[CANCER RESEARCH 63, 1454–1457, April 1, 2003]

Advances in Brief

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[1]

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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%). $BRAF^{V599E}$ 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[3] are the most common thyroid malignant tumor. Characteristic chromosomal rearrangements linking the promoter and NH_2-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. $BRAF^{V599E}$ exhibits elevated basal kinase activity and has diminished responsiveness to stimulation by oncogenic H-RAS. $BRAF^{V599E}$ also transformed NIH3T3 cells with higher efficiency than the wild-type form of the kinase, consistent with it functioning as an oncogene. Cancers with $BRAF^{V599E}$ 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 neoplasm 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 TPC1 (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 Juilliard (University of California Los Angeles, Los Angeles, CA), TPC1 cells were obtained from Sissy M Jhiang (Ohio State University, Columbus, OH), and

Received 11/27/02; accepted 2/18/03.

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[1] Supported in part by NIH Grants CA50706 and CA72597 (to J. A. F.), GCRC Grant MOIRR08084 and American Cancer Society grant RSG-03-027-01-CCE (to Y. E. N.). E. T. K. is recipient of Coordenaçao de Aperfeiçoamento de Pessoal de Nível Superior Grant BEX1891/01-4 from the Ministry of Education of Brazil, and is on leave from the Institute of Biomedical Science, University of São Paulo, São Paulo, Brazil.

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[3] The abbreviations used are: PTC, papillary thyroid cancer; MEK, mitogen-activated protein/extracellular signal-regulated kinase kinase; EPAC, exchange factor directly activated by cyclic AMP; GCRC, General Clinical Research Center; SSCP, single-strand conformational polymorphism; cAMP, cyclic AMP.

SKMel28 and NCI-HI1755 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 *BRAF* exons 11 and 15, respectively. PCR primer sequences were as follows: exon 11: 5′tctgtttggcttgacttgacttt 3′ and 5′catgccactttcccttgtagac 3′; and exon 15: 5′aaactcttcataatgcttgctctg 3′ and 5′ggccaaaaatttaatcagtgga 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^{32}P]dCTP, 1.5 mM $MgCl_2$, 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 0.6× 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 PTC samples were repeated 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 the rest purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA). Direct sequencing was performed using the BigDye v3.03 cycle sequencing kit (Applied Biosystems, Foster City, CA) in a capillary automatic sequencer (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).[4]

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 pick were directly sequenced to verify the presence of *RAS* mutation and detect the exact nucleotide change.

Detection of *RET* Rearrangements. Rearrangements of the *RET* gene were analyzed in 67 human tumor samples by Southern blot analysis. Briefly, 10 μg of DNA was digested separately with *Eco*RI, *Hind*III, *Bam*HI, and *Bgl*II (Invitrogen), electrophoresed in 0.8% agarose gel, and transferred to nylon filters (Osmonics Inc., Minnetonka, MN). Hybridization was performed with a 1-kb *Bam*HI-*Bgl*II *RET*-specific probe (20) ^{32}P-labeled using a random oligonucleotide primer kit (Amersham 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 same thymine-to-adenine transversion at nucleotide 1796, resulting in a valine-to-glutamate substitution at residue 599 (V599E). All of the $BRAF^{V599E}$ mutations were in papillary carcinomas (28 of 78: 36%; Table 1). By contrast, all 46 of the follicular neoplasms (follicular carcinomas, Hürthle cell carcinomas, follicular adenomas, and Hürthle cell adenomas) examined were wild-type. Of the four thyroid cancer cell lines examined, only the PTC line NPA had the $BRAF^{V599E}$ mutation. The TPC1 line, which is also derived from a PTC 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.

Table 1 *Prevalence of BRAF mutations in thyroid neoplasms: BRAF mutations are unique to papillary thyroid cancers*

	$BRAF^{V599E}$
PTC	28/78 (35.8%)
Follicular adenoma	0/14
Follicular carcinoma	0/10
Hürthle cell adenoma	0/12
Hürthle cell carcinoma	0/10
Cell lines	
NPA	+
PTC1	−
WRO	−

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 $BRAF^{V599E}$ 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. 1*A* is the SSCP pattern for 18 PTCs, 7 of which showed an aberrantly migrating band, which was confirmed by sequencing to correspond to $BRAF^{V599E}$. The intensity of the abnormal *BRAF* band in PTC 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 PTC, 4 carrying the *BRAF* mutation (Fig. 1*B*). On microscopical examination, all of the PTCs 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 ($BRAF^{G468A}$; Ref. 12). Despite clear resolution of the known mutant sample, all of the PTCs exhibited a normal SSCP pattern (data not shown). Nine PTC samples with normal SSCP patterns that were also negative for $BRAF^{V599E}$ 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 PTCs 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 PTC, 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 $BRAF^{V599E}$ oncogene was found only in PTCs, 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 mela-

[4] Internet address: http://www.ncbi.nlm.nih.gov/BLAST/.

