How Familial Cancer Genes and Environmentally Induced Oncogenes Have Changed the Endocrine Landscape

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The gene responsible for MEN-2, the *ret* proto-oncogene, has elucidated mechanisms of endocrine tumorigenesis. Activating mutations of this transmembrane tyrosine kinase receptor represent the first known example of an inherited oncogene. This knowledge has altered our approach to affected patients by allowing *in utero* screening and prophylactic thyroidectomy rather than provocative testing and morphologic analysis of C cell hyperplasia—will it result in eradication of medullary carcinoma of thyroid? The lessons from Chernobyl taught us how radiation can induce chromosomal rearrangements that involve the same gene. This has led to a better understanding of papillary thyroid carcinoma and provides a novel immunohistochemical marker that widens our diagnostic armamentarium.

KEY WORDS: Thyroid, Ret proto-oncogene, Medullary carcinoma, Ret rearrangements, Papillary thyroid carcinoma.

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Molecular biology has had a significant impact on the practice of endocrine pathology. This article will focus on various genetic alterations involving a single gene and a single tissue to illustrate the “bench-to-bedside” application of molecular diagnostics.

Medullary Thyroid Carcinoma is Frequently a Familial Disorder

Medullary thyroid carcinoma (MTC) may have a familial predisposition that falls into one of three types: familial medullary thyroid carcinoma alone (FMTC), multiple endocrine neoplasia (MEN) type

2A in which MTC is associated with pheochromocytomas, and MEN 2B in which the thyroid and adrenal disorders are associated with mucosal ganglioneuromas and a Marfanoid habitus. Identification of carrier status for individuals in families with predisposition is important to prevent the potentially lethal outcome of MTC. Familial MTC is often multicentric and arises from underlying C-cell hyperplasia (1). This led to the use of C-cell hyperplasia to identify familial cases; apparently sporadic tumors can represent index patients, and the protocol for examination of a thyroid with MTC included an evaluation of underlying C-cell hyperplasia. Many definitions of C-cell hyperplasia have been offered, all requiring immunohistochemistry because C cells cannot be reliably recognized with routine histologic stains. Quantitation of C cells and geographic mapping throughout the gland must be performed (1, 2). C cells are usually limited to the junction between the upper and middle thirds of the lateral lobes where they are distributed singly rather than in clusters. Increased numbers of C cells (more than seven cells per cluster), complete follicles surrounded by C cells, and distribution of cells beyond this geographic location are indicative of C-cell hyperplasia (Fig. 1). Pathologists devoted many hours to the meticulous morphometric evaluation of calcitonin-stained tissue sections. Inevitably, there were borderline specimens (3). False positive samples also resulted, because C-cell hyperplasia also can be associated with chronic hypercalcemia, follicular nodular disease, and thyroiditis (4–7).

Members of kindreds afflicted by FMTC or MEN-2 were required to undergo repeated testing to determine carrier status. This entailed serum calcitonin measurements and annual pentagastrin stimulation testing to identify early any rise in circulating hormone levels or any abnormal response that would herald the onset of disease. Pathologists were asked to evaluate thyroidectomy specimens to confirm the presence of C-cell hyperplasia in those

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FIGURE 1. A patient with familial medullary carcinoma of thyroid usually has C cell hyperplasia in the contralateral lobe. **A,** Sometimes the hyperplastic foci are obvious and the diagnosis of a familial disorder is readily proven. **B,** In some patients, C cells are numerous, but the criteria for a diagnosis of hyperplasia are difficult to prove.

who had abnormal biochemical tests. Again, the structural features did not always allow unequivocal identification of carriers of the disorder, nor was it possible to provide verification of normal status using this technology. Moreover, there were always examples of patients whose biochemistry only provided evidence after the disease had spread.

