

# Editorial: Challenging Dogma in Thyroid Cancer Molecular Genetics—Role of *RET/PTC* and *BRAF* in Tumor Initiation

*RET/PTC* oncogenes are believed to play an important role in the pathogenesis of a significant subset of papillary carcinomas of the thyroid (PTC), in particular those arising after radiation exposure, and in pediatric cancers. Chromosomal rearrangements linking the promoter and N-terminal domains of unrelated gene/s to the C-terminal fragment of *RET* result in the illegitimate expression of a chimeric form of the receptor in thyroid cells that is constitutively active (1). Several molecular forms have been identified that differ according to the 5' partner gene involved in the rearrangement, with *RET/PTC1* and *RET/PTC3* being the most common. *RET/PTC1* is formed by a paracentric inversion of the long arm of chromosome 10 leading to fusion of *RET* with a gene named *H4/D10S170*. *RET/PTC3* is also a result of an intrachromosomal rearrangement and is formed by fusion with the *RFG/ELE1* gene. *RET/PTC* is believed to be one of the key first steps in thyroid cancer pathogenesis because: 1) There is a high prevalence of *RET/PTC* expression in occult or microscopic PTC (2–4), pointing to the activation of this oncogene at early stages of tumor development. 2) Thyroid-specific overexpression of either *RET/PTC1* (5, 6) or *RET/PTC3* (7) in transgenic mice leads to development of tumors with histological features consistent with papillary thyroid carcinoma, indicating that these oncoproteins can recreate the disease in an animal model. 3) Exposure of cell lines (8) and fetal thyroid explants (9) to ionizing radiation results in expression of *RET/PTC* within hours, supporting a direct role for radiation in the illegitimate recombination of *RET*. 4) The breakpoints in the *RET* and *ELE1/RFG* genes resulting in the *RET/PTC3* rearrangements of radiation-induced pediatric thyroid cancers from Chernobyl are consistent with direct double-strand DNA breakage resulting in illegitimate reciprocal recombination (10). Moreover, the *H4* and *RET* genes, although lying at a considerable linear distance from each other within chromosome 10, are spatially juxtaposed during interphase in thyroid cells and presumably present a target for simultaneous double-strand breaks in each gene after ionizing radiation, thus giving rise to the *RET/PTC1* rearrangement (11). These data provide evidence that ionizing radiation, the major risk factor for development of papillary thyroid cancer, can directly induce *RET* recombination events and link environmental agents to tumor initiation through this genetic pathway.

The paper by Unger *et al.* (12) in this issue of the *JCEM* potentially adds a new dimension to our understanding of

Abbreviations: MEK, MAPK kinase; PTC, papillary carcinomas of the thyroid.

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the role of *RET/PTC* in thyroid cancer pathogenesis. The authors used fluorescence *in situ* hybridization with differentially labeled fluorescent yeast artificial chromosome probes complementary to the region of the *RET* gene immediately upstream of the recombination (labeled in *green*) or downstream (labeled in *red*) to detect rearrangements in interphase cells of papillary cancer specimens. A rearranged *RET* gene would manifest as a split of the *red* and *green* signals. Using this approach, they confirmed a high prevalence of *RET/PTC* rearrangements in papillary thyroid cancers from Ukrainian patients exposed to radiation after the Chernobyl nuclear accident. However, there was considerable heterogeneity within the tumor specimens, in which only a small proportion of cells harbored the rearrangement. The regions with or without *RET* rearrangements tended to cluster in different regions of the tumor. The authors took care to microdissect the tissue samples to minimize the number of nontumoral cells in the specimen. They used confocal microscopy to examine the full thickness of the paraffin section, which presumably allowed them to explore the entire volume of the nucleus for fluorescent signals. The latter is important because of the tendency of papillary thyroid nuclei to overlap, which could generate artifacts. They ruled out other technical artifacts by validating the approach in colorectal cancer tissue (which should have only wild-type *RET*) and in paraffin sections of a thyroid cancer cell line harboring a clonal *RET/PTC1* rearrangement. These data are interpreted as evidence that post-Chernobyl tumors are either of polyclonal origin, or that *RET* rearrangements are a late, subclonal event. However, there is an alternative explanation for these findings. As mentioned above, the *RET* and *H4* genes lie adjacent to each other in about 35% of normal thyroid cells, presumably due to nonrandom interactions between the respective chromosomal domains with components of the nuclear matrix (11). Hence, it is possible that, even after recombination, the two rearranged fragments of *RET* may remain contiguous in the nucleus. In this scenario, and with the fluorescence *in situ* hybridization technique used in the study by Unger *et al.*, these cells would be incorrectly scored as not having a *RET* rearrangement. In addition, the findings need to be reconciled with previous evidence that *RET/PTC* rearrangements can be detected in 19% of sporadic papillary thyroid cancers by Southern blotting (13), a low-resolution methodology that would likely not be sensitive enough to detect the rearrangement if it were only present in a small fraction of cells.

