosarcoma; the presence of scattered atypical cells and inflammatory infiltrates may suggest malignant fibrous histiocytoma. Prominent vascularization may suggest hemangioendothelioma (3,5,195).

In all three variants, mitotic figures and atypical mitoses are frequent. There is usually extensive necrosis and in some cases, necrosis may be so extensive that the only viable tumour is around blood vessels. Inflammatory infiltrates are associated with necrosis and the osteoclast-like giant cells that are occasionally found in these tumors have been shown by immunohistochemical studies to be reactive cells of monocytic/histiocytic lineage (196,197).

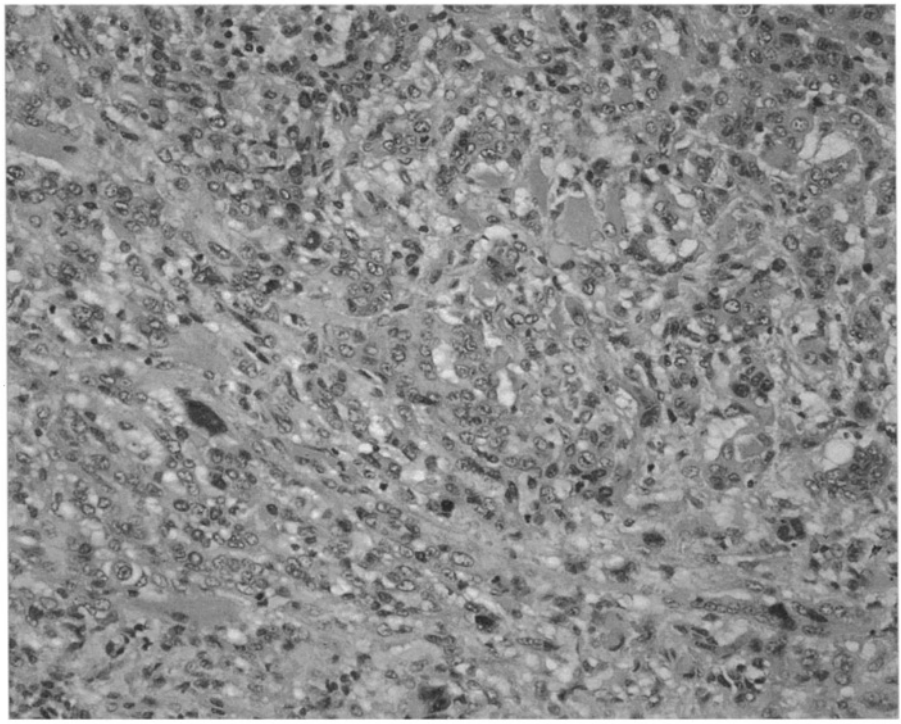

Figure 18. Anaplastic carcinoma may arise in differentiated carcinoma; it is characterized by anaplastic giant cells, prominent mitoses and geographic necrosis (not shown).

Anaplastic carcinomas are highly infiltrative. Malignant cells usually grow between residual thyroid follicles and invade skeletal muscle, adipose tissue and other perithyroidal structures. Blood vessel invasion and thrombosis with or without tumour cell involvement is frequent.

The appearances of anaplastic carcinoma on FNA are quite varied and reflect the histologic type with giant cells or squamoid cells or spindle cells. There is high cellularity, with necrosis, acute inflammation and marked cellular pleomorphism. Mitoses are often atypical and no colloid is seen.

Immunohistochemistry is useful in only a limited fashion in the diagnosis of these lesions. Most anaplastic carcinomas do not contain convincing reactivity for thyroglobulin and the few that are positive have only a weak or focal reaction (194, 197–201). This staining must be interpreted carefully, since it may reflect trapped nontumorous follicles or follicular cells, and since thyroglobulin is known to diffuse into non-follicular cells (5). The epithelial nature of the tumor cells can be verified with stains for cytokeratins but again most undifferentiated lesions are negative for this marker. Squamoid areas may exhibit reactivity for high molecular weight keratins and/or epithelial membrane antigen (EMA) (194, 197–199). CEA reactivity may be found in the centre of squamous

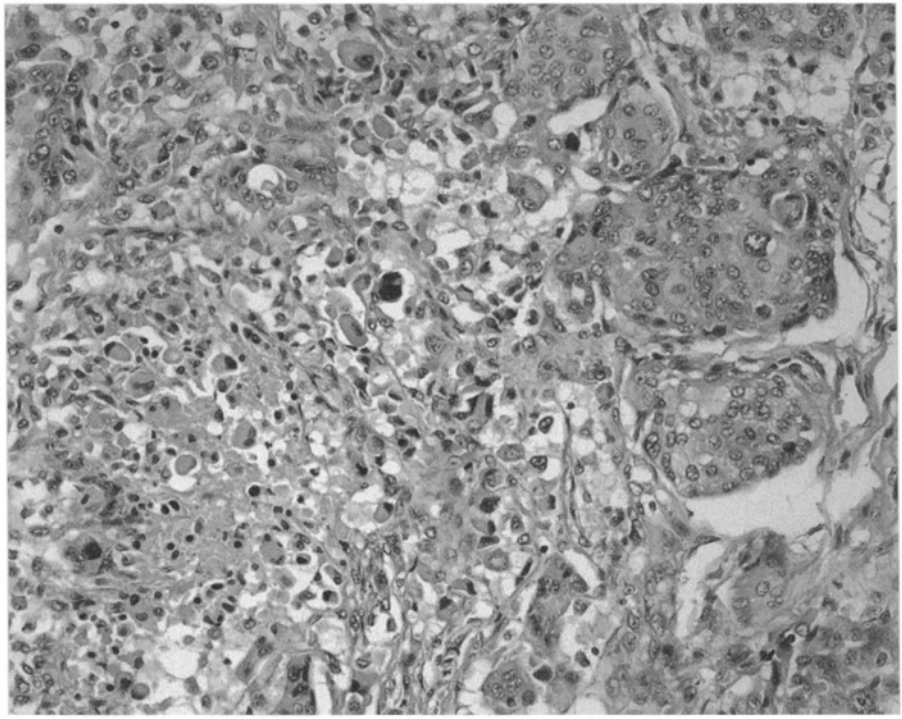

Figure 19. Some anaplastic carcinomas exhibit rhabdoid and/or sqamous morphology.

nests (194,197). Anaplastic tumours have been reported to be positive for calcitonin, but this finding should alter the diagnosis to that of medullary carcinoma (5).

p53 mutations are common in anaplastic thyroid carcinomas (202–208); since mutated forms of this tumour suppressor gene have prolonged half lives, the application of immunohistochemistry has yielded positive results in these tumours (209,210). (Chapter 8).

By electron microscopy (196,198,201,211,212), there may be formation of intercellular junctions, microvilli, and basal lamina, providing evidence of epithelial differentiation. However, many tumors do not exhibit evidence of any differentiation. Their large nuclei have prominent nucleoli and clumped chromatin; usually the cytoplasm contains only poorly developed rough endoplasmic reticulum, scattered dense bodies, lipid droplets, numerous free ribosomes, mitochondria and lysosomes. Intermediate filaments (keratin or vimentin) may form filamentous whorls that correspond to the acidophilic hyaline globules seen by light microscopy. Secretory granules are not seen in these tumours.

Most anaplastic thyroid carcinomas are aneuploid on flow cytometry; this abnormality correlates with poor outcome (162).

Some tumors do not exhibit immunohistochemical or ultrastructural markers that allow classification as epithelial malignancies. Nevertheless, the diagnosis of anaplastic

carcinoma should be favoured for pleomorphic lesions in older patients if they arise in the thyroid.

Small cell carcinomas and lymphomas constitute a common source of diagnostic error, often misclassified as anaplastic carcinomas (3,5,195,198). The former are usually poorly differentiated medullary carcinomas, which can also mimic giant cell or spindle cell anaplastic carcinomas; the latter are readily identified by staining for leukocyte common antigen (LCA) and other markers of lymphoid cells. Rarely, primary intrathyroidal thymoma may be mistaken for anaplastic carcinoma (213,214).

The reported association between well-differentiated thyroid carcinoma and anaplastic carcinoma ranges from 7% to 89% of cases, however, the lower figures are likely underestimates, attributable to inadequate sampling (3,193,194,198,215–217). The data suggest that anaplastic carcinoma originates most often in an abnormal thyroid; the tumor has a higher incidence in regions of endemic goitre and a history of goitre is reported in over 80% of cases (3,193). As stated above, nodular goitre is often the site of monoclonal proliferation, the first step in the hyperplasia-neoplasia sequence. However, it is difficult to document transformation of a benign lesion to a malignant tumor. Insular carcinoma appears to be intermediate in the spectrum, and may represent a transition form (190,217). The association of papillary carcinoma, particularly the more aggressive tall cell variant, with anaplastic tumors has also been described (3,217,218). Thyroid carcinomas can exhibit an entire spectrum of differentiation through insular to anaplastic foci. The significance of microscopic insular or anaplastic change is controversial; some people have suggested that focal microscopic dedifferentiation does not alter prognosis but others have shown that this finding alone is statistically significant as a marker of aggressive behaviour.

The factors underlying dedifferentiation in thyroid tumors remain to be established; age and radiation have been implicated (219,220). Clearly, the vast majority of well differentiated thyroid lesions do not undergo such transformation. A pattern of genetic mutations resulting in oncogene activation or loss of tumour suppressor gene activity has been proposed to correlate with the stepwise progression from adenoma to carcinoma and through the dedifferentiation process in thyroid (202,203).

MEDULLARY CARCINOMA

Medullary carcinoma of the thyroid comprises 5–10% of all thyroid carcinomas (5). This lesion is usually readily recognised because of its unusual cytologic and histologic features but sometimes special investigation is required to distinguish it from follicular lesions or other tumours, including lymphomas and/or anaplastic carcinomas.

The aspirate from medullary carcinoma has a variable appearance. The cells may be spindle-shaped, columnar or plasmacytoid; they may even exhibit oncocytic or clear cell morphology. Nucleoli and nuclear pseudoinclusions are often seen. Amyloid is identified in up to 60% of cases as homogeneous, spherical or rod-shaped extracellular material which polarises with the Pap stain or the Congo Red stain. The diagnosis is confirmed by immunostaining for calcitonin or the demonstration of secretory granules on electron microscopy.

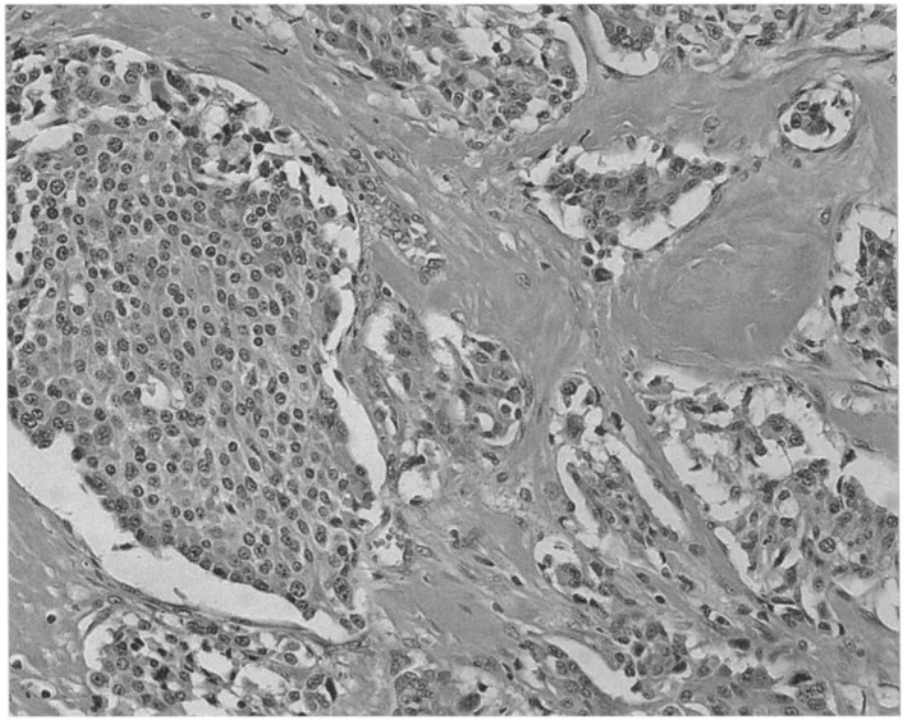

Figure 20. Medullary carcinoma of thyroid is derived from the calcitonin-producing C cells that are neuroendocrine cells. These lesions are composed of solid nests of epithelial cells with poorly defined cell borders. They often have stromal fibrosis and occasionally there is deposition of intensely eosinophilic material, amyloid, derived from the calcitonin precursor molecule.

Medullary carcinoma has a wide range of histologic appearances (2,221). Typically, the tumors are composed of sheets or more usually nests of round, polyhedral or spindle-shaped cells which may exhibit palisading at the periphery (Figure 20). The stroma is vascular. There may be prominent amyloid in the stroma, which, when present, provides a helpful diagnostic marker. However, although amyloid is present in more than half of these tumours, it may be intracytoplasmic and difficult to identify without a high index of suspicion. In addition, amyloid may also be present in occasional non-medullary thyroid carcinomas (222).

Sometimes, fixation artefact produces a pseudopapillary appearance; areas of true papillary architecture may also be found and the distinction of such lesions from papillary carcinoma can be difficult (223). A pseudofollicular appearance frequently results from entrapped nonneoplastic thyroid follicles or rounded masses of amyloid and true glandular variants have been described. Dedifferentiation results in a small cell tumour morphology, which can mimic lymphoma. Oncocytic features may predominate and make the distinction of medullary from oncocytic follicular carcinoma difficult.

Foreign body giant cells may be associated with amyloid deposits and calcification may be identified. These features may result in difficult differential diagnosis. True psammoma bodies are generally not seen in these tumours but have been reported.

Staining for amyloid can be helpful. Congo Red staining is typical and the apple-green birefringence with polarised light is diagnostic. Nevertheless, as indicated, some follicular tumors may also contain amyloid stroma.

Immunohistochemical staining represents the gold standard for the diagnosis of medullary thyroid carcinoma. These tumours express cytokeratins, chromogranin A, and NSE, but the most specific diagnostic marker is calcitonin. The number of calcitonin-positive cells varies from case to case, but the diagnosis should be questioned in the absence of calcitonin staining. The amyloid in these tumours often stains for calcitonin, likely because the amyloid protein represents deposition of a precursor of the calcitonin molecule.

These tumours also stain for carcinoembryonic antigen (CEA) and the inverse relationship between the intensity of staining for calcitonin and that for CEA may be prognostically significant: tumors containing few calcitonin-positive cells and abundant CEA immunoreactivity are said to have a worse prognosis than the well differentiated tumours with strong calcitonin immunoreactivity (224,225). CEA is not identified in follicular thyroid tumors; occasional reports of positivity are attributable to use of antibodies that react with non-specific cross-reacting antigens (226). Therefore CEA positivity indicates the presence of medullary thyroid carcinoma or other lesions such as metastatic carcinomas or thymic carcinomas.

Medullary thyroid carcinomas also produce a number of other peptides including somatostatin, derivatives of the proopiomelanocortin molecule (ACTH, MSH, ß-endorphin and enkephalin), serotonin, glucagon, gastrin, cholecystokinin, VIP, bombesin, and α-HCG (5,227–229). Calcitonin gene-related peptide (CGRP) is also identified in normal C-cells as well as medullary thyroid carcinomas. Individual tumours may express a variety of these various hormones but none have been shown to correlate with altered prognosis (230).

Ultrastructural examination confirms the presence of cells that do not form desmosomes but do show complex interdigitations of cell membranes. The cytoplasm contains characteristic membrane-bound secretory granules which usually are numerous and variable in size.

The importance of distinguishing this tumour from follicular lesions is two-fold. The first is for diagnostic classification and management considerations in the individual patient. These tumors do not preferentially take up iodine and therapy with radioactive iodine is not indicated; in contrast, expression of somatostatin receptors by some of these tumors (231) makes the octreoscan a feasible diagnostic tool to localise the primary lesion and to identify metastatic deposits (232) and somatostatin analoges may have applications in the management of disseminated disease (233). The other aspect of management involves the implications for both the patient and members of his/her family, since many of these tumours are hereditary (234).

The inherited forms of medullary carcinoma are of three types: familial medullary thyroid carcinoma alone (FMTC), multiple endocrine neoplasia (MEN) type IIA in

which MTC is associated with pheochromocytomas, and MEN IIB in which the thyroid and adrenal proliferative disorders are associated with mucosal ganglioneuromas and a Marfanoid habitus. The inheritance of all three syndromes was mapped to the pericentromeric region of chromosome 10 by linkage analysis (235–237). Subsequently, mutations in exons 10 and 11 of the *ret* proto-oncogene in patients with FMTC or MEN IIA and at codon 918 in MEN IIB (238,239) have provided a more accurate marker of germline mutation and predisposition to this disease (240). Current recommendations suggest that family members of FMTC and MEN IIA kindreds have genetic screening early in life and affected members should undergo total thyroidectomy at around the age of 5 years. This age was chosen because of the early onset of medullary thyroid carcinoma in these familial forms of the disease; metastatic tumour has been found in patients as young as 6 years of age. Affected children with MEN IIB undergo surgery even earlier (241, Chapter 24).

Sporadic medullary carcinomas also may have mutations of *ret* in the same codons as the familial disorders (239,242); the mutation involved may have prognostic value (243). The presence of *ret* mutations in sporadic tumours indicates the importance of analysing DNA from white blood cells to establish that a mutation is germ line, therefore potentially hereditary. Other oncogenes and tumor suppressor genes have not been implicated in the pathogenesis of MCT: *ras* mutations are rare, c-*myc,* and c-*erb*B are not amplified (244,245), and p53 mutations are not found in these tumors (246).

Familial forms of medullary thyroid carcinoma usually result in multicentric disease as well as multicentric C-cell hyperplasia (247). Many definitions of C-cell hyperplasia have been offered, all requiring immunohistochemistry since C cells cannot be reliably recognised with routine histologic stains. Quantitation of C cells as well as geographic mapping throughout the gland must be performed (247,248). C cells are usually limited to the central portion of the junction between the upper and middle thirds of the lateral lobes where they are generally distributed singly rather than in clusters. Increased numbers of C cells (>7 cells per cluster), complete follicles surrounded by C cells, and distribution of cells beyond this geographic location are indicative of C-cell hyperplasia. The presence of C-cell hyperplasia usually indicates an inherited disorder rather than a sporadic lesion, however, C-cell hyperplasia can also be associated with chronic hypercalcemia, thyroid follicular nodular disease, and thyroiditis (249–252).

The identification of oncogenic activation of *ret* in familial C cell disease has raised questions about the term “C cell hyperplasia”. In this disorder, unlike other familial cancer syndromes that result from inactivation of tumour suppressor genes, each affected member is born with an activated oncogene. Theoretically, then, every C cell has already undergone transformation, since it does not appear to require a second hit to knock out protective mechanisms. If this proves to be true, it will suggest that the term “C cell hyperplasia” is a misnomer, since each C cell with its activated oncogene is a transformed cell that represents a site of neoplastic potential. This remains to be proven, however, and the mechanism of tumorigenesis in C cells of the thyroid, as it unfolds, will shed further light on the biology of neoplasia.

MIXED FOLLICULAR-C CELL LESIONS

Although controversial, mixed follicular-parafollicular cell carcinomas do occur (253); these rare monomorphous tumours are composed of cells showing dual differentiation (254,255). Composite tumors are composed of two intermixed well differentiated components, one showing thyroglobulin immunoreactivity and either papillary or follicular architecture and cytology, the other with calcitonin and CEA immunopositivity (81,256). The diagnosis of a mixed or composite tumor can be convincing only in cases where metastatic disease is identified, since the identification of thyroglobulin and calcitonin in a primary intrathyroidal tumor may represent the identification of a typical medullary thyroid carcinoma with trapped nontumorous elements containing thyroglobulin, or phagocytosis of thyroglobulin by medullary carcinoma cells. Moreover, the two tumours may occur separately in the same gland and metastasise together to a regional node (257,258).

CONCLUSION

Thyroid nodules are common and their management can be difficult and controversial. Clearly, the pathologist has an important role to play in their evaluation. The use of fine needle aspiration biopsy has significantly improved our ability to identify specific high risk disorders and to facilitate their management in an expeditious and cost-effective manner. Patients who require surgery for further confirmation of the disease process rely upon the pathologist to correctly characterize their nodule and pathologists are actively involved in research to clarify the pathogenesis of thyroid disease. There are other areas of thyroid pathology that have seen uniform advances in our understanding of the pathobiology of disease. Most experts accept tall cell or columnar morphology as predictive of more aggressive variants of papillary carcinoma. The recognition of insular carcinomas as an intermediate category of "poorly differentiated carcinoma" has been validated by clinical and molecular studies. The biology of familial genetic alterations in medullary carcinoma has revolutionised patient care. Advances in our understanding of the molecular basis of thyroid cancer will allow more accurate characterization of specific subtypes of neoplasia and malignancy even on single cells obtained at fine needle aspiration biopsy. This should further enhance the usefulness of this technique and better guide the management of patients with a thyroid nodule.

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3. THYROID LYMPHOMAS

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INTRODUCTION AND HISTORICAL ASPECTS

Primary thyroid lymphomas have been recognised for many years and have been documented from the 1940s and 1950s. It was deemed to be important to recognise this entity "for it seems that about a third of the cases may be treated successfully with X-rays, followed by maintained thyroid medication" (1). Thus, the importance of separating lymphomas from its mimics, namely, chronic thyroiditis and small cell carcinoma, was evident at an early stage because of the therapeutic implications. Indeed, the histological difficulty in separating lymphoma from chronic thyroiditis and small cell carcinoma, no doubt led to the under-diagnosis of lymphoma. Even 50 years and more ago, certain peculiarities of thyroid lymphoma were apparent to pathologists: the predilection for elderly women, long survival and the tendency for similar lesions to occur in the gastrointestinal tract. These lymphomas were so characteristic that Brewer and Orr coined the term "struma reticulosa" to describe them (2). The fact that primary and secondary lymphomas could occur in the thyroid was accepted and sporadic papers on the subject, including the occasional large review, appeared in the literature (3). It was not until the early 1980s that primary lymphomas of the thyroid gland came under scrutiny again and was the centre of intense research. The introduction of the mucosa associated lymphoid tissue (MALT) concept led to the critical examination of primary thyroid lymphomas and similar appearing lymphomas with the seminal work of Isaacson and Wright responsible for the crystallization and clarification of the pathogenesis and morphology of these lymphomas (4–6). It is now clear that MALT

and lymphomas arising from these sites share morphological, immunophenotypic and molecular features to the extent that MALT-lymphomas can metastasize from one MALT site to another.

INCIDENCE

Lymphomas occurring primarily in the thyroid are decidedly uncommon, accounting for about 5% of all thyroid malignancies (7–10). This figures increases to 10% of thyroid malignancies in certain geographic locales where antecedent chronic thyroiditis is common (10, 11). Primary thyroid lymphomas constitute 2.5 to 7% of all extranodal lymphomas (12–14).

It is stated that 25 to 100% of thyroid lymphomas arise against a background of thyroiditis, either chronic lymphocytic or Hashimoto's thyroiditis (15–18). This association is so strong that the relative risk of a patient with chronic thyroiditis developing lymphoma of the thyroid is 40 to 80 times greater than the general population (14, 19, 20). The lymphomas evolve after a prolonged period, usually 20 to 30 years after the onset of chronic lymphocytic thyroiditis (14).

CLINICAL PRESENTATION

Women are more frequently affected than men with a ratio of 2.5 to 8.4: 1. Most patients are in the 50 to 80 year age range. There is usually rapid enlargement of an already existing goitre, and the mass may extend extra-thyroidally. The rapid growth and extent of invasion may result in dysphagia, hoarseness and dyspnoea (3, 16, 21). Thyroid function is usually normal but hypothyroidism has been documented in a minority of cases (11, 22). If hypothyroidism is present, it is usually due to the pre-existing thyroiditis and not due to the obliteration of thyroid parenchyma by the lymphomatous infiltrate. Very rare cases of hyperthyroidism have been encountered where rapid destruction of thyroid follicles with release of colloid and thyroid hormone into the circulation, have been implicated as causative (23, 24).

NOMENCLATURE AND TERMINOLOGY

In the last 10 or so years lymphoma classification has undergone a major revision with the appearance of the Revised European-American Lymphoma (REAL) classification (25–27) and the subsequent World Health Organization (WHO) update, refinement and minor modification of the REAL classification (28–34). After Isaacson and Wright brought the concept of MALT and lymphomas arising therefrom to prominence, the terms MALT lymphoma or lymphoma arising in MALT or "MALT-oma" have been used. The advent of the REAL/WHO classifications led to a re-appraisal, and these peculiar and characteristic lymphomas were categorized as: extra-nodal marginal zone B-cell lymphomas (MZBL) of MALT-type. This is the prototype lymphoma occurring primarily in the thyroid. Variants and other common related lymphomas will be discussed.

TYPES OF PRIMARY THYROID LYMPHOMA

As mentioned above the most morphologically distinctive and recognizable (but not necessarily the commonest variant encountered) primary lymphoma is MZBL of

Table 1. Staging of primary thyroid lymphomas

Stage IE: Primary thyroid lymphoma (PTL) with or without perithyroidal soft tissue extension,
Stage IIE: PTLs with involvement of lymph nodes on the same side of the diaphragm,
Stage IIIE: PTLs with involvement of lymph nodes on both sides of the diaphragm,
Stage IV E: PTLs with dissemination to other extranodal sites.

MALT type. Others seen include: MZBL of MALT type with large cell (blastic) transformation (mixed MZBL and diffuse large B cell lymphoma [DLBCL]), DLBCL without MZBL and a miscellaneous, heterogenous group usually consisting of single case reports of a wide variety of lymphomas that can occur in lymph nodes and any other extranodal site. These include: Hodgkin's disease, follicular lymphoma, intravascular lymphomatosis, anaplastic large cell lymphoma and T-cell lymphomas (35–42). These will not be dealt with, as their occurrence in the thyroid is the same as any other extra-nodal site or lymph node for that matter.

The high-grade lesions: mixed MZBL/DLBCL and DLBCL without MZBL are the commonest histological types of primary thyroid lymphoma that are encountered (36, 42).

STAGING

The recommended staging system is Musshoff's modification of the Ann Arbor staging system (36). See Table 1. Over 90% of patients present as stages IE or IIE.

PATHOLOGY

Gross pathology

Enlargement of the thyroid gland, either rapidly or slowly, is the commonest gross manifestation (Figure 1). This is often accompanied by extrathyroidal extension into surrounding soft tissue and skeletal muscle. The lymphomatous gland varies in its naked eye appearance: fleshy, tan, white, grey or red with a fish-flesh appearance, firm or soft, multinodular, lobulated or with diffuse deposits, solid or cystic. The cut surface may be smooth, bulging or lobular. Foci of hemorrhage and necrosis may be present imparting a mottled or variegated appearance to the cut surface. Lymphomatous involvement of the gland ranges from 0.5 to 19.5 cm (36). Uninvolved thyroid tissue, if present, may show the macroscopic features of the associated lymphocytic thyroiditis: beige in color with fibrosis and lobular accentuation. Obviously, none of these gross features are specific to lymphoma of the thyroid gland.

LIGHT MICROSCOPY

MZBL of MALT type

This type of lymphoma is probably the most histologically distinct and recognizable of all the primary thyroid lymphomas. The constituent cells are slightly larger than centrocytes and are called "centrocyte-like" cells in view of this resemblance. These cells have condensed chromatin, slightly irregular nuclear contours but are rarely, if ever, cleaved (Figure 2). The centrocyte-like cells have a propensity for permeating thyroid follicle epithelial cells giving rise to the histological hallmark of MALT lymphomas,

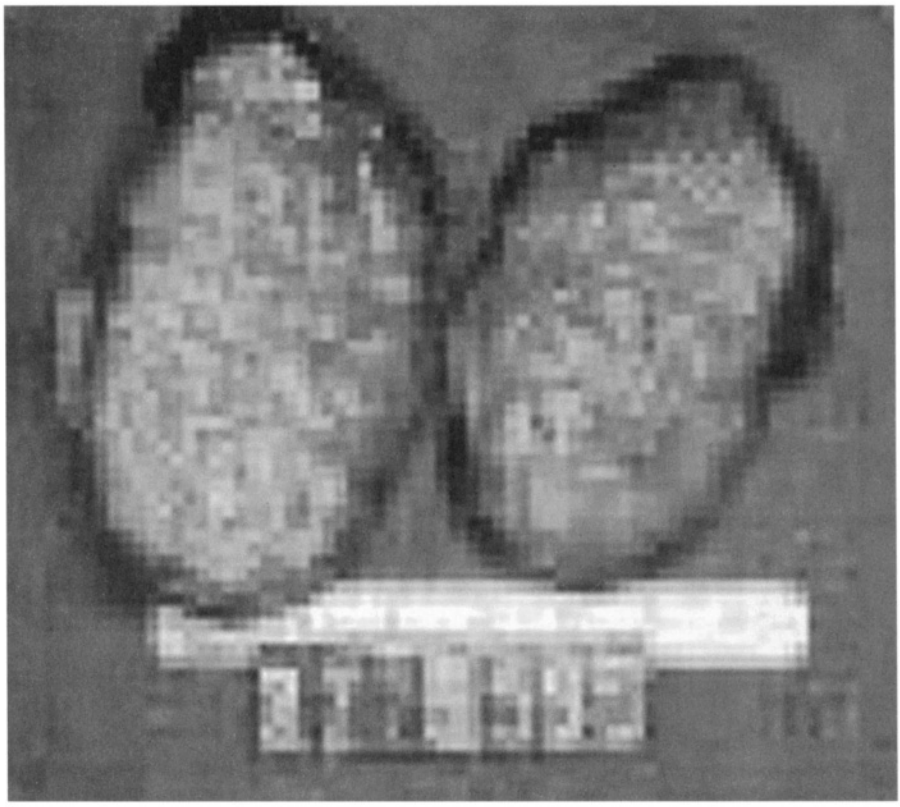

Figure 1. Gross illustration of a thyroid gland infiltrated by a lymphoma: enlarged, fleshy gland that is tan coloured.

the so-called "lymphoepithelial lesion" (Figures 3 & 4). Not only are the centrocyte-like cells seen insinuating between thyroid follicle cells but they are also found in between thyroid follicles within the stroma. The thyroid follicles bearing lymphoepithelial lesions vary in appearance from being relatively intact (Figure 5) to showing marked atrophy and destruction (Figure 6). In the more severe lymphoepithelial lesions, the follicles are markedly attenuated and may not be seen on H&E sections. Tombstones or the occasional residual epithelial cell may only be visible with a cytokeratin stain as a remnant of a previous follicle (Figure 5). Sometimes the centrocyte-like cells traverse the thyroid follicle cells and come to occupy the centres of the follicles displacing/kern-1ptreplacing the colloid, so-called "stuffed follicles" or what Derringer and colleagues have dubbed, "MALT balls" (36) (Figure 7). Other cellular components that are present in MZBL of MALT type include: plasma cells, mature, round, slightly elongated or lymphoplasmacytoid lymphocytes, monocytoid B-lymphocytes and centroblastic appearing cells (Figure 8). Indeed, the occasional eosinophil and histiocyte

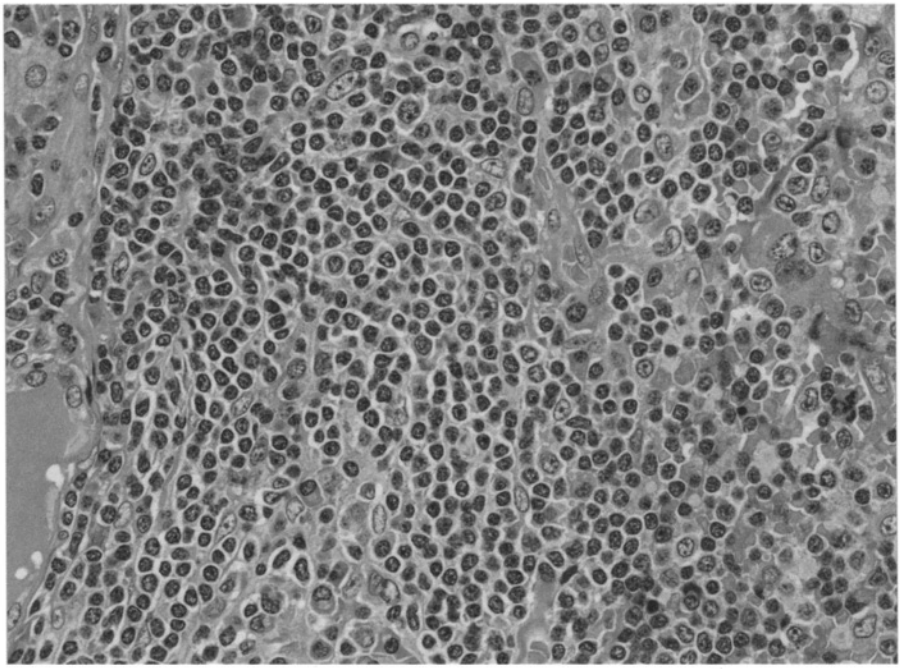

Figure 2. Centrocyte-like cells are the dominant cell type in MZBL of MALT-type. They are slightly larger than mature centrocytes and have a slightly irregular, but not cleaved, nuclear contour.

may also be present. The infiltrate between thyroid follicles is typically heterogenous and the centrocyte-like cells almost exclusively participate and form lymphoepithelial lesions. However, plasma cells can form a major component of the infiltrate and can in some instances be the dominant cell type. The plasma cells are mainly mature in morphology although bi-nucleate forms and those harboring Russell and Dutcher bodies have been encountered. The plasma cells rarely, if ever, permeate the thyroid follicle epithelium and tend to have a para-follicular distribution (Figure 9). This may be so marked in some instances that there almost appears to be a "compartmentalization" of the plasma cells away from the centrocyte-like cells and they appear as a separate infiltrate. In general, they are admixed together. The monocytoid B-cell component, made up of uniform small to intermediate cells with characteristic abundant eosinophilic to clear cytoplasm, occurs in clusters of varying size. Centroblastic cells, larger cells with vesicular chromatin and prominent nucleoli, are found scattered in amongst the other cellular constituents (Figure 10). They are not the dominant cell type nor do they form cohesive aggregates in MZBL of MALT type.

Reactive lymphoid follicles are also a common feature in this type of lymphoma (Figure 11). These are found within the neoplastic infiltrate and are not related to the associated lymphocytic thyroiditis. The follicles warrant careful examination to separate

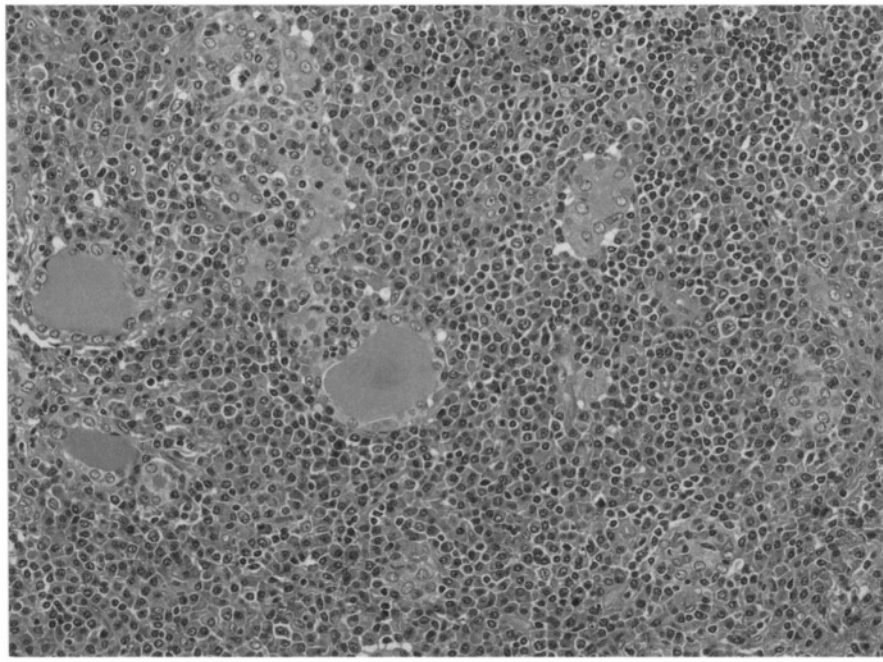

Figure 3. The centrocytes-like cells home into the thyroid follicles, traverse the epithelial cells and form lymphoepithelial lesions.

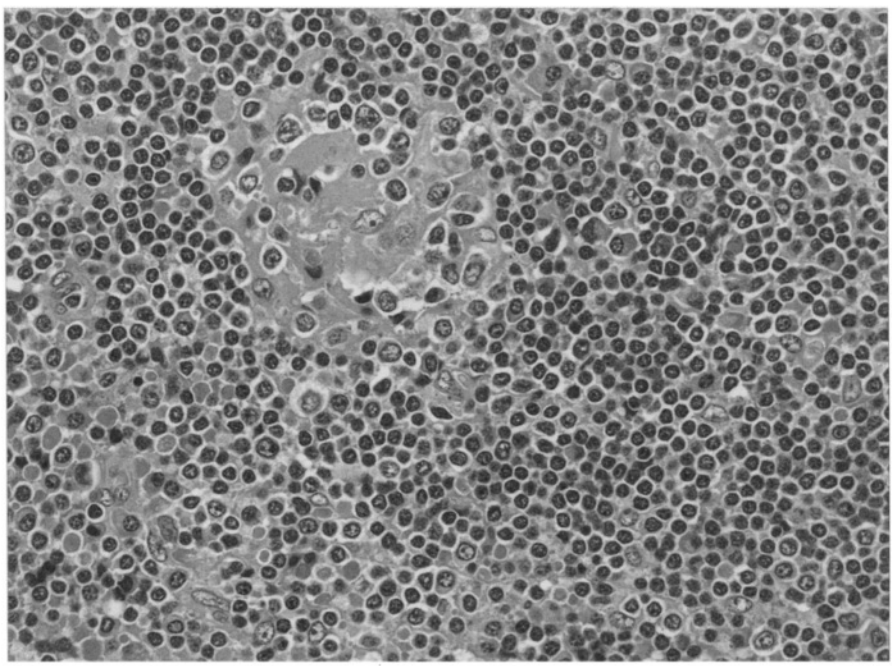

Figure 4. Under higher magnification, the centrocyte-like insinuate between and through the thyroid follicle cells with resultant disruption and destruction of the follicle.

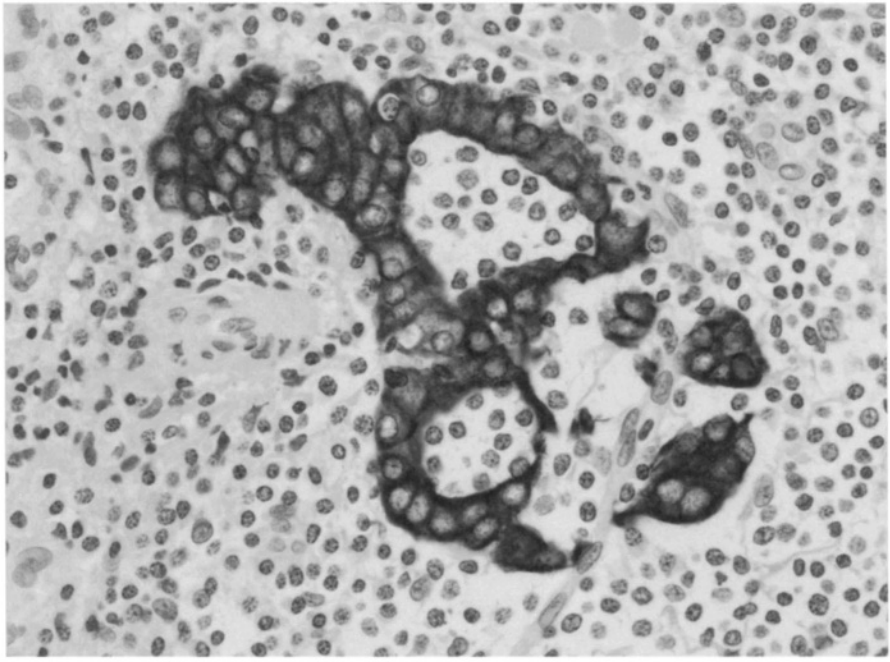

Figure 5. A cytokeratin stain highlights a relatively intact follicle that nonetheless is permeated by centrocyte-like cells, some of which are present within the follicle lumen.

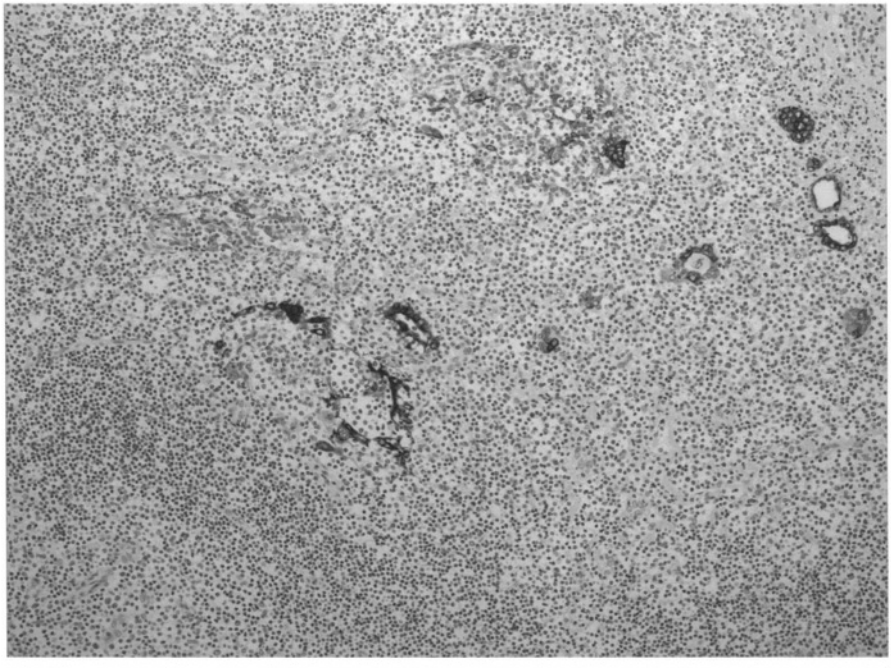

Figure 6. More destructive lymphoepithelial lesions with only remnants of thyroid follicle left. A cytokeratin stain is useful in showing residual epithelial cells within the dense lymphoid infiltrate.

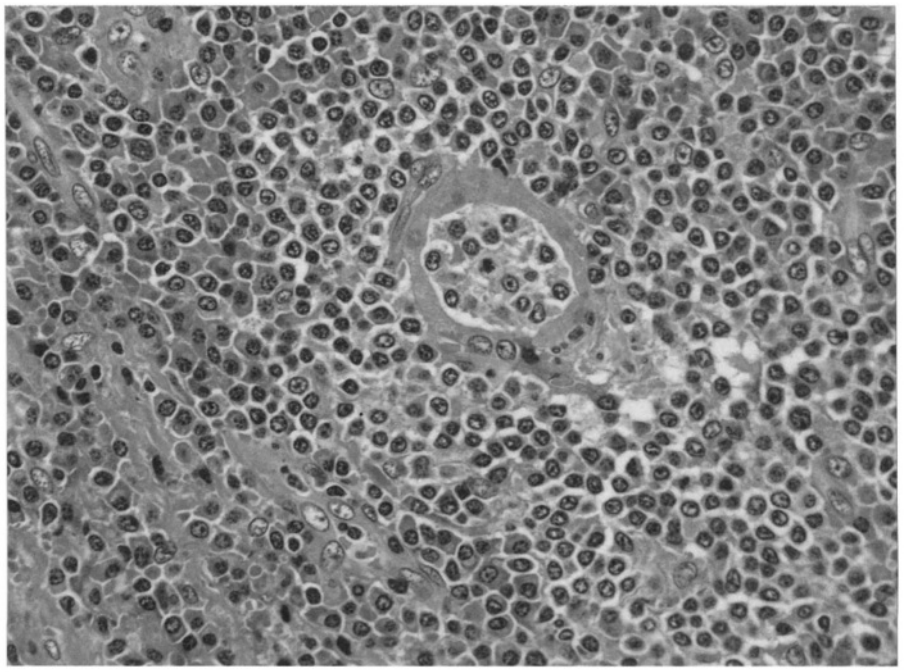

Figure 7. So-called "MALT ball" or "stuffed follicle" where the neoplastic cells reside within the lumen of the thyroid follicle.

truly reactive germinal centers from so-called "follicular colonization". In this phenomenon the centrocyte-like cells home into the germinal centers of lymphoid follicles both in the thyroid gland and draining lymph nodes. The centrocyte-like cells displace the usual cells of the germinal center and this often imparts a nodular or follicular appearance to the neoplastic infiltrate. Morphologically the possibility of follicular lymphoma needs to be separated from a MZBL of MALT type with follicular colonization.

MZBL OF MALT TYPE WITH LARGE CELL TRANSFORMATION (MIXED MZBL AND DIFFUSE LARGE CELL B-CELL LYMPHOMA [DLBCL])

For categorization as MZBL of MALT-type with large cell transformation, areas of typical MZBL of MALT-type, as described above must be histologically evident. This type of primary thyroid lymphoma accounted for just under 50% of all cases in one series (36). The presence of either MZBL of MALT-type with a component of large, blastic lymphoma cells warrants a diagnosis of mixed MZBL and DLBCL. How much large cell or high-grade lymphoma has to be present to make a diagnosis? Whilst this has not been quantified widely, but general principles suggest that confluent clusters, aggregates or sheets of large cells indicate a significant component and hence designation as MZBL of MALT-type with large cell transformation. Usually an obvious cluster

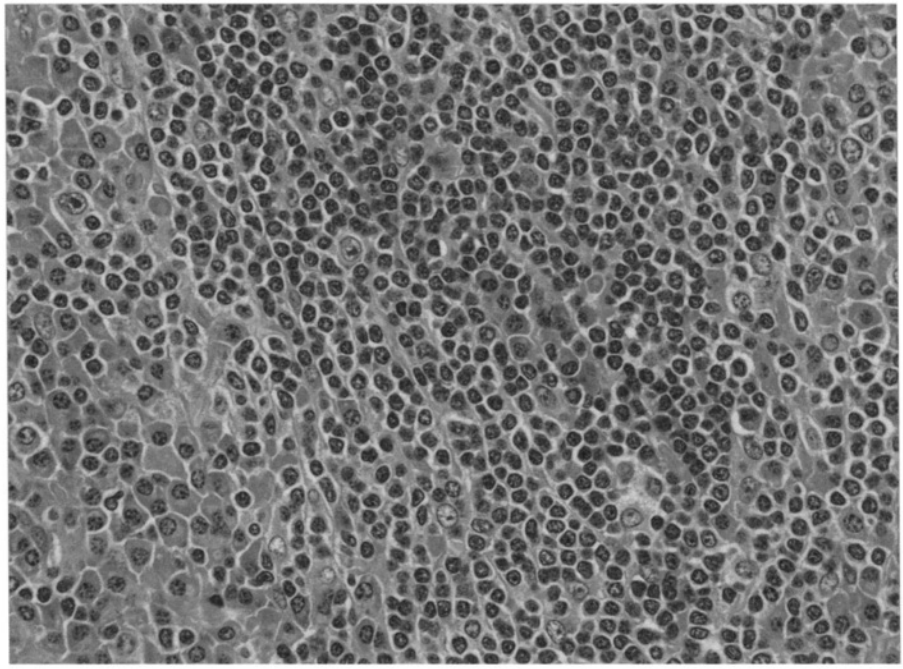

Figure 8. Other cellular components of MALT-lymphoma include plasma cells, larger centroblastic cells and occasional histiocytes. These are admixed with centrocyte-like cells in MZBL of MALT-type.

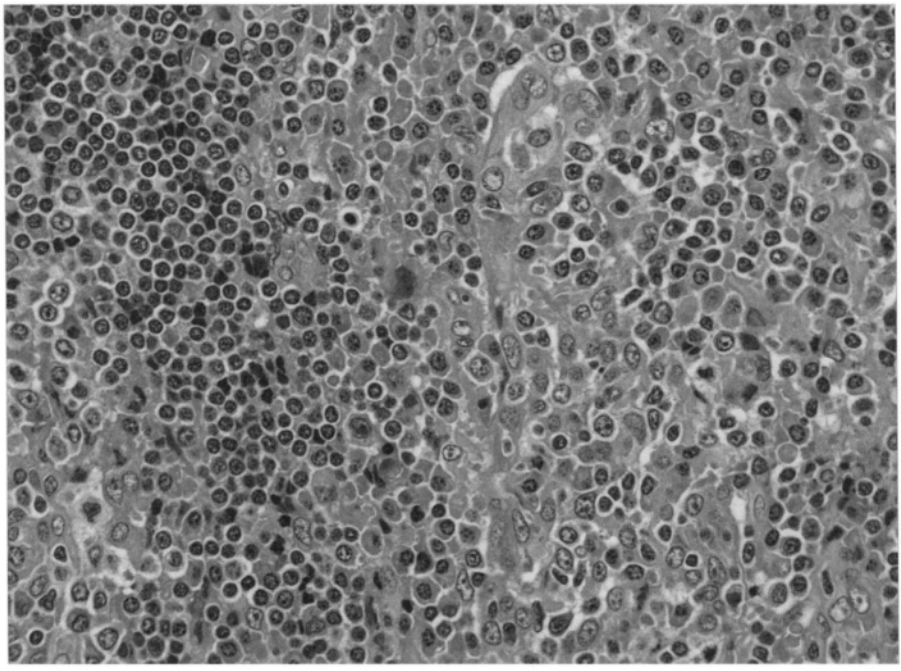

Figure 9. The plasma cell component can be seen to localize around the follicle epithelium: a para-follicular location.

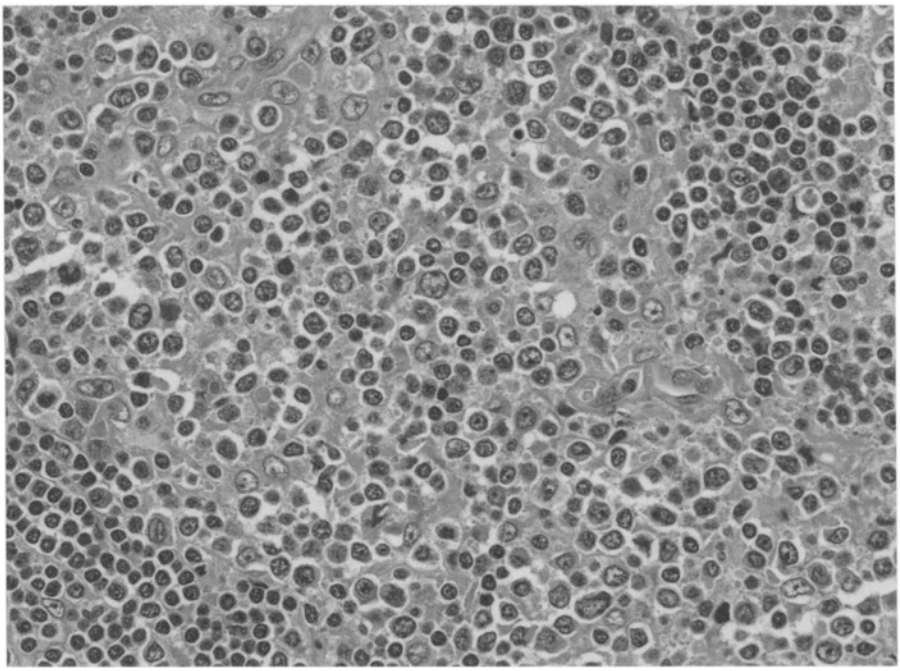

Figure 10. A cluster of centroblastic cells making this a mixed MZBL of MALT-type and DLBCL.

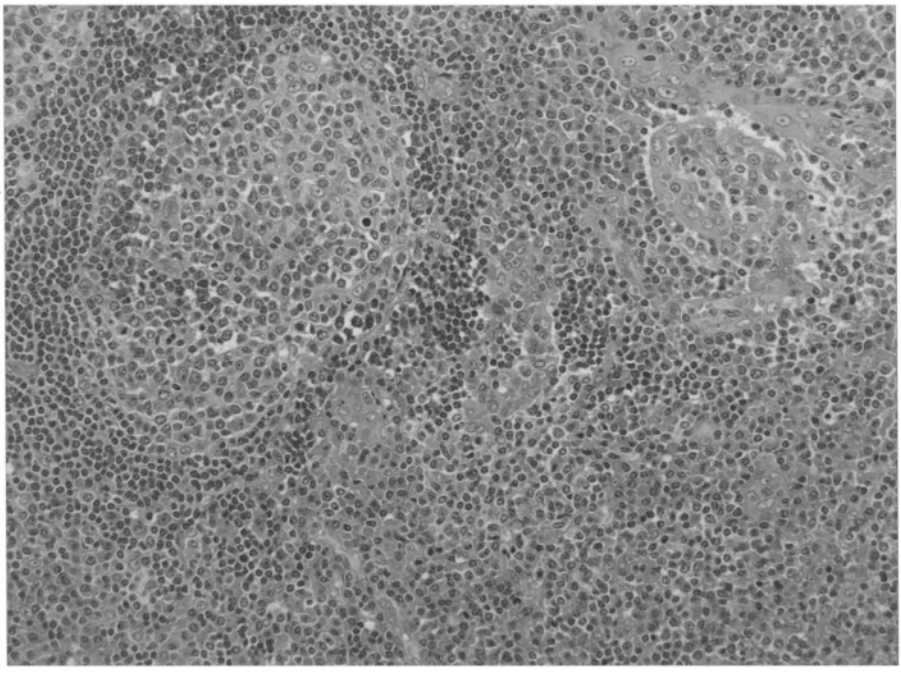

Figure 11. A reactive lymphoid follicle replete with germinal centre and mantle zone, in a MZBL of MALT-type.

or sheet or multiple areas of blast cells are encountered. In the study by Derringer and colleagues the DLBCL component accounted for at least 50% of the lymphoma (36). Thus, it would appear that the large cell component is obvious and easily discerned. The large cells may resemble centroblasts (large cells with vesicular chromatin and a small typically single nucleolus) usually, or even immunoblasts (large cells with vesicular chromatin and a prominent nucleolus) less commonly. In the series reported by Derringer et al, they even noted scattered Reed-Sternberg-like cells and cells reminiscent of the large cells seen in Burkitt's like lymphoma (36). The large cells may permeate diffusely through the thyroid with destruction of thyroid follicular epithelium or may also result in lymphoepithelial lesions (Figure 12). However, a distinct low-grade MZBL component must also be present for this lymphoma to be considered a mixed pattern.

DIFFUSE LARGE B-CELL LYMPHOMA (DLBCL) WITHOUT MZBL OF MALT-TYPE

As the name implies a histologically detectable MZBL of MALT-type as described above is not present. In a study by Skacel and colleagues, they encountered 16 cases of DLBCL without any MZBL but containing lymphoepithelial lesions composed only of large cells. These cases are more appropriately categorized as DLBCL without MZBL of MALT-type because of the absence of the latter component. Even in the absence of a low-grade component, the vast majority (85%) of DLBCL do show some of the morphological features associated with MALT-lymphoma: lymphoepithelial lesions, plasma cells, follicle colonization and monocytoid B-cells are detected although varying in number from case to case (36). In the absence of the MALT-lymphoma morphology, it is impossible to separate primary thyroid DLBCL from secondary involvement of the gland by a nodal primary. Careful examination of several sections may be required to find the morphological features of a MALT-lymphoma in an otherwise pervasive large cell infiltrate of the thyroid. The large cells of DLBCL are identical to the large cells described above.

IMMUNOHISTOCHEMISTRY

The use of immunohistochemistry in thyroid lymphomas is somewhat limited in that there isn't a characteristic or diagnostic immunoprofile. The application of LCA and cytokeratins as a first line investigation usually accomplishes the separation of lymphoma from carcinoma. MZBL of MALT-type, MZBL with blasts and DLBCL are all B-cell lymphomas and will stain positively with traditional B-cell markers (CD20, CD79a). The reactive T-cells decorate with CD3, while light chain restriction in plasma cells, which is seen in about 20% of cases, can be demonstrated with kappa and lambda stains. In some instances light chain restriction (cytoplasmic and membrane staining) can be demonstrated in the neoplastic lymphoid cells too. The neoplastic centrocyte-like cells of MALT origin are usually: CD5, CD10, CD23, cyclin D1 and IgD negative and positive for IgM. This immunophenotype reflects their origin from marginal zone B-cells in sites of chronic organ-specific inflammation caused by autoimmune disease or a specific inflammatory lesion (43–45). The use of bcl-2 immunostaining will also assist in excluding follicular lymphoma from reactive lymphoid follicles. In must be borne in

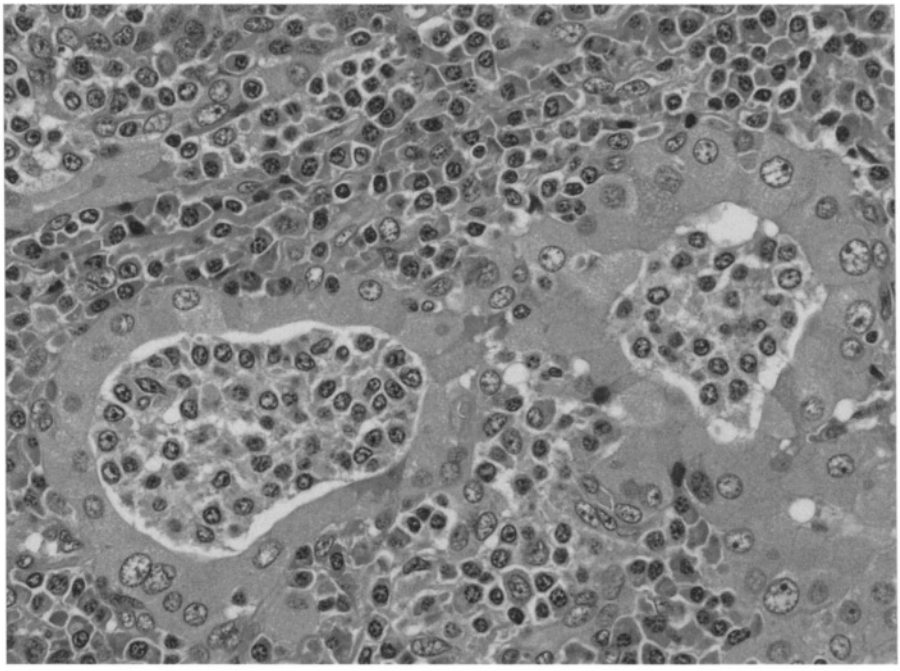

Figure 12. A MZBL of MALT-type with high-grade transformation. Here large cells have formed a lymphoepithelial lesion and occupy a thyroid follicle lumen (MALT-ball).

mind that the centrocyte-like cells in lymphoepithelial lesions and colonized follicles may also express bcl-2 protein (46, 47). Bcl-2 immunoexpression does not separate MZBL of MALT-type from Hashimoto's thyroiditis, as the centrocyte-like cells can be positive in both conditions. However, high-grade lesions lose bcl-2 immunoexpression with an accompanying increase in p53 protein expression (47).

Cytokeratin stains obviously also highlight the thyroid follicle epithelium and the degree of destruction caused by lymphoepithelial lesions. Cytokeratin staining is useful in the DLBCL cases in showing the remnants of epithelium and hence a morphological features of a MALT-lymphoma. Obviously, more directed lymphoid markers could be employed if wanting to exclude or confirm the various other types of lymphoma that may occur primarily in the thyroid gland.

CYTOGENETICS/MOLECULAR BIOLOGY

As with immunohistochemistry, there is no single cytogenetic or molecular aberration that typifies MALT-lymphomas. Within the remit of MALT-lymphomas some similar genetic findings have been noted irrespective of site, however, gastric MALT-lymphomas have been more intensively examined and have chromosomal abnormalities that tend to be commoner at this site than other MALT sites. There are some repetitive abnormalities, however. Trisomy 3 has been identified in approximately 60% of

MALT-lymphomas (48). The t (1;14) translocation involving the *bcl-10* gene, which then undergoes further mutations has been identified in 6–8% of MALT-lymphomas, especially those showing large cell transformation (49). The t (11;18) (q21;q21) translocation, leading to fusion of the *apoptosis inhibitor-2* gene and the MALT lymphoma associated translocation *(MALT1)* gene, has been found in 18–35% of MALT-lymphomas but mainly those occurring in the gastrointestinal tract and are rarely encountered in thyroid MALT-lymphomas (50). Similarly, the t(14;18)(q32;q21) translocation tends not to be present in thyroid MALT-lymphomas (50). It is clear that no single chromosomal abnormality occurs in thyroid MALT-lymphomas and that some translocations within the MALT-lymphoma group are site-specific. It is thought that the various translocations and other cytogenetic abnormalities render the lymphoid tissue unstable and permissive for the process of lymphomagenesis.

RELATIONSHIP BETWEEN LOW- AND HIGH-GRADE MALT-LYMPHOMAS

The coexistence of low- and high-grade MALT-lymphoma suggests either a pathogenetic link between the two components, or that they represent two independent *de novo* clones. The weight of evidence points to a close relationship between the low- and high-grade foci: often there is a transition noted microscopically, both immunohistochemical and molecular studies substantiate commonality and transformation of low-grade to high-grade lymphoma, and the clinical presentation of a sudden and/or rapid increase in size of an already enlarged thyroid gland (51–55). Peng and colleagues performed PCR and mutational analyses to identify clone-specific rearranged immunoglobulin heavy chain gene sequences (55). The PCR products from both the low- and high-grade foci were identical in size and direct sequencing revealed common clone-specific immunoglobulin heavy chain gene rearrangements (55).

DIFFERENTIAL DIAGNOSIS

Anaplastic (small cell) carcinoma

This type of carcinoma is likely to simulate high-grade lymphoma. Attention to the cohesive growth pattern, stromal sclerosis/desmoplasia, absence of MALT-lymphoma morphological features, especially the admixture of plasma cells, should alert one to the possibility of carcinoma. Confirmation is readily achieved by the use of epithelial immunohistochemical markers.

HASHIMOTO'S THYROIDITIS

It is important to note that similar cellular constituents (centrocyte-like and plasma cells) together with lymphoepithelial lesions are encountered in Hashimoto's thyroiditis. Hence, florid cases can be exceptionally difficult to separate from MZBL of MALT-type. It has been suggested that a dense lymphoid infiltrate with fewer intervening reactive lymphoid follicles, broad bands of centrocyte-like or clear cells and large number of lymphoepithelial lesions that diffusely efface the thyroid parenchyma, favour a diagnosis of lymphoma. In the histologically suspicious cases, the demonstration of sheets of B-cells, light chain restriction and heavy chain gene rearrangement are

ancillary features that will help in confirming the diagnosis of lymphoma. The clinical picture of sudden or rapid enlargement of the thyroid in a patient previously diagnosed as having Hashimoto's thyroiditis will also be useful. At the end of the day, there are going to be some cases (hopefully a very small number!) that clinical information, light microscopy, immunohistochemistry and molecular techniques will not be able to separate. When unsure, the possibility of lymphoma should be raised and careful follow-up of the patient should be recommended.

PROGNOSTIC FACTORS AND OUTCOME

Several prognostic factors have been advanced as indicators of poor outcome: stage greater than IE, diffuse large cell lymphoma histology, rapid clinical growth, abundant apoptosis, vascular invasion, high mitotic rate, perithyroidal extension and compressive clinical symptoms (36). The most important prognostic factor, however, is stage and this generally is in concert with the histological appearance, with MZBL of MALT-type being a low-grade lesion that is low stage with a very good prognosis and long survival.

Therapy is usually in the form of debulking surgery or combined adjuvant chemotherapy.

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4. MOLECULAR EVENTS IN FOLLICULAR THYROID TUMORS

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INTRODUCTION

The analyses of human thyroid tumor tissues have proven informative in identifying key molecular events in epithelial neoplasia. The thyroid gland gives rise to a variety of epithelial tumors that differ markedly in their biologic patterns. The accessibility of thyroid tumors provides a tractable opportunity to define mechanisms of epithelial cell transformation in a spectrum of related cancers.

Two primary issues must be considered when investigating molecular genetic alterations within human thyroid tumor groups. The first is tumor classification. Thyroid tumors are classified predominantly on the basis of morphologic features interpreted by pathologists. Morphologic features provide initial biologic and clinical information but they have been defined somewhat non-specifically in retrospective series. Thus, thyroid tumor diagnosis can be imprecise [1–4] and can create confusion when correlating molecular genetic alterations with clinical and pathologic features. Mutations that predominate in one thyroid tumor group may be identified in others and the distinction as to whether such tumors are misclassified or contain additional alterations is difficult to ascertain. A second important issue relates to mutation detection. Polymerase chain reaction-based amplification and sequencing of nucleic acids from fresh or fast-frozen tissues are most often employed. Such assays are exquisitely sensitive and prone to cross-contamination, particularly when poorly preserved or archival tissues are used. Polymerase chain reaction can even detect genetic alterations within a minute sub-fraction of tumor cells. The biologic significance of this is often unclear. Tissue

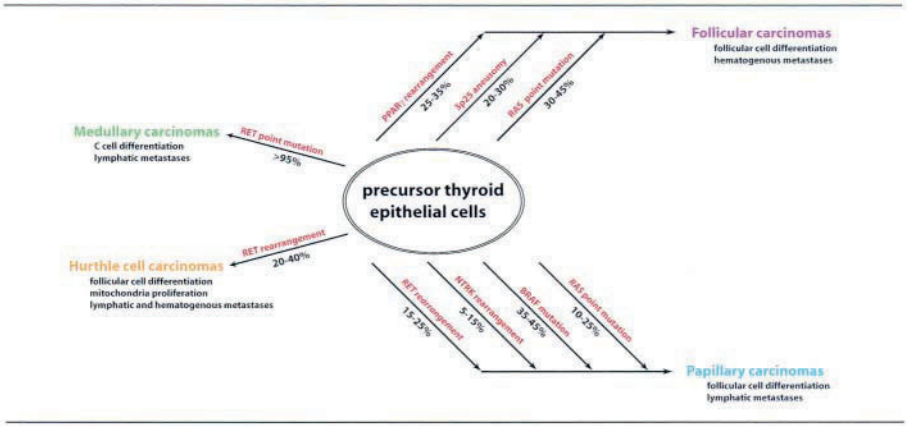

Figure 1. Histologic-Molecular Model of Thyroid Cancer Formation. Four main types of thyroid carcinoma with distinct biologic features are recognized. A subset of each type may progress to poorly differentiated and/or clinically aggressive forms. Genetic alterations that characterize these pathways and sub-pathways are shown.

composition must also be documented rigorously because thyroid tumor resections contain admixtures of tumor, normal thyroid, lymphoid, reactive and stromal elements. All such factors must be considered or erroneous results will be obtained [5–7].

This chapter begins with a histologic-molecular model of thyroid cancer formation and discusses known mutations and emerging biologic and clinical correlates in follicular thyroid tumors. A summary and comparison of thyroid carcinomas with the acute myeloid leukemias follows.

A histologic-molecular model of thyroid cancer

A model that encompasses histologic, molecular, and biologic facets of thyroid cancer formation is shown in Figure 1. At least four sub-types of thyroid cancer with distinct characteristics are recognized. Tumors within each group may progress to poorly differentiated, metastatic, and/or anaplastic forms. The thyroid carcinoma model seems unique relative to other carcinomas in several respects. First, distinct gene mutations define separate pathways of oncogenesis within the thyroid. This is different than a single linear genetic pathway envisioned commonly for other carcinomas such as those arising in the colon [8] and exocrine pancreas [9, 10]. Second, both thyroid specific and non-thyroid specific mutations characterize different thyroid carcinoma subgroups. One particularly interesting class of thyroid-specific mutations is the chromosomal rearrangements that encode gene fusions [11, 12]. Gene fusions been identified infrequently in carcinomas even though they are common in blood cell and soft connective tissue cancers [13]. Third, thyroid cancer mutations correlate with specific biologic properties. For example, *RET* and $PPAR\gamma$ rearrangements characterize papillary [14] and follicular [12] thyroid carcinomas that tend to spread via regional lymphatics or blood vessels, respectively. Distinct *RET* germ line point mutations identify different familial medullary thyroid carcinoma patients with propensities for poor

outcome and/or concomitant non-thyroid disease [15]. Thus, mutation staus provides predictive biologic information in thyroid cancer and thus may augment our current morphology-based classification and treatment schemes. Even so, it must be kept in mind that a combination ofcellular events, not single gene alterations, determines overall thyroid cancer biology. Thyroid tumors with apparently identical single gene mutations but distinct patterns of growth and/or prognoses have been reported [16–21].

PPARγ rearrangements

Somatic rearrangements in the gene encoding the nuclear receptor *PPARγ* have been identified in thyroid cancers with follicular cell differentiation, frequent encapsulation, vascular invasion and capsular penetration. These are follicular thyroid carcinomas (Figure 1). The discovery of *PPARγ* rearrangements resulted from mapping [12] of a chromosomal translocation, t(2;3)(q13;p25), which had been identified in follicular thyroid tumors [12, 22–27]. The t(2;3) rearrangement juxtaposes the promoter region and 5′ coding sequence of the *PAX8* gene on chromosome 2 with most of the coding sequence ofthe *PPARγ* gene on chromosome 3 and results in expression of a chimeric *PAX8-PPARγ* transcription factor (Figure 2).

PAX8-PPARγ is a thyroid-specific mutation and one member of a family of *PPARγ* rearrangements in follicular carcinomas. Another follicular carcinoma translocation, t(3;7)(p25;q31) [28], fuses the promoter and 5′ coding sequence of a novel transcription factor gene termed *CREB3L2* or *BBF2H7* [29] on chromosome 7 with most of the coding sequence of *PPARγ* (Figure 2). PAX8-PPARγ and CREB3L2-PPARγ (Figure 2) contain identical PPARγ sequences that include wild-type PPARγ DNA binding, ligand binding, RXR dimerization, and transactivation domains [30]. Additional putative *PPARγ* rearrangements have been detected in other follicular carcinomas [12, 22, 31, 32].

PPARγ rearrangements have been identified in 25–35% of follicular carcinomas based on studies using pathologically well-defined tissues [32–38]. *PPARγ* rearrangements [32] or *RAS* gene point mutations but not both [33] are detected early in low stage follicular carcinomas, suggesting the existence of sub-pathways of oncogenesis in follicular carcinoma (Figure 1). Such a model is further supported by distinct patterns of galectin-3 and HBME-1 protein expression in *PPARγ* rearrangement- versus *RAS* mutation-positive follicular carcinomas [33] and by an additional genetic subset of follicular carcinomas (25%) that possess 3p25 aneusomy in the absence of *PPARγ* rearrangement [32].

The mechanisms through which *PPARγ* rearrangements deregulate thyrocyte growth are being investigated and aberrations in transcription (Figure 3) and other cell functions may be involved. PAX8-PPARγ stimulates proliferation, inhibits apoptosis, and induces anchorage independent growth of human thyroid cells [39], supporting a primary role for PAX8-PPARγ in follicular cell transformation. PAX8-PPARγ also transforms NIH3T3 mouse fibroblasts in colony assays [39], demonstrating that PAX8-PPARγ can alter both thyrocyte and non-thyrocyte growth functions. PAX8-PPARγ has little ability to stimulate transcription from *PPARγ* response elements *in vitro* and also inhibits transcription mediated by wild-type *PPARγ* [12, 39], activities that fit

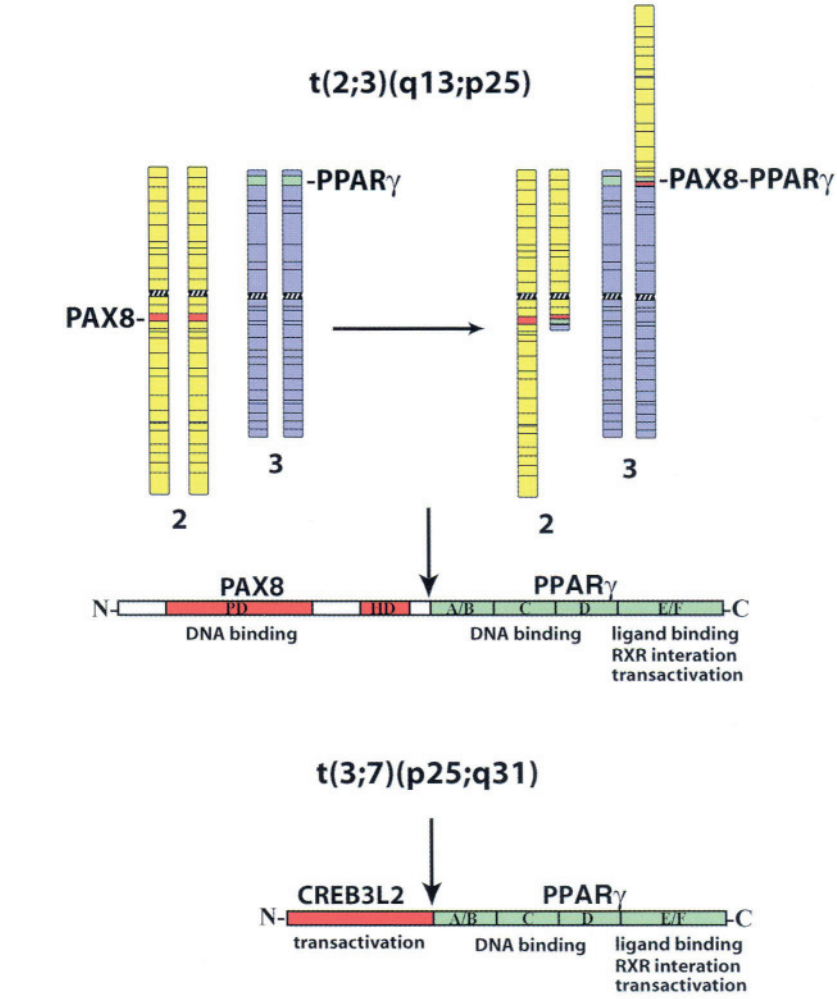

Figure 2. PPARγ Gene Rearrangements in Follicular Thyroid Carcinoma. The breakpoints of two chromosomal rearrangements, t(2;3)(q13;p25) and t(3;7)(p25;q31), have been cloned from human follicular thyroid carcinomas. Each rearrangement encodes a chimeric fusion protein that contains identical domains (A-E) of the PPARγ nuclear receptor.

well with the known tumor suppressor-like effects of wild-type PPARγ in a variety of epithelial cells [40–44]. In general, wild-type PPARγ stimulation inhibits thyroid cell growth [45, 46] and a reduction in PPARγ expression has also been noted in a significant subgroup of thyroid cancers without *PPARγ* rearrangement [32, 38]. The retinoblastoma tumor suppressor protein and cell cycle regulators may be involved [45, 47, 48].

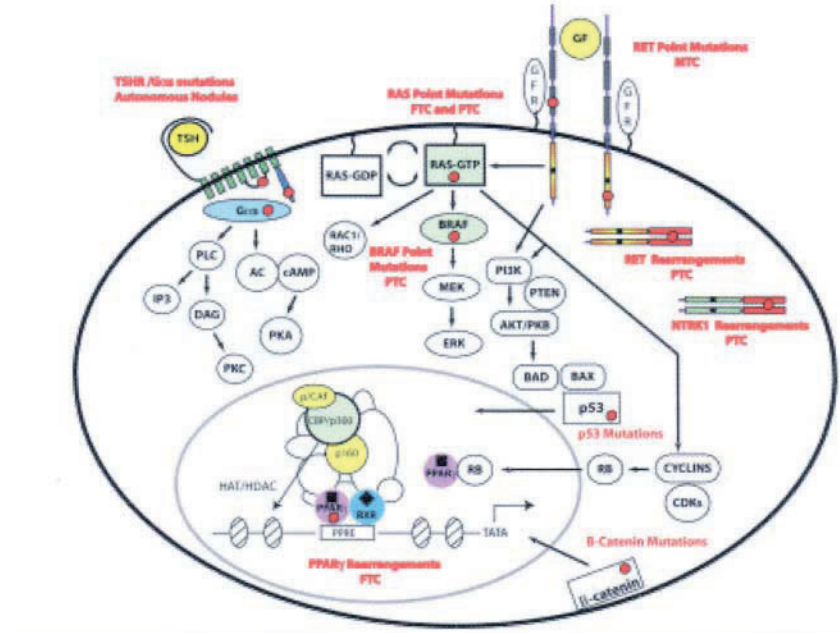

Figure 3. Molecular Pathways in Follicular Thyroid Tumors. Schematic representation of major molecular pathways involved in follicular thyroid tumors. Some, but not all, components and inter-connections of these pathways are indicated. Mutations are note in red and by red dots. Abbreviations: TSHR, thyroid stimulating hormone receptor; Gαs, guanine nucleotide stimulatory factor α; PLC, phospholipase C; IP3, inositol triphosphate; DAG, diacylglycerol; PKC, protein kinase C; AC, adenyl cyclase; cAMP, cyclic adenosine monophosphate; PKA, protein kinase A; RAC1/RHO, rac1/rho GTP binding proteins; GFR, growth factor receptor; GF, growth factor; RET, ret tyrosine receptor kinase; NTK1, ntrk1 tyrosine receptor kinase; GTP, guanine diphosphate; GTP, guanine triphosphate; RAS, ras GTP binding protein; BRAF, braf serine/threonine kinase; MEK, mitogen activated protein kinase kinase; ERK, extracellular signal regulated kinase (mitogen activated protein kinase); PI3K, phosphoinositol-3-kinase; PTEN, pten dual specificity phosphatase; AKT, akt serine/threonine kinase; PKB, protein kinase B; BAD and BAX, proapoptotic bcl-2 family members; p53, p53 tumor suppressor protein; RB, rb retinoblastoma tumor suppressor protein; CDKs, cyclin-dependent kinases; PPARγ, peroxisome proliferator-activated receptor γ; RXR, retinoid X receptor; p/CAF, CBP/p300, p160, nuclear receptor co-activators; HAT, histone acetyl transferase; HDAC, histone deacetylase complex. TATA, tata box.

Although inhibition of wild-type PPARγ by PAX8-PPARγ appears to be functionally important, the CREB3L2-PPARγ fusion protein appears to exhibit little inhibitory activity [30], suggesting that other mechanisms are also critical. PAX and CREB3L2 rearrangements have been noted in other cancers, supporting the idea that contributions of these domains in PAX8-PPARγ and CREB3L2-PPARγ are functionally important. For example, the *PAX3* and *PAX7* genes are rearranged in alveolar rhabdomyosarcoma [49–51] and *CREB3L2* is rearranged in fibromyxoid sarcoma [29]. Wild-type PAX8, a transcription factor required for normal thyroid follicular cell development [52], also possesses transforming activities *in vitro* [53].

Follicular adenomas with *PAX8-PPARγ* rearrangement have been identified at apparent lower frequency than in follicular carcinomas [33, 34, 36] and it seems most reasonable to consider these early (precursor/in situ) follicular carcinomas [32] unless genetic and/or clinical distinctions from the follicular carcinomas can be documented. *PPARγ* rearrangements are expected in at least some follicular adenomas because differential diagnosis of follicular adenomas from carcinomas is not precise. The possibility that *PPARγ* rearrangements mark a subset of follicular carcinomas, some even before histologic evidence of invasiveness is apparent, suggests that molecular analyses of fine needle aspiration biopsies may be useful to detect these follicular cancers [54]. However, the exact diagnostic utility of PPARγ rearrangements in diagnosis will not be clear until the biologic and molecular relatedness of follicular carcinomas and adenomas with *PPARγ* rearrangement is better defined. Papillary (follicular variant) and Hurthle cell carcinomas with *PPARγ* rearrangement have been observed infrequently [32, 34, 55], suggesting that these thyroid cancers arise via alternate transformation pathways (Figure 1).

Clinical and pathological characteristics of follicular carcinoma patients with *PPARγ* rearrangements have been examined. Follicular carcinomas with *PPARγ* rearrangement tend to have well-defined foci of vascular invasion and capsular penetration but not lymph node metastases [32, 33]. They also tend to present at younger patient age than follicular carcinomas without *PPARγ* rearrangement [32, 33] and progress and metastasize in some cases [23, 35]. Even so, few *PPARγ* rearrangements have been detected in anaplastic thyroid carcinomas [34, 35], which are highly aggressive cancers thought to arise from follicular and papillary carcinomas. Further studies are required to define the biologic characteristics and patterns of progression of follicular thyroid tumors with *PPARγ* rearrangement.

RET rearrangements

Somatic rearrangements in the gene encoding the *RET* receptor tyrosine kinase have been identified in a subset of thyroid cancers that exhibit follicular cell differentiation, characteristic papillary and/or nuclear morphologies, and a propensity for lymph node metastases. These are papillary thyroid carcinomas (Figure 1). Interestingly, the *RET* gene plays a fundamental role in multiple thyroid cancers. Whereas rearrangements of *RET* characterize papillary thyroid carcinomas [11, 14], germ-line *RET* point mutations characterize medullary thyroid carcinomas arising in the multiple endocrine neoplasia type 2 [56–59] and family medullary thyroid carcinoma syndromes. Thus, different *RET* mutations (rearrangements or point mutations) arising in different cellular contexts (follicular or C cell lineages) promote formation of different thyroid cancers (Figure 1). The *RET* rearrangements are discussed in detail in Chapter 12.

RET rearrangements in papillary carcinoma are thyroid-specific mutations and most often result from para-centric chromosomal inversions. For example, the *RET* gene at chromosome 10q11 is recombined frequently with other 10q loci such as *H4* in PTC1 [60] and *ELE1* in PTC3 [61, 62]. Several less frequent reciprocal translocations involving *RET* and other chromosomal loci have been described, particularly in papillary carcinoma patients exposed to radiation in the Chernobyl accident [63–65]. All

known *RET* rearrangements result in expression of cytoplasmic, chimeric fusion proteins that contain the intracellular tyrosine kinase domain of RET fused to domains of non-RET (termed RET fusion genes or RFG) genes. The extracellular cadherin-like, cysteine-rich, and transmembrane domains of RET are not retained in the RFG-RET fusion proteins.

Experiments expressing RFG-RET fusion proteins in thyroid cell lines support a central role of the RAS-BRAF-MEK-ERK pathway in neoplastic transformation of follicular cells into papillary carcinomas (Figure 3). The RFG-RET fusion proteins stimulate follicular cell proliferation and inhibit differentiation [66–70]. Apoptosis may also be altered [71]. These biologic effects are mediated by ligand-independent dimerization [72, 73], cytoplasmic relocation [73], and constitutive activation of the RET tyrosine kinase. Adaptor molecules such as Shc, Frs2, Enigma, and Grb proteins interact with RET proteins [69, 74–78] and stimulate downstream RAS-BRAF-MAPK-ERK and other signal transduction pathways.

Transgenic mouse lines engineered to express RFG-RET fusion proteins in the thyroid document their ability to promote formation of papillary carcinoma-like tumors *in vivo* [79–82]. However, these transgenic lines do not all develop thyroid tumors with high penetrance or short latency and few, if any, develop tumors that metastasize without co-expression of additional mutations, arguing that multiple alterations are required for expression of the full papillary carcinoma phenotype [66, 67, 83].

RET rearrangements have been detected in 15–25% of papillary carcinomas and have been considered specific based on RTPCR and Southern blot experiments [70, 84–91]. *RET* rearrangements appear to arise early in papillary carcinoma because they are most common in low stage and the occult/micropapillary tumors [89, 92–94]. Papillary carcinomas with *RET* rearrangements may also present at younger patient age than papillary carcinomas without *RET* rearrangements [87, 95, 96], in a manner that resembles $PPAR\gamma$ rearrangements in follicular carcinoma. Other strong clinicopathologic correlates of *RET* rearrangement include classic papillary (not follicular variant) morphology [97, 98] and the presence of lymph node spread [86, 87, 96, 98]. The ELE1-RET (PTC3) fusion protein may be more frequent in the aggressive tall cell [17] and solid [16, 20] papillary carcinoma subtypes. A significant fraction of papillary carcinomas with *RET* alterations appear to progress to poorly differentiated thyroid carcinoma [99] but few *RET* rearrangements have been detected in anaplastic thyroid cancers [84, 89].

A few recent reports have noted *RET* rearrangements, somewhat unexpectedly, in benign and malignant Hurthle cell tumors [18, 19] and in thyroid hyalinizing trabecular adenomas [100, 101]. These Hurthle cell carcinomas with *RET* rearrangements appear to have increased tendency for lymphatic spread [102], supporting a biologic connection to papillary carcinoma as well. Thus, one intriguing possibility is that the Hurthle cell tumors with *RET* rearrangement are actually papillary carcinomas with additional morphologic and perhaps biologic features. An alternate possibility that must be excluded is that the *RET* rearrangements are present in a small fraction of the tumor cells because a only combined high cycle RTPCR and nucleotide probe hybridization have so far demonstrated their presence.

NTRK1 rearrangements

Somatic rearrangements in the gene encoding the *NTRK1* receptor tyrosine kinase have been identified in 5–15% of papillary thyroid carcinomas (Figure 1). These are discussed further in Chapter 12. In essence, *NTRK1* rearrangements bear strong resemblance to *RET* rearrangements in several respects. First, both NTRK1 and RET are receptors for neurotrophic ligands [103] and are not normally expressed in follicular epithelial cells. Second, both *NTRK1* and *RET* rearrangements were identified by transfection of papillary carcinoma DNA into NIH3T3 cells [11, 14, 85]. Third, both *NTRK1* and *RET* rearrangements arise frequently from subtle intra-chromosomal inversions. Fourth, both *NTRK1* and *RET* rearrangements lead to expression of fusion proteins with constitutive tyrosine kinase activation. For example, rearrangements at 1q21 often fuse the NTRK1 tyrosine kinase domain to other proteins such as *TPM* and *TPR* [104–106]. Fifth, both *NTRK1* and *RET* rearrangements may be more frequent in younger patients and in patients with lymph node metastases [95, 96, 107]. Last, the NTRK1 and RET fusion proteins activate related signal transduction pathways in thyroid follicular cells [66, 108–111] (Figure 3). Expression of the NTRK1 fusion proteins in the thyroid of transgenic mice leads to follicular hyperplasia- and papillary carcinoma-like proliferations [112].

RAS mutations

Somatic point mutations in *RAS* genes have been detected frequently in both non-thyroid [113] and thyroid (Figure 1) cancers. This contrasts the thyroid-specific gene rearrangements involving *PPARγ*, *RET,* and *NTRK1. RAS* mutations are most common in follicular versus papillary and Hurthle cell tumors [33, 91, 114–120] and have been detected in 20–50% of follicular adenomas and carcinomas [33, 91, 119–122]. The presence of *RAS* mutations in both follicular adenomas and carcinomas is consistent with a model in which many *RAS*-initiated follicular carcinomas develop from adenoma (morphologic) precursors. Experimental evidence supports this contention in that mutated RAS is insufficient to induce a fully transformed phenotype *in vitro* [66, 123–126] or follicular carcinoma *in vivo* [127, 128]. *N-RAS* mutations appear to predominate over *K-RAS* and *H-RAS* mutations in follicular thyroid tumors and mutations in codon 61 of *N-RAS* may be the most prevalent [33, 119, 120, 129]. The possibilities that *K-RAS* mutations are more frequent in papillary compared to follicular thyroid tumors [114, 115, 130], radiation-associated carcinomas [114], and/or aggressive thyroid cancers [130] require further investigation, particularly in view of the primary role of *K-RAS* mutations in pancreatic ductal carcinomas [10, 131] that are highly aggressive.

Recent studies have correlated the clinical and pathologic features with *RAS* mutation status. Thyroid carcinoma patients with *RAS* mutations may present at older age and with larger tumors [33] and may more frequently have less differentiated, high stage cancers [130, 132–134]. Careful pathologic evaluation of classic from follicular variant papillary carcinomas has noted another potentially interesting pattern. Follicular variants seem to contain more *N-RAS* (75%) and *H-RAS* (25%) mutations and

few if any *RET* rearrangements, whereas classic papillary carcinomas seem to contain more *RET* rearrangements (30–35%) and few if any *RAS* mutations [98]. Follicular variants papillary carcinomas also had statistically lower rates of lymph node metastases and higher rates of tumor encapsulation and vascular invasion (follicular carcinoma-like features) compared to classic papillary carcinomas [98]. Thus, the existence of a morphologic and molecular "hybrid" thyroid cancer with some features of papillary and follicular carcinoma needs to be further explored.

Mouse modeling experiments have documented that *RAS* mutations are important role in tumorigenesis and tumor maintenance[128, 131, 135, 136] and RAS proteins transduce multiple stimuli from the thyroid follicular cell surface (Figure 3) as discussed further in Chapter 7.

BRAF mutations

Somatic point mutations in the *BRAF* gene have been identified recently in thyroid and other cancers [137]. *BRAF* encodes a serine/threonine kinase downstream of RAS and it transduces signals from multiple stimuli (Figure 3). A mutation that alters valine 599 to glutamic acid (V599E) in the BRAF kinase domain has been identified in 35–45% of papillary thyroid carcinomas [70, 90, 91, 120, 138–140] and in some undifferentiated/anaplastic thyroid carcinomas [90, 138]. *BRAF* mutations have been detected in few other benign or malignant thyroid tumors [70, 90, 91] and seem not to co-exist with *RAS* point mutations or *RET* rearrangements [70, 91, 138], thereby defining an additional sub-pathway in papillary carcinoma (Figure 1).

Papillary thyroid carcinoma patients with *BRAF* mutations tend to present at older age [90], at higher stage [90, 138], and with more frequent distant metastases compared to papillary carcinoma patients without *BRAF* mutation. Thus, mutated *BRAF* may define an aggressive papillary carcinoma form. In agreement with this possibility, mutated BRAF exhibits enhanced kinase activity and increased transformation efficiency compared to wild-type BRAF *in vitro* [137].