Familial MTC and MEN-2 Are Associated with Mutations of the Ret Proto-Oncogene

Using linkage analysis, the inheritance of all three syndromes was mapped to the pericentromeric region of chromosome 10 (8–11). The disease exhib-

ited an autosomal dominant inheritance pattern with age-related complete penetrance, suggesting inheritance of an activated oncogene. In seemingly unrelated work, a chimeric oncogene was found to induce classical NIH 3T3 transformation; the gene involved on chromosome 10 was named “RET” because it was “REarranged during Transfection” (12). This gene, with 20 exons in over 60 Kb of genomic DNA, encodes a transmembrane receptor tyrosine kinase (Fig. 2) that has an extracellular ligand-binding domain with homology to the cadherin genes, a cysteine-rich extracellular region that has been implicated in protein conformation and dimerization, and an intracellular split kinase (13). In 1993, mutations of the *ret* proto-oncogene were shown to segregate with disease in patients with FMTC or MEN 2A (14) and subsequently with MEN 2B (15–17), confirming the role of this gene in the development of MTC in these familial disorders. Highly specific and characteristic missense mutations in three exons of the *ret* proto-oncogene in patients with FMTC or MEN2 (14, 15) are present in affected members of families but not in individuals not at risk of the disease. The data indicate that mutations of *ret* are the underlying cause of FMTC and MEN2, providing the first example of a syndrome of inherited oncogene activation.

The function of *ret* was not clear and therefore its role in the pathogenesis of this disease remained to be proven. *Ret* expression was found to be specific to cell lineages derived from neural crest, including migrating neural crest cells, the ventral neural tube, autonomic, enteric and sensory ganglia, and thyroid C cells (18). As expected, *ret* was expressed in MTC as well as in pheochromocytomas and gangli-
oneuromas (18, 19). This pattern of expression was consistent with the disease patterns, but unexpectedly, *ret* was also found to be expressed in ureteric bud and kidney (20). A mouse model harboring a disrupted *ret* gene (*ret* “knockout” mice, *ret* $-/-$) clarified the significance of these expression data;

FIGURE 2. Schematic illustration of the *ret* proto-oncogene and its activating mutations in multiple endocrine neoplasia type 2 (MEN-2) and familial medullary thyroid carcinoma (FMTC). In MEN-2A and FMTC the mutations are usually in the extracellular cysteine-rich region encoded by exons 10 and 11; in MEN 2B there is usually an activating mutation of the kinase domain at codon 918.

the homozygous animals that lacked functional ret failed to develop an enteric nervous system and kidneys (21). This led to the recognition that inactivating mutations of ret can be implicated in some forms of Hirschsprung's disease (22, 23).

The structure of ret implicated this protein as a transmembrane receptor, but it remained an orphan receptor with no known ligand until a knockout mouse model deficient in glial-derived neurotrophic factor (GDNF) was found to exhibit a phenotype identical to that of ret $-/-$ mice (21). This led to recognition that GDNF is a ligand for ret (24, 25). It is now known that GDNF and ret form a trimeric complex with a GDNF receptor (known as GDNFR α or RETL1) that is membrane-anchored but has no signaling capacity, resulting in signaling through ret (26, 27). Another novel cell surface protein, RETL2, has been shown to mediate GDNF-dependent ret signaling (28). Thus, inactivation of GDNF or its receptors provide additional mechanisms for the development of Hirschsprung's disease.

Patients with FMTC or MEN 2A have highly specific missense mutations in the extracellular cysteine-rich domain of the receptor, mainly in exons 10 and 11; rare examples of mutations in exon 13, 14 and 15 have been described. The most common changes affect five codons for cysteine, codons 609, 611, 618 and 620 in exon 10 and codon 634 in exon 11 (Fig. 2). Targeting of these mutant forms of ret to C cells in transgenic mice results in the development of MTC (29). It has been shown that replacing cysteine with any of several other amino acids increases formation of active ret dimers, explaining the oncogenic potential of these mutations (30). Moreover, patients with MEN 2A mainly have mutations affecting codon 634, and those with a Cys634 to Arg substitution have a greater risk of developing parathyroid disease (31). These data suggest that simple constitutive activation is insufficient to develop a phenotype in tissues other than thyroid C cells, and that tissue-specific factors have an impact on the outcome of ret activation in parathyroid and adrenal. It remains to be shown whether the receptors RETL1 or RETL2 play a role in this mechanism. Moreover, the importance of tissue-specific modifiers is exemplified by the occasional "ret inactivation" phenotype seen in some examples of activating mutations, such as Hirschsprung's disease associated with MEN 2A (32) and renal hypoplasia in mice with MEN 2A activating mutations (33).

