**Minireview: RET/PTC Rearrangements and BRAF Mutations in Thyroid Tumorigenesis**

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Thyroid papillary carcinoma is the most common type of endocrine cancer. It is frequently associated with genetic alterations leading to activation of the MAPK signaling pathway. The two most frequently affected genes, BRAF and RET, are activated by either point mutation or as a result of chromosomal rearrangement. These mutations are tumorigenic in thyroid follicular cells and correlate with specific phenotypic features and biological properties of papillary carcinomas, including tumor aggressiveness and response to radioiodine therapy. Molecular inhibitors that block RET/PTC or BRAF kinase activity have shown substantial therapeutic effects in the experimental systems and are currently being tested in clinical trials. (*Endocrinology* 148: 936–941, 2007)

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**Thyroid Cancer is the most common malignancy of the endocrine system and accounts for approximately 1% of all newly diagnosed cancer cases (1). The most frequent type of thyroid malignancy is papillary carcinoma, which constitutes more than 80% of all cases. Recent years have been marked by dramatic expansion in the understanding of the molecular basis of thyroid carcinogenesis. It has become apparent that thyroid tumors, especially those of the papillary type, frequently have genetic alterations leading to activation of the MAPK signaling pathway. This crucial intracellular cascade regulates cell growth, differentiation, and survival in response to growth factors, hormones, and cytokines that interact with receptor tyrosine kinases present on the cell surface (Fig. 1) (2). Molecular alterations found in papillary carcinomas involve genes coding for the receptor tyrosine kinases, RET and NTRK1, and two intracellular effectors of the MAPK pathway, a GTP-binding protein RAS and a serine-threonine kinase BRAF. Mutation of one of these genes can be found in more than 70% of papillary carcinomas and they rarely overlap in the same tumor, suggesting that activation of this signaling pathway is essential for tumor initiation and alteration of a single effector of the pathway is sufficient for cell transformation (3–5). BRAF and RET are two most frequently affected genes and will be a focus of this review.

**RET/PTC**

Activation of the RET gene via chromosomal rearrangement has been known for almost two decades as one of the most common molecular events in papillary carcinoma (6, 7). The RET protooncogene resides on chromosome 10q11.2 and codes for a cell membrane receptor tyrosine kinase (8, 9). It contains three functional domains: an extracellular ligand-binding domain, a hydrophobic transmembrane domain, and an intracellular tyrosine kinase (TK) domain. The ligands of the RET receptor are growth factors belonging to the glial cell line-derived neutrophil factor family (10). Binding of the ligand causes receptor dimerization, autophosphorylation of tyrosine residues within the intracellular domain, and activation of the signaling cascade.

In the thyroid gland, RET is expressed at high level in parafollicular C-cells but not follicular cells, in which it can be activated by chromosomal rearrangement, resulting in the fusion of the 3' portion of the RET gene to the 5' portion of several unrelated genes, known as RET/PTC rearrangement (6, 7). At least 11 types of RET/PTC have been reported to date, formed by the RET fusion to different partners (11, 12). The partner genes share some common characteristics: they are expressed in thyroid follicular cells and therefore provide an active promoter for the expression of RET TK domain, and they contribute dimerization domains (typically one or more coiled-coil domains) that are essential for dimerization and ligand-independent activation of the truncated RET protein. Virtually all breakpoints in the RET gene occur within intron 11, leaving intact the TK domain of the receptor and enabling the RET/PTC oncprotein to bind SHC via Y1062 and activate the RAS-RAF-MAPK cascade (13). The two most common rearrangement types are RET/PTC1 and RET/PTC3, which account for the vast majority of all rearrangements found in papillary carcinomas. RET/PTC1 is formed by fusion with the H4 (D10S170) gene (7) and RET/PTC3 by fusion with the NCOA4 (ELE1, RFG, or ARA70) gene (14, 15). RET/PTC1 and RET/PTC3 are intrachromosomal paracentric inversions because both genes participating in the fusion are located on chromosome 10q (16, 17). In contrast, RET/PTC2 and other rare types of RET/PTC are interchromosomal translocations (reviewed in Refs. 18, 19).

RET/PTC is tumorigenic in thyroid follicular cells; it transforms thyroid cells in culture (20) and gives rise to thyroid carcinomas in transgenic mice (21–23). Transgenic animals with targeted thyroid expression of RET/PTC1 and RET/
PTC3 develop thyroid tumors with microscopic features recapitulating those of human papillary carcinomas. Activation of RET/PTC in cultured thyroid cells results in down-regulation of expression of thyroid-specific gene, such as thyroglobulin and sodium iodide symporter, and cell de-differentiation (24, 25). Recent findings suggest that these effects of RET/PTC activation require signaling along the MAPK pathway (13, 26) and, more specifically, the presence of the functional BRAF kinase (26, 27). Indeed, BRAF silencing in cultured thyroid cells reverses the RET/PTC-induced effects such as ERK phosphorylation, inhibition of thyroid-specific gene expression, and increased cell proliferation (26, 27). However, signaling from the wild-type RET receptor and its truncated RET/PTC forms is also known to activate a number of other pathways, particularly the phosphatidylinositol-3 kinase/AKT pathway, which may also contribute to its biological effects (28–30).