Thyroid stimulating hormone receptor and G protein mutations

Iodide uptake and thyroid hormone biosynthesis and metabolism are coordinately regulated with proliferation in thyroid follicular epithelial cells. These differentiated thyroid functions are controlled by the thyroid stimulating hormone receptor (TSHR) and its downstream signaling molecules (Figure 3) such as cyclic AMP and phospholipase C [141–143]. Somatic mutations in molecular components of the TSHR pathway have been detected in 60% or more of benign TSH-independent (autonomous/hyperfunctioning) thyroid nodules. The remaining 40% of autonomous nodules are postulated to contain undefined alterations in the same TSHR system [144]. Approximately 90% of mutations involve TSHR, often in the third intracellular loop or transmembrane regions of this seven-spanning membrane receptor [145, 146]. 5–10% of the mutations involve the G protein subunit Gsα/gsp activated by TSHR ligands [147]. Thus, constitutive stimulation of the TSHR pathway underlies most autonomous thyroid tumors [148].

Autonomous thyroid tumors usually exhibit hyperplastic morphology and transgenic mice and other animal models with an activated TSHR-Gsα/gsp-cAMP axis [149, 150] develop follicular hyperplasia and hyper-functioning thyroid tumors, supporting a fundamental role of the TSHR system. Furthermore, nodular hyperthyroidism in non-autoimmune autosomal dominant hyperthyroidism [151] and the McCune-Albright Syndrome [152] have been associated with germ-line mutations in TSHR-Gsα/gsp axis. Although chronic stimulation of the TSHR pathway promotes formation of benign thyroid nodules, this seems to provide little increased risk of thyroid cancer. Additional cellular alterations [153], potentially including the down-regulation of PPARγ[154], are apparently required.

B-catenin and *p53* mutations

Stage at presentation is a key prognostic factor in thyroid carcinoma. Mutations in the genes encoding B-*catenin,* a component of the Wnt signaling pathway [155], and *p53,* an important tumor suppressor and a sensor of genome stability, have been identified most often in advanced stage thyroid cancer. Mutations in exon 3 of B-*catenin* have been detected in 25–60% of poorly differentiated and anaplastic thyroid carcinomas [156, 157], and the expression of *B-catenin* protein is often reduced or re-localized from the plasma membrane to the cytoplasm and nucleus in these [156–158] and some follicular and papillary [157–160] thyroid carcinomas. p53 mutations have been identified mostly in poorly differentiated and anaplastic thyroid carcinomas [161–164] and they appear to interfere with differentiated functions in thyroid cells [165, 166] and promote thyroid cancer invasion and metastases in transgenic mouse models [83, 167]. The p53 pathways are discussed in detail in Chapter 8.

Aneuploidy and other chromosomal aberrations

A low level of chromosomal instability is observed in benign thyroid tumors and well-differentiated thyroid cancers such as papillary carcinoma, a moderate level of chromosomal instability is observed in follicular carcinoma, and higher levels of chromosomal instability are observed in Hurthle cell, poorly differentiated/anaplastic, and metastatic carcinomas. Thus, increased chromosomal instability and aneuploidy correlate generally with increased thyroid cancer aggressiveness. On the other hand, microsatellite instability is relatively infrequent in thyroid cancer [168–174]. Exposure to ionizing radiation increases genetic instability and thyroid carcinoma prevalence as discussed in Chapter 11.

Analyses of human thyroid tumors with conventional cytogenetics and fluorescence in situ hybridization have identified additional recurrent chromosomal abnormalities. Hyperplastic nodules from thyroid goiters often contain one or two clonal numerical changes, including trisomies of chromosomes 7, 10, 12, 17, and/or 22, whereas follicular adenomas more frequently contain three or more numerical chromosomal alterations and/or balanced chromosomal rearrangements [25, 175–178]. However, it should be kept in mind that karyotypes frequently present an incomplete picture of chromosomal content because the cultures may frequently appear diploid as the result

of contaminating normal cells. All suspected genetic alterations must be verified in primary thyroid tumor tissues.

The chromosomal regions 2p21 and 19q13 are rearranged in approximately 10% and 20%, respectively, of thyroid follicular adenomas with clonal cytogenetic aberrations. Both the 2p21 [26, 175, 179] and 19q13 [24, 175, 180] loci fuse with multiple different partner chromosomes in different follicular adenomas. The 2p21 and 19ql3 breakpoints have been mapped using follicular adenoma cell lines that contain t(2;7)(p21;p15), t(2;20;3)(p21;q11;p25), t(5;19)(q13;13), or t(1;19)(p35;q13).The 2p21 breakpoint appears to involve a novel candidate gene termed *THADA* [181, 182] and the 19q13 breakpoint a novel transcription factor gene termed *ZNF331/RITA* [183–185]. It will be informative to define the cell biologic and biochemical mechanisms of these new thyroid rearrangements.

Additional genetic imbalances have been defined in follicular thyroid tumors using loss of heterozygosity studies and comparative genomic hybridization techniques. Genetic gains predominate over losses in follicular adenomas, whereas genetic losses predominate over gains in follicular, Hurthle, and anaplastic thyroid carcinomas. The most consistent losses in follicular cancers involve chromosomes 2p [186–189], 2q [186–188], 3p [169, 174, 187–191], 7q [188, 192, 193], 9 [174, 187, 188, 194, 195], 10q [196–198], 11q [187, 189, 195, 197, 199, 200], 13q [187, 188, 196, 197], 17q [201], 18q [174, 187, 197], and 22q [187, 188, 195, 202, 203] regions. In addition to these losses, Hurthle cell carcinomas harbor deletions at 1q, 8q, 9q, 14q, and 17p [174, 194, 201]. The possibility that at least some of these genomic loci contain genes important in thyroid tumor pathogenesis is reinforced by the fact that three regions (2q13, 3p25 and 7q31) have been shown to be involve follicular carcinoma rearrangements [12, 30]. Thus, functionally important loci may be targeted by multiple genetic mechanisms.

Summary

Knowledge of the molecular events that govern human thyroid tumorigenesis has grown considerably in the past ten years. Key genetic alterations and new oncogenic pathways have been identified. Molecular genetic aberrations in thyroid carcinomas bear noteworthy resemblance to those in acute myelogenous leukemias. Thyroid carcinomas and myeloid leukemias both possess transcription factor gene rearrangements—*PPARγ*-related translocations in thyroid carcinoma and *RARα*-related and *CBF*-related translocations (amongst others) in myeloid leukemia [204]. PPARγ and RARα are closely related members of the same nuclear receptor subfamily, and the PML-RARα and PAX8-PPARγ fusion proteins both function as dominant negative inhibitors of their wild-type parent proteins [12, 205, 206]. Thyroid carcinomas and myeloid leukemias [207–210] also both harbor *NRAS* mutations (15–25% of both cancers) and receptor tyrosine kinase mutations – *RET* mutations in thyroid carcinomas and *FLT3* mutations in myeloid leukemias [211, 212]. The *NRAS* and tyrosine receptor kinase mutations are not observed in the same thyroid carcinoma or leukemia patients [209, 213], suggesting that multiple initiating pathways exist in

both. Lastly, thyroid carcinomas [214] and myeloid leukemias [209, 215] possess p53 mutations at relatively low frequency (10–15%) in patients who tend to be older and have more aggressive, therapy resistant disease. Such parallels are unlikely to occur by chance alone and argue that common mechanisms underlie these diverse epithelial and hematologic cancers.

The comparison of thyroid carcinomas and myeloid leukemias may highlight areas of thyroid cancer investigation worthy of further focus. For example, few collaborating mutations have been defined in thyroid carcinomas even though they play a clear role in myeloid leukemias [212, 216], as exemplified by *RARα* rearrangements [217, 218] and *FLT3* mutations [219] that together dictate the promyleocytic leukemia phenotype. Functional interactions between collaborating mutations are possible at multiple levels, and it is tempting to speculate that some thyroid carcinomas might develop through an unique combination or co-activation of RET and RAS and/or RET and PPARγ (and/or other) signaling systems. In fact, the ELE1-RET (PTC3) fusion protein contains the ELE1 nuclear receptor co-activator domain [220, 221] and it appears to physically associate with and inhibit wild-type PPARγ in some papillary carcinomas [222].

The similarities of the fusion proteins in thyroid carcinoma and myeloid leukemia suggest that a more directed search for fusion genes in non-thyroid carcinomas is warranted. In fact, novel fusion genes have been identified recently in aggressive midline [223, 224], secretory breast [225], and renal cell [226–232] carcinomas, although the epithelial nature of the latter is not well-documented. Interestingly, these cancers all tend to present more frequently in adolescence and young adulthood in a manner similar to thyroid and myeloid [233] malignancies that have fusion genes. The analyses of cancers that present earlier in life may enhance fusion gene recognition in other carcinoma types.

Definition and biologic characterization of the precursor cells that give rise to thyroid carcinoma will also be important. Myeloid leukemias are thought to arise from stem/progenitor cells that acquire disturbed self-renewal and differentiation capacities but retain characteristics of the myeloid lineages. Although the presence of comparable stem/progenitor cells in the thyroid are not defined, distinct thyroid cancer lineages and patterns of differentiation exist and candidate stem/progenitor cells such as the p63-immunoreactive solid cell nests [234] are apparent.

A last important area is development of molecular-based therapies for thyroid carcinoma patients resistant to standard radio-iodine treatment. Treatments for such cancers are limited and pathways defined by thyroid cancer mutations are prime targets for pharmacologic interventions with molecular inhibitors. Tyrosine kinase inhibitors [235–239] and nuclear receptor ligands [240–242] have proven dramatically effective in some myeloid leukemia patients. Various molecular inhibitors are being investigated now in thyroid cancer models [45, 243–249]. Such developments predict that the thyroid cancer model will continue to provide biologic insights into human carcinoma biology and that improved pathologic diagnosis and treatment for thyroid cancer patients sit on the not too distant horizon.

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5. MOLECULAR EPIDEMIOLOGY OF THYROID CANCER

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INTRODUCTION

Molecular biology studies have greatly enhanced our knowledge of thyroid tumorigenesis, although their impact in clinical practice is still negligible.

Most benign and malignant thyroid tumors have a monoclonal origin, suggesting that genetic events are responsible for their occurrence (34). These may involve the activation of oncogenes or the inactivation of tumor-suppressor genes. Several genetic abnormalities (point mutations or gene rearrangements) have been evidenced in human thyroid tumors (review in 14,40,53). Several *in vitro* and *in vivo* animal models, including transgenic mice that reproduce the human situations, are also available.

ONCOGENES AND THYROID TUMORS

Tyrosine kinase receptors

Growth factors act on the target cell through interaction with specific membrane receptors, some of which belong to the family of tyrosine kinase receptors. The genes encoding these receptors are frequently involved in the pathogenesis of human cancers, including thyroid cancer. Whenever uncontrolled activation of a tyrosine kinase receptor gene occurs, either through overexpression or activating mutations, increased responsiveness to growth factors or ligand-independent gene activation ensues, both of which then activate the signaling pathways downstream. Three tyrosine kinase receptor

genes are known to be associated with the pathogenesis of papillary thyroid cancer: the *met* gene through overexpression and the *ret* and *trk* genes through gene rearrangements.

Ret */PTC oncogene*

MOLECULAR BASIS OF *Ret*/PTC REARRANGEMENTS. The *ret* proto-oncogene is a 21-exon gene located on chromosome 10q11-2 that encodes a membrane tyrosine kinase receptor. The ret receptor together with the glial cell line-derived neurotropic factor (GDNF) receptor (GFRα-1), an extracellular protein tethered to the cell membrane, form a receptor for GDNF. The ret receptor may also combine with other members of the GFRα receptor family, thereby forming receptors for other peptides (artemin, neurturin, persephin). The ret protein is composed of an extra-cellular domain, with a distal cadherin-like domain and a juxta-membrane cystein-rich domain, a transmembrane domain and an intra-cellular domain with tyrosine-kinase activity. The gene is expressed in a variety of neuronal cell lineages including thyroid C cells and adrenal medulla but is not expressed in normal thyroid follicular cells.

Under normal conditions, the ret ligands induce receptor dimerization and tyrosine trans-phosphorylation of the receptor kinase domain, thus activating the pathways downstream. When the gene is mutated, ligand interaction is no longer needed for receptor activation and the downstream pathways are continuously activated: ret/PTC kinase activity promotes interaction with shc, an intermediate in the RAS-RAF-MEK-MAP kinase pathway. Inappropriate activation of this pathway induces abnormal proliferation and differentiation in many human cancers and also induces genomic instability.

Ret activation was first evidenced by transfection experiments and was initially found exclusively in papillary thyroid carcinoma (PTC). The resulting oncogene was thus called *ret*/PTC (16,18,40). All activated forms of the *ret* proto-oncogene are due to chromosomal rearrangements in which the 3′ or tyrosine kinase domain of the *ret* gene is fused with the 5′ domain of a foreign gene. The foreign gene is constitutively expressed, resulting in permanent expression of the rearranged *ret* gene. These rearranged genes have coiled-coil domains that activate the ret protein through permanent dimerization. They also lack the intracellular juxta-membrane domain that normally exerts a negative regulatory effect on ret tyrosine kinase activity. Finally, the chimeric protein lacks the extracellular and transmembrane domains and is located in the cytosol. Three major forms of the *ret* rearrangements have been identified in epithelial thyroid tumors:

Ret/PTC$_1$, is formed through an intra-chromosomal rearrangement fusing the *ret* tyrosine kinase domain to a gene designated H4, whose function is still unknown.

Ret/PTC$_2$, is formed through an inter-chromosomal rearrangement fusing the *ret* tyrosine kinase domain to a gene located on chromosome 17 that encodes the RIα regulatory subunit of cAMP-dependent protein kinase A.

Ret/PTC$_3$, is formed through an intra-chromosomal rearrangement fusing the *ret* tyrosine kinase domain to a gene designated ELE1, whose function is still unknown.

In the three major *ret* rearrangements (*ret*/PTC$_{1;2;3}$), the breakpoints of the *ret* gene are located in the same intronic region, between exons 11 and 12. Several other

ret/PTC rearrangements have been observed that differ because of the location of the breakpoint in the *ret* gene or because of the partner gene.

Recent studies have shown that the unique spatial proximity of *ret* and H4 partner genes in the nuclear matrix of thyroid cells (but not in other cell types) may be a major reason for the development of *ret* rearrangement following exposure to ionizing radiation and may explain why *ret*/PTC are found exclusively in papillary thyroid carcinomas (30).

FREQUENCY OF *ret*/PTC REARRANGEMENTS. The frequency of *ret*/PTC rearrangements in papillary thyroid carcinomas occurring in adult patients who never received neck irradiation during childhood varies between 2.5 and 35% among the different series (3,4,11). *Ret*/PTC1 and *ret*/PTC3 are the most frequent rearrangements in these tumors and *ret*/PTC2 is less frequent. Variations in the frequency and type of rearrangements could be due to differences in the geographical origins of the populations studied or in the sensitivity of the method used to detect the rearrangements. In children with sporadic papillary thyroid carcinoma without a history of radiation exposure, the incidence of *ret*/PTC-positive tumors is similar to that observed in adult patients of the same ethnic background (3,11). *Ret*/PTC rearrangements are more frequently found in papillary thyroid carcinomas occurring after exposure to ionizing radiation during childhood, due to external irradiation or to the Chernobyl accident (see below).

In patients who did not previously receive neck irradiation, rearranged *ret* genes were detected only in papillary thyroid carcinomas. All other tumors studied (thyroidal or non thyroidal) were negative for *ret*/PTC1 (48). In two series, 15–21% of thyroid adenomas were *ret*/PTC positive, but the existence of micropapillary thyroid carcinomas cannot be excluded (20).

That *ret*/PTC rearrangement is found in papillary thyroid microcarcinomas suggests that it is an early event in thyroid carcinogenesis (59). In patients with multifocal disease, diverse *ret*/PTC rearrangements were found in different tumors from the same patient, indicating that these tumors had arisen through distinct initiating events (54).

A high frequency of *ret*/PTC rearrangements has also been found in Hürthle cell papillary thyroid carcinomas (7), and in about 10% of poorly-differentiated thyroid carcinomas, demonstrating that these *ret*-positive tumors derive from papillary thyroid carcinomas (50). On the other hand, *ret*/PTC-positive tumors lack evidence of progression to undifferentiated tumor phenotypes (55).

Transfection of the *ret*/PTC1 gene in normal rat thyroid cells resulted in loss of differentiation and of TSH growth dependency. However, cells were totally transformed only after transfection with *ret*/PTC and mutated *ras* genes, suggesting that simultaneous activation of several genes is necessary for tumor progression. Transgenic mice in which the *ret*/PTC1 gene is expressed in thyroid tissue develop papillary thyroid carcinoma, which is histologically identical to the human cancer (24,49). However, in this model all thyroid cells possess the rearrangement but only a few cells give rise to tumors a few months after birth, suggesting that at least one more mutation is needed to give rise to PTC. The use of the *ret*/PTC3 gene results in a more aggressive histological and clinical behavior. This is consistent with a more aggressive type of human papillary thyroid carcinomas in which *ret*/PTC3 can be evidenced (42,56).

Trk oncogene

The *trk* proto-oncogene is located on chromosome 1. It encodes a membrane tyrosine kinase receptor for the nerve growth factor (NGF). *Trk* expression is restricted to peripheral nerve ganglia.

Activated forms of the *trk* proto-oncogene are the result of chromosomal rearrangements in which the 3′ or tyrosine kinase domain of *trk* is fused with the 5′ domain of a foreign gene (17,40). The foreign gene is constitutively expressed giving rise to permanent expression of the rearranged *trk* gene. These genes have domains that induce *trk* activation through permanent dimerization. All the breakpoints in these chimeric genes are located in the same *trk* domain.

Several rearrangements have been found in human thyroid tumors:

N-trk is formed through an intra-chromosomal rearrangement fusing the *trk* tyrosine kinase domain with the 5′ region of the non muscular tropomyosine gene.

Trk-T_1 and *trk*-T_2 are formed through fusion of the *trk* tyrosine kinase domain with the 5′ region of the translocated promoter region *(tpr)* gene.

Trk-T_3 is formed through fusion of the *trk* tyrosine kinase domain with the 5′ region of a gene called *tag* (*trk* activating gene).

Trk rearrangements have only been found in papillary thyroid carcinomas. Their frequency is lower than that of *ret*/PTC, ranging from 0 to 10% (3,5,42).

Met *oncogene*

The *met* proto-oncogene encodes a membrane tyrosine kinase receptor. Its ligand is the hepatocyte growth factor (HGF) or scatter factor (SF). HGF-SF is a potent mitogen for epithelial cells and promotes cell motility and invasion.

The *met* proto-oncogene can be activated either as a result of rearrangement with unrelated sequences (this mechanism is not found in thyroid carcinoma) or through overexpression. Overexpression of the *met* oncogene was found in about 50% of papillary thyroid carcinomas and this may be a factor in metastatic spread (9). Negative or low *met* oncogene expression has been found in the other histologic types.

A relationship has been found between *ras* and *ret* activation and *mg* overexpression in human thyroid epithelial cells. This overexpression may in turn sustain their growth through the action of HGF secreted by stromal cells.

Defects in the intracellular signaling pathway: *ras* and *b-raf*

The *ras* genes *(H-ras, K-ras* and *N-ras)* encode a 21 kD protein (p21) involved in signal transmission from cell membrane receptors to growth factors to the nucleus. The *ras* gene is activated by point mutations in codon 12 or 61 and sometimes in codon 13 or 59.

Ras mutations were initially found in up to 50% of benign or malignant thyroid tumors and was the most frequent genetic alteration found in these tumors. All three *ras* genes (H, K and N) were found to be activated at a similar frequency (11–15%) in thyroid tumors. No predominance of mutations in critical codons (12,13 or 61) or in base substitution was reported. The frequency of *ras* mutations in papillary thyroid

tumors is in general lower than in follicular tumors and varies from 0 to 60% in different series (6,25,35,52,62). In subsequent studies, controversial results were reported and a recent review found that the frequency of ras mutations was lower than initially reported: *ras* oncogene mutations were found more frequently in follicular carcinomas (34%) than in benign adenomas (19%), codon 61 being the most frequently involved; *ras* mutations are more rarely observed in other types of thyroid tumors, being present in 11% of papillary thyroid cancers (58).

The tumorigenic role of the *ras* gene in the thyroid has been studied in normal follicular cells transfected with a mutated *ras* gene. Under such conditions, follicular cell proliferation is increased and the expression of differentiation markers such as thyroglobulin, thyroperoxidase and NIS is reduced or abolished. Transgenic mice, with *ras* gene expression targeted at thyroid cells, develop both thyroid hyperplasia and papillary thyroid cancer (44), and follicular adenoma or carcinoma (47), with a decline in the expression of differentiation markers. Mutated ras proteins stimulate cell division, inhibit cell differentiation and cause genomic instability and facilitate additional mutagenic events.

B-raf gene has been found to be activated by mutation in human cancers, in 66% of malignant melanomas and in less than 15% of colon carcinomas. In melanomas, 98% of the mutations are a missence thymine (T) to adenine (A) transversion at codon 1796, resulting in a valine to glutamate substitution at residue 599 (V599E). This mutation was found in 36% and 69% of papillary thyroid carcinomas but was not found in any of the other types of follicular cell derived tumors (8, 26). Moreover, there was no overlap with *ret/*PTC*, ras* and *b-raf* mutations. Thus, the frequent activation at various points of this pathway may be a key event in the pathogenesis of papillary thyroid carcinoma.

PAX8-PPARγ1 fusion gene

Cytogenetic studies of follicular carcinomas have evidenced abnormalities in chromosomes 2 and 3 (19), and the molecular basis for a chromosomal translocation t(2; 3)(q13; p25) was recently reported. The chromosome 2q13 breakpoint lies within the coding region of the thyroid transcription factor Pax8, and the 3p25 breakpoint within the coding region of PPARγ isoform 1 (27). Pax8 (Paired Box 8) is a transcription factor that plays a role in thyroid ontogenesis and in the expression of several thyroid specific genes. PPARγ (Peroxisome Proliferator-Activated Receptor gamma) is a transcription factor belonging to the hormone nuclear receptor family. Through dimerization with RXRα (Retinoid X Receptor alpha), PPARγ plays a role in the regulation of lipid metabolism, the inflammatory process, differentiation, the cell cycle and tumorigenesis. The fusion protein consists of PAX8 paired and homeobox binding domains, and PPARγ1 DNA binding, ligand binding, dimerization, and transactivation domains. When the PAX8-PPARγ1 fusion gene was transfected to heterologous cells, it did not transactivate promoter constructs containing PPAR response elements, either alone or in the presence of troglitazone, the PPAR ligand agonist. The fusion construct did however prevent wild-type PPARγ1–mediated transactivation, indicating that it may have a dominant negative effect. This negative effect may inhibit terminal differentiation and growth suppression induced by PPARγ agonists.

The PAX8-PPARγ1 translocation was found in 26%–63% of follicular carcinomas and in 8–13% of follicular adenomas. It was not found in normal thyroid tissues, nor in nodular hyperplasia, papillary, Hürthle cell and anaplastic carcinomas (27,30,38,39).

In one series of follicular carcinomas, 86% revealed either ras (58%) or PPARγ1/PAX8 (30%) mutations, and there was no overlap between these two mutations, PPARγ1/PAX8 rearrangement was almost exclusively found in follicular carcinomas that occurred at a younger age, that were small and widely invasive; in contrast, *ras* mutations occurred with a similar frequency in both adenomas and follicular carcinomas; these carcinomas occurred at an older age, were larger and were less invasive. All these data suggest two different pathways in follicular tumorigenesis (39).

The PAX8-PPARγ1 translocation is associated with PPARγ1 protein overexpression. PPARγ1 expression is downregulated in some thyroid carcinomas (1) and is overexpressed in some tumors without a detectable PAX8-PPARγ1 translocation, which suggests that PPARγ may have other translocation partners. Indeed, a novel gene, located at 7q34 and provisionally named FTCF (follicular thyroid carcinoma fusion) was recently detected fused to the 5′ region (exons1–6) of the PPARγ1 gene, leading to expression of a FTCF-PPARγ1 fusion transcript and fusion protein.

Defects in the TSH stimulation pathway: TSH-receptor gene and *gsp* oncogene mutations

TSH stimulates follicular cell proliferation and differentiation by binding to a membrane receptor, the TSH-receptor (TSH-R). The TSH-R belongs to the receptor family with 7 transmembrane domains coupled to G proteins. These are heterotrimeric proteins, composed of three sub-units, α, β and γ. Binding of TSH to its receptor stimulates the enzyme adenylate-cyclase, through interaction with a Gs protein, that in turn increases the intra-cellular concentration of cAMP. This acts as a second messenger stimulating protein kinase A (PKA). Activated PKA phosphorylates different target proteins and particularly, the cAMP-responsive transcription factor (cAMP responsive element binding protein, or CREB) in the nucleus. Other pathways may be involved in the intra-cellular transduction of the message.

Several point mutations activating the TSH-R have been described in toxic adenomas with wide variations in frequency (from 10% to more than 80%) between the different series. Such differences may be explained by geographical variations, patient selection but also because different regions of the TSH-R were studied. Most activating mutations are found within or near the third intra-cellular loop, a region implicated in the interaction with the Gs protein. Transfection experiments have shown that the mutated TSH-R is constitutively activated, but differences exist between different mutations in terms of the extent of the increase in basal cAMP, the activation of signal transmission and response to TSH stimulation (57).

Activating point mutations in one of the 2 critical codons of the α subunit of the Gs protein gene (then called *gsp*) have been found in 7 to 38% of toxic adenomas (46, 57). As a result, mutations were found in these 2 genes in 40–60% of hyperfunctioning adenomas. It may be hypothesized that in negative tumors, alterations in another gene participating in the cAMP pathway may be responsible for the phenotype. Activating

mutations of the TSH-R have also been found in the rare hyperfunctioning follicular carcinomas with high radioiodine uptake and thyrotoxicosis (45).

In transgenic mice, gsp and TSH-R activating mutations have been demonstrated to play a role in the development of hyperfunctioning thyroid tissue. The expression of an A2 adenosine receptor gene (equivalent to TSH-R) in thyroid tissue induces diffuse thyroid hyperplasia and early hyperthyroidism (57). The expression of gsp in thyroid tissue induces focal hyperfunctioning, that is equivalent to that of a human hyperfunctioning nodule with late hyperthyroidism (33).

Activating mutations of gsp and TSH-R have also been found in hypofunctioning benign and malignant follicular thyroid tumors but at a low frequency (<10%) (45,51). In follicular thyroid carcinomas they are restricted to a subset of tumors with high basal adenylate cyclase activity. These data suggest that gsp and TSH-R mutations may participate in the tumorigenesis of some hypofunctioning thyroid tumors, by conferring a growth advantage to a cellular clone in which another yet unknown genetic alteration has already abrogated the growth-limiting mechanism, which normally down-regulates the response to cAMP (53).

Tumor suppressor genes and other genetic abnormalities

Tumor suppressor genes code for proteins that normally inhibit or restrict cell division. They become tumorigenic through loss of function and tend to act in a recessive manner. One allele is usually lost as part of a large deletion of chromosomal material, while the other allele is inactivated by a point mutation.

No genomic abnormalities were found in *Rb,* the retinoblastoma gene. However, transgenic mice with thyroid specific expression of a human papilloma virus, type E7, develop nodular goiter and subsequently papillary and follicular thyroid carcinomas (28). This protein can functionally inactivate the Rb protein, suggesting that the latter acts in the negative control of cell proliferation (14,53).

A high frequency of inactivating point mutations (22 to 83%) in the *p53* gene were observed in anaplastic but not in differentiated thyroid carcinomas (10,13,23). These data suggest that inactivation of the *p53* gene may be a key event in progression from differentiated to anaplastic carcinoma and that this alters cell differentiation. Conversely, the bcl2 protein is expressed in differentiated thyroid carcinomas but is absent in anaplastic tumors (41).

Mutations in the adenomatous polyposis colonic *(APC)* gene probably contribute to the development of thyroid cancer seen in familial adenomatous polyposis, but linkage analysis excluded the APC gene as a rare susceptibility gene for familial papillary thyroid carcinoma.

Germline inactivating mutations in the *PTEN* gene are found in 80% of patients with in Cowden's disease (multiple hamartomas, breast and follicular thyroid tumors) (12). If no hamartomas are present, *PTEN* germline mutations are found in only 5% of the families with breast and thyroid tumors. *PTEN,* the phosphatase and tensin homolog gene is an inhibitor of Akt1, a critical intermediary in several Phosphatidyl Inositol 3 (PI3) kinase signaling transduction pathway. In sporadic follicular thyroid carcinoma, mutations are rare but *PTEN* gene expression is decreased and expression

and phosphorylation of Akt are increased and this may be involved in follicular pathogenesis (43).

Linkage studies have permitted chromosomal mapping of at least 3 syndromes with a preponderance of familial PTC (29). A syndrome of familial PTC together with papillary renal neoplasia has been mapped to 1q21. This syndrome is clinically and genetically distinct from other familial tumor syndromes and is not a variant of familial papillary renal carcinoma caused by inherited activating mutations of the *MET* proto-oncogene. A familial syndrome characterized by PTC alone has been mapped to 2q21. Two different studies reported genetic linkage to 19p13.2 of a large kindred with different clinical features: in one family all thyroid tumors were oxyphilic (TCO) and many were benign; in the other one no oxyphilic changes were found and all tumors were PTC.

There are also a number of familial disorders potentially related to familial PTC, including familial goiter syndromes, one syndrome located at 14q and another at Xp22. Finally, thyroid nodules may be associated with either hypothyroidism or hyperthyroidism when gene mutations are components of pathways of thyroid metabolism or its regulation.

Loss of genetic sequences has been described in the long arm of chromosome 11 (11q13) in sporadic follicular thyroid tumors and, as described above in the short arm of chromosome 3, but only in follicular carcinomas (19,40).

Simian virus 40 (SV40) large T antigen (Tag) sequences have been detected in several human tumors, and are believed to be the result of SV40 infections. The presence of the Tag region of SV40 has been demonstrated in 66% of papillary thyroid cancer and in 100% of anaplastic thyroid cancer, as well as in normal thyroid tissue adjacent to these tumors (60). The high prevalence of SV40 footprints has been interpreted as a possible participation of this oncogenic virus in the onset/progression of specific thyroid carcinomas. Further studies are needed to understand the role of this finding in thyroid tumorigenesis.

RADIATION-ASSOCIATED THYROID TUMORS

The thyroid gland is highly sensitive to radiation during childhood, the excess relative risk per Gray being 7.7, and 88% of thyroid cancers occurring in these subjects being attributable to radiation. The irradiated thyroid gland is thus an adequate model for the study of radiocarcinogenesis.

Genetic predisposition

Several epidemiological studies have suggested a familial predisposition to developing a thyroid carcinoma after irradiation. Firstly, approximately 3–5% of patients with thyroid cancer, without previous exposure to radiation, have a familial history of the same disease (29). Secondly, when both individuals in sibling pairs were irradiated, the occurrence of thyroid tumors was concordant more often than would have been expected by chance. Thirdly, patients with one radiation-induced tumor (thyroid, salivary, neural, parathyroid) are more likely to develop another tumor than patients with comparable risk factors but who had never had a tumor. This predisposition may

be related to a defect in DNA repair mechanisms, but lifestyle risk factors may also explain some of these epidemiological findings.

Age at exposure

Epidemiological studies have shown that the carcinogenic effects of radiation are maximal during early childhood and then decrease rapidly with increasing age. This has been linked to the growth rate of the thyroid gland. Carcinogenesis is a multi-step process, and after the occurrence of a genetic abnormality, several cell divisions are needed for lesions to accumulate and for clonal expansion.

Indeed, a number of experiments in rats have shown that after thyroid exposure to radiation, the risk of developing a thyroid tumor is increased when cell proliferation is stimulated (administration of goitrogens, high or low iodine diet, partial thyroidectomy, TSH stimulation) and decreased when cell proliferation is decreased (hypophysectomy, administration of L-thyroxine). In a recent study in rats, high and low iodine diet both increase proliferation, and both induced thyroid adenomas but no thyroid malignancies occurred. Thus both a mutagenic event (radiation exposure) and increased proliferation rate are needed for the occurrence of thyroid carcinoma (2).

Ionising radiation is less carcinogenic in adults, in whom growth has already been completed: during adulthood, thyroid cell replication rarely occurs (doubling time: 8 years). In contrast, in children thyroid cells are in the process of active replication and this could allow intracellular accumulation of abnormalities that heighten the likelihood of an emerging abnormal clone of transformed cells.

Genetic abnormalities in radiation-associated thyroid tumors

Irradiation of the thyroid may directly induce *ret/*PTC rearrangements. This was found to be the case when *ret/*PTC rearrangements were induced in a dose-dependent fashion after *in vitro* irradiation of human cell lines of undifferentiated thyroid cancer (22). Chromosomal loci involved in the *ret/*PTC1 rearrangement (i.e. *ret* and H4) are juxtaposed during the interphase in normal human thyroid cells, providing a target for radiation to induce simultaneous double-stranded DNA breaks that lead to erroneous nonhomologous recombination via end-joining (37).

*Ret/*PTC rearrangements were found in 55–85% of papillary thyroid carcinomas that developed in children who had been exposed to external radiation or contaminated during the Chernobyl accident (4,11,15,21,42,56,61). In both cases, intrachromosomal rearrangements (*ret*/PTC1&3) were predominant. However, in papillary thyroid carcinomas that emerged early after the Chernobyl accident, the *ret*/PTC3 form was more frequently observed and was associated with a solid growth pattern and a more aggressive phenotype (56). In contrast, in papillary thyroid carcinoma occurring either after external irradiation or more than 10 years after the Chernobyl accident, *ret*/PTC1 was the predominant form and was associated with a less aggressive phenotype (classical papillary thyroid carcinoma and diffuse sclerosing variant). *Ret/*PTC rearrangements were also found in 11–45% of thyroid adenomas that occurred after external irradiation during childhood, and in 52% following exposure in Chernobyl (6,11).

Trk rearrangements were found at a similar low frequency in spontaneous and radiation-associated papillary thyroid carcinomas (3,5,42).

Activating mutations in the *ras* genes have been found in thyroid tumors from patients with a history of external irradiation, at a frequency similar to that observed in apparently spontaneous tumors (6). In contrast, in tumors that developed in children after the Chernobyl accident, *ras* point mutations were found in 25% of the follicular tumors but not in papillary thyroid carcinomas (36,61). In spontaneous thyroid tumors, transversions (a base change from purine to pyrimidine or vice-versa) as well as transitions (a base change from purine to purine or pyrimidine to pyrimidine) were detected in the *ras* genes. In radiation-associated tumors, only transversions were present (6). The exact mechanism of these mutations remains to be determined, but it can be postulated that they arise through an ionizing radiation-induced oxidative lesion, producing 8-OXO-dG which can pair with adenosine during DNA replication (6,53).

The frequency of activating point mutations in the $G\alpha s$ and TSH-R genes is low (<10%) in tumors occurring either after external irradiation during childhood and after the Chernobyl accident (6). *P53* gene mutations have been detected in a few papillary thyroid carcinomas occurring after external irradiation during childhood and after the Chernobyl accident. These mutations may explain the aggressiveness of some of these tumors (36).

From these data, it can be postulated that radiation may directly lead to DNA strand breaks and *ret* activation through gene rearrangements. The precise nature of possible secondary genetic events resulting in further progression is unknown.

CONCLUSION

Several conclusions can be drawn from the study of oncogenes and tumor suppressor genes in human thyroid tumors (Table 1):

Alterations of membrane tyrosine kinase receptors *(ret*/PTC, *trk, met)* are observed only in papillary thyroid carcinomas; the higher frequency of *ret*/PTC rearrangements in radiation-associated papillary thyroid carcinoma and also their discovery in radiation-associated follicular adenomas suggest that they may be directly induced by radiation exposure. *Met* overexpression may be a secondary event.

Activating point mutations of the *ras* genes are found in 11% of papillary thyroid carcinomas. *B-raf* mutations were found in 36% and 69%, with no overlap between *ret*/PTC, *ras* and *b-raf* mutations. The activation of this pathway is frequently observed in papillary thyroid carcinomas and may play a determining role in their pathogenesis.

Ras mutations are found in 20% of benign and in 30% of malignant follicular thyroid tumors. *B-raf* mutations were not found in these tumors. Other genetic abnormalities that may be facilitated by genetic instability induced by *ras* mutations are needed for tumor progression and to determine the histological type of the thyroid tumor.

PPARγ1/PAX8 translocations were found in malignant and benign follicular tumors. In one series of follicular carcinomas, 86% revealed either *ras* or PPARγ1/PAX8 mutations. However, there was no overlap between these two mutations and phenotypes associated with each of these mutations were different, suggesting two different pathways in follicular tumorigenesis.

Table 1. Frequencies (%) of genetic alterations in hypofunctioning thyroid tumors, in the absence of previous neck irradiation.

	Papillary carcinoma	Follicular adenoma	Follicular carcinoma	Anaplastic carcinoma
Tyrosine kinase receptors:				
ret/PTC rearrangement	2.5–35	rare	rare	rare
trk rearrangement	0–15	rare	rare	rare
met overexpression	50–70	rare	rare	rare
Intra-cellular signaling pathway:				
ras point mutation	11	19	34	<40
b-raf point mutation	36–69			
PPARγ-PAX8 rearrangement	NR	8–13	26–63	NR
TSH stimulation pathway:				
TSH-R mutation	rare	<10	<10	rare
gsp	rare	<10	<10	rare
p53 mutations	rare	rare	rare	25 – >80

P53 mutations are observed only in poorly-differentiated or anaplastic thyroid cancers; they play a determining role in progression from differentiated to undifferentiated thyroid carcinomas and in the dedifferentiation process.

TSH-R and Gαs activating point mutations are found in about 60% of hyperfunctioning adenomas; their role in the pathogenesis of hypofunctioning thyroid tumors has not been confirmed.

Several growth factors are overexpressed in thyroid tumors. Paracrine factors such as Fibroblast Growth Factor (FGF1 and 2) are mitogens for thyrocytes, and Vascular Endothelial Cell Growth Factor (VEGF) may play a determining role in tumor neovascularisation. Other growth factors may also play a role, such as insulin like growth factor–1 (IGF1), Epidermal Growth Factor or TGFα. Overexpression of these growth factors is believed to be secondary to other oncogenic events.

Other abnormalities may also play a role in thyroid tumorigenesis. DNA methylation is frequently abnormal in thyroid tumors and this may modify gene functions (31). The status of telomerase may be modified: follicular adenomas are telomerase-negative, and about half of papillary and follicular carcinomas are telomerase-positive. Some telomerase-negative cancers maintain telomere length by a mechanism independent of telomerase (32). Other genetic abnormalities may also exist, and deletions have been demonstrated in follicular tumors, possibly indicating the location of yet unknown tumor suppressor genes.

These data may suggest a scheme for epithelial thyroid tumorigenesis. Ongoing studies of the transcriptome and proteome will rapidly increase our knowledge in the field.

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6. GROWTH FACTORS AND THEIR RECEPTORS IN THE GENESIS AND TREATMENT OF THYROID CANCER[1]

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INTRODUCTION

The oncogenes and/or tumor suppressor genes that are implicated in the transformation and progression of the majority of thyroid neoplasms remain unknown. Mutations that have been identified in other human malignancies are restricted to a relatively small subset of thyroid neoplasms, if they are identified at all. It would appear that novel genetic alterations are implicated including the well-characterized ret/PTC rearrangements. Numerous factors have been shown to govern thyroid cell differentiation and proliferation. Indeed, increasing evidence suggests that many of these growth factors and their receptors can also be implicated in tumor cell progression in genetically transformed thyrocytes. The molecular mechanisms underlying dysregulated thyroid cell growth and their potential role in the tumorigenic pathway will be discussed.

GROWTH FACTORS AND RECEPTORS

Overview

Growth factors are polypeptides of several major families that regulate cell replication and functional differentiation by directly altering the expression of specific genes (1). They are considered to play an important role in the multistep pathway of tumorigenesis. A number of oncogene products are homologous to growth factors, their receptors,

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or enzymes that participate in the mitogenic process. In several systems, growth factors have been shown to interact with specific membrane receptors in regulating cell growth and gene expression in an autocrine or paracrine manner. Some are known to affect hormone production and some are, in turn, modulated by hormones (2). A few have been identified in the thyroid where they are considered to play a physiological role in endocrine cell regulation (3;4).

Endocrine cells including thyrocytes are the site of both synthesis and action of growth factors. A number of growth factors have been identified in endocrine cells, including insulin-like growth factors-I and -II (IGF-I, IGF-II) (5;6), epidermal growth factor (EGF) (7;8), transforming growth factor-α (TGFα) (9–11), transforming growth factor-TGF-ß, platelet-derived growth factor (12;13) and basic fibroblast growth factor (bFGF; FGF-2) (14). Growing evidence suggests that human thyroid tumor cells produce multiple peptides that regulate their own function in vitro. The relative significance of these different growth factors in human thyroid neoplasia, however, remains to be established.

THE EPIDERMAL GROWTH FACTOR FAMILY

The EGF family of ligands includes EGF, TGF-α, amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), and betacellulin (BTC) (15). An additional family of EGF-related agonists include neuregulins which include glial growth factors (GGFs), neu differentiation factors (NDFs)/heregulins, ligands for erbβ-3 and erbβ-4. It is still not very clear which specific subsets of erbB receptors become activated in response to each of these ligands.

Transforming growth factor-α

Transforming growth factor-α is expressed as a membrane-anchored protein (16) that may alter pituitary production of TSH as well as cell proliferation (17). TGFα is thought to mediate estrogen-induced cell proliferation in several tissues (18–20). Estrogen stimulation has been implicated in thyroid tumorigenesis most aptly in rodents using a number of synthetic estrogenic compounds. Using a two-stage thyroid tumorigenesis model, one week administration of N-bis(2-hydroxypropyl)nitrosamine, gonadectomized F344 rats of both sexes were implanted with fused pellets containing EB for 32 weeks (21). Thyroid gland weights were increased by EB pellet in a dose-dependent and increased the occurrence of thyroid proliferative lesions in male and female animals. These data provide suggestive evidence for the potential significance of this growth factor in thyroid tumorigenesis.

Epidermal growth factor and receptor (EGF; EGF-R)

The common receptor of EGF and TGF-α, EGF-R, is a 170-kD plasma membrane tyrosine kinase product of the protooncogene v-*erbβ*. EGF-R is over-expressed in several types of human cancers that correlate with tumor aggressiveness. In the thyroid, EGF promotes growth but may inhibit some functional parameters. The normal thyroid displays EGF and EGF-R staining that is variable, but largely cytoplasmic, for both EGF

and EGF-R(4;8;22). Nuclear positivity for EGF and EGF-R has been described in both follicular adenomas and follicular carcinomas. In marked contrast, nuclear staining has been reported as almost absent in papillary carcinomas. The absence of nuclear EGF and EGF-R in papillary carcinomas would suggest that the role played by EGF in growth control differs between papillary carcinoma and follicular adenomas/carcinomas of the thyroid (23).

Interestingly, the compound ZD6474, a low molecular weight EGF tyrosine kinase inhibitor was recently shown to have enzymatic functions on RET-derived oncoproteins. This agent blocks the *in vivo* phosphorylation and signaling of the RET/PTC3 and RET/MEN2B oncoproteins and of an EGF-R/RET chimeric receptor. This inhibition was associated with morphological reversion and prevented the growth of human PTC cell lines that carry spontaneous RET/PTC 1 rearrangements (24).

As mentioned previously, the EGF-R is one of four highly homologous tyrosine kinase receptors that include erbβ2/HER2/neu/p185, erbβ-3 (HER3), and erbβ-4 (HER4). Growing evidence in support of functional cross-talk between the different members of this receptor family is now well recognized (25). Ligand-induced stimulation can result in transphosphorylation of *neu* via EGF-R (25;26). Over-expression of a wild type EGF-R and heterocomplex formation with *neu* dramatically increases receptor autophosphorylation and binding of EGF (25;27).

Erb β-2/neu in thyroid neoplasia

The specific role of the erbβ-2 proto-oncogene in human carcinomas was investigated in human thyroid tumours including nodular hyperplasias, follicular carcinomas and papillary carcinomas (without and with tall-cell features, insular, or anaplastic de-differentiation). There was no evidence of DNA amplification of erbβ-2 gene itself. Furthermore, sequencing of the transmembrane domain revealed no activating point mutations of the of erbβ-2 gene. The level of mRNA expression, however, was variable with nearly a third of papillary carcinomas showing statistically significant elevated mRNA levels compared with corresponding normal thyroid tissue. These findings, however, did not correlate with other indicators of poor prognosis. Moreover, in contrast to the elevated mRNA levels in thyroid tumours, the level of protein staining correlated with the degree of differentiation. Normal and hyperplastic tissue being strongly positive and poorly differentiated tumours showing negative of erbβ-2 immunostaining. Thus, these studies indicate the absence of mutations or amplifications of the erbβ-2 gene in human thyroid tumours. Elevated erbβ-2 mRNA expression in some thyroid tumours was not associated with clinical features of poor prognosis. Nevertheless, the significance of the elevated mRNA levels remains unclear, as it did not result in protein overexpression. Instead, cytoplasmic erbβ-2 protein detection by immunohistochemistry appears to correlate with differentiation of human thyroid tumours and may be a feature of good prognosis. There does not appear to be a positive relationship between erbβ-2 expression and the well-characterized ret/PTC rearrangements indicating that the two events are likely to be mutually exclusive in genesis and action of these two putative thyroid oncogenes (28).

THE TRANSFORMING GROWTH FACTOR-β

Transforming growth factor (TGF)-β has been implicated in the regulation of normal and neoplastic cell function. TGF-β regulates the expression of various proteins, including p27Kip1 (p27), a cell cycle inhibitory protein. Enhancement of tumor cell growth and invasiveness by transforming growth factor-β (TGF-β) requires constitutive activation of the ras/MAPK pathway. How MEK activation by epidermal growth factor (EGF) influences the response of fully differentiated and growth-arrested thyroid epithelial cells in primary culture to TGF-β1 is not clear. The epithelial tightness was maintained after single stimulation with EGF or TGF-β1 for 48 hours. In contrast, co-stimulation abolished the trans-epithelial resistance and increased the paracellular flux of labeled inulin. Reduced levels of the tight junction proteins claudin-1 and occludin accompanied the loss of barrier function. N-cadherin, expressed only in few cells of untreated or single-stimulated cultures is increased and co-localizes with E-cadherin at adherens junctions. TGF-beta1 only partially inhibited EGF-induced Erk phosphorylation. The MEK inhibitor U0126 prevents Erk1/2 phosphorylation and abrogated the synergistic responses to TGF-β1 and EGF. These observations indicate that concomitant growth factor-induced MEK activation is necessary for TGF-β1 to convert normal thyroid epithelial cells to a mesenchymal phenotype providing evidence for the role of these growth factors in thyroid cell transdifferentiation.(29).

VASCULAR ENDOTHELIAL GROWTH FACTOR

Vascular endothelial growth factor (VEGF) also known as vascular permeability factor (VPG) exists in a number of isoforms in human and rodent tissues including VEGF206h/205r, VEGF189h/188r, VEGF165h/164r, VEGF145h/144r and VEGF121 that differ in their molecular masses and biological activities. The VEGF isoforms bind with two tyrosine-kinase receptors, KDR/flk-1 and flt-1. In addition, VEGF165 binds with co-receptor, neuropilin-1, which is expressed in human endothelial cells and several types of non-endothelial cells including solid tumors. Recent studies on the role of estrogen in the regulation of tumor angiogenesis demonstrated that this steroid induces neovascularization in parallel with early induction of VEGF and the VEGFR2- (flk-1/KDR) protein expression in both blood vessels and non-endothelial cells (30). Moreover, estrogen-induced rat pituitary tumors in Fisher 344 rats express higher VEGF164 and neuropilin-1 levels compared to control untreated rats (31). These findings suggest that over-expression of VEGF and its receptor (VEGFR-2) may play an important role in the early phases of estrogen induced tumor angiogenesis in some endocrine tissues.

FIBROBLAST GROWTH FACTORS & RECEPTORS

Fibroblast growth factors (FGFs)

Basic Fibroblast Growth Factor (now known as FGF-2) is one of an ever-expanding family of FGFs several of which possess mitogenic, angiogenic, and hormone regulatory functions (32). FGF-2 immunoreactivity was described originally in the non-hormone

producing folliculo-stellate cells of the pituitary (33). In one mouse model, estrogen-induced tumorigenesis was associated with parallel increases in the expression of a pituitary tumor transforming gene (PTTG) as well as FGF-2 (33). In turn, both PTTG and FGF-2 have been shown to be increased in mRNA expression in papillary thyroid cancer that was also associated with lymph nodal invasion and distant metastasis. These findings were upheld even after consideration of other known prognostic factors such as age and gender of the patient and size and type of the tumor (34). Similarly, increased concentrations of FGF-2 in the serum of patients with differentiated papillary thyroid carcinoma has also been reported (35).

Fibroblast growth factor receptors (FGFRs)

There are 4 mammalian FGFR genes encoding a complex family of transmembrane receptor tyrosine kinases (RTKs) (36). Each prototypic receptor is composed of 3 immunoglobulin (Ig)-like extracellular domains, 2 of which are involved in ligand binding, a single transmembrane domain, a split tyrosine kinase, and a COOH-terminal tail with multiple autophosphorylation sites. Multiple forms of cell-bound or secreted receptors are produced by the same gene. Tissue-specific alternative splicing, variable polyadenylation sites and alternative initiation of translation result in truncated receptor forms (37;38). The first two extracellular loops of FGFR1 can be secreted as soluble circulating FGF binding proteins (39) but their physiological importance remains to be established. Different FGFRs can dimerize, so that truncated forms of FGFR1 block signalling through FGFR1, 2, and 3 (40).

Structural alterations of FGFRs may play a role in human tumorigenesis. FGFR1 is highly expressed in the brain (41) but the shorter (2 Ig-domain) form of FGFR1 is more abundant in some CNS glioblastomas (42). Anti-sense targeted interruption of FGFR1 reduces malignant melanoma cell proliferation and differentiation (43). FGFR2 exon switching has been observed to accompany prostate cell transformation (44).

The expression of FGF-2 and one of its receptors FGFR1 was recently compared in differentiated thyroid cancers, normal thyroids, multinodular goiters, and Graves' disease specimens. The investigators noted that FGF-2 was significantly over-expressed in thyroid carcinomas compared with normal thyroid tissue. More interestingly, increased FGF-2 mRNA expression was independently associated with lymph nodal invasion and distant metastasis at tumor presentation (34).

The biological relevance of the FGF signaling system in thyroid cell growth has been further hinted at from genetically altered mice. Mice deficient for FGFRR2-IIIb were generated by placing translational stop codons and an IRES-LacZ cassette into exon IIIb of FgfR2. Expression of the alternatively spliced receptor isoform, FgfR2-IIIc, is not affected in these mice. The FGFR2-IIIb deficient mice, however, show dysgenesis of several non-endocrine as well as endocrine tissues including the thyroid, adrenals, pancreas, and pituitary. These findings are particularly interesting in view of the fact that FGF ligand expression is not altered with normal FGF8, FGF10, Bmp4, and Msx1 in this animal model (45).

In contrast, gain-of-function mutations in the FGFR-3 gene have been described to result in inhibition of cartilaginous cell growth in the growth plate suggesting an important growth inhibitory signal for this receptor. RT-PCR examination confirmed the expression of this growth factor in papillary thyroid carcinomas. Over-expression of FGFR-3 was successful in specific binding of 125I-FGF-2. Growth rates of cells over-expressing FGFR-3, however, were similar to those of control cells (46). Cells over-expressing FGFR3 continued to grow beyond the density of control cells. These interesting findings suggest a role for FGFR3 in thyroid cancer cell adhesion and/or invasiveness.

The nerve growth factor family

NGF is a growth factor that generally results in anti-proliferative and anti-invasive effects in neuroendocrine tumors. NGF inhibits thyrocyte invasion and reverts the effect of retinoic acid in these cells. This effect is likely mediated by an increase in adhesion to the extracellular matrix proteins laminin and collagen IV and the inhibition of cell migration. NGF also induces expression of its receptor p75 NGF receptor. This receptor can be the subject of rearrangements. Indeed, the thyroid TRK oncogenes are generated by chromosomal rearrangements juxtaposing the neurotrophic tyrosine receptor kinase type 1 (NTRK1) tyrosine kinase domain to foreign activating sequences. TRK oncoproteins display a constitutive tyrosine kinase activity in NIH3T3 cells (47). The TRK oncoproteins' signal transduction involves several signal transducers activated by the NGF-stimulated NTRK1 receptor including fibroblast growth factor receptor substrate (FRS) FRS2 and FRS3, two related adapter proteins activated by fibroblast growth factor and NTRK1 receptors, in the signaling of the thyroid TRK-T1 and TRK-T3 oncogenes. FRS2 and FRS3 are recruited and activated by TRK-T1 and TRK-T3. Expression studies show different expression patterns of the FRS adapters in normal and tumor thyroid samples. FRS3 is expressed in both normal and thyroid tumor samples, whereas FRS2 is not expressed in normal thyroid but is differentially expressed in some tumors. These data are consistent with the notion that the FRS2 and FRS3 adapter proteins may have a role in thyroid carcinogenesis triggered by TRK oncogenes and provide the basis for a new dimension of pharmaco-therapeutic possibilities.

CONCLUSIONS

Thyroid tumors are common neoplasms that exhibit a wide range of biologic behavior. Numerous factors have been shown to govern thyrocyte proliferation. In particular, hormones and growth factors likely play a role as promoters of tumor cell growth in genetically transformed cells. In some instances enhanced growth factors and their receptors may serve as survival signals for neoplastic cells. In other instances, however, abnormal forms of growth factor receptors (such as members of the EGF-R/HER2/neu) may also be important in the early stages of cell transformation and chromosomal instability consistent with the clonal composition of thyroid neoplasms. More detailed structure/function studies of growth factor/receptor

functional interactions in morphologically characterized thyroid nodules are required. It is anticipated that these studies will identify signaling patterns that will provide the basis for the development of more specific and effective pharmacotherapeutic agents.

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7. BIOLOGY OF RAS IN THYROID CELLS

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INTRODUCTION

Ras is an almost universal component of signaling pathways in vertebrates, invertebrates and yeast where it plays critical roles in development, proliferation, differentiation and survival. In the twenty years since the first identification of mutated Ras genes in human tumors, intensive effort has been devoted to understanding how Ras promotes neoplastic transformation. What has become clear is that Ras promotes transformation in multiple ways. The effects of Ras are diverse due to the significant complexity of Ras-mediated signaling pathways. Mammalian cells express multiple Ras proteins, which localize to discrete membrane microdomains and exhibit differential affinities towards downstream signaling molecules. The existence of a large number of Ras effectors, many of which are members of multi-gene families, together with extensive sites of crosstalk between Ras and other intracellular signaling pathways, further increases the complexity of Ras-mediated signaling.

Mutations in all three cellular Ras genes (H-, K- and N-Ras) have been identified in benign and malignant thyroid tumors. This has generated immense interest in elucidating the cellular consequences of Ras activation in thyroid cells. The recent discovery of B-Raf mutations in thyroid tumors reaffirms the important contribution of Ras-mediated signaling pathways to thyroid cell transformation. Interestingly, the effects of Ras in thyroid cells are unusual in several respects. Unlike primary fibroblasts where expression of activated Ras induces growth arrest, Ras stimulates proliferation in primary human thyrocytes. Recent data suggests that this may be a consequence of

cell type specific effects on cell cycle regulatory proteins. Thyroid cells are one of few cellular models where proliferation is positively regulated by cAMP. As discussed below, crosstalk between cAMP and Ras markedly influences the signaling pathways activated by Ras. Indeed, TSH has been shown to modulate the effects of Ras on differentiation, proliferation and survival. The focus of this chapter is on the effects of Ras activation in thyroid cells, including the role of cellular Ras in TSH driven proliferation and the contribution of sustained Ras activity to thyroid cell transformation.

RAS REGULATION AND SIGNALING

Ras proteins are 21kDa GTP-binding proteins that function as molecular switches, cycling between active GTP- and inactive GDP-bound states. Cellular Ras activity is regulated by the opposing action of guanine nucleotide exchange factors (GEFs) that catalyze GDP dissociation, and GTPase activating proteins (GAPs) that stimulate intrinsic GTPase activity. Multiple RasGEFs and RasGAPs co-exist in most cells, increasing the diversity of signals that regulate Ras activity. Ras proteins are localized to the plasma membrane where they are poised to respond to signals initiated by the activation of cell surface receptors. Cellular Ras activity is maintained at very low levels. In response to signals such as those elicited by growth factors and hormones, Ras becomes activated in a transient manner. For cell surface receptors with tyrosine kinase activity, receptor dimerization induces tyrosine phosphorylation, thereby creating docking sites for signaling molecules such as Grb-2 and Shc, adaptor proteins comprised of SH2 and SH3 domains. Grb-2 is associated with the RasGEF SOS in the cytosol. Recruitment of Grb-2 to the activated receptor localizes SOS to the plasma membrane in close proximity to Ras, facilitating its activation. For G protein-coupled receptors, Ras is activated through second messengers such as diacylglycerol, calcium, and possibly cAMP (Busca et al., 2000; Pak et al., 2002), as well as through heterotrimeric G protein β/γ subunit- and src-mediated pathways.

In its active conformation, Ras binds to a variety of effectors. Effectors are defined as proteins that interact selectively with the GTP-bound form of Ras, and become activated as a consequence of this interaction. Three downstream effector pathways have been characterized in the most detail (Figure 1). They include members of the Raf, PI3K and RalGDS families (reviewed in Reuther et al., 2000; Shields et al.,

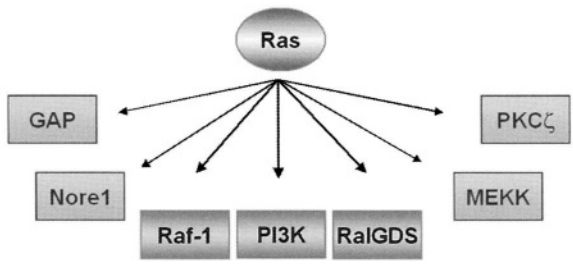

Figure 1. Ras signals through multiple downstream effectors including, but not limited to those illustrated here. In thyroid cells, Ras has been shown to signal through Raf-1, PI3K and RalGDS (shown in bold).

2000). Interaction between GTP-Ras and its first identified target, Raf-1, induces a conformational change that unmasks phosphorylation sites and anchors Raf-1 to the plasma membrane. Once this occurs, Raf-1 activity becomes Ras-independent. Active Raf binds to and phosphorylates MEK1/2 proteins, stimulating their kinase activity. MEK proteins are dual specificity serine/threonine and tyrosine protein kinases that phosphorylate and activate MAPK1/2 (also referred to as ERK1/2), protein kinases that play important roles in many cellular processes including the regulation of gene expression. In a similar fashion, binding of GTP-Ras to the p110 catalytic subunit of PI3K stimulates lipid kinase activity, increasing the production of second messenger phosphoinositide (3,4) P_2 (PIP2) and phosphoinositide (3,4,5) P_3 (PIP3). PIP3 promotes the activation of a kinase cascade that includes PDK-1, Akt (or PKB) and p70 ribosomal S6 protein kinase (p70s6k). These kinases phosphorylate numerous protein substrates with diverse roles in protein synthesis, cell proliferation and cell survival. PI3K also regulates survival through activation of Rac GTPases. Binding of GTP-Ras to RalGDS stimulates GEF activity towards the Ras-related proteins, Ral A and B. Downstream targets of Ral include phospholipase D, Rho, and Rac- and Cdc42-selective GAPs. There are a number of additional putative Ras effectors, including RasGAPs, MEKK, AF6, PKCζ and Nore1. To date, activated Ras has been shown to signal through MAPK, PI3K and RalGDS in thyroid cells, effects that are markedly influenced by cAMP, an important regulator of thyroid cell function and proliferation (Figure 2).

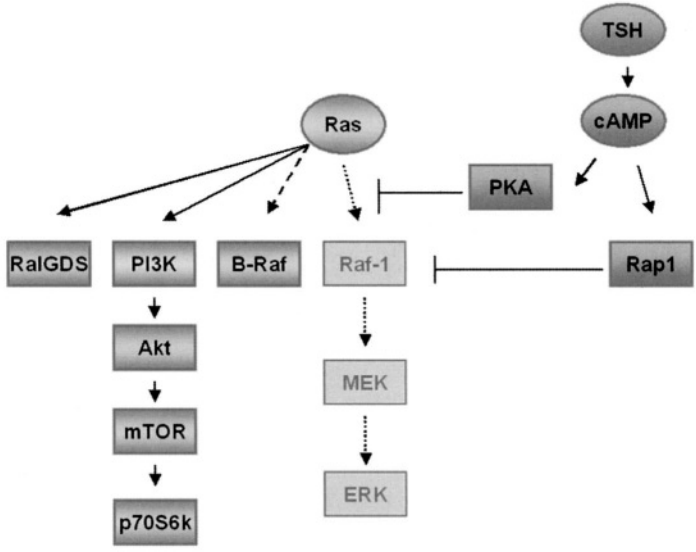

Figure 2. Ras signaling pathways are altered by TSH. TSH elevates cAMP, which activates PKA and Rap1 in thyroid cells. PKA has been reported to disrupt signaling from Ras to Raf-1 by phosphorylating the N-terminus of Raf-1, which decreases the affinity of Raf-1 for Ras. Activated Rap 1 binds to Raf-1, but does not stimulate its activity. It is not yet know whether Ras signals to B-Raf in thyroid cell (dashed line).

RAS MUTATIONS IN THYROID CANCER

A metastatic tumor is the end result of a complex series of steps involving multiple gene products. Work performed over the past decade has identified a number of gene products with putative roles in the initiation and progression of thyroid tumorigenesis. Mutations in Gsα (gsp) and the TSH receptor have been identified in hyperfunctioning adenomas. Ras mutations are prevalent in follicular carcinomas (see below). Mutations in ret, trk and met were identified in papillary carcinomas. Aberrant DNA methylation, leading to loss of expression of the p16 tumor suppressor gene, has been described in both types of cancer. Finally, mutations in p53 appear to play a role in the final dedifferentiation process. The reader is referred to several excellent recent reviews regarding the molecular basis of thyroid cancer (Jhiang, 2000; Gimm, 2001; Puxeddu et al., 2001; Fagin, 2002).

Early reports revealed that Ras mutations were particularly prevalent in benign follicular adenomas and follicular carcinomas, where estimates ranged as high as 50% (Lemoine et al., 1990; Namba et al., 1990; Suarez et al., 1990; Shi et al., 1991; Farid, 1994). The frequency of Ras mutations was initially reported to be similar in benign adenomas and follicular carcinomas, suggesting that Ras played an early role in thyroid transformation. However, more recent studies suggest that Ras mutations are less frequent than was first reported, occurring with an overall frequency of 16–19% (Esapa et al., 1999; Vasko et al., 2003). These studies also revealed a higher frequency of Ras mutations in follicular carcinomas versus adenomas, consistent with a role for Ras in malignant progression. According to recent data, mutations in codon 61 of N-Ras are the most frequent Ras mutation found in thyroid tumors (Nikiforova et al., 2003; Vasko et al., 2003). Besides Ras mutations, a significant proportion of follicular carcinomas exhibit a specific chromosomal translocation that fuses the coding regions for the paired and homeobox binding domains of the Pax-8 transcription factor to the DNA and ligand binding, dimerization and transactivation domains of PPARγ1 (Martelli et al., 2002). Interestingly, follicular carcinomas harboring both Ras mutations and the Pax-8/PPARγ translocation are extremely rare. This indicates either that both changes activate similar signaling pathways or that follicular carcinomas are comprised of at least two distinct tumor types that arise by different mechanisms (Nikiforova et al., 2003).

Although Ras mutations are infrequent in papillary thyroid carcinomas, somatic mutations in B-Raf were recently identified in these tumors (Kimura et al., 2003; Cohen et al., 2003). B-Raf mutations were discovered in a wide range of human tumors only last year (Davies et al., 2002; Rajagopalan et al., 2002). Intriguingly, mutations in B-Raf were found in cancers that typically harbor Ras mutations, such as malignant melanomas, colorectal tumors and ovarian cancers. The most frequent B-raf mutation (V599E) results in the insertion of an acidic residue close to a site of activating phosphorylation in the kinase domain. Recombinant B-RafV559E exhibits increased kinase activity, suggesting constitutive activation of signaling pathways similar to those activated by Ras. Moreover, unlike activated Raf-1 mutants that stimulate transformation through an autocrine mechanism involving Ras, the effects of

B-RafV559E on cell transformation were Ras-independent (Davies et al., 2002). The same B-Raf mutation has now been shown to be the most common genetic change in papillary thyroid carcinomas (Kimura et al., 2003). These results are striking for several reasons. First, there are three mammalian Raf proteins: Raf-1, A-Raf and B-Raf. While Raf-1 is ubiquitously expressed, A- and B-Raf exhibit a more restricted pattern of expression. Intriguingly, B-Raf is expressed in neuronal, neuroendocrine and endocrine cells. Of further interest with regard to thyroid cells, Raf proteins are important sites of integration between signals activated by Ras and cAMP. Cyclic AMP impairs the activation of Raf-1 by serum growth factors and Ras (Figure 2). In contrast, cAMP stimulates B-Raf activity (Erhardt et al., 1995; Vossler et al., 1997; Busca et al., 2000). Therefore, it is perhaps not surprising that melanoma cells harbor B-Raf mutations given their regulation by α-melanocyte stimulating hormone and related proopiomelanocortin-derived peptides that upregulate intracellular cAMP. Similarly, the identification of B-Raf mutations in thyroid tumors is particularly interesting given the growth promoting effects of chronic TSH stimulation. Despite the high frequency of Ras mutations in colorectal cancers, B-Raf mutations were found only in tumors without Ras mutations. In agreement with these results, no overlap was seen between mutations in Ras and B-Raf in papillary thyroid carcinomas (Kimura et al., 2003). These results provide strong support for the notion that B-Raf and Ras mutations are equivalent in their tumorigenic effects. Finally, RET/PTC oncogenes also signal partly through Ras (Barone et al., 2001; Castellone et al., 2003) and possibly through PDK-1 (Kim et al., 2003), a protein kinase that is also activated downstream from Ras. Strikingly, there appears to be no overlap between papillary carcinomas harboring RET/PTC, B-Raf and Ras mutations, which together account for two thirds of all papillary carcinomas. These findings underscore the significant contribution of Ras-mediated signaling pathways to thyroid tumorigenesis. In the following sections, I review what is known regarding the role of endogenous Ras, and the consequences of sustained Ras activity in thyroid cells.

ROLE OF CELLULAR RAS IN THYROID CELLS

TSH regulates the function and proliferation of thyroid follicular cells, highly specialized epithelial cells that synthesize, store and secrete thyroid hormones. Thyroid hormone biosynthesis requires the expression of cell type specific gene products, including the TSH receptor, thyroperoxidase, thyroglobulin and the sodium/iodide symporter (Damante et al., 1994). TSH regulates the expression of these genes in part through effects on thyroid-specific transcription factors such as TTF-1, TTF-2 and Pax-8 (Missero et al., 1998). The proliferation of thyroid cells is TSH-dependent (for recent reviews, see Medina et al., 2000; Kimura et al., 2001). However, for the most part, TSH acts together with insulin or IGF-I and serum to stimulate sustained proliferation. The effects of TSH on function and proliferation are reproduced by cAMP elevating agents and analogs. Positive growth regulation by cAMP is one of the unique features of thyroid cells and stands in marked contrast to the growth inhibitory effects of cAMP in many other cell types (Cook et al., 1993; Sevetson et al., 1993; Wu et al., 1993).

Compared to the effects of ectopic expression of constitutively active Ras, much less is known regarding the roles of endogenous Ras in thyroid cells. TSH-stimulated DNA synthesis was repressed following expression of dominant negative mutant Ras (Ras17N) in Wistar rat thyroid (WRT) (Kupperman et al., 1993) and FRTL-5 cells (Medina et al., 2000; Ciullo et al., 2001). Interference with Rac (Cass et al., 1999) or RhoA (Medina et al., 2002) also impaired TSH-stimulated DNA synthesis and mitogenesis, respectively. RhoA inhibition induced G1 phase cell cycle arrest in FRTL-5 cells (Hirai et al., 1997). Together, these data indicate that Ras family members play an integral role in the proliferation of rat thyroid cells. Whether Ras is required downstream from TSH/cAMP (Medina et al., 2000; Medina et al., 2000; Tsygankova et al., 2000), in addition to functioning downstream from insulin/IGF-I, is controversial (Van Keymeulen et al., 2000).

Although Ras stimulates proliferation through the Raf-1/MAPK cascade in many cells, this effector pathway does not play a major role in TSH signaling. Lamy *et al.* first reported that TSH failed to stimulate MAPK activity in canine thyroid cells (Lamy et al., 1993), in agreement with numerous studies in other cell types where cAMP inhibits growth factor stimulated-MAPK activity. TSH inhibited serum-stimulated MAPK activity in WRT cells (Miller et al., 1997) and treatment with the MEK1 inhibitor PD98059 had no effect on TSH-stimulated DNA synthesis in FRTL-5 cells (Medina et al., 2000). In addition to indicating that TSH does not signal through this cascade, these findings also suggested that Ras is unlikely to signal through the Raf-1/MAPK cascade in the presence of TSH (Figure 2), results that were later confirmed (see below). TSH stimulates p70s6k activity in WRT (Cass et al., 1998) and FRTL-5 cells (Medina et al., 2000), effects that were observed in the absence of insulin. Interference with mTOR, an upstream activator of p70s6k, impaired TSH-stimulated proliferation (Cass et al., 1998; Cass et al., 1999; Coulonval et al., 2000). As p70s6k can be activated downstream from PI3K, the role of PI3K in thyroid cell proliferation was investigated. Treatment with cell permeable PI3K inhibitors, microinjection of a dominant negative PI3K mutant, or injection of a p110-specific antibody inhibited TSH/cAMP stimulated DNA synthesis (Cass et al., 1999). Similarly, expression of dominant negative PI3K induced G1 phase cell cycle arrest in FRTL-5 cells and PI3K inhibitors blocked the stimulatory effects of TSH on cyclin E expression, a molecular marker of G1 phase cell cycle progression (Medina et al., 2000). Wortmannin and LY294002, cell permeable PI3K inhibitors, impaired TSH and insulin-stimulated DNA synthesis in canine thyroid cells (Coulonval et al., 2000) and IGF-I-stimulated proliferation in FRTL-5 cells (Saito et al., 2001). The role of PI3K in thyroid cell proliferation gained further support with the discovery that expression of PTEN, a negative regulator of PI3K, was decreased in a significant proportion of follicular neoplasms (Bruni et al., 2000), and that Akt activity is increased in follicular carcinomas (Ringel et al., 2001).

The acute effects of Ras activation in thyroid cells were first examined following microinjection of purified Ras protein into WRT cells (Kupperman et al., 1993). Microinjection of cellular or activated H-Ras protein was sufficient to stimulate DNA synthesis in quiescent cells. The ability of Ras to stimulate DNA synthesis in starved

cells was impaired by co-injection of a Raf-1 antibody or of a dominant negative MEK1 protein (Al-Alawi et al., 1995), invoking a role for the Raf/MAPK cascade in growth stimulation by Ras. When injected into cells that were treated with TSH or 8BrcAMP, however, co-injection of the Raf-1 antibody or of the dominant negative MEK1 protein failed to impair Ras-stimulated DNA synthesis. These results provided the first indication that crosstalk between TSH/cAMP and Ras influences which effector pathways are activated by Ras in thyroid cells (Figure 2). The ability of TSH to influence Ras signaling received additional strong support from Ciullo *et al.* These authors confirmed that Ras and PI3K are required for TSH-stimulated cell cycle progression in FRTL-5 cells (Ciullo et al., 2001). Importantly, they demonstrated that TSH stimulated complex formation between endogenous Ras and PI3K, whereas it impaired association between Ras and Raf-1.

SUSTAINED RAS ACTIVITY AND THYROID CELL PROLIFERATION

The identification of activating Ras mutations in thyroid tumors prompted studies of the consequences of sustained Ras activity in thyroid cells. Early gene transfer studies revealed the oncogenic potential of Ras in thyroid cells *in vitro.* Stable expression of activated H- or K-Ras in FRTL-5 cells was fully transforming. Ras-expressing cells exhibited hormone-independent proliferation, anchorage-independent growth and formed tumors in nude mice (Fusco et al., 1987). In contrast to these results, rat thyroid PC-CL3 cells were only partially transformed by Ras. *In vivo* studies demonstrated that Ras activation was insufficient for tumor formation. When injected into adult Fischer rats, a K-Ras retrovirus induced the formation of differentiated thyroid carcinomas only in goitrogen-treated animals (Portella et al., 1989). Similarly, targeted expression of activated K-Ras to the thyroid gland stimulated hyperplasia and adenoma formation, but only in rare instances tumor formation, which required goitrogen treatment (Santelli et al., 1993). In seeming contrast to these findings, expression of activated H-Ras in the thyroid gland stimulated hyperplasia and papillary thyroid tumors (Rochefort et al., 1996; Feunteun et al., 1997).

The effects of Ras on the proliferation of human thyroid cells are of enormous interest. Using retroviruses, Lemoine *et al.* demonstrated that activated H-Ras stimulates the sustained proliferation of primary human thyroid cells (Lemoine et al., 1990; Bond et al., 1994; Jones et al., 2000). The mitogenic effects of Ras were also seen following acute introduction of activated Ras protein by scrape loading or microinjection (Gire et al., 1999). The ability of Ras to stimulate proliferation in primary thyroid cells is very different from its effects in primary fibroblasts where expression of activated Ras stimulated growth arrest (Serrano et al., 1997; Olson et al., 1998). Ras-expressing human thyroid cells were morphologically transformed and exhibited anchorage-independent growth, but not tumor formation. Strikingly, after 15–25 population doublings, Ras-expressing cells ceased to proliferate and became senescent, results quite similar to the limited growth potential of benign follicular adenomas harboring Ras mutations. These data strongly support the idea that human thyroid cells harbor a cell-intrinsic mechanism that prevents unchecked proliferation, even in the face of sustained expression of activated Ras.

In human cells, Ras-stimulated proliferation was impaired by inhibition of MAPK and PI3K activity. Although both pathways were required, activation of either signaling cascade alone was insufficient to stimulate proliferation (Gire et al., 1999; Gire et al., 2000). These findings are not dissimilar from those observed in rat thyroid cells, where Ras stimulated DNA synthesis through the Raf/MAPK cascade in the absence of TSH (Al-Alawi et al., 1995).

SUSTAINED RAS ACTIVITY AND THYROID DIFFERENTIATION

Ras transformation suppressed differentiated gene expression in rat thyroid cells (Avvedimento et al., 1985; Fusco et al., 1987; Francis-Lang et al., 1992). Intriguingly, H- and K-Ras impaired differentiation in different ways. H-Ras transformation was associated with the loss of Pax-8 and TTF-2 expression, and inactivation of TTF-1 possibly through decreased phosphorylation (Francis-Lang et al., 1992; Velasco et al., 1998). In contrast, TTF-1 expression was abolished in K-Ras transformed cells (Francis-Lang et al., 1992). K-Ras has also been shown to impair the nuclear localization of PKA, thereby preventing PKA-mediated phosphorylation of nuclear transcription factors (Gallo et al., 1995). Ras-transformed human cells retain their differentiated phenotype (Lemoine et al., 1990; Gire et al., 2000), a finding consistent with the occurrence of Ras mutations in differentiated thyroid tumors. It should be noted that de-differentiation is not an obligate response of rat thyroid cells to activated Ras. Using Ras effector domain mutants that signal through discrete downstream effectors (White et al., 1995), Miller *et al.* demonstrated that Ras signaling through the Raf/MAPK cascade impaired thyroglobulin expression in WRT cells, while Ras signaling to RalGDS (Miller et al., 1998) or PI3K did not (Cass et al., 2000). Stable expression of activated MEK-1, a downstream target of Raf, failed to de-differentiate FRTL-5 cells, perhaps due to its relatively modest effects on MAPK activity (Cobellis et al., 1998). Indeed, further studies by the same authors revealed that transient expression of RasS35, the effector domain mutant that signals selectively through Raf-1, or of activated Raf-1 impaired TTF-1 activity (Missero et al., 2000). Furthermore, MEK inhibitors partially blocked the inhibitory effects of Ras on TTF-1 activity. These data indicate that activation of the MAPK cascade impairs differentiated gene expression in rat thyroid cells, and that there are likely to be additional signals through which Ras suppresses thyroid differentiation. Different effects of RhoA on differentiated gene expression have been reported. Stable expression of activated RhoA impaired thyroglobulin and TTF-1 mRNA and protein levels (Medina et al., 2002), however transient expression of activated RhoA failed to repress TTF-1 promoter activity (Missero et al., 2000). Given the inhibitory effects of Ras signaling to MAPK on thyroid differentiation, the ability of TSH to direct Ras signals to alternate effectors such as PI3K and RalGDS would allow TSH to stimulate proliferation through Ras in cells that retain their differentiated character.

RAS AND THYROID CELL SURVIVAL

Although stable expression of activated Ras in thyroid cells confers hormone and anchorage-independent proliferation, it also renders cells more sensitive to apoptosis.

Serum withdrawal induced apoptosis in H- and K-Ras-transformed, but not parental FRTL-5 cells (DiJeso et al., 1995). H-Ras-transformed WRT cells exhibit an enhanced sensitivity to apoptosis in response to deprivation of adhesion or treatment with MAPK and PI3K inhibitors (Cheng et al., 2001). Inducible expression of activated Ras in PC-CL3 cells stimulated proliferation followed by apoptosis (Shirokawa et al., 2000). Intriguingly, apoptosis was strictly dependent upon TSH or cAMP elevation; in the absence of TSH/cAMP, Ras stimulated proliferation. Although epithelial cells typically perish by anoikis following detachment, Ras stimulated apoptosis in adherent PC-CL3 cells. Interference with either MAPK or JNK impaired apoptosis, invoking a role for these signaling pathways in Ras-mediated apoptosis. Acute infection of WRT, FRTL-5 and PC-CL3 cells with an adenovirus expressing activated H-Ras also stimulated apoptosis, although in this instance cell death occurred in the presence or absence of TSH (Cheng et al., 2003).

The effects of Ras on apoptosis are not limited to rat thyroid cells. Human thyroid cells immortalized by temperature-sensitive SV40 large T antigen underwent rapid cell death following expression of activated Ras at the restrictive temperature (Burns et al., 1993). Exposure to the phorbol ester tumor promoter TPA stimulated apoptosis in human thyroid cells expressing activated H-Ras (Hall-Jackson et al., 1998). Moreover, inhibition of PI3K activity induced apoptosis in H-Ras-expressing human thyroid cells, suggesting that clonal expansion induced by Ras requires PI3K activity to suppress apoptosis (Gire et al., 2000). Together these findings indicate that apoptosis is a conserved response to acute expression of activated Ras in thyroid cells. The relative ease with which rat and human thyroid cells are selected to survive stable expression of activated Ras also indicates that secondary changes that allow for the survival of Ras-expressing thyroid cells are frequent. The elucidation of the factors that dictate whether Ras stimulates transient or sustained proliferation, growth arrest or apoptosis is an exciting area for exploration.

RAS AND GENOMIC INSTABILITY

One of the consequences of Ras mutations in human tumors is destabilization of the karyotype. Expression of activated Ras in a variety of established cell lines and tumor cells induces chromosomal aberrations including an enhanced frequency of gene amplification (Smith et al., 1995), chromosome losses and gains, aberrant chromosome segregation and centrosome amplification (Saavedra et al., 1999). Inducible expression of activated H-Ras in PC-CL3 cells stimulated the formation of micronuclei containing chromosomes and chromosome fragments (Saavedra et al., 2000). Micronuclei with whole chromosomes arise as a consequence of spindle disruption; micronuclei containing chromosome fragments are typically generated by double strand DNA breaks. Although the effects of Ras on micronuclei formation were rapid, they were observed in only a small proportion of cells, perhaps due to the presence of wildtype p53 in these cells. This raises the interesting possibility that Ras predisposes thyroid cells to the acquisition of additional mutations by inducing genomic instability. Over time, cells harboring Ras mutations would acquire additional genetic and epigenetic changes that contribute to their full transformation. In this regard, it is interesting that Ras

mutations occur at a higher frequency in follicular versus papillary carcinomas, given the higher degree of aneuploidy observed in follicular carcinomas (Fagin, 2002). It will be interesting to assess whether papillary tumors bearing B-Raf mutations exhibit similar chromosomal aberrations. Whether Ras induces genomic instability in primary human thyroid cells, which exhibit a more stable karyotype than do rodent cells, has not yet been determined.

While chromosomal instability could provide the mechanism for Ras-stimulated apoptosis, the low frequency of micronuclei formation versus the high frequency of apoptosis argues that these events are distinct (Shirokawa et al., 2000). Apoptosis was TSH-dependent, while micronuclei formation was insensitive to cAMP levels. Furthermore, the signaling pathways through which Ras stimulated micronuclei formation and apoptosis were distinct. Therefore, acute expression of activated Ras stimulates multiple signals leading to DNA damage and chromosomal instability in a small proportion of cells and apoptosis in most cells. The changes that occur in the small population of surviving cells that contribute to their survival in the presence of activated Ras remain to be identified.

CELL CYCLE DEREGULATION AND APOPTOSIS

Acute expression of activated Ras stimulated aberrant cell cycle progression followed by apoptosis in WRT cells (Cheng et al., 2003). Infection of quiescent cells with an adenovirus expressing activated H-Ras induced cell cycle progression into S phase. Rather than completing the cell cycle, the majority of Ras-expressing cells exhibited a protracted S phase and ultimately perished by apoptosis. The effects of Ras on cell cycle regulatory proteins were very different from the mitogens, TSH, insulin, and serum. Unlike mitogen treatment, which increased the expression of cyclins D1 and B, Ras rapidly decreased cyclin D1 expression, and failed to increase cyclin B expression. Excessive mitogenic signaling, for example through overexpression of E2F (Nahle et al., 2002), is a potent inducer of apoptosis, as is delayed cell cycle progression (Meikrantz et al., 1995). These findings suggest that the acute effects of Ras on thyroid cell cycle progression are aberrant, and induce an apoptotic response. Similar effects on cell cycle progression and apoptosis have been reported following expression of high mobility group (HMG) proteins in thyroid cells. Impaired expression of HMGA in PC-CL3 cells blocked Ras transformation, suggesting a role for HMGA downstream from Ras (Fedele et al., 2001). When stably overexpressed, HMGA induced aberrant cell cycle progression characterized by premature entry into S phase and delayed transition into G2/M. These aberrant effects on cell cycle progression were accompanied by apoptosis. These data support the idea that conflict between mitogenic pressure and the inability to proceed through the cell cycle generates an apoptotic signal in rat thyroid cells. The high frequency of cell death in Ras-expressing human thyroid cells at the restrictive temperature for SV40 large T (Burns et al., 1993) may reflect a similar mechanism. The deletion of thyroid cells harboring Ras mutations by apoptosis might explain why Ras mutations are not found in a higher proportion of thyroid tumors.

The effects of activated Ras on the expression of p16, p21 and p27, cyclin-dependent kinase inhibitors, in human thyrocytes (Jones et al., 2000) differed from those reported

in human fibroblasts (Serrano et al., 1997; Olson et al, 1998). In thyroid cells, acute expression of Ras decreased p27 expression, and failed to increase expression of either p16 or p21. These changes would be expected to increase cyclin-dependent kinase activity, in particular cdk-2 activity, which is potently inhibited by p21 and p27 (reviewed in Sherr et al., 1999). These alterations could contribute to the mitogenic activity of Ras in human thyroid cells. In fibroblasts, Ras stimulated p16 and p21 expression, resulting in G1 phase cell cycle arrest. Cessation of proliferation in human thyroid cells was accompanied by re-expression of p27 and *de novo* expression of p16, changes expected to impair cdk activity (Jones et al., 2000). Interestingly, when HPV E7 was expressed in these cells to inactivate growth suppression by p16 and p27, senescence was bypassed. Although the cells continued to proliferate, proliferation was accompanied by increasing cell death. Therefore, in human and rat thyroid cells, proliferative pressure in the face of signals that impair growth induces apoptosis. This would impose selective pressure for cells in which apoptosis is circumvented, for example through the increased expression of positive regulators of cell cycle progression, decreased expression of growth inhibitors or alterations in cell survival signals. Indeed, unlike acute expression of activated Ras, which abolished the expression of cyclin D1, WRT cells selected to survive constitutive expression of activated Ras exhibited increased levels of cyclin D1 (Cheng et al., 2003). The expression of cyclins Dl (Wang et al., 1998; Muro-Cacho et al., 1999) and E (Lazzereschi et al., 1998; Wang et al., 1998) are frequently increased in thyroid tumors. Decreased expression of p27 in carcinomas versus adenomas has been reported (Erickson et al., 1998; Wang et al., 1998). Methylation of the p16 promoter, resulting in reduced p16 expression, has been reported in many differentiated thyroid tumors (Ivan et al., 1996; Jones et al., 1996). Together these findings suggest that alterations in the expression of cell cycle regulatory proteins may contribute to oncogenic transformation by Ras.

CONCLUSIONS AND PERSPECTIVES

While the frequency of Ras mutations in human tumors is only 20% overall, constitutive signaling through Ras is a conserved feature of a much higher proportion of human tumors. Mutations giving rise to increased production of growth factors or sustained activation of growth factor receptors are frequent events in human tumors. Over the past year, mutations in a Ras effector, B-Raf, have been identified in several types of human cancer (Davies et al., 2002; Rajagopalan et al., 2002). Together, mutations that give rise to constitutive signaling through Ras-mediated pathways comprise a significant proportion of human tumors. The fundamental role of Ras in tumorigenesis is particularly evident in the thyroid gland where mutations in Ras, B-Raf and RET/PTC have now been identified. Expression of activated Ras in thyroid cells *in vitro* elicits morphological transformation, sustained proliferation, apoptosis and genomic instability, hallmarks of human tumor cells (Figure 3). Despite the significant advances that have been made in identifying regulators and targets of Ras, these recent advances highlight how little we know regarding the signaling pathways activated by Ras, as well as the consequences of sustained Ras activity in the thyroid cell.

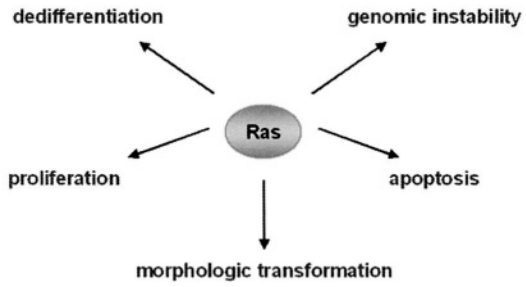

Figure 3. Ras elicits multiple effects in thyroid cells.

Overexpression studies have relied extensively on H- and K- rather than N-Ras, which appears to be a more frequent target for mutation in thyroid cancer. With accumulating evidence that individual Ras proteins localize to different cellular microdomains (Prior et al., 2001; Chiu et al., 2002; Matallanas et al., 2003) and signal in distinct ways (Maher et al., 1995; Villalonga et al., 2001; Walsh et al., 2001), it will be important to assess the specific consequences of N-*ras* activity in thyroid cells. It is interesting that N-Ras provides a survival signal in fibroblasts (Wolfman et al., 2002).

The recent discovery of B-Raf mutations in thyroid tumors paves the way for studies of the contribution of this specific Raf isoform to tumorigenesis. Although cAMP impairs Ras signaling to Raf-1, its effects on B-Raf activity in thyroid cells are largely unknown. Moreover, cAMP itself stimulates several signaling pathways with the potential to regulate B-Raf activity. Thyroid cells are a rich source of Epac, a Rap1-specific GEF (DeRooij et al., 1998; Kawasaki et al., 1998). TSH and cAMP activate endogenous Rap1 in rat (Tsygankova et al., 2001) and canine (Dremier et al., 1997) thyroid cells, effects that are PKA-independent. In other cells, active Rap1 binds to Raf-1 and B-Raf, but with differing consequences. Association between Rap1 and B-Raf stimulates B-Raf activity, whereas Rap-1:Raf-1 complexes are inactive. B-Raf is a substrate for protein kinase A, the other arm of the cAMP signaling pathway. This raises interesting avenues for regulation of B-Raf by cAMP and PKA. The signaling pathways activated by B-Raf in thyroid cells are unknown. While B-Raf binds and activates MEK1/2, it remains to be determined whether this pathway is active in TSH-treated cells.

Rap1 has been linked to TSH effects on differentiated gene expression (Tsygankova et al., 2001) and proliferation (Ribeiro-Neto et al., 2002), although the mechanism through which it elicits these effects is not clear. A limited mutational analysis failed to reveal mutations in Rap 1 or Epac in follicular adenomas (Vanvooren et al., 2001). Nonetheless, the contribution of Rap 1 to thyroid cell biology is important to pursue based on its ability to signal through B-Raf and to affect Ras-mediated signaling. Rap1 was initially isolated as K-rev1, an inhibitor of K-Ras transformation (Kitayama et al., 1989). Although Rap 1 clearly functions in Ras-independent pathways, crosstalk between Ras and Rap 1 has been shown to modulate the ability of Ras to activate

discrete effector pathways. Competition between Rap1 and Ras for downstream signaling molecules may provide a mechanism for balancing the activities of these two signaling molecules and for channeling their effects to discrete effector pathways.

The notion that Ras stimulates genomic instability, predisposing thyroid cells to the acquisition of additional mutations, promises to provide further insight into the molecular mechanisms through which Ras contributes to thyroid cell transformation. Thyroid cancer cell lines and tumors have been shown to exhibit mitotic checkpoint dysfunction, however the genetic and/or epigenetic changes responsible for this have not been identified. A recent analysis failed to reveal mutations in the candidate checkpoint genes, BUB1 or BUBR1 (Ouyang et al., 2002). The relationship between tumors harboring mutations in Ras or B-Raf to DNA damage and effects on p53 deserves further attention given genetic evidence for the acquisition of p53 mutations secondary to mutations in N-Ras in thyroid tumors (Asakawa et al., 2002). In experimental models, stable expression of activated Ras has been shown to induce p53 mutations (Chen et al., 1998). The effects of Ras on cell cycle regulatory proteins needs to be examined in more detail given the unusual effects of Ras on these molecules in human and rat thyroid cells, together with the identification of mutations in cyclins and cyclin-dependent kinase inhibitors in thyroid tumors. It is noteworthy that overexpression of cyclins D1 (Lung et al., 2002) and E (Spruck et al., 1999) has also been linked to genomic instability.