Setting aside methodological issues, the significance of whether or not *RET/PTC* rearrangements are present in all tumor cells is worth considering in greater detail. If *RET/PTC* were the first hit and the oncogene were then lost, then the

locus would have to be deleted during tumor evolution, which would most likely occur through whole-chromosome losses or large deletions. However, Unger *et al.* report that two copies of chromosome 10 were present in all cells that were evaluated, excluding this possibility. Alternatively, *RET/PTC* may occur as a later step in tumor evolution. This is certainly possible, at least in some cases. Many of the experimental data in the literature would, however, need to be reinterpreted, such as the expression of *RET/PTC* in micropapillary carcinomas, and the ability of *RET/PTC* to induce papillary carcinomas in transgenic mice. Finally, the question of whether *RET/PTC* is a clonal or subclonal event should not be viewed as sterile academic minutiae. Much to the contrary, the future development of drugs that interfere selectively with *RET* kinase activity (14) make this a clinically relevant question: *i.e.* if *RET/PTC* is a clonal change, then patients with tumors that harbor this mutation may benefit from *RET* antagonists. In contrast, if *RET/PTC* is only present in a subpopulation of cells, these therapies will likely fail.

In the past year we have witnessed an explosion of new information on thyroid cancer pathogenesis, primarily based on the discovery of mutations of *BRAF* in thyroid papillary carcinomas. There are three isoforms of the serine-threonine kinase *RAF* in mammalian cells: A-Raf, B-Raf, and C-Raf or Raf1. Raf isoforms activate the *MAPK* pathway following stimulation by Ras and are thus critical relays in the transmission of signals generated after ligand binding of membrane tyrosine kinase receptors. B-Raf has higher affinity for *MAPK* kinase (*MEK*)1 and *MEK*2, is more efficient in phosphorylating *MEKs* than other Raf isoforms (15), and is the predominant isotype in thyroid follicular cells (Mitsutake, N., L. Zhang, J. A. Knauf, and J. A. Fagin, unpublished observations). *BRAF* somatic mutations were first reported in malignant melanomas (16) and in a smaller subset of colorectal and ovarian cancers (16). A total of 98% of the mutations in melanomas resulted from thymine-to-adenine transversions at nucleotide position 1796, resulting in a valine-to-glutamate substitution at residue 600 (V600E), formerly designated as V599E. This mutation is believed to produce a constitutively active kinase by disrupting hydrophobic interactions between residues in the activation loop with residues in the ATP binding site that maintain the inactive conformation, allowing development of new interactions that fold the kinase into a catalytically competent structure (17, 18). Correspondingly, B-RAF<sup>V600E</sup> exhibits elevated basal kinase activity and transforms NIH3T3 cells with higher efficiency than the wild-type form of the kinase, consistent with it functioning as an oncogene.

The *BRAF*<sup>T1796A</sup> mutation is the most common genetic mutation in papillary carcinomas and is present in 36–69% of cases (19–26). *BRAF*<sup>T1796A</sup> mutations are not found in any other form of well-differentiated follicular neoplasm. There is practically no overlap between papillary thyroid carcinomas with *RET/PTC*, *BRAF*, or *RAS* mutations, which altogether are found in about 70% of cases (19, 25). The lack of concordance for these mutations provides strong genetic evidence for the requirement of this signaling pathway for transformation to papillary thyroid cancer. This represents a unique paradigm of human tumorigenesis through mutation of three signaling effectors lying in tandem. *BRAF* mutations

can occur early in development of papillary carcinomas, based on evidence that they are present in microscopic lesions (21). Moreover, papillary thyroid cancers with *BRAF* mutations have more aggressive properties, present more often with extrathyroidal invasion and at a more advanced clinical stage, and can give rise to undifferentiated or anaplastic carcinomas (21, 23). These data indicate that *BRAF* mutations may be an alternative tumor-initiating event in papillary thyroid cancer and that tumors with this genotype carry a less favorable prognosis.

Two papers in this issue of the *JCEM* (27, 28) explore the prevalence of *BRAF* mutations in papillary carcinomas developing after exposure to ionizing radiation in childhood during the Chernobyl nuclear reactor accident. In contrast to papillary thyroid cancers from adults, in which *BRAF* mutations are highly prevalent (on average, ~40% of cases), pediatric thyroid cancers from children living in contaminated areas exposed to ionizing radiation harbor *BRAF* mutations very infrequently: four of 34 (12%) in the series by Lima *et al.* (28) and eight of 48 (16%) in the study by Kumagai *et al.* (27). A similar finding was reported in a recently published paper in which only two of 55 (4%) post-Chernobyl cases had *BRAF* point mutations (29). Interestingly, those children presenting with cancer at an early age had a particularly low frequency of *BRAF* mutations, whereas in those that were operated on as adolescents or young adults *BRAF* alterations were somewhat more common (27). By comparison, the prevalence of *RET/PTC* rearrangements in this patient population is very high, found in 50–70% of cases (27, 28, 30, 31). As pointed out by Lima *et al.*, these data may be explainable by the nature of the environmental mutagen. In the case of post-Chernobyl cancers, ionizing radiation may predispose in particular to double-strand DNA breaks and genetic recombination, hence the high frequency of *RET/PTC*, and to a lesser extent *NTRK* rearrangements (32). In sporadic cancers arising in adults, unknown factors may predispose primarily to point mutations, primarily of *BRAF* and *RAS*. A caveat to this rationale is that papillary thyroid cancers from children not exposed to ionizing radiation also have a high prevalence of rearrangements (*RET/PTC*) (30) and a low prevalence of *BRAF* mutations (27, 28). It is possible that thyroid follicular cells from young individuals may have an intrinsic propensity to undergo recombination events and that radiation exposure simply increases their relative frequency. Alternatively, papillary cancers in sporadic pediatric cases may result from inadvertent exposure to genotoxic agents or greater susceptibility to undergo damage by them. Regardless of the genetic mechanisms, the ultimate consequence is constitutive activation of one of the components of the *RET-RAS-BRAF-MAPK* pathway, one of which is required for tumor initiation or promotion.

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