In most studies, RET/PTC is found in 20–40% of adult sporadic papillary carcinomas, although its prevalence is highly variable between different observations (reviewed in Refs. 18, 19). Apart from the geographic variability, which is likely to exist, it is likely due to the usage of different detection methods and genetic heterogeneity of papillary carcinomas. Indeed, the distribution of RET/PTC rearrangement within each tumor can vary from involving almost all neoplastic cells (clonal RET/PTC) to being detected only in a small fraction of tumor cells (nonclonal RET/PTC) (31, 32). This has to be taken into account when selecting patients for the RET receptor-targeted therapy because tumors with nonclonal RET/PTC frequently have other genetic alterations and are unlikely to respond to such a treatment.

The prevalence of RET/PTC is significantly higher in papillary carcinomas from patients with the history of radiation exposure, including those subjected to either accidental or therapeutic irradiation. Among papillary carcinomas from children affected by the Chernobyl nuclear accident, RET/PTC was found in up to 80% of tumors removed 5–8 yr after the accident and 50–60% of those removed 7–11 yr after exposure (33–37). The formation of RET/PTC1 and RET/PTC3 rearrangements after radiation exposure is likely to be predisposed by close positioning of the RET chromosomal locus to its fusion partners within the nuclei of normal thyroid cells, which would facilitate the simultaneous breakage of both genes and their end joining (38, 39). RET/PTC is also found more frequently (40–70%) in sporadic papillary carcinomas from children and young adults (35, 40–42). Overall, papillary carcinomas with RET/PTC rearrangements typically present at younger age and have a high rate of lymph node metastases, classic papillary histology, and possibly more favorable prognosis (43).

Using DNA microarray analysis of human tumors and cultured cells with inducible expression of RET/PTC, a distinct expression profile for RET/PTC has been observed (5, 44). Among several functional clusters of genes found to be activated after RET/PTC expression, many genes are involved in regulating the inflammatory and immune responses (45, 46). This suggests a link between RET/PTC signaling and inflammatory infiltrates, which are frequently seen within thyroid tumor nodules and in surrounding thyroid tissue and may play a role in promoting tumor progression and invasion.

BRAF

BRAF belongs to the family of RAF proteins (ARAF, BRAF, CRAF), which are intracellular effectors of the MAPK signaling cascade (47). Upon activation triggered by RAS bind-
ing and protein recruitment to the cell membrane, these serine-threonine kinases phosphorylate and activate MAPK/ERK kinase (MEK), which in turn activates ERK and consequent effectors of the MAPK cascade. Among the three functional human RAF proteins, BRAF has the highest basal kinase activity and is the most potent activator of MEK (47, 48).

In 2000, Davies et al. (49) implicated BRAF in carcinogenesis by reporting that point mutations within the kinase domain of the gene occur in melanomas, colorectal cancer, and several other types of human tumors. Among several mutations found, a thymine to adenine transversion in the nucleotide 1799 (T1799A) (originally described as T1796A) was the most common. It results in a substitution of a valine with a glutamic acid at residue 600 of the protein (V600E). V600E and most of other mutations within the kinase domain involve either the activation loop or the P loop (ATP binding site) and lead to constitutive activation of BRAF kinase (49). The mechanism of BRAF activation has been recently elucidated (50). In the dephosphorylated wild-type BRAF protein, the hydrophobic interactions between the activation loop and the ATP binding site maintain the protein in an inactive conformation. The V600E substitution disrupts these interactions and allows the formation of new interactions that keep the protein in a catalytically competent conformation, resulting in continuous phosphorylation of MEK (50).

More recently a high prevalence of BRAF mutation has been found in thyroid papillary carcinomas (3, 51). To date, multiple studies have confirmed that BRAF mutation is the most common even in sporadic adult papillary carcinomas and occurs in approximately 45% of all cases (reviewed in Refs. 52 and 53). Virtually all mutations found in these tumors are V600E. The other two mutation types, a point mutation K601E and an insertion V599Lns, have been reported in single-tumor cases (54, 55). In contrast to adult papillary carcinomas, pediatric tumors (both sporadic and radiation induced) have a low prevalence of BRAF mutations (0–12%) (56–58). In addition to papillary carcinomas, BRAF mutations are found in thyroid anaplastic and poorly differentiated carcinomas, typically in those tumors that also contain areas of well differentiated papillary carcinoma (59–61). In those tumors, mutant BRAF is detectable in both well-differentiated and poorly differentiated or anaplastic tumor areas, providing evidence that it occurs early in tumorigenesis and predisposes to tumor dedifferentiation.

BRAF mutations are highly prevalent in classical papillary carcinomas and the tall cell variant of papillary carcinoma but are rare in the follicular variant (52). In several studies, the presence of BRAF mutation has been found to correlate with older age of patients, more frequent extrathyroidal extension, advanced tumor stage at presentation, and tumor recurrence (59, 60, 62, 63). In the largest series of papillary carcinomas reported to date, BRAF mutation was found to be an independent predictor of tumor recurrence, even in patients with stages I and II of the disease (62). Importantly, BRAF mutations have also been associated with the decreased ability of tumors to trap I-131 and treatment failure of the recurrent disease (62, 63). However, the association between BRAF mutation and more aggressive tumor behav-ior has not been found in several other studies of human tumors (64–66).