The TSH-dependent nature and relative ease with which rat thyroid cells can be manipulated *in vitro* affords an important cell model for future studies. Transgenic and knock out animal models for discrete Ras and Raf isoforms hold enormous promise for understanding the contributions of these signaling molecules to thyroid tumorigenesis. Whatever is learned from the rodent model systems must be validated in human thyroid cells. With increasing evidence that Ras signals through discrete pathways in rodent versus human cells (Hamad et al., 2002), studies of the signal transduction mechanisms and consequences of Ras and B-Raf activity in human thyroid cells are essential. Finally, while numerous studies have examined the cellular consequences that arise following transient or stable expression of activated Ras, few studies have attempted to model both the primary and secondary adaptive changes that occur in response to sustained Ras activity in the same cells.

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8. P53 AND OTHER CELL CYCLE REGULATORS

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INTRODUCTION

Most tumor suppressor genes (whose function in cancer biology was surmised by their inactivation or deletion) turn out to be important in normal cell growth and proliferation. Inactivation of these genes opens the gates to malignant transformation driven by aberrant growth signals. In general, tumor suppressor dysfunction does not initiate genomic instability or aneuploidy, hallmarks of the origin of cancer, but they are at least as susceptible as other genes to the consequences of these destabilizing phenomena. On the other hand, these genes have a predilection to inactivation by epigenetic mechanisms, such as aberrant methylation with the progression of cell transformation.

This chapter focuses on the pathways anchored by the two "big" regulators of the cell cycle: p53 and the retinoblastoma (Rb) genes. Until recently the connections between these two networks was obscure (1). It is now apparent that they regulate and counter-regulate each other in a Ying-Yang fashion. The network centred on p53 is denser and more intricate (2,3), receiving signals from upstream modulators and downstream effectors. In addition to their respective roles at crucial check-points in the cell cycle and DNA repair, the products of both these genes appear to have roles in embryogenesis, cell differentiation and cell fate including senesce(1,4,5).

THE P53 NETWORK

P53 is a transcription factor that transactivates a large number of genes. The abundance of the p53 protein is predominantly regulated through its degradation. Compared to

the other features of its biology, our knowledge of the transcriptional regulation is less than complete. The gene is induced by single DNA breaks, radiation, UV light, some chemotherapeutic agents (3) and as demonstrated beautifully recently by interferons α/β (6). P53 tansactivation of its target genes is regulated by posttranslational mechanisms including phosphorylation, acetylation and prolyl isomeration (3,7) or by protein-protein interaction (8). P53 thus modified may select subsets of target promoters by changing its shape and affinity to bind to regulatory DNA sequences that vary among the downstream genes. Little is, however, known about the mechanisms underpinning the selection of the target genes and the divergent cell fates triggered down a given pathway (3,7). In certain situations apparently non-modified P53 can select and activate genes (6).

MDM2 and networks cross-over

P53 is ubiquinated by MDM2, directing it to the proteosomes for degradation. P53/MDM2 makes a finely balanced tandem. Thus, while p53 up-regulates MDM2 gene transcription, the MDM2 protein marks p53 for proteolysis by attaching ubiquitin to its carboxy-terminus (2,3). Furthermore phosphorylation of p53 NH2-terminus (and its activation) influences its binding affinity to MDM2, and thus its degradation (2,3). A deubiquinating enzyme can counteract the degree to which P53 is ubiquinated (9). Moreover, the transcriptional response of P53 may be regulated by the SUMO-1 modification of its carboxy-terminus (10).

And the regulation of MDM2 gets even more complex, in that it is capable of self-ubiquination and is SUMO-1 modified. The latter modification prevents MDM2, s self-ubiquination and therefore, in turn, its ability to ubiquinate P53 (11) The phosphorylation of MDM2 through the phosphatidylinositol 3-kinase (PI3K) pathway (12,13) may also enhances its ability to ubiquinate and thus regulate the cellular level of p53.

Other cues for the p53—anchored pathway also feed through MDM2. Thus growth signals from a number of oncogenes e.g. RAS, induce the ARF gene or stabilize its protein. ARF promotes the accumulation of SUMO-1 modified MDM2 and block the shuffle of MDM2 from the nucleolus to the cytoplasm, thereby stabilizing P53 (14–16).

MDM2 is also the conduit for the regulation of growth factors and allied receptors relevant to proliferation and signaling (17,18).

The influence of ARF on MDM2 function is an important link between the P53 signaling network to that centered on Rb, in that ARF gene transcription is, in turn, regulated by E2F (1). And this influence is far from being unidirectional because MDM2 (which you will recall is transcriptionally regulated by P53) binds to Rb and E2F with resulting increase in E2F gene transcription (19 for review).

Cycle arrest & apoptosis

When the cell is stressed or its function impaired, p53 abundance is increased and the protein activated to arrest cell division until such time as repairs are affected (2,3). P53 mediates the arrest of the cell cycle at the G1/S restriction point and the G2/M phase through the increasing the transcription of $p21^{WAF}$, an inhibitor of cyclin-dependent

kinases. p21 activation is another important node in the cross-communication between p53/Rb networks. p21 activation inhibits the kinases that drive cyclin D and related kinases which phosphorylate RB thus releasing E2F from its grips(1). Other genes regulated by p53 include Reprimo, which arrest the cycle at G2. Interestingly, in epithelial cells 14-3-3σ, by sequestering cyclin 1/CDK1 complexes in the nucleus can affect G2 arrest. Inhibition of 14-3-3σ allows epithelial cells to grow indefinitely (3).

When cellular damage is beyond the capability of the repair mechanisms, apoptosis is orchestrated by p53 (2,3,7). That option is apparently dependent on the abundance of p53 in the cell and is possibly tissue-specific (20). P53 homologs p63 and p73 are not relevant to tumor suppression but they are to apoptosis (21). The transcription of the Bax gene, whose product is pro-apoptotic, is enhanced by p53 apparently through the mediation of another gene (22). Other genes which promote cell death signals and that are induced by p53 include Noxa, p53 A1P1 and PIDD. A1P1 forms complexes with p53 to interact more strongly with the promoters of apoptosis-inducing genes than those involved in cell cycle arrest (8,20). P53 may induce apoptosis through a lysosomal-mitochondrial pathway that is initiated by lysosomal destabilization (23).

Another aspect of the role of p53 as a "guardian of the genome" is its function in maintaining genomic integrity. This is probably achieved by regulating nucleotide excision repair, chromosomal recombination and segregation. That p53 has an important role in DNA repair is supported by its induction of "ribonucleotide reductase" gene following DNA damage (24).

P53 inhibits the formation of new blood vessel in response to trophic factors elaborated (VGEF) by some tumors. Loss of p53 in late progression removes these restraints and allows continued tumor growth and metastases (25).

P53 IN HUMAN CANCERS

P53 is mutated in some 50% of human tumors (see http://perso.curie.fr/Thierry.Soussi,www.iarc.fr/p53/index.html and http://cancergenetics.org/p53.htm, 2,3,7). There are about 18,000 such entries in existing databases, the vast majority of which are missense mutations that apparently disable p53 tumor suppressor function. Table 1 lists the various mechanisms involved in p53 inactivation. While DNA sequence alterations (missense mutations, deletions and insertions, abnormal splicing) are more common in some malignancies compared to others, the other mechanisms shown in Table 1

Table 1. The various ways P53 may be inactivated in malignancies, *modified from (3)*

Mechanism of p53 inactivation	Consequences of inactivation
Missense mutations in the DNA-binding domain	Failure of p53 as a transcription factor
Carboxy-terminus deletions	No p53 tetramers are formed
MDM2 gene amplification	Enhanced p53 proteolysis
ARF deletion	Impaired inhibition of MDM2
Infection by some DNA viruses	Viral products inactivate p53 or increase its degradation
Failure of p53 to localize to the nucleus	p53 does not function

are limited to narrow classes of tumors. Some mechanisms e.g human papilloma virus in cervical cancer, may be related to environmental access of the carcinogen, whereas others may reflect the specific differentiated tissue environment e.g. MDM2 amplification in sarcomas and brain tumors (3). Not all potential mechanisms for inactivation of the p53 pathways have been systematically investigated for the majority of tumors.

Not only do P53 and Rb pathways cross but also the products of some DNA viruses inactivate them both (1–3).

Of the almost 18,000 p53 mutations in human malignancies, 97% are clustered in the core of the DNA-binding domain and 75% represent missense mutations. Until quite recently only a limited number of the tumor-derived missense mutations have been shown to render p53 defective. In order to address this deficiency Kato et al (7) mutated all 393 residues for all possible substitutions, examined the transactivating capacity of the products, after appropriate editing. Overall, 36% of the mutants were functionally inactive and 64% of core domain mutants fell in that category. Except for mutants at the C-terminal tetramerization domain, the functional part of p53 was concentrated between residues 96 and 286. Even within the DNA-binding core, the secondary structures were more susceptible than the connecting loops to functional disruption. By the same token, the conserved regions in closer proximity to DNA were more sensitive to mutations than conserved region not as intimately associated with DNA. At least for some substitutions, there appear to be differential effect on the transactivation of the p53 responsive genes examined (MDM2, BAX, 14-3-3σ, p53A1P1, GADD 45, Noxa, p53R2).

Interestingly, of the1266 (54.7%) mutants which could be explained by function/mutation notion, 39.1% were inactive for all 8 promoters and have never been reported in tumors, 15.6% were inactive and reported in tumors, 16.1% were reported at least once in tumors but retained wild type transactivating capacity, 1.6% were inactivating mutations but have never been reported in tumors (7) probably because they occur in p53 domains not usually studied for mutations) and finally 27.5% were inactive for only a limited number of promoters. The last category of mutants may well have partial function in tumor suppression but may show pleomorphism in their range of activity against various downstream target genes (7)

P53 mutations in thyroid cancer

The prevalence of p53 mutations (14.3%) in thyroid carcinoma overall is much lower than in common cancers (2,26). Most studies have limited mutation screening to exons 5–8. Apparent mutation hot spots were located at residues 167, 183, 213, 248 and 273, mapping to the DNA-binding core of the p53 protein (2, 26,27). Viewed in the light of recent developments (7), it is apparent that the mutations reported at the caroxby terminal of exon 8 and indeed a few within the DNA-binding core (27), do not influence the transactivating capacity of p53. Such functionally silent mutants may have been accidentally expanded during clonal selection of tumor cells. It cannot, however, be excluded that these apparently functionally silent mutants within the DNA-binding core may have minor disruptive influence on p53´s tumor-suppressive function unrelated to its transactivating capacity. In this context, the claim that homozygozity for

p53 proline 72 (a polymorphic Arg/Pro site) predisposes to anaplastic carcinoma (28) cannot be sustained. That the silent mutation rate of p53 in thyroid carcinomas was almost 120 fold that expected and 6 times the average rate of p53 silent mutations in the databases, the apparent random distribution of these mutations and distribution of multiple mutations (doublets, triplets) in accordance with Poisson's expectations suggest that p53 is particularly hypermutable in malignant thyroid tumors (2,27).

Almost a third of the mutations in p53 comprise G: C→A: T transitions at CpG dinucleotides and 5 of 6 mutation hotspots (codons 175, 245, 273 and 282) are CpG sites. ^{m}C (5-methylcystosine) is frequently converted to T by spontaneous hydrolytic deamination, forming a basis for an epigenetic mutational mechanism. These epigenetic events occur predominantly in poorly differentiated and anaplastic tumors; with one exception each all transitions at codons 273 and 248 were found in such tumors (2). Although the distribution of C→T/G→A transitions suggest that ^{m}C deamination is as likely to be time-dependent as replication- dependent (29), we speculate that it may occur at the thyroidal stem cell stage.

Even though p53 is only one of many pathways leading to thyroid cancer it appears to have a pivotal role in differentiated thyroid function, in that thyroid –specific differentiation genes are re-expressed on anaplastic thyroid cancer cells harbouring p53 mutant with transfection of wild type p53 (30).

Mutations in radiation related thyroid cancer

The prevalence of p53 mutations thyroid cancers related to radiation is no different from that in the non-irradiated tumor population (2,27). However, the frequency distribution of the mutation spectrum is radically different between two groups (Table 2). Moreover, the radiation—related thyroid tumors show higher G: C→A: T transitions rates and silent mutations than the non-radiation related thyroid tumors. Experimental radiation of thyroid epithelial cells was found to increase the rate of p53 silent mutations (31). The role of radiation in targeting mutation sites is further bolstered by the fact that none of the mutations in radiation related tumors involved CpG dinucleotides as opposed to one-quarter in the non-radiation related tumors.

Table 2. Mutation spectrum in radiation-related compared to non-radiation related thyroid carcinomas

P53 codons	Radiation-related	Non-radiation related
248		8/8
273		9/9
213	6/8	2/8
167	5/5	
183	5/5	
173		3/3
208	2/3	1/3
266		3/3

Only codons with 3 or more mutation events are considered. χ_2(heterogeneity) = 34.98, $p < 0.0005$ (df = 7)

The apical pole of thyrocytes copes with significant oxidative stress. The notion that that DNA mutagenesis is induced by oxidation of G (32) and exaggerated by radiation is attractive but is not upheld by the nature (transitions) of p53 mutations actually observed. Other mechanisms of p53 oxireduction by environmental factors (33) cannot be excluded. On the other hand given the abundance of nitrous oxide (NO) synthases in thyroid tissue (34) and documented increased NO in malignant tumors (35), it is conceivable that local NO generation may be responsible for G: C→A:T transitions in poorly differentiated thyroid carcinomas. The scenario for incriminating NO in p53 mutagenesis might, however, be different from that shown in inflammatory bowel disease and rheumatoid arthritis field (36–38).

P53 mutations in different studies

Although the number of samples exhibiting p53 mutations in individual reports in the literature is small, there are enough striking differences between them to warrant comment. Recurrence of the nature and type of mutations in series from individual centers is interesting. Thus in two series from Japan transitions at p53 codon 248 predominated, in a US study mutation were limited to transitions at codon 273, whereas in a third series (from Italy) p53 abnormalities in thyroid tumors were completely limited to frame-shift mutation, uncommon in the database as a whole (see 2 for review). If the possibility of cross-contamination is put aside, one might therefore speculate that local environmental or genetic factors contributing to the differences in mutations from different centers. Given the small numbers of mutations in each series these must remain that—speculations. The differences between radiation-related and spontaneous thyroid tumors considered above are probably on a more secure basis.

The abundance of P53 in thyroid tumors

While mutant p53 have longer half-lives and are thus more abundant in thyroid tumors harboring p53 mutations, p53 abundance may also be regulated by upstream signals in the absence of mutations (2,3,7). Cancer genes involved in malignant thyroid cell transformation mediate some of their effect by repressing cellular p53 levels (13,39). Thus the oncogene RET/PTC was found to reduce the levels of p53 and it is known that the PI3K pathway, normally repressed by the tumor suppressor PTEN, can influence p53 levels by way of phosphorylation of Mdm2 (12,13).

Although there is broad correlation between p53 mutations and immunohistochemical reactivity, documented gene mutation rates are less than estimates based on protein abundance on histology. Aberrant p53 immunoreactivity is detectable in 40–50% of poorly differentiated carcinomas, not significantly more than in anaplastic (50–60%) tumors (40,41). While, however, in undifferentiated cancer p53 expression is widespread, in poorly differentiated carcinoma it is usually observed in a lower proportion of cells and is sometimes confined to specific tumor foci with aggressive/infiltrative growth.

It is likely that a wide range of activities against downstream target genes are necessary for p53 to exert its full tumor suppressive function. This may explain why whereas p53 is frequently mutated, mutations in downstream target genes are rarely found.

MDM2 in thyroid cancer

MDM 2 is upregulated in a small percentage of malignant thyroid tumors. That this occurs, irrespective of the presence of wt p53, suggest that increased MDM2 gene transcription is in response to upstream cues and probably feed upon other targets of MDM2 (42). MDM2 is not amplified nor re-arranged in thyroid tumors. Interestingly, MDM2 transcripts abundance was related to clinical tumor staging (43).

MDM 2 is apparently more likely to be translocated to the nucleus in well-differentiated malignant thyroid tumors than in benign nodular tissue, thus sequestering p53 (44) (or driving the cell cycle via Rb/E2F). An increase in MDM 2 protein by immunocytochemistry is more frequent, found in half the tumors and is related to the expression of the anti-apoptotic protein Bcl-2 (45).

P21 in thyoid cancer

P21 is a downstream transcriptional target of p53 which inhibits Rb phosphorylation in a cyclin-dependent kinase 2-specific but not cyclin-dependent kinase 4-specific sites (46), thus setting up a pathway that uses the cell's normal regulatory machinery involving Rb phosphoryaltion. Interestingly, cyclin D1 involved in the phosphorylation of p21 may influence the cell cycle independent of the cyclin-dependent kinases by sequestering p21 (47). P21 may be induced independent of p53 and plays important roles in differentiation of such tissues as skeletal muscle (48).

P21 protein expression may be reduced in carcinomas with p53 mutations. However, this correlation is far from consistent in that p21 is detected in 1/3 of tumors irrespective of their degree of differentiation and is often co-expressed with p53, suggesting p53 independent induction of p21 in these tumors (49,50). Deletions and mutations of p21 were examined in one study only, comprising 57 thyroid tumors. Exon 2 of the p21 gene was deleted in 12.5% of papillary thyroid carcinomas. The deletion in 3/5 samples was related to a point mutation 16 bps upstream from the splice donor site and result in aberrant RNA splicing. P21 codon 31 (Ser→Arg) polymorphism was no more frequent in patients with thyroid cancer compared to controls (51). It was assumed that these mutants were functionally inactive as they mapped in the most evolutionarily conserved part of the gene and as p21 truncated beyond nt 222 were functionally inactive (52). None of the tumors haboring p21 deletion mutant carried p53 or Rb mutations (43,51, see below).

The putative tumor suppressor gene TSG101 is involved in the regulation of the cell cycle by binding to p21 and increasing its stability. The influence of TSG101 on p21 is proliferative-phase and perhaps tissue specific (53,54). TSG 101 transcripts and gene product abundance has been reported to be upregulated in papillary carcinoma (55). Interestingly, TSG101 is encoded in a chromosomal region notorious for its genomic instability and which spans another tumor suppressor gene, *FHIT,* which

is frequently deleted in all stages of thyroid tumor formation (56).The notion that frequent abnormal transcripts of TSGl01and *FHIT* reflect genomic instability and are of doubtful biologic significance (57) is likely incorrect.

THE RETINOBLASTOMA GENE NETWORK

Aberrations of the Rb gene is found in ~50% all human tumors (1) and yet it has not been as intensively studied in human cancers as have p53!. Indeed alterations in the Rb signalling pathway, by activation of positive acting components such as G1 cyclins and cyclin-dependent kinases (cdk), by inactivation of negative acting components such as cdk inhibitors and p53, or by mutations in Rb itself has been detected in virtually all human cancers (58).

Rb plays a pivotal role in the G1 checkpoint in the cell cycle, in balancing proliferation and apoptosis and in determining cell fate. It is important for the terminal differentiation of a number of tissues both in the embryonic and extraembryonic tissues (4).

Rb inhibits cell cycle advance when underphosphorylated. Once phosphorylated by two cyclin/cdk complexes Rb releases its control on the cycle. The G1 cyclins upstream of Rb are, in turn, regulated by inhibitors. By controlling the activities of certain transcription factors and thus responding genes Rb permits the cycle to progress from the G1 phase into S phase. The most prominent and important of these transcription factors are members of the E2F family (1). Two classes of cdk inhibitors interpret the responses of the cell to environmental signals: the CIP/KIP and INK4 that silence the cdks.

The G1 phase cyclins are engaged by cdk4 or ckd6 early in G1 and cdk 2 late in G1, to phosphorylate Rb thus releasing E2F to act at their cognate promoters. Early in S phase cyclinA/cdk2 complexes are activated not only to drive the cycle forward but also to terminate the transcriptional influence of E2Fs by phosphorylating them (1,59).

RB in thyroid carcinoma

Rb is deleted not only in retinoblastoma but in a large variety of sporadic tumors including osteosarcoma, carcinomas of the bladder, prostate and small cell lung carcinoma (1). Reports of Rb aberration in thyroid tumors are sparse.

We found in frame deletion of Rb exon 21 in 55% of malignant (but not benign) thyroid tumors (60). Exon 21 is an integral component of the Rb "pocket" that binds E2F and oncogenic DNA virus antigens (1). The Rb deletions were related to defective RNA splicing, although no mutations at the exon/intron junction were found (60). Both copies of the Rb gene were deleted in only one –third of the samples. Immunocytochemical analysis of pRb was, however, reduced or diminished in all positive specimens, suggesting that additional lesions in the promoter or coding sequence of Rb may have inactivated the second allele. It is noteworthy that mice with a single copy of Rb ($Rb^{+/-}$) develop neuroendocrine tumors including medullary thyroid carcinoma (61); it conceivable that a different tissue environment in adult thyroid tissue encourage somatic transformation of epithelial thyroid cells.

For technical and interpretative reasons the loss of Rb immunoreactivity in malignant thyroid tumors was not upheld earlier (see 62 for review). Since then, pRb loss turns out to be a consistent feature of papillary and follicular carcinoma but apparently not that of Hürthel cell or Warthin-like variants of the former (62,63).

The Cyclin-kinase Inhibitors

The CPI/KIP family

P21 is rarely mutated in human neoplasms and has been considered in thyroid carcinoma above under P53.

$P27^{KIP1}$ responds to cellular environmental signals such as growth factors, cell anchorage and contact inhibition. It is also rarely deleted in tumors. $P27^{KIP1}$ protein abundance is regulated post-translationally by way of phosphorylation and proteosome-mediated degradation following ubiquination (1).

$P27^{K1P1}$ protein abundance is reduced in malignant thyroid tumors and is associated with poor clinical outcomes (64,65). Its levels are, however, maintained in a subset of tumors, predominantly those with oncocytic histology including follicular variant of papillary carcinoma (66,67). The cytoplasmic accumulation of $P27^{K1P1}$ is probably related to its sequestration by cyclin D3 (68). This mechanism surmised on my part would need to be directly confirmed. This phenomenon is, however, not without precedent as p21 may be regulated by its being sequestered by cyclin D1(47).

The INK4 family

The two members of the INK4 family, INK4A and INK4B, are closely linked on 9p21, a region of the genome highly mutable in familial melanomas and pancreatic carcinomas. This region thus exhibits a high rate of LOH in tumor tissue. Human tumors contain either mutations in Rb or INK4A, which needs wild type Rb to disrupt the cell cycle. The reading frame of another gene alluded to before in the connection of the p53/Rb networks, ARF, overlaps that of INK4A (1 for review).

Mutations or deletions of INK4A and INK4B are infrequent in primary thyroid carcinomas but are common in thyroid carcinoma cell lines (69–72). Epigenetic silencing of INK4B by hypermethylation may be not an uncommon mechanism for gene silencing in clinical tumor material (72,73). Moreover, LOH at 9p21 may be a more frequent event in thyroid carcinoma than is the recorded inactivation of INK4 family members, suggesting the presence of tumor suppressor genes in the vicinity.

The cyclins

Cyclin D1 is an early G1 cell cycle progression factor (1,2,48). While cyclin D1 regulates Rb activity by way of post-translational modification, Rb enhances the specific transcription of cyclin D1 (74), thus setting up a regulatory feedback loop. Aberrant expression of cyclin D1 through gene amplification or overexpression is a common feature of several cancers. CyclinD1is often amplified only in those tumors that retain wild-type pRb (See 48). We first reported (48,75) the upregulation of cyclin D1 transcripts in $^1/_3$ of malignant thyroid tumors. Overexpression of cyclin D1, studied

in 32 tumors was not related to gene amplification or re-arrangement. Cyclin D1 overexpression was predominantly found in tumors retaining wild type Rb, a fact not emphasized enough in subsequent studies. Our findings have been amply verified at the level of cyclin D1 protein level (43,66,67,76–79) as have our suggestion that gene amplification is not involved in gene product overexpression (64,80). Cyclin D1 (and lack thereof of p27) overexpression has proved to be an important prognostic factor (43,76,78,79) in the prediction of lymph node metastases in papillary thyroid cancer. Moreover cyclin D1 overexpression in papillary microcarcinomas predicted secondaries to the regional lymph nodes (64). Constitutive activation of Ret as a result as of RET/PTC re-arrangement appears to be involved in inducing cyclin D1 over-expression (81). Cyclin D1 may also be upregulated by other oncogenes (82,83).

In well-differentiated papillary thyroid carcinomas cyclin Dl overexpression was related to that of p21 (43,77), which was apparently induced by a p53-independent mechanism (43).

The abundance of the other G1 phase cyclin, cyclin E, appeared to parallel that of cyclin D1 (66,67) and did not appear to be differentially expressed between different tumor types.

E2F

The E2F family of transcription factors is the most important downstream effectors of Rb. They bind to relevant promoters only when heterodimerized with their partner DP. The complexes show specificity for Rb or its homolgos p107 and p130. The E2F family members mediate many of Rb,s effect in embryogenesis, cell differentiation and cell fate as well as apparently functions as a transcription factor independent of Rb (1). E2F is involved in the induction of genes required in initiating and executing DNA replication, DNA repair, cell cycle progression and apoptosis (including Myc and ARF). Beyond the G1/S restriction point E2F regulate genes involved in G2/M checkpoints and mitotic regulation as well as chromosomal dynamics.

The E2F/DP complex is inactivated by cyclinA/cdk2 phosphorylation and its abundance modulated by ubiquitin—mediated degradation.

Although no mutations have been described in E2F in human tumors, it is apparent that its overexpression stimulates cell proliferation and contribute to carcinogenesis. Depending on ambient growth signals it E2F can promote apoptosis in both p53-dependent and—independent manner.

Thyroid carcinomas are reported to overexpress E2F (63,80,84). E2F appeared to be particularly expressed in oxyphilic adenomas and carcinomas (2/3 of samples) (84) compared to non-oxyphilic lesions (1/3 of samples). Anwar developed this theme further by showing that the presence of pRb and E2F can be used to sub-categorize papillary carcinomas: Hürthle and Warthin-like variants which arise within the context of Hashimoto's thyroiditis were Rb and E2F1 positive, whereas papillary carcinoma, including the follicular variant were negative for both (63). Interestingly, metaplastic Hurthle cells associated with thyroid autoimmune disease were only Rb positive. This fascinating observation (63) warrants further follow-up.

Just as with p53, E2F appears to be under negative regulation by TR and the interaction is equally complex in that when bound to E2F promoter the unliganded TR activates transcription, whereas the liganded receptor represses the transcription of S-phase specific DNA polymerase, thymidine kinase and dihydrofolate reductase genes and thus withdrawal of S phase to initiate differentiation (85). A high rate of aberrant TR in thyroid tumors (see the Chapter by Cheng) would release such restraining influence on E2F.

Further afield

It is apparent that the influence of Rb and its downstream interactors extends beyond the S phase of the cell cycle, to G2-M and hence my justification for the discussion the roles in thyroid cancer of cyclins acting later in the cycle and indeed a negative cyclin.

Cyclins A, B1 and cdc2 are factors which regulate the transition from G2 to the M phase of the cycle. Cyclin A and cdc2 were overexpressed in undifferentiated thyroid cancers, as opposed to cyclin B1 which was less frequently overexpressed in this class of tumors (86).

Recently Ito et al (87) found that the expression of cyclin G2 that negatively regulates cell cycle progression could differentiate between papillary and follicular thyroid tumors. Thus, whereas normal thyroid tissues and papillary tumors were negative for cyclin G2, it was expressed in 2/3 follicular adenomas but only 20% of follicular carcinomas.

THYROID HORMONE RECEPTORS & TUMOR SUPPRESSOR NETWORKS

Thyroid hormones have a profound influence on development, growth, energy and intermediary metabolism and, as being recently realized, in carcinognesis (See Chapter 9). Thyroid hormones acting through their receptors (TRs) can be also be modelled as a wide-ranging network with crucial nodes. Not surprisingly the p53-based network overlap that predicated on thyroid hormone action and apparently influence each other. P53 apparently binds to and modulates TRβ activity and depending on cell type and experimental system p53 was reported to repress basal or ligand-mediated activity (88,89). TR can apparently also regulate MDM2 gene transcription independently of p53 (90). The potential for thyroid hormones influencing both tumor suppressor networks discussed above is immediately obvious and is an area where more functional studies would be welcome. Cheng discusses elsewhere in this book the mutation of TR in thyroid carcinoma, I only point out that the frequent silent mutations noted in those studies echo those in p53!

CONCLUSIONS & PRESPECTIVES

Our understanding of the physiologic roles of the many nodes that interconnect within and between the p53 and Rb—based networks is less than complete. However, rapid progress is being made. In thyroid tumors much of the relevant data is predicated on the abundance of specific proteins determined immmuncytochemically. While this approach is rapid, cheap, may relate gene product abundance to histology and even

allow the construction of hypotheses on the pathways involved, it gives limited insight into the molecular mechanisms involved.

Studies of the rates and nature of p53 mutation suggest that the gene is highly mutable in thyroid tumors and specifically incriminates a commonly occurring epigenetic event in anaplastic carcinoma, rarely found in well-differentiated tumors. Another common epigenetic mechanism for tumor suppressor inactivation, hypermethylation of regulatory sequences, has been documented in INK4B within the Rb network as well as in genes in independent pathways, involved in cytoskeletal integrity (91). Rb gene is frequently truncated resulting in low levels of Rb protein, a finding that needs to be explored more widely. That p21, which is rarely inactivated in tumors, is also truncated in thyroid carcinomas through aberrant RNA splicing raises suspicions that correct RNA splicing may be impaired in thyroid tumors.

Interestingly, the only two genes within the p53 and Rb networks we found to be differentially regulated in thyroid tumors were A1P1, involved in p53 related apoptosis and cyclin D1 (Puskas L & Farid, NR, unpublished). Cyclin D1, which is important in regulating Rb function and thus cell cylce progression, is upregulated through diverse pathways. Cyclin D1 is also a "cross-over artist" because by sequestering p21 it can extend its proactive influence on to the p53 network. Cyclin D1's abundance is valuable in forecasting the outcome of thyroid carcinoma, even those in the microtumor stage of their evolution (64).

It is apparent that genes entrusted with putting the brakes on thé cell cycle, often fail in thyroid tumors. There are many areas of uncertainty or outright ignorance that need to be filled in with systematic studies of genes and gene products within these pathways.

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9.ABNORMALITIES OF NUCLEAR RECEPTORS IN THYROID CANCER

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INTRODUCTION

Nuclear receptors comprise a large family of ligand-inducible transcription factors that are critically important for growth, differentiation, development, and maintenance of metabolic homeostasis. They regulate the expression of target genes by binding to the specific DNA sequences at the promoters to mediate the biological effects. Many nuclear receptors have multiple isoforms with over-lapping functions or isoform-specific functions (1, 2). The expression of these receptor isoforms is regulated in a tissue- and development-dependent manner. A host of coregulatory proteins that influence the ligand selectivity and DNA binding capacity further modulates the transcriptional activities (3, 4).

Abnormal expression and/or aberrant functions of sex steroid nuclear receptors are known to be involved in the development and progression of such endocrine cancers as breast, ovarian, endometrium, and prostate, but less is known about the role of nuclear receptors in the carcinogenesis of the thyroid. Progress in this area has recently been made as a result of the discoveries of the fusion gene of PAX8 with the peroxisome proliferator-activated receptor γ (PPARγ; PAX8-PPARγ) in follicular thyroid carcinoma and of the spontaneous development of follicular thyroid carcinoma in the homozygous knock-in mutant mice harboring a mutated thyroid hormone β receptor (TRβ). This review will first examine the latest findings on the possible roles of several sex steroid nuclear receptors in thyroid carcinogenesis. It will then discuss the molecular actions of the mutant TRβ in carcinogenesis, particularly in relation to a unique knock-in mouse model of thyroid cancer.

ABNORMAL EXPRESSION OF ESTROGEN AND PROGESTERONE RECEPTORS IN THYROID CANCER

Thyroid carcinoma is more common in women than in men (5). For 2003, the estimate of new cases of thyroid cancer has a female predominance with a 2.9:1 ratio (6). This predominance suggests that estrogens may play a critical role in the development of thyroid carcinoma. In the past two decades, efforts have been made to demonstrate the presence of estrogen receptors (ERs) in thyroid tumors and to correlate tumor malignancy with ER expression. Using a dextran-coated charcoal method and analysis by the method of Scatchard, Miki et al. did not detect ER in the cytosol of normal thyroids, but they found a significantly higher ER in the neoplastic and hyperplastic thyroid tissues (7). Using different biochemical methods, Mizukami et al. (8) and Yane et al. (9) also showed a higher expression of ER in neoplastic thyroid lesions than in normal thyroids or in adjacent normal tissues. Lewy-Trenda examined 72 thyroid glands for the expression of ER by using immunochemical assays with anti-ER antibodies. Positive staining occurred in the nuclei of differentiated thyroid cancer cells (24%), but not in non-neoplastic cells. A small number of oxyphillic (4%) and follicular adenomas (6%) also stained positive for ERs (10).

Consistent with these findings, several studies showed that estrogens stimulate the proliferation of thyroid carcinoma cells (11–13), whereas the antiestrogen, tamoxifen, inhibits the proliferation of a tumor cell line derived from medullary thyroid carcinoma (11). These studies clearly showed that cell proliferation induced by estrogens is mediated by ERs, but little is known about the specific molecular pathways. One study suggested that activation of the mitogen-activated protein kinase by phosphorylation might be one of the key steps in the estrogen-mediated cell proliferation of thyroid cancer cells (13).

The relevance of the increased expression of ERs in thyroid tumorigenesis is not obvious, particularly given that there is no clear correlation in the extent of expression of ER to age, sex, presenting clinical or pathological features, or, in cases of carcinoma, to subsequent metastatic potential (10, 14, 15). Furthermore, the failure of several studies to detect a greater expression of ERs in thyroid tumors than in normal tissues casts further doubt on the significance of expression of ERs in thyroid tumor development and progression (16–19). It is unclear whether the discrepancy among studies is due to the sensitivity of the detection or the intrinsic variability in the expression of ERs in tumor samples. Plainly, more studies are needed to understand whether estrogens and ERs are the major factors that contribute to thyroid cancers predominance in females.

Fewer studies have investigated the roles of progesterone receptors (PRs) in thyroid carcinogenesis. Because of the interest in understanding thyroid cancer's predominance in females, the expression of PRs in thyroid tumors has been evaluated by means of ligand binding assays, enzyme immunoassays, and/or immunohistochemistry. In a few limited studies, the presence of PR and ER was assessed concurrently in the same samples. In 135 thyroid lesions that included papillary, follicular, medullary, and Hurthle cell carcinomas, van Hoeven detected the presence of PR in 51% of the cases, with the highest abundance in papillary carcinomas, particularly in male patients and women older than 50 years (15). In that same study, ER was found in 46% of the

samples. In other studies, however, a higher frequency of ER than PR was found in papillary carcinomas (7, 10, 14). Similar to the findings for ER, no correlations were observed between the expression of PR and age, sex, tumor size, presence of capsular or vascular invasion, or lymph node status (10, 14). Still, how the expression of PR is involved in the development of thyroid cancer has not been assessed.

ALTERED EXPRESSION OF THE RETINOIC ACID RECEPTORS IN THYROID CANCER

Retinoic acids (RAs) are essential for many biological processes including proliferation, development, differentiation, carcinogenesis, and apoptosis. These biological effects are mediated through their receptors (RARs). The retinoids, both the natural and synthetic analogs, have been shown to be effective in preventing several cancers in experimental animals and in reversing pre-neoplastic lesions in humans (20, 21). Whether the retinoids could be effective in re-differentiating thyroid cancer cells to be amenable to radioiodide or TSH-suppressive T4 therapy has prompted several investigators to study the expression of RAR in cancer cell lines and tissues. Using Northern blot analysis, del Senno found that the expression of RARα mRNA was lower in thyroid carcinoma cells than in normal thyroid follicular cells. Moreover, del Senno demonstrated that RA reduces the proliferation and function of thyroid follicular cells (22, 23). These findings were confirmed in a larger study. Using immunohistochemistry and Western blotting, Rochaix et al. compared the expression of RARβ in 40 normal/benign tissues, 16 papillary carcinomas, and two follicular carcinomas, RARβ immunostaining was detected in the nuclei, but was limited to the normal epithelial thyroid tissue. A dramatic decrease in RARβ immunostaining was observed in all 16 papillary carcinomas, but in only one follicular carcinoma (24).

Because the feasibility of retinoid-induced differentiation therapy in thyroid cancer hinges on functional RARs, Schmutzler et al. not only examined the expression of mRNA in several human thyroid carcinoma cell lines and tissues, but also assessed the ligand and DNA binding activities (25). Functional RARs were clearly detectable in the two human thyroid carcinoma cell lines (FTC-133 and FTC-238) and two anaplastic thyroid carcinoma cell lines (HTH74 and C643). Intriguingly, variable levels of mRNA were observed in these cell lines, an observation probably indicative of dysregulation of receptor expression in thyroid cancer (25). These results suggest the heterogeneity in the expression of RARs and the association of the dysregulation of the expression of RAR with thyroid carcinogenesis. However, the available expressed functional RAR seems to be able to respond to RA treatment. In a pilot study, patients with advanced thyroid cancer and without the therapeutic options of operation or radioiodide therapy were treated with 13-cis-retinoic acid (1.5 mg/kg body weight daily for 5 weeks). Overall, tumor regression was observed in 19 patients (38%). However, response to retinoid therapy did not always correlate with increased radioiodine uptake (a re-differentiation marker), and so other direct antiproliferative effects could also be involved (26). These encouraging clinical findings warrant additional studies on the RA-based treatment of thyroid cancer. At present, however, little is known about either the molecular mechanisms by which the expression of RAR

is dysregulated during thyroid carcinogenesis or the RA-induced–redifferentiation of follicular cells. Elucidation of these mechanisms should help in the design of an effective treatment of thyroid carcinomas that uses the retinoids.

ABNORMALITIES OF THYROID HORMONE RECEPTORS IN THYROID CANCER

The thyroid hormone receptors (TRs) mediate the pleiotropic activities of the thyroid hormone (T3) in growth, development, and differentiation and in maintaining metabolic homeostasis. The two TR genes, α and β, are located on human chromosomes 17 and 3, respectively. Alternative splicing of the primary transcripts gives rise to five major TR isoforms ($\alpha 1$, $\alpha 2$, $\beta 1$, $\beta 2$, and $\beta 3$). TR$\alpha 1$, TR$\beta 1$, TR$\beta 2$, and TR$\beta 3$ differ in their lengths and amino acid sequences at the amino terminal A/B domain, but they bind T3 with high affinity to mediate gene regulatory activity. By contrast, TR$\alpha 2$, which differs from the other TR isoforms in the C-terminus, does not bind T3, and its precise functions have yet to be elucidated. The expression of TR isoforms is tissue-dependent and developmentally regulated (1, 2).

Early evidence to suggest that mutated TR could be involved in carcinogenesis came from the discovery that TR$\alpha 1$ is the cellular counterpart of the retroviral v-erbA that is involved in the neoplastic transformation leading to acute erythroleukemia and sarcomas (27, 28). The oncogenic role of v-erbA was subsequently demonstrated in mammals in that male transgenic mice overexpressing v-erbA developed hepatocellular carcinoma (29).

In recent years, increasing evidence suggests that aberrant expression and mutation of the TR genes could be associated with human neoplasias. Somatic point mutations of TR$\alpha 1$ and TR$\beta 1$ were found in 65% (11/17 tumors) and 76% (13/17 tumors), respectively, of human hepatocellular carcinomas. Many of these mutated TRs have lost T3-binding activity and exhibit aberrant DNA-binding activity (30). Aberrant expression and mutations of TR genes were also found in renal clear cell carcinomas (31). Cloning of TRs from 22 renal clear cell carcinomas and 20 surrounding normal tissues identified somatic mutations in 32% and 14% of cloned TR$\beta 1$ and TR$\alpha 1$ cDNAs, respectively (32). Most of the mutations were localized in the hormone-binding domain that leads to loss of T3-binding activity and/or impairment in binding to TREs. Similar to the mutated TRs detected in hepatocellular carcinoma (30, 33), the mutated TRs identified in renal clear cell carcinomas exhibit dominant negative activity (32). These studies suggest that mutated TR plays an important role in the development of these human cancers.

Abnormal expression and somatic mutations of TRs in thyroid cancer

Similar to the expression levels reported for ER, PR, and RAR, an altered expression of TRs was detected in thyroid carcinomas. Comparison of the mRNA expression levels of TR isoforms in normal, hyperplastic, and neoplastic human thyroid tissues indicated that TRβ mRNA is significantly lower in papillary and follicular carcinomas than it is in normal thyroid. No differences, however, were found in the expression levels of TR$\alpha 1$ and TR$\alpha 2$ mRNA (34, 35). These findings suggest an association of the reduced expression of TR$\beta 1$ mRNA with the development of thyroid carcinomas.

These studies, however, did not determine whether TRβ1, TRα1, and TRα2 were altered at the protein level.

In addition to the reduced expression of TRβ1 mRNA, a lower expression of TRα1 mRNA was found in 16 papillary thyroid carcinomas from Polish patients. The TRβ1 and TRα1 protein levels, however, were higher in cancerous tissues than in nearby healthy tissues, an indication of the complexity in the regulation of TR expression in these tumors (36). To understand the nature of TRs in these papillary thyroid carcinomas, cDNAs were cloned concurrently from both the tumor lesions and the healthy thyroids as controls. Sequence analyses indicated that 93.8% and 62.5% of papillary thyroid carcinomas had mutations in TRβ1 and TRα1, respectively. In contrast, no mutations were found in healthy thyroid controls, and only 11.1% and 22.2% of thyroid adenomas had mutations in TRβ1 and TRα1, respectively. Functional analysis indicated that these mutated TRs lose their transactivation function and exhibit dominant negative activity (36).

The reduced expression of TRβ1 mRNA in papillary thyroid carcinomas was further confirmed in a more recent study of 16 Japanese patients (37). In contrast to the Polish patients, no amino acid-substitution-mutations were detected in the TRβ1 cDNAs cloned from these papillary thyroid carcinomas. The reasons for the different propensity in the mutations of the TRβ gene in these two groups of patients are not entirely clear. One possibility is that the Polish patients were from the post-Chernobyl population and that radiation exposure is a contributing factor to the high frequency of TR mutations. Indeed, five of the 16 Polish patients with mutated TRs were in their teens when the Chernobyl accident occurred. One of 16 patients received radiation treatment during her childhood because of another disease. The age of other patients ranged from 32–58 years old at the time of the Chernobyl accident (Monika Puzianowska-Kuznicka; personal communication). The validation of this hypothesis would require a cohort study with a larger number of patients and a detailed knowledge of irradiation dose received by the patients.

Another possibility is that the propensity of mutations of TRβ1 in papillary thyroid carcinomas could be affected by the patient's ethnic origin. Genetic variation between different populations occurs frequently. For example, a wide variation in the frequency of RET/PTC rearrangements, a hallmark of papillary thyroid carcinoma, has been reported, ranging from a few percent in Japanese (38) and Saudi Arabian patients (39), to 18.8% in Italian patients (40), to 70% in New Caledonian and 85% in Australian patients (41). The frequency of polymorphisms associated with thyroid diseases also differs in Japanese and Caucasian populations (42). Clarification of the issue of whether genetic background affects the frequency of TRβ1 mutations in papillary thyroid carcinoma awaits additional analyses in patients with different ethnic origins.

Germline mutations of the TRβ gene in thyroid cancer: lessons learned from a unique mouse model of thyroid carcinogenesis

So far, the TR mutants identified in human cancers including thyroid carcinoma are somatic mutations. A knock-in mouse that harbors a gerrnline mutation of the TRβ gene has been created (43). The mutation was targeted to the TRβ gene locus

Table 1. Histologic progression of thyroid neoplasia in 5–14 month-old $TR\beta^{PV/PV}$ mice

Hyperplasia	Capsular invasion	Vascular invasion	Anaplasia	Metastasis
27/27(100%)	23/27(85%)	20/27(74%)	10/27(37%)	7/27(26%)

via homologous recombination and the Cre/loxP system. The mutation is called PV (TRβPV mouse) after a patient with the mutation who suffers from the disease known as resistance to thyroid hormone (RTH) (44, 45). RTH is a syndrome characterized by the elevated levels of circulating thyroid hormone that are associated with non-suppressible TSH. Some of the clinical features include attention-deficit hyperactivity disorder, mental retardation, short stature, decreased -weight, tachycardia, and hearing abnormalities (44, 45). PV has a unique mutation in exon 10, a C-insertion at codon 448, which produces a frame shift of the carboxyl-terminal 14 amino acids of TRβ1. *In vitro* studies revealed that PV has completely lost T3-binding activity, lacks transcriptional capacity, and exhibits potent dominant negative activity (46). Extensive characterization of the phenotype indicates that the TRβPV mouse faithfully reproduces the human RTH (43). This TRβPV mouse provides a valuable model for clarifying the role of germline mutations of the TRβ gene in carcinogenesis.

In addition to the phenotypes of RTH, homozygous TRβPV ($TR\beta^{PV/PV}$) mice exhibited the phenotype of age-dependent increased mortality. By the age of about 10 months, 50% had died, and by the age of 14–15 months, all mice were dead. In contrast, the heterozygous ($TR\beta^{PV/+}$) mice did not exhibit such abnormalities. Morphological examinations of the moribund $TR\beta^{PV/PV}$ mice indicate that as these mice aged, they spontaneously developed thyroid carcinoma (47). Histological evaluation of thyroids of 27 moribund $TR\beta^{PV/PV}$ mice showed capsular invasion (85%), vascular invasion (74%), anaplasia (37%), and metastasis to the lung and heart (26%) but not to lymph nodes (Table 1).

Representative examples of the pathological features of capsular invasion (Panel A), vascular invasion (Panel B), anaplasia (Panel C), and metastasis to the lung (Panel D) are shown in Figure 1. The histological features and the metastatic patterns indicate that the thyroid carcinoma developed in $TR\beta^{PV/PV}$ mice is follicular. Thus $TR\beta^{PV/PV}$ mice provide the first animal model for studying the molecular genetics underlying follicular thyroid carcinogenesis.

Using microarrays consisting of 20,000 mouse cDNAs, Ying et al. recently profiled the global alterations in gene expression in the thyroids of $TR\beta^{PV/PV}$ mice at 6 months of age, at which time metastasis had begun (48). They found that 185 genes were up-regulated (2- to 17-fold) and 92 were down-regulated (2- to 20-fold). The majority (~60%) of these altered genes are unnamed. Functional clustering of named genes with reported functions (100 genes) indicated that ~39% were tumor-, metastasis/invasion-, and cell cycle-related. Importantly, several tumor-related genes, such as cyclin D1, pituitary tumor transforming gene-1, cathespin D, and transforming growth factor α, that have been reported to be over-expressed in human thyroid

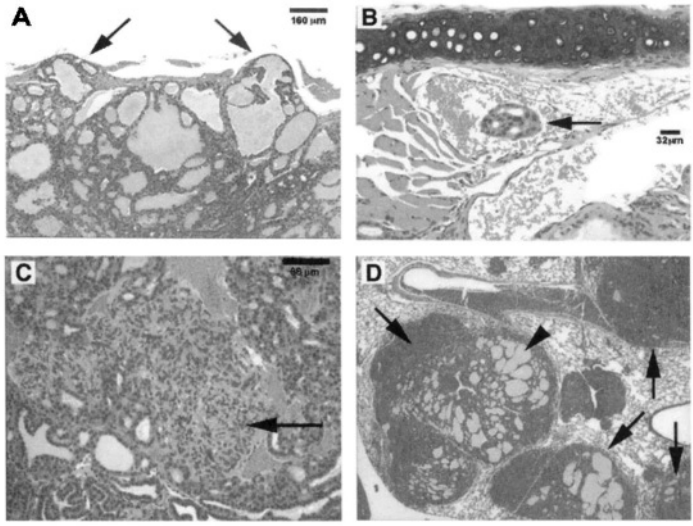

Figure 1. Pathological features in thyroid glands and metastasis in the lung of $TR\beta^{PV/PV}$ mice. Histologic sections from tissues of $TR\beta^{PV/PV}$ mice showed evidence of capsular invasion in thyroid (A) (arrows), vascular invsion in thyroid (B)(arrows), anaplasia in thyroid (C) and metastatic thyroid carcinoma lesions in lung (arrow).

cancers were found to be activated in the arrays (49–53). Analyses of the gene profiles suggested that the signaling pathways mediated by TSH, peptide growth factors, transforming growth factor-β, tumor necrosis factor-α, and nuclear factor κB were activated, whereas pathways mediated by peroxisome proliferator-activated receptor γ (PPARγ) were repressed (48). These findings suggest that the expression of the TRβ mutant directly and indirectly alters multiple signaling pathways that could contribute to the development of thyroid cancer and that thyroid carcinogenesis is mediated by multiple genetic events.

The frequent occurrence of the somatic mutations in several human cancers (30, 32, 36, 54, 55) and the development of follicular thyroid carcinoma in $TR\beta^{PV/PV}$ mice (47) raise the question of whether PV could function to initiate carcinogenesis. On the basis of observations that $TR\beta^{PV/PV}$ but not $TR\beta^{PV/+}$ mice develop follicular thyroid carcinoma, it is unlikely that PV could act alone to initiate thyroid carcinogenesis. One of the significant differences in phenotypes between $TR\beta^{PV/PV}$ and $TR\beta^{PV/+}$ mice is that the circulating serum TSH concentration in $TR\beta^{PV/PV}$ mice is ~275-fold higher than that in $TR\beta^{PV/+}$ mice (43). TSH is the main regulator of thyrocyte differentiation and proliferation, and the possibility that it is an initiator of thyroid carcinogenesis has been intensively studied (56, 57). Recent clinical and biochemical studies, however, do not support the role of TSH as an initiator of follicular carcinoma (58, 57). Additional genetic changes need to occur for the transformation of the hyperproliferative thyroid cells to cancer cells. On the basis of these considerations, it is reasonable to propose

that mutation of the two alleles of the TRβ gene could be one of the genetic changes leading to the transformation of the hyperproliferative thyroid cells to cancer cells. This hypothesis needs to be tested in future studies.

ABNORMALITIES OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ IN THYROID CANCER

PPARγ is a nuclear receptor that is involved in a wide range of cellular processes including adipogenesis, inflammation, atherosclerosis, cell cycle control, apoptosis, and carcinogenesis (59, 60). PPARγ mRNA is abundantly expressed in adipose tissue, large intestine, and hematopoietic cells, and it is moderately expressed in kidney, liver, and small intestine (61). It was recently found also to express in the thyroid (48). PPARγ inhibits cell growth, and one of the mechanisms in inhibition of cell proliferation is by reducing E2F/DP DNA-binding and transcriptional activity (62). Consistently, activation of PPARγ signaling by its ligands has been shown to block cell proliferation of various malignant cells and, in some cases, to induce differentiation and apoptosis (63–68). Ohta et al. reported that PPARγ mRNA is expressed in human papillary thyroid carcinoma cell lines (69). Significant, but variable expression of PPARγ mRNA was detected in four of the six cell lines studied. Consistent with findings in other cancer cell lines (63–68), cell proliferation was inhibited and apoptosis was induced by treatment with troglitazone. Ohta et al. also found that troglitazone significantly reduced tumor growth and prevented distant metastasis of BHP18–21 tumors in nude mice *in vivo* (69). In a more recent study, Martelli et al. also evaluated whether PPARγ is involved in the growth regulation of normal and tumor thyroid cells (70). No mutations were detected in PPARγ exons 3 and 5 in human thyroid carcinoma cell lines and tissues. The growth of PPARγ-expressing thyroid carcinoma cells was inhibited by treatment with PPARγ agonists, but no growth inhibitory effect was observed in NPA cells by PPARγ agonists that did not express PPARγ. Growth inhibition induced by PPARγ agonists or by overexpression of the PPARγ gene in thyroid carcinoma cells was associated with increased p27 protein levels and apoptotic cell death (70).

$TR\beta^{PV/PV}$ mice provide an unprecedented opportunity to study the role of PPARγ in thyroid carcinogenesis *in vivo.* Using quantitative real-time PCR and Northern blotting, Yin et al. found that the expression of PPARγ mRNA was repressed 50%–60% in the thyroids of $TR\beta^{PV/PV}$ mice at the ages of 4, 6, and 12 months (71). Immunohistologic analysis demonstrated that the expression of PPARγ protein in the primary lesions of $TR\beta^{PV/PV}$ mice was less than that in the thyroids of wild-type mice and was not detectable in the metastasis in the lung (unpublished results), an indication that the expression of PPARγ protein remained low during thyroid carcinogenesis. Moreover, PV was found to abolish ligand (troglitazone)-dependent transcriptional activity of PPARγ in primary cultured thyroid cells from wild-type mice (71). The PV-induced transcriptional repression could be due to PV's competition with PPARγ for binding to the peroxisome proliferator-activated receptor response element (PPRE) present in the PPARγ downstream target genes. Indeed, gel shift assay showed that the *in vitro* translated PV protein could bind to PPRE. This notion is supported by the

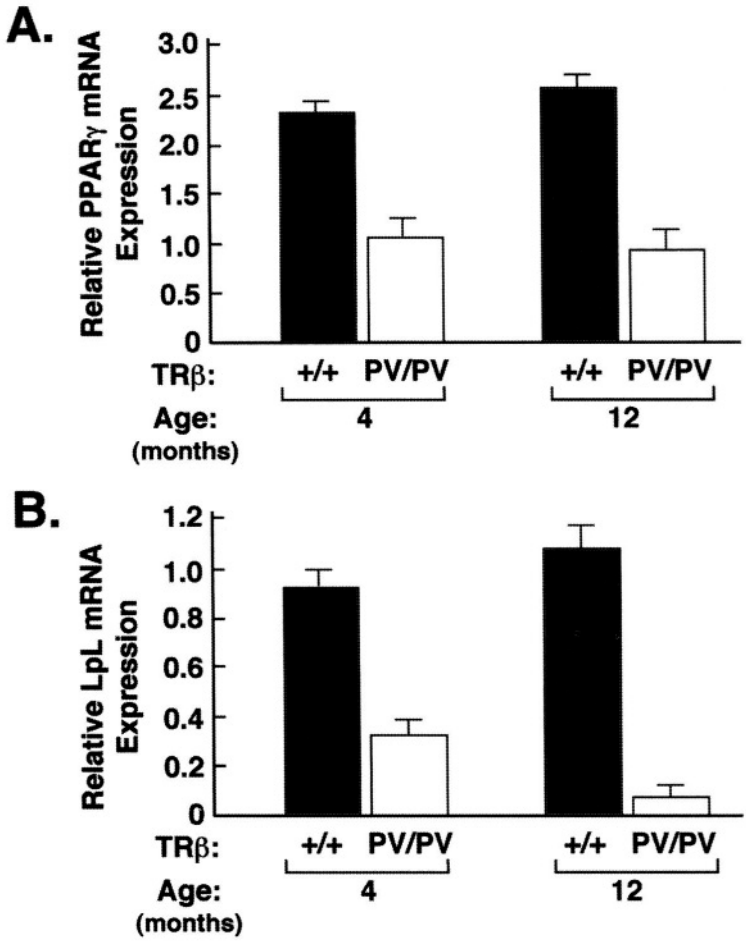

Figure 2. Camparison of the expression of PPARγ and lipoprotein (LpL) mRNA in the thyroids of $TR\beta^{PV/PV}$ and wild-type mice at different ages by quantitative real-time PCR. Relative expression levels of PPARγ(A) and LpL(B) mRNA in the thyroid glands were determined using age matched wild-type and mutant mice at the ages of 4 and 12 months as marked. The data are expressed as mean ± SD($n = 4$).

finding that the lipoprotein lipase (LpL) gene, a known PPARγ downstream target gene (72), was repressed ~5-fold, as shown by cDNA microarrays (48). Subsequent analyses by quantitative real-time PCR further demonstrated that the expression of the LpL gene was down-regulated (Panel B; Figure 2) concurrently with PPARγ mRNA (Panel A; Figure 2) in the thyroid glands of $TR\beta^{PV/PV}$ mice at the ages of 6 and 12 months, thus confirming the repression of PPARγ signal pathways during thyroid carcinogenesis (71). These results indicate that reduced expression of PPARγ mRNA and repression of its transcriptional activity are associated with thyroid carcinogenesis

and raise the possibility that PPARγ can be tested as a potential molecular target for prevention and treatment of follicular thyroid carcinoma.

That the attenuation of the PPARγ signaling pathways is associated with the development and progression of follicular thyroid carcinoma is also supported by the findings that the PAX8-PPARγ rearrangement occurs frequently in human follicular thyroid carcinomas, less frequently in adenomas, but not at all in papillary thyroid carcinomas (73–76). Even though the molecular actions of the PAX8-PPARγ rearrangement, particularly in its relation to the thyroid follicular carcinoma, has yet to be clarified, it is known that the fusion of PAX8, a thyroid transcription factor, to the amino terminus of PPARγ results in the loss of the transcriptional activity of PPARγ (73). Moreover, PAX8-PPARγ protein acts to inhibit thiazolidinedione-induced transactivation by PPARγ in a dominant negative manner (73). Taken together, these studies suggest that suppression of PPARγ signaling is closely linked to the development and progression of follicular thyroid carcinoma.

CONCLUDING REMARKS

Studies in the past few decades have clearly established that nuclear receptors play significant roles in the development and progression of several endocrine tumors, such as breast and prostate cancers. Progress in understanding the role of nuclear receptors in thyroid carcinoma lags behind that in breast and prostate cancers. Studies so far indicate that altered expression of ER, PR, RAR, TR, or PPARγ is associated with thyroid carcinomas. More studies are warranted to clarify the functional consequences of altered expressed receptors and to elucidate their signaling pathways in relation to carcinogenesis of the thyroid. These efforts will not only advance our understanding of the molecular genetics of thyroid cancer, but also provide opportunities to develop novel strategies for prevention and treatment.

The discovery that $TR\beta^{PV/PV}$ mice spontaneously develop follicular thyroid carcinoma indicates that, in addition to altered expression of nuclear receptors, mutation of nuclear receptors is another abnormality that could contribute to thyroid carcinogenesis. It is currently unknown whether, in addition to the TRβ gene, mutations of other nuclear receptors could also contribute to the development and progression of thyroid cancer. The finding that the $TR\beta^{PV/PV}$ mouse can be used as an animal model of follicular thyroid carcinoma opens the door to further study of the molecular genetic events underlying carcinogenesis, to identifying signature genes during different stages of tumor progression for clinical diagnosis, and to the testing of drugs and other treatment modalities.

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10. MATRIX METALLOPROTEINASES IN THYROID CANCER

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INTRODUCTION

Cancer is a multistage disorder in which sequential and cumulative genetic aberrations lead to malignant cell transformation (1–2). Approximately 50% of cancer mortality results from invasion and metastasis. Tumor cell invasion and metastasis is a complex multistep process that involves the degradation of extracellular matrix (ECM) proteins by matrix metalloproteinases (MMPs), an important step in the process of cancer invasion and metastasis. Correlation between MMPs overexpression and cancer metastasis have been repeatedly made by numerous studies. Malignant cells rely on these proteinases to disrupt basement membranes, invade surrounding tissues and metastasize to different organs. It is now apparent that not only tumor cells but also non-malignant stromal cells actively participate in the proteolytic degradation of ECM. Tissue inhibitors of metalloproteinases (TIMPs) act as negative regulators of MMPs and it has been shown that they can prevent the spread of cancer in animal models by preserving ECM integrity (3–4).

Matrix metalloproteinases, also called matrixins, constitute a family of zinc–dependent endopeptidases. Twenty-eight members of this family have been identified. Collectively, MMPs play important roles in ECM homeostasis, mediating such normal physiological processes as embryogenesis, organ morphogenesis, reproduction, angiogenesis, and tissue resorption and remodeling (5). The proteolytic activities of MMPs are tightly regulated by endogenous inhibitors, α-macroglobulins, and tissue inhibitors of metalloproteinases (TIMPs) (5). Any disruption of this fine balance can contribute

to the pathogenesis of serious diseases such as arthritis, periodontal disease, and cancer metastasis (6).

THE MMP FAMILY AND STRUCTURE

At present, the human MMP family consists of 23 structurally related members (Table 1). Historically, the MMPs were divided into subgroups of collagenases, gelatinases, stromelysins, membrane-type MMPs, and other novel MMPs, on the basis of their specificity for ECM components. As the list of MMP substrates has grown and several MMPs can degrade a number of different ECM components, a sequential MMP numbering system has been adapted, and the MMPs are now grouped according to their structure. There are eight distinct structural classes of MMPs: five are secreted and three are membrane-type MMPs (Figure 1) (7).

MMPs are produced and secreted by a number of cell types, including fibroblasts, smooth muscle cells, and endothelial cells. They share several highly conserved domains, including an N-terminal propeptide domain that contains a "cystein switch" sequence that enfolds the zinc atom of the catalytic site to maintain the latency of pro-MMPs, a catalytic domain with a zinc binding site and a conserved methionine, and a C-terminal hemopexin-like domain linked to the catalytic domain by a proline rich hinge region. The catalytic domain contains a zinc binding motif HEXXHXXGXXH, in which the three histidine residues represent the three zinc ligands and the glutamic residue the active site. The hemopexin domain contains a single Cys-Cys bond and plays a role in substrate recognition (for example, it is required for collagenases to cleave triple helical interstitial collagens), interaction with TIMPs, and binding of the enzyme to ECM or cell surface (4–5).

The substrates of MMPs are primarily insoluble proteins of ECM, including interstitial and basement membrane collagens, glycoproteins such as laminin, fibronectin, vitronectin, tenascin and elastin as well as proteoglycans. However, more recent data demonstrate that certain MMPs can degrade proteins other than ECM proteins. Many cell membrane bound precursors of growth factors (TGF-α, TGF-β), growth factor receptors (FGF receptor 1, HER2/neu, HER4) and cell adhesion molecules (CD 44, E-cadherin, αv integrin) have been reported to be MMP substrates. For example, MMP-11 can cleavage of insulin-like growth-factor-binding protein (IGF-BP) to release IGFs (8); MMP-12 can proteolytically process plasminogen to generate angiostatin, an inhibitor of angiogenesis (9); MMP-2 and MMP-9 can proteolytically activate TGF-β and promote tumor invasion and angiogenesis (10); and finally, cleavage of the αv integrin subunit precursor by MMP-14 enhances cancer cell migration (11). Although the significance of these observations is not entirely clear, they reflect the complex nature of MMPs in cancer progression.

REGULATION OF MMP ACTIVITY

The activities of MMPs are regulated at three major levels: transcriptional regulation, activation of latent MMP, and inhibition/deactivation by endogenous inhibitors such as α-macroglobulins and TIMPs.

Table 1. The matrix metalloproteinase (MMP) family

MMP subgroup	MMP	Domain class*	Common name (s)
Collagenase			
Collagenase-1	MMP-1	B	fibroblast collagenase, tissue collagenase, interstitial collagenase
Collagenase-2	MMP-8	B	neutrophil collagenase, granulocyte collagenase, PMN collagenase
Collagenase-3	MMP-13	B	todpole collagenase
Collagenase-4	MMP-18	B	found in Xenopus, no human homologue is known
Stromelysins			
Stromelysin-1	MMP-3	B	transin-1, proteoglycanase, procollagenase-activating protein
Stromelysin-2	MMP-10	B	transin-2
Stromelysin-3	MMP-11	D	
Matrilysins			
Matrilysin	MMP-7	A	matrin, PUMP1, small uterine metalloproteinase
Matrilysin-2	MMP-26	A	endometase
Gelatinases			
Gelatinase A	MMP-2	C	72-kDa gelatinase, 72-kDa type IV collagenase, neutrophil gelatinase
Gelatinase	MMP-9	C	92-kDa gelatinase, 92-kDa type IV collagenase
Membrane-type MMPs			
MT1-MMP	MMP-14	F	MT-MMP1
MT2-MMP	MMP-15	F	MT-MMP2
MT3-MMP	MMP-16	F	MT-MMP3
MT4-MMP	MMP-17	G	MT-MMP4
MT5-MMP	MMP-24	F	MT-MMP5
MT6-MMP	MMP-25	G	MT-MMP6, leukolysin
Other MMPs			
Metalloelastase	MMP-12	B	Macrophage elastase, macrophage metalloelastase
RASI-1	MMP-19	B	
Enamelysin	MMP-20	B	
XMMP	MMP-21	E	homologue of Xenopus XMMP
CMMP	MMP-22	B	found in chicken
Femalysin	MMP-23	H	cysteine array MMP
(no trivial name)	MMP-27	B	
Epilysin	MMP-28	D	
McoI-A	No designation	B	found in mouse
McoI-B	No designation	B	found in mouse
75-kDa gelatinase	No designation	C	found in chichen

*see Figure 1

MMP mRNA levels can be induced by a wide variety of chemical agents (e.g. phorbol esters), growth factors (e.g. epidermal growth factor, EGF), hormones (e.g. thyroid hormone, relaxin) cytokines (e.g. interleukin-1, IL-1 and tumor necrosis factor-α, TNF-α), and physical stress. They may also be down-regulated by suppressive factors such as transforming growth factor-β, retinoic acids and glucocorticoids (5,12). The promoter regions of several MMP genes (MMP-1, MMP-3, MMP-7, MMP-9, MMP-10, MMP-12, and MMP-13) contain some common regulatory DNA

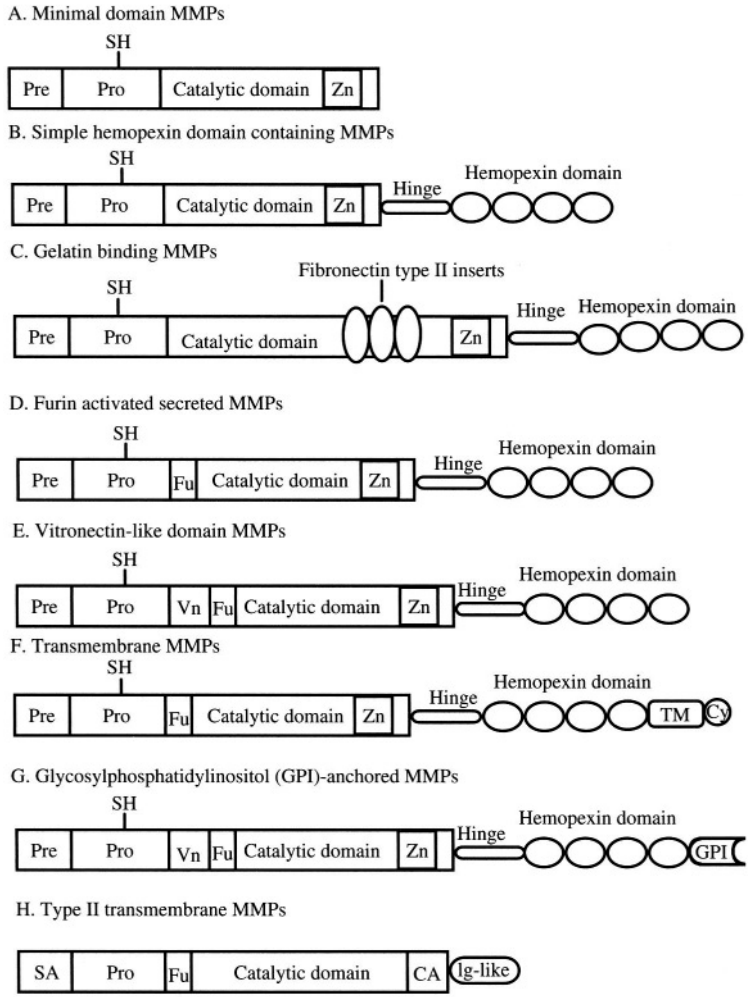

Figure 1. Matrix metalloproteinases groups.
Matrix metalloproteinases (MMPs) can be classified into eight distinct groups by their domain structure, five of which are secreted and three of which are membrane-type MMPs (MT-MMPs). Secreted MMPs: The minimal-domain MMPs contain an N-terminal signal sequence (Pre) that directs them to the endoplasmic reticulum, a propeptide (Pro) with a zinc-interacting thiol (SH) group (from cysteine) that maintains them as inactive zymogens and a catalytic domain with a zinc-binding site (Zn). In addition to the domains that are found in the minimal domain MMPs, the simple hemopexin domain MMPs have a hemopexin-like domain—that is connected to the catalytic domain by a hinge (H), which mediates interactions with tissue inhibitors of metalloproteinases, cell-surface molecules and proteolytic substrates. The first and the last of the four repeats in the hemopexin-like domain are linked by a disulphide bond. The gelatin-binding MMPs contain three inserts that resemble collagen-binding type II repeats of fibronectin (Fi) and is responsible for the specific binding to gelatins and collagens. The furin-activated secreted MMPs contain a recognition motif for intracellular furin-like serine proteinases (Fu) between their propeptide and catalytic domains that allows intracellular activation by these proteinases. This motif is also found in the vitronectin-like insert (Vn) MMPs and the membrane-type MMPs (MT-MMPs). MT-MMPs: MT-MMPs include transmembrane MMPs that have a C-terminal, single-span transmembrane domain (TM) and a very short cytoplasmic domain (Cy), and the glycosylphosphatidylinositol (GPI)-anchored MMPs. MMP-23 represents a third type of membrane-linked MMP. It has an N-terminal signal anchor (SA) that targets it to the cell membrane, and so is a type II transmembrane MMP. MMP-23 is also characterized by its unique cysteine array (CA) and immunoglobulin (Ig)-like domains instead of the hemopexin domain. Adapted from Ref. 7.

sequences. Two important elements for transcriptional regulation are an AP-1 binding site for AP-1 transcription factors which comprise of members of the FOS and JUN family of transcription factors, and a PEA-3 element that binds ETS transcription factors. The AP-1 site, located approximately 70 bp upstream from the transcriptional start site, has been considered to play an important role in the transcriptional activation of the MMP promoters, whereas interaction between AP-1 and PEA-3 site is necessary for basal transcription and trans-activation by cytokines and growth factors. The DNA binding and trans-activation of both AP-1 and ETS transcription factors are regulated by mitogen-activated protein kinases (MAPKs) (12). Interestingly, AP-1 site is not present in the promoter region of MMP-2, a critical metalloproteinase involved in cancer metastasis, and MMP-14, which is involved in the activation of MMP-2 (13). Another transcriptional control of MMP expression is the presence of naturally occurring sequence variation or single nucleotide polymorphisms (SNPs) in the promoters of MMP genes (14). These genetic polymorphisms have been shown to have allele-specific effects on the MMP promoter activities, e.g. an insertion of a guanine at position –1607 in the MMP-1 gene promoter creates the core sequence (5'-GGA-3') of a binding site for the ETS transcription factors. The 2G allele has a higher transcriptional activity in melanoma cells and is associated with more invasive tumors (15).

All MMPs are synthesized as prepro-enzymes. Most MMPs are secreted as inactive, latent pro-MMPs, with the exception of MT-MMPs, which are membrane bound and localize at the cell surface. Since MMP activation occurs after secretion into the extracellular milieu, an important control point for MMP activity is the proteolytic cleavage of pro-MMPs. It has been demonstrated that serine proteases such as trypsin, plasmin, or urokinase initiate activation of MMPs from the zymogen form (16). Some MMPs can also activate other members of the family. A good example is the activation of pro-MMP-2 at the cell surface by MMP-14 and TIMP-2 (17): TIMP-2 binds MMP-14 at its amino terminus and pro-MMP-2 at its carboxyl terminus, which allows an adjacent, non-inhibited MMP-14 to cleave the bound pro-MMP-2. MMP-14 does not fully activate MMP-2 and another already activated MMP-2 is required to remove a residual portion of the MMP-2 propeptide (18). Pro-MMP2 can also be activated by MMP-15 through TIMP-2 independent mechanism (19). Although most MMPs are activated outside the cells by serine proteases or other activated MMPs, MMP-11, MMP-28, and the MT-MMPs can also be activated by intracellular furin-like serine proteases before they reach the cell surface (20).

A final and important control point of MMP activity is the inhibition of activated enzymes by endogenous inhibitors. The main inhibitor of MMPs in tissue fluids is α-macroglobulin, an abundant plasma protein (21). α-Macroglobulin binds to MMPs and the MMP/α-macroglobulin complex then binds to a scavenger receptor and is irreversibly cleared by endocytosis. In a similar way to α-macroglobulin, thrombospondin-2 forms a complex with MMP-2 and facilitates scavenger-receptor-mediated endocytosis and clearance (22). By contrast, thrombospondin-1 binds to pro-MMP-2 and -9 and directly inhibits their activation (23–24). Curiously, thrombospondin-1 has also been reported to increase MMP-2 and -9 activation (25).

Another group of endogenous MMP inhibitors are TIMP family of inhibitors. At present, four structurally related members have been characterized (TIMP-1, TIMP-2, TIMP-3, and TIMP-4) with 40–50% sequence identity at the ammo acid levels (26). TIMPs are small, low molecular weight proteins (20–30 kDa). They differ in tissue-specific expression and ability to inhibit various MMPs. They reversibly inhibit active MMPs with relatively low selectivity by occupying the catalytic domain of activated enzymes (27–28). The TIMP/MMP complex is a tight binding, non-covalent complexes with a stoichiometric 1:1 molar ratio. Unlike TIMP-1, TIMP-2, and TIMP-4, which are secreted in soluble form, TIMP-3 has a unique association with ECM. Studies with Timp-2-deficient mice indicate that the dominant physiological function of TIMP-2 is activation of MMP-2 (29). Apart from inhibiting MMPs, TIMP-3 has been shown to promote apoptosis whereas TIMP-1 is active in blocking apoptosis and overexpression of TIMP-2 protect cancer cells from apoptosis (30–32).

MMPs AND TIMPs IN THYROID CANCER PROGRESSION AND METASTASIS

The expression and activity of MMPs are increased in many types of human cancer, and this correlates with advanced tumor stage, increased invasion and metastasis, and shortened survival. Many studies show a negative association between MMPs activity and prognosis (7). MMP-2 and MMP-9 are of particular importance in tumor cell invasion, because they degrade type IV collagen, the main structural component of the basement membrane. Tumor cells expressing high levels of these enzymes are highly metastatic. Cancer cells are not the only source of MMPs. Stromal cells are also participated in the production of MMPs (20). MMPs that are secreted by stomal cells can still be recruited to the cancer cell membrane, e.g. MMP-2 mRNA is expressed by stromal cells of human breast cancers, whereas MMP-2 protein is found on both stromal and cencer cell membranes (33). It has been shown that cancer cells can stimulate tumor stromal cells to produce MMPs in a paracrine fashion through secretion of cytokines, growth factors, and EMMPRIN (extracellular matrix metalloproteinase inducer). EMMPRIN is an intrinsic plasma membrane glycoprotein produced in high amounts by cancer cells, which stimulates local fibroblasts to synthesize MMPs (34). Tumor cell interactions with fibroblasts via EMMPRIN leads to fibroblast-induced local degradation of basement membrane and ECM components, thus facilitating tumor cell invasion. It has been shown that MMP-9 production in tumor infiltrating macrophages play a critical role in angiogenesis and progressive growth of human ovarian tumors in mice (35). Stromal cells and their products have been reported to even cause tumorigenic transformation of adjacent epithelial cells (36).

Earlier studies have shown that invasion by cultured human follicular thyroid carcinoma is correlated with increased production of beta 1 integrins and MMPs (37). Correlation between MMPs and ECM degradation is further demonstrated by the study of plasmin activation system in metastatic follicular thyroid carcinoma cell lines (38). As mentioned earlier, plasmin is a serine protease involved in the activation of MMPs. Plasmin is generated from plasminogen by urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA). UPA-mediated plasminogen activation is an important pathway in tumor invasion and can be inactivated by

plasminogen activator inhibitors (PAI-1 and –2) (39). Decreased activity of PAI-1 is associated with greater ECM degradation in follicular thyroid carcinoma cell lines (38).

Overexpression of MMP-2, and MMP-9 has been found in thyroid carcinomas and is correlated with large tumor size, high intrathyroid invasion, presence of lymph node metastasis, and advanced disease stage (40). A more comprehensive study of MMPs profile involving seven secreted MMPs (MMP-1, -2, -3, -7, -8, -9, and -13) and three membrane-bound MMPs (MMP-14, -15, and-16) demonstrates that the major MMPs produced in papillary thyroid carcinomas are MMP-2 and MMP-14 (41). The pro-MMP-2 activation and the expression of MMP-14, known to activate pro-MMP-2 at the cell surface, are considerably higher in carcinomas with lymph node metastasis than those without metastasis. MMP-15 expression is confined to 26% of cases. MMP-2, MMP-14, and MMP-15 are immunostained in both carcinoma and stromal cells (41). In a separate study, increased MMP-2 expression is found in follicular and anaplastic thyroid carcinomas, but not in follicular adenomas (42). Interestingly, MMP-2 mRNA expression is restricted to fibroblasts in the stroma adjacent or close to invading tumor cells (42). MMP-1 expression is significantly greater among follicular and papillary thyroid carcinomas compared to benign lesions. However, there is no relationship between MMP-1 expression and invasion, metastasis, or disease recurrence (43). Both carcinoma and stromal cells have been shown to express MMP-1 (43–44). A recent cDNA and tissue microarray study shows that MMP-11 is up-regulated in 67% of papillary thyroid carcinoma tissues (45).

Both TIMP-1 and TIMP-2 expression are increased in thyroid carcinomas, and are correlated with large tumor size and advanced disease stage (40,46), which seem to be contradictory to the role of TIMPs as inhibitors of tumor cell invasion and metastasis. Further study shows stronger TIMP-1 immunostaining in the stromal cells surrounding the tumor, suggesting that the high levels of TIMP-1 transcripts in advanced stage of thyroid carcinoma are likely represent a stroma response to tumor cell invasion. Overexpression of TIMP-1 by gene transfer has resulted in a significant suppression of invasive potential of NPA cells, a poorly differentiated thyroid carcinoma cell line (46). Reduced TIMP-1 expression has been shown in recurrent papillary thyroid carcinoma when compared to non-recurrent carcinomas (43). Apparently, tumor invasion is not dependent on the absolute levels of TIMPs or MMPs. It is the balance between TIMPs and MMPs that determines the potential of thyroid tumor invasion and metastasis. Indeed, the molar ratio of total amounts of MMPs:TIMPs is significantly higher in the thyroid carcinoma samples than in the adenoma and normal samples (41).

Many MMP genes are transcribed at low or undetectable levels in normal thyrocytes. Analysis of MMPs and TIMPs expression *in vitro* demonstrates that MMP-1, -2, -9, -14, and TIMP-1, -2, -3 mRNA are present in normal thyrocytes, malignant thyroid cells and thyroid-derived fibroblasts. The basal levels of MMP-1, -9, and -14 are much lower in thyrocytes than in malignant thyroid cells and thyroid-derived fibroblasts, whereas high basal levels of MMP-2, TIMP-1, -2, and -3 are found in all three cell types without striking difference (47–48). IL-1 can upregulate MMP-1and MMP-9 mRNA in all the cell types through activating nuclear factor of κB (NF-κB), and has no significant effect on TIMPs, MMP-2, and MMP-14. TNF-α, also acting via

NF-κB passway, can stimulate MMP-9 mRNA expression in malignant thyroid cells and thyroid-derived fibroblasts. EGF, acting via protein tyrosine kinase, can only stimulate MMP-1 expression in malignant cells (49). Phorbol—myristate acetate (PMA, an active phorbol ester) can induce MMP-1, MMP-9 and TIMP-1 mRNA in all the cell types, MMP-14 in malignant thyroid cells and thyroid-derived fibroblasts (47–49). Since PMA, acting via protein kinase C (PKC), can induce *c-jun* and *c-fos* gene expression in human thyroid cells, and their gene products are AP1 transcriptional factors (50), it is likely that PKC is involved in the induction of MMP transcription. Although thyroid-stimulating hormone (TSH) has no significant effect on the basal MMP-1, or TIMP-1 mRNA levels, it can cause a dose-dependent inhibition in PMA or EGF-induced MMP-1 mRNA in malignant cells, and PMA-induced MMP-1 and TIMP-1 mRNA in benign thyroid cells. The repressive action of TSH on MMP-1 mRNA can be mimicked by the forkolin and 8-bromo-cAMP, and can be abrogated by a protein kinase A (PKA) inhibitor, H-89, suggesting that it is PKA-mediated (49). MMP-11, -13, and -18 genes are thyroid hormone responsive genes. Although they have not been shown to be involved in thyroid cancer, they have distinct functions during frog embrogenesis (51).

Several studies have shown that high serum levels of MMP-2, MMP-9, and TIMP-1 are associated with tumor invasion and poor survival in several types of cancer (52–54). Thus, they may be used as prognostic markers in cancer patients. Higher levels of MMP-2 and TIMP-2 are detected by ELISA in peripheral blood of thyroid cancer patients when compared to normal control, and increased blood levels of MMP-3 and MMP-9 appear to be associated with medullary thyroid cancer (55). It remains to be determined whether serum levels of MMPs and TIMPs can be used as diagnostic or prognostic markers for thyroid carcinoma.

MMP INHIBITION IN ANTICANCER THERAPY

Given that MMPs play important role in tumor invasion and metastasis, inhibition of MMPs activity has been the focus of much anticancer research and clinical trials. Pharmaceutical industries have invested considerable effort over the past decade to develop safe and effective MMP inhibitors for use in cancer patient. Three classes of synthetic MMP inhibitors have been developed (Table 2): the collagen peptidomimetics which mimic the collagen amino-acid sequence near the collagenase cleavage site; the collagen non-peptidomimetics which are synthesized based upon the conformation of MMP active site; and the tetracycline derivatives which inhibit the activity of MMPs without antibiotic activity (13, 56–57). Numerous preclinical studies using these MMP inhibitors in cancer models have demonstrated their effectiveness to delay primary tumor growth and inhibit experimental metastasis. Initiation of treatment when tumor burden is minimal has a more profound effect on tumor growth inhibition than at the time of large tumor bulk. Despite of positive preclinical results in the use of MMP inhibitors, most clinical trials have not yielded significant beneficial effects in patients with advanced cancer (57). In the case of BAY12-9566, alarming reports show significantly poorer survival for groups treated with the drug than for placebo-treated group.

Table 2. The matrix metalloproteinase inhibitors for cancer therapy

Inhibitor	Structure	Specificity
Marimastat (BB-2516)	Peptido mimetic	Broad spectrum (2nd generation of BB-94)
Batimastat (BB-94)	Peptido mimetic	Broad spectrum (e.g. MMP-1, 2, 3, 7, 9, 12)
Tanomastat (Bay 12-9566)	Non-peptido mimetic	Broad spectrum (e.g. MMP-2, 9, 11, 13, 14)
Prinomastat (AG3340)	Non-peptido mimetic	Broad spectrum
BMS-275291	Non-peptido mimetic	Broad spectrum
MMI 270 (CGS27023A)	Non-peptido mimetic	Broad spetrum
Metastat (COL-3)	Tetracycline derivative	Gelatinases (MMP-2, 9)

In view of the disappointing results of synthetic MMP inhibitors in clinical trials, we and other investigators have recently explored the potential applications of TIMP gene overexpression for cancer gene therapy (58–60). Antitumor effects have been shown following systemic or local delivery of TIMP-1, TIMP-2, and TIMP-3 genes in animal models (60–63). However, stimulation of mammary tumorigenesis has been reported following systemic TIMP-4 gene delivery. TIMP-4 has been shown to up-regulate Bcl-2 and Bcl-X(L) protein and inhibit apoptosis in human breast cancer cells (64). Given the multifunctional nature of TIMP proteins, further preclinical studies will be needed before initiation of clinical gene therapy trial in patients with cancer.

CONCLUSIONS

As compared with tumors from other organs such as lung, colon, and breast, a limited number of studies have been carried out so far on the involvement of MMPs and TIMPs in thyroid tumorigenesis. Based upon the available data, it is clear that MMPs, especially MMP-2 and MMP-9, and TIMP-1 are involved in thyroid tumor invasion and metastasis. Although TIMP-1 can reduce the invasive potential of thyroid cancer cells *in vitro,* therapeutic intervention *in vivo* has not been attempted yet in animal models to inhibit thyroid tumor growth, invasion, and metastasis, using either synthetic MMP inhibitors or TIMPs gene therapy. Clearly, more studies are needed to fully appreciate the important roles of MMPs and TIMPs in thyroid cancer.

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11. THE MOLECULAR PATHWAYS INDUCED BY RADIATION AND LEADING TO THYROID CARCINOGENESIS

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INTRODUCTION

The association between ionizing radiation and thyroid cancer is well established. It was first proposed in 1950 in children who received X-ray therapy in infancy for an enlarged thymus (1). During the following decades, numerous reports have documented an increased incidence of thyroid neoplasms in children after external radiation for different benign conditions of the head, neck and thorax (2). Since the early 1960s, when the use of radiotherapy for benign conditions was abandoned, the incidence of radiation-associated thyroid malignancy in children gradually decreased (3). Currently, radiation therapy for malignancy continues to be a source of radiation-associated thyroid cancer (4). An increased risk of thyroid cancer has also been linked to environmental irradiation. This was documented in survivors of atomic bomb explosions in Japan in 1945(5), and in residents of the Marshall Islands exposed to fallout after detonation of a thermonuclear device on the Bikini atoll in 1954 (6). In the U.S., exposure to radioiodines from atmospheric nuclear tests in Nevada in the 1950s has been suggested to lead to an excess of thyroid neoplasms (7, 8). In April 1986, an accident at the Chernobyl Nuclear Power Station in the former USSR produced the most serious environmental disasters ever recorded and led to a dramatic increase in the frequency of childhood thyroid cancer in contaminated areas of Belarus, Ukraine, and western Russia (9, 10). This tragic disaster has created one of the most striking paradigms of radiation-induced thyroid tumors and allowed significant progress in the understanding of the molecular pathways induced by radiation. In this chapter, I

review the genetic events and molecular mechanisms underlying radiation carcinogenesis in the thyroid gland.

RET/PTC REARRANGEMENTS

Over the last decade, rearrangements of the RET proto-oncogene have been identified as the most common genetic event in thyroid tumors associated with radiation exposure.

The RET proto-oncogene is located on chromosome 10q11.2 and encodes a cell membrane receptor tyrosine kinase (11, 12). The receptor consists of three functional domains: an extracellular domain containing a ligand-binding site, a transmembrane domain, and an intracellular domain that includes a region with protein tyrosine kinase activity. The ligands for RET receptor are neurotrophic factors of the glial cell-line derived neurotrophic factor (GDNF) family, including GDNF, neurtulin, artemin, and persephin (13). Binding of a ligand causes the receptors to dimerize, leading to autophosphorylation of the protein on tyrosine residues and initiation of intracellular signaling cascade. Wild-type RET is expressed in neuronal and neural-crest derived tissues including thyroid parafollicular C-cells, but not in thyroid follicular cells. In thyroid follicular cells, RET can be activated by fusion to different constitutively expressed genes. The product of this rearrangement is a chimeric oncogene named RET/PTC (PTC for papillary thyroid carcinoma).

Structure of RET/PTC oncogenes

Since the original report on RET activation by rearrangement in papillary thyroid carcinomas (14), three major types of the rearrangement have been identified: RET/PTC1, RET/PTC2, and RET/PTC3 (Figure 1). All of them are formed by

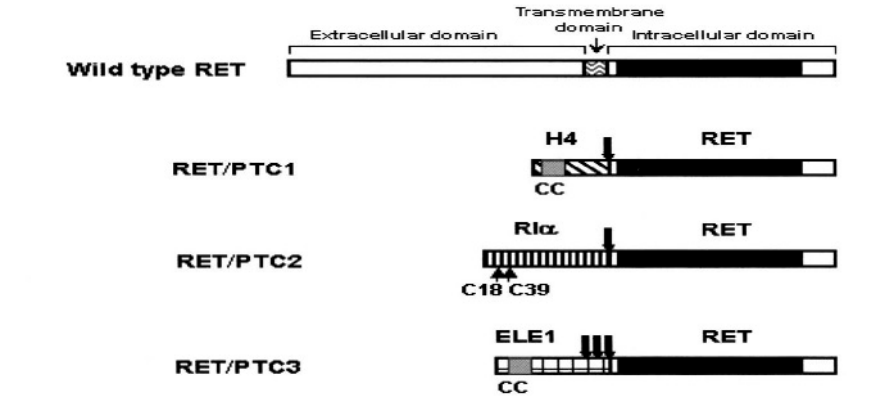

Figure 1. Schematic representation of the wild type RET gene and three major types of RET/PTC rearrangement. The 3′ portion of RET participating in the fusion encodes the tyrosine kinase domain (black box) but lacks the transmembrane and extracellular domains. The genes fused with RET encode dimerization domains, either coiled-coil domain (CC) or cysteine residues forming disulfide bonds during dimerization (C18, C39), allowing ligand-independent dimerization and activation of the truncated RET receptor. Block arrows indicate breakpoints.

fusion of the tyrosine kinase domain of RET to the 5′ portion of different genes. In RET/PTC1, RET is fused to the H4 (also known as D10S170) gene (14) and in RET/PTC3 to the ELE1 (RFG or ARA70) gene (15, 16). RET/PTC1 and RET/PTC3 are paracentric inversions since both genes participating in the rearrangement are located on chromosome 10q (17, 18). In contrast, RET/PTC2 is formed by reciprocal translocation between chromosomes 10 and 17, resulting in RET fusion to the 5′ terminal sequence of the regulatory subunit RIα of the cyclic AMP-dependent protein kinase A (Figure 1).

Recently, several novel types of RET/PTC have been described, most of them in papillary carcinomas from patients with the history of radiation exposure. Five novel types (RET/PTC5, RET/PTC6, RET/PTC7, RET/KTN1, RET/RFG8) were found in post-Chernobyl tumors (20–23), and two other in tumors from patients subjected to therapeutic external radiation (RET/PCM-1, RET/ ΔRFP) (24, 25). All novel types of RET/PTC are translocations and resulted from the fusion of the intracellular domain of RET to heterologous genes located on different chromosomes (Figure 2).

