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Concomitant *BRAF*^{V600E} Mutation and *RET/PTC* Rearrangement Is a Frequent Occurrence in Papillary Thyroid Carcinoma

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Background: The tyrosine kinase receptors/RAS/RAF/MAPK cascade is a site of mutational events associated with thyroid carcinogenesis. Some studies suggest the reciprocal exclusion of different oncogenes in the mitogen-activated protein kinase cascade, whereas others suggest that *BRAF* mutations and *RET* rearrangements can simultaneously occur in sporadic cases. The aim of this study was to determine the prevalence of concomitant *BRAF*^{V600E} mutation and *RET/PTC* rearrangements in the same tumor and its association with some clinicopathological features.

Methods: The percentage of mutant *BRAF* alleles and the presence of *RET/PTC* rearrangements were determined by means of pyrosequencing and Southern blot analysis of reverse transcription polymerase chain reaction products in a series of 72 conventional papillary thyroid carcinomas (PTCs). Then, the associations between clinicopathological characteristics and mutation status were assessed.

Results: *BRAF*^{V600E} alleles were present in 32 out of 72 PTCs (44.4%) in the range of 5.1–44.7% of total *BRAF* alleles. *RET/PTC* was present in 26 tumors (36.1%). Concomitant subclonal *BRAF* and *RET/PTC* were demonstrated in 14 PTCs (19.4%), and none of the oncogenes was detected in 22 tumors (30.5%). Only *BRAF*^{V600E} was associated with a more advanced tumor staging.

Conclusions: The present study demonstrates that concomitant *BRAF* mutation and *RET/PTC* rearrangement is a frequent event in PTC.

Introduction

BRAF^{V600E}, the most common genetic mutation occurring in papillary thyroid carcinoma (PTC), and the less common *RAS* mutations, *RET* (*RET/PTC*) and *trk-1* (*TRK*) rearrangements, are believed to play an important role in the pathogenesis of PTC (1–6). The products of these genes are constitutively activated tyrosine kinase (RET/PTC, TRK), serine-threonine kinase (BRAF^{V600E}), or small GTP-binding proteins (RAS), which are components of the mitogen-activated protein kinase (MAPK) cascade. The in-tandem organization of all these altered genes participating in the same signaling pathway highlights the importance of the MAPK pathway in thyroid cell transformation. The hypothesis that *RET/PTC*, *BRAF*, and *RAS* mutations can overlap in a single PTC has been debated for a long time; it was ruled out and has finally been accepted as a rare occurrence. *RET/PTC* rearrangements were demonstrated by immunohistochemical staining in a relevant fraction of *BRAF*-mutated PTC (6). However, this conclusion has been questioned because the anti-RET antibody used could have stained the small amount of RET protein produced by thyroid cells, instead of the RET/PTC chimera. At the same time, other studies did not confirm these results and concluded that *BRAF* mutation and *RET/PTC* rearrangements were alternative events in PTC (5,7). This topic was taken up again by other studies, concluding that only rare PTCs harbor both oncogenes and that the presence of a dual mutation is associated with a higher rate of recurrence (8). RET/PTC and BRAF^{V600E} are oncogenes with a recognized pathogenetic role in thyroid carcinogenesis (9–12). *RET/PTC* is a PTC-restricted oncogene generated by an interchromosomal rearrangement (13). Interphase fluorescence *in situ* hybridization demonstrated that *RET/PTC* rearrangements can occur only in a fraction of PTC cells, indicating that these tumors can be composed of a mixture of cells with and without a *RET* rearrangement (14). Recently, it has been demonstrated that *BRAF*^{V600E} can also occur as a subclonal or oligoclonal event in PTC; this brings about significant implications for thyroid carcinogenesis and challenges the efficacy of targeted therapies (15,16). These findings show that multiple mutations can

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be found in PTC cells, acting as a stimulus to study how often $BRAF^{V600E}$ mutation and *RET/PTC* rearrangements are concomitant in PTC and whether tumors with both oncogenes are associated with specific clinicopathological features.

