High Prevalence of BRAF Mutations in Thyroid Cancer: Genetic Evidence for Constitutive Activation of the RET/PTC-RAS-BRAF Signaling Pathway in Papillary Thyroid Carcinoma

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Abstract

Thyroid papillary cancers (PTCs) are associated with activating mutations of genes coding for RET or TRK tyrosine kinase receptors, as well as of RAS genes. Activating mutations of BRAF were reported recently in most melanomas and a small proportion of colorectal tumors. Here we show that a somatic mutation of BRAF, V599E, is the most common genetic change in PTCs (28 of 78; 35.8%). BRAFV599E mutations were unique to PTCs, and not found in any of the other types of differentiated follicular neoplasms arising from the same cell type (0 of 46). Moreover, there was no overlap between PTC with RET/PTC, BRAF, or RAS mutations, which altogether were present in 66% of cases. The lack of concordance for these mutations was highly unlikely to be a chance occurrence. Because these signaling proteins function along the same pathway in thyroid cells, this represents a unique paradigm of human tumorigenesis through mutation of three signaling effectors lying in tandem.

Introduction

PTCs are the most common thyroid malignant tumor. Characteristic chromosomal rearrangements linking the promoter and NH₂-terminal domains of unrelated gene(s) to the COOH terminus of RET resulting in constitutively activated chimeric forms of the receptor (RET/PTC) are the genetic hallmark of this type of cancer. RET/PTC rearrangements are thought to be tumor-initiating events (1) and are specific to PTC. They are found in the majority of pediatric PTCs (2, 3) and in most tumors from patients exposed to external radiation during childhood (4), or to ionizing radiation after environmental disasters such as the Chernobyl nuclear reactor accident (3, 5, 6). However, in PTC from adult patients, prevalence of RET/PTC rearrangements is much lower, usually between 5 and 30%, and quite variable in different reported series. Rearrangements of the tyrosine kinase receptor TRK have also been observed but are extremely rare (7). Activating mutations of RAS, commonly found in follicular thyroid neoplasms, are also seen in a small subset of PTCs (8). Taken together, these known oncogenes only account for a small fraction of adult PTCs, which in most cases are not associated with an identifiable genetic defect.

Constitutive activation of RET/PTC kinase activity promotes the interaction with Shc, an intermediate in the RAS pathway. RET-mediated transformation of NIH3T3 cells requires signaling via SHC-RAS-RAF-MEK (9). In mammalian cells, there are three isoforms of the serine-threonine kinase RAF: ARAF, BRAF, and CRAF or RAF1, with different tissue distribution of expression (10). Although all of the RAF isoforms activate MEK phosphorylation, they are differentially activated by oncogenic RAS. In addition, BRAF has higher affinity for MEK1 and MEK2, and is more efficient in phosphorylating MEKs than other RAF isoforms (11). BRAF somatic mutations were reported recently in 66% of malignant melanomas (12), and in <15% of colorectal (12, 13) and ovarian cancers (12). 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 599 (V599E). This mutation is believed to mimic the phosphorylation in the activation segment by insertion of an acidic residue close to a site of regulated phosphorylation at serine 598. BRAFV599E exhibits elevated basal kinase activity and has diminished responsiveness to stimulation by oncogenic H-RAS. BRAFV599E also transformed NIH3T3 cells with higher efficiency than the wild-type form of the kinase, consistent with it functioning as an oncogene. Cancers with BRAFV599E had no mutations in RAS, presumably because of lack of cooperativity between these activating mutants, consistent with their transforming properties being relayed through the same signaling pathway (12). Here we report that BRAF mutations are the most common genetic abnormality associated with thyroid papillary carcinomas and not present in any of the other types of differentiated follicular neoplasms we tested. Moreover, PTC had mutations in RET/PTC, RAS, or BRAF, with no overlap among them. These data provide genetic evidence that thyroid cell transformation to papillary cancers takes place through constitutive activation of effectors along the RET/PTC-RAS-BRAF signaling pathway.