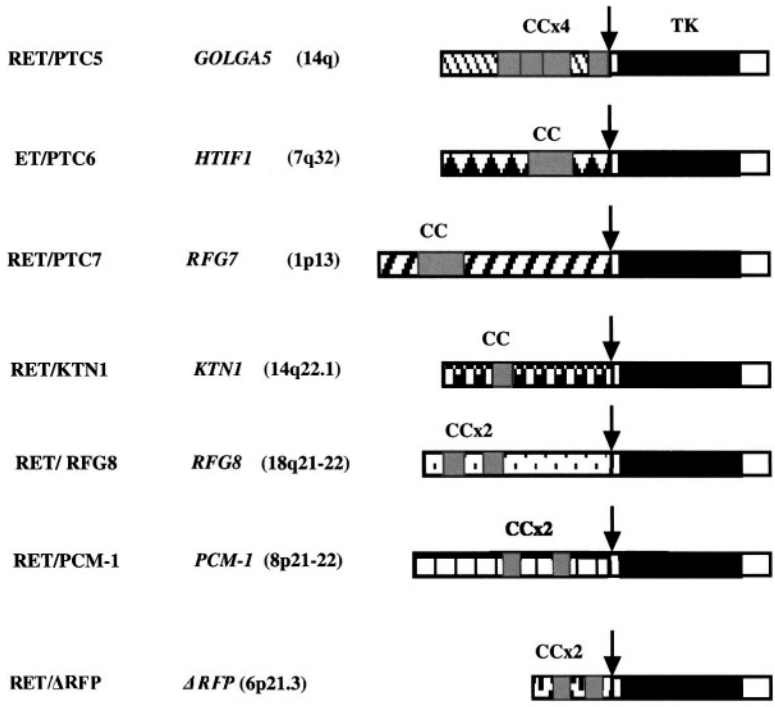

Figure 2. Schematic representation of the novel RET/PTC types found in papillary carcinomas associated with radiation exposure. Each fusion involves the tyrosine kinase (TK) domain of RET and the 5′ portion of different genes that encode one or more putative coiled-coil domains (CC) essential for dimerization and RET TK activation. Chromosomal location of the RET fusion partners is shown in brackets. Arrows indicate breakpoints.

The genes fused with RET are constitutively expressed in thyroid follicular cells and drive the expression of the chimeric RET/PTC oncogene. In addition, these partners provide a dimerization domain essential for ligand-independent activation of the RET tyrosine kinase (26, 27). It allows ligand-independent dimerization of the chimeric protein and autophosphorylation of the truncated RET receptor. Indeed, it has been demonstrated that tyrosines 1015 and 1062, which are autophosphorylated in the wild-type RET only upon ligand binding, are constitutively phosphorylated in the RET/PTC chimeric protein (28). The ligand-independent activation of the RET tyrosine kinase is considered essential for the transformation of thyroid cells (29).

Another important role of the genes fused with RET is in determining a subcellular localization of the chimeric protein which lacks the transmembrane domain and cannot be anchored to the cell membrane. In RET/PTC3 protein, for instance, the N-terminal coiled-coil domain of ELE1 (RFG) not only mediates oligomerization of the receptor and chronic kinase activation, but is also responsible for the compartmentalization of the chimeric protein at plasma membrane level, where most of the normal ELE1 (RFG) protein is distributed (30). Thus, different types of RET/PTC chimeric proteins, which have a similar RET tyrosine kinase portion but different N-terminal domains, may be distributed in varies cytoplasmic compartments, allowing them to interact with distinct sets of signaling proteins. This may provide an explanation for some variations in biological properties recently found between different RET/PTC types (reviewed in (31).

RET/PTC prevalence in sporadic and radiation-associated tumors

The prevalence of RET/PTC in papillary carcinomas varies significantly in different studies and geographic regions. In the U.S., the five largest series of adult papillary carcinomas showed the frequency ranging from 11% to 43% (32–36), with the cumulative incidence of 35%. A comparable rate of 30–40% have been found in series from Canada (37)) and Italy (34, 38, 39). In other regions, a wide variation in frequency of RET/PTC has been reported, ranging from 3% in Saudi Arabia (40) to 85% in Australia (41). A higher overall prevalence of RET/PTC has been noted in papillary carcinomas from children and young adults (38, 42–44). Among sporadic tumors of all age groups, RET/PTC1 is the most common type, comprising up to 60–70% of all rearrangements, whereas RET/PTC3 accounts for 20–30% of positive cases and RET/PTC2 for less than 10% (32, 36, 39).

An unusually high incidence of RET/PTC rearrangements has been documented in papillary carcinomas from patients with the history of radiation exposure, including those subjected to either accidental or therapeutic irradiation (Table 1).

In children affected by the Chernobyl nuclear accident, 67–87% of papillary carcinomas removed 5–8 years after exposure and 49–65% of tumors removed 7–11 years after the accident harbored RET/PTC (42, 45–48). Remarkably, in tumors developed less than 10 years after the accident, RET/PTC3 was the most common type, whereas those developed after the longer latency had predominantly RET/PTC 1 (Table 2). In patients subjected to therapeutic X-ray irradiation for benign or malignant conditions, the prevalence of RET/PTC was also significantly higher than in the general

Table 1. Prevalence of RET/PTC rearrangements in radiation-induced thyroid cancer

	Total RET/PTC	RET/PTC1	RET/PTC2	RET/PTC3
Environmental Radiation (post Chernobyl)				
Klugbauer et al. (46)	8/12 (67%)	17%	0	50%
Fugazzola et al. (45)	4/6 (67%)	0	17%	50%
Nikiforov et al. (42)	33/39 (87%)	16%	3%	58%
Smida et al. (47)	32/51 (63%)	24%	0	26%
Rabes et al. (48)	94/191 (49%)	25%	0	20%
Therapeutic Radiation				
Bounacer et al. (49)	16/19 (84%)	74%	0	21%

Table 2. RET/PTC prevalence after chernobyl: correlation with the latency period*

Latency period	Total rearrangement positive	RET/PTC1	RET/PTC3	Novel RET/PTC types
≤10years	40/61 (66%)	23%	60%	13%
>10 years	60/130 (46%)	65%	23%	5%

* Modified from Rabes et al. (2000) (48) with permission.

population, being reported at 52–87% (49–51). Exposure to ionizing radiation not only results in a higher prevalence in RET/PTC, but also promotes the fusion of RET to the unusual rearrangement partners, since seven out of eight novel RET/PTC types have been detected in tumors associated with radiation exposure. The novel types comprised up to 13% of all rearrangements found in post-Chernobyl tumors (48) (Table 2).

The fact that post-Chernobyl tumors arising shortly after exposure had mostly RET/PTC3 and those after a longer latent period harbored predominantly RET/PTC1 suggests at least two possibilities. First, radiation may be more effective in inducing RET/PTC3, whereas other factors (still unknown), responsible for the majority of thyroid cancers that are not associated with radiation exposure, lead mostly to RET/PTC1. Second, it is conceivable that radiation is equally efficient in generation of both types, but tumors initiated by RET/PTC3 have a higher growth rate and manifest several years earlier. The latter possibility is supported by the animal experiments demonstrating that RET/PTC3 transgenic mice develop more malignant phenotype and metastatic disease as compared to RET/PTC1 animals (52, 53), and by recent *in-vitro* functional studies showing that RET/PTC3 is a more potent activator of MAPK signaling pathway and more efficient in promoting proliferation of cultured thyroid cells than RET/PTC1 (54).

The occurrence of RET/PTC has been observed after high-dose irradiation of human undifferentiated thyroid carcinoma cells (55) and of fetal human thyroid tissues transplanted into SCID mice (56, 57). In both studies, the rearrangements were

detected by RT-PCR as soon as 2 days after exposure. In addition, fetal thyroid cells revealed both RET/PTC 1 and RET/PTC3 7 days after irradiation, while only RET/PTC 1 persisted and was detectable 2 months later (57). The effective dose of radiation in both studies was high (50 Gy) and lethal for dividing cells, and the cells irradiated in the study by Ito and colleagues (55) were already highly transformed and hence more susceptible to develop secondary genetic defects. Nevertheless, these observations suggest that radiation exposure may lead to the generation of RET/PTC rearrangements in thyroid cells.

Potential mechanisms of RET/PTC generation after radiation exposure

The high prevalence of RET/PTC in post-Chernobyl children and in populations affected by other types of environmental and therapeutic exposures as well as the *in-vitro* studies provide strong evidence for the association between ionizing radiation and RET/PTC rearrangement. The role of radiation appears particularly important for the RET/PTC3 type, since its high prevalence was uniquely associated with papillary carcinomas developed shortly after the Chernobyl accident. The mechanisms of RET/PTC generation in thyroid cells after radiation have been a subject of extensive study over the last years.

Analysis of the breakpoint sites revealed no long-sequence homology between the DNA regions involved in RET/PTC1 fusion in sporadic tumors (58) and in RET/PTC3 in post-Chernobyl carcinomas (59, 60), establishing that these rearrangements result from illegitimate rather than homologous recombination. When the breakpoint sites within the RET and ELE1 genes were mapped and analyzed in 12 post-Chernobyl tumors with RET/PTC3, it appeared that in each tumor the relative position of a breakpoint in the RET gene corresponded to the location of a breakpoint in the ELE1 gene (60). Specifically, after aligning the genes in opposite orientations, the breakpoints were located just across from each other in 5 (42%) tumors (Figure 3, A), while in other tumors they could be aligned by sliding one gene with respect to another (Figure 3, B, C). Similar pattern could be deducted from another study where the breakpoints in 22 post-Chernobyl tumors with RET/PTC3 were characterized (61). Such predilection for the breakpoint site in one gene to correspond to the breakpoint site located within the certain region of the other gene suggests the presence of a stable spatial relationship between these two chromosomal loci within the nucleus (Figure 3).

If the interaction between these loci exists, it should involve folding of chromosome 10 where both RET and ELE1 genes reside separated by a linear distance of ~18 Mb. This is conceivable since one of the levels of DNA packaging involves chromatin arrangement into loops of different size attached to a chromosomal backbone (62). Therefore, although two chromosomal loci are located at a considerable linear distance from each other, they may be closely spaced in the interphase nucleus because of their location at specific areas of chromosomal loop(s).

Indeed, it has been recently demonstrated that chromosomal regions containing the RET and H4 genes are non-randomly positioned with respect to each other in the interphase nuclei of normal thyroid follicular cells (63). Utilizing fluorescence in situ hybridization, two-dimensional distances between RET and H4 were measured in

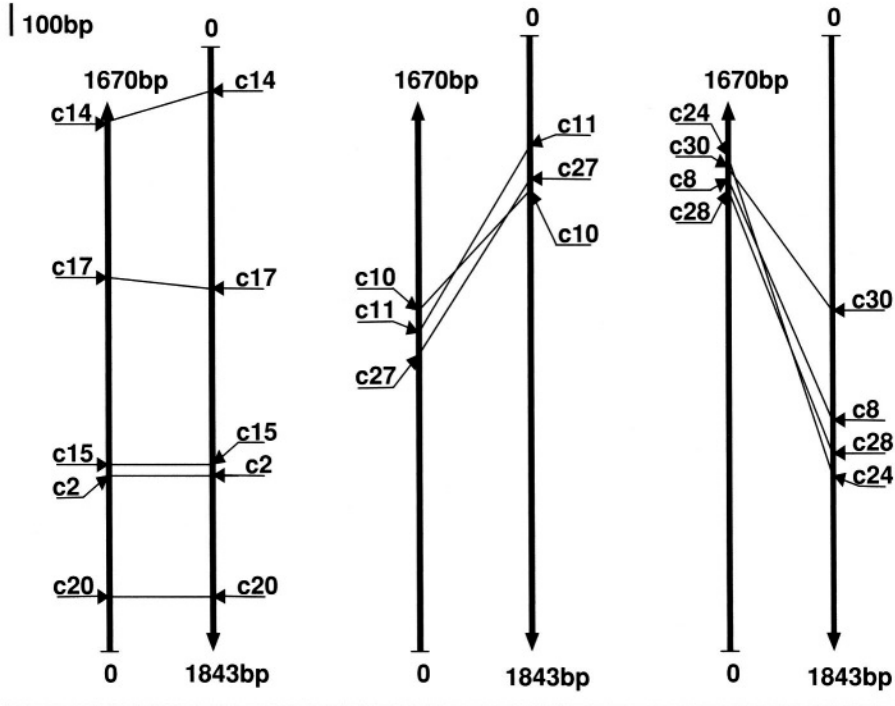

Figure 3. Alignment of the breakpoint sites involved in *RET*/PTC3 in post-Chernobyl tumors demonstrating three patterns of correspondence between the position of breakpoints in each tumor (A, B, C). Modified from Nikiforov et al. (1999) (60) with permission.

the interphase cells and compared with a theoretical Rayleigh model that describes a distribution of distances between two points of linear polymers that fold in a random manner. Previous studies have shown that interphase distances between random loci on the same chromosome conform to the Rayleigh distribution (62, 64). Indeed, in the control experiment, distances between the RET and D10S539 loci, the latter located on chromosome 10q between RET and H4 and is not known to participate in the rearrangement, were found to follow the Rayleigh distribution (Figure 4) (63). As for the RET and H4 distances, they showed a strong deviation from the Rayleigh model, primarily due to the loci either juxtaposed or closer than expected, indicating a non-random manner of RET and H4 interaction. In addition, as many as 35% of primary cultured thyroid cells had at least one pair of RET and H4 genes juxtaposed. These data suggest that generation of RET/PTC rearrangements in thyroid cells may be in part due to the structural organization of chromosome 10, resulting in non-random interaction and spatial approximation of these potentially recombinogenic DNA loci (Figure 4).

It remains unclear, however, whether RET/PTC is a direct consequence of DNA breaks induced by ionizing radiation, or it forms indirectly, after DNA damage has

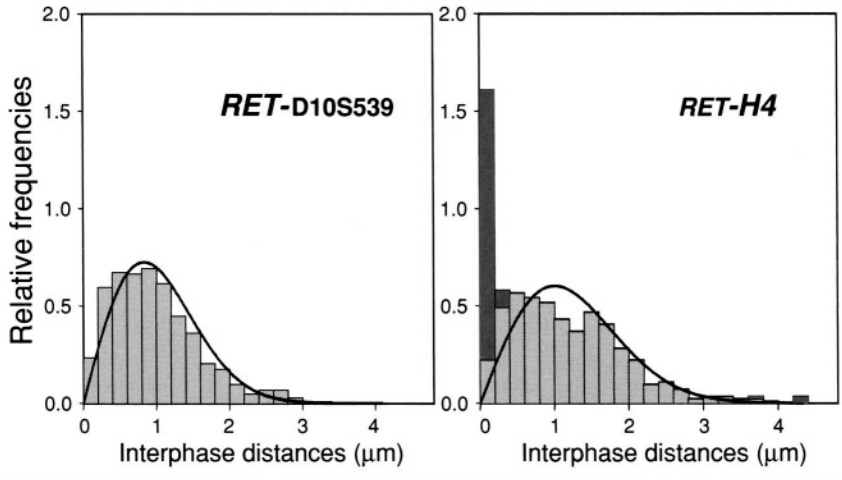

Figure 4. Distribution of interphase distances between RET and D10S539 (A) and RET and H4 (B) in cultured normal thyroid cells as compared with the theoretical Rayleigh distribution (solid line). Dark bars indicate the RET – H4 distances that were in excess over the number expected based on the Rayleigh distribution. From Nikiforova et al. (2000) (63).

been repaired. Important information in this respect can be obtained by mapping and characterization the breakpoint sites involved in the fusion. Thus, if the rearrangement occurred indirectly (as a result of activation or disruption of the recombination machinery), the breakpoints are expected be located within recombinase signal sequences at both participating loci, have similarity in nucleotide composition, or cluster at certain specific hypersensitive DNA regions. However, as it had been convincingly demonstrated in post-Chernobyl tumors with RET/PTC3, the breakpoints within and surrounding ELE1 intron 5 and RET intron 11 were distributed randomly, with no breakpoints occurring at exactly the same base or within an identical sequence in any of 41 tumors reported in three different series (59–61). The breakpoints exhibited no particular nucleotide sequence or composition. In one study, no evidence of AT-rich regions, fragile sites, recombination-specific signal elements, or other target DNA sites (i.e. chi-like motifs, heptamer/nonamer signal sequences) implicated in illegitimate recombination in mammalian cells was found (60). These results favor the direct induction of RET/PTC as a result of random double-strand DNA breaks, rather than disruption of the recombination machinery. However, another study proposed an alternative mechanisms and found at least one topoisomerase I site exactly at or in close proximity to all breakpoints, suggesting the role of DNA breaks induced by this enzyme in the generation of RET/PTC3 (61).

PAX8-PPARfl REARRANGEMENT

Recently, a PAX8-PPARγ fusion has been identified in follicular thyroid carcinomas with cytogenetically detectable translocation t(2;3)(q13;p25) (65). This rearrangement

leads to an in-frame fusion of the PAX8 gene, which encodes a paired domain transcription factor, with the peroxisome proliferator-activated receptor (PPAR) γ gene. The structure and biological properties of the fusion gene are discussed in Chapter 4. In the original report, PAX8-PPARγ was detected in 5 out of 8 (63%) follicular carcinomas (65), whereas in the larger follow-up series, the prevalence was 26–35% (66, 67). However, when this alteration was studied separately in follicular carcinomas from patients without a history of radiation and in those exposed to radiation, PAX8-PPARγ fusion was found in 5 out of 12 (42%) sporadic tumors and in all three (100%) tumors associated with radiation (68). Despite the small number of follicular carcinomas in the latter group, this finding points towards the possible association between radiation exposure and PAX8-PPARγ rearrangement. Although papillary carcinoma is by far the most common type of thyroid tumors associated with radiation, an increased risk of follicular carcinoma development has also been documented in these populations (69). Therefore, it is likely that radiation exposure may promote the development of follicular tumors through the generation of PAX8-PPARγ rearrangement in a similar way it involves in the initiation of papillary thyroid carcinogenesis via RET/PTC rearrangement.

RAS MUTATIONS

Point mutations of the three RAS genes, N-RAS, H-RAS, and K-RAS, have been found with various prevalence in thyroid follicular and papillary tumors. The initial reports have suggested that K-RAS mutations in follicular carcinomas may be associated with radiation exposure, since they constituted 3 out of 4 (75%) RAS mutations in patients subjected to radiation, in contrast to 6 out of 25 (25%) RAS mutations in sporadic tumors (70). However, the subsequent studies have not confirmed such an association (Table 3). Indeed, RAS mutations were found in 30% of thyroid tumors from patients with a history of external irradiation and in 42% of sporadic tumors, and similar prevalence of the K-RAS gene mutations was observed in the two groups (71). In a series of post-Chernobyl tumors, no RAS mutations was detected in 33 papillary carcinomas; whereas 3 follicular adenomas and 1 follicular carcinoma harbored N-RAS codon 61 mutation (72). Similar findings were observed in another series of 31 post-Chernobyl papillary carcinomas (73). Two RAS mutations detected in 44

Table 3. Prevalence of RAS mutations in radiation-induced thyroid tumors

Therapeutic Radiation	
Wright et al. (70)	4/12 (33%) after radiation, 3/4 K-RAS (75%) in follicular carcinomas
	25/68 (37%) in non-radiation group, 6/25 (25%) K-RAS
Challeton et al. (71)	10/33 (30%) after radiation
	36/86 (42%) sporadic, similar prevalence of K-RAS mutations
Environmental Radiation (Post-Chernobyl)	
Nikiforov et al. (72)	0/33 in papillary carcinomas
	3/7 in follicular adenomas
	1/1 in follicular carcinoma
Suchy et al. (74)	2/44 in papillary carcinomas (none in codons 12, 13, or 61)
Santoro et al. (73)	0/23 in papillary carcinomas

Table 4. Prevalence of p53 mutations in radiation-induced papillary thyroid cancer

Therapeutic radiation	
Fogelfeld et al. (75)	4/22 (18%) after radiation 0/18 in control group
Environmental Radiation (post-Chernobyl)	
Hillebrandt et al. (76)	6/26 (23%)
Nikiforov et al. (72)	2/33 (6%)
Smida et al. (77)	5/31 (16%)
Santoro et al. (73)	0/35
Total post-Chernobyl	13/125 (10%)

post-Chernobyl papillary carcinomas in another study were not in critical codons 12, 13, or 61 of the gene (74). These data indicate that activating mutations of the RAS genes do not play a significant role in radiation-induced papillary carcinomas. Similar conclusion can be made for thyroid follicular tumors, the prevalence of RAS mutation in which shows no correlation with the history of radiation.

P53 MUTATIONS

Missense point mutations in exons 5–8 of the p53 tumor suppressor gene have been detected in 4 out of 22 (18%) papillary carcinomas developed in the patients with a history of childhood irradiation to the head and neck area (75). Several studies have reported the prevalence of p53 mutations in post-Chernobyl tumors. In one series, PCR-SSCP analysis revealed two (6%) somatic mutations, both in exon 5, in a series of 33 papillary carcinomas (72). One of those mutations was a missense mutation and another was a silent mutation, as detected by nucleotide sequencing. Other studies reported a 0–23% prevalence of mutations in the critical exons of the p53 gene in pediatric post-Chernobyl papillary carcinomas (Table 4) (73, 76, 77). Despite some variation in the results between these observations, the overall prevalence of p53 mutations in this post-Chernobyl population appears approximately 10%. This indicates that inactivation of the p53 tumor suppressor gene has only a limited role in radiation-induced thyroid carcinogenesis.

OTHER GENETIC EVENTS

A number of other genetic events have been explored as the possible mediators of radiation-induced carcinogenesis in the thyroid gland. Mutations of the GSP and TSH receptor genes were found to play either no role or rarely present in tumors associated with external radiation (71) and in post-Chernobyl tumors (73, 78). It has been recently suggested that mutations in mitochondrial DNA, particularly large-scale deletions, were significantly more frequent in radiation-induced post-Chernobyl tumors in comparison to sporadic neoplasms, and the frequency of these alterations correlated with the levels of radioiodine contamination in the areas of patients' residency (79). Recently, point mutation at nucleotide 1796 of the BRAF gene has been

identified as the most common genetic alteration in papillary carcinomas from the general population (80). However, our preliminary data suggest that this mutation is rare in post-Chernobyl papillary carcinomas (Nikiforova et al., manuscript in preparation).

Several types of genomic instability have also been studied in radiation-associated thyroid tumors. These studies were important in the light of the reports of a higher rate of germline mutations at minisatellite loci in children born from parents exposed to radiation after Chernobyl (81, 82). However, post-Chernobyl thyroid carcinomas revealed either no significant minisatellite or microsatellite instability (83), or low rate of microsatellite mutations (84).

CONCLUDING REMARKS

A large volume of information on the genetic alterations in thyroid tumors, generated over the last decade, points towards a significant difference in the molecular pathways involved in the sporadic and radiation-induced carcinogenesis in the thyroid gland. Thus, it appears that the molecular landscape of sporadic papillary and follicular carcinomas is dominated by point mutations, such as those of the BRAF and RAS genes, whereas large-scale chromosomal abnormalities, such as RET/PTC and PAX8-PPARγ rearrangements, are the most common abnormality in radiation-induced tumors. Since ionizing radiation is known to produce single strand and double strand DNA brakes, it is likely that radiation-induced DNA damage plays a direct role in promoting carcinogenesis through the generation of these chromosomal abnormalities.

Significant progress has been achieved in the understanding of why, despite a random distribution of radiation-induced DNA damage, some common cancer-associated chromosomal rearrangements are very specific. It is likely that spatial proximity of potentially recombinogenic chromosomal loci in the nuclei of normal human cells predisposes to these recurrent genetic alterations. It remains unclear, however, whether rearrangements occur directly by mis-rejoining of radiation-induced DNA breaks or they are stimulated by radiation exposure in an indirect way. Some data exist in support of both mechanisms, and further studies are required to explore this important question in more detail. This is crucial for better understanding of the mechanisms of radiation-induced carcinogenesis in humans, and thyroid cancer represents a unique model to address it.

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12. TRK ONCOGENES IN PAPILLARY THYROID CARCINOMA

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INTRODUCTION

The NTRK1 gene encodes the high affinity receptor for Nerve Growth Factor, and its action regulates neural development and differentiation. Deregulation of NTRK1 activity is associated with several human disorders. Loss of function mutations cause the genetic disease Congenital Insensitivity to Pain with Anhidrosis (CIPA). Constitutive activation of NTRK1 has been detected in several tumor types. An autocrine loop involving NTRK1 and NGF is responsible for tumor progression in prostate carcinoma and in breast cancer. Somatic rearrangements of NTRK1, producing chimeric oncogenes with constitutive tyrosine kinase activity, have been detected in a consistent fraction of papillary thyroid tumors.

The topic of this review is the thyroid TRK oncogenes; the modalities of activation, the mechanism of action, and the contribution of activating sequences will be discussed.

NTRK1 proto-oncogene

NTRK1 (also known as TRKA) is the prototype of a family of genes which also includes NTRK2 (TRKB) and NTRK3 (TRKC), encoding tyrosine kinase receptors for the neurotrophins of the Nerve Growth Factor (NGF) family. NGF is the preferred ligand for NTRK1, brain-derived neurotrophic factor and NT4/5 are ligands for NTRK2, and NT3 is the ligand for NTRK3. Interestingly, NT3 is also capable of binding to NTRK1 and NTRK2, although with low affinity (Barbacid, M., 1995).

All the neurotrophins bind also the p75 low affinity receptor, which belongs to the TNF receptor family, and is devoid of kinase activity (Kaplan, D. R. et al., 2000).

Neurotrophins are responsible for the survival, differentiation and maintenance of specific population of neurons in the developing and adult nervous system (Davies, A. M., 1994). In particular, the NGF/NTRK1 signaling supports survival and differentiation of sympathetic and sensory neurons responsive to temperature and pain. In addition to its neurotrophic functions, NGF also stimulates proliferation of a number of cell types such as lymphocytes, keratinocytes and prostate cells (Otten, U., et al., 1989; Di Marco, E. et al., 1993; Djakiew, D. et al., 1991).

NTRK1 was originally isolated from a human colon carcinoma as a transforming oncogene activated by a somatic rearrangement that fused a non-muscle tropomyosin gene to a novel tyrosine kinase receptor (Martin-Zanca, D. et al., 1986). Cloning of the full length gene (Martin-Zanca, D. et al., 1989) and identification of the NGF as a ligand occurred few years later (Kaplan, D. R. et al., 1991).

The NTRK1 gene is located on chromosome 1q21–22 (Weier, H.-U. G. et al., 1995) and consists of 17 exons distributed within a 25 kb region (Greco, A. et al., 1996). The NTRK1 receptor is a glycosylated protein of 140 kDa, comprising an extracellular portion, including Ig-like and Leucine rich domains for ligand binding, a single transmembrane region, a juxta-membrane domain, a tyrosine kinase domain and a C-terminal tail. Following NGF binding, NTRK1 undergoes dimerization and autophosphorylation of five tyrosine residues (Y490, Y670, Y674, Y675, and Y785). Activated receptor initiates several signal transduction cascades, including the Mitogen Activated Protein Kinase (MAPK), the phosphatidylinositol 3-kinase (PI3K) and the PLC-γ pathways. These signaling cascades culminate in the activation of transcription factors that alter gene expression (Kaplan, D. R. et al., 2000).

NTRK1 in human diseases

Deregulation of NTRK1 activity is associated with several human diseases. Mutations affecting different NTRK1 domains are associated with CIPA (Congenital Insensitivity to Pain with Anhidrosis), a rare recessive genetic disease characterized by loss of pain and temperature sensation, defects in thermal regulation and occasionally mental retardation (Indo, Y. et al., 1996). CIPA is the consequence of a genetic defect in the differentiation and migration of neural crest elements. By studying the effects of different CIPA mutations on NTRK1 biochemical and biological properties, the molecular mechanisms responsible for the disease have been unveiled. CIPA mutations cause inactivation of the NTRK1 receptor by at least three different mechanisms, such as complete inactivation, protein processing alteration, and reduction of receptor activity (Greco, A. et al., 1999; Greco, A. et al., 2000; Miranda, C. et al., 2002a; Miranda, C. et al., 2002b).

NTRK1 gain of function mutations have been described in some human tumors. Activation through genomic rearrangements producing chimeric oncogenes has been detected in a consistent fraction of human papillary thyroid carcinoma, and it will be described later. A 75 amino acids deletion in the extracellular domain of the NTRK1 receptor, resulting in a mutated protein with in vitro transforming activity, has been

detected in a patient with acute myeloid leukaemia, indicating that constitutive activation of the NTRK1 receptor may contribute to leukemogenesis (Reuther, G. W. et al., 2000). In human neuroblastoma, expression of NTRK1 is a good prognostic marker, suggesting that lack of NTRK1 expression contributes to malignancy, presumably because it results in the loss of signaling pathways important for growth arrest and/or differentiation of the neural crest derived cells from which these tumors originate (Brodeur, G. M. et al., 1997). In prostatic carcinoma an autocrine loop involving NGF and NTRK1 is responsible for tumor progression (Djakiew, D. et al., 1991), and tumor growth can be blocked by NTRK1 kinase inhibitors (Weeraratna, A. T. et al., 2001). Recently, an autocrine NGF/NTRK1 loop has been demonstrated in breast cancer cells, suggesting that it could represent a potential therapeutic target (Descamps, S. et al., 2001). It is interesting to outline that, at variance with other RTKs such as Met, FGFR, Kit and RET, no oncogenic activation of NTRK1 by point mutations have been found in human cancer. Moreover, the introduction of missense mutations releasing the oncogenic potential of different RTKs showed a distinct effect on NTRK1 receptor. This suggests that, despite the high degree of conservation of certain amino acid residues, the NTRK1 receptor diverges from other RTKs in terms of tridimensional structure and have a distinct auto-inhibitory mechanism (Miranda, C. et al., 2002c).

Thyroid TRK oncogenes

Papillary thyroid carcinoma (PTC) is the most frequent neoplasia originating from the thyroid epithelium, and accounts for about 80% of all thyroid cancers (Hedinger, C. et al., 1988). A consistent fraction (50%) of PTC harbors chromosomal alterations causing somatic rearrangements, and consequent oncogenic activation, of two RTK genes, namely RET and NTRK1 (Pierotti, M. A. et al., 1996). For a long time RET and NTRK1 rearrangements represented the only known genetic alterations in PTC. Recently, mutations of BRAF, alternative to RTK rearrangements, have been reported by different groups, and they represent the most frequent alteration in PTC (Kimura, E. T. et al., 2003; Cohen, Y. et al., 2003; Soares, P. et al., 2003).

Both RET and TRK oncogenes have been discovered in our laboratory by DNA transfection/focus formation assay in NIH3T3 cells, starting from papillary thyroid tumor DNA. Transforming activity correlated with the presence of human RET and TRK sequences in the mouse transfectants DNA (Bongarzone, I. et al., 1989); this provided the basis for the isolation and characterization of the chimeric oncogenes, containing the receptor tyrosine kinase domain preceded by activating sequences from different genes.

Several TRK oncogenes have been isolated from thyroid tumors, differing in the activating genes (Figure 1). The TRK oncogene, identical to that first isolated from colon carcinoma, and containing sequences from the TPM3 gene on chromosome 1q22–23 (Wilton, S. D. et al, 1995), has been frequently found in thyroid tumors (Butti, M. G. et al., 1995). TRK-T1 and TRK-T2 derive both from rearrangement between NTRK1 and TPR gene on chromosome 1q25 (Miranda, C. et al, 1994); however, they display different structure, especially for the different TPR portion

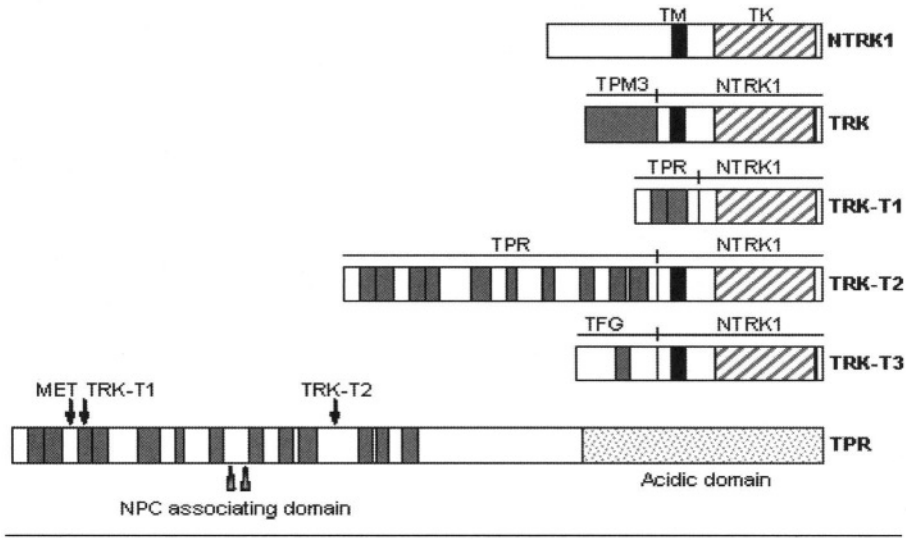

Figure 1. Schematic representation of NTRK1, TRKs and TPR proteins. In the oncoproteins the portions contributed by NTRK1 and activating sequences are indicated. The gray portions represent coiled-coil domains. On TPR the break sites of Met, TRK-T1 and TRK-T2 oncoproteins are indicated by arrows. TM: transmembrane domain, TK: tyrosine kinase domain, NPC: nuclear pore complex.

(Greco, A. et al., 1997; Greco, A. et al., 1992). TRK-T3 is activated by TFG, a novel gene on chromosome 3q11–12 (Greco, A. et al, 1995). All TRK oncogenes but TRK-T1 retain the NTRK1 transmembrane domain. TRK oncogenes display constitutive, ligand-independent tyrosine kinase activity.

In experimental models TRK oncogenes recapitulate the biological effects of the NTRK1 receptor upon NGF stimulation. In fact, they induce morphological transformation of NIH3T3 mouse fibroblasts, and neuronal-like differentiation of PC12 cells (Greco, A. et al., 1993b). The mechanisms by which TRK oncogenes mediate their effects have been in part elucidated (Figure 2). TRK oncoproteins interact to and activate PLCγ Shc, FRS2, FRS3, IRS1 and IRS2. All these molecules, except PLCγ, are recruited by the same tyrosine residue, corresponding to Tyr490 of NTRK1, most likely in a competitive fashion. Such interaction site is crucial for oncogenic activity, in so far its mutation to phenylalanine completely abrogate TRK oncogene biological activity. Moreover, by using a Shc dominant-negative mutant unable to recruit GRB2, we showed a crucial role of Shc adaptor in TRK-T3 biological activity. Conversely, mutation of the PLCγ interaction site did not affect the oncogenic activity. It is worth noting that our studies on TRK oncogenes allowed the identification of novel proteins interacting with NTRK1 kinase, such as IRS1, IRS2 and FRS3 (Miranda, C. et al., 2001; Roccato, E. et al., 2002; Ranzi, V. et al., 2003).

The capability of TPM3, TPR and TFG to activate chimeric tyrosine kinase oncogenes is not restricted to NTRK1; in fact, they have been found fused to other kinase genes. TPM3 and TFG were reported to fuse to ALK in anaplastic large cell lymphoma

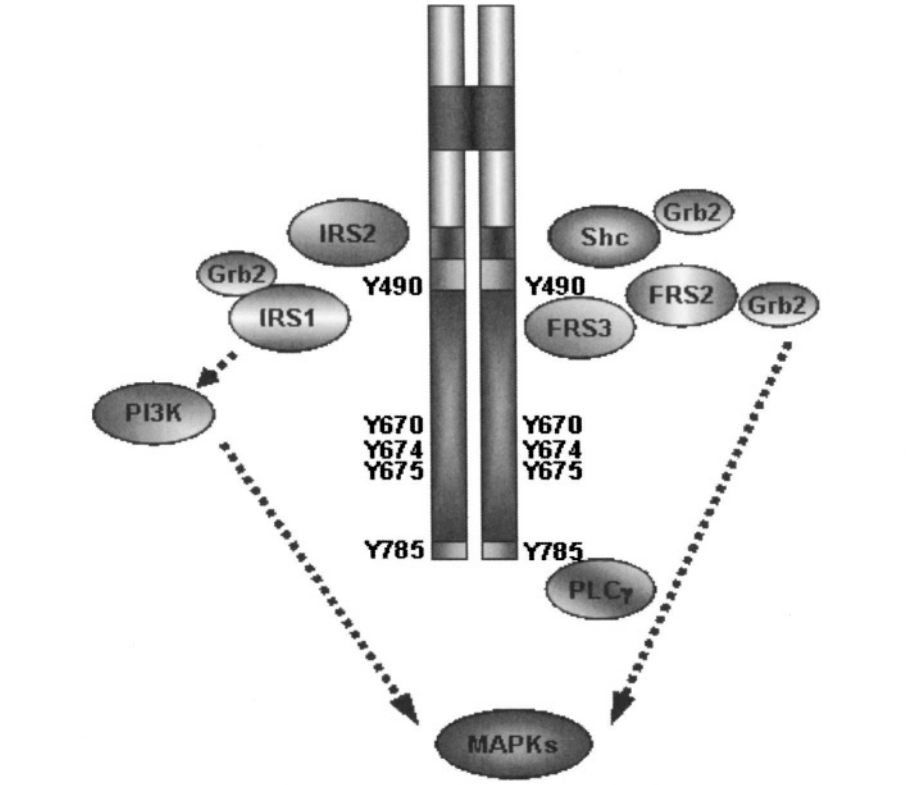

Figure 2. TRK oncogenes signaling pathways. Tyrosine residues are indicated with the number of the corresponding aminoacids of NTRK1.

(Hernandez, L. et al., 2002; Lamant, L. et al., 1999). TPR was first identified as part of the MET oncogene in HOS cells, fused to the TK domain of the hepatocyte growth factor receptor (Park, M. et al., 1986); subsequently it was detected fused to the raf oncogene during the transfection of a rat hepatocarcinoma (Ishikawa, F. et al., 1987). Interestingly, TPR and TFG were first identified in rearranged, oncogenic versions. TPM3 gene encodes a non-muscle tropomyosin isoform. TPR gene encodes a large protein of the nuclear pore complex; recent studies have shown that TPR is a phosphorylated protein involved in mRNA export, through the formation of complexes with different interacting proteins (Shibata, S. et al., 2002; Green, D. M. et al., 2003). TFG encodes a protein of still unknown function.

Role of activating sequences in TRK oncogenic activation

Despite the diversity in structure and function, all the NTRK1 activating proteins contain coiled-coil domains that promote protein dimerization/multimerization. Coiled-coil domains are characterized by heptad repeats with the occurrence of apolar residues

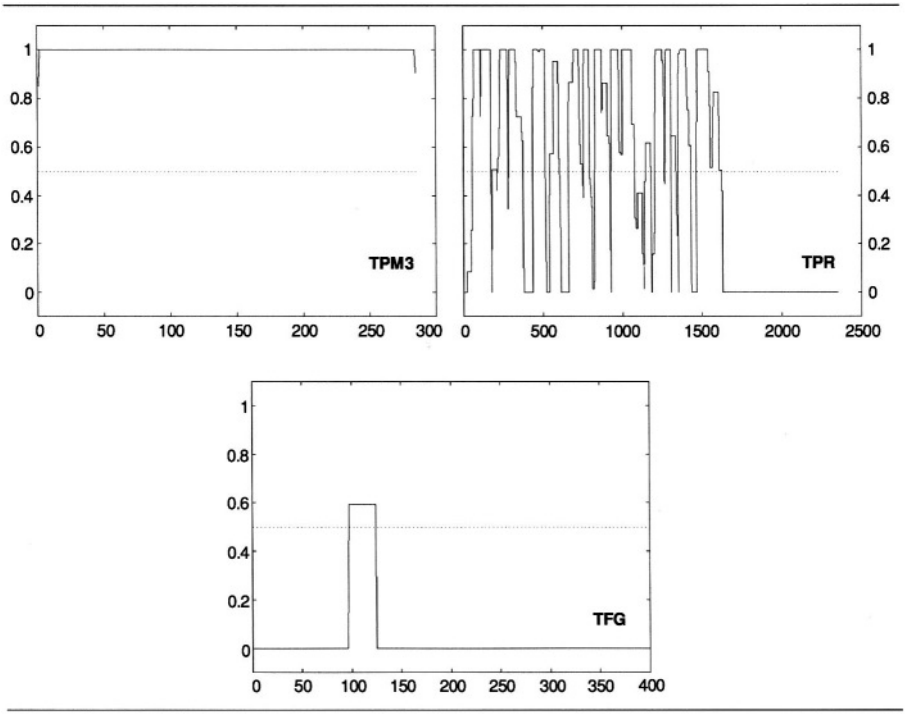

Figure 3. Prediction of coiled-coil domains in TPM3, TPR and TFG with the use of Paircoil program (Berger B., PNAS vol 92, 1995. pag. 8259–8263). The vertical scale represents relative coiled-coil probability; the horizontal scale represents amino acids number.

preferentially in the first (*a*) and fourth (*d*) positions (Lupas, A., 1996; Lupas, A. et al., 1991). This confers to the proteins the capability to fold into α-helices that are wound into a superhelix. In Figure 3 the coiled-coil domains detected in TRK activating sequences by sequence analysis with the COIL program are shown.

TPM3 contains numerous, overlapping coiled-coil domains. Several coiled-coil domains are present in TPR, and two of them fall in the region contained in MET and TRK-T1 oncogenes. It has been reported that mutations within the first coiled-coil domain drastically reduces MET transforming activity (Rodrigues, G. A. et al., 1993). TFG contains a single coiled-coil domain, of approximately three heptads, shorter than typical coiled-coil domains (Figures 3 and 4). However, the presence of a hydrophobic residue in position *a,* would increase the strength of association, despite the short length (Greco, A. et al., 1995). The contribution of TFG coiled-coil domain to TRK-T3 oncogenic activation has been elucidated by studies employing mutants where the domain was either deleted or mutated at leucine residues in position *d* of each heptad. We have demonstrated that coiled-coil domain plays a crucial role in TRK-T3 oncogenic activation by mediating oncoprotein complexes formation, an essential step for tyrosine kinase activation. Our studies support the model by which coiled-coil

domains mediate protein oligomerization of RTK oncogenes leading to constitutive, ligand-independent tyrosine kinase activity (Greco, A. et al., 1998). The TFG coiled-coil domain is predicted to fold into trimers. By size-exclusion chromatography we have demonstrated that the wild type TRK-T3 protein is part of high molecular weight complexes, compatible with the assembly of six oncoproteins molecules, or including other proteins. However, the precise composition of TRK-T3 complexes remains to be determined (Roccato, E. et al., 2003).

Studies on receptor and non receptor tyrosine kinase chimeric oncogenes have demonstrated that the importance of activating genes is related to the coiled-coil domains which mediate the activation above reported. However, an attractive hypothesis is the possibility that activating sequences may contribute with other functions, apart from dimerization. In addition, since the oncogenic rearrangements disabled one allele of the activating gene, the reduced activity of the corresponding protein could play a role in thyroid carcinogenesis. In this respect the thyroid TRK-T3 oncogene represents an attractive model for at least three reasons: 1) the activating portion is provided by TFG, a gene coding for a novel protein whose function remains to be unveiled; 2) the TFG portion contained in TRK-T3 display a single, short coiled-coil domain, corresponding to 14% of the aminoacid residues; 3) TFG might interact with other proteins (see later).

After our initial isolation as part of the TRK-T3 oncogene, the normal TFG counterpart was cloned and characterized (Figure 4).

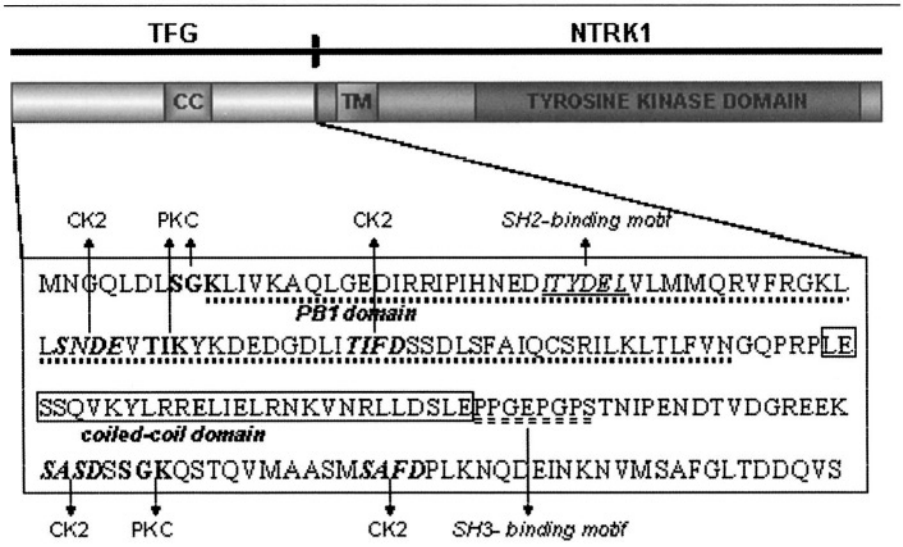

Figure 4. Schematic representation of the TRK-T3 oncogene. The portions contributed by TFG and NTRK1 are indicated. In the box the TFG aminoacidic sequence is reported; specific domains and consensus sites are indicated. CC: coiled-coil domain; TM: transmembrane domain; CK2: putative phosphorylation site for CK2; PKC: putative phosphorylation site for PKC.

TFG gene is ubiquitously expressed in human adult tissues and it is conserved among several species, including C. *e*legans. In addition to the coiled-coil domain, the TFG protein also contains putative phosphorylation sites for PKC and CK2, glycosilation sites, as well SH2- and SH3-binding sites (Mencinger, M. et al., 1997). Several of these sites are identical in TFG proteins from different species, indicating that the protein might be involved in basic cell processes (Mencinger, M. et al., 1999). We have recently identified a PB1 domain, which encompasses almost entirely the TFG N-terminal portion (Roccato, E. et al., 2003). PB1 is a novel protein module mediating protein oligomerization: in fact it is capable of binding to target proteins containing PC motifs and, as recently discovered, other PB1 domains (Terasawa, H. et al., 2001; Nakamura, K. et al., 2003). Based on the peculiarity of TFG, we have recently focused our interest on the role of sequences outside the coiled-coil domain in TRK-T3 oncogenic activation. On the whole our studies demonstrate that the regions outside the coiled-coil domain give a great contribution to TRK-T3 activation. When deleted, complexes formation is unaffected; however transforming activity is reduced to different extent. More detailed information was provided by studies employing point mutants: transforming activity was significantly reduced by mutating a putative SH2-binding motif, whereas it was abrogated by the mutation of the conserved Lys residue within the PB1 domain. These evidences strongly support the notion that proteins interacting with TFG might play a role in TRK-T3 oncogenic activity (Roccato, E. et al., 2003). The identification of such proteins will give an important contribution to the definition of the modalities by which TRK-T3 triggers thyroid carcinogenesis, as well as to the discovery of TFG normal function.

Genomic features of NTRK1 oncogenic rearrangements

The NTRK1 genomic rearrangements present in the tumor DNA have been cloned and characterized (Figure 5) (Greco, A. et al., 1997; Greco, A. et al., 1995, Butti, M.G. et al, 1995). All the breakpoints fall within a NTRK1 region of 2.9 Kb, showing a GC content of 58.8% (Greco, A. et al., 1993a). The NTRK1 rearrangements are balanced; in fact, in addition to the oncogenic rearrangement containing the 3′ moiety of the receptor, the reciprocal product of the rearrangement, containing the 5′ portion of NTRK1 fused to the 3′ portion of the activating genes, was present in tumor DNA.

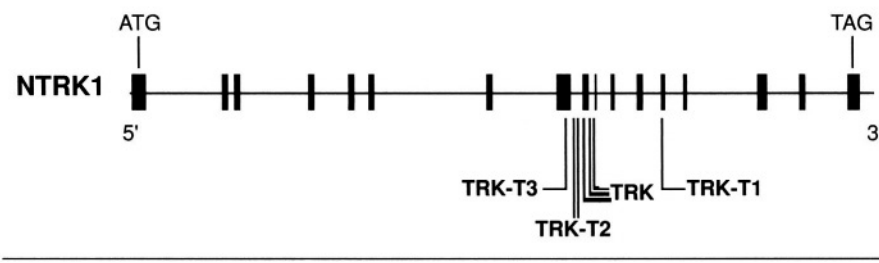

Figure 5. Genomic structure of the NTRK1 gene. The break sites of the different TRK oncogenes are indicated.

Sequencing of the breakpoint regions showed no homologies between the joined extremities. This suggests that the Non Homologous End Joining (NHEJ) mechanism, which requires little or not sequence homology, could be involved in the generation of TRK oncogenes. The NHEJ pathway is activated by ionizing radiations, and this is consistent with the association of PTC with therapeutic or accidental radiation exposure. Analysis of the breakpoint regions in different tumors demonstrated that all the rearrangements are conservative, involving deletion, insertion or duplication of only few nucleotides. In a tumor carrying the TRK-T2 oncogene a peculiar rearrangement was found, with the 5′ end of NTRK1 joined to sequences from chromosome 17; however, such additional rearrangement does not contribute to oncogenic activation (Greco, A. et al., 1997).

No cytogenetic studies are available for tumors carrying NTRK1 rearrangements; therefore the type of chromosomal rearrangement generating TRK oncogenes is not documented. A t(1 ;3) translocation is most likely responsible for the generation of TRK-T3 oncogene (TFG/NTRK1 rearrangement). For TRK, TRK-T1 and TRK-T2, produced by rearrangements with genes located on the q arm of chromosome 1, similarly to NTRK1, three mechanisms of rearrangement can be postulated: deletion, inversion, and reciprocal translocation between the two chromosome 1 homologues. The presence in the tumor DNA of the reciprocal products of the rearrangement allowed us to exclude the deletion. Sequence data recently available in public databases unveiled that NTRK1 has transcriptional orientation opposite to that of TPM3 and TPR. Therefore, intrachromosomal inversion is the only mechanism capable to produce TRK, TRK-T1 and TRK-T2 oncogenes (Figure 6).

The thyroid epithelium is very prone to chromosomal rearrangements. These include the RET and NTRK1 oncogenes in PTC, and the PAX8/PPARγ fusion gene associated with follicular thyroid tumors. This predisposition to gene rearrangements is a peculiarity of thyroid epithelium, at variance with other epithelia, and the understanding of the molecular basis underlying such predisposition represents a fascinating topic. Recently, Nikiforova et al (2000) have shown that, in thyroid interphase nuclei, RET and H4 loci display a distance reduced with respect to other cell type, and suggested that this spatial contiguity may provide the structural basis for the generation of the thyroid RET/H4 (PTC1) oncogene. It is very important to assess whether or not this attractive model also apply to NTRK1 and its partners, rearranging genes. Another possibility is that the high frequency of chromosomal rearrangements in thyroid tumors might reflect the thyrocyte intrinsic capability to repair DNA DSBs, either spontaneous or induced. Yang et al (1999) showed that human thyrocytes exposed in vitro to ionizing radiations failed to induce apoptosis; instead, they showed a significant increase of DNA end-joining activity. In this respect the analysis of the DNA repair kinetics and the status of the enzymes involved in DNA repair in human thyrocytes deserve to be investigated.

CONCLUSIONS

Rearrangements of RET and NTRK1 are frequently detected in human papillary thyroid carcinoma. TRK oncogenes involve different activating genes containing

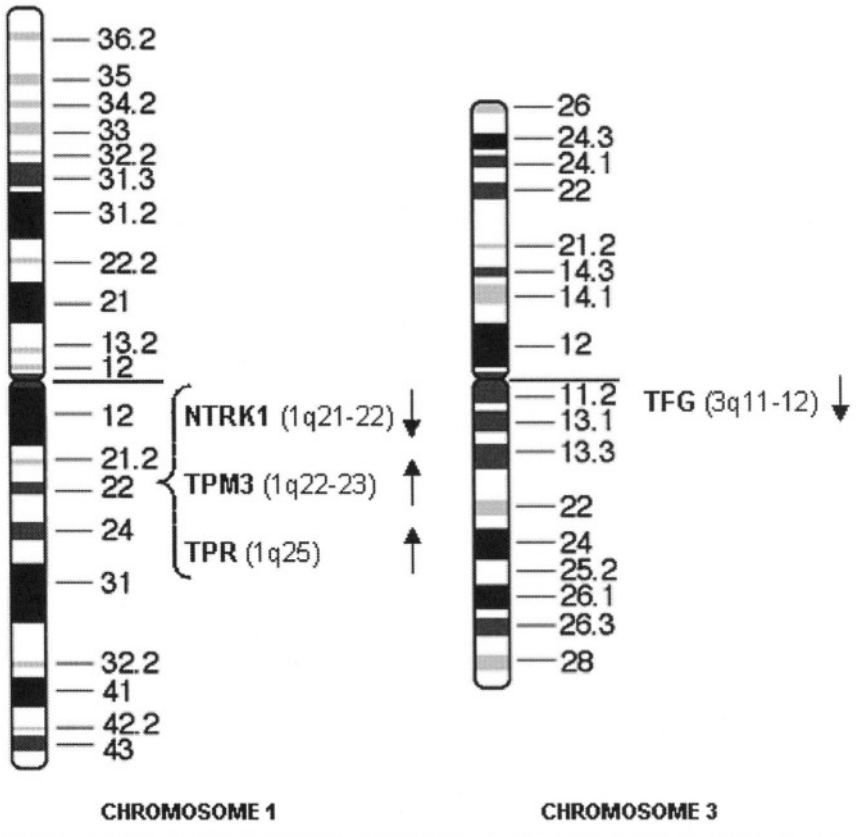

Figure 6. Ideogram of chromosomes 1 and 3 showing the localization of NTRK1, TPM3, TPR and TFG genes. The arrows indicate the transcriptional orientation.

coiled-coil domains chat mediate protein dimerization and consequent tyrosine kinase activation. However, also regions outside the coiled-coil domain contribute to oncogenic activation, with modalities presently under investigation. The high proneness of thyroid epithelium to chromosomal rearrangements might reflect structural and enzymatic properties of thyrocytes with respect to DNA repair. The identification and study of such properties will elucidate the mechanisms responsible for the generation of thyroid oncogenes.

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13. THYROIDAL IODIDE TRANSPORT AND THYROID CANCER

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INTRODUCTION

The current treatment for metastatic papillary and follicular thyroid carcinomas, consists of total thyroidectomy followed by administration of radioiodide for the ablation of any remaining thyroid cancer cells or metastases (1). Radioiodide treatment of thyroid cancer has been employed for over 60 years (2), and this is the most effective targeted and curative radiotherapeutic modality available for any cancer. Radioiodide also destroys any remaining normal thyroid tissue, thus increasing the sensitivity of subsequent ^{131}I scanning and serum Tg measurements for the detection of recurrent or metastatizing disease. This is because, if normal thyroid cells remained after thyroidectomy, they would tend to prevent cancerous cells from being detected by either method. The success rate of this treatment is impressive: the mortality of patients with metastatic thyroid cancer who are treated with ^{131}I is just 3%, as opposed to 12% for those who are not treated (3). Side-effects resulting from this therapy, such as mild sialadenitis, are minimal (3); in most instances they resolve within a few weeks of termination of the treatment.

Two key characteristics of the thyroid contribute to the success of this approach. First, thyrocytes, both normal and cancerous, exhibit a remarkable ability to actively transport iodide (I^-). Thus, when radioiodide is administered, it is actively taken up almost exclusively by thyrocytes without affecting other cells. This makes radioiodide therapy a distinctively specific targeted method that delivers radiation from within the cancerous cells themselves. Even though I^- transport activity is significantly lower in the

majority of cancerous thyrocytes than in normal ones, the activity remains high enough for accumulated radioiodide to destroy cancerous thyrocytes as well. The decreased ability of cancerous thyrocytes results in the presence of "cold nodules" at tumor sites on thyroid scintigraphic scans. Second, although the thyroid is physiologically crucial, its function can be fully restored after thyroidectomy by thyroid hormone substitutive therapy, thus keeping patients in a euthyroid state.

It has long been well known that active I^- transport is a key attribute of differentiated thyrocytes, as I^- is essential for thyroid hormone biosynthesis. The Na^+/I^- symporter (NIS) is the plasma membrane glycoprotein that mediates active I^- transport from the bloodstream into the cytoplasm of thyrocytes. Using expression cloning in *Xenopus laevis* oocytes, our group isolated the cDNA encoding NIS from rat-thyroid-derived FRTL-5 cells (4). On the basis of a high degree of homology with the rat NIS cDNA, the human, mouse, and pig NIS cDNAs were subsequently cloned (5, 6, 7). NIS-mediated active I^- transport has also been documented in a few other tissues, including salivary glands, gastric mucosa, and lactating (but not non-lactating) mammary gland (8, 9). These findings and the generation of high affinity anti-NIS Abs have led to a thorough molecular characterization of NIS (10) (11)and to the analysis of both thyroidal and extrathyroidal I^- transport in health and disease (9, 12–14).

FUNCTION AND STRUCTURE OF NIS

NIS couples the inward translocation of Na^+ down its electrochemical gradient to the simultaneous inward "uphill" translocation of I^- against its electrochemical gradient. NIS activity is inhibited by the "classic" competitive inhibitors perchlorate and thiocyanate (9, 15–18). Two Na^+ are transported per each I^- (19). The Na^+ gradient that provides the driving force for I^- uptake is maintained by the Na^+/K^+ ATPase. In the thyroid, both NIS and the Na^+/K^+ ATPase are located on the basolateral side of the thyroid follicular cells, facing the blood supply (20). Rat NIS (rNIS) is a 618-amino acid protein (relative molecular mass 65,196) (4); both human and pig NIS, which contain 643 amino acids each, are highly homologous (75.9% and 74.2%, respectively) to rNIS (6, 7). Based on extensive experimental testing, we have proposed a NIS secondary structure model with 13 transmembrane segments (Figure 1) (12). The amino and carboxy termini face extra- and intracellularly, respectively (10). NIS is a glycoprotein; three of its Asp residues (225, 485, 497) are glycosylated in the endoplasmic reticulum (21). However, glycosylation is not essential for proper NIS function, as indicated by the observation that a non-glycosylated NIS protein is properly targeted to the plasma membrane and displays I^- transport activity with an identical K_m value (~20–30 μM) to that of wild-type (WT) NIS (21). The ca 70-amino acid hydrophilic carboxy terminus is the main phosphorylated region of the protein (22). Freeze-fracture electron microscopy studies of NIS-expressing *Xenopus laevis* oocytes revealed the appearance of 9-nm intramembrane particles corresponding to NIS (19). The size of these particles suggested that NIS may function as a multimeric protein. Recent co-immunoprecipitation experiments indicate that NIS is indeed an oligomer (23). A putative leucine zipper motif constituted by leucines at positions 199, 206, 213 and 220 may be the structural basis for NIS oligomerization (4).

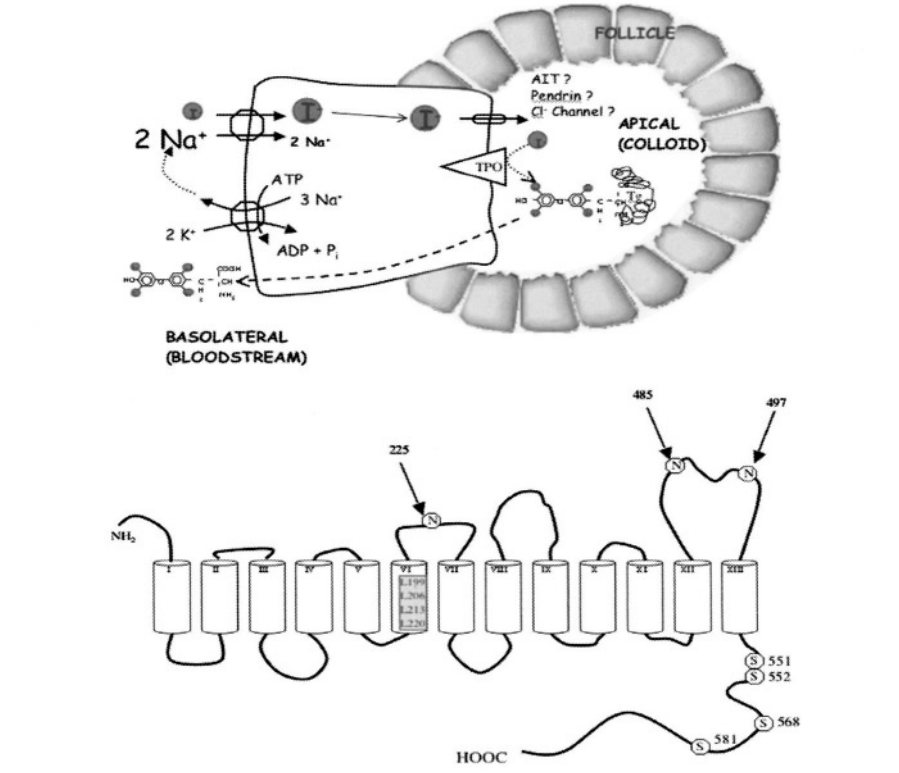

Figure 1. Iodide transport and biosynthetic pathway of thyroid hormones T_3 and T_4 in the thyroid follicular cell. Thyroid follicles are comprised of a layer of epithelial cells surrounding the colloid. The basolateral surface of the cell is shown on the left side of the figure, and the apical surface on the right. Active accumulation of I^-, mediated by the Na^+/I^- symporter (NIS) [top circle], driven by the Na^+ gradient generated by the Na^+/K^+ ATPase [bottom circle]; once I^- effluxes towards the colloid [cylinder], (TPO) [triangle] catalyzes the organification of I^- on the thyroglobulin (Tg) molecule. Dotted line pointing from the apical to the basolateral surface indicates endocytosis of iodinated Tg, followed by its phagolysosomal hydrolysis and secretion of thyroid hormones.
Secondary structure model of NIS. Transmembrane segments are numbered with Roman numerals I-XIII. The N-terminus faces the extracellular milieu and the C-terminus the cytosol. N-glycosylation sites are indicated by arrows and the leucine zipper motif in the VI transmembrane segment is shaded gray. Serines on the C-terminus are indicated.

NIS EXPRESSION IN THYROID CANCER

Given that most thyroid cancers exhibit decreased or absent radioiodide accumulation, the prevailing expectation for a long time was that NIS expression would be found to be decreased or absent in cancerous thyrocytes. The first investigations addressing this issue, carried out using RT-PCR and showing lower mRNA levels in cancerous than in normal thyrocytes, seemed to confirm these expectations (24–28). RT-PCR is an easy-to-perform and very effective technique to detect mRNA expression even in very small tissue samples. However, determinations of mRNA levels by either RT-PCR or

Northern blot analysis provide no information on RNA stability. In addition, mRNA levels of proteins like NIS, with long half-lives and complex posttranscriptional regulation, do not necessarily correlate with actual protein expression (29). Immunoblot analyses to directly assess protein expression would address this limitation; however, this requires significantly larger tissue samples, which are not often available from human specimens.

Immunoblot analyses may provide satisfactory quantitative and qualitative information on NIS protein expression and some posttranslational modifications, but not on subcellular distribution. The subcellular localization of NIS is particularly significant because, as pointed out earlier, NIS is functional *only* when it is properly targeted to the plasma membrane. Hence, immunohistochemical analysis of NIS expression in thyroid cancer was carried out (20, 30, 31). In addition to revealing the subcellular distribution of NIS, immunohistochemistry offers the advantage that NIS protein expression in the carcinomatous tissue can be compared to the surrounding normal tissue in the same thyroid gland. Surprisingly, immunohistochemical studies of NIS protein expression in thyroid cancer have shown that as many as 70% of thyroid cancers actually exhibit NIS protein overexpression (Figure 2B), as compared to the surrounding normal tissue, although in these cancerous cells NIS is mainly located in intracellular membrane compartments rather than in the plasma membrane. NIS was absent only in about 30% of the cases (20, 31). Thus far, no NIS mutations resulting in impaired protein expression or altered plasma membrane trafficking have been identified in thyroid cancer (32). These findings have had a significant impact on research approaches aiming to improve the effectiveness of radioiodide therapy, since they emphasize the importance of stimulating NIS targeting to and/or retention at the plasma membrane rather than stimulating NIS expression at the transcriptional level.

UNDERSTANDING NIS REGULATION IN HEALTH AND DISEASE MAY IMPROVE THE EFFECTIVENESS OF RADIOIODIDE TREATMENT

TSH and I^- are the two main factors that regulate NIS expression: TSH stimulates and I^- decreases it. Hence, TSH stimulation and I^- depletion of residual thyroid carcinoma tissue are the two most important modulators routinely used to optimize radioiodide treatment. To achieve maximum therapeutic effect, thyroidectomized patients must have TSH levels above 30 mU/l and must have been on a low I^- diet for two weeks prior to initiation of radioiodide treatment (1).

TSH has long been known to be a key regulator not only of NIS expression but also of thyroidal I^- uptake (i.e., NIS activity). No thyroidal NIS expression is observed in hypophysectomized rats (because of the lack of TSH), but thyroidal NIS expression is restored as early as 24 h after treatment with TSH. In intact (i.e., non-hypophysectomized) rats, treatment with the I^- organification inhibitor propylthiouracil causes elevated TSH levels, which in turn lead to higher NIS expression than in control animals (10). TSH regulates NIS expression at both the transcriptional and posttranscriptional levels. Several groups have demonstrated that TSH upregulates I^- transport by a cAMP-mediated increase in NIS transcription, while withdrawing TSH causes decreased cAMP levels and diminished NIS transcription (33).

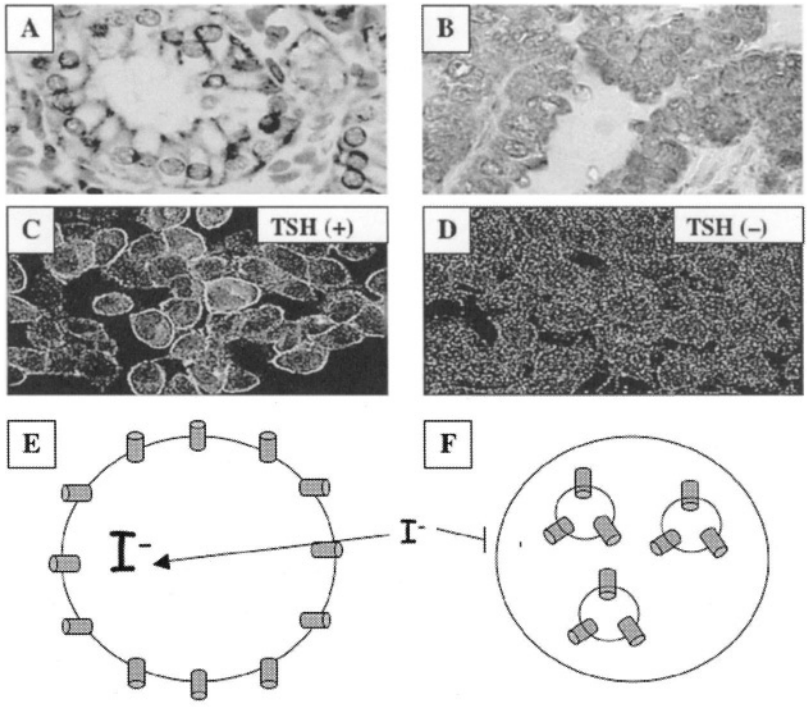

Figure 2. **A**: NIS immunohistochemistry in Graves' disease. NIS is localized in the basolateral plasmamembrane **B**: NIS immunohistochemistry in follicular carcinoma shows the intracellular localization of the significantly overexpressed NIS protein **C**: Indirect immunofluorescence analysis of NIS localized in the plasma membrane of FRTL-5 cells kept in the presence of TSH, **D**: intracellular NIS localization in TSH deprived FRTL-5 cells **E**: schematic representation of NIS plasma membrane localization and iodide transport in FRTL-5 cells kept in the presence of TSH **F**: schematic representation of NIS localized in the intracellular membrane compartments in TSH deprived FRTL-5 cells, resulting in lack of iodide transport.

The detailed analysis of the rat and human NIS promoters has confirmed the significant role of Pax8 in NIS expression (34–36). In rat, the proximal NIS promoter was found to contain a TTF1 binding site and a TSH-responsive element where a putative transcription factor NTF-1 (NIS TSH-responsive factor 1) interacts (39). NIS upstream enhancer (NUE-2495 to -2260) contains two Pax-8 binding sites and a degenerate CRE (cAMP-responsive element sequence), which are essential for full TSH cAMP-dependent transcription of NIS (34). Interestingly, during chronic TSH stimulation when the catalytic subunit of PKA is downregulated, cAMP is still able to stimulate NIS transcription, indicating the existence of both PKA-dependent and independent mechanisms (34). Recently, a thyroid-specific far-upstream (–9847 to –8968) enhancer in the human NIS gene – highly homologous to the rat NUE – has been reported. It contains putative Pax-8 and TTF-1 binding sites and a CRE-like sequence. The TTF-1 binding site is not required for full activity (35, 36).

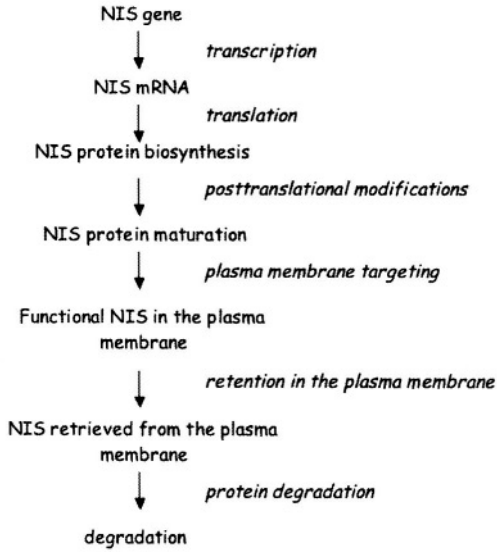

Figure 3. Multiple levels of NIS regulation.

FRTL-5 cells are rat-thyroid-derived, well-differentiated normal thyroid epithelial cells that grow in media supplemented with TSH. These cells are frequently used as an *in vitro* model system to study TSH regulation. In FRTL-5 cells, NIS expression is TSH dependent. Kaminsky *et al.* (37) observed that, in the absence of TSH in the medium, intact FRTL-5 cells did not transport I^-, whereas membrane vesicles prepared from the same TSH-deprived cells surprisingly maintained their I^- transporting ability. This suggested that mechanisms other than transcriptional might also operate in regulating NIS activity in response to TSH. Riedel *et al.* (22) investigated this phenomenon in detail. They observed that in the absence of TSH, there was no *de novo* NIS synthesis in FRTL-5 cells, while previously synthesized NIS was redistributed from the plasma membrane to intracellular membrane compartments. (Figure 2 C,D,E,F) These authors also demonstrated that NIS has a long half-life: 5 days in the presence and 3 days in the absence of TSH. Considering the TSH regulation of NIS expression and the long half-life of NIS, it is clear that NIS mRNA levels alone are not a good indicator of actual NIS protein levels. Instead, NIS protein levels must be assessed directly with anti-NIS Abs. In addition, it is also essential to keep in mind that NIS protein expression, in turn, does not necessarily correlate with NIS activity, because such factors as subcellular distribution of NIS to the plasma membrane play a key role in NIS function; hence, it is crucial to quantitate NIS activity (Figure 3).

TSH modulates NIS phosphorylation

The mechanism by which TSH regulates the subcellular distribution of NIS is unknown. Phosphorylation has been shown to be implicated in the activation and

subcellular distribution of several transporters (38–40). NIS has several consensus sites for kinases, including those for cAMP-dependent protein kinase, protein kinase C, and casein kinase-2 (9, 22). Furthermore, TSH actions in the thyroid are mainly mediated by cAMP. All these points raised the possibility that phosphorylation might be involved in the regulation of NIS subcellular distribution. When FRTL-5 cells were labeled with ^{32}Pi, lysed, and immunoprecipitated with anti-NIS Ab, it was observed in the autoradiogram that NIS was phosphorylated, independently of the presence of TSH in the culture medium (22). The phosphopeptide map obtained after NIS digestion with trypsin was markedly different when TSH was present from that when TSH was absent (22).

The predominant phosphorylated region of NIS was determined by treatment of the immunoprecipitated symporter with CNBr. CNBr cleaves polypeptides at methionine residues. The anti-NIS Ab generated against the last 16 amino acids of NIS recognized an 11-kDa polypeptide observed also by autoradiography. The densitometric quantitation of the autoradiogram indicated that the major phosphorylation region of NIS is the carboxy terminus (22). Moreover, TSH increased the phosphorylation level of the COOH terminus of NIS ~16-fold. For the identification of which of the serine residues within the COOH terminus are phosphorylated, S551, S552, S568, and S581 (Fig. 1) were replaced individually and simultaneously with alanine. Significantly, the replacement of the four serines of the COOH terminus promoted phosphorylation of threonines in NIS, suggesting that there is an important biological pressure to preserve phosphorylation of NIS at the COOH terminus (22). Future experiments should elucidate whether NIS phosphorylation is involved in trafficking and/or retention of NIS at the plasma membrane.

Regulation of NIS expression by I^-

As indicated above, I^- itself, the substrate of NIS, also regulates NIS expression, but the mechanism of this regulation is less clear than that of TSH. For over 60 years, it has been known that I^- organification and, consequently, thyroid hormone biosynthesis, are blocked when the intracellular concentration of I^- rises to a certain threshold. This phenomenon (i.e., the inhibition of I^- organification by a high concentration of I^-) is called the Wolff-Chaikoff effect, and it has been used to block thyroid function in hyperthyroid patients (41, 42). I^- also suppresses I^- transport in a time- and dose-dependent manner, an effect that has been investigated by several groups both *in vitro* and *in vivo* (43–45). As I^- transport decreases, the intrathyroidal I^- concentration falls, the inhibition of organification is relieved, and thyroid hormone synthesis resumes; thus, by downregulating its own I^- transport, the thyroid "escapes" from the inhibitory effect of I^- overload. Grollmann *et al* (45) investigated the effect of I^- preincubation on I^- uptake in FRTL-5 cells, and found that I^- preincubation of these cells suppresses I^- uptake in a dose- and time-dependent manner. These authors observed decreased I^- transport after a 2-h incubation with 100 μM of NaI (45)

With the availability of the NIS cDNA and anti-NIS Abs, the inhibitory effect of I^- on its own transport in the thyroid has been partially reinvestigated by several groups. In dog, Uyterspot *et al* (46) found that I^- inhibited both TPO and NIS

mRNA expression, but no protein levels were measured. In FRTL-5 cells, Spitzweg *et al* (47) and Eng *et al* (48) published somewhat contradictory results. Spitzweg *et al* (47) reported a 50% decrease in I^- uptake. However, in these uptake studies, the specific activity of the radioactive I^- used in the transport measurements was diluted out by preincubation with unlabeled I^-, which results in its intracellular accumulation. Without taking this factor into account, the interpretation of these findings is uncertain. These authors also reported a decrease in NIS mRNA levels, but did not determine NIS protein. In contrast, Eng *et al.* (48) did not find decreased NIS mRNA levels after I^- preincubation. Instead, they found that both the levels and the half-life of the NIS protein were significantly decreased. Hence, Eng *et al.* (48) concluded that I^- regulates its own transport in FRTL-5 cells mostly by posttranscriptional mechanisms. Surprisingly, the same authors found that both NIS mRNA and protein levels were decreased *in vivo* in response to the administration of I^- (49). Evidently, a thorough molecular examination of the regulatory effect of I^- on I^- transport simultaneously assessing NIS expression, subcellular localization, and kinetic properties is required to understand the intriguing role of I^- in its own transport.

Effect of spatial organization of thyroid cells

It is clear from earlier studies that the spatial organization and apical-basolateral polarization of thyroid epithelial cells significantly influence their functions, including I^- transport, I^- organification, and protein expression. Roger *et al* (50) isolated human thyroid epithelial cells from normal subjects and grew the cells in the presence or absence of serum. They observed that, whereas cells grown in the presence of serum formed monolayers, in the absence of serum the cells formed aggregates. Following TSH stimulation, cells in aggregates exhibited more avid I^- transport than cells in monolayers. Interestingly, this TSH stimulatory effect was abolished by the addition of serum to the medium. Takasu *et al* (51) showed that, in porcine thyroid cells, polarity is important for I^- uptake, and a follicular structure is required for I^- organification. Kogai *et al* (52) have recently reinvestigated the effect of spatial organization of thyrocytes on NIS expression and function. They showed that TSH upregulates both NIS mRNA and protein levels in 2- and 3-dimensional human thyrocyte primary cultures, but a significant increase in I^- uptake occurs only in 3-dimensional structures.

SPECIFIC CONSIDERATIONS RELATED TO RADIOIODIDE TREATMENT

A prerequisite for the success of radioiodide treatment is the retention of the radioisotope for a sufficiently long time so the necessary dose is delivered to destroy the malignant tissue. The retention time of radioiodide in thyrocytes is determined by I^- uptake and I^- efflux. At steady-state conditions, I^- accumulation reflects the equilibrium between the rates of influx and efflux (Figure 4).

In the healthy thyroid gland, NIS mediates the active accumulation of I^-, whereas the mechanisms involved in I^- efflux are poorly understood (see "I^- efflux: pendrin and AIT" below). I^- organification – i.e., the TPO-mediated iodination of the tyrosine residues on the thyroglobulin molecule – occurs on the colloidal surface of the apical membrane. Iodinated thyroglobulin molecules remain in the colloid, surrounded

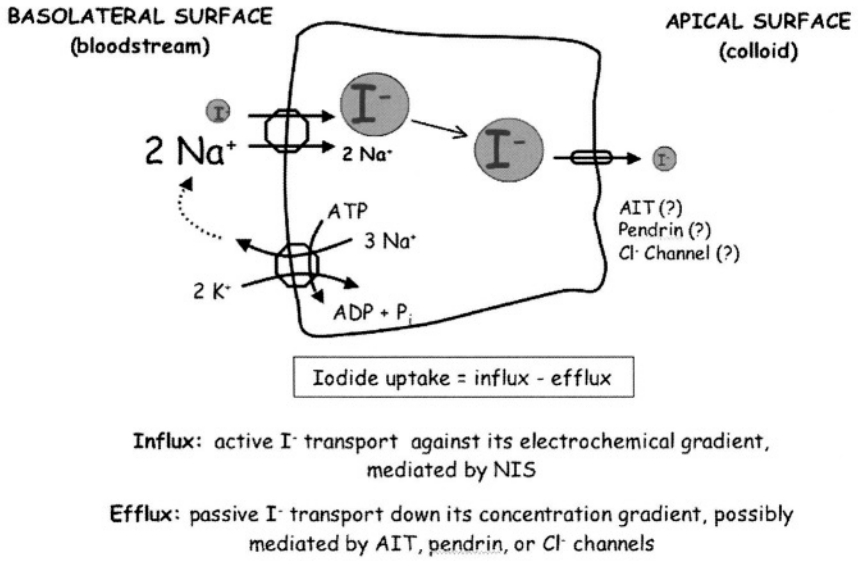

Figure 4. Determinants of iodide accumulation.

by the thyroid epithelial cells, thus increasing the radioiodide retention time in the thyroid gland. In contrast, in thyroid cancer, the typical follicular architecture of normal thyroid tissue is not conserved, as the malignantly transformed epithelial cells lose their polarity (53). Hence, these cells display no well-defined colloidal space, and as a result, thyroglobulin leaks out into the extracellular space and the bloodstream. Most differentiated thyroid cancers exhibit TPO protein expression, but at levels lower than those considered normal (54–56); TPO gene mutations have also been reported in some differentiated thyroid carcinomas (57). Furthermore, earlier studies in humans showed impaired or absent I^- organification in thyroid cancer (58), underscoring the loss or reduction of organification in thyroid cancer.

More recently, the organification effect in radioiodide retention time has been assessed *in vivo* in non-NIS-expressing tumors into which both rat and human NIS have been introduced under the control of tissue-specific promoters. Since these tumors do not express TPO, they do not organify I^-. Cho *et al* (59) reported radioiodide retention time greater than 24 h in hNIS-expressing xenografted human glioma cells in rats, and observed a longer survival in animals with NIS-expressing tumors versus control animals with non-NIS-expressing tumors. Spitzweg *et al* (60) introduced NIS in a recombinant adenovirus into a human prostate carcinoma cell line under the regulation of the prostate specific antigen (PSA) promoter. They reported a retention time of 5.6+/–1.4 h in NIS-expressing prostate carcinoma xenografts in nude mice and a remarkable decrease (over 80%) of the size of these xenografts after a single ip injection of 3 mCi ^{131}I. Dingli and colleges (61), for their part, expressed NIS in a myeloma cell line using a transcriptionally targeted lentiviral vector, where the therapeutic or reporter

gene is under the control of minimal immunoglobulin promoter and enhancer elements (immunoglobulin κ-light chain enhancer elements). These authors also investigated the so-called bystander effect. β-particles emitted during the decay of ^{131}I can travel a distance of 0.2–2.4 mm. Therefore, the isotope is capable of destroying "bystanding" non-NIS-expressing cells. Dingli *et al.* (61) also treated myeloma xenografts containing variable numbers of radioiodide-transporting, NIS- and non-NIS-transduced tumor cells. The result was striking: all tumors in which 50–100% of the cells expressed NIS had completely regressed two weeks after a single dose of 1 mCi ^{131}I.

The above results provide strong evidence against the widely held notion that radioiodide therapy is likely to be ineffective in non-thyroidal cells that, while functionally expressing NIS (whether endogenously or by targeted transfection), lack the ability to organify I^-. The reasoning was that the absence of organification resulted in the isotope not being retained in the cells for a sufficiently long time. Yet, in the mentioned studies (59, 61, 62), radioiodide treatment was effective even in the ***absence*** of I^- organification.

If organification is not essential for radioiodide therapy to be effective, sufficient iodide uptake mediated by NIS and slow I^- efflux are the requirements for successful radioiodide therapy.

I^- efflux: pendrin and AIT

Several groups have tried to identify the mediator of apical I^- efflux in thyroid epithelial cells. Two recently cloned molecules are the main candidates: pendrin and the apical iodide transporter (AIT) (Figure 1).

In 1997, a gene defective in Pendred syndrome (PDS) was identified by positional cloning (63). Pendred syndrome is characterized by sensorineural (most often prelingual) deafness and goiter with defective organification. In PDS, goiter can develop at any age or may be absent, whereas deafness is generally present (63). Pendrin has been localized on the apical membrane of the thyroid epithelial cells by immunohistochemistry (64). In heterologous expression systems, pendrin has been shown to transport iodide, chloride, formate, and nitrate (65).

The organification defect characteristic of Pendred syndrome was attributed to defective pendrin-mediated apical I^- transport into the colloid, where organification occurs (Figure 1). Surprisingly, although the recently generated Pds-knockout mice are completely deaf, they do not exhibit a pathologic thyroidal phenotype (66); therefore, pendrin's function as the apical I^- transporter remains to be further investigated.

By means of a PCR cloning strategy based on NIS-sequence homologies, a 610-amino-acid protein-coding gene was recently cloned from a human kidney cDNA library. The newly identified protein shares both a strikingly high identity (46%) and similarity (70%) to hNIS (67). This protein, called the human apical iodide transporter (hAIT), has been localized to the apical membrane of thyroid epithelial cells; however, a thorough molecular and kinetic characterization is required to unequivocally establish whether hAIT mediates "downhill" movements of I^- from the cytosol to the colloid.

AIT (SLC5A8) expression in thyroid carcinomas has not yet been investigated. Interestingly, Li *et al* (68) found, while screening hypermethylated sequences in colon

carcinoma cell lines and human colon carcinoma tissues, that the hAIT gene is heavily methylated and hAIT mRNA expression is decreased or absent. Reintroducing AIT into colon cancer cell lines harboring methylated endogenous AIT suppressed their ability to form colonies in soft agar and xenograft tumors in athymic mice. Based on their observations, the authors suggested that AIT could play a role as a tumor suppressor in colon cancer (68).

STUNNING

Stunning is the decrease in radioiodide uptake in thyroid tissue caused by previous exposure of the tissue to a tracer dose of the radioisotope for dosimetry. Stunning was first described almost 50 years ago (69), and since then, several clinical studies have been carried out to investigate its deleterious effect on radioiodide therapy. Before treatment with radioiodide, patients usually undergo diagnostic and dosimetric studies. After administering a tracer dose of radioiodide, the percentage of radioiodide uptake in the tumor tissue is determined and used as a basis to calculate the appropriate therapeutic dose. However, stunning is often observed, so that the percentage of radioiodide uptake measured upon administration of the therapeutic dose is significantly lower than that when the tracer dose was first administered for dosimetry. It is hypothesized that stunning is caused by radiation damage to the cells from the previously administered tracer dose. When stunning occurs, the delivered radioiodide therapeutic dose is insufficient for successful ablation of the malignant tissue (70). While most studies have investigated stunning *in vivo* during the treatment of patients (71), Postgard *et al* (72) recently reported an *in vitro* study revealing more about the possible mechanism of ^{131}I stunning. These investigators used porcine thyroid cell primary cultures grown on a filter in a bicameral chamber. The apical and basolateral media were separated by the cells assembled into a monolayer. Thyroid cells kept in TSH- and methimazole-supplemented medium were exposed to different amounts of ^{131}I. Stable iodide (10 nM) was administered to control cells. Transepithelial ^{125}I transport (from the basal to the apical membrane) was evaluated after a 3-day washout period. Iodide transport decreased 50% with a 3-Gy absorbed dose, and it was almost completely inhibited with an 80-Gy dose. The transepithelial electrical resistance of the cell monolayer was unchanged, showing that the integrity of the epithelial monolayer remained intact. The presence of perchlorate – the competitive inhibitor of I^- transport – during ^{131}I incubation, partially prevented the reduction of ^{125}I transport. Considering that there was no cell damage from radiation exposure and that the same amount of stable iodide had no effect on transport, these authors concluded that decreased I^- transport after ^{131}I exposure was most probably the result of a direct effect of radiation on thyroid function.

IMPROVING I^- TRANSPORT IN THYROID CANCER

To achieve an optimal therapeutic effect with maximum radioiodide uptake in thyroid carcinoma metastases, TSH has to be above 30 mU/l in thyroidectomized patients, who must be on a low iodide diet for two weeks prior to treatment (1, 73).

Radioiodide uptake in thyroid cancer could be increased by stimulation of NIS activity. Experimental therapies aiming at restoring NIS function in thyroid cancer have concentrated only on increasing NIS transcription. However, as mentioned earlier, TSH and I^- are the main regulators of NIS expression and plasma membrane targeting; therefore, high TSH and low I^- levels are optimal for upregulating NIS expression and cell surface targeting. Clearly, elucidation of the mechanisms involved in NIS targeting to and retention at the plasma membrane may result in novel therapies for thyroid cancer treatment, since NIS is overexpressed in 70% of thyroid cancers but not properly targeted to the plasma membrane (20).

Several groups have attempted to induce NIS transcription in non-NIS-expressing thyroid carcinoma cell lines. Transcriptionally inactive promoter regions often contain hypermethylated CpG rich regions (5-methylcytidine immediately followed by guanidine). These methylated sites of the DNA were found to bind specifically to histone-deacetylase complexes. The N-terminal lysines of unacetylated histones are positively charged and interact with DNA phosphates preventing the binding of transcription factors. When the positive charge of the N-terminus is neutralized via acetylation of the lysines, their electrostatic interaction with the DNA is disrupted, making the binding of transription factors to the DNA possible. Therefore, inhibiting histone deacetylase activity and/or demethylating CpG-rich promoter regions would initiate transcriptional activity.

As the hNIS promoter has CpG-rich regions, Venkataraman *et al.* (74) hypothesized that hypermethylation of the hNIS promoter resulting in transcriptional failure could be responsible for decreased or absent NIS expression. These authors were able to restore hNIS mRNA expression in 4 out of 7 cell lines using 5-azacytidine and sodium butyrate treatment. The increase in NIS mRNA transcritpion correlated with demethylation of the untranslated region in the first exon of the hNIS gene. They also investigated NIS mRNA expression by Northern blot and methylation status of the hNIS promoter in proximity to the TATA box in human thyroid tumors. NIS mRNA expression was observed in 16 out of 22 carcinomas, including papillary, follicular, and anaplastic subtypes. These findings suggest that, in these cases, posttranscriptional mechanisms are probably responsible for decreased I^- uptake. In the six non-NIS-mRNA-expressing papillary carcinomas, the hNIS promoter was strongly methylated.

Kitazono *et al.* (75) reported increased NIS mRNA expression detected by Northern blot and quantitative RT-PCR in four human thyroid carcinoma cell lines (two follicular and two anaplastic) *in vitro* after treatment with depsipeptide, a histone deacetylase inhibitor. This increase in NIS mRNA expression was accompanied by an increase in I^- uptake.

Zarnegar *et al.* (76) used another histone-deacetylase inhibitor, trichostatin, in papillary, Hurthle, and follicular carcinoma-derived cell lines, and found increased NIS mRNA expression by quantitative PCR. NIS protein levels and I^- uptake activity were not determined.

Other investigators have tried to achieve redifferentiation of thyroid cancer cells with *trans*-retinoic acid (tRa) treatment in thyroid carcinomas to restore radioiodide uptake. Schmutzler *et al* (77) were able to upregulate NIS mRNA expression by growing

follicular thyroid carcinoma-derived cell lines in media supplemented with 1 μM tRa for one week, but no effect was observed on either protein expression or I^- uptake ability. Surprisingly, the same treatment decreased I^- uptake activity in the highly differentiated FRTL-5 cell line (78).

CONCLUDING REMARKS

The role of thyroidal I^- transport in the treatment of thyroid cancer is difficult to overestimate. For over 60 years, the administration of radioiodide to thyroid cancer patients after thyroidectomy has been the most effective internal targeted anticancer radiotherapy available, on account of the unique specificity of NIS. Radioiodide therapy is not only effective and specific, it is also remarkably free of severe side effects. This article shows how, upon isolation of the NIS cDNA and the characterization of the NIS molecule, considerable strides have been made in our understanding of NIS regulation at all levels, including biosynthesis, biogenesis, half-life, targeting, and subcellular localization. These advances considerably increase our potential ability to manipulate the system to optimize the effectiveness of radioiodide treatment. In addition, the discovery that NIS is expressed endogenously in breast cancer has raised, for the first time, the realistic prospect of effectively applying radioiodide therapy in extrathyroidal cancers that express NIS endogenously. Finally, recent studies on the transfer of the NIS gene to cancers that otherwise lack endogenous NIS expression, have opened the door to the possible use of radioiodide therapy in these cancers as well.