Materials and Methods

Tumor samples

After obtaining the consent of the patients, and with approval from the institutional review boards, cytological samples were collected using a 22-gauge needle syringe, passed four to five times. Needle material was used to prepare a smear for cytological examination, and then the needle was washed out with 5 mL of normal saline into a collection tube, and centrifuged. The pellet was resuspended in TRI Reagent buffer (Sigma) and stored at −20°C until nucleotides were extracted. Cytological smears were classified according to the British Thyroid Association (17). Postsurgery tumor tissues were examined by hematoxylin and eosin staining and classified according to the WHO histopathological typing (18). Cytological samples diagnosed as conventional PTC by histological examination were retrieved for DNA and RNA extraction, according to the TRI Reagent manufacturer's recommendations. The final pellet was resuspended in 10 μL diethyl pyrocarbonate water.

Detection of BRAF mutation

Pyrosequencing was performed as described previously (15). Briefly, DNA was amplified by polymerase chain reaction (PCR), processed to obtain single-stranded DNA, and hybridized to sequencing primers, and a sequencing-by-synthesis reaction of the complementary strand was automatically performed on a PSQ 96MA instrument (Biotage). The coefficient of variation for the percentage of the mutant allele, calculated from quadruplicate pyrosequencing analysis, was 0.5% (range 0% to 1.4%). The cutoff was set at 5%, corresponding to the mean percentage of normal tissues plus 2 standard deviations. Eight benign nodules were used as negative controls. Two tumors with concomitant Hashimoto's thyroiditis were not excluded from the study because *BRAF* mutational analysis is not hampered by the presence of lymphocytes infiltrating the thyroid tissue (19,20).

Detection of RET/PTC rearrangement

mRNA was reverse transcribed with SuperScript III (Invitrogen) in a 20 μL reaction volume with random primers. RNA integrity and the efficiency of the reverse transcription reaction was confirmed by reverse transcription PCR for thyroglobulin mRNA. *RET/PTC-1* and *RET/PTC-3* were detected by Southern blot analysis on reverse transcription PCR products as described, using appropriate primers and oligoprobes specific for the *TK* domain, *H4*, or *ELE1* (Table 1) (21).

Statistics

Statistical analyses included ANOVA, chi-square test, and simple and rank correlation analysis. The *p*-value was considered statistically significant when <0.05 and borderline significant when >0.05 and <0.10. Data presentation includes prevalence of categorical data and mean or median with range of continuous data, as appropriate.

Results

Mutation analysis of BRAF in PTC cytological samples

Genomic DNA from cytological samples of 72 conventional PTC was amplified by PCR and subjected to pyrosequencing for the *BRAF* status (Table 2). By setting a 5% cutoff, 8 benign nodules displayed 100% wild-type *BRAF* (data not shown). $BRAF^{V600E}$ was detected in 38 (44.4%) PTCs in the range of 5.8–45.8% (Fig. 1). The mean and the median percentage of mutant alleles in the $BRAF^{V600E}$-positive tumors were 22.7% and 18.8%, respectively.

Analysis of RET/PTC rearrangement and concomitant occurrence of BRAF mutation

RET rearrangements were searched in RNA extracted from 72 cytological samples. Total RNA extracted from fine-needle aspirates was analyzed by Southern blot on reverse transcription PCR for the presence of *RET/PTC1* and *RET/PTC3* rearrangements. A *RET* rearrangement was detected in 26 (36.1%) PTCs (Table 2): 9 and 19 tumors were positive for *RET/PTC1* and *RET/PTC3*, respectively. In two tumors, both rearrangements were detected. $BRAF^{V600E}$ was detected in the genomic DNA of 14 out the 26 (54%) *RET/PTC*-positive samples (Figs. 2 and 3). The *BRAF* mutation was present as a subclonal or oligoclonal occurrence in the range of 6–37.5%. Mutational analysis of *BRAF* and *RET/PTC* showed that a genetic alteration was found in 50 (69.5%) PTCs, a dual mutation was detected in 14 (19.4%) PTCs, and a concomitant $BRAF^{V600E}$ occurred in 14 out of 26 tumors harboring *RET/PTC* (53.8%). None of the oncogenes was detected in 22 tumors (30.5%).