The involvement of BRAF mutation in tumor initiation and dedifferentiation as well as its correlation with more aggressive tumor characteristics has also been suggested by studies of transgenic mice with thyroid-specific expression of V600E BRAF (67). These animals developed papillary carcinomas with high penetrance, and microscopic features of these tumors closely recapitulated those seen in human papillary carcinomas. Importantly, these tumors frequently revealed invasion of blood vessels, thyroid capsule, and perithyroidal skeletal muscle, all of which are characteristics of more aggressive behavior in humans, and showed multifocal progression to poorly differentiated carcinoma.

Recently another mechanism of BRAF activation has been identified. It involves inversion of chromosome 7q that leads to an in-frame fusion between BRAF and the AKAP9 gene (68). The AKAP9-BRAF protein contains the protein kinase domain and lacks the autoinhibitory N-terminal portion of BRAF. It exhibits elevated basal kinase activity, stimulates ERK phosphorylation, and induces transformation of NIH 3T3 cells, consistent with its role as an oncogene. This fusion is rarely found in sporadic papillary carcinomas and is more common in tumors associated with radiation exposure. Yet another mechanism of BRAF activation in thyroid tumors may involve the increase in the gene copy number through numerical gains of chromosome 7 or as a result of gene amplification. This has been found in a significant portion of thyroid follicular tumors of both conventional and oncocytic (Hurthle cell) types but not in papillary carcinomas (69).

**Targeted Therapies**

The high frequency of RET/PTC and BRAF mutations in papillary carcinomas and their role in tumor initiation and dedifferentiation make them the logical targets for anticancer drug therapy. Inhibitors of different kinases along the MAPK signaling pathway are available and have shown substantial therapeutic effects in thyroid cells, at least in the experimental systems, and are currently being tested in clinical trials.

Several TK inhibitors have been shown to block oncogenic RET/PTC signaling. An anilinoquinazoline ZD6474, an inhibitor of the vascular endothelial growth factor receptor-2, has been found to effectively block phosphorylation and signaling of RET/PTC3 and RET/MEN2B proteins (70). It induced growth arrest of human papillary carcinoma cell lines carrying RET/PTC1 and prevented tumor formation in nude mice after injection of RET/PTC3-transformed fibroblasts (70). Two other small-molecule TK inhibitors, the pyrazolopyrimidine compounds PP1 and PP2, have been tested and found to be effective in therapeutic concentrations in blocking RET/PTC signaling in vivo and abolishing its tumorigenic effects in experimental animals (71, 72).

After identification of BRAF mutations as the most common genetic alteration in papillary carcinomas, the efforts have focused on the development of BRAF inhibitors. They would be particularly valuable because of the well-proven role of mutant BRAF in tumor dedifferentiation and its likely association with tumor recurrence and resistance to the conventional radiiodine therapy. Moreover, because BRAF

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function is crucial for signaling and transforming abilities of RET/PTC oncogenes. BRAK inhibitors may be effective in tumors with either BRAF or RET alterations, although this prediction remains to be proven in the clinical setting.

Of several known multikinase inhibitors, the BAY 43–9006 compound effectively blocks the wild-type BRAF and mutant V600E BRAF kinase activity (50). BAY 43–9006 has been found to inhibit the BRAF signaling and growth of all thyroid cell lines carrying the mutant BRAF (73). It also retarded the growth of the anaplastic carcinoma cell line xenografts in nude mice, and large areas of necrosis were found in the xenografts after the treatment of animals for 5 d (73). The inhibition of growth was mainly a cytostatic effect due to the cell arrest in G1 phase, and more profound cell death could be mediated by the inhibition of other kinases, especially those involved in angiogenesis. BAY 43–9006 cytostatic effect has also been found in cells carrying the activated forms of RET, including RET/PTC3 (74).

The effects of thyroid cell treatment with two new inhibitors of RAF kinases, AAL-881 and LBT-613, have also been studied (75). Both compounds were found to block the MAPK signaling and growth of rat thyroid cells and human thyroid tumor cell lines harboring the V600E BRAF and RET/PTC1 oncogenes. Suppression of the growth of BRAF mutant tumor xenografts in nude mice was also noted. However, some of these anticancer effects may be due to off-target effects because they also occurred in the absence of inhibition of MEK and ERK phosphorylation (75, 76).

Additional therapeutic target along the MAPK pathway are located downstream of BRAF. In the recent study, MEK inhibitor CI-1040 has been found to abrogate tumor growth in BRAF mutant xenografts derived from various tumor types (77). An increasing number of novel compounds are being discovered, which, together with the progress in clinical testing of the available agents, provide hope for new effective therapies for thyroid cancer based on the inhibition of RET/PTC, BRAF, and other kinases along the MAPK signaling cascade.

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