In contrast to the wide variety of mutations associated with FMTC and MEN 2A, patients with MEN 2B have a characteristic mutation of codon 918 in exon 16. This genetic alteration replaces a methionine (ATG) with a threonine (ACG) (15, 16). This mutation in the kinase region alters the substrate

specificity of the kinase, resulting in tyrosine autophosphorylation on new sites as well as phosphorylation of new downstream targets. Activated wild-type ret binds Shc, Grb-2, PLC- γ Crk and Nck; in contrast, the mutant form phosphorylates proteins that predominantly bind Crk, Nck, including the cytoskeletal protein Paxillin (34). A number of *de novo* cases of MEN 2B have been reported with this mutation, and the same mutation is frequent in sporadic MTC (16, 35) countering the proposed common ancestral origin theory of this highly specific mutation.

Genetic Screening Is Indicated to Prevent MTC

Mutational analysis now offers an accurate marker of predisposition to the various diseases (36, 37). The screening approach most widely applied involves direct sequencing of exons 10 and 11 from PCR-amplified products for MEN 2A (38, 39) and the simple and more rapid technique of restriction endonuclease digestion for MEN 2B (40). The lethal component of the three syndromes is, indeed, MTC, and prevention of this disorder significantly improves prognosis. Current recommendations suggest that family members of kindreds have genetic screening early in life and affected members should undergo total thyroidectomy at around the age of 5 years (41). This age was chosen because of the early onset of MTC in these familial forms of the disease; metastatic tumor has been found in patients as young as 6 years of age.

Sporadic medullary carcinomas also may have mutations of ret in the same codons as the familial disorders (15, 42); the mutation involved may have prognostic value (43). The presence of ret mutations in sporadic tumors indicates the importance of analyzing DNA from white blood cells to establish that a mutation is not simply somatic but rather involves the germ-line and is therefore potentially hereditary.

In this disorder, unlike other familial cancer syndromes that result from inactivation of tumor suppressor genes, each affected member is born with an activated oncogene. The current trend of using molecular diagnosis to identify carriers of this genetic, autosomal dominant disorder has led to the recommendation of prophylactic thyroidectomy in childhood; this will almost certainly drastically improve the prognosis of the familial forms of this malignancy.

Ret is also Implicated in Papillary Thyroid Carcinoma

The identification of the ret proto-oncogene was rapidly followed by the identification of a novel

tumor-specific rearrangement of this gene in papillary thyroid carcinoma (44), long before it was recognized as the gene for MEN-2. There are now at least five members of this family of gene rearrangements that are specific to papillary carcinoma. These ret/PTC oncogenes 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 proto-oncogene (45–50) (Fig. 3). The rearrangements are common in radiation induced tumors (50–54). Ret/PTC-3 has been analyzed to prove the role of radiation in chromosomal breakage and illegitimate recombination (55). Sporadic papillary carcinomas also harbor ret/PTC rearrangements, most commonly ret/PTC-1, followed by ret/PTC-3 and occasionally ret/PTC-2; the etiology of these rearrangements in sporadic papillary carcinomas is not known (56–59).

Ret/PTC Gene Rearrangements Are True Oncogenes

The tumorigenic role of ret/PTC gene rearrangements was shown using classical NIH 3T3 transformation assays. Moreover, transgenic mouse models have proven the tumorigenicity of these fusion proteins (60–62). However, these animal models have utilized the thyroglobulin gene promoter to drive expression at very high levels.

Mechanisms of Ret/PTC Oncogenesis in Thyroid Follicular Epithelium

The rearrangements result in translocation of the fusion protein to the cytoplasm and constitutive tyrosine kinase activation (63). Western blotting identifies dimers of ret/PTC-1, providing a mechanism of autophosphorylation and kinase activation,

FIGURE 3. The three most common ret/PTC gene rearrangements are shown. The top portion represents the intact ret receptor, with an extracellular region (EC), a transmembrane domain (TM) and a tyrosine kinase domain (TK). The rearrangements involve a breakpoint at amino acid 713 and encode the distal portion of ret including the tyrosine kinase. The upstream portions are regulated by the promoters of other genes, H4, R1α and ele, and encode fusion proteins as shown.