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14. MOLECULAR SIGNALING IN THYROID CANCER

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INTRODUCTION

Molecular signaling – or signal transduction – is central to our understanding of the core biological processes in any type of cancer. Defining the responses of cancerous cells to environmental and endogenous signals, and comparing them to those exhibited by their counterpart normal parental cells can provide valuable insight into the intimate mechanisms underlying malignancy formation, progression, invasion and spread to distant sites (metastasis). Further, detailed knowledge of cancer cell signaling allows us to envisage molecular strategies, upon which novel anticancer therapies will be founded. Ideally, within the context of a particular cancer, our understanding of where and how signal transduction pathways become deranged should enable us to design therapies targeted to the "diseased" elements of the relevant pathway(s). Moreover, the delineation of the evolution of such molecular derangements at each step along the oncogenic transformation process could provide us with the opportunity to intervene at early or intermediate stages of cancer development, i.e., prior to the emergence of irreversible genomic instability, which usually accompanies the transition of a microscopic (or *in situ*) malignancy to the phenotypes of invasive macroscopic carcinoma and metastatic disease (1). In many cases, alterations in molecular signaling in cancer cells are etiologically linked to the oncogenic process. Undoubtedly, the oncogenic potential of a molecule along a signaling pathway can be released through multiple genetic mechanisms (e.g., point mutation or over-/underexpression), ultimately leading to tumor formation. However, it is also true that a number of (qualitative or quantitative) changes

in signaling pathway molecular elements could merely represent an epiphenomenon of the oncogenic process, and, hence, the potential significance of the existing descriptive data on such changes needs to be scrutinized carefully and with due circumspection.

All the above notions are highly relevant to thyroid cancer (TC). Indeed, during the past decade, a rapidly evolving body of knowledge has been accumulated on signaling pathways in TC, and their significance in its pathogenesis and progression. An excellent example of the complexity and interconnectedness of such pathways is the thyrotropin (TSH)-dependent signaling system. Although most of the elements of molecular signaling in TC cells are shared with those existing in normal thyrocytes, some are certainly unique to TC cells, such as protein products of fusion genes (*RET/PTC* and *Pax-8/PPARγ*, as presented in the Chapters 4 & 7 respectively. Additionally, other shared elements that are expressed in both normal and malignant thyroid tissues may be altered in specific ways (e.g., overexpressed or mutated) in malignant thyrocytes, a sound example being protein products of mutationally activated *ras* or *b-raf* genes, expanded upon in Chapter 7. Of note, as the thyroid follicular cell is an endocrine cell, it possesses a wide variety of "identity-specific" signaling systems, which are pertinent to the multitude of its endocrine functions and are correlated with its status of differentiation. Specific alterations in these endocrine function-related systems can accompany malignant transformation (e.g., loss of thyroglobulin [Tg] or sodium-iodide symporter [NIS] expression), and usually coexist with derangements in signaling pathways unrelated to the endocrine character of the cell, as commented upon in the contributions in Chapters 13 & 17.

In this chapter, we make an effort to present the currently accumulated knowledge in this important field by appropriately categorizing the various pathways studied to-date, and summarizing the molecular (known or suspected) derangements along these pathways. We will focus our contribution on carcinomas arising from the follicular epithelial cells (thyrocytes), i.e. papillary, follicular and anaplastic TC's (PTC's, FTC's and ATC's, respectively). Further, we will restrict our review to membrane receptor-associated signaling systems. Intracellular (including nuclear) receptor signaling is also an integral part of cell regulation, as recently highlighted by the role of the Pax-8/PPARγ oncoprotein in FTC's (see Chapter 4) and the presence of functional estrogen and thyroid hormone receptors in PTC's and FTC's (refer to Chapter 9), but we will not comment on this subject as it is exhaustively dealt with by other contributors.

In broad terms, signaling essentially begins with the signal molecule/ligand-sensing receptors, and is based on modulation of the activity of "downstream" pathways (or cascades) that are dependent upon the activation of the aforementioned receptors. In order to place some degree of conceptual order in an unwieldy body of data, we have attempted to categorize signaling in TC cells that occurs via activation of plasma membrane receptors and their downstram effector systems, i.e., (i) G-protein coupled receptors (GPCR's) and associated proteins and (ii) enzyme-coupled receptors and downstream pathway elements. Of note, although ion channels and various symporters (e.g., NIS) are expressed in TC, to-date, the only definitive demonstration of

a functional ion channel-coupled membrane receptor signaling system pertains to the presence of muscarinic acetylcholine (calcium channel-coupled) receptors in immortalized, yet not truly malignant, thyroid cell lines (2, 3). Moreover, signal sensing and propagation in TC cells also occur through miscellaneous, not yet fully elucidated mechanisms, for example, those responsible for responses of thyrocytes to generic environmental cellular insults, such as hypoxia (4) or hydrogen peroxide/reactive oxygen species (5); additionally, it is believed that thyroglobulin (6, 7) or inorganic iodide (8) can initiate specific cellular effects. In this chapter, we will not comment on these mechanisms, the details of which remain unknown as of yet.

The major signaling systems operative in TC cells and their interrelationship with other important elements controlling thyrocyte growth, apoptosis, and differentiation are summarized schematically in Figure 1, shown at the end of the chapter.

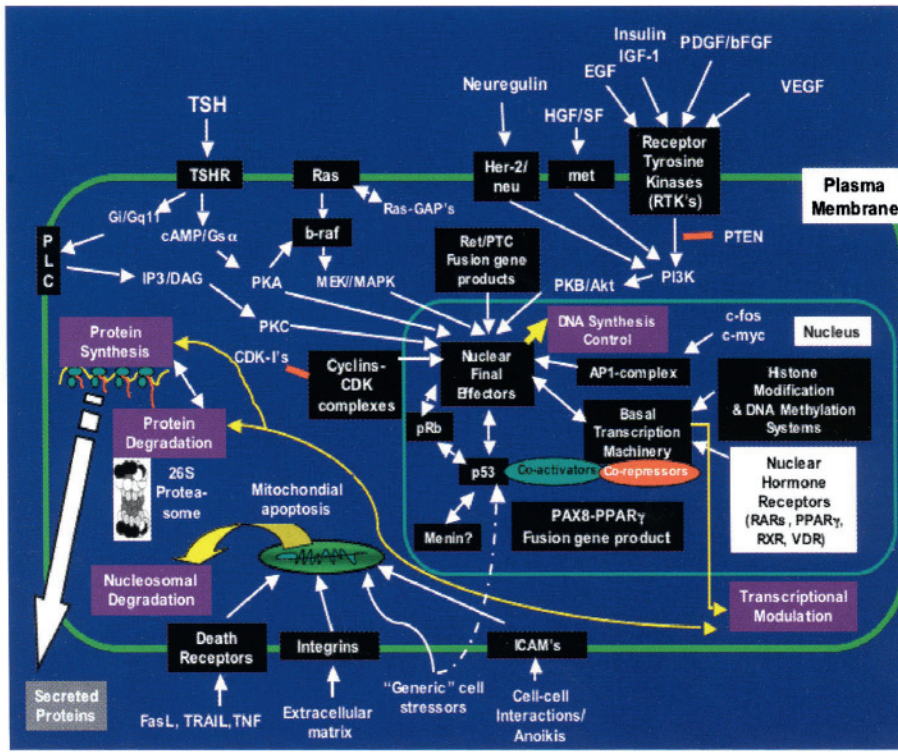

Figure 1. Membrane receptor-dependent signaling pathways involved in the regulation of thyrocyte growth, function, and differentiation. Cross-interacting circuits have been omitted for clarity purposes. Changes in specific elements of the above pathways are intimiately associated with TC development, propagation, "aggressiveness", morphologic phenotype, and acquisition of metastatic potential. For more details, refer to the text. All abbreviations are defined in the text, as well as in the List of Abbreviations. Boxes of different colors designate functional categories of the various molecules or pathways.

ELEMENTS AND PATHWAYS OF MOLECULAR SIGNALING VIA PLASMA MEMBRANE RECEPTORS IN TC

G-protein coupled receptors (GPCR's) systems, associated proteins, and downstream effectors

TSH receptor (TSHR) and G-proteins

The *par excellence* thyroid-specific GPCR is the TSHR. It is a typical member of the GPCR family containing seven-transmembrane (TM) domain receptors, and transduces the signal of ambient TSH to the thyrocyte (9). Its ligand, TSH, upon binding onto TSHR activates thyroid function, proliferation and differentiation via activation of both G-protein- and inositol triphosphate (IP3)/phospholipase C (PLC)-dependent pathways.

The trophic role of the TSHR system is considered highly significant; in fact, suppression of endogenous pituitary TSH production by thyroid hormone treatment in patients with TC following thyroidectomy is considered standard therapy in this malignancy, leading to decreased morbidity and mortality, at least for well-differentiated TC's (as reviewed in [10]). TSH also possesses a lesser, inhibitory effect on TSHR signaling, as chronic stimulation of this system leads to down-regulation of TSHR expression (11). Additionally, TSH exposure can also desensitize TSHR-dependent signaling by activating a G-protein-coupled kinase (GRK), which phosphorylates TSHR (12). The phosphorylated TSHR then attracts arrestins, which are proteins inhibiting the G-protein dependent signaling cascade (see below) (13, 14). Of note, TSHR expression may be lost (or at least severely diminished) in some TC's; consequently, the growth of these tumors is not expected to be modulated by ambient TSH, i.e., in the clinical context, to respond to TSH-suppressive therapy with exogenous thyroid hormone (15). Although activating TSHR mutations represent the molecular basis of several autonomously functioning benign thyroid adenomas (reviewed in [16] and [17]), such mutations have only very rarely been reported in TC (18–20). Indeed, most authorities believe that TSHR-activating mutations play a minimal role in the development of TC (21, 22). Transfection of TSHR in cell lines derived from ATC's -and consequently transplanted in nude mice- has led to decreased growth rate and tumor formation *vs.* wild type (non-transfected) cells (23), suggesting highly complex interactions of the TSHR signaling system with other pathways gorverning cell growth, apoptosis and differentiation in TC.

The TSHR is associated with submembranic proteins, the G-proteins, which are responsible for the downstream delivery of its signal. Each G-protein consists of an α–subunit and a $\beta\gamma$–subunit dimer. The predominant type of the α-subunit in thyrocytes is the $G_s\alpha$ variant. In the resting state, G-proteins bind guanosine diphosphate (GDP) via their α-subunit, while their three subunits α, β and γ are tightly bound in a trimer configuration. Upon binding of TSH on TSHR, GDP is dissociated from the α-subunit and replaced by GTP, thus leading to dissociation of the subunit from the β– and γ– subunits, and the formation of an active α–subunit. The latter binds to "downstream" effectors and modulates their action, primarily activating adenylyl cyclase (AC), leading to cyclic adenosine 3′,5′-monophosphate (cyclic AMP or cAMP)

production (see below). The subunit possesses intrinsic GTPase activity, hydrolyzing bound GTP to GDP, thus, rendering itself inactive. In that state, the α–subunit is able to re-associate with the β– and γ–subunits, rendering the conformation of the αβγ complex to its "baseline" inactive state, and, thus, terminating the action of the ligand (TSH) (24). Other submembranic moieties, such as the RGS proteins (*R*egulators of *G*-protein Signaling), are capable of accelerating the hydrolysis of GTP to GDP, also enhancing the kinetics of termination of the hormone signal (25). Hyperactivity of $G_s\alpha$ can ensue from mutations at either of two "hot spots", i.e. residues Arg201 or Gln227. In a functional sense, these are inactivating mutations, as they abrogate the enzymatic GTPase function or alter the affinity for GTP/GDP, the final result being "locking" the G-protein in its GTP-bound active conformation. At this point, G_s becomes an oncogene or *gsp* (*G*-stimulatory *p*rotein).

$G_s\alpha$ mutations have been described in some autonomously functioning thyroid benign adenomas (26), but seem to be extremely rare in TC (27). The above observations are corroborated by the fact that the thyroid manifestations of McCune-Albright syndrome, a sporadic genetic disease caused by a post-zygotic activating mutation of the $G_s\alpha$ gene (28), classically have included benign thyroid neoplasms (mainly follicular adenomas), and not TC (29). Interestingly, however, clear-cell TC has been recently described in a single case of a patient with McCune-Albright syndrome. In this patient's tumor, malignant thyrocytes enriched for the mutant allele for $G_s\alpha$ (30, 31). Furthermore, in transgenic mouse models, sustained activation of the TSHR/G_s pathway generated by expression of mutant $G_s\alpha$ (32), cholera toxin (33), or a G_s-coupled A2 adenosine receptor (34) caused thyrocyte hyperplasia and hyperfunction, but not cancer, suggesting that this pathway alone is not sufficient to cause malignant transformation.

In addition to stimulation of $G_s\alpha$, TSHR activation also induces stimulation of G_i, as demonstrated by TSH-dependent inhibition of AC in certain *in vitro* systems. This inhibition partially opposes the stimulation of AC through $G_s\alpha$, and can be relieved by pertussis toxin (35). Activation of the G_i/G_0 system via stimulation of the P2-purinergic receptor by extracellular adenosine triphosphate (ATP) in thyroid FRTL-5 cells induces activation of phospholipase A_2 (PLA_2) (36). PLA_2 hydrolyzes the *sn*-2 ester bond of cellular phospholipids, producing a free fatty acid and a lysophospholipid, both of which are lipid signaling molecules. The activation of this signaling pathway is a point of "cross-talk" with pathways activated by mitogens and growth factors (see below). The free fatty acid produced is frequently arachidonic acid (AA, i.e., 5,8,11,14-eicosatetraenoic acid), the precursor of the eicosanoid family that includes prostaglandins, thromboxanes, leukotrienes and lipoxins (reviewed in [37]). It remains unknown whether this G-protein-dependent PLA_2 activation is operative in TC cells. Moreover, TC cells express in variable amounts prostaglandin-endoperoxide H synthase-2 (38), the enzyme responsible for the generation of prostaglandin H2 (PGH2) by arachidonic acid. Of note, PGH2 and its metabolite thromboxane A2 (TXA2) has been implicated in cancer progression and emergence of metastatic potential in assorted malignancies (39). With regard to the role of other G-proteins in thyroid oncogenesis, although it was initially suggested that neither G_q nor G_{11} played a critical role in TC

development (40), subsequent studies showed that a novel $G_{q/11}$-coupled receptor, the metastin receptor, is overexpressed in PTC, and activates the mitogen-activated protein kinase (MAPK) in the ATC-derived cell line ARO. Of note, metastin signaling does not involve Akt/protein kinase B (PKB) (41). No data exist on the role in TC development of other G-proteins to which the TSHR couples, such as G_{12}, or G_{13} (42).

Finally, proteins known as arrestins have been shown to exert potent regulatory control upon GPCR-dependent signaling, usually inducing termination of G-protein activation. Thus, changes in their expression level or activity can have profound effects upon TSHR signaling (43). The amount of β-arrestin-2 was found to be increased in hyperfunctioning "hot") thyroid nodules, but decreased in hypofunctioning ("cold") thyroid nodules, as compared to the neighboring perinodular, normal-appearing tissue (44). For more details on other regulators of the intrathyrocyte cAMP levels within the context of the complex TSHR-dependent signaling system, we refer the reader to two recently published excellent reviews on this subject (45, 46).

Adenylyl cyclase, protein kinase A, and CREB

As alluded to above, TSHR signaling eventually leads to AC activation and production of cAMP. A subset of TC's has been shown to manifest increased AC activity. Earlier studies had shown that this phenomenon was not due to an increase in the number or affinity of the TSHR's (47), but to quantitatively greater amount of G-proteins in TC cells (48). Of interest, the exact opposite phenomenon has been observed in a subgroup of TC's, i.e. decrease in the cAMP production capacity of the malignant *vs.* normal thyrocytes, probably due to defective "coupling" of the TSHR to the G-protein in these tumors (49). Along the TSHR-dependent cascade, cAMP activates protein kinase A (PKA).

PKA belongs to a large family of proteins, whose members are heterotetramers, consisting of two regulatory (R-) subunits and two catalytic (C-) subunits. When the protein is in the fully complexed configuration (i.e., exists as the tetramer), it is inactive. Binding of cAMP to the R-subunits causes dissociation of the C-subunits. Once liberated from the inhibition of the R-subunits, the catalytic sites on the two dissociated C-subunits phosphorylate serine and threonine residues of various acceptor proteins, both in the cytoplasm and the nucleus. In the nucleus, the acceptor proteins are transcription factors, able to modulate the rate of DNA transcription of cAMP-responsive genes (50) (see below). Moreover, several PKA variants are themselves able to translocate to the nucleus, in order to exert their actions. Because there are four R-subunit isoforms (RI, RI, RII, RII), and three C-subunit isoforms (α,gβ, and γ), assorted combinations of holoenzyme complexes with different functional properties exist, a feature that confers considerable complexity to the cAMP/PKA signaling system. Although there is paucity of activating mutations of PKA C-subunits in TC (51), inactivating mutations of the PKA RIα subunit gene, which – when they occur in the germline – are associated to a rare multiple neoplasia syndrome named Carney complex (52), has been identified in a subgroup of TC's (53). It is believed that in these malignancies, the PKA RIαgene (*PRKAR1A*) seems to be functioning as a tumor suppressor.

In turn, TSH is also able to control the magnitude of its own signal along this pathway, by increasing the activity of the cyclic nucleotide phosphodiesterases (54, 55), as well as the expression level of certain PKA-anchoring proteins on subcellular organelles (56), events which prevent nuclear entry of PKA (56).

In thyrocytes, PKA phosphorylates a number of substrates, including the p85 phosphoprotein, leading to enhancement of the interaction between phosphatidylinositol-3-phospokinase (or PI3 kinase, PI3K) and p21/Ras (see below). Simultaneously, cAMP can also inhibit Raf1 kinase signaling by decreasing Raf1 availability to Ras. Hence, under conditions of strong cAMP activation, it is believed that PI3K-dependent signaling is favored (57). In addition to the above moleculart interactions, activated PKA also phosphorylates other proteins with fundamentally important functions, such as CREB (*c* AMP-*R*esponsive *E*lement *B*inding protein). CREB is a nuclear transcription factor that belongs to the large family of leucine zipper (b-ZIP) DNA binding proteins. CREB binds to cAMP response elements (CRE's) on the promoter of cAMP-responsive genes. CREB expression is reduced in human "toxic" thyroid adenomas (58), but unchanged in non-functioning (scintigraphically "cold") thyroid adenomas, when compared to normal thyroid tissue (59). On the other hand, CREB expression is markedly downregulated in TC, but this decrease in the amount of CREB seems unrelated to the functional state of differentiation of the malignancy, as assessed by NIS expression levels (60). Additionally, PKA phosphorylates the following nuclear target substrates, which – of note – are all CREB analogues: inducible cAMP early repressor (ICER; a.k.a. *c* AMP-*R*esponsive *E*lement *M*odulator or CREM), and activating transcription factors (ATF)-l and -3. (60). Phosphorylated CREB binds to a protein called CREB-binding protein (CBP), which in turn interacts with the transcription factor TFIIB to recruit RNA polymerase II onto the preinitiation complex, thus promoting transcription of defined genes (reviewed in [61]). Although PKA can also also directly phosphorylate nuclear transcription factors AP-2 and junD (62), the role of these PKA-dependent effectors in TC initiation and progression remains unknown to-date.

Finally, TSH-dependent cAMP accumulation can lead to phosphorylation of high mobility protein (HMG)-14, which is a nuclear factor associated with transcriptionally active chromatin, thus altering its interactions with nucleosomes (63). The role of HMG-14 on transcriptional regulation has been recently reviewed in (64).

Phospholipase C and protein kinase C

TSHR activation can also result in stimulation of pathways dependent on the γ-isoforms of phospholipase C (PLC-γ), via coupling of the receptor to members of the $G_{q/11}$ family (34). The level of plasma membrane PLC activity is increased in neoplastic thyroid tissue, with the level of increase correlating to the degree of tumoral de-differentiation (65). PLC stimulation results in hydrolysis of phosphatidylinositol 4,5-biphosphate (PIP_2), which generates IP_3 and diacylglycerol (DAG). IP_3 increases the concentration of intracellular ionized calcium (iCa^{++}) by favoring its release from the endoplasmic reticulum, while DAG activates protein kinase C (PKC). In turn, PKC, which under certain conditions can also activated directly by iCa^{++}, phosphorylates

several target proteins (66). With regard to PKC itself, a naturally occurring inhibitor of the PCK isoenzyme PKCε has been found in one TC cell line (WRO), whereas transfection of normal thyroid cells with this inhibitor, generated a neoplastic phenotype (67). Moreover, a selective impairment in PKCε was noted in several human PTC's and FTC's (68). Both these observations seem to be consistent with a protective role of PKC against the development of TC. In general, activation of the PKA pathway in thyroid tissue antagonizes the PI3-iCa^{++}-PKC pathway. A pertinent example of this antagonism in TC cells is the differential effects of the activation of PKA *vs.* PKC on the expression of two transcription factor proto-oncogenes, namely *c-fos* and *c-jun* (69).

Miscellanea

There are at least another four TSH-independent G-protein-mediated signaling pathways that could be putatively active in TC. These signaling systems are progressively better characterized, and are dependent on the following receptors: the adrenergic receptor (AR), the P1-purinergic (adenosine) receptor, the endothelin (ET) receptor, and (possibly) the ghrelin receptor (GHS-R1a).

In more detail, the expression level β2-AR is increased in TC, in proportion to the extent of de-differentiation (70). The functional significance of the β-AR system in controlling TC growth remains unknown, although it has been suggested cAMP overproduction due to preferential activation of the β-AR *vs.* the TSHR system may lead in growth inhibition in selected TC cell lines (71). Normal thyroid tissue also expresses α1-AR and α2-AR (72, 73). Constitutive activation of α1B-AR in transgenic mice leads to TC development with invasive features, probably via simultaneous activation of AC and PLC (74).

Regarding the effects of adenosine on cAMP accumulation in thyroid cells, such effects have been shown in earlier studies (75) and have been subsequently verified (76). Further, transgenic mouse models of thyroid hypertrophy have been developed with targeted overexpression of the adenosine-A2a receptor (reviewed in [77]). The above notwithstanding, the exact role of the adenosine receptor signaling system in native human TC's remains unknown.

ET-1, a potent vasoconstrictor, mitogen, and infammation mediator, also has effects upon thyroid cells. These were initially thought to be independent of cAMP or cGMP generation, and were mainly expressed as an ET-1-dependent decrease in Tg production *in vitro* (78). More recent studies showed that ET-1, secreted by porcine thyrocytes, was acting in an autocrine manner to inhibit TSH-induced iodine metabolism (79). It is assumed that in TC the two major subtypes of ET receptors, i.e., ET_AR and ET_BR, are coupled to their cognate effector systems by several distinct types of G-proteins, as is the case in other malignancies (reviewed in [80]), although this issue has not been specifically addressed in TC systems. Very recently, ET-1 and ET_AR overexpression has been observed in TC, commensurate with a mitogenic role of ET-1 (81).

Ghrelin, the endogenous ligand for the growth hormone secretagogue-receptor (GHS-R1a) (82) is also present in minute amounts in normal fetal (yet not adult) thyroid tissue (83). Ghrelin protein and mRNA are present, in variable amounts, in both

benign and malignant thyroid tumors. TC tissues expressed ghrelin binding sites, and ghrelin induced dose-dependent inhibition of growth in TC cell lines. Nevertheless, specific GHS-R1 a expression has not been convincingly demonstrated in either benign or malignant thyroid tissues (84).

Finally, receptors for both glucagon (85), and vasoactive intestinal peptide (VIP) (receptor subtype VPAC-1) (86) have been identified in normal thyroid tissue, but (to-date) not in TC. At this point, there is a paucity of data regarding the expression of other neuropeptide receptors in either normal or neoplastic thyroid tissue.

Enzyme-coupled membrane receptor systems

Receptor-tyrosine kinases (RTK's) and downstream effectors

RTK's are membrane receptors that have intrinsic tyrosine kinase (TK) activity and, hence, can independently phosphorylate tyrosine residues. These receptors can phosphorylate downstream intracellular substrate proteins or can undergo auto-phosphorylation. These modifications can result in the provision of docking sides for a variety of SH2-domain – containing proteins, which then bind either onto to the phosphorylated RTK itself or phosphorylated downstream effectors (87). Downstream molecules along RTK-dependent pathways include the elements of the Ras – *M*itogen-*A*ctivated *P*rotein (MAP) kinase (MAPK) pathway, the PI3 kinase (PI3K)-protein kinase B (PKB)/Akt system, phospholipase C-γ (PLC-γ), and GTPase-activating proteins (GAP's) (Reviewed in [88]).

SIGNALING DEPENDENT ON RECEPTORS FOR INSULIN AND GROWTH FACTORS. Insulin and insulin-like growth factor-1 (IGF-1) acting via the IGF-1 receptor (IGF-1R) synergize with TSH to promote follicular cell growth (89). Several patterns of expression have been also reported for IGF-1-binding proteins (IGFBP)-1 and -4 in thyroid tumors, thus adding further layers of complexity in the IGF-1 signaling pathway in thyrocytes (90). Of note, recent studies in transgenic mouse models of TC have suggested an important role of IGFBP-3 and –5 in the development of benign thyroid nodules, possibly through paracrine mechanisms (91). IGF-1R's are overexpressed in well-differentiated PTC's, but not in poorly differentiated TC's or ATC's, whereas insulin receptors (IR's) are greatly overexpressed in all TC histologic types, with a trend for higher values in de-differentiated tumors. As a consequence of IR overexpression, high amounts of IR/IGF-1R hybrid receptors, which bind IGF-1 with high affinity, have been detected in TC (92). Further, a novel autocrine loop involving IGF-2 and the IR-A isoform has been reported recently (93). Recently, IGF-1 has been shown to increase the expression of vascular endothelial growth factor (VEGF) in TC cells, via both AP-1/ hypoxia inducible factor-1α (HIF-1α)- and PI3K-dependent mechanisms, with obvious implications on the role of IGF-1 in peritumoral angiogenesis (94).

Several other signal molecules (growth factors), such as basic fibroblast growth factor (bFGF) (95, 96), VEGF (97), epidermal growth factor (EGF) (98, 99), and transforming growth factor-α (TGF-α) (100) have also been shown to stimulate thyroid epithelial growth, while nerve growth factor (NGF) (101) seems to inhibit such growth. EGF,

well as phorbol esters, activate the PKC pathway, and may either stimulate thyrocyte growth or antagonize the effects of TSH, depending upon the system studied (99, 100). Although TC cells may rarely produce colony-stimulating factors (CSF) (102), to-date no cognate receptors for these factors (CSF-1R or FMS, the protein product of the *c-fms* proto-oncogene) have been identified in either normal or neoplastic thyroid tissue (103). Various reports have proposed the role of activating mutations, amplification or abnormal glycosylation patterns of assorted RTK's, including EGFR, various subtypes of NGFR, and platelet-derived growth factor receptor (PDGFR) in TC, which have been summarized in a recent review (104).

TC cells also express a variety of other RTK's, including c-erbB-2/HER-2/neu (104), FGFR-1 (or Flg) and FGFR–3 (105, 106), hepatocyte growth factor receptor (HGFR)/c-met (107), a spliced variant of c-ret (108), NTRK-1 (a.k.a. trkA, or p75/LNGFR) (109, 110), and VEGFR (existing in various subtypes and isoforms thereof, e.g. KDR, Flt-1, Flt-4, Tek, etc.) (111, 112). Specifically for the type-1 VEGFR (Flt-1), the level of its expression has been found to correlate with size in pediatric patients with PTC (113). With regard to HGFR expression, occult PTC's ("microcarcinomas") have been shown to overexpress this receptor, thus indicating that HGFR signaling may be involved in early stages of PTC formation (114), as well as also be associated with multicentricity, a common feature of PTC's (115). Interestingly, the level of HGFR expression was inversely correlated with the tendency of the tumors for angioinvasion (116), as well as risk for metastasis and clinical recurrence (117). Recently, the HGFR system has been shown to be universally and specifically active in PTC's, with STAT3 (rather than PI3K-dependent molecules) utilized preferentially as the intermediate effector molecule (118). Further, the levels of another RTK, KIT, the stem-cell factor receptor (SCFR, i.e., the protein product of the oncogene *c-kit*) (119) have been shown to be decreased in TC's, predating the decrease in thyroid-specific markers observed during de-differentiation (120, 121).

The progressive elucidation of the initial and intermediate steps along the RTK-dependent signal transduction pathways have led to the identification of the Grb2 adaptor molecule and Sos protein, a nucleotide exchange factor, as well as other downstream molecules, such as Shc, FRS-2, and insulin receptor substrate-1 (IRS-1) (122). These discoveries have led to new hypotheses on the tumorigenic potential of activating mutations of such molecules in TC, as well as their "cross-talk" with other oncoproteins, such as the RET/PTC molecules (123).

RAS AND THE MAPK KINASE/MAPK PATHWAY. Ligand-induced stimulation of RTK's results in the activation of the Ras—*M*itogen-*A*ctivated *P*rotein (MAP) kinase pathway, via modulation of Shc and Grb2/Sos (see above). Ras is the protein product of the *p21/ras* oncogene and has intrinsic GTPase activity. In its activated form, Ras is able to recruit other kinases to the cell membrane, where they are in turn activated themselves. This leads to transmission of the growth signal to the genome via nuclear transcription factors (reviewed in [124]). Indeed, Ras leads to activation of the Raf kinase family (including Raf-1 and B-raf), and subsequently the successive activation of MAPK kinase (MAPKK or *M*AP/*E*RK *K*inase [MEK]), MAPK (or *E*xtracellular signal-*R*egulated *K*inase [ERK]), and p90 *R*eceptor-activated *S*ignal Cascade *K*inase

(p90/RSK) (reviewed in [125]). The latter intermediate enzymes ultimately transmit the growth signal to final nuclear effectors, which remain largely unknown, although MAPK activation has been shown to modulate the activity of the AP-1 complex (*c-fos/c-jun*), the *c-myc* gene (126), and members of the Ets family of transcription factors (127) in assorted (non-thyroid) cell systems.

The nuclear transcription factors/oncoproteins c-fos and c-myc do not seem to play a significant role in the initial stages of thyroid oncogenesis, as no mutations, deletions, rearrangement, or amplification involving these genes have been described to-date in TC (128, 129). The level of expression of c-fos seems to be increased in PTC's and FTC's (130). The oncogene *c-myc* is a reliable proliferative marker in a variety of neoplasms, hence, the level of its expression in TC has been positively correlated with the degree of tumor de-differentiation, as well as poor prognosis (130). The oncogene *ets-1* is also expressed in various types of TC in the context of both human tissue and cell lines (131). More recently, it has been shown that induction of ETS-1 and ETS-2 oncoproteins is required for thyroid cell transformation, and leads to modulation of *c-myc* activity (132).

Ras and selected RTK's can also lead to activation of p38/MAPK, a serine/threonine kinase responsible for the mediation of stress-activated cell responses, such as "generic" responses to ionizing radiation, ultraviolet light, heat, and osmotic shock (reviewed in [133]). Of note, the p38 MAPK kinase pathway can be activated by cytokines and other inflammatory mediators, although the isoenzymes involved are different than those pertinent to the Ras/RTK-induced activation. The final effectors of p38 MAPK signaling include ribosome protein S6 kinase-B (RSK-B), as well as nuclear proteins, such as CREB and ATF (134). The p38-α isoenzyme is expressed in normal thyroid tissue (135). Although activation of this isoenzyme though Ras, RTK's and cytokines is suspected in TC, it has not been demonstrated firmly. Interestingly, p38/MAPK is activated by TSHR in immortalized thyroid cells via a cAMP/PKA/Rac1 pathway (136).

The significance of the ras signaling pathway in TC is reviewed extensively herein in Chapter 7. We would just like to mention that downstream from ras are the Raf kinases, with main representatives being Raf-1 and b-Raf (the product of the *BRAF* gene). Very recently, mutations in the *BRAF* gene have been demonstrated as the most common genetic change in the tumors of adult patients with PTC. A single type of mutation was found, leading to mutation of the Val599 residue (137), and formation of the $BRAF^{V599}$ oncoprotein. Additionally, BRAF mutations have been found in ATC's and poorly-differentiated variants of PTC's (thought to emanate from de-differentiation of pre-existing PTC's), but not in FTC's (of any histologic grade) or follicular adenomas (138). These recent findings indicate that *BRAF* activation may be one of the key determinants of the expression of the PTC phenotype.

SIGNALING DEPENDENT ON PHOSPHOLIPIDS AND OTHER LIPID MOIETIES. In addition to activation of the Ras-MAPK pathway, RTK's can also activate PI3K signaling, leading to the generation of D-3-phosphoinositides, such as phosphatidylinositol-3,4,5-trisphosphate (PIP3) (reviewed in [139]). A fact that renders the picture more

complex is that PI3K can also be stimulated by both integrin-dependent cell adhesion and GPCR's (139); this type of PI3K activation has not been studied to-date in TC. D-3-phosphoinositides are able to recruit a subset of signaling proteins with pleckstrin homology domains (PHD's) to the submembranic space, where they are activated. Examples of such proteins include: Akt/Protein Kinase B (PKB), PDK1, intracellular protein tyrosine kinases (such as the members of the Tec family), GTP-binding "exchange factors" (such as Grp1 and Rac), and numerous Ras-associatcd adaptor proteins (such as GAB-1) (reviewed in [140]). Ultimately, these proteins initiate complex sets of events that control protein synthesis, actin polymerization, cell survival, and cell cycle entry.

In regards to the modulation of PI3K-dependent signaling, the following comments are pertinent: The *PTEN* (or *MMAC1 /TEP1*) gene encodes a dual specificity phosphatase with high homology to tensin, which appears to function as a tumor suppressor in patients with Cowden disease (141). The latter is an inherited syndrome characterized by the development of a wide variety of malignancies and hamartomas of ectodermal, mesodermal, and endodermal origin. Patients with Cowden disease characteristically develop FTC's (142). *PTEN* appears to negatively control the PI3K signaling by dephosphorylating the D-3-position of phosphoinositides (143). The *PTEN* gene is inactivated in up to 10% of sporadic follicular neoplasms, including a similar proportion of sporadic FTC's (144). Based on the background presented above, inactivation of *PTEN* should lead to enhancement of PI3K signaling. Indeed, this is the case in Cowden disease. In the context of sporadic TC's, increased levels of phosphorylated total Akt were identified in FTC's, but not PTC's, when compared with normal tissue. Levels of Akt-1 and -2 proteins and Akt-2 mRNA were elevated only in FTC's. Additionally, in assorted TC cell lines, Akt-1,-2, and -3 proteins were expressed, total Akt was activated by insulin (via PI3K), and inhibition of PI3K activity reduced cell viability, suggesting that Akt activation may play a significant role in the etiopathogenesis and/or progression of TC (145). Interestingly, although overactivity of Akt (through constitutive myristoylation) leads to a definite growth advantage, it does not seem to be sufficient for the induction of thyrocyte de-differentiation (146).

Activation of the PI3K pathway results in putative second lipid messengers other than glycolipipids, i.e., sphingolipids. In assorted non-thyroidal malignancies, various growth factors can effectively induce sphingomyelinase activation, and subsequent sphingomyelin cleavage to yield ceramide and phosphocholine. In turn, these molecules can exert complex cellular actions (reviewed in [140] and [147]). In human TC cell lines, exogenous C2-ceramide was capable of activating *c-JUN N*-terminal *K*inase (UNK), leading to apoptosis (148). More recently, lysophosphatidic acid, via binding to its cognate high-affinity receptor edg 4 (a GPCR), has been reported to promote growth in thyroid cell systems, in synergism with TSH. Significant overexpression of edg4 has been observed in both PTC's and FTC's *vs.* normal or goitrous thyroid tissue (149).

SIGNALING VIA FUSION ONCOPROTEINS WITH TK ACTIVITY. Although formally not a part of membrane-associated RTK systems, TC cells express specific intracellular fusion proteins that are produced in an aberrant fashion and possess TK activity. These

are the protein products of the *RET/PTC* and *trk* oncogenes. The pathways dependent on these oncoproteins and their significance in TC are reviewed extensively in the Chapters 11 & 12, as well as in (150) and (151). Of note, activated RET/PTC and Trk proteins can interact with both Shc and Grb2 adaptor proteins, thus establishing "cross-talk" with RTK-dependent signaling cascades (152).

Tyrosine kinase (TK)-associated receptors (TKAR's) and downstream effectors

TKAR's represent a large family of receptors, which lack an intrinsic TK domain, but are able to activate various intracellular TK's following ligand binding, thus leading *indirectly* to the phosphorylation of downstream target substrates.

CYTOKINE SIGNALING AND THE JAK-STAT PATHWAY. The major TKAR-dependent systems pertinent to thyroid growth and differentiation are those related to cytokine signaling. This is a rapidly expanding field, but suffice it to say that an ever-increasing list of interleukins (IL's) is expressed or secreted by TC cells in *in vitro* systems (153–158), with their secretion being modulated by retinoids (159) or TSH (160) in selected cases. These immune peptides have important actions upon the regulation of cell cycle entry, induction of mitotic arrest in TC (161, 162), and modulation of TC cell responses to cytotoxic agents (163).

Activation of cytokine receptors leads to phosphorylation and activation of Janus kinases (Jak), usually via prior phosphorylation of the gp130 glycoprotein. Jak's subsequently phosphorylate and activate differentially members of the signal transducer and activator of transcription (STAT) proteins, eventually resulting in the regulation of transcription of specific genes, along a cascade known as the Jak-STAT pathway (reviewed in [164]).

Specifically for TC, IL-6 and its receptor (IL-6R) have been shown in less than 50% of PTC's studied (118). Interestingly, the downstream effector of the IL-6R system, STAT-3, seemed to be much better correlated with HGFR (MET) expression in these PTC's (118). At any rate, STAT-3 activation may be a great part of the mechanism relevant to the establishment of the specific morphologic phenotype in PTC's, as it is present in virtually 100% of these malignancies (118). Following activation by phosphorylation, STAT's translocate to the nucleus (165), where they can bind to three different classes of DNA sequences, IL-6-like cytokine response elements, interferon-activated sequences (GAS) and *sis*-inducible elements (SIE's) (166) on specifically targeted gene promoters.

TNF AND APOPTOSIS-RELATED MOLECULES. Closely related to TKAR's are the members of the tumor necrosis factor (TNF) receptor (TNFR) family. These heterotrimeric receptor systems are responsible for induction of apoptosis (or programmed cell death), and, hence, are also collectively known as "death receptors". Downstream effectors of the death receptor-dependent signaling cascades include the following (reviewed in [167] and [168]): (i). the adaptor molecules, which are interacting directly with the death receptors via pairs of molecular domains, known as the death domain (DD), death effector domain (DED), and caspase recruitment domain (CARD). Examples of adaptor molecules include: the Fas-associating death domain protein/mediator of receptor-induced toxicity-1 (FADD or MORT1), the TNFR1-associated death

domain protein (TRADD), and the receptor-interacting protein (RIP). Activation of the adaptor molecules leads to subsequent conversion of the pro-enzyme of caspase-8 (a.k.a. FLICE) to its active form (see below). This pathway can be blocked by FLICE-inhibitory proteins (FLIP's); (ii). the caspases, a family of at least 14 *c*ysteinyl *asp*artate-specific prote*ases*, the prototypal members of which is the interleukin-1β-converting enzyme (ICE). Caspases constitute the common final pathway of all apoptotic signals, as they virtually dismantle the cell (leading to cell lysis); and (iii). the proteins of the Bcl-2 (for B-cell lymphoma leukemia-2) family. This family has diverse members that can have either pro-apoptotic or anti-apoptotic properties. Signaling through bcl-2 and other anti-apoptotic members of this protein family eventually involves inhibition of caspases, thus leading to promotion of cell survival (169).

The role of these receptor systems in apoptosis regulation in thyroid tissues has been recently reviewed exhaustively (94, 170). Without delving in depth, the following points are pertinent: TNF-α is detectable in PTC's, although its presence in normal thyrocytes remains debatable (171, 172). In addition, functional receptors for TNF-α have been shown in several TC cell lines (173). Activation of TNFR induces the activation of stress-activated protein kinase (SAPK) (a variant of p38-MAPK) (174), and c-*J* un *N*-terminal *k*inase (JNK) (175). Upon activation, JNK translocates to the nucleus and enhances the transcriptional activity of transcription factors, among which are c-jun and ATF-2 (176).

The significance of thyroidal TNFR-dependent signaling has been dwarfed recently by the crucial ability of death receptors other than the TNFR in controlling thyrocyte apoptosis. Indeed, one of these "death receptors", namely Fas (or CD95/Apo-1), seems to be of seminal importance in promoting apoptosis in assorted cell systems. The interaction of Fas with its ligand (FasL) initiates a number of processes along the pathway of cell death, via activation of caspases (177, 178). Whether normal thyrocytes constitutively express significant amounts of FasL remains controversial (179), while they have been shown not to express Fas (at least in the presence of ambient TSH) (180, 181). The identification of a possible Fas-FasL interaction in thyroid follicles during the development of Hashimoto thyroiditis, leading to thyrocyte apoptosis (179, 182), renders this system of immediate relevance for TC formation. In fact, apoptosis in TC could ensue either via infiltrating cytotoxic lymphocytes or as part of the natural course of rapidly growing tumors outstripping their growth resources. Induction of Fas expression in malignant thyrocytes can lead to their destruction via the coupling of Fas to either its constitutively expressed ligand *in situ* or FasL derived from cytotoxic lymphocytes in the vicinity of the malignant cells (183). FasL has been demonstrated in abundance in both PTC's and FTC's, and may help these cancers evade the immune system by eliminating Fas-sensitive tumor-infiltrating immunocytes. Additionally, FasL may have prognostic implications in PTC, as, when expressed in high levels, it is associated with a more aggressive phenotype in this subtype of TC (94, 184). Of note, recently it has been demonstrated that loss of FADD expression, along the Fas-dependent cascade, is strongly associated with TC development (185). Regarding other death receptors in TC, TNF-related apoptosis-inducing ligand (TRAIL/Apo2L), through cognate TRAIL receptors, has been proven to induce strong cytotoxicity in various TC cell lines (186).

With regard to the final effectors of death receptor signaling in TC, the following comments are of pertinence: Both Bcl-x (anti-apoptotic mioiety) and Bax (pro-apoptotic moiety) have been detected with high frequence in FTC's. In these tumors, the levels of expression of Bax and Bcl-2 are inversely correlated with the degree of cellular dedifferentiation; the converse is true for Bcl-x (187, 188). In contrast, another study showed (paradoxically?) increased expression of Bcl-2 in ATC's, which are by definition undifferentiated malignancies, in which the level of pro-apoptotic moieties would have been expected to be low (189). Thus, the relationship of the expression the final effector molecules of the apoptotic pathways with the degree of TC differentiation has not been firmly established. The expression pattern and significance of other elements of the death receptor pathways in TC, including various caspases and mitochondrial effectors, remain largely unknown.

Serine-threonine kinase (STK) receptors and downstream effectors

The ligands for this group of receptors are members of the transforming growth factor-β (TGF-β) superfamily. The STK receptor-dependent signaling systems play a seminal role during embryonic development, but also participate in adult tissue homeostasis (reviewed in [190]). The expression of several members of this family of ligands, and their cognate receptors, has been identified in both normal and neoplastic thyroid tissues, including TGF-β (191–194), activin (191, 195), and bone morphogenetic proteins (BMP)'s (196).

STK receptors consist of a single TM domain, with STK activity within their intracellular part, which is activated upon receptor dimerization. Two types (I and II) of STK receptors have been described, the only known function of type II receptors being the activation of type I receptors via phosphorylation (reviewed in [197]). The "downstream" signal transduction cascade includes activation (via phosphorylation) of cytoplasmic SMAD proteins (SMAD-1 to -8), which then translocate to the cell nucleus and regulate the transcription of a variety of specific target genes (198). The STK receptor-dependent signaling system is under tight control by multiple other intracellular regulators, as this pathway is not only relevant to transduction of selected hormonal signals, but is also closely implicated in the initiation and promotion of tumorigenesis, as demonstrated *in vitro* for assorted malignancies (reviewed in [199]).

Specifically in TC, loss of the cell growth inhibition induced by TGF-β leads to loss of differentiated phenotype of thyrocytes *in vitro* (200, 201), while SMAD-2 expression is downregulated in FTC's, even in the presence of intact TGF-β receptors (192). In ATC-derived cell lines, despite the great degree of cell de-differentiation, all elements of the TGF-β-dependent signaling cascades have been demonstrated as intact. Nevertheless, in one of the cell lines studied, there was indication of a novel mechanism for TGF-β insensitivity, with escape from its growth inhibiting effects, despite maintainance of expression of TGFR and SMAD proteins (202). A very recent study showed maintenance of normal levels of SMAD-4 in cell lines derived from PTC, FTC and ATC, and upregulation of SMAD-7 in the ATC-derived cell line ARO (203). Regarding the mechanism of TGF-β-induced growth inhibition (eventually leading to apoptosis) in normal thyrocytes, it is believed that it involves reduction in the levels of p27/kip1 (a cyclin-dependent kinase [cdk] inhibitor). This reduction in

p27/kip1 is overridden in malignant thyrocytes by NF-κB activation (204). Of note, p53 also modulates cellular responses to TGF-β in TC *in vitro* systems (205). Finally, TGF-β1 affected IGF-1-stimulated IRS-1 phosphorylation and its association with Grb2 protein, as well as decreased the activation of the adaptor protein CrkII and its association with the IGF-1R, actions that provide the basis for "cross-talk" between RTK- and STK-dependent signaling cascades in TC (206).

COMMENTARY

TC is one of the most well studied endocrine neoplasms at the molecular level. It also expresses a remarkable repertoire of ligands, cognate receptors, and downstream effectors, thus representing a "model malignancy" for the investigation of the molecular pathobiology of cancer cell signaling. In this chapter, we focused specifically on the description of the signal transducing receptors at the level of the cell membrane, their cognate ligands, the post-receptor downstream elements propagating the signal, as well as known or suspected "final effectors" of each pathway. We also presented in global terms the functional connections among various key molecules, as well as the current theories on the relevance of these signaling systems to TC formation, progression, and biological behavior. It is our belief that intimate knowledge of the elements of these systems and their interaction with other constituents of the cell proliferation machinery (cell cycle, protein synthesis and degradation, gene transcription) increases their potential as future targets for therapy, especially in the context of TC patients harbor disease currently refractory to standard treatment modalities.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
AC	adenylyl cyclase
AR	adrenergic receptor
ATC	anaplastic thyroid carcinoma
ATF	activating transcription factor
ATP	adenosine triphosphate
bcl	B-cell lymphoma leukemia
BMP	bone morphogenetic proteins
b-ZIP	leucine zipper
cAMP	cyclic adenosine 3′,5′-monophosphate
CARD	caspase recruitment domain

CBP	CREB binding protein
cdk	cyclin-dependent kinase
CRE	cAMP response element
CREB	cAMP-responsive element binding protein
CREM	cAMP-responsive element modulator (a.k.a. ICER)
CSF	colony-stimulating factor
CSF-1R	CSF-1 receptor (the protein product of the *c-fms* proto-oncogene)
DAG	diacylglycerol
DED	death effector domain
DD	death domain
EGF	epidermal growth factor
EGFR	epithelial growth factor receptor
ET	endothelin
ETR	endothelin receptor
FADD	Fas-associating death domain protein (a.k.a. MORT-1)
FAP	familial adenomatous polyposis
FasL	Fas ligand
(b)FGF	basic fibroblast growth factor
FLIP	FLICE-inhibitory protein
FTC	follicular thyroid carcinoma
GAP	GTPase-activating protein
GAS	interferon-activated sequence
GDP	guanosine diphospate
GF	growth factor
GH	growth hormone
GHS-R1a	ghrelin receptor-1a
GPCR	G-protein coupled receptor
GRK	G-protein-coupled kinase
gsp	G-stimulatory protein
GTP	guanosine triphosphate
HIF-1α	hypoxia inducible factor-1α
HGFR	hepatocyte growth factor receptor (c-met product: MET)
HMG	high mobility protein
iCa^{++}	intracellular ionized calcium
ICE	interleukin-1 – converting enzyme
ICER	inducible cAMP early repressor (a.k.a. CREM)
IFN	interferon
IGF-1	insulin-like growth factor-1
IGF-1R	IGF-1 receptor
IGFBP	IGF-1 binding protein
IL	interleukin
IP3	inositol triphosphate
IR	insulin receptor
IRS	insulin receptor substrate

JAK	Janus kinase
MAPK	mitogen-activated protein kinase
MORT-1	mediator of receptor-induced toxicity-1 (a.k.a. FADD)
NGF	nerve growth factor
NGFR	NGF receptor
NIS	sodium-iodide symporter
PDGFR	platelet-derived growth factor receptor
PGH2	prostaglandin H2
PHD	pleckstrin homology domain
PI3K	phosphatidylinositol-3-phospokinase
PIP_2	phosphatidylinositol 4,5-biphosphate
PKA	protein kinase A
PKB	protein kinase B (AKT)
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PPAR	peroxisome proliferator-activated receptor
PTC	papillary thyroid carcinoma
RGS	regulators of G-protein signaling
RIP	receptor-interacting protein
RSK-B	ribosome protein S6 kinase-B
RTK	receptor tyrosine kinase
SAPK	stress-activated protein kinase
SCFR	stem cell factor receptor (c-kit product: KIT)
SIE	*sis*-inducible element
STAT	signal transducer and activator of transcription
STK	serine-threonine kinase
Tg	thyroglobulin
TGF	transforming growth factor
TC	thyroid cancer
TK	tyrosine kinase
TKAR	tyrosine kinase (TK)-associated receptor
TM	transmembrane
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TRADD	TNFR1- associated death domain protein
TRAIL	TNF-related apoptosis-inducing ligand (a.k.a. Apo2L)
TSH	thyroid stimulating hormone (thyrotropin)
TSHR	TSH receptor
TK	tyrosine kinase
TXA2	thromboxane A2
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide

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15. GENE EXPRESSION IN THYROID TUMORS

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INTRODUCTION

Endocrinologists and pathologists would welcome a simple reliable test of the nature and potential of thyroid nodules at the first encounter. Even with satisfactory fine needle aspirations a definitive cytological diagnosis may not be always be possible and prognostication is thus limited. As discussed in other chapters in this book (2 & 16), several molecular markers examined in surgical specimens have been proposed to be specific for histologic types of thyroid tumors and/or malignancy. None, however, is diagnostic.

In the event the approaches to examine a limited number of molecular markers at a time are by means not robust enough to apply to the cytological harvest of thyroid FNA. Because of the very nature of malignant transformation (see Chapter 1), it is expected that gene products in many cellular pathways would be involved, only some of which turn out to be tissue-specific or tumor-subtype specific.

The exploration of the transcriptome of tumors or normal tissue during development, or following treatment with drugs or hormones holds much promise to the better understanding of physiologic and pathologic processes (1–4).

This chapter discusses the application of this technique to thyroid tumors.

DNA MICROARRAYS

In essence, cDNA or oligonucleotides are spotted onto glass slides, silicon wafers or nylon membranes and are then exposed to florescently-labelled mix of RNA (or cDNA made thereof) from biological specimens. Each DNA latches onto the RNA or cDNA

that matches its sequence. Based on the location and intensity of the signal the source gene and its activity can be detected. Many protocol refinements and software programs have been introduced to ensure internal consistency, reproducibility, statistical analysis, gene annotation and ontological linkages of the huge amounts of raw data (3,4).

THE APPLICATIONS OF DNA MICROARRAYS

DNA microarray is a tool most commonly used to monitor levels of gene expression levels. DNA chip technology can be helpful in documenting DNA copy number, DNA/protein interactions and genetic polymorphisms (4). It holds promise in the search for gene promotor regions and screening DNA/chromosomes for gene expression (4).

One of the first and still most used tools applied to microarray data visualization is hierarchical clustering. In one dimension, sample output is grouped to similarity and in the other according to the overall similarity of expression across samples. An important objective of this approach is to identify similarly regulated genes across specimens examined (3). Sophisticated computational treatment of the data has, however, failed to establish this "guilt by association" as a valid spin-off from microarray analysis (5). An evolutionary approach identifying orthologs that have retained their function goes a long way to answering the criticism of the identification of co-expressed genes by microarrays (6).

Cluster analysis of differentially expressed genes has, nevertheless, been helpful in a number of areas of clinical medicine, particular in oncology (3,7,8). Hierarchical clustering has been helpful in:

1. Tumor classification or subclassification
2. Identification of potentially important genes characterizing a tumor, susceptibility to drugs and metastatic potential
3. Identification of new drug targets to provide new therapeutic tools.
4. Identifying biomarkers for establishing or confirming diagnosis and outcome of therapy.

Microarray technology as currently used has provided novel insight into B-cell lymphoma, breast cancer, melanoma and other human malignancies (2,8–11)

GENE EXPRESSION PROFILING IN THYROID CANCER

A limited number of studies have reported on DNA microarray studies in thyroid cancer: two on follicular tumors, one on papillary carcinoma (PTC) and only one that examined a range of benign and malignant thyroid disease. The numbers of samples analyzed in each study was small to moderate in size. Most of the studies quoted are, however, robust and pass muster for the stringent rules stipulated for reporting of gene expression studies (4).

Papillary thyroid carcinoma

Huang et al. (12) used oligonucleotide DNA chips containing more than 12,000 genes to profile 8 papillary carcinomas and matching normal thyroid tissue. They found the

expression of 8 genes to be suppressed in 7/8 samples and that of 19 genes in 6/8 samples. The genes whose expression was suppressed fell in a number of categories: tumor suppressor genes (e.g. *bcl-2, gas-1 and fos-B*), thyroid metabolism (e.g. *dio-1, dio-2, tpo*), cell adhesion (*dpt and fgl-2*), fatty acid binding (*apo-B* and *fabp-4*) and signal transduction (*stc-1* and *itpr-1*).

24 genes were overexpressed in all 8 papillary thyroid cancer samples and an additional 22 genes in 7/8 specimens. Among the genes found to be overexpressed were several previously reported and include fibronectin-1, the *met* oncogene, *dipeptidylpeptidase IV,α-1-antitrypsin*, *keratin-19* and *galectin 3.* Other overexpressed genes fell in the categories of cellular adhesion/extracellular matrix, cytoskeleton, growth factors and their receptors as well as those involved in signal transduction.

Several genes found to be over-expressed in papillary carcinoma were not previously reported in any neoplasia or the thyroid and include: *ADORA1* (adenosine A1 receptor), *SCEL* (sciellin), *ODZ1* (Odz 1, Drosophila), *PROS1* (vitamin K-dependent plasma protein S), *KIAA0937, CST6* (cystatin E/M), *SDC4* (ryudocan core protein), *P4HA2* (propyl-4-hydroxylase alpha (II) subunit), *DUSP6* (dual specificity phosphatase 6), *TSSC3* (tumor suppressing subtransferable candidate 3). Changes in gene expression of selected genes were confirmed by multiplex semiquantitative RT PCR and consistent and highly correlated patterns of gene expression pattern in tumor samples relative to normal was also verified by hierarchial cluster analysis. The latter aspect of the results was unexpected given the previously noted heterogeneity of individual gene expression in PTC.

Specificity for PTC of two gene products (Cbp/p300-interacting transactivator [*CITED*1] and surfactant, pulmonary–associated protein B [*SFTPB*]) previously associated with other neoplasia was explored by immunoflorescence in a large number archival of PTC tissue and other thyroid malignant tumors (12).

Follicular tumors

Using the same array system as did Huang et al. (12), Barden et al. (13) compared RNA from 10 follicular adenomas with those from 9 follicular carcinomas (FTC), two minimally invasive, one poorly differentiated and one Hürthle cell carcinomas. The authors identified 105 genes whose expression significantly differed between adenomas and carcinomas (overexpressed in one or the other). They found that many previously unidentified genes contributed to the distinction between adenomas and carcinomas. Interestingly, very few of the genes suppressed or overexpressed in follicular tumors were identified as important in the Huang et al. study (12).

The authors (13) chose 5 genes with >3 fold overexpression for further verification of expression by semiquantitative RT-PCR and in the case of one gene, product extracellular matrix metalloproteinase inducer (EMMPRIN), by Western blotting of the extracted protein. The gene products overexpressed in follicular carcinomas compared to adenomas were adrenomedullin, autotoxin, EMMPRIN, transforming growth factor β II receptor and the *met* oncogene. *Met* was previously reported to be relevant to thyroid carcinogenesis (12,14, also see below). Adrenomedullin is important in growth and survival of several human cancers whereas autoxin promotes tumor cell

growth and angiogenesis. EMMPRIN is a surface glycoprotein that is associated with metastatic behavior.

Aldred et al. (15,16) selected genes found to be differentially regulated in 19 FTCs. They chose genes mapping to regions of loss of heterozygozity (LOH), previously reported in FTC (15). They also monitored the downregulation of peroxisome proliferator-activated receptor gamma [PPAR γ] (16). Because of the questions asked, the authors focussed on down-regulated genes. In contrast to the initial study from this group (12), there was not enough material to test the samples in duplicate nor was there enough paired normal thyroid tissues for samples. The authors had, therefore, to respectively normalize their results to mean intensity and to carry out pairwise comparisons between all normal and tumorous thyroid tissue (15).

Three genes coordinately downregulated, caveolin-1, caveolin-2 and GDF10/BMP3b were further studied on the basis of their localization to two chromosomal regions, 7q31.1 and l0q11.1, that commonly show LOH in FTC. The authors also selected for further analysis two additional genes (glypican-3: Xq26.1 and a novel chordin-like:Xq22) involved in bone morphogenesis signalling and possible interaction with *GDF10.* Each of the 5 genes was downregulated in at least 15/19 of samples by RT-PCR. The authors followed in greater detail the relevance of *caveolin-1,* thought to be involved in the regulation of the dual-specificity phosphatase PTEN, suppressed in FTC. They found that it is the β isoform of *calveolin-1* that is specifically downregulated and that the reduced expression is specific to FTC, including insular and Hürthle-cell varieties (15). On the other hand, the expression of the 3 genes involved in bone morphogenesis signalling were downregulated also in benign adenomas and multinodular goitre and glypican-3 in PTC suggesting that they are early events in pathologic thyroid cell growth.

In apparently the same set of tumors, Aldred et al (16) found that whereas only 2/19 FTC exhibited the *PAX8/PPAR*γ re-arrangement (see Chapter 4), the majority (13/17) of the remainder showed greatly reduced expression of PPARγ by microarray and semiquantitative RT-PCR. Reduction of PPARγ immunoreactivity was found not only in FTCs but also in Hürthe cell carcinomas and PTCs. Down regulation of PPARγ is probably related to repression by one or more upstream regulatory protein, as the results could not be explained on the basis of gene deletion, mutation or hypermethylation of the PPARγ regulatory sequences.

A spectrum of thyroid tumors

We have used human cDNA microarray constructed in-house by spotting previously PCR-amplified and purified gene-specific samples to study RNA from a range of thyroid tissues. We examined samples from multinodular goitre (MNG), Graves' disease, Hashimoto's thyroiditis, papillary carcinoma, follicular carcinoma and follicular adenoma and compared these to normal perinodular thyroid tissue. Hierarchical cluster analysis (Figure 1) showed clear separation of various clinical pathological entities according to variation in the expression of 1322 genes, many of which were unidentified at the time.

We selected 26 genes whose expression showed variation in thyroid tumors for further examination using semiquantitative RT-PCR. Given that we used a completely

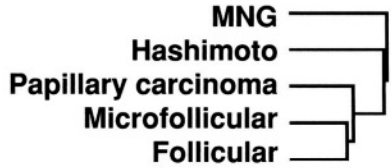

Figure 1. Hierarchical clustering of 1322 genes separates the thyroid diseases studied. Specific clusters were arrived at according to thyroid disease. The closest relatives were mircofollicular adenomas and follicular carcinoma, while papillary carcinoma exhibited some relationship to this subfamily. Multinodular goiter and Hashimoto's thyroiditis were distinct from each other and from other thyroid disease types.

Hierarchical cluster analysis was done using the Omniviz Gene Expression software package (Omniviz Inc.)

different panel of genes than did Huang et al. (12), we found remarkable agreement in genes differentially regulated in PTC. The expression of some of these genes was, however, also regulated in other thyroid tissues studied, thus (and as expected) *met* expression was increased in FTC, as was that of *galacetin3* whereas that of *cartilage glycoprotein3* was overexpressed in adenomas, MNG and FTC. That of Cpb/p300-interacting protein was overexpressed in FTC but 5 fold less in PTC.

Type I iodothyronine deiodinase which is down-regulated in PTC was enhanced in FTC and benign nodular tissue, *CRAB1 cellular retinoic add binding protein* expression was also reduced in adenoma, FTC and MNG but less than in PTC whereas *fatty add binding protein* 4, suppressed in PTC, appears to up-regulated in MNG.

We found the C8FW phosphoprotein, regulated in the thyroid gland by TSH and EGF (17), to be specifically overexpressed in FTC and so was *proprotein convertase subtilisin/kexin 2.* The latter is a furin involved in the processing of prohormones (18) but which is also involved in the digestion of pre-metalloproteases and cadherins and might be involved in tumor invasion and metastatic behavior. S100 calcium binding protein (*mts 1* or metastasin), the subject of intense recent interest in carcinogensis (19), was specifically increased in PTC. By contrast the *small GTPase Rap1* (20), which is involved in the regulation of cell-adhesion and Glycerol kinase 2 (testis specific), a key enzyme in adipose tissue metabolism are increased in FTC.

The 15 Kda selenoprotein (21) whose abundance is modified in transformed cells was increased in PTC but not in FTC and less so in MNG.

We found *secreted frizzled related protein* (22,23), that is involved in the regulation of wnt signalling, to be specifically reduced in PTC. In contrast, the expression of *tight junction protein* 1, important in maintaining cellular polarity (24) was curiously suppressed specifically in FTC. *Eosinophil-derived neurotoxin,* which plays a role in allergic reactions and has an anti retroviral activity is reduced in all tumorous tissues studied (25,26). We speculate that it may be involved in evasion by tumors of immune cells. *Keratin, Type II cytoskeletal 7* important in maintaining cellular structure is reduced in all except follicular adenomas (27). In an independent study (28), we found that the cannabinoid receptor 2 (*CNR2*) as well as *met* to relevant to the metastatic behavior of anaplastic thyroid carcinoma.

CONCLUSIONS AND PERSPECTIVE

The study of gene expression by microarray technology (just as the earlier use of serial analysis of gene expression [SAGE, 29]) has yet to yield the answer to the proposition posited at the beginning of this chapter. While the protocols for performing gene expression are relatively easy, the analysis and interpretation of the results are time consuming and may be difficult to interpret in our current state of knowledge of functional genomics. Independent corroboration of differential expression is necessary by different techniques and, in our opinion, by replication in different sample sets in the same or independent laboratories. Some of the studies reviewed have limited themselves to circumscribed issues e.g overexpression of genes in two sets of tumors one benign and one malignant (13) and generalization from any of the studies is hampered by the small number of samples examined in each. Given the diverse pathways to malignancy it would be naïve to adopt a "candidate gene panel" approach in trying to develop robust tests to diagnose malignancy in a thyroid nodule, predict histology and subsequent tumor behavior and response to therapy.

A concerted multicenter effort in which large numbers (30–50) of thyroid tumors whose histology has been verified by expert thyroid pathologists is necessary to provide us with the data-base that can satisfy our diagnostic needs. The application of statistical methods such as that described by Wright et al. (30), will allow comparison of the results from the bank of tumors proposed above to previously studied tumors whose array results are in the public domain, and from tumors that may be independently studied in the future to hone in on a limited number of genes important in tumor classification, prognostication and adjuvant therapy targeting. The validity of these genes (probably 15–20) can then be prospectively validated in a large series of FNA specimens.

It is also important to reflect in a broader sense that genes overexpressed in a tumor, with poor prognosis for example, may not represent an appropriate target for therapeutic intervention or for targeted drug design. On the other hand, we may have to look to pathways upstream from those genes for the most vulnerable targets, usually enzymes or receptors.

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16. ANIMAL MODELS OF THYROID CARCINOGENESIS

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INTRODUCTION

Thyroid nodules affect approximately 20% to 45% of the population during their lifetime, but only a minority of nodular goiters bear a clinically relevant malignant potential. A simple diagnostic approach solving this problem does not exist. Cytological evaluation after fine needle aspiration obtained from thyroid nodules allows only for the detection of thyroid carcinoma in 80% to 90% of the cases. Thus, better methods predicting the malignant potential of thyroid nodules and/or diagnosing existing malignancies are urgently needed. To solve this problem the first step was designating animal models of thyroid carcinogenesis that help to understand this process in more detail. Last century's investigations in this field showed that the thyroid gland serves as a useful experimental model for understanding tumor formation not only in endocrine systems but, in epithelial tissues in general. Since the mid-1930s, the study of experimental carcinogenesis in rats, mice, hamsters, guinea pigs, sheep and swine has been focus of attention. In vivo, the growth of the follicular epithelium is controlled by a single tropic stimulus, the thyroid-stimulating hormone (TSH), which is secreted by the anterior pituitary gland at a rate dependent on the serum concentration of thyroid hormones (T3 and T4). Inhibition of this feedback loop by reduction or abolition of T3/T4 production leads to an increase in serum TSH. This initially induces an uniform hyperplasia of thyroid follicular epithelium, the first step of tumorigenesis. The discovery of the thyrostatic effect of some naturally occurring substances and the subsequent development of numerous synthetic preparations with the same action

gave an impetus to and provided an opportunity for the systematic investigation of morphological and functional changes in thyroids of experimental animals.

HISTORICAL OVERVIEW

In 1909, McCoy was the first to systematically investigate thyroids of animals. He searched for tumors in 23,000 wild rats, but failed to detect them. Eight years later, Bullock and Rhodenburg identified nine tumors in 4,300 rats. In 1926, Slye at al found 12 so-called carcinomas in 51,700 mice. All these tumors were spontaneous tumors. After the description of goiter in man (Marine 1924), the era of experiments in the field of thyroid carcinogenesis started sporadically with the investigations of Wegelin (1928), Hellwig (1935), and Hercus & Purves (1936). In the following time, each decade was influenced by leading research groups. In the 1940s, Purves, Bielschowsky, Kennedy and Griesbach (New Zealand, Germany) described the role of low iodine intake and continued the investigation of other positive goitrogenic agents. Kennedy, therefore, synthesized a quantity of allylthiocyanate (mustard oil), from a glucoside in mustard seeds, which were also goitrogenic. This compound was too toxic to be fed to rats, but when treated with ammonia, it was converted to allylthiourea and could be incorporated in the rat diet. It was shown that such drugs are goitrogenic, as is a deficiency of iodine, because they block the production of thyroid hormone. In the 1950s-1970s, Lindsay, Chaikoff (USA) and Doniach (U.K.) described the effects of external and internal irradiation on thyroid tumor production and gave evidence of a dose-dependent relationship. In the 1980s, Hesch, von zur Mühlen (Germany) and Dumont (Belgium) searched for tumor-initiating mechanisms through hormone dysbalances (TSH vs. TRH) and detected morphological changes by electron microscopy. Williams and Wynford-Thomas (U.K.) clarified the functional role of TSH and described differential gene expression (*ras*) in experimental thyroid tumors for the first time (Lemoine et al 1988). In the 1990s, Brabant, Dralle and Hoang-Vu (Germany) conducting short-term and long-term studies, investigated the regulatory mechanisms of thyroid tumorigenesis and described the changes of histology, ultrastructure, function, and proliferation in detail. In the present time, experiments performed by Japanese and Russian groups (Hirose, Hoshi, Hiasa, Nadolnik) focus on testing the carcinogenic potential of several parts of nutrition and diverse environmental factors.

THE PROBLEM OF TUMOR CLASSIFICATION

All the difficulties encountered in the classification of human thyroid tumors have to be faced in an attempt to classify thyroid tumors in animals, particularly in the rat. In early experiments, the well-known absence of clear-cut histological criteria for distinguishing reactive hyperplasia from neoplasia or benign from malignant tumorous growth has been reflected in publications describing corresponding lesions in the rat. In most cases, the authors have tried to apply the nomenclature of human pathology to the lesions observed in the rat thyroid. This approach has not only some easily recognizable advantages, but also some disadvantages. On the one hand, the use of similar terms would provide an opportunity for conducting a comparative analysis of

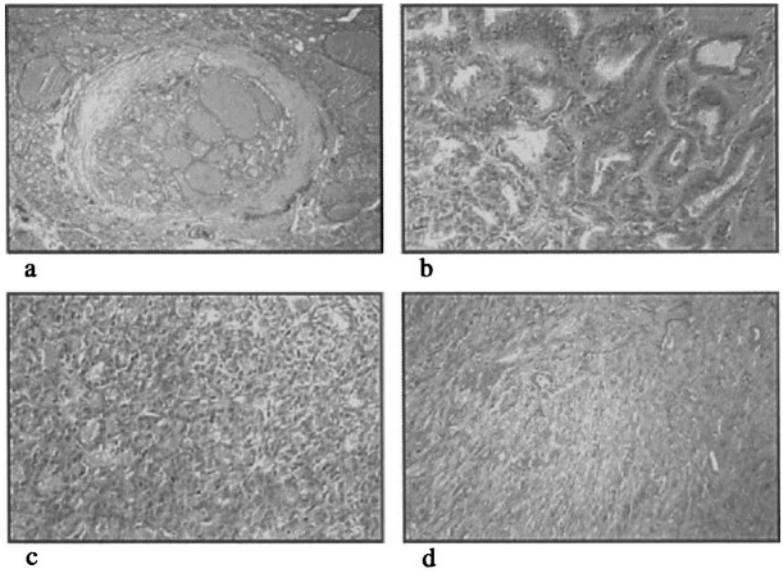

Figure 1. Experimental thyroid tumors in rats induced by x-ray irradiation: Low power view of a follicular adenoma with surrounding capsule (a), high power view of papillary carcinoma (b), follicular carcinoma (c) and squamous cell (epidermoid) carcinoma.

experimental and clinical observations. On the other hand, the same terms might be, and have already been, applied to morphological lesions that are superficially similar, but whose biological behavior is different. The broadest definition applicable to both benign and malignant neoplasms was formulated by Axelrad and Leblond (1955). It fits in with the criteria applied to rat tumors by most experimentalists and can be paraphrased as follows: a pathological change in the rat thyroid can be regarded as a neoplasm when it is focal; it is distinct from the rest of the gland cytologically and architecturally and shows evidence of progressive growth. As to the definition of such terms as "adenoma" and "carcinoma", the wording suggested here is based on the above definition of a neoplasm and on personal experience gained in studying the peculiarities of behavior of rat thyroid tumors.

An encapsulated epithelial neoplasm without evidence of invasive growth or distant metastasis can be considered an adenoma (Figure 1a). A carcinoma in the rat thyroid is an epithelial neoplasm of any histological structure that shows destructive invasive growth, which leads to metastasis to the body (Figure 1b–d). Apart from clear proof of local invasive growth, there is no convincing evidence of malignant growth except the demonstration of metastases. The tendency to overestimate the malignancy of rat thyroid neoplasms and to diagnose them as carcinomas simply on the grounds of their "malignant" appearance is discernable in many publications. The classification given below, was drawn up under consideration of certain data on embryogenesis of this gland, heterogeneity of its epithelial cell population, and the already mentioned

peculiarities of normal growth of the thyroid epithelium. However, to make this classification more practicable and comparable with that of WHO for human tumors microscopic morphology rather than histogenesis has been selected as a basis:

Benign tumors:

Follicular adenoma (including microfollicular, polymorphofollicular and trabecular adenoma), papillary adenoma, simple solid adenoma, light-cell solid adenoma, and squamous cell (epidermoid) cystadenoma.

Malignant tumors:

Follicular carcinoma (including microfollicular and polymorphofollicular carcinoma), papillary carcinoma, solid carcinoma (including small cell, polymorphous solid and light-cell solid carcinoma), squamous cell carcinoma, sarcomas and mixed tumors (carcinosarcoma).

This classification does not include such entities as leiomyoma, hemangioma, lymphoma, teratoma, neurogenous tumors, and some other neoplasms that have been observed in humans and several animal species other than the rat. An attempt has also been made to avoid the use of proper names that have already led to some diagnostic confusion (for instance Hürtle cell tumor or Lindsay tumor). Of the characteristic and predominant histological patterns observed in neoplastic epithelial nodules, the three most common ones (follicular, solid and epidermoid) were selected to designate the main categories of tumors. As mentioned earlier, the proliferation of rat thyroid epithelial cells in solid aggregations must be considered a normal feature. The listed tumors are rarely found in their pure morphological form. Most experimental tumors in animals represent virtually different transitional variations in between these artificially separated entities. The vast majority of follicular neoplasms contain solitary or numerous foci of solid cell nests, and it is the rare solid tumor that does not show areas of follicular structure. An introduction of all the subdivisions covering even the most frequent transitional forms of tumors would make this classification useless. An attempt to classify the endless variety of histological pictures produced by physiological shifts in this correlation is hardly justifiable.

SPONTANEOUS THYROID TUMORS IN ANIMALS

Roe (1965) defined a spontaneous thyroid tumor as a neoplasm that had developed without any influence exerted by internal or external carcinogens. Data on the incidence of spontaneous thyroid tumors in animals are contradictory. McCoy (1909) and Woolley & Wherry (1912) were the first to systematically investigate thyroids of animals. They searched for tumors in 23,000 and 100,000 wild rats, but failed to detect them. In 1917, Bullock and Rhodenburg firstly described nine tumors found in 4,300 rats; in 1926, Slye at al detected 12 so-called carcinomas in 51,700 mice. They are seen more often in laboratory rats, but have a predilection for older animals. Many of these tumors are derived from the C-cell component of the thyroid gland. Lindsay et al (1968) coined the term "naturally occurring carcinoma of the rats thyroid" for these medullary tumors. In the following years, many studies described spontaneous tumors in various rat strains: For example, van Dyke (1944) reported on nine cystadenomas in 16 Wistar rats that died at the age of more than 800 days. According to other reports,

the incidence of spontaneous thyroid neoplasms in Wistar rats is much lower (about 5%; Bielschowsky 1953, van Dyke 1953). In a large group of female Sherman rats kept for more than two years, Axelrad and Leblond (1955) came across only one solid nodule that was composed of light cells. In 1960, Isler et al showed that approximately 40% of female Sherman rats had developed small nodules of light cells at the age of 14 months. In a careful study of serial sections of thyroid glands from Sprague-Dawley rats (mean age 637 days), Thompson & Hunt (1963) observed C-cell tumors in 39% of animals. In contrast, Schardein et al (1968) recorded only 20 follicular adenomas in 5,086 Sprague-Dawley rats. Boorman et al (1972) reported on 123 cases of naturally occurring medullary carcinomas among 334 WAG/Rij rats, 84% of which were older than two years. Lindsay et al (1968) investigated various rat strains and found medullary carcinomas in 19% of Wistar, 22% of Fischer, 22% of Sprague-Dawley, and 40% of Long-Evans rats. Hamsters were also occasionally used for experimental tumorigenesis; Pour et al (1976) found spontaneous thyroid adenomas in 5–10% and carcinomas in 1 % of untreated Syrian hamsters. Summarizing the data of the above-mentioned studies, one can draw the following conclusions: the vast majority of spontaneous thyroid tumors so far observed in rats maintained under conventional conditions, i.e., not subjected to factors that either continuously increase the output of TSH or have direct carcinogenic effects, is represented by solid neoplasms. These tumors appear as nodules of different sizes, composed of several varieties of large, oval or polygonal pale cells that neither form follicular structures nor produce colloid. Such nodules usually grow slowly, despite the fact that growth is infiltrative in many cases. The incidence of the neoplasms increases with age. Most of them are revealed in two-year-old or even older rats. The origin of spontaneous solid thyroid tumors in rats has been attributed to the intrafollicular light cells (Askanazy/Hürthle-cells) and to the parafollicular C-cells. In normal rats, spontaneous tumors of follicular, adenomatous and papillary pattern have been observed much less frequently than the above mentioned neoplasms, and in these rare cases, they were consisted of colloid cysts or small hyperplastic nodules.

EXPERIMENTAL INDUCTION OF THYROID TUMORS

Methods for inducing thyroid tumors in animals can be subdivided into two groups according to the mechanism of action. The first group comprises methods based on the application of substances with a direct oncogenic effect on thyroid cells, i.e., proper carcinogenic agents. The methods in the second group aim primarily at establishing a hormonal imbalance that, in turn, will lead to tumor development. Such a division, however, is rather artificial. Some known carcinogenic agents with a direct mechanism of action may also produce profound and irreversible hormonal disturbances that lead to thyroid carcinogenesis. Vice versa, many of the agents used to disturb the hormonal balance may exert a direct carcinogenic effect.

Tumor induction by elevation of TSH

As a result of numerous investigations, a consistent concept of experimental thyroid tumor pathogenesis was established to explain the tumorigenic effect of antithyroid drugs (Bielschowsky 1955). According to this concept, the first stage in the

development of thyroid tumors is inhibition of hormone production by the thyroid tissue under the influence of goitrogens. The second stage is the sustained intensification of synthesis and release of TSH. Continuous excessive secretion of TSH is assumed to be one of the basic pathogenic factors responsible for thyroid tumor development.

Goitrogen-induced tumors

Experimental goitrogen–induced tumorigenesis began with the observation that prolonged feeding of a diet containing plants of the Brassica species produced goiter in rats (Hercus & Purves 1936) leading to a high yield of adenomas (Griesbach et al. 1945). Kennedy (1942) suggested that the active agent in the rape seed was a urea derivative. Numerous investigations have previously revealed that in the thyroid the family of thiourea derivatives is both goitrogenic and carcinogenic. For example, Paschkis et al (1948), Kuzell et al. (1949), Clausen (1953), Wollman (1961), and Grundman & Seidel (1965) used thiouracil; Doniach (1950), Christov (1968), and Jemec (1977) used methylthiouracil (MTU); Van Dyke (1953) and Sellers & Schonbaum (1957) used propylthiouracil (PTU); Ulland et al. (1972), Graham et al. (1975), and Arnold et al. (1983) used ethylenethiourea (ETU); tetramethylthioura (TMTU) was used by Stula et al. (1979). Although there is some variation in the goitrogenic activity of the different thioureas, subsequent development of tumors appears to be a uniform finding for all members of this family of thiourea derivatives. They all inhibit steps in hormone synthesis (coupling of iodotyrosines, iodination of thyrosines and monoiodotyrosines) and some, such as PTU, also inhibit deiodination. Daily intake of 5–l0mg PTU or 10–20mg MTU is considered an optimum dosis for tumor development. Another compound with goitrogenic and carcinogenic activity is the herbicide aminotriazole (ATA). Jukes & Shaffer (1960) and Napalkov (1967) found a similarly high tumor frequency (25% adenomas) in rats of both sexes following lifelong 0.01% ATA administration. Tsuda et al. (1976) and Steinhoff et al. (1983) were able to produce carcinomas in Wistar rats. ATA has turned out to be an experimental goitrogen because the level of general toxicity was lower, than that observed in the thiourea group (Gibson & Doniach 1967). In contrast to mice and rats, hamsters appear to be relatively resistant to tumorigenesis caused by goitrogenic agents. Steinhoff et al. (1983) showed that irrespective of the amount of dosage given to hamsters ATA produced no increase in thyroid neoplasia, while PTU produced thyroid hyperplasia but no neoplasia (Kirkman 1972). However, MTU is reported to produce adenomas and carcinomas in hamsters with frequency and latent intervals similar to those in rats and mice (Akimova et al. 1969, Christov & Raichev 1972). In addition, Hellwig & Welch (1963) described the development of thyroid tumors in 15% of guinea pigs after 14 months PTU intake.

Tumor induction by low-iodine intake

Thyroid tumors have been induced in rats by prolonged over-stimulation of the gland with endogenous TSH only. This method involves maintaining the animals in a state of chronic iodine deficiency. The first observations of rats kept in such a state can be traced back to Bircher (1910, 1911). Since then, the method has been perfected by several groups (Hellwig 1935, Bielschowsky 1953, Isler 1959, Al-Saadi 1968 a.o.), but

the most detailed examination was performed by Axelrad & Leblond (1955). These authors found that the changes seen in the thyroid gland and pituitary gland with a low-iodine diet are identical to those seen under long-term goitrogen administration. Thyroid adenomas could be induced in almost all experimental rats when maintaining an iodine-restricted diet with daily intake of about $0.7\mu g$ for two years. However, the malignancy of the follicular neoplasms that arise in the rat thyroid as a result of chronic iodine deficiency is questionable. In all cases reported so far, the frequency of carcinomas originating from follicular epithelium is clearly lower in rats kept on a low-iodine diet than in animals treated with goitrogens. In rats, iodine deficiency is a much more effective tumor promoter than is a carcinogen, suggesting that a similar relationship may exist in human populations (Ward & Oshima 1986). In C3H/Hey strain-mice, Schaller & Stevenson (1966) used low-iodine diet to induce benign and malignant thyroid tumors; after one year of treatment, carcinomas developed in 14%. In hamsters, Fortner et al. (1959) found well-differentiated, metastatic follicular tumors in 18% of females, but no lesions in males after a 70-week iodine-deficient diet.

Tumor growth after partial thyroidectomy

Although it has been claimed that subtotal thyroidectomy is a potent method that raises the level of trophic stimulus to the thyroid, the yield of tumors in animals treated in this way is lower than in animals given a low-iodine diet or long-term goitrogen (Domach 1970). Doniach & Williams (1962) and Goldberg et al. (1964) induced 14% adenomas and 4% carcinomas in Lister rats 15 months after 85%-excision of thyroid mass. In contrast, Ird (1968) found that subtotal thyroidectomy alone did not increase the incidence of tumors above that seen in control rats, and that surgery reduced the incidence of tumors in rats treated with MTU (25% vs. 74%). The relatively low yield of tumors obtained by this method may be explained by the fact that owing to surgery most part of the target gland responsible for the trophic stimulus is removed. This, of course, reduces the population of cells that might undergo neoplastic change.

Tumor induction by ionizing radiation

Radiation affecting the thyroid gland is possible via two routes: external or internal. External administration of radiation is achieved by using either X- or gamma-emitting radiation sources. Theoretically, this route has the advantage of permitting delivery of a precisely calculable amount of rads (Gray), but suffers from several practical problems. Firstly, given the size of the thyroid of the rat or mice, it is difficult both to localize the target and to avoid damaging the surrounding tissue. Secondly, it is also essential, but difficult, to avoid unirradiated parts of the thyroid, leading to an overestimation of the dose delivered. These problems may be solved by lightly anaesthetizing animals and by carefully placing a lead collar with a small window over the neck region, both to protect the surrounding tissue and to hold the animal in position. Internal irradiation is usually given in form of sodium salts of I^{131} or I^{125} by intraperitoneal or intrathyroidal injection. This method is much more convenient than external irradiation, although different problems arise. Accurate calculation of the dose received is difficult, since it is dependent both on percentage uptake and retention of the isotope. The amount of

Table 1. Review of the literature: incidence of thyroid tumors in rats following a single irradiation with radio-iodine (I^{131})

Dosage (μCi I^{131})	Age (days)	Latent period (month)	Adenoma (%)	Carcinoma (%)	Authors
1	—	6	0		Lindsay et al. (1968b)
1	—	12	1		Lindsay et al. (1968b)
1	—	24	2		Lindsay et al. (1968b)
1.1	4	12	26		Doniach (1969)
2.9	4	12	39		Doniach (1969)
5	70	15	50		Doniach (1953)
5	—	6	0		Lindsay et al. (1968b)
5	—	12	9		Lindsay et al. (1968b)
5	—	24	12		Lindsay et al. (1968b)
5.5	4	12	48		Doniach (1969)
10	42–84	32	33	16	Lindsay et al. (1957)
25	42	12	11	3	Lindsay et al. (1966)
25	56	24	96	26	Potter et al. (1960)
25	42–84	32	35	15	Lindsay et al. (1957)
30	70	15	50		Doniach (1953)
30	84	15	18		Doniach (1956)
100	70	15	0		Doniach (1953)
100	42–84	31	0	10	Lindsay et al. (1957)
200	42–84	32	13		Lindsay et al. (1957)
400	42–84	28	0.7		Lindsay et al. (1957)

isotope retained by the gland is dependent on the biological, as well as on the physical, half-life. The biological half-life is difficult to predict and may vary with the strain of animal species, diet and sex. Even the surrounding temperature has been shown to influence iodine uptake (Doniach 1950).

Tumor growth following internal radio-iodine application

The destructive effect of variable amounts of radioactive iodine on the normal thyroid was shown for the first time in 1942 by Hamilton & Lawrence in the dog and rabbit and in 1948 by Findlay & Leblond in the adult rat. Experimentally induced neoplasms of the rat thyroid following I^{131}-irradiation were first produced by Doniach (1950). He reported that the administration of 32μCi I^{131} significantly increased the formation of adenomas, as compared with untreated groups. In 1951, Goldberg & Chaikoff showed that a single dose of I^{131} would cause benign and malignant tumors after a period of 1.5–2 years. Since these initial experiments, several pieces of evidence have shown that as little as 5μCi I^{131} is tumorigenic to the rat thyroid, and that the administration of doses ranging from 5–400μCi I^{131} can produce benign and malignant neoplasms. However, with increasing dose, a larger percentage of cells is sterilized, thus reducing the number of tumors produced (Table 1). The optimal carcinogenic radiation dose to young adult rats is approximately 5–50μCi I^{131}. One important factor affecting the dose-response curve for tumor production is the age at which radiation is given. Doniach (1969) showed that after administration of 2.9μCi I^{131} at birth tumor yield was similar to that following 30μCi I^{131} given to adult rats. Taking into account the weight

Table 2. Review of the literature: incidence of thyroid tumors in rats following a single bilateral irradiation with X-rays

Dosage (Gy)	Age (days)	Latent period (month)	Adenoma (%)	Carcinoma (%)	Authors
1	70	18–20	14		Doniach (1974)
2.5	70	18–20	10		Doniach (1974)
3	10	15	38		Christov (1978)
3	60	15	15		Christov (1978)
4	40	13	70		Boltze et al. (2002)
4	40	25	80		Boltze et al. (2002)
5	70	18–20	0	6	Doniach (1958)
5	56–84	24	18	4	Lindsay et al. (1961)
10	56–84	24	54	22	Lindsay et al. (1961)
11	92	15	23	8	Doniach (1956)
20	56–84	24	75	25	Lindsay et al. (1961)

of the animals, his results do not reveal any increased susceptibility of the newborn rat to the carcinogenic action of radioactive iodine compared with adults. There is also no marked difference in weanling rats (28 days) as compared with young adults (70 days) (Doniach 1957).

Tumor induction after external X-ray irradiation

Experimentally, the effects of external irradiation on thyroid tumor production have mostly been assessed in rats. In several studies, doses ranged from 1 Gy to 20Gy, including various intermediate doses. Table 2 shows that the optimum dose for the development of tumors is dependent on the age at which the radiation is initially administered and, obviously, the age at which the animal is examined. For 3-month-old rats, the optimal dose for thyroid tumor induction using only X-rays lies between 5 and 10Gy. A lower dose of X-rays given to the neck region of 10-day old rats has induced more tumors with a shorter latent period than the same dose given to 60-day-old animals (Christov 1978). This increase in tumorigenicity in younger animals is possibly due to the higher mitotic index of the thyroid in younger rats.

Comparisons of X-ray and I^{131} doses, which are able to produce thyroid tumors have shown a ratio of 1:8 to 1:10 (Doniach 1956, 1963). Abbatt et al. (1957) investigated the inhibition of goitrogenesis in rats, produced by varying doses of X-rays and radioactive iodine. After giving either 30μCi I^{131} or 10 Gy of X-rays their results showed a similar effect. They suggested that compared with I^{131} the apparent ten-fold increased sensitivity of the thyroid to X-rays as may be due to the overall uneven distribution of radiation with I^{131} so that some follicles possibly receive a smaller amount than those absorbed by others.

Thyroid tumorigenesis induced by chemical carcinogens

In 1942, Esmarch was the first to use chemical carcinogens for the production of thyroid tumors in experiments. He applied methylcholanthrene directly to thyroid glands of rats. The direct application of other carcinogenic polycyclic hydrocarbons

was studied later and, as expected, such an approach produced more sarcomas and squamous cell carcinomas than adenocarcinomas. Money & Rawson (1950, 1965) found that administration of dimethylbenzanthracene either directly to the thyroid gland or by subcutaneous injection did not significantly alter the incidence or type of produced tumors.

Tumor induction by aromatic amines and azo dyes

Thyroid adenomas can be produced by systemic administration of acetylaminofluorene (AAF, formerly used as an insecticide), which can also produce a high yield of tumors in other organs of rats, including mammary gland, liver, kidney, intestine and uterus (Bielschowsky 1944). Murthy (1980) found that continuous administration of the dye intermediate, 4,4′-methylene-bis-(N,N-dimethyl)-benzenamine (MDBA), to F344 rats of both sexes produced follicular tumors after 80 weeks. The incidence was higher in animals receiving larger doses, and it was in these animals only that hyperplastic changes were seen in the thyroid. Murthy et al. (1985), taking up earlier studies of 4,4′-oxydianiline (ODA) that had shown evidence of a goitrogenic and carcinogenic effect on the thyroid in rodents (Hayden et al. 1978), in whom it also caused a high incidence of liver tumors. They reported that the incidence of thyroid hyperplasia and neoplasia was high in animals given 0.04%–0.05% ODA in the diet. The earliest follicular tumor was seen at 28 weeks, and after two years, neoplasms were present in 86% of the survivors of these groups. Thyroid hyperplasia and changes in thyrotroph population in the pituitary glands of rats with follicular tumors suggested that the carcinogenic effect of ODA was at least partly mediated through an elevation of TSH.