Materials and Methods

Sample Selection and DNA Isolation. We analyzed 124 snap-frozen tumor samples including 78 papillary carcinomas, 10 follicular carcinomas, 12 Hürthle cell carcinomas, 14 follicular adenomas, and 10 Hürthle cell adenomas. Hürthle or oncocytic cell tumors are derived from follicular cells and are thought to represent a distinct variant of follicular neoplasms. Tissues were obtained from the Department of Pathology at the University Hospital in Cincinnati with the help of the University of Cincinnati GCRC Tissue Procurement Facility, or through the Tissue Procurement Facility of the Cedars-Sinai Medical Center GCRC. Normal thyroid tissue from the same patients was also available in 6 papillary carcinoma cases. In addition, the following human cancer cell lines were studied: NPA and TPCI (PTC), WRO (thyroid follicular carcinoma; Ref. 14), SKMel 28 (melanoma), and NCI-H1755 (non-small cell lung cancer). NPA and WRO cells were originally obtained from Guy Juillard (University of California Los Angeles, Los Angeles, CA), TPCI cells were obtained from Sissy M Jhiang (Ohio State University, Columbus, OH), and...
SKMel28 and NCI-H1755 were from the American Type Culture Collection. DNA from tumor samples was extracted using phenol-chloroform method as described previously (15) and from cell lines with the Puregene DNA isolation kit (Gentra Systems, Minneapolis, MN).

**Detection of BRAF Mutations.** Mutations of BRAF reported recently in melanomas and colorectal cancers are confined to exons 11 and 15 (12, 13).

DNA samples were screened by SSCP for mutations within these regions, as well as sequencing of gel-extracted and/or whole-sample PCR products. Primer pairs were designed flanking primer sequences were as follows: exon 11: 5'ctgcttggctgctgtgct 3' and 5'catgctccctccctgacccg 3'; and exon 15: 5'aatcctgaaatgttcgct 3' and 5'gccaaataatcattgga 3'. Amplifications were carried out for 35 cycles with annealing temperatures optimized for each primer pair. Twenty-five µL PCR reactions were performed on 100 ng genomic DNA, 7.5 pmol of each primer, 100 µM deoxynucleoside triphosphates, 5 µCi [α-32P]dCTP, 1.5 mM MgCl2, Platinum TaqDNA polymerase high fidelity (Invitrogen, Carlsbad, CA), and buffer. SSCP analysis was performed using a method reported previously (16). The PCR reaction mixture was diluted in DNA gel-loading buffer (95% formamide, 10 mM NaOH, 0.25% bromphenol blue, and 0.25% xylene cyanol), denatured by incubating at 94°C for 5 min, placed on ice, and loaded onto a 0.6% mutation detection enhancement gel solution (BioWhittaker Molecular Applications, Rockland, ME) with 10% glycerol. Gels were run with 0.6X Tris-borate EDTA buffer at 8W for 7–10 h at room temperature. Autoradiography was performed with an intensifying screen at ~70°C for 12–24 h. All of the PCR reactions from PT samples were replicated at least twice.

**Sequencing.** Genomic PCR products or aberrant SSCP bands cut directly from dried gels were sequenced. PCR reactions in 50 µL of final volume were performed as described above but with omission of radioactive nucleotide. A 2-µL aliquot was run on an agarose gel to verify the adequacy of the reaction, and then purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Direct sequencing was performed using the BigDye v3.0 cycle sequencing kit (Applied Biosystems, Foster City, CA) in a capillary automatic sequence analyzer (ABI PRISM 3100 Genetic Analyzer; Applied Biosystems) at the Cincinnati Children’s Hospital DNA Core Facility. Sequence comparisons were carried out using the BLAST Program (17).