and the dimerization has been attributed to the leucine zipper region of H4 in ret/PTC-1 (64). Expression of ret/PTC-1 has been shown to impair expression of a differentiated phenotype in a rat thyroid epithelial cell line; this has been attributed to down-regulation of mRNA and protein of the thyroid transcription factor Pax-8 and to altered function of the thyroid transcription factor TTF-1 (65). More importantly, from a diagnostic perspective, ret/PTC-1 alters the nuclear envelope and chromatin structure to account for nuclear features of papillary carcinoma in a ras independent fashion (66).

Ret/PTC-2 involves the Type Iα regulatory subunit of cAMP-dependent protein kinase (RIα). In this rearrangement, dimerization requires the N terminus of RIα and results in activation sites that bind SH2 domains of PLCγ and Grb10; however, these interactions are not required for mitogenesis induced by this rearrangement (67). In contrast, another binding site involving Tyr 586 outside the tyrosine kinase domain interacts with the second of three LIM domains of *Enigma* and was also identified as a phosphorylation-independent interaction site. *Enigma*, a 455-residue protein, was discovered because of its interaction with the insulin receptor through its C-terminal LIM domain. The interaction with *Enigma* has been shown to be responsible for mitogenic signaling by ret/PTC-2.

Ret/PTC Gene Rearrangements in Human Papillary Thyroid Carcinoma

Ret/PTC gene rearrangement appears to be an early event in tumor development, because the rearrangements have been identified with highest prevalence in occult papillary microcarcinomas (68, 69). The data presented above indicate that ret/PTC rearrangements are indeed transforming *in vitro* and tumorigenic *in vivo*, but the identification of these gene rearrangements in microscopic carcinomas that are not clinically relevant indicates that they are not potent carcinogens and it is likely that other genetic events are required to promote aggressive tumor growth.

The true incidence of ret/PTC gene rearrangements in clinically significant papillary carcinomas remains controversial and unknown (58), largely because DNA analysis has not proven reproducible and RNA analysis has yielded evidence of variable levels of expression that may require higher sensitivity than previously thought (69). Although early reports identified a frequency of rearrangements as low as 2.5%, most recent reports confirm that ret/PTC expression is identified in approximately half of these tumors (69).

Ret/PTC Expression May Be A Prognostic Marker in Papillary Thyroid Carcinoma

These rearrangements have been implicated as predictive of aggressive behavior in pediatric radiation-induced papillary carcinomas after the Chernobyl disaster (70). In patients with radiation-induced solid variant papillary carcinoma, the prevalence of ret/PTC-3 is unusually high, suggesting a possible correlation between this rearrangement and a distinct phenotype (71).

In sporadic papillary carcinomas the levels of expression of the rearranged genes are highly variable (69). It has been suggested that high levels of expression may predict a more aggressive course of lymphatic dissemination (57), specifically in young patients with no other adverse clinical or morphologic parameters (58). The rearrangements are not expressed, however, in the most aggressive papillary carcinomas, such as the tall cell variant or papillary carcinomas with insular dedifferentiation (58). It is therefore recognized that ret/PTC oncogene activation defines a subset of papillary thyroid carcinomas that lack evidence of progression to poorly differentiated or undifferentiated phenotypes (72, 73).

Ret Expression Is A Diagnostic Marker in Papillary Thyroid Carcinoma

Under normal circumstances, ret is expressed in thyroid C cells and is activated by point mutations in disorders involving C cells. Ret is not expressed in normal thyroid follicular epithelium. However, when involved in the gene rearrangements known as ret/PTC-1 through -5, that are unique to papillary thyroid carcinoma, the product of the various rearrangements can be identified by immunohistochemical staining for ret (69). The commercial antisera to ret are directed against the carboxy terminus of the protein and this portion of ret is included in the fusion proteins that result from ret/PTC gene rearrangements. Therefore application of this method can identify the expression of a ret/PTC gene rearrangement (Fig. 4). Although RT-PCR for ret/PTC mRNA may be more sensitive, immunostaining is a practical approach to identify tumors that harbor these rearrangements.