Tumor growth induced by nitrosamines

Diisopropanolnitrosamine (DIPN) is one member of a family that belongs to the carcinogenic nitrosamines, i.e., postulated intermediates of the parent compound di-n-propylnitrosamine, with broad-spectrum activity in most species tested. When given weekly subcutaneous injection to Sprague-Dawley rats for life, there was up to 50% incidence of thyroid tumors, with short latent intervals of 23 weeks for adenomas and 26 weeks for carcinomas (Mohr et al. 1977). N-bis-(2-hydroxypropyl)nitrosamine (DHPN) can also initiate thyroid tumors, although the frequency is lower in the absence of a promoting agent. Obviously, there is a difference in the sensitivity of rat strains to this agent: equitoxic doses resulted in thyroid tumors in 50% of Sprague-Dawley rats and in 20% of MRC rats (Mohr et al. 1977, Pour et al. 1979). N-nitrosobis (2-oxopropyl)amine (BOP) is a nitrosamine closely related to DHPN, with a high yield of thyroid tumors in rats: Pour & Salmasizadeh (1978) found 50% incidence in MRC rats following a single dose, and 60% after weekly treatment for life. When given in equitoxic doses, BOP produced thyroid neoplasms in 60% and DHPN in 20% of MRC rats (Pour et al. 1979). In 1986, Pour also showed that intrauterine exposure of hamsters to BOP results in 50% incidence of thyroid adenomas in female animals, but 0% in male animals; this is of particular interest as in adult hamsters, the carcinogenic effect is almost entirely confined to the pancreas.

Tumor induction by nitrosoureas

Thyroid carcinogenesis with N-nitroso-N-methylurea (NMU) was first described by Jobst (1967). He treated rats during their intrauterine development and after birth with weekly injections and found thyroid carcinomas in some of the survivors. NMU (3 injections of 30mg/kg) was given to Wistar rats by Thomas & Bollman (1974), who reported a 100% incidence of follicular cell carcinomas after 7–8 months. Takizawa & Nishihara (1971) reported the induction of a thyroid carcinoma in a female rat given the powerful neurocarcinogen N-nitrosobutylurea (NBU). N-ethyl-N-nitrosourea (ENU) has been extensively applied as a transplacental neurocarcinogen in several species, but the use of high doses in young rats can produce thyroid tumors, mostly of encapsulated papillary type, in a small portion (Stoica & Koestner 1984). Warzok et al. (1977) and Napalkov et al. (1981) reported that transplacental administration of ENU to dogs causes not only early development of thyroid tumors in a minority of cases, but also to late tumors.

Studies with combination of tumor-inducing factors

The methods of thyroid tumor induction outlined above, i.e., treatment with antithyroid or carcinogenic substances, restriction of iodine consumption and irradiation, are often used in various combinations. For instance, pre-irradiation of the thyroid significantly increased the tumor development in rats kept on a low iodine diet (Nadler et al. 1969). Similar effects have been observed in experiments with the combining irradiation with antithyroid drugs or certain chemical carcinogens (Lindsay 1969). The combination of antithyroid drugs or a low-iodine diet with chemical carcinogens (AAF was usually used) also resulted in accelerated tumor developmemt (Bielschowsky 1944, Hall & Bielschowsky 1949, Axelrad & Leblond 1955). Hall (1948) made the interesting observation that even relatively small doses of AAF given for a period as short as 1 week were sufficient to produce an enhancing effect on tumorigenesis, and this effect was still observed when goitrogen treatment with allylthiourea was delayed for up to 18 weeks. There is evidence that AAF causes neoplastic progression of thyroid epithelium only under conditions of excess TSH stimulation. Otherwise no detectable effect was observed by Bielschowsky & Griesbach (1950) a finding not confirmed by other studies (Grundmann & Seidel 1965). It is possible that owing to a toxic alteration of hepatic cells normally responsible for hormone degradation AAF itself might indirectly raise TSH levels. Hiasa et al. (1982) found that treatment of rats with either repeated small doses of DHPN or treatment with aminotriazole (ATA) did not produce tumors, but combination therapy with both agents could yield neoplasms in up to 100% of animals after 12 weeks. PTU has also been used successfully in combination with DHPN by this group (Kitahori et al. 1984). Several studies have given evidence of the promoting effect of barbiturates on thyroid carcinogenesis by DHPN. Whereas single or multiple administrations of DHPN alone resulted in 0% tumors at week 20 in rats, additional treatment with barbital for 12 weeks produced thyroid neoplasms in 45% of animals; phenobarbital treatment was even more effective, leading to an incidence of up to 100% of animals at week 20 (Hiasa et al. 1983). Schaffer & Müller (1980) found

invasive thyroid tumors by 16 weeks and pulmonary metastases by 30 weeks after 3-times NMU injection in combination with long-term MTU treatment. Similar results were obtained by combining NMU with PTU in F344 rats (Milmore et al. 1982), and NMU with phenobarbital (Tsuda et al. 1983). Ohshima & Ward (1984) found that iodine-deficient diet was also an efficient promoter for NMU-initiated tumors, with 100% incidence of thyroid tumors in F344 rats after 20-week treatment. Even without the additional influence if iodine-deficient diet, NMU treatment resulted in 10% incidence of adenomas after 20 weeks, 70% incidence after 33 weeks, and 10% incidence of carcinomas after 33 weeks. Diwan et al. (1985) used NMU in combination with barbiturates and demonstrated that the incidence and multiplicity of thyroid tumors are greater in male rats, because in this sex phenobarbital seems to be a more effective promoter, as shown by the higher incidence of liver tumors in male animals in the same experiment.

Long term study for investigating changes in morphology, function and proliferation of thyrocytes after varying nutrition iodine and external radiation in rats

Several studies mentioned above have addressed the question of a predominant iodide-dependent regulation in the development of hyperplasia and proliferation of thyroid follicle cells *in vivo,* or have reported a major contribution of TSH in this respect. However, most of these studies, based on severe iodide depletion, used a short observation period for defining iodide-dependent effects. Only few reports have dealt with the ontogenesis of morphological and hormonal changes during moderate long-term iodine deficiency, which more closely parallels the situation of humans in an iodine-deficient area. The long-term effects of iodine excess in humans have not been studied in detail, but recent reports suggest that iodine excess also induces goitrogenesis and benign thyroid tumors.

One study (Boltze et al. 2002) aimed at systematically monitoring the influence of a long-term increase or decrease in daily iodine supply on the morphology of the thyroid of rats. To develop a reproducible model of thyroid tumorigenesis, this treatment was combined with short-term external radiation of the thyroid using known environmental hazards. It is expected that such a model helps to define the relevant genetic alterations causing thyroid tumor formation in a subsequent step, and may thus contribute to the diagnosis of thyroid adenomas.

Experimental design

Three groups of 80 male Sprague-Dawley rats (28d old), each differing in daily iodine supply, were investigated: 1. normal iodine intake (7000ng Iodine/100g body weight/day)(In), 2. low iodine diet (420ng Iodine/100g bw/d (I-) and 3. high iodine diet (72000ng Iodine/100g bw/d. On day 40, the thyroid region of half of each group was externally irradiated with a single dose of 4Gy X-rays (InR, I-R, I+R). Weekly, the animals were monitored as for their body weight, and blood samples for determining TSH, T3 and T4 were obtained. Of each of the six groups, 10 animals were killed at weeks 15, 35, 55 and 110; the thyroids were removed and investigated by histology

Table 3. Number of benign and malignant thyroid tumors after and without nutrition pretreatment and radiation ($n = 10$)

Group and tumors	55th week	110th week
Normal iodine		
In		
B	2 dermoid cyts	3 dermoid cysts
C	0	0
P	0	0
InR		
B	7 adenomas	8 adenomas
C	0	0
P	0	1 squamous carcinoma
Iodine deficiency		
I-		
B	5 adenomas	9 adenomas
C	0	0
P	0	0
I-R		
B	7 adenomas	9 adenomas
C	0	2 FTC / 3 PTC
P	1 adenocarcinoma	1 adenocarcinoma
Iodine supplementation		
I+		
B	5 adenomas	9 adenomas
C	0	0
P	0	0
I+R		
B	9 adenomas	10 adenomas
C	0	3 FTC / 5 PTC
P	0	1 squamous carcinoma

B, benign thyroid tumors; C, thyroid carcinomas; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma. Parathyroid carcinomas (P) were also induced: squamous cell carcinomas of the cervical soft tissue and adenocarcinomas of salivary glands.

and immunohistochemistry. The following parameters were detected: follicles/mm^2, colloid diameter, index of fibrosis, proliferation rate and number of tumors.

Results

Iodine-dependent changes without radiation:

Iodine deficiency led to lower daily growth rates and a significantly lower final mean body weight of 430g (I-) vs. 501g (In) vs. 475g (I+). The growth process was finished after 18 weeks in I+ and after 21 weeks in In and I-. Long-term iodine deficiency significantly decreased plasma T3 and T4 concentrations after week 9. In contrast, the high iodine diet did not change thyroid function. All changes manifested themselves in alterations in thyroid morphology. Iodine deficiency was associated with significantly large, but fewer, follicles, whereas the high iodine diet led to a significant decrease in the diameter, but to an increase in the number of follicles. The mitotic activity of thyrocytes was very low under normal iodine intake conditions ($<1 \pm 0.2\%$). Not only iodine deficiency, but also higher iodine intake significantly increased proliferation

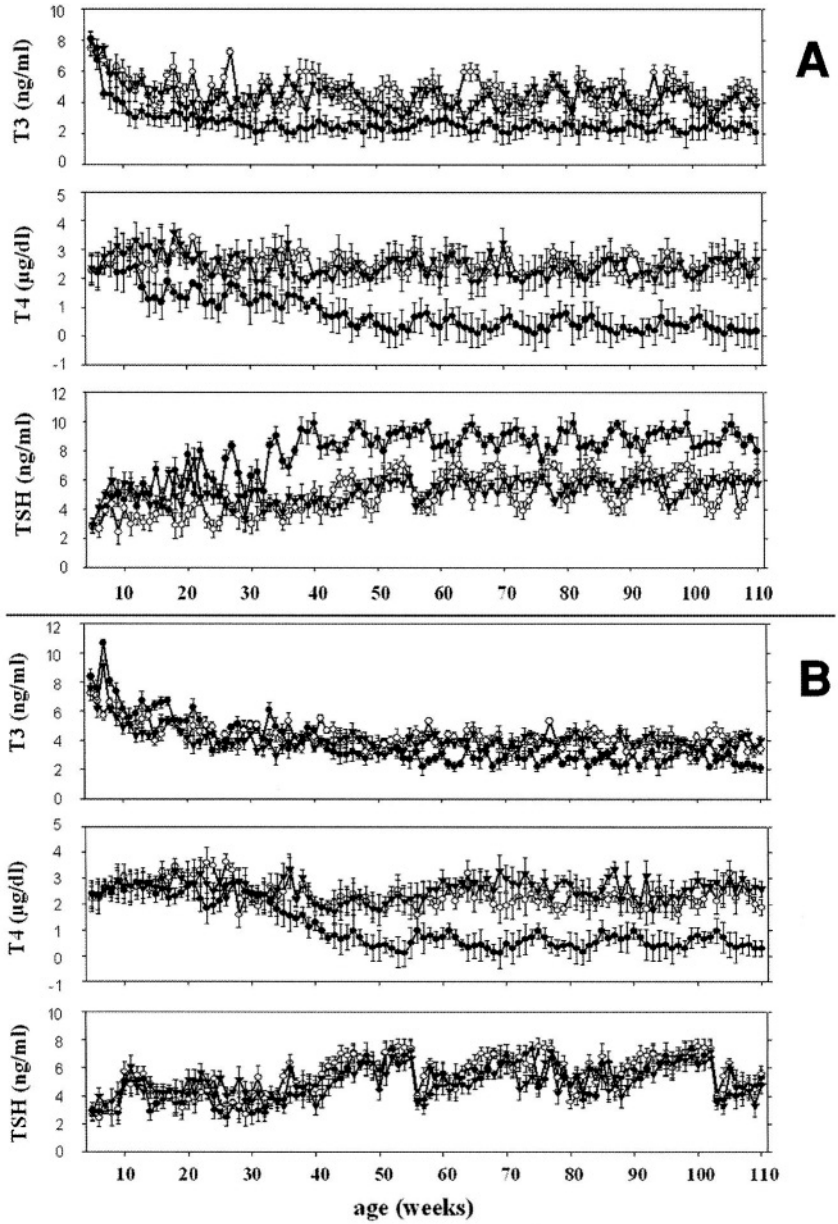

Figure 2. Changes of T3, T4 and TSH plasma concentrations in rats without (A) and after radiation with 4Gy (B) under normal nutrition iodine conditions (white circle (○)), iodine deficiency (black circles (●)) and iodine supplementation (black triangles (▲)).

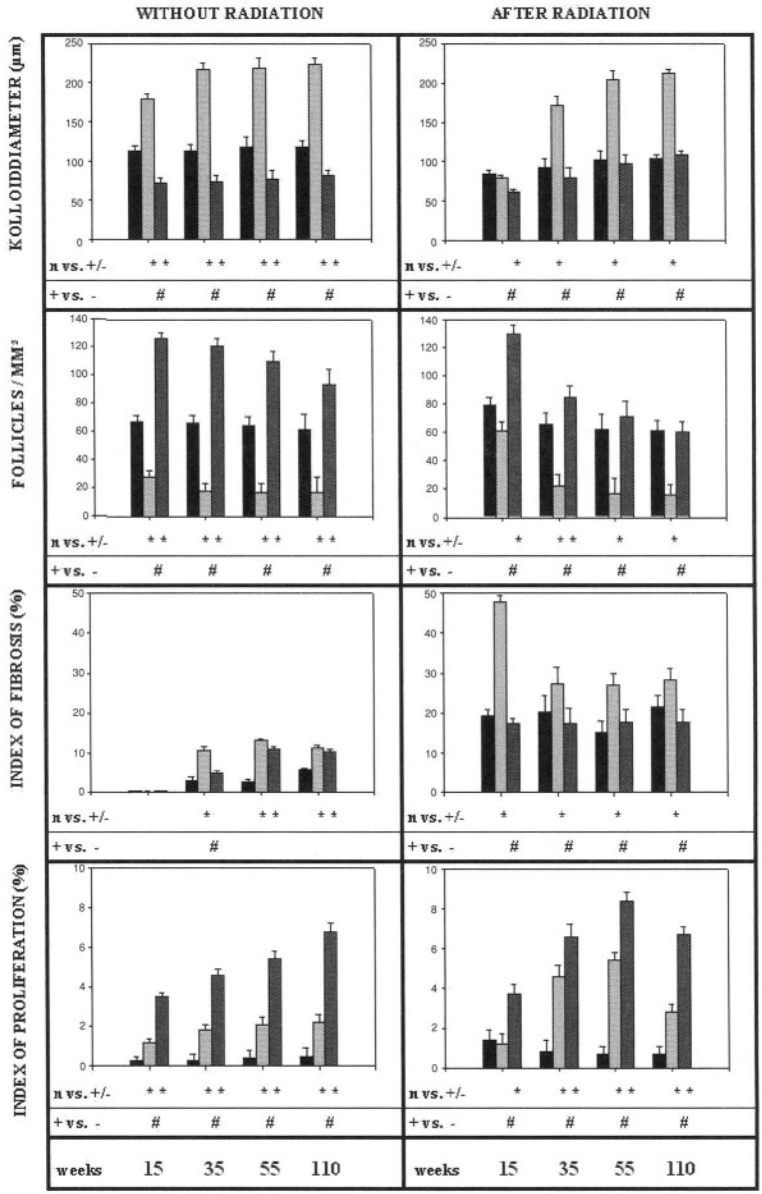

Figure 3. Histological data of the thyroids after 15, 35, 55 and 110 weeks under normal iodine conditions (In; black bars ■), iodine deficiency (I-; gray bars ▒) and iodine supplement (I+; dark-grey bars ▓) with or without irradiation (n = 10; mean±SEM).
(* = statistical significance In vs. I- or I+, $p < 0.05$; # = statistical significance 1+ vs. I-, $p < 0.05$)

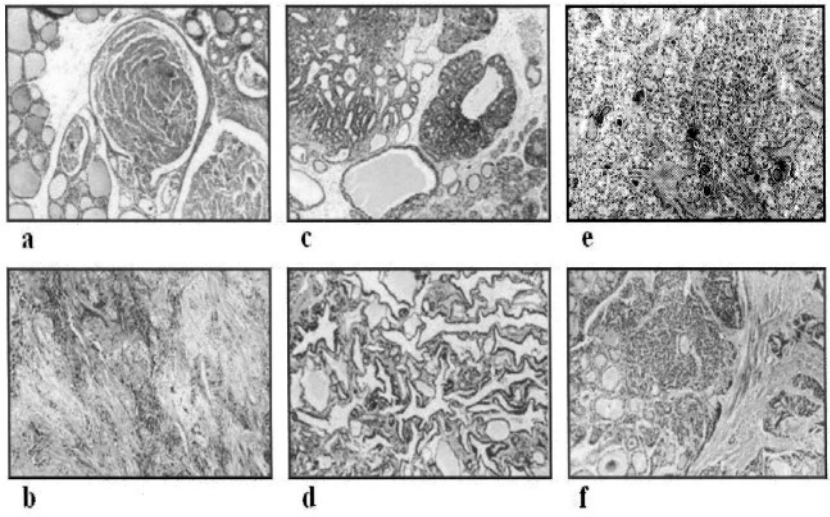

Figure 4. Histological examples of tumors induced after 4Gy radiation of the thyroid region under normal nutrition iodine conditions (A and B), iodine deficiency (C and D) and iodine supplementation (E and F; magnification x100). A, Metaplasia of squamous epithelium with dermoid cysts (55 weeks). B, Squamous cell carcinoma of the cervical soft tissue beside the thyroid gland (55 weeks). C, Adenocarcinoma of a salivary gland (glandula submandibularis; 55 weeks). D, Follicular thyroid carcinoma with a bizarre pattern (110 weeks). E, Follicular thyroid carcinoma, small follicular/insular type (110 weeks). F, Papillary thyroid carcinoma, predominant follicular pattern, sporadic papillae (110 weeks).

rates. At week 55 and 110, all non-irradiated animal groups were free of malignant tumors, and benign tumors were not detected until week 55.

Iodine-dependent changes after radiation:

After radiation, there were increases in T3 and T4, and a significant decrease in TSH in the group with iodine deficiency. The hormone concentrations of the normal iodine and high iodine groups were not significantly altered. In all groups, thyroid weight was not significantly influenced by radiation. Histologically, the most important finding in irradiated low iodine diet thyroids was the total destruction of follicles observed at week 15. After this destruction, a short-term increase in T3 (7th week) was measured. After the 55th week, a complete restitution of the follicle structure was seen. In contrast to the sole manipulation of iodine intake, radiation treatment led to a higher number of benign tumors, starting 55 weeks after having changed nutritional iodine supply, and to malignant tumors after 110 weeks. Parathyroid carcinomas were also induced: squamous cell carcinomas of the cervical soft tissue and adenocarcinomas of the salivary glands. The thyroid carcinomas were solitary tumors, their size ranged between 0.1 and 1.5mm. Neither local lymph node metastasis nor distant metastasis was found.

Conclusions

This animal model clearly supports the concept that in thyroid carcinogenesis, there is a very long latency period between the mutational event and the developement

of malignant changes. This contradicts previous studies using a higher stimulation of thyrocyte proliferation by iodine deficiency, where malignancies were detected after much shorter time intervals (Axelrad & Leblond 1955). Large doses of iodine may induce thyroid carcinomas (Correa & Welsh 1960). We showed that mild iodine excess is not necessarily associated with the formation of thyroid malignant neoplasms, but when combined with a mutagen, carcinomas arise with high frequency. These data on mild forms of high iodine intake thus put a note of caution to a long term-use of high iodine. It was shown that euthyreosis is best protection against thyroid cancer before environmental hazards are effective.

The well-defined setting in these experiments clearly demonstrates that mutational lesions acquired by radiation are clinically silent over a long period of time. It is tempting to use such a model to search for candidate genes altered by mutagens, but which are not changed in thyroid adenomas found under control conditions. The definition of such changes may then have important implications for the characterization of the malignant potential of a given adenoma well before cytological or histological changes occur.

FUTURE OF ANIMAL MODELS INVESTIGATING THYROID CARCINOGENESIS

In the last 75 years, very different models investigating thyroid carcinogensis have been developed. The concept of initiation and promotion of carcinogenesis is well demonstrated by the studies reviewed in this chapter. The initiation step may be produced by diverse agents, including ionizing radiation and many classes of carcinogens. The action of these agents is promoted by raising the level of trophic stimulation (TSH), which can also be achieved in a variety of ways (for instance goitrogen therapy or low-iodine diet). All these models successfully described the changes in morphology and function of thyrocytes during carcinogenesis. However, the time in which these kind of models were used is over. Therefore, in the last ten years, such studies were published only sporadically. The molecular basis of thyroid neoplastic processes involved in experimental tumors is now being elucidated by investigations of the changes associated with developing tumors, and also by the reconstruction of the tumor phenotype through the introduction of genes into thyroid cells. In the last few years, many new methods (including chip- and array technology, or proteomics) have been developed to clarify tumor-related mechanisms and to search for tumor-associated genes. The tumor induction models mentioned above can serve as the basis for yielding material of different stages of thyroid carcinogenesis. A factor limiting these forthcoming gene expression studies is the small amount of relevant material. The use of more sensitive methods will solve this problem in future.

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17. DIAGNOSTIC MOLECULAR MARKERS IN THYROID CANCER

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INTRODUCTION

Thyroid cancer is the commonest classical endocrine tumor, accounting for approximately 1% of all cancers. The diagnosis of thyroid cancer is typically made on cytopathologic features on fine needle aspiration or histological features on surgical samples. Following treatment of patients with thyroidectomy, and in some cases radioiodine, patients are monitored for disease recurrence using a variety of scanning modalities and serum thyroglobulin. The accuracy of both the preoperative testing and postoperative monitoring is excellent in many cases; however, there are some important deficiencies that have led to the development new tools for clinical use. Specifically, the application of molecular methods to the analysis of pathology and blood samples has led to the development of highly sensitive markers for the diagnosis of new cases of thyroid cancer, and in the evaluation of patients for recurrent disease. In this review, the molecular analysis of thyroid nodules, lymph nodes and peripheral blood as adjunctive tests for thyroid cancer will be discussed.

PREOPERATIVE EVALUATION OF THYROID NODULES

Thyroid nodules are extremely common with prevalence rates approaching 50–60% of adults under 60 years old. Because only approximately 5% of thyroid nodules are malignant, accurate pre-operative characterization of thyroid nodules is critical in selecting patients appropriate for surgical thyroidectomy. Fine needle aspiration (FNA) is the single most important diagnostic procedure in the evaluation of thyroid nodules.

Table 1. Molecular markers for thyroid nodules and lymph nodes

Potential diagnostic markers for thyroid nodules and lymph nodes	
Telomerase	GLUT-1
Galectin-3	CA 19-9
Thyroid peroxidase	CD 15
Thyroglobulin	HBME-1
Oncofetal fibronectin	CD 30
ret/PTC oncogenes	CD 57
Pax8/PPARγ oncogene	CD 97
B-Raf mutations	Leu-7
Nm23	Epithelial Membrane Antigen
High mobility Group I (Y) Protein	Cyclooxygenase 2
Ceruloplasmin	Cytokeratin 19
Survivin	Cytokeratin 20
	TSH Receptor Hypermethylation

For small, solid nodules, experienced cytopathologists can accurately distinguish most benign nodules and papillary cancers. However, cytological features do not distinguish benign from malignant follicular neoplasms, and cystic papillary thyroid cancers are a common cause of false negative results. Importantly, only 15% of cytological follicular neoplasm will ultimately be follicular carcinomas; therefore, 85% of individuals that undergo surgery for these nodules will have done so unnecessarily. Finally, by its nature, cytopathologic interpretation of FNA samples is subjective. For these reasons, the application of molecular analysis to better characterize thyroid nodule cytologic samples has been an area of intense interest.

With the advent of methods, such as reverse transcriptase-polymerase chain reaction (RT-PCR), in which tiny amounts of samples are suitable for analysis, and increases in the number of antibodies suitable for immunocytochemistry, the possibility of improving FNA-based characterization of thyroid nodules is now possible. In the initial section of this review, several of the most carefully studied molecular markers (Table 1) for thyroid FNA will be discussed.

Telomerase

Telomeres are chromosomal end structures, consisting of tandem repeats of TTAGGG that play a critical role in the protection of chromosomes during cell division and are important in chromosome positioning during replication (1). Chromosomes typically lose about 50 to 200 nucleotides of telomeric sequence from chromosomal ends per cell division because DNA polymerase is unable to replicate the ends of linear DNA. The resultant progressive shortening of chromosomes as cells divide has been described a cellular "biological clock"; once the chromosomes are shortened to a critical length through telomeric loss, cell growth stops and apoptosis is induced. Therefore, preservation of chromosomal end length during division would be expected to retard this natural "aging" of cells and result in continuous cell growth.

Telomerase is an enzyme that extends telomeres, thereby preserving chromosomal length. Structurally, telomerase is a ribonucleic acid-protein complex containing a catalytic component, the human telomerase reverse transcriptase (hTERT) (2). Telomerase expression and activity have been identified in immortalized human cell lines and cells and in bone marrow cells that normally divide, but not in normal human adult epithelial cells. However, expression of telomerase and demonstration of telomerase activity using a PCR-based assay (TRAP; Telomeric Repeat Amplification Protocol) has been described in a variety of malignancies and other dividing cells, such as germinal cells of the ovary and testis (1). Based on these results, detection of telomerase activity and expression of hTERT have been explored as potential distinguishing markers for the presence of malignant thyroid cells.

Saji et al. (3) evaluated surgical pathology samples from thirty papillary thyroid cancers, three benign nodules and ten normal thyroid specimens for telomerase activity by TRAP and found that 67% of the malignant tissues had telomerase activity compared to 0% of the benign nodules. In this study, 64% of the papillary thyroid cancers that had a non-diagnostic preoperative FNA were positive for telomerase activity. Haugen et al. (4) and Onoda, et al. (5) obtained similar results when they investigated surgical thyroid specimens.

The ability of telomerase help differentiate follicular adenoma from carcinoma was reported by Umbricht et al. (6) who studied frozen tissue samples from patients undergoing thyroidectomy for follicular neoplasm on FNA. TRAP assays were performed on 44 follicular thyroid tissue specimens and 22 normal thyroid tissue samples. The authors reported a sensitivity of 100% and a specificity of 76% for detecting follicular carcinoma. The false positive samples occurred mostly in tumors that also had lymphocytic infiltration, as lymphocytes are known to have detectable telomerase activity. However, it is possible that the presence of telomerase activity in histologically benign specimens may represent an early step in the development of an invasive tumor. This issue of potentially creating an assay that is more sensitive than the clinical gold standard is a problem for many RT-PCR-based assays.

De Deken et al. (7) demonstrated a decrease of telomere length as well as increased variability in the telomere size in benign nodules without measurable telomerase activity, as compared to normal thyroid tissues. These data suggest that the benign nodular cells may have progressed through more mitotic divisions than the adjacent normal tissue and therefore may be closer to their limit for further growth, consistent with benign tumor growth.

The cloning of the human telomerase reverse transcriptase cDNA allowed for RT-PCR analysis of expression of its mRNA in clinical samples. This created an opportunity to design a more user-friendly telomerase assay that could be applied to FNA samples. Saji et al. (8) studied 19 malignant and 18 benign thyroid surgical samples for evidence of hTERT gene expression by RT-PCR. HTERT mRNA was detected in 15 (79%) of the malignant and 5 (28%) of the benign tumors; all 5 benign lesions with demonstrable hTERT gene expression had lymphocytic infiltration on final pathology. HTERT mRNA was not detected in any of the normal thyroid specimens. The

results correlated with TRAP assay results from the same samples. Similar results were reported by the same authors using thyroid FNA samples (9).

Fine needle aspiration specimens have also been investigated for telomerase activity using TRAP assay. Sebesta et al. (10) failed to show any additional usefulness of measuring telomerase activity in a small study of FNA samples. It is likely that RT-PCR of hTERT is more sensitive than TRAP assay due to the logarithmic amplification inherent in RT-PCR, thus accounting for a greater sensitivity when used to analyze FNA samples.

The frequency of telomerase-positive results, either by RT-PCR or TRAP assay for papillary thyroid cancer varies between studies. For example, some report that as many as 67% of papillary thyroid cancers are positive (3), while others indicate a much lower percentage of about 20% (11). The small number of cases in many of these studies makes interpretation quite difficult. Similarly, results regarding the association between telomerase activity and tumor aggressiveness also vary; some studies demonstrate a correlation between telomerase activity and tumor progression (5, 12) while others do not (3). It is clear that larger, more extensive studies are needed before telomerase can be considered an effective diagnostic tool.

Galectin-3

Galectin-3 is a member of the lectin family that regulates the functions of its protein targets by interacting with attached galactose-containing glycoprotein side chains. As a group, galectins regulate cell growth and differentiation, intercellular recognition and adhesion, as well as malignant transformation. Galectin-3 levels have been directly correlated with metastatic potential in fibrosarcoma and melanoma cell lines. In vitro studies have indicated that expression of galectin proteins is elevated in thyroid cancer cell lines and microarray analysis has demonstrated increased levels of galectin-3 in papillary thyroid cancers (13) This led to their investigation as molecular markers used to distinguish between benign and malignant thyroid tissues.

Xu, et al. (14) evaluated protein derived from 41 surgical thyroid specimens for galectin-1 and galectin-3 expression by Western blot. Elevated levels of both proteins were demonstrated in thyroid cancer compared to normal thyroid tissue. Similarly, normal thyroid tissue did not express galectin 1 or 3 by immunohistochemical analysis, but high levels of both proteins were detected in both papillary and follicular thyroid cancers, and in regional nodal metastases. A second group evaluated 41 malignant and 35 benign thyroid tissue specimens (15) for both galectin-3 protein and RNA levels. Galectin-3 expression was identified in 18 of 18 of papillary cancer samples, 4 of 8 follicular cancers, 2 of 3 poorly differentiated cancers, 5 of 5 anaplastic cancers, 3 of 6 medullary, and 1 of 1 Hurthle cell cancers. By contrast, none of the normal or benign nodular tissues expressed galectin-3, other than those with lymphocytic infiltration. Levels of galectin-3 mRNA appeared to correlate with protein levels in papillary cancers and normal tissue (15).

To determine if galectin-3 immunocytochemistry could be applied to FNA samples, Orlandi, et al. (16) evaluated FNA and surgical pathology specimens from 64 patients who had undergone thyroidectomy whose preoperative diagnosis was

malignant (n = 15), indeterminate (n = 37), and benign (n = 12). The final histologic diagnosis included 18 papillary and 17 follicular cancers, as well as 29 follicular adenomas. All papillary thyroid cancers expressed galectin-3 in both FNA and surgical specimens. For the follicular cancers, immunoactive galectin-3 was detected in all surgical specimens in a heterogeneous pattern, and in all but 3 FNA samples. By contrast, only 3 of 29 benign follicular adenomas expressed galectin-3.

In a more recent study (17), different antibodies against human galectin-3 were used in an immunohistochemical study of thyroid surgical specimens (13 benign and 62 malignant). Immunoactive galectin-3 was most prevalent in the papillary thyroid cancers (33 of 45), but some benign lesions were 3 of 8 benign adenomas demonstrated immunoactive galectin-3.

Finally, Bernet, et al. (18) applied quantitative RT-PCR to galectin-3 analysis to determine if a particular "cut-point" of galectin-3 gene expression correlated best with malignancy. In this study, markedly elevated levels of galectin-3 mRNA were identified in papillary cancers compared with normal tissue. There was no difference between the galectin-3 mRNA levels in follicular adenomas and carcinomas.

Based on the above results, galectin-3 immunocytochemistry seems to be a promising new marker of thyroid cancer that could be applied to FNA analysis. It appears that classic molecular approaches, such as quantitative RT-PCR may not be helpful for the conundrum of follicular neoplasm FNA results. However, additional studies are still required.

Thyroid peroxidase

Thyroid peroxidase (TPO) is a thyroid-specific enzyme that catalyzes iodide oxidation, thyroglobulin iodination, and iodothyronine coupling. Reduced expression of TPO impairs thyroid follicular cell function correlates with a loss of differentiated thyroid function and has been well described in thyroid cancer cell lines and tumor samples. Thus, immunohistochemical staining for TPO expression and molecular analysis of the TPO gene have been studied for use as diagnostic tools for thyroid cancer.

DeMicco, et al. reported a retrospective study of 150 FNA samples including 125 benign tissues (19), and demonstrated that 113 of 125 benign lesions were characterized by immunoactive TPO in more than 80% of cells while <80% of the cells expressed TPO in all 25 malignant lesions. Thus, using this level of TPO-expressing cells as a positive, they reported a sensitivity of 100% and a specificity of 90%.

Christensen, et al. reported their prospective experience using this method in 124 consecutive FNAs using the same anti-TPO primary antibody (20). In their hands, TPO immunohistochemistry (>80% cut-off) correctly identified all cases of cancer. Only one benign follicular adenoma was identified as malignant by this immunohistochemical criterion. These investigators concluded that TPO immunohistochemistry of FNA samples using the 80% cut-off values has a sensitivity of 100% and a specificity of 99%. These results are obviously subjective and may be antibody dependent.

Because germline mutations of the TPO gene that cause functional loss of TPO activity cause of congenital hypothyroidism, loss of heterozygocity (LOH) at the TPO gene locus has been implicated as a cause of the organification defect typical of benign

and malignant thyroid tumors. However, in a study of 40 hypoactive thyroid nodules (21), LOH of the TPO gene was noted in only 6, making this an unlikely method for evaluating thyroid nodules preoperatively.

Thus, it appears that immunostaining for thyroid peroxidase may be a valuable addition to the analysis of FNA samples. Studies with additional available antibodies may be useful from a practical standpoint.

Oncofetal fibronectin

Fibronectins are high-molecular-weight glycoproteins found in the extracellular matrix. Oncofetal fibronectin is characterized by the presence of the oncofetal domain (IIICS domain), which is absent in normal fibronectin. Overexpression of this variant of fibronectin has been demonstrated in many epithelial cancers and it has been studied as a molecular marker of malignancy. Several investigators have evaluated the utility of the oncofetal fibronectin mRNA as a marker of thyroid malignancy.

Higashiyama, et al. (22) evaluated 19 malignant and 33 benign surgical thyroid specimens by competitive RT-PCR and demonstrated elevated levels in papillary and anaplastic cancers versus benign tissues. Levels were variable in follicular carcinomas and were not clearly different from follicular adenomas. The same group also reported detection of oncofetal fibronectin mRNA on surgical samples using in situ hybridization and reported similar results (23).

Takano et al. (24) examined 72 FNA samples (23 normal, 14 adenomatous goiters, 13 follicular adenomas, 3 follicular carcinomas, 18 papillary carcinomas and 1 anaplastic cancer) for expression of oncofetal fibronectin mRNA using RT-PCR. 95% of the papillary or anaplastic carcinomas by cytology also expressed oncofetal fibronectin mRNA compared to only 4% (n = 109) of benign specimens. In contrast, none of the 6 follicular tumors expressed oncofetal fibronectin. Fifty of these patients underwent surgery, based on the results of the surgical histology, oncofetal fibronectin RT-PCR was 97% sensitive and 100% specific. These results are similar to Higashiyama, et al. as all but one cancer sample included in this study was papillary. These results suggested that oncofetal fibronectin mRNA amplification was an accurate marker of papillary, but not follicular carcinoma. A potential cause of false positive results is the expression of oncofetal fibronectin in thyroid fibroblasts (25). Despite the fibroblast data, the results of the immunhistochemical and molecular studies suggest that measurement of oncofetal fibronectin expression may be useful as an adjunctive test for identifying papillary thyroid carcinoma.

Ret/PTC

Ret/PTC oncogenes are genomic rearrangments that couple the tyrosine kinase domain of the Ret receptor to different 5′ regions leading to aberrant expression and activation of Ret. To date, there are 8 Ret/PTC proteins, however, the prevalence is greatest for Ret/PTC 1, 2, and 3. Translocations involving Ret are particularly prevalent in papillary carcinomas that develop following exposure to radiation. Because

these rearrangements are largely limited to thyroid carcinomas, the expression of PTC oncogenes has been studied as molecular markers for thyroid malignancy.

In a study of 73 thyroid specimens from which both FNA and surgically obtained tissue was available, Cheung, et al. (26) evaluated the presence of PTC1-5 by RT-PCR. Only Ret/PTC 1, 2 or 3 were detected in the samples; Ret/PTC translocations were not detected on FNA and surgical samples from 39 benign tissue samples, including 11 follicular adenomas, 25 nodular hyperplasia's and 3 Hashimoto's thyroiditis cases. In contrast, Ret/PTC1, 2, or 3 expression was detected in 17 FNA samples and 21 surgical specimens derived from 33 malignant thyroid tumors. Of importance, this molecular method was more accurate than routine cytopathology in these samples.

Conflicting results were reported by Elisei, et al. (27) who studied 154 patients referred to surgery for FNA-characterized benign nodules (n = 65) or papillary thyroid cancer (n = 89). Expression of Ret/PTC-1 and Ret/PTC-3, the most common Ret/PTC oncogenes, was identified in both benign and malignant nodules. RET protein expression has been evaluated by immunohistochemistry in papillary thyroid cancers (28). Overall, expression of Ret was heterogenous and was demonstrated in regions of cellular atypia in both malignant and benign lesions. Thus, based on these data, it appears that Ret/PTC may not be helpful in pre-operative diagnosis due to a relatively low prevalence in many populations with papillary thyroid cancer and potential issues with specificity. However, more studies are needed to clarify a role for Ret/PTC rearrangement or Ret overexpression in the diagnosis of thyroid nodules.

Pax8-PPARγ

Kroll, et al. (29) identified a chromosomal translocation t(2;3)(q13;p25) causing a fusion gene between Pax8 and the peroxisome proliferator activated receptor gamma (PPARγ) in follicular thyroid carcinomas. Specifically, 5 of 8 follicular cancers expressed the fusion gene, while all of the 20 follicular adenomas, 10 papillary thyroid carcinomas and 10 other benign nodules did not express the rearranged gene, suggesting that detection of Pax 8-PPARγ fusion gene expression might accurately identify follicular carcinomas preoperatively.

The specificity of the Pax 8-PPARγ may not be complete, as other groups (30, 31) have reported expression of PAX8–PPARγ in benign follicular adenomas, albeit at a lower frequency than follicular carcinomas. The importance of expression of Pax8-PPARγ in follicular adenomas on malignant transformation is uncertain. It has been speculated that overexpression of PPARγ alone, even in the absence of a defined chromosomal rearrangement, may be a marker of malignant transformation. Detection of PPARγ overexpression by immunohistochemistry appears to be more sensitive, but also, less specific for detection of follicular carcinoma (31).

B-Raf

Mutations in the serine-threonine kinase, B-Raf have been described in 35–70% of papillary thyroid carcinomas, with almost no overlap with other known oncogenes

or other benign or malignant thyroid lesions (32–34). Because this mutation appears quite specific for papillary thyroid cancer, and it is limited to two specific mutations, detection of the mutations has been proposed as an adjunctive test for FNA analysis (32). This method would likely be useful only for papillary thyroid cancer detection, however.

Nm23

Re-expression of the Nm23 tumor suppressor gene has been demonstrate to reduce the metastatic potential of malignant cells in-vitro and reduced expression of Nm23 occurs in aggressive forms of breast cancer (35) . In thyroid tissues, the interesting finding of increased expression has been demonstrated, primarily in stage IV papillary cancers and anaplastic carcinomas (36). Farley et al. (37) also evaluated 34 thyroid tumors, including 4 follicular adenomas, 19 papillary carcinomas, 6 follicular carcinomas and 5 medullary carcinomas for Nm23 mRNA levels. In this study, overexpression of Nm23 was noted in follicular and medullary cancers, although there was overlap between benign and malignant samples. Similarly, Berthau, et al. (38) reported that immunocytochemical analysis of Nm23 protein expression did not accurately distinguish between benign and malignant lesions. Mechanistically, the finding that overexpression of nm23, rather than reduction of loss of nm23 expression were demonstrated suggests an alternative function for this protein in thyroid cancer (39).

High mobility group I(Y) protein—HMGI(Y)

The high mobility group I (HMGI) proteins are nuclear proteins that regulate chromatin structure and function. HMGI(Y) is particularly highly expressed during embryogenesis, and its reexpression has been described in cancers, but not in normal adult tissues. Chiappetta et al. (40) reported evaluated expression of HMGI(Y) protein by immunohistochemistry on 358 thyroid tissue samples. HMGI(Y) was detected in 18 of 19 follicular carcinomas, 92 of 96 papillary tumors and 11 of 11 anaplastic cancers, but in only 1 of 20 hyperplastic nodules, 44 of 200 benign follicular adenomas and 0 of 12 normal thyroid tissue samples. HMGI(Y) mRNA was detected in 4 of 4 malignant tumors while eight benign FNA samples (6 follicular adenomas and 2 normal thyroid tissue) were negative. Thus, HMGI(Y) may be a potentially useful diagnostic tool for thyroid cancer that warrants further identification.

Ceruloplasmin

Because ceruloplasmin, a copper transport protein that shares homology with lactoferrin (a molecular marker for several tumor types), it has been investigated as a tumor marker in thyroid cancer. Tuccari et al. (41) evaluated 56 surgical thyroid specimens for ceruloplasmin expression by immunohistochemistry. None of the 15 follicular adenomas expressed ceruloplasmin, while two of two Hurthle cell tumors, all 21 follicular, and all 6 papillary carcinomas were positive. All of the medullary thyroid cancers were negative for ceruloplasmin, as was the normal thyroid tissue surrounding the thyroid cancers. The functional role of ceruloplasmin in thyroid tumors as its potential role as a marker for malignancy require further clarification.

Cytokeratins

Cytokeratins are structural proteins found in all epithelial cells; several types of keratins have been identified with altered expression patterns in malignancies. In thyroid cancer, immunocytochemical expression for prekeratin was detected in papillary thyroid cancer but not normal thyroid tissues, follicular adenomas and follicular thyroid carcinomas (42). With the development of more specific antibodies that identify cytokeratin subtypes, a more comprehensive evaluation was able to be performed. Schelfhout et al. (43) used monoclonal antibodies against cytokeratin 8, 18 and 19 to characterize cytokeratin expression in different thyroid histologies. Of these, cytokeratin-19 was overexpressed 12 of 12 papillary cancers, while follicular cancers, follicular adenomas, colloid nodules and normal thyroid tissue were negative or had only weak staining. The authors concluded that staining with antibodies against cytokeratin 19 is a useful diagnostic tool for papillary thyroid cancer. However, these promising results were not able to be confirmed. Sahoo, et al. (44) evaluated 35 surgical thyroid specimens for cytokeratin 19 expression. Although papillary cancers tended to display more intense staining than other tumors, the presence or absence of immunoactive cytokeratin 19 did not distinguish the tumor histologic subtypes. Technical issues could account for the discrepant results and further studies are needed. Cytokeratin 20 has also been evaluated in lymph nodes and peripheral blood of patients with medullary and follicular cell-derived thyroid cancer (see below).

GLUT 1

Because malignant cells typically are characterized by an increased rate of glucose utilization, overexpression of glucose transporters has been identified in malignancies, particularly overexpression of Glut-1. In thyroid cancer, Haber, et al. (45) reported the absence of immunoactive Glut-1 in 38 benign thyroid tissues, but its presence in 9 of 17 papillary, 2 of 6 follicular and 2 of 2 anaplastic cancers. These results suggest that Glut-1 could be potentially useful marker of malignancy. These results concur with clinical studies that demonstrate enhanced glucose uptake using [18F]-2-fluorodeoxyglucose (FDG) PET in aggressive thyroid tumors with a worse prognosis (46). Thus, determination of Glut-1 expression levels may be important both diagnostically and prognostically in thyroid cancer.

CA 19-9 and CD15

CA 19-9 and CD 15 (Leu-M1) are have markers for a variety of epithelial tumors and Hodgkin's disease, respectively, that have been evaluated in thyroid cancer. Immunohistochemical expression of both CA19-9 and CD15 were identified in benign thyroid tumors and in papillary carcinomas, suggesting these would not be useful markers in the clinical setting (47).

HBME-1

In contrast to CA 19-9 and CD 15, HBME-1, a tumor suppressor gene whose product is involved in signal transduction, has been reported to have a pattern of expression

suggesting it would be a potential marker of papillary thyroid cancer (48). Of importance is a recent report that demonstrated that papillary and follicular cancers with apocrine or Hurthle cell features, respectively, have distinctly lower levels of HBME-1 expression than more typical papillary and follicular tumors (49). The biological impact of this finding is uncertain. Mase, et al. recently published data demonstrating that HBME-1 expression was detected in 23% of follicular adenomas, 27% of benign goiters, but in 85% of follicular and 97% of papillary cancers (50). Based on these results, HMBE-1 is a potentially useful marker FNA samples, although follicular carcinomas require initial evaluation and the papillary cancer data require confirmation.

CD30

The CD30 antigen (Ki-1) is a cytokine receptor that is expressed in activated B and T lymphocytes, but not normal adult epithelial cells. Its expression has been demonstrated in Hodgkin's disease and Burkitt's lymphoma. The presence and distribution of both CD30 and the CD30 ligand in the thyroid were investigated using immunohistochemistry in 131 thyroid specimens and 6 normal thyroid glands (51). Normal thyroid tissue did not express CD30 or the CD30 ligand including tissue adjacent to benign nodules or follicular cancer did not express either molecule, while tissue adjacent to papillary and medullary cancer expressed CD30 ligand. Of thyroid tumors examined, 20% of follicular adenomas showed coexpression of CD30 and CD30L, while 7% of the follicular, 33% of the anaplastic, 76% of the papillary and 67% of the medullary cancers expressed both proteins. The overlap in expression between benign and malignant thyroid tissues may ultimately limit the use of this marker in identifying thyroid cancer, however, the regulation of these proteins may be very interesting for thyroid cancer biology.

Epithelial membrane antigen and Leu-7 (CD57)

Epithelial membrane antigen (EMA) is a glycoprotein that is expressed by malignant epithelial cells, while Leu-7 is an antigen expressed by immune cells whose expression has been demonstrated in a variety of tumors. Cheifetz et al. (52) evaluated the expression of these proteins in 40 benign and malignant nodules by immunohistochemistry of surgical specimens. For EMA, 16 of 22 malignant (73%) and 5 of 18 benign (28%) tumors were positive, and Leu-7 expression was detected in 20 of 22 malignant tumors and 6 of 18 benign tumors, both of which were significantly different statistically. Leu-7 expression as a marker of thyroid malignancy was also evaluated by Khan, et al. (53) who found that 95% of 83 malignant and 21% of 77 benign surgical specimens were positive. This results in an overall sensitivity of 98% and a specificity of 82%, but, as with other immunhistochemical markers, differences in the intensity and distribution of the staining were noted.

Cyclooxygenase-2

Cyclooxygenase type 2 (Cox-2) is a highly inducible enzyme in the phospholipase A2 pathway that appears to be involved in carcinogenesis. Cox-2 mRNA and protein

levels are upregulated in many epithelial cell-derived malignancies. Similarly, in thyroid cancer, Cox-2 gene and protein expression are also elevated both in surgical and FNA samples (54). These data suggest that in addition to being a treatment target, Cox-2 mRNA and/or protein levels could distinguish benign from malignant thyroid tumors.

Hypermethylation of the TSH receptor

Gene silencing can occur through a variety of mechanisms. One of the most common is hypermethylation of CPG islands in promoter regions that cause reduced expression of genes. This phenomenon has been shown to occur in thyroid cancer. Xing, et al. (55) demonstrated that detection of TSH receptor gene methylation by PCR was an accurate adjuct in the evaluation of thyroid tumors. Further work in this area is required to determine if this method is useful in a clinical setting.

MOLECULAR MARKERS OF TUMOR RECURRENCE OR PROGRESSION

The use of highly sensitive molecular tests to identify recurrent or progressive disease using tissue and/or tumor-specific markers have been used to detect metastases in bone marrow, lymph nodes, peripheral blood, and other sites. Methods employed include RTT-PCR amplification of tissue or tumor-specific transcripts or isolation of cancer cells directly using cell sorting. These approaches are particularly attractive for thyroid cancer because, in comparison to other solid tumors, initial therapy of thyroid cancer frequently results in the removal and ablation of all thyroid tissue, making both tumor and tissue-specific markers useful for early diagnosis. Several markers have been applied to nodes (Table 1) and peripheral blood (Table 2).

Lymph node recurrence

The most common sites of tumor metastases in thyroid cancer are local-regional lymph nodes, particularly for papillary cancer. These metastases are frequently present at diagnosis and can be difficult to isolate and eradicate. Standard approaches to diagnosis of local nodes include the level of elevation of serum thyroglobulin concentrations, the presence of abnormally sized or appearing nodes on anatomic imaging often with abnormal cytology on FNA, or iodine uptake in an extrathyroidal location. The diagnosis of metastatic thyroid cancer within a node frequently is confirmed by FNA, but

Table 2. Published diagnostic peripheral blood markers for thyroid cancer

Marker	References
Thyroglobulin	(66–77, 82–85, 87,88)
Thyroid Peroxidase	(67, 74, 84)
Ret/PTC Oncogenes	(67)
Cytokeratin 20	(58)
TSH Receptor	(76)
Human Kallikrein 2	(86)

this method is difficult for small nodes in the neck bed where the amount of aspirated tissue may be small. To enhance diagnostic sensitivity, there has been an interest in developing RT-PCR based approaches to amplify thyroid-specific transcripts from node FNA for both thyroid cancer derived from for papillary and follicular thyroid cancer and for medullary thyroid cancer.

Arturi, et al. (56) reported their experience using RT-PCR amplification of thyroglobulin and TSH-receptor mRNAs from nodal tissue obtained by FNA of 46 lymph nodes and compared them to cytopathology, thyroglobulin immunoassay of the aspirate fluid, and final histopathology. RT-PCR detected thyroid transcripts in 41 of 41 histopathologically confirmed metastatic tumor samples, including 45% that were inadequate or false negative by standard cytopathology. Similar results were obtained by Gubala, et al. (57) who reported their experience in 70 nodes aspirated from 60 patients with suspected thyroid cancer recurrence. Taken together, these data confirm that thyroid-specific mRNAs can be amplified from nodes in patients with metastatic thyroid cancer, that false positives from ectopic transcription in lymphocytes appears to be uncommon using these particular primers, and the overall accuracy may be adequate for clinical use. Weber, et al. (58, 59) used a slightly different approach, amplifying cytokeratin 20, an epithelial cell tumor marker, mRNA using RT-PCR from nodes suspected of harboring metastatic differentiated thyroid cancer. In comparison to cytokeratin 20 immunhistochemistry and cytology, the molecular diagnostic approach was more sensitive.

This group has also reported similar data for patients suspected to have recurrent medullary thyroid cancer in cervical nodes. The report that amplification of cytokeratin 20 and preprogastrin mRNA, a marker of neuroendocrine tumors, by RT-PCR demonstrated enhanced sensitivity and specificity over routine cytology (60, 61). These results, in combination with detection of medullary cancer-related mRNAs in peripheral blood of patients suggest this approach may be useful for patients with medullary cancer (62).

The importance of detecting metastases earlier has not been clarified in thyroid cancer, a disease that typically follows an indolent course. However, for patients with malignant melanoma, amplification of tyrosinase mRNA from sentinel lymph node tissue removed at surgery correlates with development of metastatic melanoma and subsequent prognosis (63, 64). With time, it is likely that early detection will result in better prognosis. The development of markers of aggressiveness, such as p53 mutation analysis, may provide additional predictive data that will help clinicians stratify patients for appropriate treatment paradigms. Other markers derived from cDNA array analysis may also be particularly useful in the future.

Detection of distant metastases

The most frequently employed tests for monitoring patients with thyroid cancer for tumor recurrence are measurements of circulating serum thyroglobulin concentrations and radioiodine scanning, both of which rely on thyroid-specific gene transcription or function. Non-thyroid specific monitoring methods include ultrasound, magnetic resonance imaging, computed tomography, positron emissions tomography,

and physical examination. Thyroid-specific monitoring, rather than tumor-specific monitoring is particularly useful for patients treated with thyroidectomy and radioiodine ablative therapy who are, theoretically, devoid of all thyroid tissue, benign or malignant.

The development of more sensitive and specific thyroglobulin assays has led to increased dependence on this test in monitoring paradigms. The ease of a simple blood test and the lack of exposure to radiation are two advantages of this method. However, there are several important limitations of serum thyroglobulin monitoring; 1) circulating autoantibodies directed against thyroglobulin (anti-thyroglobulin antibodies) interfere with clinical assays in approximately 20% of patients, and 2) stimulation of thyroglobulin transcription and release with either endogenous or exogenous thyrotropin (TSH) is required for adequate clinical sensitivity (65). There has therefore been an interest in developing new assays for thyroid cell detection that are not altered by antibodies and are sensitive enough to not require TSH stimulation.

Qualitative thyroid mRNA assays

Ditkoff, et al. (66) reported results from 100 individuals including 87 with thyroid cancer, 6 with benign thyroid disease (nontoxic goiters), and 5 normal subjects following total thyroidectomy (except normal subjects). Total RNA was isolated from the macrophage layer of peripheral blood, and, using RT-PCR amplification of thyroglobulin mRNA, they detected thyroid transcripts in blood from 9 of 9 patients with metastatic thyroid cancer, but from only 7 of 78 patients thought to be free of disease, and no patients having surgery for benign disease or normal control subjects. Detailed clinical information was not included regarding the clinical status of the patients and TSH levels were not reported. However, these investigators clearly demonstrated that thyroglobulin mRNA could be amplified from peripheral blood and that its presence appeared to correlate with stage of disease.

Tallini, et al. (67) subsequently reported data using different RT-PCR assays for detection of thyroid transcripts from peripheral blood. In this study, the investigators evaluated 44 patients including 24 with thyroid cancer (16 with metastases and 8 free of disease), either pre-operatively, postoperatively, or at both time points for peripheral blood expression of thyroglobulin, thyroid peroxidase, and the RET/PTC1 thyroid oncogene. 56% of the patients with either local or distant metastases had positive assays, compared to 63% of those thought to be free of disease. Of those thought to be free of disease that had positive assays, 80% had cervical adenopathy at diagnosis and were felt to be at high risk of tumor recurrence. Of the patients with benign disease, 2 of 20 patients had a positive mRNA assay, both of which reverted to negative after surgery. The in vitro sensitivities of this assay were approximately 50 cells/ml of blood. Technically, these authors isolated total RNA from whole blood drawn into EDTA-containing tubes and did not isolate a buffy coat layer.

Ringel, et al. (68) also developed a thyroglobulin mRNA assay designed for detection of circulating thyroid cells. The method employed in this study used whole blood placed directly into an RNA-stabilization solution and resulted in a more sensitive assay. In this study, 87 individuals with thyroid cancer were evaluated. Thyroglobulin

mRNA was detected in all 14 cervical or distant metastases during L-T4 therapy, while 65% of patients with thyroid bed uptake and 20% of patients with no uptake had detectable thyroglobulin mRNA. These data suggested both a high sensitivity and lower specificity of the assay than the prior studies. Of concern was that similar to the patients with multinodular goiter analyzed by Tallini et al. circulating thyroglobulin mRNA was detectable in all of the normal subjects evaluated and in 20% of athyreotic patients. These results raised the possibility that thyroglobulin may not represent a truly thyroid-specific transcript and that this more sensitive assay detected ectopically transcribed of thyroglobulin in non-thyroid cells. Alternatively, the assay could have been detecting very early minimal residual or recurrent disease.

Additional data have been published from many groups using similar qualitative approaches to amplify thyroglobulin and other mRNA transcripts from peripheral blood. The results have been remarkably variable, with some groups demonstrating excellent correlation between tumor stage and results (69–72), while others demonstrate no correlation with tumor stage (73–75). Several have concluded that the assay is more useful for papillary rather than follicular cancer (69), while others have demonstrated optimal screening by combining thyroglobulin mRNA with new highly sensitive thyroglobulin immunoassays (71). Taken together, nearly all groups have confirmed the presence of circulating thyroglobulin mRNA in peripheral blood of normal subjects, and in a subset of athyreotic patients, suggesting that ectopic transcription of thyroglobulin or splice variants of thyroglobulin can be detected.

The importance of assay methodology has been highlighted in several recent studies. Bojunga, et al. (73) reported data using low and high sensitivity qualitative thyroglobulin mRNA assays in patients with thyroid cancer. Using a lower sensitivity assay, they detected circulating thyroglobulin mRNA in 69% of patients with metastatic disease, 46% of patients with thyroid cancer thought to be free of disease, 25% of patients with benign thyroid disease and 18% of control patients. The more sensitive assay increase sensitivity modestly, but resulted in the complete loss of specificity. Gupta, et al. (76) created PCR primers designed to carefully avoid amplification of all known splice variants of thyroglobulin and the TSH receptor. Using these PCR primers, these authors reported detection of thyroid transcripts in 83% of thyroid cancer patients with positive compared to 5% of patients with negative radioiodine scans. All normal volunteers were negative. The specificity was slightly greater for TSH mRNA detection rather than thyroglobulin mRNA detection. Similarly, Savagner, et al. (77) designed thyroglobulin primers that amplified known splice variants and others that did not. They determined that the splice variants account for approximately 1/3 of the total amplified thyroglobulin mRNA, and that when the primers that do not amplify the region are used, the results correlated with the volume of thyroid tissue and TSH concentration. Taken together, these data clearly demonstrate the importance of methodology in performing these assays, and in proper evaluation of the published data. Differences in sensitivity could be due to the method of sample collection, storage of samples between the phlebotomy and RNA isolation, the specific method for reverse transcription and the PCR primers employed.

Quantitative thyroid mRNA assays

Due to the subjective nature of PCR and the apparent discrepancy in the results of studies using qualitative RT-PCR systems, there has been interest in attempting to quantify peripheral blood RT-PCR assays in order to define a clinically relevant level of detection. The advent of real-time quantitative PCR has enabled testing of this approach in clinical trials. Similar to quantitative RT-PCR, the methodological issues are considerable, particularly when attempting to detect very rare transcripts within a particular sample. Other major issues when considering quantitation of RNA is normalization to a control transcript. Traditionally, normalization to glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) or beta actin has been employed; however, tremendous variability in these control transcripts has been reported (72–74). An alternative is normalization to total RNA (18S), while others have chosen not to normalize transcripts at all and normalize to the original blood volume (78–80). This also may not be an accurate method and the use of a "geometric" panel of markers has recently been suggested (81). Thus, it is apparent that normalizing to different control transcripts clearly will alter the reported results and, to date, no standard method has been applied by all laboratories; however, it appears clear that normalizing to a single "housekeeping" gene such as GAPDH or beta actin is likely not appropriate for these samples (81).

Wingo, et al. (82) reported the first quantitative thyroglobulin mRNA assay. In this study, total RNA was derived from peripheral blood samples and the assay was extensively tested. Calibration assays revealed interassay variability of 17–22% due primarily to RNA stability, RNA handing and the reverse transcriptase reaction. The assay displayed reproducible results over a three log concentration range. Ringel, et al. (83) subsequently used this assay to analyze peripheral blood RNA from 107 patients with thyroid cancer; including 84 during L-T4 therapy, 14 following L-T4 withdrawal, and 9 before and after thyroxine withdrawal. Twenty-three patients had circulating anti-thyroglobulin antibodies. Using an arbitrary cut-point to identify patients as either positive or negative for detection (36 PCR cycles), thyroglobulin mRNA measurement assay was more sensitive than thyroglobulin immunoassay, but was less specific at detecting the presence of local and distant metastases. In addition, while there was a statistical correlation between the level of thyroglobulin mRNA and the presence of thyroid tissue on scan, the level of thyroglobulin mRNA did not correlate well with stage of disease. Importantly, the assay appeared to be unaffected by circulating anti-thyroglobulin antibodies, suggesting that perhaps Thyroglobulin mRNA could be used as an adjunctive test to identify patients with recurrent or residual thyroid tissue in the presence of anti-thyroglobulin antibodies. However, the authors cautioned that there was significant overlap between the patients with positive results without definable disease and those with disease, a factor which may limit the usefulness of this particular assay method in clinical practice. Thus, for individual patients, the absolute value of thyroglobulin mRNA did not appear to be diagnostically useful, but the presence or absence of thyroglobulin mRNA might be useful. In addition, similar to other studies, even using a cut-point, a significant minority (38%) of patients with no evidence of

disease had positive results and many had detectable values below the cut-point. The relevance of an isolated thyroglobulin mRNA level is uncertain as it might reflect a false positive result from ectopic expression, or the presence of bona fide residual thyroid tissue.

Savagner, et al. (77) developed a quantitative assay for measurement of thyroglobulin mRNA in peripheral blood. In this study, the cut point of a positive or negative assay was determined to be the amount of circulating prostate specific antigen mRNA as a control transcript, no internal normalization was performed and results were reported per total RNA amount. The results in this study were similar to those of Ringel, et al. in that using a mean value, there was a statistical correlation with the absence or presence of residual or recurrent thyroid tissue, but there was significant overlap between all groups for individual data.

Similar to the experience with qualitative thyroglobulin mRNA assays, variable results have also been reported with the quantitative approach. Some of these differences are methodological (different primers, use of DNase I, normalization), inherent in the assay method (instability of RNA), while others may be interpretive. Takano, et al. (84) performed a study evaluating thyroglobulin mRNA from peripheral blood and similar to Ringel, et al. identified this transcript in all patients. Unlike the prior study, they were not able to correlate levels with stage of disease. However, in this study, the normalization was performed in a different manner (GAPDH), different PCR primers were utilized, and DNase I treatment was not performed, all different from Ringel, et al. Takano, et al. (84) also report similar data amplifying thyroid peroxidase (TPO) as a tumor marker, results that did not agree with those of Roddiger, et al. (74) who reported a better correlation using TPO mRNA amplification than thyroglobulin mRNA in patients with thyroid cancer. Eszlinger, et al. (85) also did not demonstrate correlation between thyroglobulin mRNA levels and the presence or absence of thyroid tissue. They evaluated several different methods of blood collection and also describe important differences in results depending on the types of tubes used for phlebotomy and the time between the sample collection and RNA isolation. These authors used a new set of primers and normalized to beta actin, factors that distinguish their method from others. To further clarify the importance of recognition of assay differences between groups, Span, et al. (75) used the same thyroglobulin PCR primers as earlier reports and were not able to confirm a relationship between stage of disease and level of thyroglobulin mRNA. However, distinct from those reports, the authors used a different method of RNA isolation and normalize their results to beta actin, both important differences in assay methods that can alter results.

Tumor-specific mRNA assays

Additional markers, such as cytokeratin 20 and human kallikrein 2 mRNA amplification have recently been reported to have potential diagnostic benefit for thyroid cancer patients (58, 86). These are not thyroid-specific, but may be cancer-specific. These preliminary data require confirmation, but may be an interesting alternative approach to molecular diagnosis of metastatic disease.

Thus, based on these data, it seems that there is clear evidence of ectopic expression of thyroglobulin, or at least splice variants of thyroglobulin in non-thyroid tissues. Assay quantitation to "subtract out" this amplification is of uncertain value due to differences in the reported methods and the challenges of normalization of results. Further study and clarification of these issues, in particular, the use of primers that do not amplify splice variants, determination of the best processing protocol for blood RNA isolation, and whether an appropriate form of normalization exists are required before a clear assessment regarding the clinical usefulness of this approach to molecular diagnosis can be made.

SUMMARY

The use of molecular assays to analyze clinical tissues in the diagnosis and management of thyroid cancer, similar to other tumors, will likely allow for more accurate characterization of the aggressiveness of individual tumors and may allow for the early diagnosis of recurrence. The application of these methods to thyroid nodules and nodal metastases is less encumbered by difficulties arising from amplification of transcripts in non-thyroid cells. For these tissues, these assays are likely to be used clinically in the near-future. New data arising from cDNA arrays identifying novel markers of malignancy or tumor aggressiveness make this a growing area of interest. The use of molecular assays in diagnosing distant metastases is more problematic due to issues with ectopic expression of either full length or splice variants of genes thought to be thyroid-specific. Assay quantitation is a complex problem owing to variability in the level of expression of "housekeeping" genes and the variety of phlebotomy and RT-PCR methods reported. Additional research in this area is clearly required before a recommendation can be given regarding clinically applicability of these tests.

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18. THYROID CANCER IMAGING

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INTRODUCTION

Molecular imaging in thyroid cancer using nuclear medicine methods is based on specific cellular characteristics. These characteristics can be derived from common cell features, but can also be based on specific properties of thyroid cancer cells. While in the diagnosis of thyroid cancer these methods have not found great potential, many applications can be found in treatment and follow up of the papillary, follicular and medullary thyroid carcinoma patients. In anaplastic thyroid carcinoma the experience with nuclear imaging is scarce, but the clinical relevance in this aggressive tumor is low.

The broad spectrum of radioactive tracer methods is associated with a great variety in sensitivity and specificity. This variation is partly based on cellular or tumor cell characteristics but also can be explained by the different technical factors and techniques. For example, where radioiodine imaging is among the cornerstones of thyroid cancer treatment, this tracer is of limited value in medullary thyroid cancer. This difference illustrates the importance of the specific cell characteristics that governs uptake of radiotracers. C-cells do not take up radioiodine, while follicular thyroid cells do. Another example can be found in the uptake of the tracer 18 FluoroDeoxyGlucose (FDG), which can be used in conjunction with the Positron Emission Tomography (PET) technique. Uptake of this tracer is based on the glucose metabolism that is present in benign and malignant cells. However, the demand for glucose is considerably higher in malignant cells, which results in higher tracer uptake and adequate

imaging of thyroid cancer lesions. Also other nuclear imaging techniques have their additional value in the diagnosis and sometimes treatment of thyroid cancer. For some tracers the discovery of its value in the diagnosis of thyroid cancer is a matter of serendipity and the mechanisms of action are not always fully understood.

In this chapter nuclear medicine tracers methods, commonly used in thyroid cancer patients, will be reviewed, with a special emphasis on the general uptake mechanism, followed by the method of scanning and the clinical applications.

IODINE

General mechanism

The synthesis of thyroid hormone depends on the supply and metabolism of iodine in the thyroid gland and on the synthesis of thyroglobulin (a receptor protein for iodine). Iodine is taken up by the thyroid follicular cells as inorganic iodide and is transformed through a sequence of metabolic process into thyroid hormones (thyroxine (T4) and triiodothyronine (T3)).

The recommendations of the World Health Organization (WHO) for the iodine intake is 90–200 μg/day (90 μg/day for the newborn, 200 μg/day for the pregnant and lactating women) to maintain growth, development and normal thyroid function (1). The average daily dietary intake of iodide varies greatly per area or country. An average of 190–300 μg iodide per person is ingested daily in the United States. In Europe the average daily intake varies greatly from 50 μg (Belgium) to 430 μg (Great Britain) (2,3). About 60 to 80 μg of iodide is taken up daily by the thyroid from the circulating pool that ranges from 250 to 750 μg. If this extrathyroidal iodide pool is labeled with radioactive iodine (^{131}I or ^{123}I), the percentage of uptake of this tracer in 24 hours (8 to 35%) gives a dynamic index of the thyroid gland activity. The total iodide content of the thyroid gland averages 7500 μg, virtually all of which is in the form of iodothyronines (secretory products of the thyroid gland). In a steady state condition 60 to 80 μg (approximately 1% of the total) iodide is released from the thyroid gland daily. Of this amount 75% is secreted as thyroid hormones, and the remainder is free iodide. The large ratio of iodide stored in the form of hormone to the amount of tuned over daily, can protect the individual from the effects of iodide deficiency for about 2 months (4).

Iodide is actively transported into the thyroid follicular cells against chemical and electrical gradients, the iodide trapping. The site of active iodide transport in thyroid follicular cells is the basolateral membrane. The transport of iodide across this membrane is linked to the transport of sodium (Na+/I symporter (NIS)), generated by Na+/K+-ATPase as the driving force. Iodide trapping is stimulated by the thyroid-stimulating hormone (TSH).

Once in the thyroid follicular cell, iodide moves to the apical surface of the cell and seems to be translocated across the apical membrane by the chloride/iodide transporter molecule pendrin (encoded by PDS-gene) into the lumen (colloid) of the follicle cell (5,6,7). The function of pendrin in the thyroid is currently not precisely determined (5,6,8). Once in the follicular lumen, iodide is immediately incorporated into tyrosine

residues of thyroglobulin (a glycoprotein synthesized on the rough endoplasmatic reticulum). Within the follicle thyroglobulin is iodinated and via monoiodotyrosine (MIT) and diiodotyrosine (DIT) T_4 and T_3 are formed.

Radioactive iodide (^{131}I and ^{123}I) can be used to visualize the thyroid gland and to measure the iodide trapping function. Only the follicular and the papillary variants of thyroid carcinomas, together called differentiated thyroid carcinoma (DTC), have the ability to concentrate radioiodine. Nevertheless, the iodine metabolism is profoundly altered in DTC. Iodine uptake is quantitatively decreased compared with the uptake in normal thyroid tissue. Furthermore, the iodine organification process is defective in thyroid cancer tissue, resulting in shorter biological half-life within the thyroid. Thyroid hormone synthesis is also usually absent. These abnormalities in organification and hormone synthesis are related to decreased NIS-expression and peroxidase genes and the impairment of the pendrin- PDS gene pathway (7). PDS-gene and pendrin expression seems to be dramatically decreased only in DTC (6, 7).

Stimulation of TSH will induce uptake of in tumors that are able to concentrate radioiodine and increase Tg production by all tumor tissues, even in lesions unable to concentrate radioiodine (9). In order to be detectable by gammacamera imaging, lesions must have a critical combination of size and tracer uptake. Thus, the ability to visualize thyroid cancer remnants or metastatic tissue with radioiodine depends on several factors: a critical cell mass; the activity of the iodine trapping and organification mechanisms and incompletely defined mechanism (e.g. pendrin) that export or clear iodine from the cells.

Delivery of radioiodine to the thyroid tissue therefore requires stimulation by high level of endogenous TSH, induced after an adequate period of withdrawal from thyroid hormones causing hypothyroidism.

Iodine isotopes

Several iodine isotopes play an important role in nuclear medicine, as well for in vivo imaging as for in vitro investigations. Radioiodine can be used as a tracer itself, but is also very suitable to label other molecules. Three kinds of iodine isotopes, including iodine-123 (^{123}I), iodine-125 (^{125}I) and iodine-131 (^{131}I), are widely applied in nuclear medicine. ^{123}I and ^{131}I are used for imaging, ^{125}I is unsuitable for imaging, but often used for in in vitro applications and radiolabeling of other substances. Iodine-124 (^{124}I) is a positron emitting isotope, which is suitable for positron emission tomography (PET) imaging. A summary of the properties of these iodine isotopes is presented in Table 1.

Table 1. Characteristics of iodine isotopes

Iodine isotopes	$T^1/_2$	Decay	Energy (keV)	Application
^{123}I	13 hours	EC, γ	159	diagnostic
^{124}I	4.1 days	$\beta+$	511	PET
^{125}I	60 days	EC, γ	28 + 35	radioimmunoassay
^{131}I	8.04 days	β^-	330 (max)	therapeutic
		γ	364	diagnostic

For diagnostic purposes the gamma emissions are important. The distribution of the radiopharmaceutical inside the body can be externally measured through imaging with gamma cameras. For therapeutic purposes the beta energy emission is important because of the destructive character in tissue. The path length of the beta particle depends on the energy and ranges from several mm to 1 cm.

Iodine-131

An important property of ^{131}I is that it can both be used for imaging purposes (high energy gamma ray) as well as for therapeutic purposes (medium-energy beta emission) while most of other radionuclides only have diagnostic utility. ^{131}I has however sub-optimal imaging characteristics, including high energy gamma ray (364 keV), which is not optimal for most gamma cameras. The long half-life (8.04 days) and high beta emission limit the administered dose for diagnostic purpose only. The path length of the beta particle is about 0.5 mm, the toxic effects are limited to the thyroid tissue, with therefore sparing of adjacent normal tissue. The normal biodistribution of iodine includes salivary glands, stomach and renal tract including the bladder.

The long half-life is advantageous in the detection of functioning metastatic thyroid cancer lesions, because imaging can be done for many days after administration. This enables long take up periods in metastatic tissue and adequate clearance of background activity.

The radiation dose delivered by ^{131}I concentrated in a tissue depends on two factors: the radioactive concentration (the ratio between total uptake and the volume of functioning tissue) and the effective half-life (time after which the radioactivity in the tissue has decreased by a factor of 2). The effective half-life is related to the physical half-life and the biological half-life, which is related to the elimination of ^{131}I from the concentrating tissue.

In normal thyroid tissue the concentration is about 1 to 2% of the administered ^{131}I activity per gram and effective half-life is about 8 days. Functional thyroid cancer tissues concentrate under favourable condition about 0.1 to 0.5% of the administered ^{131}I activity per gram and the effective half-life is shorter than 3 days (9).

Iodine-123

Like ^{131}I the chemical behavior of ^{123}I is identical to that of stable iodide. The half-life of ^{123}I is 13.2 hours. ^{123}I decays by electron capture and is a lower energy gamma emitter (159 keV) compared with ^{131}I. Therefore the resulting imaging quality is better than ^{131}I. In addition, ^{123}I delivers a lower radiation dose to the thyroid tissue due to the absence of beta particle emission which may prevent a possible 'stunning' effect (discussed below). The major disadvantages of ^{123}I are the high cost due to the facts that it is produced by cyclotron, the limited availability and furthermore, the short half-life.

Iodine-124

While the radioisotopes ^{123}I and especially ^{131}I are used on a wide scale in diagnosis and treatment of all thyroid disorders, the positron emitting isotope ^{124}I, which is suitable for PET, has received little attention. Chemically identical to non-radioactive iodine, this

isotope would allow thyroid cancer imaging using the high resolution PET technique (10). ^{124}I,however, is difficult to obtain and only available at specific research centers, as it is produced in a cyclotron. The isotope has a relatively low yield ofradiation (positron yield 23%) suitable for imaging, but also emits other high-energy gamma radiation that increases the radiation to the thyroid (when present) almost to the (therapeutic) level of ^{131}I. In addition, the high-energy byproducts may deteriorate image quality. For these reasons clinical use has been minimal. ^{124}I has been used for dosimetric purposes or thyroid volume measurements (11, 12, 13, 14, 15). Recent development of combined PET-CT scanners with a single gantry, may increase clinical application in thyroid cancer patients, as detailed anatomical information is combined with the location of iodine positive tissue (16). The clinical value, for example, as compared to ^{131}I scintigraphy, is currently unknown.

Iodine-125

^{125}I decays by electron capture and gamma emission. The very low energy gamma emission (28–35 keV) and the long half-life (60 days) of ^{125}I make this radionuclide less suitable for in-vivo application. The very low energy gamma ray is to weak to be detected by gamma cameras. However, ^{125}I is extremely suitable for in-vitro application. It is a common agent for use in radioimmunoassay.

Scan method

Patient preparation

Thyroid stimulating hormone (TSH), produced by the pituitary is essential for stimulation of thyroid cells for optimal imaging with radioiodine. There are two ways to prepare a patient for radioiodine imaging: thyroid hormone withdrawal or administration ofrecombinant human TSH (rhTSH) during thyroid hormone therapy. Standard thyroid hormone medication (l -thyroxine, T4) withdrawal is usually 4–6 weeks until the serum TSH is greater than 30 mU/l to permit maximum stimulation of thyroid tissue. L-triiodothyronine (T3, Cytomel) replacement therapy (25 μg BID or TID) can be given the first 4 weeks of a 6 weeks withdrawal due to the short half-life and the immediate effects of L-triiodothyronine. The transient thyroid hormone suppletion withdrawal is associated with morbidity of hypothyroidism and therefore decreases the quality of life and diminishing productivity (17).

Recombinant human TSH (rhTSH) prevents the profound symptoms of hypothyroidism as a consequence ofthyroid hormone withdrawal. rhTSH increases serum TSH concentration sufficiently to stimulate thyroidal ^{131}I uptake and release of thyroglobulin (Tg) while patients are still taking thyroid hormone medication. The recommended protocol of rhTSH is two intramuscular injections of 0.9 mg given on 2 consecutive days followed by 148 MBq (4 mCi) ^{131}I on the third day and a WBS and Tg measurement on the fifth day. Whole body images were acquired after 30 minutes of scanning or after 140,000 counts. This is necessary because 4 mCi ^{131}I after rhTSH has about the same effect as 2 mCi given in the hypothyroid state with reduced renal clearance and raised ^{131}I body retention (18,19).

However, it must be emphasized that there is so far few experience concerning this issue especially on the long term effects on outcome, so the application of rhTSH in the diagnostics still a matter of discussion (20).

Diagnostic ^{131}I WBS

TECHNIQUES OF SCANNING. Imaging is performed using high-energy collimator. The bladder must be emptied before imaging. Supine anterior and posterior images of the neck, chest, abdomen and pelvis are acquired. Anatomic landmark or transmission scans using cobalt marker can be helpful in the interpretation of the images. Additional or delayed images can be obtained in patients with atypical findings on the scans.

INTERPRETATION. The correct interpretation of the radioiodine images is crucial in the therapy management of thyroid cancer. It requires knowledge and understanding of the normal biodistribution of radioiodine. Radioiodine uptake in the choroid plexus, nasal mucosa, salivary glands, mammary glands, gastric mucosa, gastrointestinal tract and urinary tract including bladder should be considered as physiological. These tissues contain like thyroid tissue NIS-transporter. Diffuse iodine uptake in the liver can also be seen on the post-treatment scans when there is functioning thyroid due to the incorporation of radioiodine into thyroid hormones which are degraded in the liver by de-iodination and conjugation. Uptake of radioiodine outside the above mentioned organs should be considered as residual and/or metastatic thyroid tissue (true positive) or as contamination (false positive) (21,22).

Clinical application

Pretherapeutic diagnostic scintigraphy

The goal of the diagnostic scan after total or near-total thyroidectomy is to quantify the residual thyroid and detect metastatic disease. It is also included as part of the follow-up procedures. The ablative or therapeutic dose of ^{131}I used for treatment can be based on the results of the diagnostic scan. The diagnostic WBS is usually acquired 48–72 hours after administration of a diagnostic dose of ^{131}I during hypothyroid state.

Performing diagnostic ^{131}I scans before ablation therapy (23) or during follow-up, up is controversial (24).

The reason to perform no pre-ablative diagnostic ^{131}I scintigraphy is, that it is known that nearly all patients show residual neck uptake after (near) total thyroidectomy. And some believe that low diagnostic dose of ^{131}I may impair the thyroid remnants uptake of the subsequent ablative dose of ^{131}I, the so-called stunning effect. This issue will be discussed further on. Carlisle et al. (22) support performing diagnostic scans prior to therapy for several reasons. First, patients with undectectable Tg and a normal diagnostic scan after total thyroidectomy need not to be treated with ^{131}I. Second, a correct treatment ^{131}I dose can be determined when the extent of the disease is known.

Discussions are continuing concerning performing diagnostic scans before ^{131}I treatment in patients with elevated serum Tg. Cailleux et al. (24) suggest that diagnostic scanning need not to be done when serum Tg is higher than 5 ng/ml and one rather should considered therapy and posttherapeutic scan after thyroid hormone withdrawal.

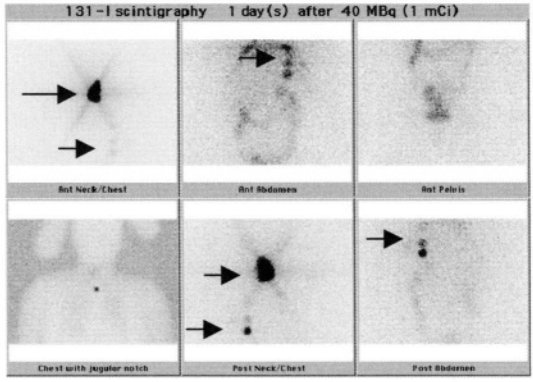

Figure 1.1. Pre-therapeutic diagnostic ^{131}I-WBS 1 day after 40 MBq in a 37-year-old patient with papillary thyroid carcinoma with elevated serum Tg after total thyroidectomy. It shows intense uptake in the neck (arrow) and uptake in the lung (arrow). Normal biodistribution in the gastrointestinal tract and bladder. This patient was subsequently treated with ablative dose of 1850 MBq ^{131}I.

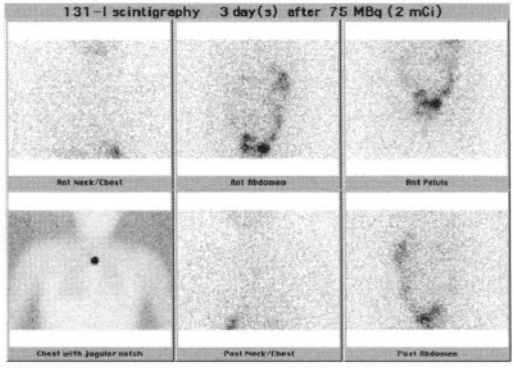

Figure 1.2. Posttherapeutic ^{131}I-WBS 10 days after a treatment dose of 5550 MBq in the same patient with papillary thyroid carcinoma with persistent elevated serum Tg and a negative diagnostic ^{131}I-WBS 3 months after ablative dose of 1850 MBq. This posttherapeutic ^{131}I-WBS was also negative, with only normal biodistribution in the gastrointestinal tract and bladder.

Pacini (25) suggest that diagnostic scanning is of low usefulness when the serum Tg-off T4 is undetectable after initial therapy.

Posttherapeutic (diagnostic) scintigraphy

A consensus for optimal dose of ^{131}I for ablation has not been reached. Some preferred a dosimetric approach by blood and whole-body and quantitative dosimetry to define the ablative dose. The majority use a standard fixed dose, which can range from 1110 MBq to 7400 MBq (30 mCi–200 mCi), depending on tumor characteristics (18,26), because of its simplicity and safety.

The timing of the acquisition of a post-therapy scan can vary widely. The interval varies from 1 day to 10 days after a therapeutic dose. However, shorter time interval allows less time for soft tissue clearance of radioiodine resulting in a relatively higher soft-tissue background which could make ^{131}I foci less visible and difficult to detect (27,28). More lesions are identified on the post-therapy scans than on the diagnostic scans. Carlisle (22) have found a discrepancy of 10% which alter the treatment management in 5% of the cases. These findings were similar with that of Fatourechi (29). The reasons of detecting more disease on the post-treatment scans compared with the diagnostic scans are probably due to the higher therapeutic doses and the longer time delay (22).

Post-therapy scans are most likely to yield important information when the serum Tg is elevated in a patient who is clinically disease-free with negative diagnostic scans or other conventional radiologic imaging (19,30).

Stunning

The timing and the amount of the diagnostic and therapeutic ^{131}I dose are controversial. There has been controversy concerning whether radiation of the diagnostic dose really has a suppressive effect on the uptake of subsequent therapeutic ^{131}I, the so-called stunning-effect (31). For extensive review of stunning see chapter 11.

The issue whether stunning is a real phenomenon and its clinical relevance/consequence is questionable (32). Our retrospective evaluation of 158 patients, who received a high-dose diagnostic scan with 370 MBq (10 mCi) because of a negative low-dose diagnostic scan with 74 MBq (2 mCi) ^{131}I, demonstrates that diagnostic ^{131}I scan with 74 MBq (2 mCi) is sufficient for correct clinical decision making with regards to further radioiodine treatment, when combined with Tg-off measurements. In 98% of the patients a 370 MBq (10 mCi) dose of ^{131}I for diagnostic WBS had no additional value (33).

rhTSH

The yield of ^{131}I scans seems to be slightly lower with rhTSH than following thyroid hormone withdrawal (17), although another study mentioned a similar diagnostic yield (34). ^{131}I scanning itself provides complementary information besides the measurement of Tg after withdrawal (24) or after rhTSH (35). For now it is unclear which specific patient group will have benefit of this follow-up policy with rhTSH (20).

The retrospective review of Robbins (35) showed no significant difference in the rate of complete ablation between a group of patients who were prepared with rhTSH or by thyroid hormone withdrawal. Other reports mention the effectiveness of rhTSH in ablative therapy (26,36). However, in the study of Menzel (37) there is a significant reduction in the effective half-life of ^{131}I in patients after rhTSH-stimulated TSH before radioiodine therapy compared with patients after endogenous stimulated TSH. Although the use of rhTSH in the follow up patients with thyroid cancer is proposed (38,39) proper prospective data concerning rhTSH applications are still very poor or even lacking (40).

Lithium

Lithium has an inhibitory effect on the release of iodine from the thyroid but does not change the uptake. The mechanism by which lithium inhibits the secretion of thyroid hormone is not well understood. In vitro, lithium decreases the droplet formation of the colloid of thyroid follicular cells, which is a reflection of a decreased pinocytosis of colloid from the follicular lumen (41). The efficiency of proteolytic digestion of thyroglobulin may also be impaired. For this feature lithium may be useful as an adjuvant for ^{131}I therapy of thyroid cancer.

However, there are very few experiences concerning the application of lithium in thyroid cancer. Only in one study was shown, that lithium prolonged the biological and effective half-lives and increased the accumulation of ^{131}I by 50% in tumors and 90% in thyroid remnants.

Thus, it is in tumors that are less likely to respond to ^{131}I therapy that lithium may be most useful but further experience is required (42).

Retinoic acid

Retinoic acids are biologically active metabolites of vitamin A. They play an important role in the morphogenesis, differentiation and proliferation of many cells (43,44). Retinoic acid has been used for cancer treatment due to their growth and differentiation effects.

Dedifferentiation changes can occurred in differentiated thyroid cancer. This is accompanied by loss of thyroid-specific function and loss of iodide uptake, which makes the therapy with radioiodine inaccessible. It seems that retinoic acids have the potential for redifferentiating therapy in these advanced stage of thyroid cancer (43,44). Nevertheless, the therapeutic effects of isotretinoin in thyroid cancer is so far very disappointing and further controlled clinical trials are required (45).

Sodium iodide symporter (NIS)

The human NIS gene is localized on chromosome 9p12–13.2. NIS is an integral protein of the basolateral membrane of thyroid gland follicular cells. Uptake of iodide from the interstitium into the cell through the NIS-transporter is an active process.

NIS-expression is inversely related to the degree of differentiation of thyroid cancer cells. NIS is more expressed in differentiated thyroid cancer and often negative in less well-differentiated thyroid cancer. Elucidating of the molecular mechanism of NIS expression in thyroid cancer might have the potential in enhancing the diagnostic and therapeutic management since thyroid cancer tissues with NIS expression take up more ^{131}I and subsequent show a high rate of response to radioiodine therapy than those without NIS expression (46,47) (see also chapter 11).

Blind therapy of ^{131}I

After total thyroidectomy and radioiodine ablation, an elevated serum Tg level as well as positive diagnostic radioiodine scanning, are good indicators of the presence of persistent, recurrent or metastatic thyroid cancer (48,49). However, there is a management

dilemma in case of negative diagnostic radioiodine scanning and an elevated serum Tg. Negative diagnostic radioiodine scanning may be caused by factors such as an insufficient rise in serum TSH or iodine contamination (50). Another explanation for negative diagnostic scanning is dedifferentation of the tumor leading to a loss of its iodine trapping ability while Tg production is still preserved. Finally, the presence of microscopic metastases that are too small to be visualized with a diagnostic ^{131}I dose, which can cause false negative scans. Nowadays in patients with negative diagnostic radioiodine scanning, an empirical therapeutic dose between 100–300 mCi ^{131}I, followed by a posttherapy whole-body scan (WBS) is advocated (24,28,38,51,52). The purpose of this approach is twofold. First, posttherapy radioiodine scanning after high-dose ^{131}I treatment is believed to be the most sensitive tool for localizing residual disease not shown by diagnostic scanning with 2–5 mCi ^{131}I (28,53,54). Thus detected residual disease can be treated with other forms of therapy, such as surgery or radiotherapy. Second, small metastases not seen on diagnostic scanning may accumulate sufficient ^{131}I after high-dose ^{131}I treatment, leading to a relevant reduction in tumor load. Several studies have shown a drop in serum Tg after high-dose ^{131}I treatment in patients with negative diagnostic radio-iodide scanning (54,55). Serum Tg remained the same or Tg increased (28,56). Since patient numbers in these studies are small and follow-up data are scarce, it is still unclear whether such high-dose ^{131}I treatment after negative diagnostic radio-iodide scanning is of benefit for the patient. Recently, several reports were published that show no additional effect of high-dose ^{131}I therapy (52,57,58), except for limited cases as lung metastases (52). High-dose ^{131}I treatment in patients with negative diagnostic ^{131}I WBS and detectable serum Tg during hypothyroidism can be used as a diagnostic and prognostic tool (59).

18 FLUORODEOXYGLUCOSE (FDG)

General mechanism

The glucose analogue FDG is a tracer of glucose metabolism, and enters cells by the same mechanisms both in benign and malignant tissue. However, the energy metabolism of malignant cells is considerably less efficient than the metabolism in their benign counterparts (60). For example anaerobic glycolysis is strongly increased in malignant cells, which is associated with less energy (ATP) production per molecule of glucose as compared to the energy production resulting from the citric acid cycle. Therefore, the need for glucose molecules and FDG is strongly increased in malignancy, which is the basis for the preferential uptake of FDG in malignancy. FDG is intracellularly phosphorylated by a hexokinase into FDG-6-phosphate, which is not further metabolized, in contrast with glucose-6-phosphate. In addition, the FDG-6-phosphate cannot leave the cell again, and the compound is therefore trapped intracellulary. The final accumulation of FDG-6-phosphate is proportional to the glycolytic rate of the involved cell. In some tissues however, the level of phosphatase activity may be variable, and FDG accumulation in liver, kidney, intestine, muscle and some tumor cells may be lower. Apart from the increased glycolysis, it has been demonstrated that levels of

transmembrane glucose transporters (e.g. the GLUT-1 transporter) and possibly also of some hexokinase isoenzymes are also increased in malignancy and relate to FDG uptake (61,62,63,64). On the one hand, the uptake mechanism of FDG with selective irreversible trapping of the tracer in malignant tissue is ideal for Positron Emission Tomography (PET) imaging, which has generated the increasing clinical application in oncology. On the other hand, it can be understood that FDG uptake does not exclusively occur in malignant tissues, as also benign tissue requires glucose. Especially activated macrophages, as present in infection and inflammation, are known to accumulate much FDG, sometimes to a degree that interferes with oncological image interpretation (65,66).

Scan method

In PET imaging radioactive tracers are used that emit positrons. After positron emission, the positron annihilates with a ubiquitous electron, which causes emission of two 511 KeV photons, precisely under an 180 degree angle. These photons are simultaneously detected by a ring of detectors, which are the main component of the PET camera.