**Detection of RAS Mutations.** Sixty-seven human tumor samples were analyzed for point mutations in codons 12/13, and 61 of the N-RAS, H-RAS, and K-RAS genes using LightCycler (Roche) fluorescence melting curve analysis. Briefly, 100 ng of DNA from each tumor was amplified with primers flanking codons 12/13 or 61 of each RAS gene using a hybridization probe format followed by fluorescence melting curve analysis (18, 19). All of the PCR products that displayed a deviation from normal (placental DNA) melting curve pattern were analyzed by fluorescence melting curve analysis. Briefly, 100 µL of DNA was digested separately with EcoRI, HindIII, BamHI, and BglII (Invitrogen), electrophoresed in 0.8% agarose gel, and transferred to nylon filters (Osmonics Inc., Minnetonka, MN). Hybridization was performed with a 1-kb BamHI-BglII RET-specific probe (20) labeled using a random oligonucleotide primer kit (Amer sham Biosciences, Piscataway, NJ). The researchers screening tumors for BRAF mutations and those genotyping for RET/PTC or RAS were blinded to their respective results until all of the experiments were concluded.

**Results.** Altogether we identified 28 cancers with mutations in exon 15 of the BRAF gene of 124 samples studied. All had the thymine-to-adenine transversion at nucleotide 1796, resulting in a valine-to-glutamate substitution at residue 599 (V599E). All of the BRAFV599E mutations were in papillary carcinomas (28 of 78; 36%; Table 1). By contrast, all 46 of the follicular neoplasms (follicular carcinomas, Hurthle cell carcinomas, follicular adenomas, and Hurthle cell adenomas) examined were wild-type. Of the four thyroid cancer cell lines examined, only the PT C line NPA had the BRAFV599E mutation. The TP C1 line, which is also derived from a PT C and is known to have the RET/PTC1 rearrangement, was wild-type for BRAF. The WRO cell line, derived from a follicular thyroid carcinomas did not have the BRAF mutation.

A representative SSCP gel for exon 15 of BRAF is shown in Fig. 1. As a positive control we used the melanoma cell line SKMel28, which, as reported, has the BRAFV599E mutation (12). Both SKMel28 and NPA cells had a similar conformer pattern, consistent with the presence of only the mutant BRAF allele (Fig. 1, A and B), which was confirmed by sequencing (Fig. 1, bottom panel). Also shown in Fig. 1A is the SSCP pattern for 18 PTCs, 7 of which showed an aberrantly migrating band, which was confirmed by sequencing to correspond to BRAFV599E. The intensity of the abnormal BRAF band in PT C samples was consistently less than that of the wild-type allele. This is likely because of admixture of stromal tissue within the tumor sample, a known histopathological feature of this type of cancer. Mutations were somatic, as determined by analysis of 6 normal thyroid tissues from patients with PT C, 4 carrying the BRAF mutation (Fig. 1B). On microscopical examination, all of the PT Cs positive for BRAF mutations were of classic papillary histotype.

We found no mutations in exon 11 of BRAF in any of the 128 thyroid tumor samples or in the cell lines. SSCP gels were run under multiple conditions, using DNA from the NCI-H1755 non-small cell lung cancer cell line as a positive control (BRAFV599E; Ref. 12). Despite clear resolution of the known mutant sample, all of the PT Cs exhibited a normal SSCP pattern (data not shown). Nine PT C samples with normal SSCP patterns that were also negative for BRAFV599E were randomly selected for sequencing, and all were found to be wild-type for exon 11.

A total of 67 papillary thyroid carcinomas were also analyzed for mutations in the known hot spots in the three RAS genes, as well as for RET/PTC rearrangements. The relative distribution of mutations of these genes in this large sample cohort is shown in Table 2. BRAF was the most commonly mutated gene. Of those tumors positive for BRAF (22 of 67; 33%), none were positive for either RAS or RET/PTC. Of those that were RET/PTC positive (11 of 67; 16%), none had either BRAF or RAS mutations. Finally, none of the 11 PT Cs with RAS mutations had BRAF or RET/PTC mutations.