Immunohistochemical staining allows rapid and clinically useful detection of this marker of papillary carcinoma, which is present in up to 80% of occult papillary microcarcinomas and approximately 50% of clinically detected lesions (69). This genetic alteration provides a marker of papillary carcinoma that has practical application to distinguish borderline lesions that can mimic follicular adenoma. Staining is totally negative in normal follicular epithelial cells. It should be noted, however, that occasional thyroids with Hashimoto's thyroiditis have been reported to harbor

FIGURE 4. The histologic criteria for the diagnosis of papillary can be controversial, as in the tumor shown in (A). Ret immunohistochemistry identifies the C terminus of the products of ret/PTC gene rearrangements (see Fig. 3) and allows identification of tumors that harbor these molecular markers of papillary carcinoma.

ret/PTC gene rearrangements in the absence of documented malignancy (69, 74).

Ret/PTC has Implications for Multifocal Papillary Thyroid Carcinoma

Papillary carcinomas may be multifocal; this has been interpreted as reflective of intraglandular lymphatic dissemination and has been used as one rationale for total thyroidectomy in patients with low risk disease. The finding of an occult papillary microcarcinoma in the opposite lobe of a patient with clinical papillary carcinoma may precipitate more aggressive therapy. However, occult papillary microcarcinomas are common in autopsy thyroids and are identified in up to 24% of the population (75). The opposing view, therefore, holds that these represent incidental findings of no clinical significance. The detection of different ret/PTC rearrangements (*e.g.*, ret/PTC-1 *versus* ret/PTC-3) in multifocal lesions, even in the majority of patients with clinically significant papillary carcinomas (69), supports the interpretation that these represent multifocal primary lesions rather than intrathyroidal dissemination in most patients.

Ret/PTC has Implications for Hürthle Cell Thyroid Carcinoma

Hürthle cell tumors are generally classified either as a separate and unique category of thyroid neoplasms

(76) or as subtypes of follicular lesions (77, 78). In either case, they are classified as benign Hürthle cell adenomas or malignant Hürthle cell carcinomas. These two entities are distinguished based on the identification of capsular or vascular invasion or on the presence of metastatic disease (76–78). However, they remain controversial because of their sometimes unexpected behavior (77, 79, 80).

Several papers have described papillary carcinomas composed of Hürthle cells (81–85). These lesions all have papillary architecture. However, the concept of papillary carcinoma has been expanded to identify well-delineated lesions with follicular architecture and characteristic nuclear features, including elongation, hypochromasia with peripheral margination of chromatin, micronucleoli, and irregular contours with grooves and cytoplasmic pseudoinclusions (76, 78). One might expect that there are also Hürthle cell tumors that would be classified as Hürthle cell adenoma but are truly follicular variant papillary carcinomas composed of Hürthle cells; however, the nuclear hyperchromasia of Hürthle cells may mask the features on which the diagnosis would rely. Using *ret*/PTC gene rearrangements as a marker of papillary differentiation, it has been shown that such tumors do exist and that tumors with *ret*/PTC gene rearrangements tend to have lymph node metastases (86). This molecular marker is a better predictor of this biologic behavior than morphologic identification of invasion. Therefore the use of *ret*/PTC as a diagnostic marker supports subclassification of Hürthle cell tumors into three groups: Hürthle cell adenoma, Hürthle cell carcinoma, and Hürthle cell PTC.

CONCLUSION

The use of molecular diagnostics has changed the endocrine landscape. The Pathologist is seeing less examples of medullary carcinoma as we diagnose familial cases by genetic screening and apply prophylactic thyroidectomy to prevent this disease. We no longer need to analyze thyroids for C cell hyperplasia to determine whether a patient is a carrier of genetic predisposition.

The applications of molecular diagnostics can make the diagnosis of borderline lesions of thyroid more scientific: tumors with *ret*/PTC gene rearrangements can be classified as papillary carcinoma if they harbor such a genetic abnormality. This technology will have implications for future diagnosis. This applies to surgical pathology (87, 88) and to the preoperative evaluation of thyroid aspirates (89). Moreover, the identification of tumor-specific gene rearrangements may have application to the follow-up care of patients with thyroid cancer.

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