Mutational analysis and clinicopathological parameters at diagnosis

PTC patients were divided into four groups according to the mutations detected in the tumor: $BRAF^{V600E}$ only, *RET/PTC* only, $BRAF^{V600E}$ + *RET/PTC*, and no mutations (Table 3). None of the groups were significantly associated with sex, age at diagnosis, tumor volume, lymph node metastasis, or

Table 1. Primers and Probes Used for *RET/PTC* Screening

	Size (bp)	*Primer sequences (5′–3′)*	*Gene*	*Oligoprobes (5′–3′)*
RET/PTC-1	165	For: GCTGGAGACCTACAAACTGA Rev: GTTGCCTTGACCACTTTTC	*H4* *TK*	GGCACTGCAGGAGGAGAACCGCGA- B GGAATTCCCTCGGAAGAACT-B
RET/PTC-3	242	For: AAGCAAACCTGCCAGTGG Rev: CTTTCAGCATCTTCACGG	*Ele1* *TK*	GGTCGGTGCTGGGTATGTAAGGA-B GGAATTCCCTCGGAAGAACT-B

Table 2. Mutational Analysis of Cytological Aspirates from 72 Conventional Papillary Thyroid Carcinomas

Gene	n	%
$BRAF^{V600E}$	38	44.4
$BRAF^{V600E}$ only	24	33.3
RET/PTC	26	36.1
RET/PTC only	12	16.6
$BRAF^{V600E}$ + *RET/PTC*	14	19.4
None	22	30.5

extrathyroidal extension. Stage IV was more frequently observed in patients with tumors harboring only $BRAF^{V600E}$ ($p=0.067$). The association between advanced stage and $BRAF^{V600E}$ became significant when tumors with this oncogene were compared to all others ($p=0.014$) (Table 4). When we considered only BRAF-mutation–positive tumors, the percentage of $BRAF^{V600E}$ alleles was directly correlated with disease stage ($R^2=0.326$ in Spearman rank correlation, $p<0.001$) (Fig. 4). Tumors with a ≥30% of $BRAF^{V600E}$ alleles were more frequently in stage IV than tumors harboring the mutation with a percentage of alleles in the interval of 5.1%–30% ($p=0.031$). Tumors with dual mutations were equally distributed among stages.

Discussion

Many alterations of specific genes have been identified in thyroid cancer cells, that is, *BRAF, RAS, RET, TKR-1, H4-PTEN, β*-catenin, anaplastic lymphoma kinase, and *p53*, indicating that multiple genetic alterations are involved in the development and progression of this type of cancer (22–24). These mutations generate constitutively activated kinases or inactivate tumor suppressors that can cooperate and transform the thyroid cell into a less differentiated one, with a higher propensity of thyrotropin-independent proliferation. In addition to genetic alterations, other genetic mechanisms with a role in carcinogenesis can be found in thyroid tumors. These include epigenetic modifications, gene amplifications, and copy-number gains, all of which lead to aberrant gene transcription (24–26). Most of these genetic alterations operate on three main signaling pathways involved in cell proliferation and survival and protein synthesis: the RAS–RAF–MEK–MAPK, the PI3K–AKT–mTOR, and the calcium–calmodulin

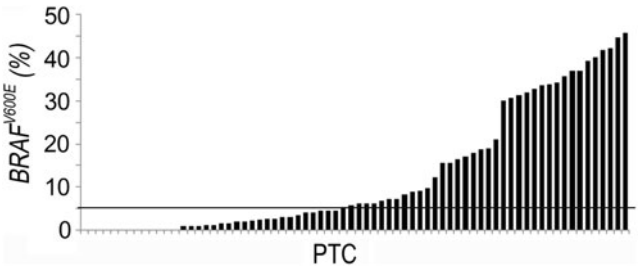

FIG. 1. Pyrosequencing analysis of *BRAF* in cytological samples. Genomic DNA from 72 PTC cytological samples was amplified by polymerase chain reaction and analyzed by pyrosequencing. Cutoff was set at 5%. SD was less than 8%. PTC, papillary thyroid carcinoma; SD, standard deviation.