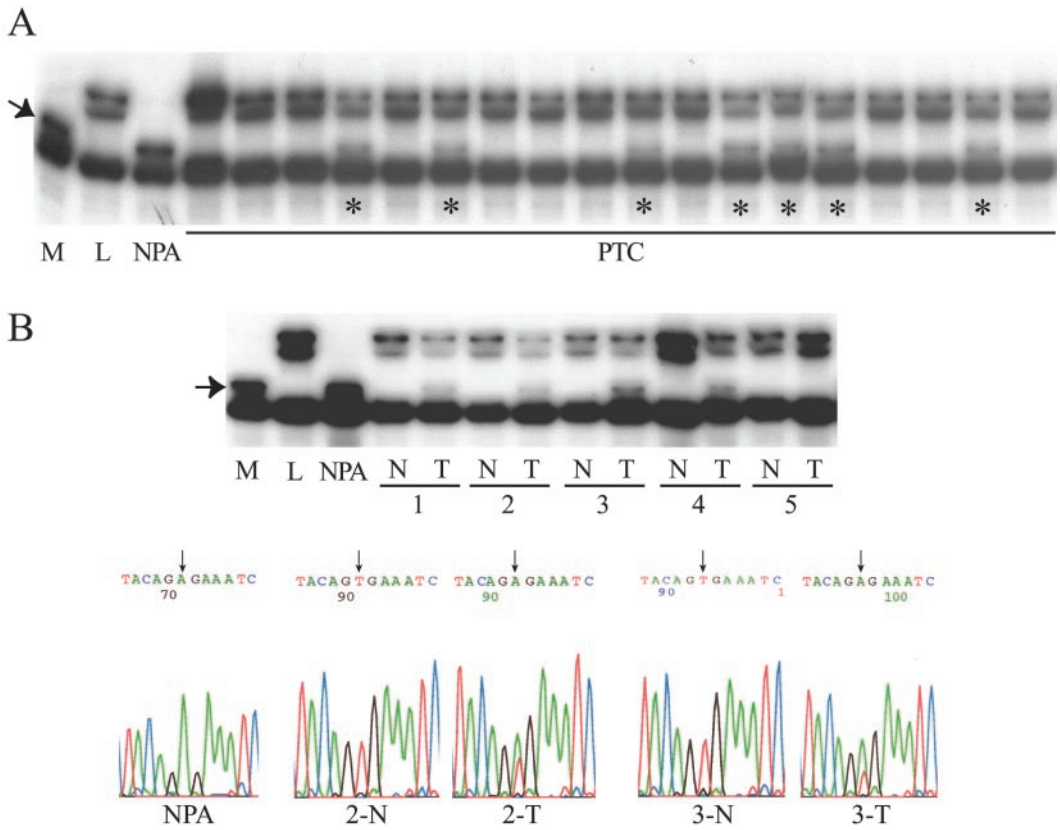

Fig. 1. *A*, SSCP analysis for *BRAF* exon 15 mutations in papillary thyroid carcinomas. *M*, SKMel 28 melanoma line, Positive control ($BRAF^{V599E}$); *L*, normal subject blood lymphocytes; *NPA*, papillary carcinoma cell line. DNA from SKMel 28 and NPA lines had identical band shifts. Several papillary carcinoma tissue samples also had a similar band patterns. Samples with an abnormal band pattern are indicated by ∗. Aberrant bands were confirmed to have the *BRAF* mutation (V599E) by direct sequencing. *B*, the *BRAF* mutation in PTCs is somatic. *Top*, aberrant bands are shown in tumors, but not normal thyroid tissue, of cases 1–4. The papillary carcinoma for case 5 is wild-type. *Bottom*, sequence chromatogram of PCR product from normal tissue and corresponding tumor for cases 2 and 3. Tumors displaying altered SSCP banding pattern present a double peak (↓) at position 1796 in *BRAF* exon 15. This thymine (*T*) to adenine (*A*) transition introduces a substitution of amino acid valine to glutamic acid at codon 599 (V599E).

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 oncogenic 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 significant 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 transformed 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.

Table 2 *Lack of overlap among BRAF, RAS, and RET/PTC mutations in papillary carcinomas*

	Mutation prevalence	BRAF	RAS	RET/PTC
BRAF	32.8%	22/67	0	0
RAS	16.4%	0	11/67	0
RET/PTC	16.4%	0	0	11/67
Total	65.6%			

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 juxtaposition 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 Hürthle 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.

Acknowledgments

We thank Massimo Santoro for the RET-specific probe used for Southern blot analysis.

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Cancer Res 2003;63:1454-1457.