FDG uptake occurs rapidly after administration, and due to the uptake mechanism, the amount of FDG that is taken up in tumor tissue, increases over time. Due to excretion of FDG, which causes clearance of 'background' uptake, and the decay of the radioactivity ($T^1/_2$ = 110 min) the optimal moment for imaging is generally considered to be 60–90 min after tracer administration.

For precise patient preparation and image protocols we refer to dedicated PET papers or books (67). Briefly, patients are generally injected with FDG in a fasting condition and after oral prehydration. The injected dose varies between 2-8MBq/kg. The scan duration for a whole body scan varies largely, but is in general 30–60 min.

Clinical application

Papillary and follicular thyroid carcinoma

FDG PET is not considered to be a useful method in the primary diagnosis of thyroid cancer. Although this issue has not received much study, the uptake of FDG in thyroid cancer in general appears to be low, and image interpretation may suffer from interfering uptake in benign tumors, such as follicular adenoma. In addition, the diagnosis can nearly always be obtained by other diagnostic methods.

Much more data are available to underscore the value of PET in the follow-up of thyroid cancer patients, such as to detect recurrences or metastases, especially in cases where metastases do not trap radioiodine. Interestingly, there appears to be a complementary uptake of FDG and radioiodine, which has been termed the 'flip-flop' phenomen. This means that some metastases within the same patient that do not trap radioiodine may accumulate FDG, and metastases that do not trap FDG, accumulate radioiodine. Some lesions accumulate both tracers. This observation was first described by Joensuu (68). It might be explained by the different degree of tissue

differentiation. Well-differentiated thyroid cancer tissue has retained its iodine trapping capabilities, but is metabolically inactive, causing uptake of radioiodine and no or minimal FDG accumulation. Less differentiated thyroid cancer tissue, as may develop during treatment, loses its iodine trapping capability and becomes metabolically more active. This results in FDG positivity and iodine negativity. For this reason, most PET research has focussed on detection of thyroid cancer metastases in radioiodine negative patients with increased thyroglobulin levels, which currently seems to be the best clinical application.

In a recent meta-analysis the value of FDG PET in papillary and follicular thyroid cancer both in patients with negative radioiodine scans and in patients with known neoplastic foci was determined (69). They selected 14 studies that met quality criteria as described by the Cochrane Methods Group on Screening and Diagnostic Tests. Although general evidence levels appeared to be low, precluding quantitative summary, all these studies claimed a positive role for PET, especially in the group of patients with negative radioiodine scans. Sensitivity for finding tumor locations of PET varied between 70 and 95%, and specificity was between 77 and 100%. Considerable heterogeneity existed, however, in the pre-PET data risk profile, such as patient selection criteria concerning variations in TNM stage, Tg levels, radioactive radioiodine dose and levels of TSH. Although troubled by severe methodological problems, the performance of FDG PET appeared to be superior to 99mTc-Sestamibi or Tc99m-furifosmin, and probably Tl-201 scintigraphy. Also the impact on overall clinical outcome of PET was difficult to assess, but, due to the general slow disease progression, that may be true for many diagnostic studies in thyroid cancer.

A frequently observed issue whether PET should be performed during the hypothyroid state (e.g. after thyroid hormone withdrawal) or in euthyroid state (during thyroxine treatment). In a study van Tol (70) better performance of PET in hypothyroid state was found, but the issue is not clearly settled.

Furthermore, it has been hypothesized that exogenous TSH stimulation with rhTSH increases FDG uptake by differentiated thyroid cancer and seems apparently more accurate than FDG-PET under suppression, in terms of number of detected lesions and tumor/background contrast (71). In a small study this hypothesis has been confirmed (18).

Medullary thyroid cancer

Nearly all imaging modalities (Ultrasonography, CT, MRI, scintigraphy using In-111-octreotide, Tc99m-DMSA-V, MIBG) have limited sensitivities (40–70%) compared to the apparently very high sensitivity of the calcitonin tumor marker (72). Although the clinical course of metastatic medullary thyroid cancer can be mild in some patients, others develop clinically relevant metastases (in liver, bone, lungs) that remain undetected until a relatively late stage. Earlier detection of metastases during follow up after primary treatment might therefore have relevant therapeutic implications. Results of FDG PET studies in MTC demonstrate slightly better performance (sensitivity around 75%—specificity 79%) as compared to other imaging modalities, but patient selection probably influences these results (73,74).

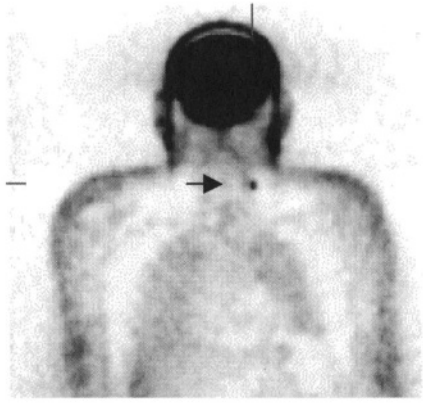

Figure 2. 50-year-old patient with papillar thyroid carcinoma, negative posttherapeutic ^{131}I-WBS 10 days after a treatment dose of 5550 MBq, thyroglobulin 25 ng/ml. FDG PET coronal slice showing a small lesion in the right neck (arrow) that proved to be a small metastasis of papillary thyroid cancer.

Other PET tracers

Similar to other neuroendocrine tumors as described above, uptake of FDG appears to be low, and theoretically radiolabeled amino acids might perform better in these calcitonin producing tumors (75). Preliminary experience using C11-methionine does not seem to confirm this expectation (76). Recent experience with the catecholamine precursor amino acid ^{18}F-DOPA and PET appears to be more promising. In a small group Hoegerle found more lymph node metastases of MTC using ^{18}F-DOPA PET than with any other modality (77). Also ^{18}F-DOPA PET was reported to be able to detect a medullary thyroid cancer lesion in a MEN2a patient (78).

THALLIUM-201 CHLORIDE

General mechanism

Thallium-201 (Tl-201) is a potassium analogue. This positively charged ion is actively transported over the cell membrane by an ATP-dependant sodium/potassium transport system and localizes non-specifically in thyroid cells, as well as in other tissues with high cellularity and high perfusion. Additional mechanisms ofentry have not been excluded. Originally Tl-201 has been developed for myocardial perfusion imaging, for which it is still routinely used, but it also accumulates in kidney, stomach, liver, spleen, testes, salivary glands, large bowel and in thyroid tissue (79). Several reports have suggested that comparison between early and delayed Tl-201 images could distinguish between benign and malignant thyroid diseases, but usually results in benign and malignant tissues overlap and have not lead to sound clinical application (80,81).

Isotope characteristic

Tl-201 is an isotope that decays by electron capture to Mercury-201. Mercury 201 emits characteristic gamma rays of 68–90 keV and much smaller amount of gamma

rays of 135 keV and 167 keV. The half-life of Tl-201 is 73.1 hours. Tl-201 is normally administered as thallium chloride and rapidly disappears from the blood with a half-life between 30 seconds and 3 minutes. Peak uptake in the thyroid occurs 5–10 minutes after injection.

The relative long half-life and the poor physical characteristic of the radiopharmaceutical limit the injected dose (3–5 mCi). The low photon energy causes a relatively large radiation burden and is also less suitable for imaging, because of scattered and absorbed radiation. This results in low image quality.

Scan method

Techniques of scanning

Tl-201 imaging for thyroid cancer does not require withdrawal of thyroid hormones or restriction of iodine intake. Imaging, using a low-energy collimator, is usually performed 10–20 minutes after intravenous injection of Tl-201, because at that time tumor/background ratio is highest. The accumulation in tumoral tissues remains constant between 20–60 minutes. Supine anterior and posterior whole-body scans are acquired. Additional or delayed images can be obtained 3–4 hours postinjection to differentiate malignant tissues (slower washout) from benign tissues (82).

Clinical application

Papillary and follicullar thyroid carcinoma

The primary value of Tl-201 is the analysis of patients with a negative ^{131}I scan and elevated thyroglobulin levels. Several studies discuss the usefulness of Tl-201 in the localization of metastatic disease (83,84,85,86). The combination of ^{131}I with Tl-201 scintigraphy resulted in a sensitivity for recurrent tumor of 90–100% at a specificity of 95%–100%. Adding also the information from thyroglobulin measurements even further increases diagnostic yield. Tl-201 alone generally depicts approximately half of all thyroid cancer lesions. Few reports have specifically addressed the value of Tl-201 scintigraphy in patients with negative ^{131}I scans.

Tl-201 scintigraphy seems to be most value in the localization of local metastases and in mediastinal lymph nodes. Sensitivity has been reported between 55–94%, specificity between 82–97% (85,86,87). The large variety in sensitivity can be attributed to the different methods of disease confirmation, the variability in location of metastases and the different selection of the patients.

Although most published studies were performed with planar images, SPECT imaging shows a 25% increase in sensitivity, especially for chest, neck and micronodular pulmonary metastases (88).

In the small study of Shiga et al. (89) Tl-201 scintigraphy seems to provide similar information in the detection of metastatic lesions after total thyroidectomy compared with FDG-PET.

Medullary thyroid carcinoma

There have been several studies of Tl-201 uptake in medullary thyroid carcinoma, most of them with a limited number of patients and in comparison with DMSA-V or

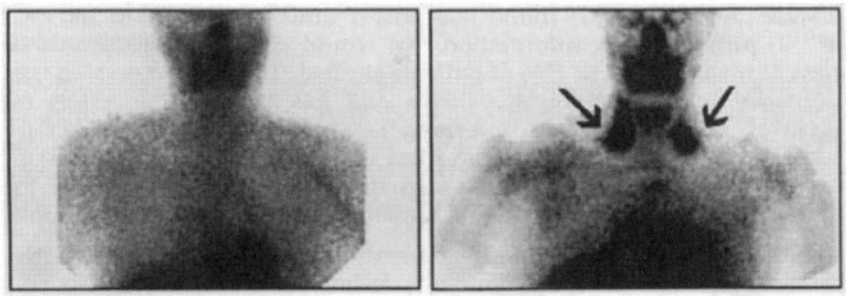

Figure 3. ^{201}Tl neck and chest scans. Normal scan taken when Tg was 4 ng/mL and ^{131}I scan was negative. Repeat ^{201}Tl scan when Tg was 75 ng/mL. Note uptake in the apices of the lungs (arrows). Iodine scan remained negative.

MIBG. The non-specific uptake of this tracer in background tissues and low tumoral uptake probably cause the relatively low sensitivity. DMSA-V scintigraphy has shown to be clearly superior to Tl-201 (90). Another study showed Tl-201 superior to MIBG. Although non-specific, Tl-201 may be be useful in individual clinical setting.

Although only limited comparison data of FDG PET and Tl-201 scintigraphy are available, but FDG PET is considered to be clearly superior.

TECHNETIUM-99M-SESTAMIBI (METHOXY-ISOBUTYL-ISONITRILE)

General mechanism

Technetium-99m-sestamibi (Tc-99m-sestamibi) is a lipophilic cationic agent that primary localizes in the mitochondria.Tc-99m-sestamibi accumulates in the mitochondria secondary to a negative potential of the mitochondria. Tc-99m-sestamibi uptake is driven by a negative transmembrane potential and up to 90% of the intracellular tracer is found in the mitochondria. The uptake is an energy dependant process. Tc-99m-sestamibi is also a substrate for the transmembrane P-glycoprotein drug efflux pump (91).

The affinity for mitochondria probably generates the specific uptake in Hürthle cell carcinoma, which is often poorly iodine concentrating, but rich in mitochondria. Similar to Tl-201, Tc-99m-sestamibi uptake in thyroid cancer cell is independent of TSH stimulation, although one report mentioned a TSH dependent uptake (92).

Isotope characteristic

Tc-99m-sestamibi is obtained by elution from a Tc generator, which contains the parent isotope molybdenum-99 (Mo-99). Mo-99 is a radionuclide with a half-life of 66 hours. The isolated 140 keV gamma emission of Tc-99m is ideally suited for gamma camera imaging. The 6 hr half life is very convenient for radiopharmaceutical production on a day to day basis. The commercial production of generators, which can be eluted up to 1 week, make Tc99m very easily available. These factors make Tc-99m the most used radioisotope in nuclear imaging in general. A kit preparation for radiolabeling of Tc-99m to sestamibi is commercially widely available.

Scan method

Tc-99m-sestamibi scintigraphy does not require any patient preparation and no thyroid hormone withdrawal. The favorable scan characteristics and the relative short half-life of Tc-99m (6 hours) in comparison to 131I (8 days) enable the use of relatively larger doses which increases image quality. This is a clear advantage over Tl-201.

Imaging is usually performed early, 10–30 minutes after tracer administration, and repeated 3 hours later. Others only image 60 minutes p.i., which might be less sensitive.

Clinical application

Tc-99m-sestamibi is only applied in papillary and follicular thyroid carcinoma.

The application of Tc-99m-sestamibi scanning is in patients with a negative 131-scan and an elevated thyroglobulin. In many instances Tc-99m-sestamibi has shown predilection to concentrate in the same abnormal sites as Tl-201.

Tc-99m-sestamibi scanning is particularly sensitive for the detection of nodal metastases. One study reported more sensitivity than high doses ^{131}I (93), another found the lowest sensitivity for lung metastases (94). Especially in high risk patient with a negative ^{131}I scan a combination of MIBI and ultrasound may be useful in detecting lymph node metastases (95).

Other Tc-99m based tracers

The data about the clinical application in thyroid cancer and value of Tc-99m Tetrofosmin, Tc-99m Pertechnate, Tc-99m Furifosmin is very limited and the results are conflicting. All three tracers are characterized by accumulation in the mitochondria by a different and not clearly understood mechanism.

Of the limited published data on Tc-99m tetrofosmin high sensitivities are mentioned (>85%) (96,97) in papillary and follicular thyroid carcinoma. No additional value is found in medullary thyroid carcinoma (90).

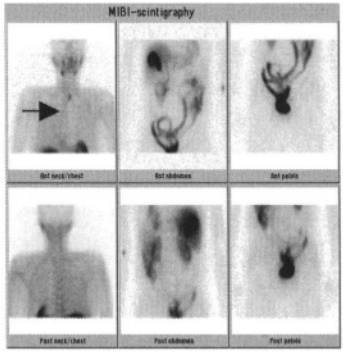

Figure 4. Technetium-99m-sestamibi scan 15 minutes after injection of 730 MBq in a 85-year-old patient with recurrent papillary thyroid carcinoma. It shows uptake in the neck (arrow). The ^{131}I-WBS was negative.

INDIUM-111 DTPA—PHE-1-OCTREOTIDE

General mechanism

Somatostatin receptors are present on many neuroendocrine tissues, both benign and malignant, also including normal thyroid cells and thyroid carcinoma cells. These receptors are the basis of scintigraphy using the radiolabelled somatostatin analogue, In-111-octreotide, which is an important diagnostic tool in neuroendocrine tumors in general. Five somatostatin receptors subtypes have been isolated. Somatostatin and its synthetic analogues act through specific binding to these receptors subtypes. Each subtype has its own tissue distribution pattern, and has specific pharmacological properties. After ligand—receptor interaction signals are transmitted (using G-proteins) to adenylate cyclase pathway, resulting in inhibitory effects on cell growth and proliferation. Normal thyroid tissue shows expression of all somatostatin receptor subtypes except somatostatin receptor subtype (SST) 2 in one study (98), while others found a high expression of SST3 and SST5, and only a weak expression of SST1 and SST2 (99). Thyroid cancer tissue shows a different SST expression. Papillar and follicular tumours have high expression of SST3, 4 and 5, while in Hürthle cell and medullary thyroid tumours SST2 expression is present.

Apart from different tissue distribution, ligand affinity also varies. The well-known somatostatin analogue, octreotide, has the highest affinity for SST2 and lower affinities for SST3 and SST5 and no affinity for SST1 and SST4. The lack or the low density of SST2 receptor is presumably the reason that only half of all thyroid cancers can be visualized by scintigraphy using radiolabelled octreotide (100).

Isotope characteristic

Indium-111 (In-111) has a half-life of 67.5 hours and is cyclotron-produced. It predominantly emits gamma photons with gamma-energy of 171 keV and 247 keV. Indium is coupled to the octreotid peptide using DTPA as a chelator (In-111-DTPA), and the ready-to-inject compound is commercially available. The tracer is also called In-111-pentreotide.

Scan method

Thyroid hormone suppression can be continued during scanning, although one study has shown a small increase in detection of positive lesions after thyroid hormone withdrawal (101). Whole-body imaging is performed 24 hours after intravenous injection of 200 MBq of In-111-octreotide, using a medium energy collimator. Laxation of patients is often performed to facilitate intestinal clearance. Normal distribution consists of intense uptake in kidneys and spleen, minor uptake in liver and intestine. Endocrine organs such as the thyroid and pituitary gland can also often be seen. Minor non-specific uptake can be observed in inflammatory lesions. Additional images, lateral views and/or SPECT, of the neck and upper abdomen improve the detection of smaller or equivocal lesions in those areas. Delayed images can be obtained 48 hours postinjection, mostly because of interfering accumulation of radioactivity in the bowel to differentiate pathological from physiological uptake.

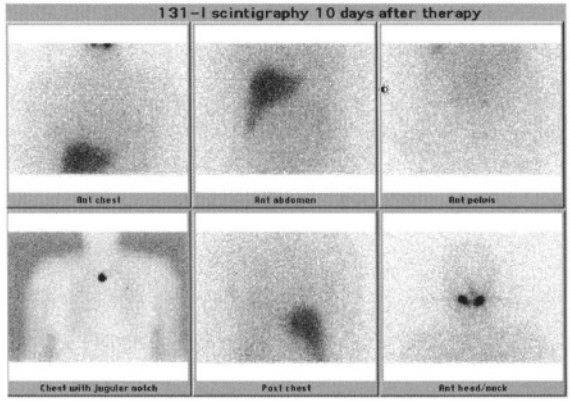

Figure 5.1. Negative posttherapeutic ^{131}I-WBS 10 days after a treatment dose of 5550 MBq in a 63-year-old patient with DTC with persistent elevated serum Tg and negative diagnostic ^{131}I-WBS 3 months after the ablative dose of 5550 MBq. Normal biodistribution in the liver and salivary glands. This patient showed on the first pre-ablative diagnostic ^{131}I-WBS, 6 weeks after total thyroidectomy, uptake in the neck and was subsequently treated with an ablative dose of 5550 MBq; the posttherapeutic ^{131}I-WBS 10 days after the ablative dose also showed uptake in the neck.

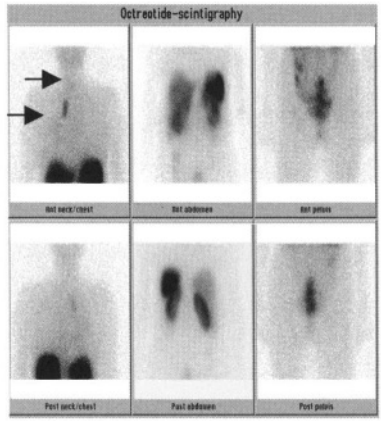

Figure 5.2. Indium-111-octreotide scan 24 hours after injection of 185 MBq in the same patient with DTC with persistent elevated serun Tg and negative ^{131}I-WBS. The indium-111-octreotide scan clearly shows uptake in the right lung (arrow) and faintly uptake in the neck (arrow).

Clinical application

Papillary and follicular thyroid carcinoma

In-111-Octreotide scintigraphy is especially useful in patients with 131-I negative scans and clinical suspicion on persistent tumor activity, as confirmed with recent studies (101,102,103). The uptake of In-111-Octreotide broadens the ability of the application of radiolabeled somatostatin analogues in general. High doses of Yttrium-90 (beta

emitting) or the In-111 (gamma emitting) DOTA chelated somatostatin analogues have been applied in both patients with papillary and follicular thyroid cancer and with medullary thyroid cancer for therapeutic reasons. Currently response rates are around 35%. (104).

Medullary thyroid carcinoma

In medullary thyroid carcinoma In-111-octreotide scintigraphy can have a complementary value in individual cases. However, the somatostatin receptor density and the number of receptors appears to be lower in medullary thyroid carcinoma in comparison to other neuro-endocrine tumors. Also some medullary thyroid tumors can produce somatostatin which may be competitive in the receptor binding (105). However, In-111-octreotide can be a useful radiotracer for the detection of metastatic or recurrent medullary thyroid carcinoma. Especially in cases with minimal residual disease, as can be found by persistently elevated calcitonin levels, In-111-octreotide scintigraphy showed more tumor localisation than conventional techniques (106,107). Liver metastases are slightly less more difficult to visualise (108) because of the non specific background uptake in the liver. In-111 octreotide has the similar sensitivity (±80%) to CT and MRI for the detection of recurrent or metastatic medullary thyroid carcinoma (109).

META-IODOBENZYLGUANIDINE (MIBG)

General mechanism

Metaiodobenzylguanidine (MIBG) is a norepinephrine analogue that can be radiolabeled with ^{131}I or ^{123}I. MIBG is an aralkylguanidine, with combines the benzylgroup of bretylium and the guanidinegroup of guanethudine with an idione on the meta place. By competition with the energy dependent transport mechanism of norepinephrine MIBG is taken up in cells (110). Evidence has been found that probably a sodium-dependent and a sodium-independent uptake system is present. Differential expression of the uptake systems, may be responsible for the variations of the kinetic parameters for both norepinephrine and MIBG in different tumor cells. (111). It is especially sensitive in (nor)epinephrine producing tumors, such as pheochromocytoma, neuroblastoma or paraganglioma. In vitro experiments have shown that MIBG may act as a substrate for chromaffin granules. The vesicular mono-amine transporter (VMAT) type 2, that has extensively been expressed in pheochromocytomas seems to be responsible for MIBG transport and tumor visualization (112,113).

Scan method

MIBG can be radiolabeled with both ^{123}I or ^{131}I. Because of the unfavorable imaging characteristics and the higher radiotion dose, ^{123}I-MIBG is mostly used, although the ^{131}I labeled variant allows imaging up to several days after administration. In general thyroid uptake of (in small quantities liberated from the tracer) free iodine is blocked by administering non radioactive iodine or perchlorate at the time of injection. Imaging is generally performed 24 hrs after tracer administration. A large variety of medications

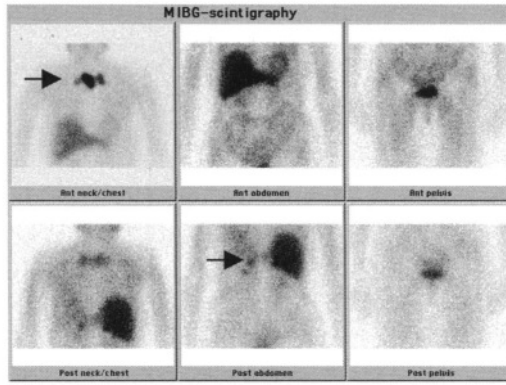

Figure 6. I-123 MIBG whole body scan 24 hours after injection of 185 MBq in a 78-year-old patient known with MTC, hyperparathyroidism and suspected of pheochromocytoma (MEN-2a). It shows intense uptake in the neck with bilateral paratracheal expansion (arrow); it also shows uptake in the left adrenal region (arrow).

may have some impact on tumoral uptake (e.g. alpha receptor blocking agents). Whole body and spot imaging may be supplemented by SPECT imaging.

Application

There is no place for MIBG scintigraphy in patients with differentiated thyroid cancer, but in medullary cancer the method may be helpful.

In medullary thyroid cancer, MIBG scans are positive in only a limited number of patients with medullary thyroid carcinoma with mentioned sensitivities of 12–30% (114,115).

A pitfall in imaging arises when liberated free iodine localizes in thyroid remnants (in the rare patients that were not ablated after thyroidectomy), but this can usually be differentiated from uptake in medullary thyroid cancer metastases. In cases of doubt Tc-pertechnetate imaging can be helpful.

A number of patients with medullary thyroid cancer have been treated with 131I-MIBG and a palliative response has been reported in 50% of the patients (116). Pentavalent 99m Technetium Dimercaptosuccinic Acid (99mTc (V)DMSA).

General mechanism

Pentevalent Tc-DMSA (called DMSA-V) is derived from DMSA, but includes the Tc99m label in a 5+ molecular charge, instead of 7+, as is the common chemical form of Tc99m. 99mTc(V) DMSA is not taken up by the normal thyroid gland, but can be applied in the diagnosis of medullary thyroid cancer due to an increased turnover of calcium and phosphate ions.The compound localizes in a number of tumours. The precise mechanism is not well known, uptake may be related to the intracellular phosphate concentration (117). 99mTc(V) DMSA exists in three isomeric forms and the biodistribution of the individual isomers differs from the whole radiopharmaceutical.

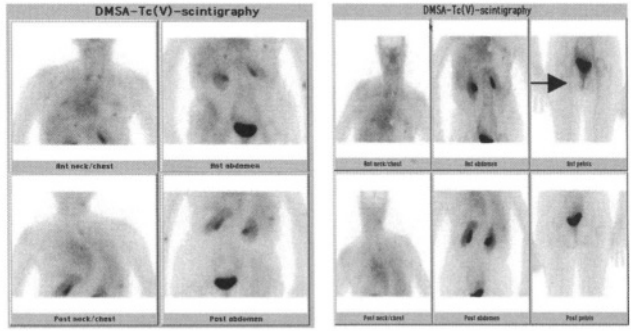

Figure 7. Tc-99m(V)-DMSA scan 2 hours after injection of 290 MBq in a patient known with MTC (MEN-2a) with persistent elevated serum calcitonin. The scan shows uptake in the neck, chest and in the right proximal femur (arrow). This patient also has severe scoliosis of the thoracal spine.

Scan method

Images are acquired 2–3 hours after injection. SPECT imaging of suspected areas may be helpful to improve the sensitivity of tumour detection.

The normal biodistribution is seen after 2 hours in the nasal mucosa and faintly in the skeleton, with breast uptake in women. Excretion is through the kidneys, liver uptake is not prominent. Some blood pool activity may also be present.

Clinical application

99mTc(v) DMSA is not commercially available in the United States but is well used in other countries. Tumor lesion sensitivity is reported to be between 50 and 95% (118,119).

RADIOLABELED ANTI CARCINOEMBRYONIC ANTIGEN ANTIBODY

General mechanism

Serum calcitonin and carcinoembryonic antigen (CEA) are used as tumormarkers in medullary thyroid carcinoma (MTC).

The specific positive immuno-histochemical property of a positive staining for calcitonin and carcinoembryonic antigen (CEA) and the expression of CEA levels at the surface of the cells were the basis for the development of specific anti-CEA monoclonal antibodies, the so-called radioimmunoscintigraphy, to image patients with MTC. Various anti-CEA antigen antibodies can be labeled with 99m-Tc, 111-In, ^{123}I or ^{131}I. The disadvantages of using monoclonal antibodies include the low tumor-background ratio and the forming of human anti-mouse antibodies (HAMA) making repeated studies difficult.

Scan method

The injected dose depends on the radionuclide the antibodies are labeled with, just like the timing of scanning and the choice of collimator (120,121,122). Injected activity

are 555–1110 MBq (15–30 mCi) for 99m-Tc labeled antibodies, 74–370 MBq (2-10 mCi) for ^{131}I labeled antibodies and 111–185 MBq (3–5 mCi) for 111-In labeled antibodies. With the 99m-Tc labeled anti-CEA antibodies planar and SPECT imaging of the neck/chest, abdomen and pelvis can be acquired 4 and 24 hours postinjection with an LEHR-collimator. With the 111-In labeled anti-CEA antibodies imaging can be performed up to 72 hours postinjection with a medium energy collimator. Imaging of ^{131}I labeled anti-CEA antibodies can be performed 4 hours and up to 7 days postinjection. Blood pool activity may be prominent, kidney and bone marrow uptake can be seen.

Clinical application

Reported lesion based sensitivity of various anti-CEA antibodies in medullary thyroid carcinoma is around 70–100%, for both known and occult disease (121,122,123). However, only a limited number of groups have published about these results. Apart from the limited application in medullary thyroid cancer, the anti-CEA antibodies scan has been extensively used in the detection of metastatic colon cancer, where it appears to have a sensitivity of 50–70% and provides clinically relevant information, especially in combination with CT scanning.

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19. PAST, PRESENCE AND FUTURE OF THYROID-STIMULATING HORMONE (TSH) SUPERACTIVE ANALOGS

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INTRODUCTION

In recent years many new engineered protein therapeutics are being developed and tested in clinical trials (Marshall et al. 2003). Many early protein drugs showed limited applicability due to short half-life or low affinity to their receptors. Recombinant DNA technology permitted engineering protein molecules with specific chemical and biological characteristics. It is now anticipated that engineered hormone analogs will largely exceed clinical efficacy of existing products, including recombinant human TSH (rec-hTSH; Thyrogen^{TM}, Genzyme). Preclinical development of superactive TSH analogs can be divided into three phases: the TSH structure-function phase, the discovery phase and the optimization phase. It is apparent that detailed structure-function studies of TSH provided important foundation for rational modifications of human TSH molecule. This chapter describes the past, presence and future of superactive analogs of TSH with high receptor binding affinity. Such superactive analogs of TSH are expected to provide not only more efficacious diagnostic methods, but should also serve as indispensable tools in management of thyroid carcinomas with low TSH receptor number, impaired coupling and deficient ligand binding.

TSH STRUCTURE-FUNCTION

TSH is a key protein controlling thyroid function through its interaction with TSH receptor in thyroid. TSH is a member of glycoprotein hormone family produced by basophiles in the anterior pituitary (Pierce and Parsons 1981; Hearn and Gomme

2000). Human TSH is a heterodimer composed of two non-covalently linked subunits, α- and TSHβ-subunit. α-Subunit contains 92 amino acids and a sequence identical in all human glycoprotein hormones. TSHβ-subunit contains 118 amino acids and is unique for human TSH. The high-resolution structure of homologous to TSH human chorionic gonadotropin (hCG) has revealed that both subunits contain a central cystine-knot motif and three loops: two β-hairpin loops (*L*1 and *L*3) on one side of a cystine-knot and a long loop (*L*2) on the other (Lapthorn et al. 1994). The long loop in the α-subunit includes two-turn α-helix. The cystine-knot is made up of three central disulfide bonds, where one of the disulfide bonds threads through a ring formed by two other disulfide bonds. Similar to other glycoprotein hormones (LH, FSH and hCG), TSH hetero-dimers are stabilized by a unique segment of the β-subunit termed "seat-belt", because it wraps around the α-subunit. In light of the common α-subunit and 38% sequence identity between the hCGβ- and hTSHβ-subunit, homology modeling of hTSH was performed and showed expected similarities in the global conformation of these two hormones (Szkudlinski et al. 1996). Accordingly, assignment of disulfide bonds in bovine TSH β-subunit revealed bonding analogous to hCG (Fairlie et al. 1996). Thus, in hTSH β-subunit, three disulfide bonds (2–52, 27–83 and 31–85) form cystine-knot motif that determines the core structure, two disulfide bonds (19-105, 88-95) are involved in "seat-belt" formation and one (17–67) links two β-hairpin loops. Such structural features result in an increased interaction between two subunits and provide stability of heterodimer in physiological conditions.

Three carbohydrate chains constitute 15–25% of TSH molecular weight. The human α-subunit contains two carbohydrate chains linked to asparagine 52 and asparagine 78, and the human TSH β-subunit contains one carbohydrate chain attached at asparagine 23. Such asparagine-linked oligosaccharides are complex-type structures displaying notable hormone-, species-, source- and production-dependent differences in their core and terminal residues. Differences in oligosaccharide structure result in physiological heterogeneity of pituitary and recombinant TSH (Szkudlinski et al. 1993). It has been very well established that co-translational attachment of site-specific oligosaccharide chains is highly important in subunit folding, dimerization, TSH dimer secretion, stability, plasma half-life and bioactivity.

As reviewed previously, TSH contains several important domains that are tightly conserved among different species or homologous hormones (Grossmann et al. 1997; Szkudlinski et al. 2002). Even minor modifications of such domains result in decreased expression, impaired receptor binding and bioactivity. These domains located within a "composite binding domain" proposed by Lapthorn et al. (Lapthorn et al. 1994) include: α-helix (α40–46), αLys51, αAsn52, the α-carboxyl terminus (α 88–92), α33–38, "the Keutmann loop" (TSHβ31–52) and the "seat belt" in the β-subunit (TSHβ88–105) (Szkudlinski et al. 1996; Grossmann et al. 1997) (Figure 1). In addition to the stabilizing role of the "seat-belt", recent studies involving β-subunit chimeras have shown that this region is critical in conferring glycoprotein hormone specificity (Grossmann et al. 1997). Additional functionally critical residues have been identified

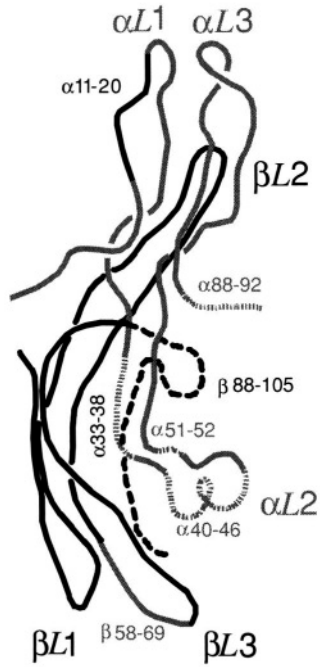

Figure 1. The schematic drawing of hTSH structure. For clarity the carbohydrate chains are not shown. The α-subunit backbone is shown as a gray line, the β-subunit chain as a black line. Important domains are marked directly within the line drawings. The peripheral β-hairpin loops are marked: α*L*1, α*L*3 in the α-subunit and ß*L*1 , ß*L*3 in the ß–subunit. Two long loops are α*L*2 with α-helical structure and ß*L*2 or the "Keutmann loop."

by studies of patients with mutations in TSHβ subunit gene [see (Szkudlinski, et al. 2002)].

Several other domains involved in the modulation of TSH and gonadotropin function have been described in the last decade. Studies employing combination of alanine with proline scanning mutagenesis have revealed the importance of α-helical conformation (α40–46) in TSH bioactivity (Szkudlinski et al. 1996). Further, the 11–20 region in the α-subunit with a cluster of basic residues [(K-K/R–K—K/R); lysine(K), arginine (R)] present in all vertebrates except hominoids (apes and humans) has been recognized as an important motif in the evolution of TSH and gonadotropin bioactivity in primates (Szkudlinski et al. 1996; Szkudlinski et al. 2002). Selective elimination of basic residues in this domain of α-subunit resulted in a major decrease of receptor binding affinity and bioactivity of TSH in higher primates. Conversely, reconstitution of such basic motif resulted in a major increase in bioactivity of human TSH. It provided the first evidence that introduction of basic residues in selected sites in peripheral loops may permit design of a new class of TSH analogs (see below).

RECOMBINANT HUMAN TSH

Recombinant human TSH (rec-hTSH; Thyrogen®, Genzyme) has been produced in a large-scale bioreactor using Chinese hamster ovary (CHO) cells stably transfected with TSH genes (Cole et al. 1993). Since CHO cells, unlike the pituitary thyrotroph cells, have no capacity to add penultimate *N*-acetylgalactosamine or terminal sulfate, rec-hTSH is predominantly composed of oligosaccharide chains terminating in sialic acid. Rec-hTSH carbohydrate isoforms are similar to the sialylated forms of hTSH that are increased in primary hypothyroidism. Rec-hTSH has longer plasma half-life compared with normal pituitary hTSH. However, in several in vivo studies the bioactivity of rec-hTSH was found comparable to that of pituitary-derived hTSH (Cole et al. 1993; Szkudlinski et al. 1993; East-Palmer et al. 1995).

After cloning of human TSH beta gene (Wondisford et al. 1988) NIH researchers led by Bruce Weintraub initiated a collaborative research agreement with the Genzyme Corporation to produce and test rec-hTSH as a diagnostic agent to stimulate ^{131}I uptake in patients with differentiated thyroid carcinoma. An initial Phase I/II clinical trials showed that rec-hTSH is safe and efficacious in stimulating ^{131}I uptake and thyroglobulin secretion (Meier et al. 1994). Subsequent Phase III and confirmatory phase III trial with more than 100 patients indicated that rec-hTSH is almost as sensitive as conventional thyroid hormone withdrawal, but leads to considerable improvement of the quality of life because it avoids the symptoms of hypothyroidism (Ladenson et al. 1997). These major studies and case reports, including description of a patient with papillary thyroid carcinoma and hypopituitarism who had metastasis detected only after administration of rec-hTSH, exemplified the diagnostic potential of rec-hTSH (Ringel and Ladenson 1996). Thyrogen® has been approved by US FDA in 1998 for use in conducting thyroid scanning and thyroglobulin testing in the follow-up of patients with well differentiated thyroid carcinomas. Although many clinical studies demonstrated that the use of rec-hTSH is an effective strategy to increase ^{131}I uptake, rec-hTSH is considered not entirely equivalent to endogenous hTSH induction by thyroid hormone withdrawal (Utiger 1997; Robbins et al. 2002; Emerson and Torres 2003). Therefore, novel recombinant hTSH analogs with enhanced bioactivity may become long awaited component of improved follow-up, ablation of remnants and metastases in differentiated thyroid carcinoma.

DISCOVERY AND OPTIMIZATION OF SUPERACTIVE TSH ANALOGS

Protein engineering using recombinant DNA methods started in 1982, after the first results of oligonucleotide-directed mutagenesis had been published. Despite numerous site-directed mutagenesis studies, successful examples of engineering proteins with improved receptor binding affinity are quite rare. The first superactive analogs of hTSH with significant increases in receptor binding affinity, in vitro and in vivo bioactivity were constructed in 1994 (Table 1).

It was recognized previously that human TSH binding to TSH receptor is relatively a low-affinity interaction resulting in an extended 4–5 log unit competition curve for

Table 1. Chronology of superactive analog development and related projects

Year (Institution)	Achievements/Studies
1994 (NIH)	First TSH superactive analogs with single amino acid substitutions
1995 (NIH)	Superactive analogs with combined substitutions in TSH and hCG
1996 (NIH)	Initial superactive analogs of LH and FSH
1996 (NIH)	Biological activity of modified free-alpha subunit
1996–1998 (UMBI)	Rescuing "loss of function" mutations
1996–2001 (UMBI)	Studies on the mechanism of TSH receptor activation

TSH binding, with IC_{50} in high nanomolar range. However, Scatchard transformation of the equilibrium binding data produced a two-component curve which translates into a high and low affinity binding site, cellular bioassays always required low ionic strength buffer to achieve adequate sensitivity and reliability (Willey 1999). Although reasons for relatively low affinity of human TSH-TSH receptor interaction were not completely understood (Rommerts et al. 1992), in order to circumvent low affinity of human TSH all bindings studies were performed using ^{125}I-labeled bovine TSH. Interestingly, bovine and rat TSH were previously found 10–100 fold more potent than human TSH and such differences were observed at human and rodent TSH receptors, both in vitro and in vivo (Rapoport and Seto, 1985; East-Palmer et al. 1995). Thus, TSH from rodents was considered more bioactive than human TSH, but the exact mechanism of such differences were unknown. Some investigators attributed differences to variable purity, carbohydrate structures or other posttranslational modifications of pituitary TSH preparations.

Our studies on the role of carbohydrate residues in TSH bioactivity performed at NIH from 1990 to 1993 indicated that species- or production-dependent variability of TSH carbohydrate residues could not provide adequate explanation for observed differences in bioactivity between human TSH and TSH from various non-primate species (Szkudlinski et al. 1993). While exploring the role of carbohydrate residues by using subunit hybrids we observed that [(bovine alpha) – (human TSH beta)] heterodimer is at least 10-fold more bioactive in vitro that human TSH or [(human alpha—bovine TSH beta)] heterodimer (Szkudlinski, Thotakura - unpublished data). These and other studies led Szkudlinski et al. in 1994 to substitute various amino acids in human alpha subunit according to the amino acid sequence of the bovine alpha subunit. After the first three "gain of activity" mutations were identified (Q13K, P16K, Q20K) more detailed sequence alignments, homology modeling and sequencing of subunit genes in various species, including primates, were performed. This resulted in an identification of additional targets within the sequence of both subunits including residue 14 in the alpha and 69 in the TSH beta subunit. Further development included combination of single mutations, alternative substitutions with arginines or histidines as well as studies on cooperative effects of different substitutions. hTSH with quadruple mutations in the α-subunit (Q13K+E14K+P16K+Q20K) and an additional replacement in the hTSHβ-subunit (L69R) showed 95-fold higher potency and more than

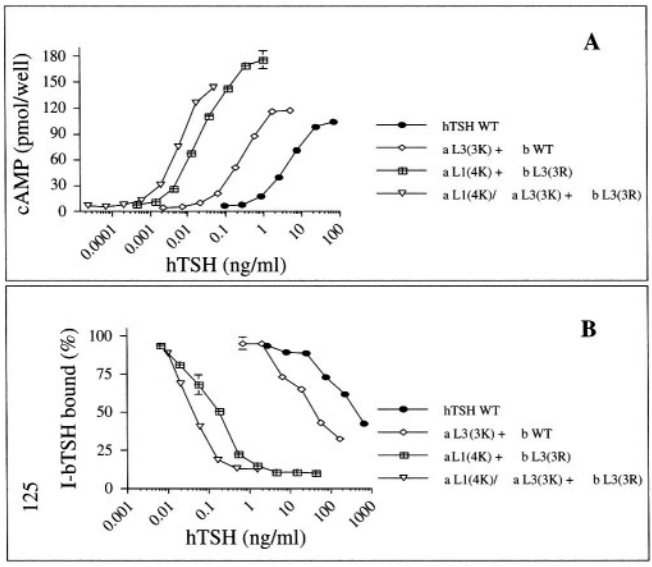

Figure 2. Superactive Analogs of TSH. Reprinted from Leitolf et al. (Leitolf et al. 2000). Gradual increase in the in vitro bioactivity (A) and receptor binding (B) for mutants with one, two and three engineered peripheral loops. Combination of the αL3 loop analog "with our previously optimized αL1-βL3 loop combination (Grossmann, et al. 1998) results in a further gain of hormone potency.

1.5-fold increase in efficacy compared to the *in vitro* bioactivity of the wild-type hormone (Szkudlinski et al. 1996). Moreover, the combination of these 4 mutations in the α-subunit with 3 mutations in the β-subunit (I58R+E63R+L69R) resulted in an analog with greater than 1000-fold increase in receptor binding and in vitro bioactivity and 100-fold increase in the in vivo activity (Grossmann et al. 1998). Subsequently seven new site-specific "gain-of-activity" mutations were identified. Four in the αL3 loop (S64K, N64K, G73K, A81K) and three in the βL1 loop (Leitolf et al. 2000) (Figure 2). TSH analogs with optimized combinations of these substitutions are significantly more potent and efficacious than any known species of TSH and hold great promise as a second generation therapeutic forms of recombinant TSH. The relative increase in potency and efficacy (Vmax) of superactive analogs in comparison to the unmodified hormone are assay system dependent as illustrated in Figure 3. Because of the specious argument that increasing dose of the unmodified hormone should result in comparable to analog maximal effect, it is important to emphasize that such possibility is limited to extremely sensitive (rarely physiological) systems with high receptor number and/or highly efficient coupling.

MECHANISM OF ENHANCED BIOACTIVITY OF TSH ANALOGS

A long-standing postulate held that charge-charge interactions are of major importance in the interaction between TSH and TSH receptor (Rees Smith et al. 1988). Similar

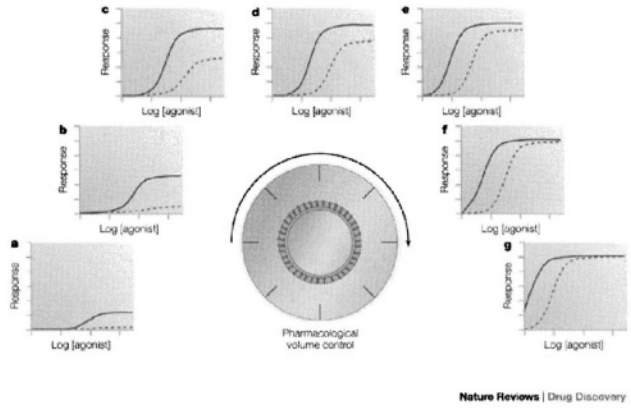

Figure 3. Schematic of a range of dose-response curves to two agonists (Kenakin 2003). Various degree of difference in potency and efficacy (Vmax) is dependent on assays sensitivity determined by coupling efficiency and receptor number. In poorly coupled systems (assay **a**), the lower-efficacy agonist (dotted line) shows almost no response. In intermediate-sensitivity assays (**b–d**), both are partial agonists, whereas in high-sensitivity assays (**f** and **g**) both are full agonists.

interactions between additional basic residues in TSH superactive analogs and specific acidic residues in the receptor, yet to be fully characterized, are likely involved in prolonged dissociation rates and enhanced receptor activation and signaling.

Largely unpublished studies indicated that new TSH superactive analogs are receptor specific and inactive at related LH and FSH receptors; analog concentrations up to 1000 fold higher than activating TSH receptors did not result in detectable stimulation. TSH analogs showed their enhanced activity at the TSH receptor from different species (i.e. human, rat, mouse), suggesting that various changes described during mammalian evolution of TSH receptor (Kaczur et al. 2003) are probably not involved in the phenomenon of analog "superactivity." In addition, an enhanced in vitro activity of TSH analogs was observed in normal media and in buffers with various salt concentrations. The effect of substitutions on the TSH in vitro bioactivity was in most cases highly correlated with their effect on receptor binding affinity. TSH superactive analogs are also more potent than standard rec-hTSH stimulators of inositol phosphate pathway. The difference in bioactivity between TSH analogs and wile-type hormone was demonstrated using cells with TSH receptor and largely depleted pool of the negatively charged cell surface proteoglycans. This further indicated that the mechanism of enhanced activity is not dependent on their interaction with heparan sulfate proteoglycans recognizing various motifs of basic residues (Cardin and Weintraub 1989; Bozon et al. 1998).

Although the exact mechanism of interaction of TSH analogs with TSH receptor has not yet been fully clarified specific interaction modes of TSH analogs with TSH receptor have been discovered. Our initial model of TSH-TSH receptor interaction was generally supported by "charge-reversal mutagenesis" and other approaches (Grossmann et al. 1997; Costagliola et al. 2002; Smits et al. 2003)) (Figure 4).

Figure 4. Schematic configuration of TSH-TSHR complex. Two parallel β-hairpin loops of the α-subunit (αL1, αL3) are located in the lower part of the model and may participate in the interaction with C-terminal portion of extracellular domain and the extracellular loops of the receptor transmembrane domain. Two loops of the β-subunit (βL1, βL3) are shown in the upper part with proposed binding site within the concave of leucine rich-repeats modeled by Kajava et al (Kajava et al. 1995).

Analogous amino acid substitutions in the α-subunit were also performed in hCG, LH and FSH. Although significant increases in respective gonadotropin affinities for their cognate receptors were observed, in contrast to TSH analogs, differences were smaller for both single and combined substitutions (Szkudlinski et al. 1996). Consequently the effects of single mutations were more difficult to detect and only selected combinations were later confirmed at various laboratories (Heikoop et al. 1999).

FUTURE PERSPECTIVES OF TSH ANALOGS

The opportunities and challenges facing clinical development of rec-hTSH analogs are now being realized more clearly. Thyrogen® (wild-type recombinant human TSH) produced in Chinese hamster ovary cells exhibits relatively low affinity to the TSH receptor that translates into limited clinical efficacy. Despite quite apparent advantages of TSH superactive analogs, further development and clinical trails of such analogs are now dependent on commitment of biotech industry for a relatively small "thyroid market" (Table 2).

Table 2. Potential uses of superactive analogs of TSH

Clinical	Differentiated thyroid cancer follow-up (TSH analog stimulated thyroglobulin testing and radioiodine scanning) Differentiated thyroid cancer treatment (TSH analog induced radioiodine ablation) Large euthyroid goiter treatment (TSH analog induced radioiodine ablation) TSH analog stimulation tests (testing thyroid reserve, identifying "warm" thyroid nodules, detecting thyroid hemiagenesis, etc.) Management of patients with TSH receptor mutations associated with low ligand binding, impaired receptor expression or coupling TSH receptor-mediated delivery of therapeutic agents to the thyroid cancer cell (Figure 5)
Laboratory	^{125}I-hTSH analog in TSH binding inhibition (TBI) assay for autoantibodies to the TSH receptor (Kakinuma et al. 1997) and immunoassays (Ribela et al. 1996) TSH analog-stimulated thyroglobulin (Tg) mRNA testing in thyroid cancer patients with positive or negative anti-Tg antibodies
Basic Science	Structure-function studies of TSH-TSH receptor interaction, studies of TSH receptors in non-thyroidal tissue (Szkudlinski et al. 2002)

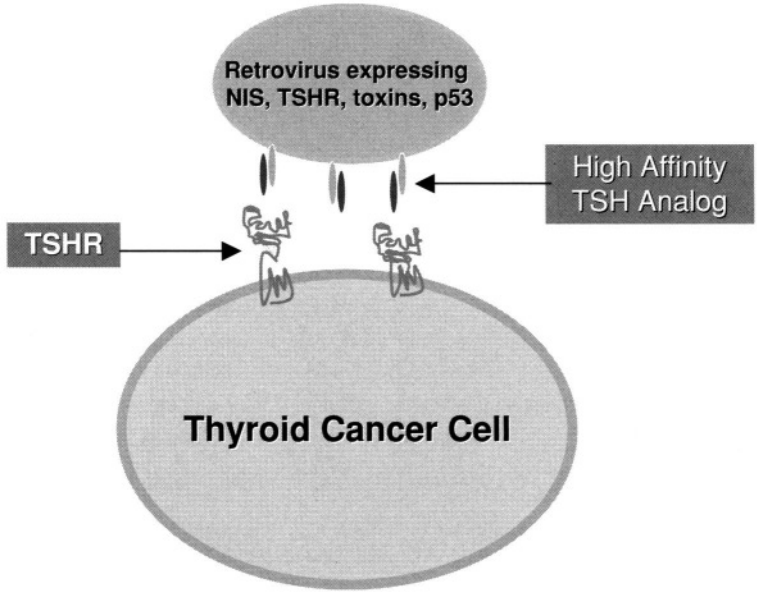

Figure 5. TSH receptor-mediated delivery of various therapeutic agents to the thyroid cancer cell. One of many possible scenarios includes restoration of cancer cell differentiation using high affinity interaction between TSH analog and largely depleted pool of TSH receptors. Another approach involves TSH analog-mediated targeted killing of thyrocyte originated carcinomas. TSHR, TSH receptor, NIS, sodium iodide symporter.

Since structural data on TSH receptor and other G protein-coupled receptors (GPCRs) are very limited, designing small molecules binding to TSH receptor that do not bind to non-targeted receptors poses a major problem. Although GPCR microarrays may narrow the number of useful agonistic compounds it is not clear if selected

small molecule can be devoid of aberrant binding to other receptors that can lead to serious side effects (Fang et al. 2003). Therefore, TSH and TSH receptor structure based analogs appear far more promising. Recent studies on the mechanism of TSH receptor activation and their constitutive activity provide additional leads for the design of high affinity antagonist and inverse agonists (Gudermann et al. 1996; Zhang et al. 2000; Wonerow et al. 2001; Rodien et al. 2003).

Interestingly, another member of glycoprotein hormone family—thyrostimulin was recently identified by comparative genomic analysis (Hsu et al. 2002) and shown to be capable of activating TSH receptor (Nakabayashi et al. 2002). Structure-function studies of thyrostimulin and its analogs should provide new insights into mechanism of TSH receptor activation. Although, the intrinsic bioactivity of highly purified thyrostimulin should be studied in more details, it is possible that third or fourth generation TSH analogs will include TSH-thyrostimulin chimeras with their respective superactive components.

Current research, development pipelines and clinical trials in progress clearly suggest that the decade of recombinant proteins with a native amino acid sequence is coming to an end. The pharmaceutical industry and biotechnology companies are increasingly exploring new types of engineered proteins including superactive hormones. Diagnostic and therapeutic use of such engineered hormones with improved characteristics, tailored to specific patient needs (long and short acting variants) should represent an important milestone in medicine of 21st century.

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20. PATHOBIOLOGY OF ANTINEOPLASTIC THERAPY IN UNDIFFERENTIATED THYROID CANCER

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INTRODUCTION

Undifferentiated thyroid carcinoma is a descriptive term often applied to the rare subset of thyroid cancers classified as anaplastic; however, there is a broad spectrum of tumors which show varying degrees of differentiated function and clinical aggressiveness. Among thyroid carcinomas derived from the thyroid follicular cell, differentiated functions include: expression and membrane localization of the sodium/iodide symporter (NIS; enabling intracellular concentration of iodide), expression of thyrotropin receptors (permitting both stimulation of the cell by rising thyrotropin levels and suppression of the cell by decreasing thyrotropin levels), organification of internalized iodide (enhancing iodide retention), and production of thyroglobulin (clinically useful as a specific tumor marker in thyroidectomized patients). Additional clinical features common to differentiated thyroid cancers include: a slow growth rate, limited metastatic potential (usually only local lymphatic spread in the majority of cases), and the ability of the host to tolerate a significant tumor burden for extraordinary lengths of time. As each of these functional features are lost and clinical aggressiveness is enhanced, therapeutic options decrease while, at the same time, the clinical situation becomes more desperate (1). This is epitomized by anaplastic carcinomas with median survival measured in months despite the most assertive therapeutic efforts (2, 3).

Although fewer than 400 cases of anaplastic thyroid carcinoma are expected in North America in a year, many-fold more patients will manifest poorly differentiated metastatic thyroid cancers with sufficient loss of differentiated function to make classical

treatments (surgery, radioiodine, and thyroid hormone suppression of thyrotropin) ineffectual. Also, disseminated medullary thyroid carcinomas and rare histologies, such as mucoepidermoid carcinomas and angiosarcomas, have no known effective systemic therapies and are usually lethal. Patients with these tumors need active antineoplastic agents that can evoke better outcomes without intolerable morbidity. To that end, it is necessary to critically review the clinical experience with current antineoplastic agents, address known mechanisms for resistance to these agents, and consider alternative therapeutic approaches to systemic therapy.

SYSTEMIC CHEMOTHERAPEUTICS, BY CLASS OF AGENT

Antimetabolites

Antimetabolites encompass compounds that have sufficient structural similarity to naturally occurring intermediates critical to the synthesis of key molecules, such as RNA and DNA, as to interfere with normal metabolism and take advantage of metabolic differences between normal and malignant cells for therapeutic specificity. Although most antimetabolite chemotherapeutic agents are nucleoside analogs, interfering with nucleic acid synthesis, some may also interfere with other critical cellular processes, such as glycosylation, organelle activities, or phospholipid synthesis. These drugs have proven most useful in hematological malignancies. Pyrimidine analogs, hindering the synthesis of cytidine, thymidine, or uridine, include the fluoropyrimidines (notably 5-fluorouracil) and cytidine analogs (cytarabine and gemcitabine). There is some preclinical single agent activity with these compounds in anaplastic thyroid carcinomas, but somewhat greater promise for their contribution to combination chemotherapy. Purine analogs, interfering with adenosine and guanosine synthesis, include the thiopurines (6-mercaptopurine and 6-thioguanine) and the adenosine analogs (fludarabine, pentostatin, and cladribine); however, these agents have not proven useful in solid tumors. Methotrexate is the most clinically useful antifolate compound, although agents such as raltitrexed (ZD1694) and edatrexate have been showing promise. These agents inhibit dihydrofolate reductase, depleting cells of reduced folates and resulting in decreased purines, thymidylate, amino acids (methionine and serine), and effects on gene methylation (4, 5).

Among the pyrimidine analogs, the only agent with any clinical experience in thyroid carcinoma is 5-fluorouracil. In all cases, it was used as part of a drug combination, with dacarbazine in advanced medullary thyroid carcinoma for modest partial responses in three of five patients (6), in combination with many drugs for a rare partial response in anaplastic carcinoma patients (7), and with three other agents in 49 patients with poorly-differentiated non-anaplastic thyroid cancers resulting in negligible clinical activity (8). This poor effect was presaged in preclinical studies using poorly differentiated or anaplastic thyroid cancer cell lines (9), although concomitant Bcl-2 antisense nucleotides (10), but not radioiodine (11), seem to enhance antineoplastic effects. Initial anaplastic thyroid cancer monolayer studies with gemcitabine were promising (12, 13) but were not followed by confirmatory reports using xenograft model systems, suggesting this agent to be less impressive than originally suggested. These deficiencies

may be remedied by using a novel multimeric gemcitabine preparation (14); however, confirmatory studies have not been published for at least three years. Likewise, an early cell culture study using (E)-2′-fluoromethylene-2′-deoxycytidine (MDL-101,731) in anaplastic cells (15) has no documented follow-up. Methotrexate is not usually considered to have clinically useful activity, although two clinical reports of its benefit in anaplastic thyroid cancer were made up to thirty years ago (16, 17). Unique cell lines may exhibit monolayer inhibition (18) but antifolate therapy is not usually considered useful for these tumors, even in combination therapy.

Alkylating agents

The first alkylating agent was the nitrogen mustard mechlorethamine, based on serendipitous observations made on sulfur mustard gas used in World War I. This class of drugs encompasses a wide range of structures with a common endpoint of forming covalent adducts on cellular DNA (5). Many of these agents differ sufficiently in their properties as to prove useful in combination with other agents of the same class. They are generally considered to be cell cycle phase non-specific, although the more rapidly proliferating cells are most sensitive. The group of nitrogen mustards, besides mechlorethamine, includes cyclophosphamide, melphalan, chlorambucil, and ifosfamide. Although a preclinical xenograft study suggested single agent cyclophosphamide to have significant activity against ATC (19), this has not been seen in clinical trials (8, 20), despite rare activity in combination with 5-fluorouracil and bleomycin (21, 22). Nitrosoureas comprise another group within this class, including BCNU (carmustine), CCNU (lomustine), MeCCNU (semustine), and streptozotocin. As with the nitrogen mustards, these agents are not useful in ATC as single agents and have only rare partial responses in combination with multiple additional chemotherapeutic drugs (23, 24). The next group of alkylating agents, the aziridines, includes triethylenemelamine, thio-tepa, mitomycin C, hexamethylmelamine, and diaziquone, as well as the related compounds, procarbazine, dacarbazine (DTIC), and temozolomide. This group is notable for the absence of published information regarding activity of these compounds in any thyroid cancer, aside from dacarbazine for medullary carcinomas. The remaining agents are classified as alkyl sulfphonates and consist of busulfan and treosulfan, neither of which is associated with treatment of thyroid cancers.

Platinum-based agents

Cisplatin has been in clinical use as an antitumor agent for over 30 years. Although the precise mechanistic pathways of activity are still unclear, the primary target is DNA. This agent has proven effective in a number of solid tumors, particularly in combination with other agents. Additional analogs include carboplatin and oxaliplatin, as well as other drugs in development. There is some evidence that failure of expression of wild-type p53, a tumor suppressor gene usually mutated or not-expressed in poorly-differentiated thyroid cancers, results in resistance to cisplatin therapy (25, 26). This may be part of the explanation for the absence of significant benefit of monotherapy with this class of agents in anaplastic thyroid carcinomas. Cell culture studies show minimal antineoplastic activity in ATC cell lines, except for sequential application after

gemcitabine (13). One veterinary study of cisplatin monotherapy for canine thyroid cancer suggested reasonable antineoplastic activity (27); however, translation of this to human disease is problematic. Consequently, most clinical application of platinum-based agents for ATC involve combination therapy, most often with doxorubicin-related drugs (24, 28–31). One possible factor affecting tumor sensitivity may relate to proliferation rate, possibly accounting for increased responsiveness of poorly differentiated thyroid cancers when patients were mildly hypothyroid causing thyrotropin stimulation of tumors (32). However, few solid tumors proliferate as rapidly as ATC and tumor dedifferentiation often results in loss of thyrotropin-dependent growth control. Although overall response was low, one study suggests enhanced response of advanced thyroid cancers to the combination of cisplatin with doxorubicin compared with doxorubicin monotherapy (31). Newer modes of delivery, using liposomal platinum complexes, will need to be assessed in clinical trials, since it may be that drug delivery and distribution problems underlie the failure to achieve significant levels of clinical response.

Topoisomerase inhibitors

Topoisomerases are critical nuclear enzymes that cut DNA strands, permitting adjustment of their topological structure during transcription, replication and recombination. Single DNA strands are cut by topoisomerase I and double strand cuts are made by alpha and beta isotypes of topoisomerase II. Inhibition of these enzymes leads to cellular death. Antitumor drugs, which inhibit one or more topoisomerase types, are among the most useful clinical agents.

There are three groups of topoisomerase II inhibitors, arranged by mechanism of activity (4). Group 1 consists of DNA intercalating compounds (anthracyclines, amsacrines, and ellipticines) that stabilize the cleavable complex of cut DNA strands but prevent religation. Group 2 agents do the same, without intercalating into DNA (epipodophyllotoxins), while group 3 compounds inhibit topoisomerase II activity, but do not intercalate into DNA nor stabilize the cleavable complex (including: dioxopiperazine, mebarone, and suramin). Generally, the level of topoisomerase II expression in a tumor positively correlates with the response of the tumor to these agents. Topoisomerase I inhibitors inhibit the religation of the cut DNA strand, trapping the enzyme in a covalent complex with DNA. These agents include camptothecin, irenotecan (CPT-11), and topotecan.

Anthracyclines, particularly doxorubicin, have been used often in thyroid carcinomas, particularly in poorly-differentiated tumors and ATC. Although some older published reports suggested this to be an active agent (20, 33–35), complete responses are rare, with partial responses achieved at the expense of significant toxicity. Anthracycline monotherapy is most often used in the context of doxorubicin radiosensitization for external beam radiotherapy, based upon early reports on ATC patients (36, 37). Doxorubicin is more typically employed in the context of combination chemotherapy, frequently administered with cisplatin (20, 24, 31, 38, 39). Other anthracycline agents have also been tested as single agents in thyroid carcinoma patients, but have not proven

effective (40) and are most commonly used, as for doxorubicin, in combination with other agents (32, 41).

Mitoxantrone is another group 1 topoisomerase II inhibitor that belongs to the class of anthracenedione antibiotics and seems to have similar toxicities as the anthracyclines. As a single agent, mitoxantrone has little activity in ATC (42), although it may have some benefit as part of combination chemotherapy (43). An additional group 1 agent, actinomycin D, has been evaluated by in vitro testing in ATC cell lines, suggesting that this agent is active in some lines (44), but it has not proven noteworthy in clinical studies.

The podophyllotoxins, particularly etoposide (VP-16) and teniposide, constitute much of the group 2 topoisomerase II inhibitors. Analysis of the expression of topoisomerase II alpha, a target of these inhibitors, shows higher levels in ATC, tall cell variant papillary carcinomas, and Hurthle cell carcinomas than in less aggressive varieties of thyroid cancer (45). This seems to suggest that podophyllotoxins would be useful for these types of thyroid cancer however, aside from rare complete responses (17), these agents have not proven active as single agents or in combination therapy (8, 46). Suramin is the only member of the group 3 topoisomerase II inhibitors that has been evaluated in ATC. Despite potent activity in monolayer and spheroid cell cultures, the same cell lines grown as xenografts had enhanced growth with suramin treatment (47) making this an unlikely candidate for clinical trials.

Topoisomerase I inhibitors inhibit the DNA religation step which traps topoisomerase I in a covalent complex and results in cytotoxicity. These are derived from camptothecin and include topotecan and CPT-11 (irinotecan). Oncologic investigations suggest that these agents work best in combination with cisplatin or its analogs; however there are no studies evaluating these agents or the combination in thyroid carcinomas.

Additional antitumor antibiotics

Bleomycin has effective tumor cytotoxic activity caused by its ability to fragment DNA. There is a long history of clinical use of this agent in aggressive and poorly differentiated thyroid carcinomas. Although there is some activity, it has not proven sufficient to suffice for single agent therapy (34, 48, 49). The most useful combination therapies have included bleomycin with doxorubicin, either by themselves (50, 51), with cisplatin (24, 52), with vincristine (17), or with vincristine and melphalan (53). Mitomycin C is an antibiotic agent that is a bioreductive alkylating compound with no effective application to thyroid carcinoma therapy.

Antimicrotubule agents

Compounds that bind tubulin have proven quite effective as antineoplastic agents. They cause mitotic arrest, even at doses too low to affect tubulin polymerization (54). Agents that stabilize microtubules are taxanes, paclitaxel (taxol) and docetaxel (taxatere), and epothilones A and B. Microtubule-destabilizing agents include the Vinca alkaloids (vincristine, vinblastine, vindesine, and vinorelbine), dolastatins, and combretastatins (combretastatin A4 phosphate).

Paclitaxel, a taxane, has proven to be the most effective single agent in ATC. This was first demonstrated in preclinical trials using ATC monolayers and xenografts (55), then in a phase 2 clinical trial (56). When administered as a weekly intravenous infusion of 175 mg/m^2 over one hour, response rates to paclitaxel exceed 50 percent. Unfortunately, although this has prolonged survival, it has not been sufficient to prevent the eventual lethality of this disease. There have not been any published clinical reports of combination chemotherapy for ATC that includes taxanes. In preclinical studies of ATC cell lines, the combination of manumycin, a farnesyltransferase inhibitor, and paclitaxel causes increased cytotoxicity more than either agent alone (57). Later evaluation in xenograft models suggests that manumycin inhibits tumor vascularity as a component of its antineoplastic activity (58). Epothilones have not been evaluated in ATC, with the exception of an apparent enhancement of epothilone B uptake and cytotoxicity in ATC xenografts treated with imatinib mesylate (STI571) (59). This is likely consequent to imatinib effects upon tumor vasculature (60, 61) rather than a direct effect of this tyrosine kinase inhibitor on ATC cells (KB Ain, unpublished data).

The Vinca alkaloids, first extracted from the Madagascar periwinkle, bind to both high and low affinity binding sites on tubulin that are distinct from taxane binding sites and cause inhibition of microtubule assembly. This causes inhibition of mitotic spindle function and results in a block in metaphase, although it is likely to involve additional mechanisms (5). None of these compounds have proven useful as monotherapy; however, vincristine in combination with doxorubicin and bleomycin (17, 62), with melphalan added to the combination (53), in combination with doxorubicin and 5-fluorouracil (63), or in combination with cisplatin and mitoxantrone (43) seems to have some moderate clinical activity. The only other agent of this group to be studied in thyroid cancer, vindesine, had no appreciable activity in combination with doxorubicin and cisplatin (29).

Additional microtubule-destabilizing drugs include the dolastatins and combretastatins. Dolastatin 10 and dolastatin 15, including some analogs, have been used in clinical trials, but have not been included in published trials in thyroid cancers. On the other hand, combretastatin A4 phosphate has been associated with an unexpected durable complete response in a patient with anaplastic thyroid carcinoma (64), prompting further evaluation and trials (reports pending). This drug has primary antineoplastic effects in ATC similar to those of paclitaxel (65) and likely has an additional mechanism of activity as a vascular targeting agent to disrupt the irregular vessels that feed tumor growth (66).

MECHANISMS OF RESISTANCE TO CHEMOTHERAPEUTIC AGENTS

Drug efflux proteins

Increased efflux of chemotherapeutic agents from tumor cells is a well-characterized mechanism of resistance to these agents. Membrane protein pumps, responsible for drug efflux, are typically members of the ATP-binding cassette (ABC) superfamily. These proteins are capable of transporting a wide range of compounds, serving a physiological role in benign tissues and, when over-expressed in malignancies, a contribution

towards chemotherapy resistance. P-glycoprotein, a product of the *MDR1* gene on chromosome 7, is found in many normal human tissues serving to pump hydrophobic amphipathic drugs and metabolites (67). High levels of expression are seen in tumors derived from tissues which normally express P-glycoprotein (68) as well as in tumors from other tissues, particularly those expressing Ras and mutant p53 proteins (69). Inhibitors of P-glycoprotein activity, such as verapamil, are capable of restoring the cytotoxicity of chemotherapy drugs in these tumors at dosages previously without such effect (70). The next major subset of the ABC superfamily includes the Multidrug Resistance Protein (MRP) group, consisting of 6 identifiable homologous members (MRP1—MRP6). These are organic anion transporters physiologically expressed in a range of normal human tissues (71). MRP1 and MRP2 are clearly over-expressed in a number of malignant tissues, high expression of MRP3 and MRP5 can be found by screening panels of different tumor cell lines (72), malignant over-expression of MRP4 can be seen in lung cancers (73), but similar studies of MRP6 have not been reported. Recent investigations suggest that high levels of MRP4 (74) and MRP5 (75) expression result in resistance to nucleoside analog drugs; however, the efflux of a wide range of glutathione-conjugated chemotherapy drugs, via MRP1 and MRP2 (also known as cMOAT, the canalicular multispecific organic anion transporter), has been well-characterized for over a decade. Lung resistance related protein, LRP (also known as the human major vault protein), is a transporter of chemotherapeutic drugs into intracytoplasmic vaults and is also associated with resistance to these drugs (76).

Normal thyroid tissues are known to express both MRP1 and LRP transporters, but not P-glycoprotein (77). This pattern of expression is similar to the one seen in multiple ATC cell lines and tissues with nearly all having MRP1 and LRP transporters, but P-glycoprotein being rarely expressed (78–81). This pattern of expression may account for the greater sensitivity of ATC to paclitaxel, since MRP1 is less effective at transporting paclitaxel than P-glycoprotein (82). There are no published reports on ATC expression of any other MRP analogs to MRP1.

Additional mediators of drug resistance

Decreased expression of topoisomerases or mutations of its genes may be responsible for resistance to topoisomerase inhibitors. Evaluation of topoisomerase II alpha expression and gene sequences in 10 ATC cell lines and 3 tumor samples failed to find any evidence of reduced expression or gene mutation (81), making this an unlikely cause of multidrug resistance. Alternatively, increased chemotherapy drug metabolism by glutathione S-transferase, may contribute to multidrug resistance (83). A recent study suggests that enhanced activity of this enzyme in thyroid carcinoma patients may contribute to their chemotherapy drug resistance (84).

The tumor suppressor gene, p53, is a critical mediator of tumor cell apoptosis in response to DNA damage from cytotoxic agents. Mutations of the p53 gene, or epigenetic loss of expression, are extremely common in poorly differentiated thyroid carcinomas, particularly ATC (25, 85). Experimental transfection of wild-type p53 into ATC cell lines with adenovirus vectors has been shown to significantly enhance sensitivity to a variety of chemotherapeutic agents (86, 87). Likewise, targeting Bcl-2

gene expression in ATC cells by transfection with antisense oligonucleotides, removes a potent inhibitor of the BAX protein, a critical component of p53-mediated apoptosis. This also served to enhance chemosensitivity of these cells to paclitaxel, cisplatin, docetaxel, doxorubicin, mitomycin C, and 5-fluorouracil (10). Effects of alterations of the p53 pathway are not always predictable and related interventions may not always provide expected results (88).

THE FUTURE

Anaplastic thyroid carcinomas are almost always distantly metastatic by the time the primary tumor is discovered. For this reason, despite heroic efforts with primary surgery and radiotherapy, mortality is usually inevitable. Any hope for success at enhancing survival is contingent upon effective systemic chemotherapies. At this time, high-dose taxanes are the most useful agents; however, they are insufficient to alter eventual disease mortality. Although much progress is being made with antiangiogenic and tumor vascular-targeting agents, it seems unlikely to effect major benefits without new potent cytotoxic drugs. A two-pronged approach, attacking chemotherapy resistance mechanisms and developing new antineoplastic drugs, appears to be critical. In the meantime, ATC patients should be enrolled, whenever possible, in sufficient phase 1 and 2 clinical trials to permit accurate assessment of potential new therapies. Likewise, considering the rapid lethality of these tumors and cross-applications to other aggressive malignances, ATC is an appropriate cancer model for creative translational research programs. These approaches provide hope for eventual successful therapeutic strategies.

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21. GENE THERAPY FOR THYROID CANCER

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INTRODUCTION

The original concept of gene therapy is to treat and cure diseases caused by a known monogenic defect by introducing and expressing a normal copy of the mutated or deleted gene into the host cells. In this regard, gene therapy for cancer should be aimed at correcting gene alternations in cancer cells, that is, replacement of tumor suppressor genes and inactivation of oncogenes. However, cancer gene therapy has evolved in somewhat different directions. These include (i) transfer of suicide genes that convert inactive prodrugs into cytotoxic compounds, (ii) transfer of genes coding immunostimulators such as cytokines and chemokines to enhance anti-tumor immunity, (iii) transfer of genes coding anti-angiogenic factors to inhibit angiogenesis in solid tumors, (iv) transfer of drug resistant genes into normal hematopoietic stem cells to render them resistant to high-dose myelosuppressive chemotherapeutic agents. These strategies do not constitute "gene-replacement therapy" as defined above, and might instead be called "DNA therapeutics" for instance (1). Cancer gene therapy can be defined simply as "the transfer of nucleic acids into cancer or normal cells to eliminate or reduce tumor burden".

Genes can be introduced into target cells *ex vivo* and placed back into the host or directly into target cells *(in vivo).* Viral or non-viral vectors are used to facilitate the transfer of genes into target cells. This chapter discusses recent advances in gene therapy of the thyroid cancer field. Attention is focused on the therapeutic genes used.

STRATEGIES USED FOR THYROID CANCER GENE THERAPY

Silencing of oncogenes

According to the original concept of gene therapy, that is, "gene-replacement therapy", we may speculate that correction of a mutated or an aberrantly overexpressed oncogene might reverse malignant phenotype. On the other hand, some may contend that since cancers generally arises as the culmination of a multiple process that involves a variety of somatic gene alternations (see Chapter 1 for more detail), it is impossible to correct all the genetic abnormalities, as neither to restore normal gene function in every cancer cells with currently available vectors.

Several mutations or overexpression of oncogenes have been identified in thyroid cancers. The former includes RAS mutations and RET gene rearrangements in follicular and papillary carcinomas, respectively (2), and the latter overexpression of c-myc and high mobility group I (Y) protein [HMG I (Y)] in some thyroid cancers with highly malignant phenotypes (3, 4). Theoretically, suppression of gene expression can possibly be achieved with antisense, ribozyme, intracellular single-chain antibodies or RNA interference. For instance, suppression by antisense method of expression of c-myc and HMG I (Y) protein is reported to induce growth inhibition and cell death, respectively, in thyroid cancer cell lines with overexpression of a respective gene (3, 4).

Replacement of tumor suppressor gene

Among numerous mutations in different tumor suppressor genes so far identified in distinct types of cancers, the gene for tumor suppressor p53 (5) is well known to be frequently mutated in anaplastic, not well-differentiated, thyroid carcinoma (6–8). These mutations are closely associated with de-differentiation of thyroid cancer, and therefore thought to be the late event in thyroid carcinogenesis.

One can expect that introduction of wild type (wt)-p53 gene into thyroid cancer cells defective in normal p53 might reverse malignant phenotype or induce re-differentiation. Indeed it has been reported that reintroduction of wt-p53 by stable transfection into p53-defective follicular cell-derived thyroid cancer cell lines and a medullary thyroid cancer (MTC) cell line led to cell cycle arrest and growth inhibition (presumably the cells expressing p53 at relatively low levels survived) (9–16). Re-expression of wt-p53 is accompanied by chemosensitization, radiosensitization and re-appearance of the differentiated markers such as TPO, TSHR and PAX8 (9–11,14,15). Besides, of interest, despite *in vitro* cell growth inhibitory, not cell-killing, effect of wt-p53 in an anaplastic thyroid cancer cell line FRO, FRO cells stably expressing wt-p53 exhibits poor tumorigenicity in nude mice (16). Thus, tumors can not grow more than a few mm in a diameter. Tumors are found to be in an angiogenesis-restricted dormant state, that is, growth of FRO cells is counterbalanced with apoptotic cell death induced by anti-angiogenic effect of wt-p53. Wt-p53 appears to exert more complex anti-cancer actions than expected from *in vitro* data.

In contrast, however, high level expression of wt-p53 achieved with recombinant adenovirus clearly induces apoptotic cell death *in vitro* (17, 18). Furthermore, in *in vivo* experiments in nude mice, intratumoral injection of adenovirus expressing wt-p53

(1×10^9 pfu/tumor) into pre-established FRO tumors almost completely inhibited tumor growth and induced a small but significant tumor reduction when combined with doxorubicin (18). Of interest, it has recently been shown that a histone deacetylase inhibitor (depsipeptide) increases p53 transcriptional activity and thereby p21 expression, a downstream target of p53, and leads to enhancement of p53's anti-tumor effect (19). Since anaplastic thyroid cancer is highly aggressive and refractory to conventional treatments, p53 gene therapy may be a promising new strategy for this type of cancer.

Suicide gene/prodrug

Genes whose products convert a relatively nontoxic prodrug into its toxic form are referred as "suicide genes" (20). Herpes simplex virus-thymidine kinase (HSV-TK), a most widely used suicide gene product, phosphorylates a prodrug ganciclovir (GCV) ~ 1000-fold more efficiently than mammalian TK. The resultant GCV monophosphate is further phosphorylated by the mammalian enzyme to GCV triphosphate, which inhibits DNA polymerase and is thus cytotoxic. *E. coli* Cytosine deaminase converts nontoxic 5-fluorocytosine to toxic 5-fluorouracil (5-FU) by deamination, which blocks thymidylate synthetase and mRNA transcription. Deoxycytidine kinase phosphorylates and activates a number of anti-neoplastic nucleotide analogues including cytosine arabinoside (Ara-C). *E. coli* Nitroreductase converts a prodrug CD 1954 to its toxic form. The *in vitro* efficacy of all these combinations was confirmed in several thyroid cancer cell lines (21, 22). Nishihara *et al.* (21) have also demonstrated HSV-TK/GCV-mediated radiosensitization in thyroid cancer cells. Although it is impossible to transduce a therapeutic gene into every cancer cells *in vivo* with currently available vectors, non-transduced cells can be killed by neighboring transduced cells, a phenomenon called "bystander effect". Phosphorylated GCV can be transferred from transduced cells to adjacent non-transduced cells through gap junctions and phagocytosis of apoptotic vesicles of dead cells by live tumor cells. Induction of active, local immune response against tumors may participate in *in vivo* bystander effect. In addition, 5-FU, phosphorylated Ara-C and toxic CB1954 can also be secreted and taken up by surrounding cells. In this regard, these latter three combinations seem to be more efficacious than HSV-TK/GCV (22). Nevertheless, most of thyroid cancer gene therapy has been performed with HSV-TK/GCV.

HSV-TK and GCV system exerts their cytotoxic effect on not only proliferating cells (including cancer cells) but also metabolically active, non-proliferating cells such as normal thyroid cells (23, 24). It is therefore necessary to target expression of HSV-TK to cancer cells. One of the methods for targeting is transcriptional control of therapeutic gene expression. Although no thyroid cancer-specific promoter has been identified, several tissue-specific promoters are available [thyroglobulin (Tg), calcitonin (CT), *etc.*]. The preliminary experiments suggesting the potential usefulness of Tg promoter for thyroid cancer gene therapy have been performed *in vitro* with normal, differentiated rat thyroid cell line FRTL5 by Zeiger *et al.* (25). Subsequently with the retrovirus vector and transformed rat FRTC cells, a model for differentiated thyroid cancer cells, Braiden *et al.* (26) have demonstrated the feasibility of Tg promoter and

HSV-TK/GCV system *in vitro* and *in vivo.* Zhang *et al.* (27, 28) have recently demonstrated not only the efficacy but also the safety of Tg promoter with adenovirus *in vivo.* Thus intravenous (i.v.) injection of adenovirus containing HSV-TK gene under the control of Tg promoter did not induce liver damage (the main target organ for i.v. adenovirus).

However, the activities of tissue-specific promoters are generally weaker than those of constitutive viral promoters [*eg.,* human cytomegalovirus (CMV) promoter]. To overcome this drawback, use of Cre-*lox*P system is one option (29). In this study, two adenovirus vectors were constructed; one contained the expression cassette of Tg promoter and Cre recombinase gene, and the other of CMV promoter and HSV-TK gene which were interrupted with two *lox*P sequences flanking the neomycin-resistance gene. When these two adenoviruses were co-infected into the cells in which Tg promoter is active, Cre was expressed from Tg promoter in the first vector, and excised the neomycin-resistance sequence and placed HSV-TK gene under the control of the CMV promoter in the second vector, which exhibited the enhanced therapeutic effect as compared with the combination of Tg promoter and HSV-TK gene. However, it should be noted here that the need of double infection might curtail the therapeutic-efficacy when multiplicity of infection (MOI) is low or tumor cells are resistant to adenovirus infection. In addition, Takeda *et al.* (30) have reported the higher efficacy of a tandemly repeated Tg promoters.

Another problem for use of tissue-specific promoters is loss of tissue-specific promoter activities in poorly differentiated and anaplastic thyroid cancer, making Tg promoter useless for treatment of these types of thyroid cancer. Two studies suggest the potential usefulness of thyroid-related transcription factors to re-activate Tg promoter in thyroid cancer cell lines with no Tg expression, but data are somewhat different (30, 31). Chun *et al.* (31) have shown enhancement of Tg promoter by co-transfection of thyroid transcription factor-1 (TTF-1) and PAX-8, while TTF-1 alone was sufficient in studies by Shimura *et al.* (32). The different cell lines used (ARO and WRO cells *versus* FRT and BHP15-3 cells) may explain these different results. Further studies will be necessary to clarify this controversy, a very important point considering clinical trial in the future.

Furthermore, Kitazono *et al.* (33, 34) have found that histone deacetylase inhibitors (depsipeptide and sodium butyrate) enhanced activity of Tg promoter or Tg enhancer/promoter and this effect was further augmented by a cAMP analogue in thyroid cancer cell lines with no Tg expression.

Similar studies with HSV-TK/GCV and a tissue-specific promoter (CT promoter) have also been performed in MTC with a rat MTC cell line, 6–23 (clone 6), and a human MTC cell line, TT, *in vitro* and with 6–23 cells *in vivo* (35, 36).

Finally, Soler *et al.* (37) have used nitric oxide synthase II (NOS II) gene as a suicide gene for treatment of MTC. NOS II produces NO which is the main mediator of the tumoricidal action of activated macrophage. Despite an extremely low gene transfer efficiency (~1 %), injection of the naked plasmid containing CMV promoter and NOS II cDNA into orthotopically established MTC tumors led to tumor growth inhibition,

suggesting marked bystander effect probably due to NO diffusion. Thus NOS II gene can also be used as a suicide gene in cancer gene therapy.

Enhancement of tumor immunity

Cancer cells can be recognized as a foreign by host immune system. However, this anti-tumor immune response is usually not strong enough to eradicate tumors. The mechanisms of insufficient anti-tumor immunity include loss of expression of major histocompatibility complex (MHC) antigens and/or co-stimulatory molecules on cancer cells, and secretion of immuno-suppressive cytokine(s) (*e.g.,* TGF-β) from cancer cells. Systemic administration of cytokine(s) can be used to enhance immune response to tumor antigens, but is always accompanied by undesirable side effects. To overcome these problems, cDNAs coding cytokines, MHC or co-stimulatory molecules have been introduced into tumor cells to make tumor cells more immunogenic and reduce the toxicity. Two approaches are usually employed; immunization with transduced (and irradiated) autologous tumor cells or *in situ* gene delivery into an established tumor mass.

To my knowledge, the first article describing immune-gene therapy against thyroid cancer is one by Lausson *et al.* (38). They showed that rat MTC cells stably expressing interleukin-2 (IL-2) injected subcutaneously or orthotopically were completely rejected in syngeneic rats. This anti-tumor effect appeared to involve the recruitment of CD8+ T lymphocytes. Subsequently, DeGroot and his colleagues performed extensive studies on immuno-gene therapy for MTC with cytokines in rat and mouse MTC models. Tumorigenicity of mouse MTC cells infected with adenovirus harboring IL-2 gene (under the control of CMV promoter) was shown very poor in syngeneic immuno-competent mice (39). Established long lasting immunity was demonstrated by re-challenge with parental MTC cells in protected mice. Cell-mediated cytotoxic assays showed that both cytotoxic T lymphocytes and NK cells play a role. Loss of tumorigenesis of MTC cells infected with adenovirus expressing IL-2 in severe combined immune deficiency mice also indicates involvement of NK cells in this anti-tumor immunity (39). In *in vivo* situations where adenovirus expressing IL-2 (1×10^9 pfu in mice and 2×10^9 pfu in rat) was injected into pre-established MTC tumors, 70% and 43%, respectively, of the small tumors (<30 mm^3 in mice and <100 mm^3 in rat) were eradicated, but all the large tumors (>30 mm^3 in mice and >100 mm^3 in rat) showed stabilization in size (40, 41). They have also addressed the safety issue of intratumoral inoculation of adenovirus harboring IL-2 gene under the control of constitutive CMV promoter (41). Despite detection of dissemination of inoculated adenovirus from tumor to liver, no liver dysfunction was observed except mild pathological change (lymphocyte infiltration) even when constitutive viral promoter was used to drive IL-2 expression, suggesting that direct injection of adenovirus expressing IL-2 (and presumably other cytokines) can be safe. Their recent studies have demonstrated that IL-12 appears to be more efficacious than IL-2 (42). In a rat MTC model, the cure rate was 100% in smaller tumors (<100 mm^3) injected with 1×10^9 pfu adenovirus expressing IL-12, *versus* 43% complete eradication with 2×10^9 pfu adenovirus

expressing IL-2 in the aforementioned report (41). Seventy-eight % of large tumors ($>100\ mm^3$) was also eradicated (*versus* 0% in case of IL-2). Furthermore, they showed intravenous injection of adenovirus coding IL-12 was safe when IL-12 gene expression was confined to MTC tumors (and thyroid parafollicular C cells) by using the modified CT promoter comprised of two tandemly arranged tissue-specific enhancer elements and a minimal proximal CT promoter (43). More recently the efficacy of adenovirus expressing IL-12 has also been shown in thyroid follicular cancer (44).

In addition, enhanced effect of the combined HSV-TK/GCV and IL-2 has also been demonstrated by three groups (45–47). For example, in studies by DeGroot's group (46), the complete eradication of pre-established MTC tumors was induced in 63% of mice treated with adenovirus expressing HSV-TK and IL-2, 38% with adenovirus expressing IL-2 and 12% with adenovirus expressing HSV-TK (all 2×10^9 pfu).

Finally, although no thyroid tumor rejection antigens have yet been identified, the possibility of preprocalcitonin ((PPCT) as a tumor rejection antigen in MTC has been investigated by a means of DNA immunization (48). Co-delivery of PPCT and granulocyte-macrophage colony-stimulating factor genes induced cellular and humoral immune responses against PPCT, suggesting a potential of DNA immunization as a novel immunotherapeutic treatment for MTC.

Selectively replicative virus (Oncolytic virus)

As shown above, recombinant adenovirus is being widely used as a vehicle for gene delivery in cancer gene therapy. *In vivo* therapeutic efficacy of non-replicative adenovirus is however limited mainly because of its low infectivity and poor gene delivery to a solid tumor. Use of replicative adenovirus is thus a potential candidate to overcome this issue (49). ONYX-015 is such an adenovirus with a deletion in E1B 55 kD gene and reportedly replicates selectively in the cells defective in p53 gene (50), although this p53 mutation-selective replication has been disputed (49). Intratumorally (or i.v.) injected ONYX-015 first infects to a small fraction of tumor cells, in which virus replicates and induces cell death (cytopathic effect), and then virus progeny released infects to surrounding tumor cells. Portella *et al.* (51) have recently demonstrated anti-tumor effect and chemosensitivity of ONYX-015 in several thyroid cancer cell lines defective in wt-p53. They have also addressed the safety of this virus using a rat normal thyroid cell line PC C13. However, one should be cautious for these data, because human adenovirus does not usually replicate well in non-human (*eg*., rodent) cells. Indeed we have previously found that selectively replicative adenovirus can replicate and produce progeny in normal human thyroid cells in culture. Further, there is no difference in viral replication between anaplastic thyroid cancer cell line FRO and FRO cells stably expressing wt-p53, suggesting that adenovirus replication appears independent from p53 status (our unpublished data). These data does not exclude the use of replicative adenovirus for thyroid cancer treatment, rather indicate that this type of oncolytic virus can be used for both differentiated and anaplastic thyroid cancers. In this case, there is a need to strictly control virus replication in order to avoid undesired viral spread. For example, we have used Cre-*lox*P system and p53-responsive promoter to control E1A protein expression, which is essential for adenovirus replication (52). This may be a

promising means to restrict virus replication to anaplastic thyroid cancer. Tg promoter can also be used to express E1A proteins in differentiated thyroid cancer (53).

However, it is usually impossible to completely eradicate tumors with replicative adenovirus alone. Therefore, multimodality treatment with other antitumor agents might be necessary. Indeed, replicative adenovirus has also been reported to work synergistically with chemotherapy. Also replicative adenovirus can be armed with a therapeutic gene such as a suicide or a cytokine gene (54).

Antiangiogenic factors

It is well known that solid tumors can not growth more than a few mm^3 without oxygen and nutrient supplied from blood (55), suggesting that new vessel formation (called angiogenesis) is a prerequisite for solid tumor growth. Therefore, inhibition of angiogenesis might be a promising strategy for cancer treatment. Numerous antiangiogenic factors have so far been isolated such as angiostatin, endostatin, *etc.* Since these agents act basically on normal vascular endothelial cells, resistance to these agents can not be easily induced. To my knowledge, only one study describes the effect of an antiangiogenic factor gene on thyroid cancer; thrombomodulin-1 inhibits angiogenesis and growth of FRO tumors (16). As mentioned above, this article also demonstrates the ability of wt-p53 to induce anti-angiogenesis-mediated dormancy.

Iodide transporter

Active influx and efflux of iodide in the thyroid gland are mediated by sodium iodide symporter (NIS) and chloride-iodide transporter (Pendrin), respectively (56, 57). Failure of iodide concentration in some differentiated and most anaplastic thyroid cancers are generally due to decreased or loss of NIS expression. Therefore, targeted expression of NIS gene in thyroid cancers with no or little NIS expression (and also non-thyroid cancers) would offer the possibility of radioiodide therapy (58, 59). Shimura *et al.* (60) have first shown the significant iodide concentration in transformed rat thyroid cancer cells genetically engineered to express NIS. However, no or weak efficacy was demonstrated in *in vivo* studies (61, 62) because of rapid efflux of iodide. Co-expression of thyroid peroxidase has recently been demonstrated to augment iodide retention in the cells by iodide organification (63). In addition, a histone deacetylase inhibitor (Trichostatin A) has been reported to increase NIS expression and decrease Pendrin expression (64). In contrast, expression of endogenous NIS in breast cancer can be diagnostically and therapeutically useful (65). Further studies will be needed to investigate the possibility of NIS gene for cancer gene therapy.