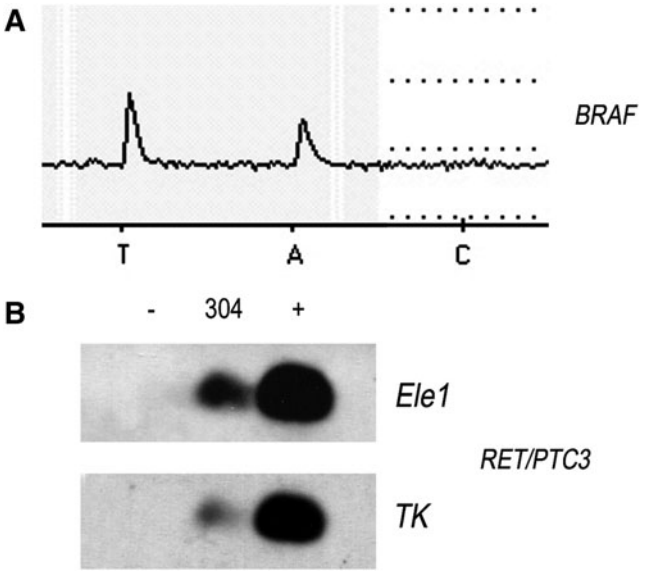

FIG. 2. *BRAF* and *RET/PTC* analysis of a PTC cytological sample. Genomic DNA and RNA extracted from a fine-needle aspirate of a PTC were analyzed by pyrosequencing for *BRAF* and Southern blot for *RET/PTC3,* respectively. $BRAF^{V600E}$ represented 37% of *BRAF* alleles **(A)**. Hybridization bands with probes for *Ele1* and *TK* domain of *RET* were clearly visible **(B)**. 304, PTC sample analyzed; − and +, negative and positive PTC controls, respectively.

pathway. Some of the genetic mutations identified appear to be selective for these pathways, while others do not. $BRAF^{V600E}$ is downstream of RAS; thus, it activates the MAPK pathway and does not directly activate the PI3K–AKT-mTOR pathway. Other genetic alterations present in thyroid tumors, such as RET/PTC and RAS mutations, can simultaneously activate multiple pathways (27–29). A paradigm of cancer development is the sequential gain of advantageous genetic alterations (30). After the initial event, further alterations can occur, are selected for, and those useful for the cell are retained. Similar to colorectal, pancreatic, and renal cancers,

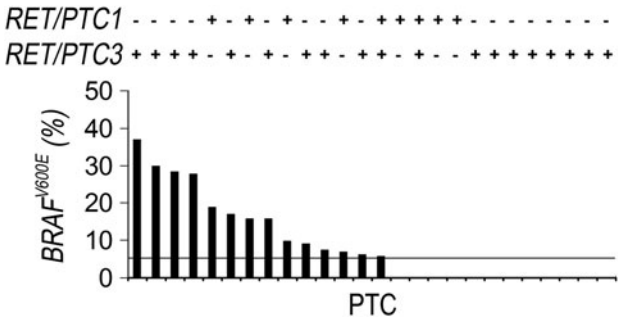

FIG. 3. Pyrosequencing analysis of $BRAF^{V600E}$ in *RET/PTC*-positive PTC. Genomic DNA from cytological aspirates of 26 PTC harboring *RET/PTC1* and/or *RET/PTC3* rearrangements was analyzed by pyrosequencing for the presence of $BRAF^{V600E}$. *RET* rearrangements were determined by Southern blot analysis of reverse transcription polymerase chain reaction products. Bars indicate the percentage of mutated *BRAF* alleles in each sample. SD was less than 8%.