**Discussion.** In this article we show that a mutation in BRAF, in all likelihood somatic, is the most common genetic change in adult PT C, the most frequent type of endocrine malignant tumor. A single type of mutation was found, consistent with findings in human melanomas and colorectal carcinomas (12, 13). The BRAFV599E oncogene was found only in PT Cs, and not in benign or malignant follicular neoplasms, indicating that this genetic event is likely to be one of the key determinants of the papillary cancer phenotype. In the original reports about this new oncogene, there was essentially no overlap between melan-
nomas harboring RAS and BRAF V599E mutations (12, 13). This was interpreted as evidence that the constitutively active function of this particular BRAF mutant could not be additionally driven by onco-
genic RAS, which was also demonstrated experimentally (12). Here we show that two-thirds of PTCs harbored mutations of RET, RAS or BRAF, but in no single case was there a mutation in two of these genes. Of the two PTC cell lines studied, one had a mutation in RET/PTC (TPC1) and the other of BRAF (NPA). The human tumor data are unlikely be a chance occurrence and have potentially signif-
ificant implications. They point to the centrality of the RET-SHC-RAS-
BRAF pathway in thyroid cell transformation to papillary cancer, although they do not entirely negate a potential contribution of other effectors systems cooperative with either RET/PTC or RAS in thyroid cells. Constitutive activation of RET kinase is known to result in autophosphorylation of Y1062 in the COOH terminus of the receptor, which couples to SHC-RAS and drives transformation by RET in NIH3T3 cells (9). Although this residue also mediates signaling along this pathway in rat thyroid cell lines, neither RET nor RAS are sufficient by themselves to induce thyroid cell transformation in vitro (21). However, after co-overexpression of both of these effectors in rat thyroid cells, clones with a completely undifferentiated and trans-
formed phenotype were observed (21). Taken together, these data and the human tumor genetic evidence presented here support the notion that activating mutations of components of the RET-RAS-BRAF pathway are required but not sufficient for development of PTC.

Whether RET and one of these two downstream effector mutants may cooperate in the development of more aggressive tumors is possible but unlikely based on our data and on the fact that RET/PTC rearrangements are uncommon in undifferentiated thyroid cancers. Until this report, PTC was known to be associated primarily with rearrangements of genes coding for the tyrosine kinase receptor RET, and less commonly TRK. In this series, RET/PTC mutations were seen in 16% of PTCs, which is quite consistent with data in the literature from patients without documented history of radiation exposure (22). As stated, one of the major risk factors for development of PTC is a history of prior exposure to radiation, and it is these particular tumors that have a high prevalence of rearranged oncogenic forms of RET (3, 23, 24). Radiation-induced RET/PTC chimeric genes have been proposed to form because of direct double-strand DNA breaks resulting in illegitimate reciprocal recombination (25) favored by spatial jux-
phase of the participating loci during interphase in thyroid cells (6). However, the great majority of patients with PTC do not have a history of radiation exposure. The fact that point mutations of BRAF and RAS may account for many of these provides a more plausible genetic mechanism for generation of these tumors in the general population.

It is notable that BRAF mutations are common in melanomas and thyroid cancers, because growth of melanocytes and thyrocytes is...
positively regulated by cAMP. In both cell types, cAMP activates MEK1 and extracellular signal-regulated kinases through mechanisms that may differ but that converge on BRAF. In thyroid cells, cAMP activates RAP1 guanine nucleotide exchange possibly via EPAC (26), whereas in melanocytes cAMP activates RAS through a yet-unidentified exchange factor (27). A similar cAMP-RAS mediated pathway has also been proposed in thyroid cells (28). Regardless, BRAF is thought to be the key RAF isoform transducing the cAMP-dependent growth signal in both these cell types (27, 29), which may account for their vulnerability to transformation by activating mutations of this particular kinase.

This study was initiated with the presumption that follicular adenomas or carcinomas would be good candidates to harbor BRAF mutations, because ~25% of them have RAS mutations (8, 30). Therefore, the fact that we did not find BRAF mutations in follicular or Hurthle cell neoplasms was a notable and unexpected result. It is tempting to speculate based on these genetic data that the dominant type of RAS downstream effector pathway used may be important in thyroid tumor fate, with RAS acting via BRAF predisposing to PTC, and RAS through yet-unknown effectors favoring transformation to follicular neoplasms.

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References


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