CONCLUSIONS

I here summarized the recent articles regarding gene therapy for thyroid cancer. Although there have been tremendous progresses in this field in the last decade, there is unfortunately no published report on clinical trial of gene therapy for thyroid cancer [except one patient treated with ONYX-015 (66)]. Patients with thyroid cancer, particularly those with anaplastic and medullary cancers, will hopefully benefit from gene therapy approach in the near future.

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22. FAMILIAL PAPILLARY THYROID CARCINOMA

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INTRODUCTION

Prior to 2000 papillary thyroid carcinoma (PTC) was considered by most to be a sporadic disorder without familial predisposition. In contrast to this traditional teaching and as understood early on by Dr. Nadir Farid [1], approximately 5 percent of all PTC are familial. The evidence that supports this familial susceptibility is reviewed here and potential clinical implications are discussed. In addition, PTC may be a relatively infrequent component of other familial tumor syndromes. Although recent findings strongly support a familial PTC predisposition, the final proof will require the identification of the susceptibility genes. There is not yet convincing evidence to suggest that other nonmedullary thyroid carcinomas (follicular thyroid carcinoma, anaplastic thyroid carcinoma, and insular thyroid carcinoma) are familial.

EVIDENCE FOR AN INHERITED SUSCEPTIBILITY TO PTC

It is reasonable to suggest that any malignancy may have a familial predisposition. Cancer is caused by multiple gene mutations that are acquired over time by the cancer progenitor cell. Although these are usually somatic mutations, it would not be surprising if the first gene mutation was inherited (germline mutation). Family members possessing this hypothetical gene mutation would be at increased risk for developing PTC. Such a hypothetical susceptibility gene could persist in the population. It takes years to decades for the thyroid cancer progenitor cell to develop into a malignancy, since it must acquire other necessary gene mutations. Even then the malignancy is slow

growing. If such an inherited gene mutation did not disrupt other essential functions, then those individuals carrying this susceptibility gene mutation would not be at any reproductive disadvantage. By chance alone the gene mutation could persist within a population. Therefore, one can make a theoretical argument that a familial predisposition to PTC may occur.

Epidemiological studies, pedigree analysis, and pathology studies all provide evidence for a familial susceptibility to PTC. Although no single type of study is sufficient to prove a familial susceptibility, taken as a whole, the evidence is strong. This evidence led investigators with access to large kindreds to perform linkage studies that further support a familial predisposition to this disorder. Interestingly, the linkage studies suggest that familial PTC (fPTC) is a heterogeneous disorder caused by more than one susceptibility gene.

Epidemiologic studies have consistently found that first-degree relatives of those with PTC have a 4 to 10 fold increased risk of PTC [2–7]. Most other malignancies in these same studies do not show this familial association. Therefore, it seems unlikely that the observed PTC association is due to an ascertainment bias. Other interpretations of this association include a predisposition caused by an environmental exposure. It seems unlikely that this would be an unusual environmental factor such as radioactive iodine released from nuclear tests, since the association has been observed in multiple studies on different continents and is not limited to populations with the greatest exposure to radiation. This does not exclude the possibility that the susceptibility gene may act by increasing the risk of malignancy as a result of exposure to a more common environmental factor.

A number of large kindreds with fPTC have been described [8–15]. These kindreds are further evidence for a familial predisposition to PTC. Against this interpretation, it can be argued that these kindreds represent the rare association of multiple sporadic thyroid carcinomas, and that the number of affected family members has been exaggerated by ascertainment bias. That is, once two family members have been identified an aggressive search for thyroid carcinoma in other family members may identify microscopic (<1 cm) papillary thyroid carcinomas that have no clinical significance, and, as opposed to large PTC (>1 cm), are relatively common at post mortem examination. However, this is probably not the case, since the PTC within kindreds differs from sporadic PTC in two subtle characteristics. First, fPTC generally presents at a younger age than sporadic disease [16]. Second, there is a greater prevalence of multifocal disease in fPTC than in sporadic PTC [16, 17]. Multifocal disease within the thyroid suggests that a predisposing factor (possibly an inherited genetic susceptibility) is present. Finally, analyses of large kindreds with genetic linkage studies have identified statistically significant associations of PTC with specific chromosomal regions, and these are discussed in the next section. For all these reasons it seems likely the familial association of PTC does not represent the rare association of sporadic PTC, but represents a true familial predisposition.

In summary, the epidemiologic observation of an increased incidence of PTC in first degree relatives of PTC subjects, the presence of large kindreds in which affected

members have a tendency to develop PTC at a relatively young age, and the pathologic finding of multifocality taken together suggest that some cases of PTC are caused by an inherited susceptibility gene mutation. It should be noted that others have interpreted these results to suggest that the familial clustering of PTC indicates that fPTC is a polygenic disease caused by relatively more common but less disruptive gene polymorpisms when the associate by chance in a single kindred [18]. These two hypotheses are not mutually exclusive.

LINKAGE ANALYSIS AND THE CHROMOSOMAL LOCI OF PUTATIVE FPTC SUSCEPTIBILITY GENES

Many tumor susceptibility genes are discovered through the genetic analysis of large kindreds. Genetic analysis is particularly useful for identifying tumor susceptibility genes that were of unknown function. The first step in this genetic analysis is to determine the chromosomal location of the tumor susceptibility gene by linkage analysis.

Linkage analysis has been applied to large fPTC kindreds and statistically significant linkage of fPTC to specific chromosomal regions has been identified (Table 1). In linkage studies a statistically significant association is generally agreed to occur when the odds ratio of the probability of affected subjects carrying the same genetic polymorphism and unaffected subjects not carrying this polymorphism is one in one thousand or greater. The results are summarized as a log of the odds ratio or LOD score, so that a LOD score of 3.0 or greater is considered statistically significant. Interestingly the results between studies are discordant suggesting more than one susceptibility gene.

A large kindred with fPTC and benign thyroid nodules with the distinct pathologic finding of eosinophilia (TCO) has been mapped to 19p13.2 with a maximum LOD score of 3.0 [19]. Eosinophilia refers to the staining of the cytosol by eosin, is often caused by a large cytoplasmic population of mitochondria, and in the thyroid these are often referred to as Hurthle cells. Interestingly, other fPTC kindreds also link to this region (19p13), but the fPTC in these kindreds were not associated with eosinophilia [20]. Since the tumors of these fPTC kindreds are pathologically distinct, it is possible that there are two different susceptibility genes at this locus. Alternatively, there may be a single susceptibility gene this locus and an additional modifier gene contributes to the eosinophilia in one kindred.

Table 1. Familial papillary thyroid carcinoma—summary of linkage analyses

Disorder	Clinical description	Linkage locus	Reference
TCO	Oxyphilic PTC and benign oxyphilic nodules Autosomal Dominant with Partial Penetrance	19p13.2	[19]
PTC at 19p13	PTC without oxyphilia Autosomal dominant	19p13	[20]
FNMTC	Autosomal Dominant with Partial Penetrance	2q21	[21]
fPTC/PRN	PTC enriched with PRN Autosomal Dominant with Partial Penetrance	1q21	[22]

In another group of 80 fPTC kindreds a familial nonmedullary thyroid cancer susceptibility gene referred to as *FNMTC* has been mapped to the long arm of chromosome 2 (2q21). The maximum multipoint LOD score in all families was 3.07, and this increased to 4.17, when the 17 pedigrees with the follicular variant of PTC were analyzed alone [21]. As with the previous linkage analysis, the susceptibility gene at this locus has not been identified.

Our studies identified the familial association of PTC and papillary renal neoplasia PRN (both adenomas and carcinomas) in a large kindred. This disorder, which is designated fPTC/PRN (OMIM #605642), has been mapped to the long arm of chromosome 1 (1q21) with a multipoint single kindred LOD score of 3.58 [22]. Therefore, this syndrome is both clinically and genetically distinct from other fPTC disorders. There are a number of other neoplasms in this large kindred including benign thyroid adenomas, germ cell neoplasms premenopausal breast carcinomas and renal oncocytoma that occur in subjects that carry the affected allele. Unfortunately there are not enough genetically affected individuals to determine if these non-PTC neoplasms are components of the fPTC/PRN syndrome with low penetrance, or if they are just sporadic events in a large kindred. In this regard, it is of interest that epidemiology studies have identified an increased incidence of premenopausal breast carcinoma in PTC subjects [23]. One interpretation of this finding is that the use of I-131 in thyroid carcinoma subjects predisposes to breast carcinoma. Alternatively, our results support the hypothesis that an inherited susceptibility is responsible for this association. It may be that the *fPTC/PRN* gene predisposes to other malignancies.

Frequently sporadic tumors and familial tumors may be caused by mutations of the same susceptibility genes. For example, activating *RET* mutations are inherited in multiple endocrine neoplasia type 2 and develop spontaneously in sporadic medullary thyroid carcinoma. We have reviewed of the gene abnormalities of sporadic PTC to determine if these occur in genes that map to the linkage regions of the familial PTC syndromes. The neurotrophic tyrosine kinase receptor type 1 (NTRK1; *TRK; TRKA*) that is located at 1q23.1and *RET* that is located at 10q11.2 are both rearranged in sporadic PTC. These rearrangements effect illicit expression of these tyrosine kinases in the thyroid follicular cell. Activating *BRAF* (7q34) mutations also occur in sporadic PTC [24] and activating mutations of *hRAS* occur in sporadic follicular thyroid neoplasms [25]. Other genes contributing to the pathogenesis of follicular neoplasms include *PTEN*(10q23.31), *PAX8* (2q13), and *PPARG1* (3p25). Of these seven genes, *PAX8, hRAS* and *TRK* are potential candidates for fPTC based upon their chromosomal location. The sequence analysis of *PAX8* in the FNMTC kindreds that map to 2q21 has not been reported. Completion of the human genome project indicates that *TRK* is telomeric to the *fPTC/PRN*locus. Therefore, it is not the *fPTC /PRN* susceptibility gene. Sequence analysis of *hRAS* in fPTC/PRN indicates that the known activating mutation of *hRAS* does not cause this disorder [26]. Interestingly LOH, normally a rare event in PTC, has been observed in about 10 percent of sporadic PTC in the region near 1q21 [27]. This finding suggests that there may be a tumor suppressor gene for

PTC at the *fPTC/PRN* locus at 1q21. In summary, the genes causing sporadic PTC are not located within the fPTC loci, suggesting that the fPTC genes are distinct from the genes that cause sporadic PTC.

CLINICAL FEATURES OF FPTC AND IMPLICATIONS FOR PATIENT CARE

The clinical features of fPTC are beginning to emerge and are compared with the clinical features of sporadic PTC in Table 2. Loh first summarized the clinical features based upon a review of available published kindreds [16]. A more recent study from Japan has reported similar results [17]. Although these results are probably reliable, final confirmation of their accuracy must await the identification of the fPTC susceptibility genes, so that the genetically affected individuals can be unequivocally distinguished from those that are genetically unaffected.

The evaluation of large kindreds suggests that inheritance is autosomal dominant with partial penetrance, although it is possible that modifying genes play an important role. As with sporadic PTC, women are affected more frequently than men. Multifocal disease is more common in fPTC than in sporadic PTC, and the age of onset is somewhat younger in fPTC (mean = 38 y) than in sporadic PTC (mean = 48 y) [16, 28]. However, Uchino did not find an age difference between familial and sporadic PTC [17]. There seems to be a greater incidence of benign thyroid nodules associated with fPTC than with sporadic PTC [17]. There may be other malignancies associated with fPTC. Epidemiologic studies suggest an increased incidence of breast carcinoma in individuals with PTC [23], and one large kindred with fPTC is enriched in PRN and possibly other malignancies [9]. Although it has been, suggested that fPTC may be more aggressive than sporadic PTC [30], the differences on a whole seem to be modest. There are occasional kindreds in which fPTC seems to be more aggressive than sporadic PTC with some subjects dieing from this disorder. A more recent study does suggest a greater incidence of recurrent disease with fPTC than with sporadic PTC, but no increased incidence of death [17].

Two known familial tumor syndromes are associated with an increased incidence of PTC [29]. The frequency of PTC in familial adenomatous polyposis (FAP) is about

Table 2. Clinical characteristics of familial and sporadic papillary thyroid carcinoma (approximate ratios and percentages)

Characteristic	Familial PTC	Sporadic PTC
Age of Onset	mean = 38 [16] mean = 49.1	mean 45–50 [28] mean 48.5 [17]
Female:Male Ratio	2:1 [16]	3:1 [16]
Multifocal PTC	41% [17]	29% [17]
Recurrence	16% [17]	10% [17]
Death	rare	rare
Benign thyroid nodules	42% [17]	30% [17]
Associated malignancies	papillary renal neplasia (selected kindreds) [29]	? breast carcinoma [23]

10 times as great as the incidence expected for sporadic PTC. In the Cowden syndrome (multiple hamartoma syndrome) there is an increased incidence breast carcinoma, follicular thyroid carcinoma, and to a lesser degree PTC.

The clinical characteristics of fPTC may modify the evaluation and treatment of fPTC patients. Clinicians should review the family history carefully in subjects with PTC, since it is anticipated that about 5 percent of all PTC subjects will have a familial predisposition to this disorder. FAP and the Cowden syndrome should be excluded. There is no role for prophylactic thyroidectomy as there is in the MEN2 syndromes, since fPTC usually is a relatively slow growing malignancy rate and since asymptomatic carriers cannot be unequivocally identified. Unfortunately, except in the very largest kindreds, genetic studies will not help to identify asymptomatic carriers. We do know that any kindred members with affected first-degree relatives are at 50 percent risk of carrying the susceptibility gene. There is debate as to how aggressively these individuals at risk should be followed. Children do not need to be followed closely, since PTC rarely occurs before puberty. After puberty, yearly neck examinations are a reasonable screening tool. Some clinicians prefer to perform an ultrasound examination of the thyroid in addition to the physical examination. The disadvantage of this approach is that it is likely to identify minor abnormalities that have little clinical significance, but, because of a strong family history, may lead to unnecessary thyroidectomy. For now, the use of ultrasound for screening should be left to the discretion of the individual clinician.

SUMMARY AND CONCLUSIONS

Over the last decade, several lines of evidence have been accumulated that support the existence of fPTC susceptibility genes. Preliminary clinical characteristics of fPTC have been identified, and linkage studies have identified the chromosomal locations of putative fPTC susceptibility genes. A logical clinical approach to fPTC is emerging.

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23. RET ACTIVATION IN MEDULLARY CARCINOMAS

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INTRODUCTION

RET gene encodes a receptor tyrosine kinase acting as the subunit of a multimolecular complex that binds four distinct ligands and activates a signaling network crucial for neural and kidney development.

Different alterations of *RET* are associated to five diseases. *RET* is the susceptibility gene for the inherited cancer syndrome multiple endocrine neoplasia type 2 (MEN2), which includes MEN2A, MEN2B and familial medullary thyroid carcinoma (FMTC), as well as a major susceptibility gene for the syndrome characterized by the congenital absence of enteric ganglia, the Hirschsprung's disease (HSCR). Finally, somatic tumor-specific rearrangements of RET gene, which originate constitutively activated fused proteins, have been found in a consistent fraction of papillary thyroid carcinomas.

This review focuses on *RET* alterations in medullary thyroid carcinoma, a rare malignancy of thyroid gland present either in sporadic or MEN2-associated hereditary forms.

RET

RET gene and RET proteins

The human *RET* gene is located on chromosome 10q11.2 (Ishizaka, Y. et al., 1989) and comprises 21 exons. Homologues of *RET* have been identified in higher and lower vertebrates, as well as in *Drosophila melanogaster* (Hahn, M. et al., 2001).

Table 1. *RET* related pathologies

Disease	Genetic alteration	Pathogenic mechanism
PTCs	Chromosomal rearrangements	Constitutive TK activity
MEN2A	Germline point mutations in the Cys-rich domain	Constitutive disulfide linked dimerization
MEN2B	Germline point mutations in RET TK domain	Altered substrate specificity Constitutive TK activity
FMTC	Germline point mutations:	
	– in the Cys-rich domain	Constitutive dimerization
	– in RET TK domain	Constitutive TK activity? Altered substrate specificity?
HSCR	Germline mutations (deletions, insertions, frame shift, nonsense or missense):	
	– in RET extracellular domain	Impairment of RET cell surface expression
	– in RET TK domain	Partial loss of RET TK activity
	– in RET C-terminus	Impairment of binding of docking proteins

RET gene was identified in 1985 as a novel oncogene, following transfection of NIH3T3 cells with DNA from a human T-cell lymphoma (Takahashi, M. et al., 1985). The transforming gene resulted from a recombination event between two unlinked DNA sequences, which occurred during the transfection process; hence the name *RET,* for '*re*arranged during *tr*ansfection'. The resulting chimaeric gene encoded a fusion protein comprising an amino-terminal region that displayed a putative zinc finger motif fused to a tyrosine kinase domain. Subsequently, the name *RET* has been retained to designate the gene coding for the tyrosine kinase protein of the fused oncogene. Rearrangements of *RET* with different genes are found frequently in papillary thyroid carcinomas (RET/PTCs) (Grieco, M. et al., 1990; Santoro, M. et al., 1992). On the other hand gain-of-function mutations of *RET* cause sporadic thyroid and adrenal cancers (Lindor, N. M. et al., 1994; Beldjord, C. et al., 1995; Komminoth, P. et al., 1996) as well as cancer syndromes, such as multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B) and familial medullary thyroid carcinoma (FMTC) (reviewed in Mulligan, L. M. et al., 1995a; Goodfellow, P. J. et al., 1995; Pasini, B. et al., 1996). Interestingly, loss-of-function mutations of the same RET gene cause Hirschsprung's disease (HSCR) or colonic aganglionosis (reviewed in Amiel, J. et al., 2001) (Table 1).

RET encodes a transmembrane tyrosine kinase displaying a structure similar to that of other receptor tyrosine kinases (RTKs), comprising extracellular, transmembrane and cytoplasmic domains.

The large extracellular portion, preceded by a typical cleavable signal sequence of 28 aminoacids, has no similarity with other RTKs and contains a conserved cysteine-rich region close to the cellular membrane and a more distal region with homology to the cadherin family of cell adhesion molecules (Takahashi, M. et al., 1988; Schneider, R., 1992; Iwamoto, T. et al., 1993; Takahashi, M. et al., 1989). Cadherins are Ca^{2+}-dependent cell—cell adhesion proteins and their adhesive properties depend on a domain of about 110 amino acids tandemly repeated in the extracellular region. RET

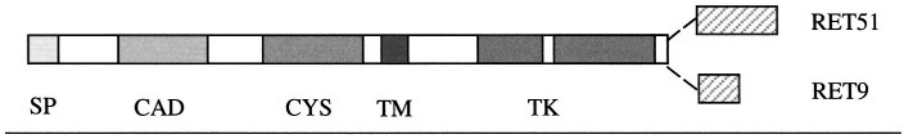

Figure 1. RET protein.
Schematic representation of the two RET isoforms, RET9 and RET51, with the signal peptide (SP), cadherin-like (CAD), cysteine-rich (CYS), transmembrane (TM), and tyrosine kinase (TK) domains.

comprises four tandemly repeated cadherin-like domains and binds specifically to Ca^{2+} ions (Anders, J. et al., 2001), supporting the hypothesis of RET as a distant member of the cadherin superfamily. RET is the only member of this superfamily containing an intrinsic tyrosine kinase domain, suggesting that RET may have arisen by the recombination of an ancestral cadherin with a protein-tyrosine kinase (Anders, J. et al., 2001).

Twenty seven of 28 cysteine (Cys) residues in the cysteine-rich domain are conserved among species suggesting a critical role for these residues in formation of intramolecular disulfide bonds and thus in determining the tertiary structure of RET proteins (Takahashi, M. et al., 1988; Iwamoto, T. et al., 1993). A single transmembrane domain of RET is followed by an evolutionary conserved tyrosine kinase (TK) domain, which is interrupted by a 27 amino acids kinase insert (Takahashi, M. et al., 1987).

The *RET* gene is alternatively spliced to yield two main protein isoforms of 1072 (RET9 or short isoform) or 1114 (RET51 or long isoform) amino acids (Tahira, T. et al., 1990) differing at the C-terminus region, by displaying 9 or 51 unrelated aminoacids (Figure 1). The RET9 and RET51 isoforms are evolutionary highly conserved over a broad range of species, suggesting that distinct isoforms can exert different roles in physiological functions of RET (Carter, M. T. et al., 2001).

RET is the signaling component of a multiprotein receptor complex involving members of two distinct groups of proteins: a soluble ligand belonging to the glial cell line-derived neurotrophic factor (GDNF) family and a glycosyl-phosphatidylinositol (GPI)-membrane anchored co-receptor belonging to GDNF family receptor α (GFRα). RET remained an orphan receptor until 1996 when GDNF was identified as the ligand of RET (Durbec, P. et al., 1996; Trupp, M. et al., 1996; Vega, Q. C. et al., 1996; Treanor, J. J. et al., 1996). Four members in the GDNF family ligands have now been characterized: Glial cell line-derived neurotrophic factor (GDNF), neurturin (NRTN), artemin (ARTN) and persephin (PSPN) (Figure 2). They represent a new subclass of the transforming growth factor-β (TGF-β) superfamily. They are secreted as disulfide-linked dimers, function as homodimers and are all neuronal survival factors (reviewed in Baloh, R. H. et al., 2000; Saarma, M., 2000). GFRα molecules do not display intracellular domains but are anchored to the cell membrane via a glycosyl-phosphatidylinositol (GPI) linkage. The ligands GDNF, neurturin, artemin and persephin use GFRα1, GFRα2, GFRα3 and GFRα4, as the preferred receptors, respectively (reviewed in Airaksinen, M. S. et al., 2002).

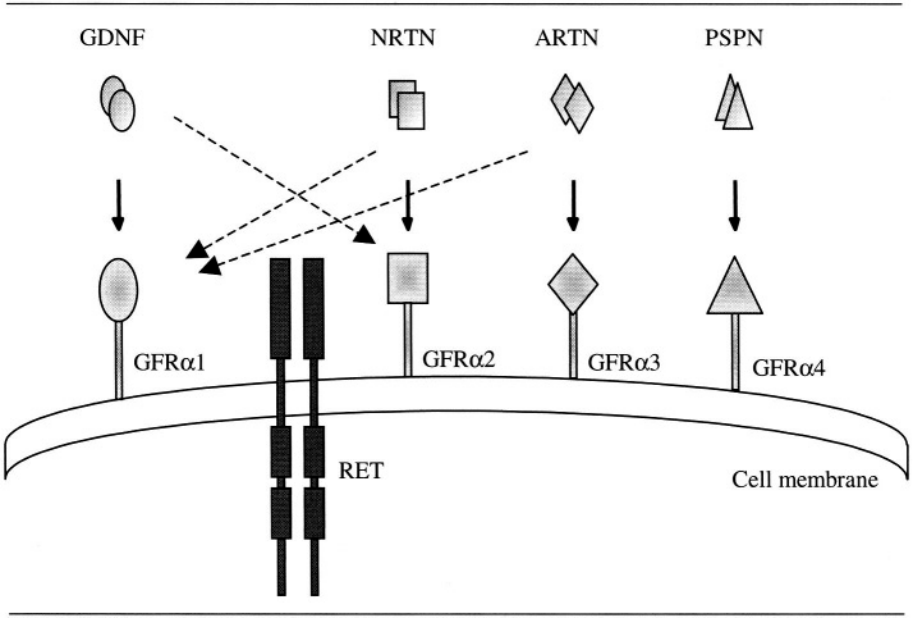

Figure 2. GDNF family ligands with their receptors.
Homodimeric GDNF family ligands activate the transmembrane RET tyrosine kinase by binding with high affinity to different GPI-linked GFRα receptors. Binding of ligand-GFRα complex to RET triggers its homodimerization, phosphorylation and intracellular signaling. The complete lines represent preferred functional binding; dotted lines may not be significant *in vivo*.

The GDNF and GFRα family members show both distinct and overlapping expression patterns (Baloh, R. H. et al., 2000), suggesting that activation of RET by formation of ligand-receptor complexes is a tightly regulated process. GDNF can also signal via GFRα1 in a RET-independent manner (Poteryaev, D. et al., 1999; Trupp, M. et al., 1999). It was recently demonstrated that in hippocampal and cortical neurons GDNF/GFRα1 make a complex with neural cell adhesion molecule (NCAM) and induce activation of Fyn and FAK promoting migration and axonal growth independently of RET (Paratcha, G. et al., 2003).

Role of RET signaling during development

The RET signaling has a critical role in the development of the enteric nervous system (ENS) and kidney, as attested by the similar and peculiar phenotype of mice with null mutations in *RET, GDNF* and *GFRα* genes. They all show severe defects in enteric innervation and renal differentiation (Schuchardt, A. et al., 1994; Sanchez, M. P. et al., 1996; Cacalano, G. et al., 1998). During vertebrate embryogenesis *RET* is expressed in the developing excretory system, in all lineages of the peripheral nervous system (PNS) and in motor and catecholaminergic neurons of the central system (CNS), including ventral midbrain dopaminergic neurons (Avantaggiato, V. et al., 1994; Durbec, P. et al., 1996; Marcos, C. et al., 1996; Pachnis, V. et al., 1993; Trupp, M. et al., 1997; Tsuzuki, T.

et al., 1995; Young, H. M. et al., 1998). Despite the widespread expression of *RET* in the nervous system of vertebrates, mutations of this locus affect, albeit drastically, only a subset of PNS ganglia. Thus, loss of function mutations of *RET* in humans lead to Hirschsprung's disease, a condition characterized by the absence of enteric ganglia from the terminal colon (Edery, P. et al., 1994; Romeo, G. et al., 1994).

Organ culture experiments and transgenic approaches have shown that RET signaling triggered by GDNF is essential for the initial ureteric budding and subsequent branching of kidney during mammalian embryogenesis (reviewed in Sariola, H. et al., 1999). In the mammalian kidney, the ureteric bud (UB), which expresses RET, induces epithelial differentiation of the nephrogenic mesenchyme, which expresses GDNF and, in turn, promotes branching of the bud. The co-receptor GFRα is expressed by both the nephrogenic mesenchyme and the UB. Thus, GDNF/RET signaling regulates the reciprocal inductive interactions between the UB and the nephrogenic mesenchyme. Recent data indicate that only RET9 seems to be critically important for kidney morphogenesis and enteric nervous system development, whereas RET51 appears dispensable (de Graaff, E. et al., 2001). However, RET51 has been suggested to be related to differentiation events in later kidney organogenesis (Lee, D. C. et al., 2002). Besides neuronal tissues and kidney, GDNF was recently implicated in sperm differentiation. GDNF is expressed by Sertoli cells, and RET and GFRα are displayed by a subset of spermatogonia including the stem cells for spermatogenesis (Meng, X. et al., 2000). Gene-targeted mice with one *GDNF*-null allele show depletion of spermatogenic stem cells, whereas mice overexpressing GDNF accumulate undifferentiated spermatogonia. Thus, GDNF contributes to the paracrine regulation of spermatogonial self-renewal and differentiation. The regulatory functions of GDNF/RET signaling in kidney morphogenesis and spermatogenesis indicate that the dosage of GDNF has both quantitative (e.g. number of branches from the UB) and qualitative (e.g. cell lineage determination of spermatogonia) dose-dependent effects in the target tissue. Thus, the expression of RET and GFRα on a cell defines the target cell type for GDNF, while the quality and nature of the response are regulated by the dosage of the ligand (reviewed in Sariola, H., 2001).

RET signaling

Ligand stimulated wild-type RET, as well as constitutive active oncogenic RET mutants, are phosphorylated at specific cytoplasmic tyrosine residues (Liu, X. et al., 1996; Coulpier, M. et al., 2002). Tyrosine autophosphorylation is required for downstream RET signaling. Recent studies have shown differences between the two isoforms, RET9 and RET51, in the intracellular signaling (Lorenzo, M. J. et al., 1997; Borrello, M. G. et al., 2002; Tsui-Pierchala, B. A. et al., 2002) as well as in the ligand-induced activation of RET inside or outside the lipid rafts (Paratcha, G. et al., 2001), which are detergent-insoluble sphingolipid and cholesterol-rich lipid microdomains that exist as phase-separated "rafts" in the plasma membrane (reviewed in Simons, K. et al., 1997; Brown, D. A. et al., 1998). Lipid rafts may be considered highly specialized signaling organelles, which assist to compartmentalize different sets of signaling molecules at both sides of the plasma membrane allowing them to interact

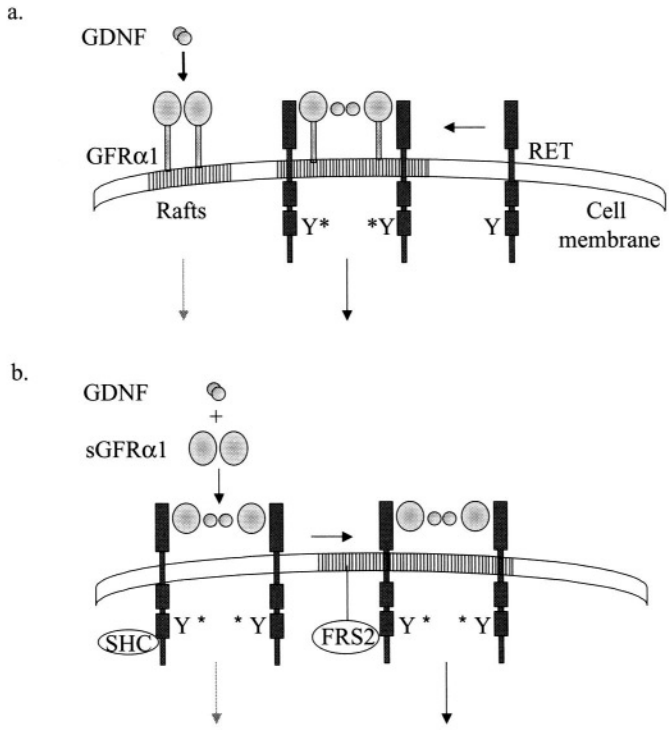

Figure 3. *Cis versus trans* signaling of RET activation.
(a) In the *cis* model of RET activation, GPI-anchored GFRα dimers, located into lipid rafts, first bind a GDNF dimer with high affinity. RET is then recruited to the GDNF-GFRα complex within the raft compartment. (b) GDNF binds to soluble GFRα in the *trans* model of RET activation. The GDNF-GFRα complex is then presented to RET, triggering RET activation. Activated RET preferentially associates with SHC outside the rafts, and predominantly associates with FRS2 within the rafts. Unphosphorylated (Y) and phosphorylated (Y*) tyrosine residues are indicated.

in a strictly regulated manner. GPI-linked GFRα proteins cluster into lipid rafts and recruit RET to lipid rafts after GDNF stimulation (Figure 3a). This recruitment of RET to rafts (through *cis* signaling) is independent on RET kinase activity. An alternative model for RET activation has been suggested by Paratcha and coworkers (Paratcha, G. et al., 2001; Manie, S. et al., 2001) (Figure 3b). Previously, soluble forms of GFRα were shown to be able to bind GDNF family ligands and to activate RET *in trans* (Jing, S. et al., 1996; Treanor, J. J. et al., 1996). A biologically active, soluble form of GFRα has been detected in the conditioned medium of neuronal and glial cell cultures (Worley, D. S. et al., 2000; Paratcha, G. et al., 2001). In the alternative pathway for RET activation, GDNF binds to soluble GFRα. The GDNF-GFRα complex is then presented to RET, triggering RET activation (through *trans* signaling). Transactivated RET is located initially outside the raft compartment and subsequently recruited to the lipid compartment through a process requiring the catalytic activity

of the receptor. Of note, this differential compartmentalization of activated RET can trigger different signaling pathways. Raft-located RET preferentially associates with the adaptor FRS2 leading to sustained ERK activation, whereas RET located outside the rafts associates with SHC and leads to transient activation of ERK and activation of PI3K/AKT pathway (Paratcha, G. et al., 2001).

The intracellular domain of RET contains 14 tyrosine residues in the long and 12 in the short isoform, the latter lacking two tyrosine residues in the C-terminus. Interactions of RET with a number of downstream targets have been identified (Figure 4).

Phosphorylated tyrosine residues Tyr905, Tyr1015 and Tyr1096, the latter long isoform-specific, have been identified as docking sites for the adaptor proteins GRB7/GRB10, Phospholipase-Cγ and GRB2, respectively (Pandey, A. et al., 1995; Pandey, A. et al., 1996; Borrello, M. G. et al., 1996; Alberti, L. et al., 1998). Tyr1062 is a multidocking site interacting with a number of transduction molecules: SHC, FRS2, IRS1/2, DOK proteins, ENIGMA and PKCα (Durick, K. et al., 1996; Arighi, E. et al., 1997; Lorenzo, M. J. et al., 1997; Kurokawa, K. et al., 2001; Melillo, R. M. et al., 2001; Hennige, A. M. et al., 2000; Grimm, J. et al., 2001; Andreozzi, F. et al., 2003). The binding of SHC, FRS2, IRS1/2 and DOK to Tyr1062 is dependent on phosphorylation of this residue and is mediated by PTB or SH2 phosphotyrosine binding

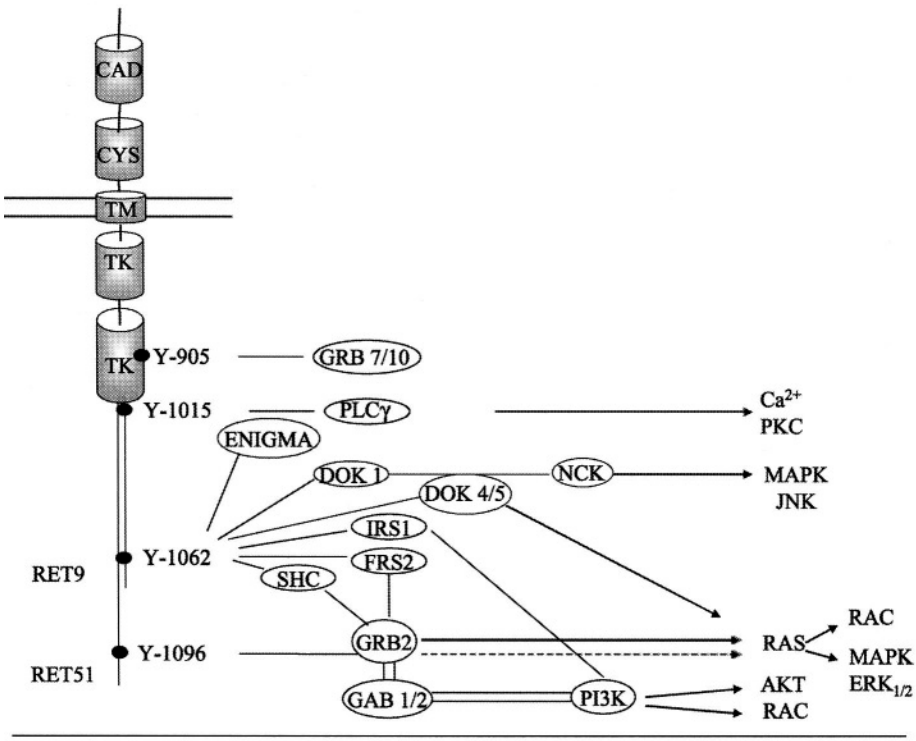

Figure 4. RET signaling pathways.

domains. In contrast, the binding to Tyr1062 of ENIGMA, a PDZ-LIM protein, is phospho-independent. Furthermore, ENIGMA binds specifically RET9, since short isoform-specific aminoacid residues +2 to +4 to Tyr1062 are required for interaction with ENIGMA (Borrello, M. G. et al., 2002).

Tyr1062-associated adaptor proteins contribute to activation of several downstream signaling pathways such as the RAS/ERK, PI3K/AKT, p38MAPK, JNK and ERK5 (Besset, V.et al., 2000; Hayashi, H. et al., 2000; Hayashi, Y. et al., 2001; Segouffin-Cariou, C. et al., 2000; Kurokawa, K. et al., 2003).

Tyr1096 is the most C-terminal phosphorylated tyrosine residue and it is unique to the long isoform (Liu, X. et al., 1996). The Tyr1062 multidocking site, although common to both isoforms, is only two residues amino-terminal to the C-terminal RET splice site, which alters the context of this residue between RET9 and RET51. Accordingly, Tyr1062 in short and long isoform does appear to have differential interactions with SHC and ENIGMA proteins (Lorenzo, M. J. et al., 1997; Borrello, M. G. et al., 2002). In addition, the two isoforms, though sharing identical extracellular domains, do not associate with each other and show different tyrosine phosphorylated associated proteins in sympathetic neurons (Tsui-Pierchala, B. A. et al., 2002).

MEDULLARY THYROID CARCINOMA

Medullary thyroid carcinoma (MTC) is a rare tumor of the thyroid gland. It was recognized as an unique entity by Hazard in 1959 (Hazard, J. B. et al., 1959); previously it was often classified as undifferentiated thyroid carcinoma.

MTC comprises approximately 5–10% of all thyroid malignancies. At variance with other thyroid malignancies, deriving from the follicular thyroid cells, MTC arises from parafollicular C cells (Williams, E. D., 1966). C cells derive from the neural crest and are able to secrete the hormone calcitonin (CT), a specific tumor marker for MTC. Calcitonin is in fact a useful marker in the follow up of treated patients and was used for screening the individuals predisposed to the hereditary form of the disease. RET analysis has now replaced CT testing to diagnose MEN2 carrier state.

About 75–80% of MTCs are sporadic (sMTC) and the remainder 20–25% are hereditary (hMTC) (Farndon, J. R. et al., 1986). In contrast to the sporadic cases which are characterized by a unifocal clonal tumor cell population, the heritable variants generally emerge from a multifocal origin (reviewed in Eng, C., 1999; Hazard, J. B., 1977).

In 1959, Hazard and coworkers first recognized medullary thyroid carcinoma as a distinct tumor (Hazard, J. B. et al., 1959). Sipple first described the association of medullary thyroid cancer, pheochromocytoma and parathyroid adenoma in 1961 (Sipple, J. H., 1961), and the syndrome was later termed "multiple endocrine neoplasia type 2" by Steiner and coworkers (Steiner, A. L. et al., 1968). The association between medullary thyroid carcinoma, pheochromocytoma, and multiple mucosal neuromas was described by Williams and Pollock in 1966 (Williams, E. D. et al., 1966) and confirmed by others. This syndrome was named MEN2B in 1975 by Chong and coworkers to be distinguished from the Sipple's syndrome (now called MEN2A) (Chong, G. C. et al., 1975).

Table 2. Clinical subtypes of MEN2

Disease	Characteristic features
MEN2A	Thyroid C-cells tumor Pheochromocytoma Parathyroid hyperplasia/adenoma
FMTC	Thyroid C-cells tumor
MEN2A with cutaneous lichen amyloidosis	MEN2A and cutaneous lesions
MEN2A/FMTC with Hirschsprung's disease	MEN2A/FMTC with intestinal aganglionosis
MEN2B	Thyroid C-cells tumor Pheochromocytoma Intestinal/mucosal ganglioneuromatosis Habitus marfanoid

Three clinically different types of MEN2 are now distinguished: MEN2A, MEN2B, and familial medullary thyroid carcinoma (FMTC), all transmitted in autosomal dominant fashion (Table 2).

They vary in aggressiveness of MTC and spectrum of disturbed organs. Their common feature is the thyroidal C cell hyperfunction with the occurrence of medullary thyroid carcinoma. MTC that occurs in hereditary forms is usually multifocal and bilateral. MEN type 2A is characterized by medullary thyroid carcinoma (MTC), pheocromocytoma in about 50% of cases, and parathyroid hyperplasia or adenoma in about 20–30% of cases. MEN2A accounts for over 75% of MEN2 (Eng, C. et al., 1996a; Ponder, B. A. J., 2001). MEN2B is the most distinctive and aggressive of the MEN2 variants and it is characterized by earlier age of tumor onset and by developmental abnormalities which include intestinal ganglioneuromatosis, atypical facies and marphanoid habitus. FMTC is characterized by the presence of MTC alone in at least four family members. FMTC is considered the least aggressive of the three MEN2 subtypes. In MTC families in fact, the tumor usually develops at a later stage of life and its course is more benign (Farndon, J. R. et al., 1986).

Several rare variants of MEN2 include MEN2A with cutaneous lichen amyloidosis (Nunziata, V. et al., 1989; Donovan, D. T. et al., 1989), and MEN2A or FMTC with Hirschsprung's disease (Verdy, M. et al., 1982).

RET ACTIVATION IN INHERITED AND SPORADIC MEDULLARY THYROID CARCINOMAS

RET mutations in MEN2 syndromes

In 1991, genetic linkage analyses mapped putative loci for MEN2 syndrome to a small interval on chromosome 10q11.2 and few years later *RET* gene was identified as the susceptibility gene for these syndromes (Donis-Keller, H. et al., 1993; Mulligan, L. M. et al., 1993b; Carlson, K. M. et al., 1994; Eng, C. et al., 1994; Hofstra, R. M. et al., 1994). Unlike other cancer syndromes, which are associated with inactivation of tumor suppressor genes, MEN2 arises as a result of activating mutations of the *RET* gene. This was the first time that predisposition to a cancer syndrome was associated with the activation of an oncogene rather than inactivation of a tumor suppressor gene (reviewed in Frischauf, A. M., 1993).

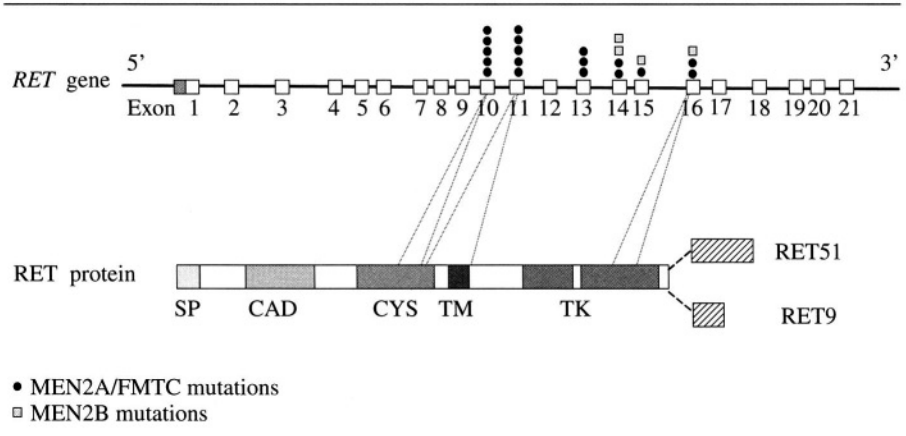

Figure 5. Schematic diagram of the *RET* gene and RET protein showing the location of MEN2 mutations.

Germline point mutations of *RET* are responsible for the inheritance of all the autosomal dominant MEN2 cancer syndromes MEN2A, MEN2B and FMTC (Figure 5 and Table 3).

The correlation between the various RET mutations and the development of thyroid carcinomas has been proved in fibroblast (Santoro, M. et al., 1995; Borrello, M. G. et al., 1995) and in a variety of different transgenic models (Asai, N. et al., 1995; Michiels, F. M. et al., 1997; Reynolds, L. et al., 2001; Acton, D. S. et al., 2000).

RET in multiple endocrine neoplasia type 2A (MEN2A)

Missense mutations of the RET gene have been found in the constitutional DNA of virtually all MEN2A families. These mutations affect the cysteine-rich extracellular domain of RET, each converting a critical cysteine residue (Cys) to another aminoacid at codons 609, 611, 618, 620, (exon 10) or at codons 630, 634 (exon 11) (see references in Table 3). They account for 98% of all mutations associated with MEN2A; the most common mutation, accounting for over 80% of all mutations associated with MEN2A, affects codon 634 and converts this cysteine into an arginine in about half of cases. Rarer duplication/insertion mutations associated with MEN2A have been described in exon 11 (Hoppner, W. et al., 1997; Hoppner, W. et al., 1998) resulting in the insertion of three or four aminoacids including a cysteine residue within the cysteine-rich domain. *De novo* cases of MEN2A have been associated with two new germline mutations (at both codon 634 and 640) on the same RET allele (Tessitore, A. et al., 1999).

RET-MEN2A oncoproteins display constitutive kinase activity consequent to ligand-independent dimerization. It is postulated that the cysteine residues are normally involved in intramolecular disulfide bonds. The disruption of a Cys by mutation may render the partner Cys available for aberrant disulfide bonding with other mutant

Table 3. *RET* mutations in MEN2 syndromes and sMTC

Exon	Codon number	Amino acid change	Phenotype	Reference
10	603	Lys to Gln	sMTC	Rey JM, 2001
	609	Cys to Arg	MEN2A/FMTC	Mulligan LM, 1995b; Eng C, 1996
		Cys to Tyr	MEN2A	Mulligan LM, 1994
		Cys to Ser	MEN2A	Igaz P, 2002
	611	Cys to Tyr	MEN2A	Mulligan LM, 1995b; Eng C, 1996
		Cys to Trp	MEN2A/FMTC	Donis-Keller H, 1993
		Cys to Gly	FMTC	Mulligan LM, 1995b; Eng C, 1996
		Cys to Ser	MEN2A/FMTC	Nishikawa M, 2003
	618	Cys to Phe	MEN2A	Mulligan LM, 1995b; Eng C, 1996
		Cys to Ser	MEN2A/FMTC	Donis-Keller H, 1993; Morita H, 1996
		Cys to Gly	MEN2A	Mulligan LM, 1995b; Eng C, 1996
		Cys to Arg	MEN2A/FMTC	Donis-Keller H, 1993; Marsh DJ, 1994
		Cys to Tyr	MEN2A/FMTC	Donis-Keller H, 1993
		Cys to Stop	MEN2A	Mulligan LM, 1995b; Eng C, 1996
	620	Cys to Arg	MEN2A/FMTC	Donis-Keller H, 1993
		Cys to Tyr	MEN2A	Donis-Keller H, 1993
		Cys to Phe	MEN2A	Mulligan LM, 1995b; Eng C, 1996
		Cys to Ser	MEN2A	Oishi S, 1995
		Cys to Gly	MEN2A	Mulligan LM, 1993b
11	630	Cys to Phe	FMTC	Komminoth P, 1995
	634	Cys to Arg	MEN2A	Donis-Keller H, 1993; Mulligan LM, 1994
		Cys to Tyr	MEN2A/FMTC	Mulligan LM, 1994
		Cys to Phe	MEN2A/FMTC	Mulligan LM, 1994
		Cys to Gly	MEN2A	Mulligan LM, 1993b
		Cys to Trp	MEN2A	Mulligan LM, 1994
		Cys to Ser	MEN2A/FMTC	Mulligan LM, 1993b; Eng C, 1996
	634 and 640	Cys to Arg and Ala to Gly	*de novo* MEN2A	Tessitore A, 1999
	639 and 641	Ala to Gly and Ala to Arg	sMTC	Kalinin VN, 2001
13	768	Glu to Asp	FMTC/sMTC	Eng C, 1995
	790	Leu to Phe	MEN2A/FMTC	Berndt I, 1998
	791	Tyr to Phe	FMTC	Berndt I, 1998
14	804	Val to Leu	FMTC	Bolino A, 1995
		Val to Met	FMTC	Fattoruso O, 1998
	804 and 806	Val to Met and Tyr to Cys	*de novo* MEN2B	Miyauchi A, 1999
	778 and 804	Val to Ile and Val to Met	FMTC	Kasprzak L, 2001
15	883	Ala to Phe	*de novo* MEN2B	Smith DP, 1997
	891	Ser to Ala	FMTC	Hofstra RMW, 1997
16	918	Met to Thr	MEN2B	Carlson KM, 1994
			sMTC	Eng C, 1994; Hofstra RMW, 1994
	922	Ser to Phe	sMTC	Kalinin VN, 2001

Exon	Base pairs duplication	Codon number	Amino acids inserted	Phenotype	Reference
8	9 bp	531–532	EEC	hFMTC	Pigny P, 1999
11	12 bp	634–635	HELC	hMEN2A	Hoppner W, 1997
	9 pb	636–637	CRT	MEN2A	Hoppner W, 1998

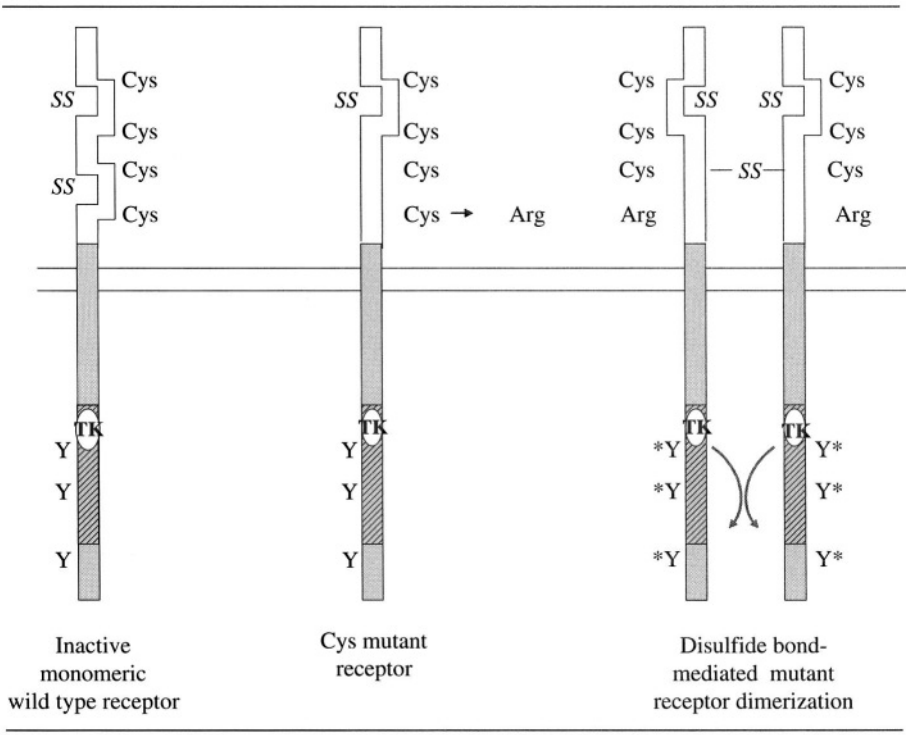

Figure 6. Mechanism of disulfide bond-mediated RET dimerization.
Intramolecular and intermolecular disulfide bonds (SS), unphosphorylated (Y) and phosphorylated (Y*) tyrosine residues are indicated.

RET molecules thus leading to constitutive receptor dimerization and hence activation (Figure 6) (Asai, N. et al., 1995; Santoro, M. et al., 1995; Borrello, M. G. et al., 1995).

Accordingly, transgenic mice in which the *RET*9 gene carrying the MEN2A-C634R mutation was expressed under the control of the human calcitonin promoter or under the MoMuLv LTR, developed C-cell tumors resembling human MTC (Michiels, F. M. et al., 1997; Kawai, K. et al., 2000).

RET mutations at codons 609, 618, and 620 lead to constitutive RET activity by causing ligand-independent disulfide-bridged homodimerization as "classical" RET-C634R mutation but, in addition, they markedly decrease the cell surface expression of RET (Carlomagno, F. et al., 1997; Chappuis-Flament, S. et al., 1998; Ito, S. et al., 1997).

Some tumors in MEN2 display a second hit, a somatic mutation involving the *RET* gene in the tumor clone precursor cell; the activated *RET* allele is amplified by chromosome 10 duplication in some tumors, or the normal *RET* allele is deleted in some others (Huang, S. C. et al., 2000).

RET in multiple endocrine neoplasia type 2B (MEN2B)

Most MEN2B cases (95%) are caused by the M918T mutation (exon 16) (Carlson, K. M. et al., 1994). Other rarer (5%) intracellular mutations involve codon 883 (exon 15) in the RET tyrosine kinase domain (Smith, D. P. et al., 1997).

The M918T substitution is also found in sporadic MTC (reviewed in Jhiang, S. M., 2000), with M918T mutation-positive tumors often displaying a more aggressive phenotype. Recently, a double mutation at codons 804 and 806 has been found in a Japanese patient that had clinical features characteristic of MEN2B (Miyauchi, A. et al., 1999). The M918T mutation does not cause constitutive dimerization but activates RET by an intramolecular mechanism (Santoro, M. et al., 1995). The methionine at codon 918 is highly conserved in receptor tyrosine kinases and it maps in the P+1 loop of the kinase domain that is predicted to interact with the protein substrate. A threonine is found at the equivalent position in cytosolic tyrosine kinases, and the two kinase classes (receptorial and cytosolic) have different signaling specificity (Marengere, L.E. et al., 1994). Accordingly, it was demonstrated that the MEN2B-M918T mutation changes the substrate specificity of RET kinase. Indeed, RET-MEN2B displays phosphorylation of intracellular proteins as well as autophosphorylation sites different from RET-MEN2A (Santoro, M. et al., 1995). Thus, the shift of RET autophosphorylation sites and of RET intracellular substrates, rather than the modest rise of RET kinase activity (Borrello, M. G. et al., 1995), may be crucial for the oncogenic activity of RET-MEN2B and responsible for its specific neoplastic activity (Santoro, M. et al., 1995). Furthermore, GDNF stimulation seems to be necessary for the full activation of RET-MEN2B (Bongarzone, I. et al., 1998).

It is not known how the A883F affects RET function. However, residue 883 is located in a subdomain of RET that defines substrate preference (Smith, D.P. et al., 1997) thus suggesting that the alteration of substrate specificity may be the common etiologic thread that underlies the pathogenesis of MEN2B. The production of a mouse model of MEN2B by introduction of the corresponding mutation into the *RET* gene demonstrated that heterozygous mutant mice displayed several features of the human disease, including C-cell hyperplasia and pheochromocytoma, while homozygous displayed more severe thyroid adrenal disease as well as male infertility. Only homozygous mice did develop ganglioneuromas of the adrenal medulla and enlargement of the associated sympathetic ganglia (Smith-Hicks, C.L. et al., 2000).

RET in familial medullary thyroid carcinoma (FMTC)

FMTC mutations can be found either in the extracellular or in the tyrosine kinase domain of RET. The ones occurring in the extracellular RET domain are the same mutations also associated with MEN2A: substitution of cysteines 609, 611, 618, 620 (exon 10), 630 and 634 (exon 11). The mutations in the tyrosine kinase domain occur at residues 768, 790, 791 (exon 13), 804, 844 (exon 14) or 891 (exon 15) (see references in

Table 3). Rare mutations have been recently reported, such as a 9-base pair duplication in exon 8 in a FMTC family (Pigny, P. et al., 1999) or mutations at codons 778 and 804 on the same RET allele which are associated with both FMTC and prominent corneal nerves (Kasprzak, L. et al., 2001). FMTC mutations occurring in the intracellular RET domain were thought to be infrequent and only a few number of families bearing the RET mutation within exons 13, 14 and 15 have been described. However, in the past two years the frequency of detection of these mutations has increased (Niccoli-Sire, P. et al., 2001) due to more accurate analysis and screening. No data are yet available on the mechanisms of activation of FMTC mutations occurring in RET tyrosine kinase domain. Patients with RET mutations in exons 13, 14 and 15 exhibit a mild C-cell disease phenotype (Berndt, I. et al., 1998; Fattoruso, O. et al., 1998) confirmed by *in vitro* studies. In fact, mutant RET proteins carrying mutations at residues 768, 804 or 891 display lower transforming activity (Pasini, A. et al., 1997; Iwashita, T. et al., 1999) compared with RET substitutions at codons 634, 918 or 883 strongly associated with MEN2A and MEN2B, respectively. Computer modeling has suggested that the E768D substitution modifies the kinase activity of the receptor by altering the substrate specificity or the ATP-binding capacity (Pasini, A. et al., 1997). As for its location, also the substitution at position 804 may exert an activating effect by altering the kinetics of interactions with normal cellular substrates or by modifying the range of substrates that are phosphorylated (Bolino, A. et al., 1995; Eng, C. et al., 1995; Iwashita, T. et al., 1999; Pasini, A. et al., 1997).

Recent studies (Feldman, G. L. et al., 2000; Brauckhoff, M. et al., 2002) have reported cases of patients harboring RET germline mutations in exons 14 and 15 (at codons 790, 791, 804) resulting in papillary microcarcinoma. Rey and colleagues (Rey, J. M. et al., 2001) also described the case of a kindred in which a novel single point germline RET mutation (K603E in exon 10) cosegregates with medullary and papillary thyroid carcinomas (PTCs). Despite the low number, these observations suggest that there might be a correlation between the occurrence of PTC and RET germline mutations in exons 13 and 14 that may play a role in pathogenesis of PTC. Of note, PTC seems to be present just in patients with low penetrance RET germline mutations. It remains an open question whether the simultaneous occurrence of inherited MTC and PTC is coincidental or the result of partly common pathogenic pathways. Reynolds and coworkers (Reynolds, L. et al., 2001) found the co-existence of MTC and PTC in transgenic mice expressing the long isoform of RET-MEN2A and suggested that this might be due to the possible existence of an ultimobranchial stem cell of endodermal origin, which gives rise to a subset of both thyroid follicular cells and C-cells (Kovacs, C. S et al., 1994).

MEN2/HSCR paradox: the same mutation of RET with both gain and loss of function

In 20 to 30% of families with a mutation at cysteine residues 609, 611, 618 or 620 and no additional mutations within the coding sequence of *RET,* MEN2A and FMTC associate with HSCR (Mulligan, L.M. et al., 1994; Attie, T. et al., 1995; Borst, M. J. et al., 1995; Nishikawa, M. et al., 2003). The occurrence of HSCR in MEN2A/FMTC

pedigrees is difficult to be explained with a gain-of-function mutation in RET, which is typical for the MEN 2 mutations. RET proteins containing these specific cysteine mutations have been shown to translocate to the cell surface with low efficiency (Ito, S. et al., 1997; Chappuis-Flament, S. et al., 1998). Moreover, a kinase activity under the threshold required for cell survival (Takahashi, M. et al., 1999), or an inability to respond to GDNF, and protect RET-expressing cells from apoptosis (Mograbi, B. et al., 2001) have also been proposed as explanations. The mutation of each cysteine involved in MEN2A/FMTC promotes the aberrant formation of disulfide-linked RET homodimers, causing a constitutive activation of RET. However, mutation of Cys634 is approximately fivefold more strongly activating than mutations of cysteine 609, 611, 618, or 620 (Chappuis-Flament, S. et al., 1998). As a result, the low level of the RET covalent dimers resulting from mutations of cysteine 609, 611, 618, or 620, might be sufficient to activate the RET signaling pathways in the thyroid C-cell and chromaffin cell, leading to hyperplasia or tumor formation. On the other hand, insufficient RET protein is available in the developing enteric nervous system, thereby leading to HSCR. However, the fact that HSCR and MEN2A/FMTC are associated in only a fraction of families with a mutation at codon 609, 611, 618, or 620, indicates that other genetic or environmental factors might influence the clinical expression of the enteric phenotype (Mulligan, L. M. et al., 1994; Decker, R. A. et al., 1998).

RET in sporadic MTC (sMTC)

The majority of MTC cases (75%) have no associated family history. However, it has been found recently that 3–7% of sporadic cases represent occult or *de novo* MEN2 cases as determined by finding germline RET mutations (Wohllk, N. et al., 1996).

Somatic RET mutations are found, however, in 30 to 70% of true sporadic medullary thyroid carcinomas and, rarely, in pheochromocytomas. Mutation at the codon 918 is largely predominant but also mutations at the cysteine codons 609, 611, 620, 630, 634 were found in sporadic MTC cases. Recently three new somatic missense mutations (at codons 639, 641 and 922) of the *RET* gene associated with sporadic MTC have been described (Kalinin, V. N. et al., 2001). The functional significance of RET mutations, however, is unclear. They have been shown to be present heterogeneously, suggesting that they have occurred during tumor evolution rather than being the initiating step (Eng, C. et al., 1996b). In addition, loss of heterozygosity (LOH) has been found in up to 30% of tumors on chromosomes 1p, 3p, 3q, 11p, 13q, 17p, 22q (Mulligan, L. M. et al., 1993a).

RET-MEN2A and RET-MEN2B signaling

The functions of RET in normal and tumorigenic cells are mediated by a complex series of downstream interactions. RET-activated pathways, such as AKT pathway, have been shown to be important for cell signaling mediated by both ligand-dependent and -independent activation of RET and to have a role in cell survival, proliferation and oncogenic transformation by all RET oncogenic forms (reviewed in Manie, S.

et al., 2001). However, RET-MEN2A is considered a constitutively activated kinase, whereas RET-MEN2B appears to be an activated kinase with in addition altered catalytic properties (Santoro, M. et al., 1995; Borrello, M. G. et al., 1995). Consistent with this fact, differences in cell signaling triggered by each of these oncogenic forms have been emerging. Phosphorylation of Tyr864 and Tyr952 is crucial for transformation triggered by RET-MEN2B while Tyr905 phosphorylation is essential for transformation promoted by RET-MEN2A (Iwashita, T. et al., 1996). Mutation of Tyr1062 multidocking site markedly impaired the transforming activity of all MEN2 mutants (Iwashita, T. et al., 1996). However it was reported that the level of phosphorylation of Tyr1062 is increased in cells expressing RET-MEN2B compared to those expressing RET-MEN2A, resulting in enhancement of activation of ERK and PI3K/AKT pathways (Salvatore, D. et al., 2001). In addition, DOK1, a Tyr1062-associated docking protein activating JNK pathways, more strongly binds RET-MEN2B than RET-MEN2A protein (Murakami, H. et al., 2002). Consequently, enhanced signaling via Tyr1062 has been suggested to be involved in MEN2B phenotype. Furthermore, recent studies obtained by differential display analysis of gene expression in cells expressing either RET-MEN2A or RET-MEN2B mutant proteins identified genes predominantly induced by either mutations (Watanabe, T. et al., 2002).

DIAGNOSIS AND MANAGEMENT OF MEN2

MEN2 gives a unique model for early prevention and cure of cancer and for stratified roles of mutation-based diagnosis of carriers. MTC is the first neoplastic manifestation in most MEN2 kindreds because of its earlier and overall higher penetrance. In older MEN2A series, with treatment initiated after the identification of a thyroid nodule, MTC progressed and showed 15–20% mortality (Kakudo, K. et al., 1985). The impact of carrier diagnosis before adulthood has been proven in long-term studies with measurement of serum calcitonin (CT). Early thyroidectomy has lowered the mortality from hMTC to less than 5%. However, the longest follow-up period for prospective CT screening is less than 25 years (Gagel, R. F. et al., 1988). Moreover, it is probable that improved management of pheochromocytoma has decreased the rate of premature mortality in MEN2 even more than has the improved management of MTC. Syndromic morbidity is more severe, and mortality is earlier in MEN2B than in MEN2A. Recognition of the most highly aggressive MTC in MEN2B and recognition of the possibility for early carrier detection have led to thyroidectomy in MEN2B far earlier than before. Like pheochromocytoma in MEN2A, pheochromocytoma in MEN2B has been virtually eliminated as a major cause of death because of improved management.

Multifocal C-cell hyperplasia is a precursor lesion to hMTC; the progression from C-cell hyperplasia to microscopic MTC is variable and may take many years (Papotti, M. et al., 1993). Metastasis may be in the central and lateral, cervical, and mediastinal lymph nodes or more distantly in lung, liver, or bone. The aggressiveness of MTC correlates with the MEN2 variant syndrome and with the mutated *RET* codon. The primary secretory product of MTC is CT, which is important only as an excellent tumor marker (Gagel, R. F. et al., 1988; Pacini, F. et al., 1994). CT values (basal or

stimulated by pentagastrin, calcium, or both) are nearly always elevated with MTC. Similarly, elevated CT values after surgery are generally the first sign of persistent or recurrent disease.

Prevention or cure of MTC is by surgery; success is mainly dependent upon the adequacy of the initial operation. Therefore, surgery for MTC should be performed, if possible, before the age of possible malignant progression. Unfortunately, standard chemotherapeutic regimens have not been proven beneficial in patients with metastatic MTC, and the tumors are not very sensitive to x-ray or thermal radiation therapy (reviewed in Brandi, M. L. et al., 2001).

MEN2 carrier determination is one of the few examples of a genetic test that mandates a highly effective clinical intervention. Consensus was reached at the MEN97 Workshop that the decision to perform thyroidectomy in MEN2 should be based predominantly on the result of *RET* mutation testing, rather than on CT testing (Lips, C. J., 1998). Sequencing of DNA for *RET* mutations is indeed effective and widely available. 98% of MEN2 index cases have an identified *RET* mutation, and testing in no MEN2 family has excluded the *RET* locus. A limited number of MEN2-associated mutations, involving *RET* exons 10, 11, 13, 14, 15, and 16, have been identified. Thus, only these exons must be tested routinely. If this is negative, the remaining 15 exons should be sequenced.

RET inactivating mutations account for approximately half of cases of familial Hirschsprung's disease (HSCR). It is thus surprising that activating mutations of *RET* codons 609, 618, and 620 have also been associated, albeit rarely, with MEN2A and HSCR. In addition, there have been rare cases of HSCR with exon 10 mutations identical to those found in hMTC. Germline mutation analysis of *RET* (containing codons 609, 618, and 620) is indicated in all children with HSCR. In those rare cases with potential activating mutation at one of these codons, consideration should be given to prophylactic thyroidectomy, and parents and other first degree relatives should be screened. *RET* codon 918 mutations, like those in MEN2B, have been reported in several children with colonic ganglioneuromatosis. In children with this disorder and a codon 918 mutation or other *RET* activating mutation, consideration should be given to prophylactic thyroidectomy.

The specifically mutated codon of *RET* correlates with the MEN2 variant, including the aggressiveness of MTC. Children with MEN2B and/or *RET* codon 883, 918, or 922 mutation are classified as level 3 or as having the highest risk from aggressive MTC and should have thyroidectomy within the first 6 months and preferably within the first month of life.

Children with any *RET* codon 611, 618, 620, or 634 mutation are classified as level 2 or as having a high risk for MTC and should have thyroidectomy performed before the age of 5 years. Total thyroidectomy should be performed.

Children with *RET* codon 609, 768, 790, 791, 804, and 891 mutations are classified as level 1 or as having the least high risk among the three *RET* codon mutation stratification categories. They, too, should have a total thyroidectomy. The biological behavior of MTC in patients with these mutations is variable, but, in general, MTC

grows more slowly and develops at a later age than with the high risk mutations. Variations between members of the same family regarding the clinical presentation of the MEN2 disease and the age at onset might be related to factors such as polymorphisms that increase susceptibility to the syndrome including variation in pathological phenotype. Identification of these factors is one of the key problems in medical genetics. Recently, the two *RET* polymorphisms G691S and S904S have been suggested to have a role as a low penetrance risk factor of MEN2A (Robledo, M. et al, 2003).

Therefore, because of DNA-based testing, many MEN2 carriers should undergo total thyroidectomy before expressing MTC. However, basal and stimulated CT testing are still useful indexes of tumor mass to screen for or monitor MTC before or after thyroid surgery.

Gene therapeutic approaches for inhibition of oncogenic RET signaling

In recent years, new therapeutic approaches as alternative strategy in MTC surgical treatment have been investigated. The inhibition of tyrosine kinase activity by small cell-permeable molecules is a promising tool to target oncoproteins and has already reached clinical application for Erb/HER subgroup of receptor (reviewed in Mendelsohn, J. et al., 2000; Dancey, J. E. et al., 2001). The availability of inhibitors specific for RET oncoproteins might provide new tools to highlight the physiologically and pathologically activated pathways involved. The tyrosine kinase inhibitors STI571, genistein, and allyl-geldanamycin selectively inhibited cell growth and RET tyrosine kinase activity of MTC cells *in vitro* in a dose manner (Cohen, M. S. et al., 2002). Carlomagno and colleagues (Carlomagno, F. et al., 2002) showed that the pyrazolo-pyrimidine PP1 blocks tumorigenesis induced by RET/PTC cytoplasmic oncoproteins by inhibiting RET enzymatic activity and its transforming effects. Carniti and coworkers (Carniti, C. et al., 2003) additionally demonstrated that along with the inhibition of tyrosine phosphorylation, PP1 induces proteosomal destruction of the activated membrane-bound RET receptors associated with MEN2 syndrome.

Another approach is to generate molecular mimetics directed toward specific mutations of the RET oncogene. The soluble ectodomain of RET carrying the C634Y-MEN2A substitution has been recently shown to inhibit *in vitro* the membrane-bound RET-MEN2A receptor by interfering with its dimerization and to compete with the wild-type RET for ligand binding (Cerchia, L. et al., 2003).

As another option for MTC treatment by gene therapeutic means, the transductional targeting of vectors to tumor cells might provide promising new alternatives in terms of selectivity and safety. The feasibility of efficient introduction of toxic transgenes into tumor cells by adenoviral (Ad) vectors would allow new aspects in tumor treatment, either alone or in combination with established therapeutic approaches. Infection of human MTC-derived cells with adenoviral-dominant-negative RET (Ad-dn-RET) were shown to inhibit oncogenic RET signaling and growth of MTC cells (Drosten, M. et al., 2002). Moreover, using transplanted human MTC cells as an *in vivo* MTC model in nude mice, adenoviral transduction of dn-RET was shown to be sufficient to achieve a strong inhibition of tumor growth accompanied by a significant increase in animal survival (Drosten, M. et al., 2003b). Modified vector capsid would allow lower

viral loads to be administered and thereby reducing virus-related toxicity (reviewed in Wickham, T. J., 2003). The development of selective peptides for different tumor cell types and their incorporation into the vector capsid demonstrate the eligibility of this approach (reviewed in Nicklin, S. A. et al., 2002).

Taken together, multiple possibilities have been and continue to be employed for improving gene therapy for inhibition of oncogenic RET signaling and, in general, for MTC. It is hard to judge whether gene therapy will totally replace surgical intervention as the primary treatment for MTC, especially after early-stage diagnosis of inherited disease. However, recurrent or metastatic disease is difficult to manage, and in these cases gene therapy may soon lead to an improvement in standard therapies. With some improvements in vector design in terms of safety and efficacy, gene therapy may soon help to overcome many obstacles in cancer therapy, especially with the emerging knowledge of molecular mechanisms of cancer (reviewed in Drosten, M. et al., 2003a).

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24. FROM GENES TO DECISIONS

EVOLVING VIEWS OF GENOTYPE-BASED MANAGEMENT IN MEN 2

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INTRODUCTION

The genomic revolution of the past 20 years has led to the identification of the genetic mechanisms that are responsible for a wide variety of human cancers, and has made a huge impact on our ability to recognize, diagnose and, more recently, treat patients with these diseases. Genetic diagnosis of heritable forms of cancer holds out the potential for presymptomatic detection in familial cases and raises the possibility of prophylactic treatments that would decrease morbidity and mortality and improve the quality of life for those affected. While the potential impact of these advances is huge, to date, our understanding of the underlying genetic events that contribute to the familial cancers has only allowed us to significantly modify our management of the disease in a few instances. The paradigm for such genetically based molecular management is the multiple endocrine neoplasia type 2 (MEN 2) syndromes.

Multiple endocrine neoplasia type 2 (MEN 2)

As described in previous chapters (1), MEN 2 is an inherited cancer syndrome characterized by medullary thyroid carcinoma (MTC) and its precursor lesion, C-cell hyperplasia. These phenotypes are clinically recognizable in >90% of all cases (2). Traditionally, MEN 2 has been divided into three disease subtypes, based on the presence of other associated phenotypes. The most common subtype, MEN 2A accounts for about 85% of MEN 2 cases. In addition to MTC, MEN 2A is characterized by pheochromocytoma (PC), tumours of the adrenal chromaffin cells, in about 50% of

cases, and hyperparathyroidism (HPT) in 15–30% of individuals. The most aggressive of the MEN 2 subtypes, MEN 2B, occurs in about 5% of cases and has a median age of tumour onset that is 10 years earlier than other forms of MEN 2 (<10 years) (3, 4). Approximately 50% of MEN 2B are *de novo* cases with no previous family history (5). As in MEN 2A, PC occurs in about 50% of individuals with MEN 2B but HPT is rare and patients also have a variety of other developmental anomalies such as buccal neuromas, marfanoid habitus, ganglioneuromas of the gut, and thickened corneal nerves (3, 6). The final disease subtype, familial MTC (FMTC) is characterized only by thyroid tumors and has no other associated anomalies. This disease form is the least aggressive MEN 2 subtype and may have a lower penetrance and later disease onset (7). As a result, FMTC families are frequently small and may be phenotypically quite difficult to distinguish from MEN 2A families in which cases of PC or HPT have not yet manifested. Because of this, stringent definitions have been suggested in which the diagnosis of FMTC requires a minimum of 4 (8, 9) or even 10 (10) family members with MTC in the absence of other phenotypes.

RET and the genetics of MEN 2

MEN 2 is inherited as an autosomal dominant disease and, as a result, all first-degree relatives of an affected individual are at 50% risk of inheriting the disease causing mutation. Although they differ in phenotype and aggressiveness, all three MEN 2 subtypes are caused by mutations of the *RET* (Rearranged in Transfection) oncogene (8, 9). *RET* encodes a cell surface receptor tyrosine kinase normally required for development of neuroendocrine cell types, the peripheral nervous system, and kidney (1, 11). *RET* mutations are identified in more than 95% of all MEN 2 families and there is no evidence of families in which the MEN 2 phenotype is not linked to *RET*. In each case, RET mutations are single amino acid substitutions that result in inappropriate activation of the RET receptor (12, 13). Mutations are clustered in "hot spots" in the extracellular domain (exons 10 and 11) or in the tyrosine kinase domain (exons 13–16) of the receptor (8, 9) (Figure 1).

Because >99% of mutations occur in only 10 codons of RET, direct DNA testing in MEN 2 is simple, widely available, and very efficient and is recommended for all at-risk individuals (Discussed below).

Although all MEN 2 subtypes are associated with *RET* mutations, specific mutations confer much higher risks for some phenotypes. For example, mutations of *RET* codon 634 are strongly correlated with HPT and PC and thus, not surprisingly, represent 85% of MEN 2A mutations (14, 15). In MEN 2A, mutations generally alter specific cysteine residues in the extracellular domain of RET (residues 609, 611, 618, 620, 634), resulting in a ligand-independent constitutively activated molecule (Figure 1). The mutations found in patients with FMTC have a broader range of functional effects, and may include both the same type of mutations as those seen in MEN 2A as well as mutations in the tyrosine kinase domain (residues 768, 804, 891) that appear to alter ATP binding (16). More than 95% of MEN 2B patients share the same amino acid substitution (Met918Thr) in the binding pocket of the RET kinase

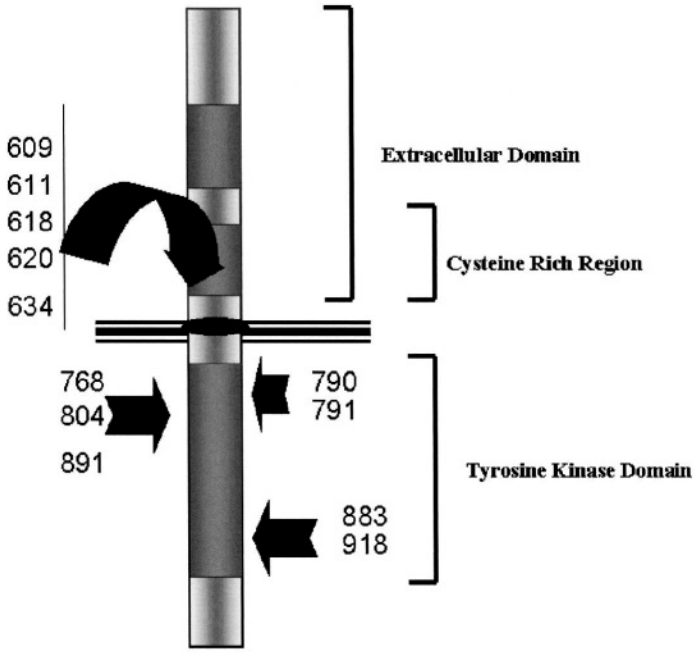

Figure 1. Schematic diagram of the RET receptor showing the relative positions of the more common mutations found in MEN 2.

domain (8, 9), although rare mutations of codon 883 are also detected (17, 18). Both mutations appear to alter the substrates of RET, thereby changing the downstream signals it sends (16) (Figure 1). The strong associations of specific mutations with each of the disease phenotypes can provide us with an additional tool for predicting patient prognosis and guiding management strategies (Discussed below).

In contrast to the activating *RET* mutations found in MEN 2, inactivating mutations of RET are identified in patients with Hirschsprung disease (HSCR), a congenital abnormality of gut innervation[19]. HSCR mutations are found throughout the *RET* gene and result in reduced levels of functional RET protein (20, 21). In rare cases, both the MEN 2 and HSCR phenotypes are associated with a single *RET* mutation (22, 23). These are generally single amino acid substitutions found in cysteine residues in the extracellular domain of RET (exon 10) (22). These oncogenic mutations may be as frequent as 1% in the HSCR population (24).

Diagnosis and prediction of MEN 2

Diagnosis in the "pre-genotyping" era

Before the identification of disease causing mutations in *RET,* MEN 2 disease status in at-risk individuals was established by biochemical screening. Generally, this involved

measurement of calcitonin peptide release by C-cells in response to a provocative agent, such as pentegastrin or calcium (25). Elevated levels of calcitonin indicated the presence of an increased number of C-cells (C-cell hyperplasia) or of MTC and these individuals would be offered prophylactic thyroidectomy. This strategy, while clinically important, had several drawbacks, not the least of which was that diagnosis was dependent on identification of early hyperplastic changes and frequently was not made until MTC or even metastatic disease was already present (26, 27). Further, a negative screen result did not indicate that a patient did not carry the MEN 2 disease mutation, only that they had no detectable disease at that time. Thus, repeated screening of all at-risk individuals was required annually, or at regular intervals, in order to detect all MEN 2 cases. As a result, 50% of at-risk individuals who had no MEN 2 mutation would be repeatedly and unnecessarily screened. Because every at-risk individual needed to be repeatedly tested, biochemical screening for MEN 2 was a relatively costly strategy. Further, after several negative tests compliance could be a significant issue. For those undergoing regular testing, borderline or difficult to interpret biochemical screening results occasionally resulted in unnecessary thyroidectomy in individuals who did not carry the MEN 2 mutation (28). The incidence of false positives may have been as high as 5–10% (10).

In individuals with a confirmed diagnosis of MEN 2 additional screening for other associated phenotypes such as PC and HPT was required. Patients would be screened for PC on a regular basis by measurement of plasma metanephrines or levels of catecholamines or metanephrines in 24 h urine collection (29). A positive screen would lead to unilateral or, if necessary bilateral adrenalectomy. In general, prophylactic adrenalectomy would not be recommended due to risks from adrenal insufficiency (30). HPT is rarely symptomatic in MEN 2 but can be detected by measurement of calcium or parathyroid hormone levels. Biochemical screening for PC and HPT is recommended annually for individuals diagnosed with MEN 2.

MEN 2 and *RET* mutation testing

The identification of *RET* mutations as the underlying cause of MEN 2 has changed the management of the individual and family with MEN 2 significantly. Genetic testing, scanning the *RET* codons known to be frequently mutated, is now the preferred method for confirming the clinical diagnosis of MEN 2 and is considered the standard of good practice (9, 10, 31). The rate of false negative (2–5%) (10) and false positive (<0.1%) results in DNA testing is a dramatic improvement over biochemical screening methods. Individuals at-risk for MEN 2 should now be screened at birth or at the earliest possible time for *RET* mutations and the results should provide the basis for recommending thyroidectomy. Prophylactic thyroidectomy for individuals carrying a germline *RET* mutation has dramatically reduced morbidity and mortality due to MTC, and perceived quality of life is much better in individuals at risk for MTC than for those at risk of other cancers where diagnosis and management are less clear cut (32).

Because the basis and expectations associated with genetic testing need to be clearly understood in order for at-risk individuals to understand the implications of a *RET* mutation test and for them to use that information to make informed decisions about disease management, it is essential that all DNA testing be accompanied by appropriate genetic counseling. This would generally take the form of pretest counseling to explain the implications and risks of the test and one or more post-test counseling sessions involving delivery of results and discussion of their implications as necessary.

INDICATIONS FOR *RET* MUTATION TESTING. Screening for *RET* mutations is now the basis of all management strategies for MEN 2, replacing reliance on biochemical screening. In families where the disease causing mutation has already been established by previous screening of affected individuals, all at-risk individuals should be genetically screened for the familial mutation. Individuals who do not carry this mutation are not at risk of MEN 2 and can be excluded from further testing. Individuals with the mutation are at high risk for MEN 2 phenotypes and should be managed accordingly. If diagnosis is made in a child, thyroidectomy should be performed before age 5 for MEN 2A and FMTC families and before age 6 months in individuals with MEN 2B mutations which are associated with earlier tumour development (10).

Early, genetically based identification of mutation carriers permits surgery before the usual onset of malignant disease and carries the optimal possibility of preventing metastatic disease. Older patients diagnosed with *RET* mutations will be offered thyroidectomy as soon as possible accompanied by biochemical monitoring for the presence of metastatic disease. Mutation positive individuals will be monitored throughout life for other tumour types or anomalies associated with their specific MEN 2 subtype, as described above. The regime for these screening protocols may, in theory, be modified based on the occurrence and age of onset of these phenotypes in other family members. However, variability in these, even within a single family, suggests that caution should be used when relaxing screening protocols (33, 34).

If an individual represents a new case/family diagnosed for MEN 2, *RET* mutation screening should be performed in a known affected family member. This also holds true for all individuals diagnosed with apparently sporadic MTC, as 1–7% of these have germline *RET* mutations and represent new MEN 2 families (35). Interestingly, early studies had suggested that germline *RET* mutations were very rare in sporadic PC but recent studies showing they may occur in up to 5% of cases (36) have suggested that all patients with these tumours should also be screened for *RET* mutations (10). Initial screening must include *RET* exons in which mutations are most frequently found, (exons 10, 11, 13–16) however these may be prioritized based on the patient phenotype. For example, 85% of MEN 2A families have mutations in exon 11 and almost 95% of MEN 2B cases have mutations of codon 918 in exon 16. If mutations of these exons are not identified a broader screen, including all *RET* exons may be necessary. Once a *RET* mutation is identified, all other at-risk family members may be

screened specifically for that change and individuals carrying the mutation are managed as described above.

In a few instances, a *RET* mutation may not be identified in a putative MEN 2 individual. These cases are rare and frequently, although not always, involve families that are quite small with few or a single affected individual. In the past, some of these families may have resulted from conservative diagnosis of FMTC in families that did not have clear features of MEN 2. For example, studies have shown that up to 5% of the population may have C-cell hyperplasia but do not have *RET* mutations nor a true MEN 2 phenotype (28). Recent studies suggest that at least some of these cases may be related to mutations of the succinate dehydrogenase subunit D gene (chromosome 11q23) and not to *RET* (37).

This highlights the necessity of accurate and unambiguous clinical diagnosis in cases where *RET* mutations have not been identified. If families with clearly defined MEN 2 but no *RET* mutation have sufficient confirmed affected family members available, linkage analysis using polymorphic sites in or near the *RET* gene (chromosome 10q11.2) may provide an alternative to direct mutation detection. In this method, a haplotype for a series of polymorphisms over the region of *RET* which presumably includes a disease mutation may be constructed based on the genotype of multiple affected family members (38–41). Inheritance of this "disease haplotype" can be used to identify individuals who have also inherited the predicted *RET* mutation and are therefore assumed to be MEN 2 carriers. This method is less amenable to diagnosis than is direct mutation testing since it is dependent on the availability of a suitable family structure, the participation of multiple family members and relies on the assumption that the clinical diagnosis is correct in suggesting MEN 2.

Where suitable family members are not available, or a haplotype cannot be constructed, MEN 2 families without *RET* mutations will be treated as they were before the advent of DNA mutation testing, using repeated biochemical screening of all at-risk individuals and offering surgery at the first sign of a positive test result.

WHO SHOULD BE OFFERED SCREENING? *RET* mutation screening is considered the standard of care for all individuals at-risk for MEN 2, irrespective of their age. Ideally, at-risk individuals in known MEN 2 families would be screened for *RET* mutations at birth or shortly thereafter. This is somewhat different from mutation testing in many other cancer syndromes where minors are not automatically screened. In the case of MEN 2, the penetrance of the disease is very high (>90% have clinically detectable disease) and onset is very young, the earliest recognition of MEN 2B being reported in children under age 5 (24, 42, 43) necessitating very early detection to allow presymptomatic intervention. Further, unlike many other cancer syndromes of childhood, there are clear, well tolerated, prophylactic options available which are demonstrably valuable in decreasing the burden of the disease in affected individuals. The advent of early childhood mutation detection has greatly reduced the incidence of MTC in families with known MEN 2 and will, with time, virtually eliminate the morbidity and mortality associated with metastatic disease in known MEN 2 families.

RET mutation screening is also recommended for individuals with sporadic MTC and also PC, although there has been no indications that *RET* mutations contribute to sporadic HPT (44). In each case, it is important to remember that *RET* mutations can occur somatically in these tumours (45–49) and that these should not be confused with germline MEN 2-*RET* mutations.

Approximately 1% of individuals with HSCR have a *RET* mutation in exon 10 which may also confer risk for MEN 2 phenotypes (24). While this may seem rare, *RET* mutations in HSCR are widely distributed throughout the gene and exon 10 mutations, primarily in codons 609, 618 and 620, represent one of the largest clusterings of mutations. Because of the oncogenic risk associated with these mutations, RET exon 10 mutation screening is recommended for all children diagnosed with HSCR. Individuals identified with any of these mutations should be treated as a potential MEN 2 case and screening of at-risk family members and surgical intervention should be offered.

Diagnosis and management: The evolving genetic contribution

While the advent of genetic testing for *RET* mutations has significantly improved our ability to manage patients and families with MEN 2, the potential exists for additional refinement that may improve the prospects even further. Several exciting options for this refinement are currently being evaluated and the accumulating body of experience with *RET* genotype and phenotype are allowing us to improve the accuracy of disease prediction.

Genotype-phenotype associations in genetically based management

Our 10 year experience of *RET* mutation and MEN 2 disease phenotype correlation has shown us that not all *RET* mutations carry equal associated risk and that the traditional definitions of disease phenotype may in future not be as useful to us as genetically or mutation based disease risk estimates.

We have long known that some *RET* mutations conferred higher risk of PC (e.g. Cys634Arg or Met918Thr) (8, 9) while others are associated with a less aggressive disease phenotype (e.g. Glu768Arg or Val804Met). Recent studies have begun to investigate the potential of using specific *RET* mutation data to guide management strategies. Three general categories of high, medium, and low risk *RET* mutations may be defined based on their relative penetrance, the specific disease phenotypes associated with them, and the aggressiveness of these phenotypes (Table 1) (10, 31). Mutations in the highest disease risk group include those found in MEN 2B (Met 918Thr, Ala883Phe). The lowest risk mutations are those found most frequently in FMTC and those associated with later disease onset (Table 1) (50).

These risk groupings are an attractive tool to supplement clinical diagnosis for defining individuals with high risk who need early thyroidectomy and/or stringent biochemical screening regimes. By defining a subgroup of higher risk patients genetically predisposed to more, or more severe, disease phenotypes we may be able to focus health care resources more effectively and reduce patient stress associated with disease management. In future, the specific *RET* mutation found in a patient is likely, to act

Table 1. Relative risk groups in MEN 2 associated with specific *RET* mutations

Relative risk	Mutant RET codons	Disease phenotype	MTC characteristics	Recommended MTC management
High	918, 883	MEN 2B	Earliest onset Aggressive tumours	Thyroidectomy < 6 months
Moderate	611, 618, 620, 634	FMTC, MEN 2A	Early onset	Thyroidectomy <3 years
Lowest	609, 768, 804, 891	FMTC, MEN 2A	Later onset, relatively indolent	Thyroidectomy <3 years[1]

[1]Management of this risk group remains controversial (10). In some centers, later surgery is recommended based on the age of onset seen in other family members.

as a guide to determine the frequency of screening required and the age at which surgery should be offered to mutation carriers. We may, for example, be able to reduce the frequency of screening for PC in individuals with mutations rarely associated with PC (e.g. Glu768Arg or Val804Met), or use a more relaxed age for thyroidectomy in patients with mutations and a family history consistent with later onset tumours. Such genotype-based management must be used with caution however, as there is still some variability in clinical presentation of MEN 2, even in families/individuals carrying the identical *RET* mutation. Aggressive, early onset cases can occur in families with mutations that have elsewhere been shown to be quite indolent (33, 34). It is clear that genotype-based management strategies will still rely heavily on the clinical diagnosis and the specific pattern of the disease within a family to optimize a program of surgery and biochemical screening appropriate to the specific genotype and phenotype of each patient.

Disease associated RET haplotypes in sporadic MTC and PC

The genotype at the *RET* locus may, in future, also provide us with some predictive information on the occurrence of sporadic tumours. The majority of MTC and PC (>75%) occur sporadically, without any associated family history (36, 51). However, recent studies have suggested that some haplotypes for polymorphic variants surrounding the *RET* locus are associated with increased risk for these sporadic tumours (39, 52). These risk haplotypes may reflect a combination of variants that themselves confer risk or may be a simple marker for another risk contributing gene lying upstream of *RET* and not yet identified. In the future, this information may be useful in predicting individuals at risk for sporadic tumours or in establishing the risk of recurrence in family members of individuals with sporadic disease.

SUMMARY CONCLUSIONS

Diagnosis and management of MEN 2 has evolved considerably since the identification of the underlying disease mutations in the *RET* proto-oncogene. Presymptomatic detection and prophylactic surgical intervention are now the accepted standard of care. The strong correlation of disease phenotype and mutation genotype has already also

allowed us to develop mutation-guided management strategies to optimize time of intervention and schedule follow-up and management. As our understanding of the depth of these correlations increases we look forward to better refining our management regimes to fit both the best care requirements and the quality of life needs of the MEN 2 patient.

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