Table 3. Mutational Status and Clinicopathological Characteristics of Papillary Thyroid Carcinomas

	All patients	*$BRAF^{V600E}$ only*	*RET/PTC only*	*$BRAF^{V600E}$ + RET/PTC*	*No mutations*	p
N	72	24	12	14	22	
Male sex, %	23.6	20.8	25	28.6	22.7	0.975
Age, mean (median)	47.6 (46)	48.7 (46)	48 (44)	52 (47.5)	43.5 (35.5)	0.335
Size (mL), mean (range)	14.6 (0.01–179.5)	16.9 (0.04–87.7)	11.4 (0.52–65.4)	14.4 (1.0–65.4)	13.9 (0.01–179.5)	0.896
Lymph node metastasis, %	54.2	66.6	41.7	57.1	45.5	0.213
Extrathyroidal extension, %	52.8	54.2	41.7	71.4	45.5	0.213
AJCC stage, %						
I	45.8	33.3	50.0	28.6	68.2	0.067
II	8.3	0	8.3	14.3	13.6	
III	9.7	8.3	16.7	14.3	4.5	
IV	36.1	58.3	25.0	42.8	13.6	

AJCC, American Joint Committee on Cancer.

thyroid cancer progression from the well-differentiated to the undifferentiated histotype is characterized by the accumulation of genetic alterations (31,32). Although the simultaneous activation of multiple signaling pathways with different biological activities might represent an advantage for tumor cells, the benefits of a constitutive activation of components lying in tandem in the same signaling are questionable. For this reason, the initial finding of mutual exclusion of $BRAF^{V600E}$ and RET/PTC or RAS mutations in PTC was not surprising (5,7). However, not all studies agree on this finding, and some showed evidence that *$BRAF^{V600E}$* and *RET/PTC* dual mutation can rarely occur in well-differentiated PTCs, whereas the coexpression of *$BRAF^{V600E}$* and *$NRAS^{61}$* or *$KRAS^{13}$* has been reported in 2 out of 64 PTCs (1,6,8,16). The findings presented here demonstrate that a dual mutation of *$BRAF^{V600E}$* and *RET/PTC* is not a rare event in well-differentiated PTCs (19.3%), and that about half of the tumors harboring *RET/PTC* also include *$BRAF^{V600E}$*. The demonstration of the subclonal occurrence of *RET/PTC* and *$BRAF^{V600E}$* in PTCs has significant conceptual implications for thyroid carcinogenesis, as well as clinicopathological implications (14,16, 33,34). These oncogenes might initiate PTC tumorigenesis or, instead, they might occur after the development of a thyroid tumor. Regardless of whether these are secondary genetic events in PTC tumorigenesis or secondary genetic alterations, these events prevail; therefore, *RET/PTC* and *$BRAF^{V600E}$* are no longer selected for and get lost; intratumor heterogeneity leads to the possibility that *$BRAF^{V600E}$*, *RET/PTC*, and *RAS* mutations can coexist in the same tumor in different cells. At present, based on the results of this study, it is not possible to discriminate whether *RET/PTC* and *$BRAF^{V600E}$* are present in the same cell or alternatively in different cells of the same tumor. Melanoma shares some common features with thyroid cancer, including the frequent occurrence of *BRAF* and *RAS* mutations. The genetic analysis of individual melanoma cells of the same tumor revealed that these two mutations can sporadically coexist in the same melanoma in different cells (35). The finding that, in the 14 PTCs with a dual mutation, *$BRAF^{V600E}$* was always present as a subclonal or oligoclonal occurrence in the range of 6–37.5% suggests that, similar to the situation in melanoma, the two mutations are not in the same cell. If the two mutations do not lie in the same cell, they still can cooperate to modify the tumor environment, modulating cell-to-cell interaction and angiogenesis trough the release of soluble factors (36,37). *RET* rearrangements and *BRAF* point

Table 4. *$BRAF^{V600E}$* and Clinicopathological Characteristics of Papillary Thyroid Carcinomas

	$BRAF^{V600E}$	*$BRAF^{wild\ type}$*	p
N	38	34	
Male sex, %	23.7	23.5	0.100
Age, mean (median)	49.9 (38.4)	45 (39.5)	0.144
Size (mL), mean (range)	16.2 (0.04–87.7)	13.6 (0.01–179.5)	0.334
Lymph node metastasis, %	24 63.1	15 44.1	0.105
Extrathyroidal extension, %	23 60.5	15 44.1	0.163
AJCC stage, %			
I	12	21	0.014
II	2	4	
III	4	3	
IV	20	6	

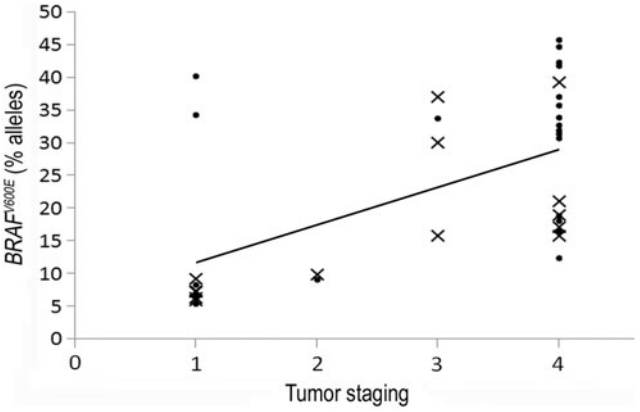

FIG. 4. Distribution of tumor staging according to the percentage of *$BRAF^{V600E}$* alleles. Thirty-eight PTCs harboring a *BRAF* mutation in the range of 5.1–45.8% mutant alleles are reported. *BRAF* mutation status was determined by pyrosequencing in duplicate measurements. SD was <8%. Full dots, PTC-positive only for *$BRAF^{V600E}$*; ×, PTCs with *$BRAF^{V600E}$* and *RET/PTC* dual mutation.

mutations have been associated with different etiologic factors. *RET/PTC* rearrangements have a strong association with the exposure to ionizing radiation, whereas $BRAF^{V600E}$ has been associated with environmental factors in volcanic areas (38,39). This cohort of patients lives in a geographic area characterized by the presence of two volcanoes, Mount Vesuvio and Campi Flegrei, and it cannot be excluded that this peculiar environmental situation is responsible for the high frequency of dual mutations observed in PTC (19%). The presence of a *BRAF* mutation in PTC has been associated with a more aggressive PTC histotype, advanced tumor stages at diagnosis, higher recurrence, and mortality (15,40). Only a few studies have investigated the association between *RET/PTC* and clinicopathological features. In a large study, PTCs harboring *RET/PTC* mutations exhibited a well-differentiated phenotype, were not associated with extrathyroidal extension, lymph node or distant metastases, or advanced tumor stage, and did not progress to undifferentiated carcinomas (41). A different study on 54 recurrent thyroid carcinomas revealed a high rate of *BRAF* and *RET/PTC* dual mutations associated with recurrent PTCs, suggesting that tumors with dual mutations underwent a positive selection and are aggressive (8). In the present cohort of 72 PTCs, advanced tumor stage was more frequent in patients with tumors harboring $BRAF^{V600E}$ and it was associated with a high percentage of mutant alleles. *RET/PTC*, alone or associated with $BRAF^{V600E}$, did not represent a risk factor for a more aggressive disease. This does not exclude other effects of *RET/PTC* on tumor biology and clinical behavior. For instance, benign thyroid nodules harboring *RET* rearrangements exhibit a faster growth than those with wild-type *RET* (42). Although the *BRAF* mutation has been recognized as a helpful prognostic marker for thyroid cancer, the utility of *RET/PTC* is still questionable, and further studies with a larger sample number are still needed.

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Author Disclosure Statement

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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