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$BRAF^{V599E}$ Mutation Is the Leading Genetic Event in Adult Sporadic Papillary Thyroid Carcinomas

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Activating mutations of *BRAF* have been identified in a variety of human cancers, most notably melanomas and papillary thyroid carcinomas (PTCs). The aim of the present study was to disclose the role of *BRAF* mutations in thyroid carcinoma development.

Seventy-two thyroid tumors, including 60 PTCs, six follicular adenomas, five follicular carcinomas, and one anaplastic carcinoma, were studied. *BRAF* mutation screening focused on exon 15 and exon 11 of the gene by single-stranded conformational polymorphism and sequence analysis. Search of RET/PTC expression was conducted with the RT-PCR technique.

The molecular genetic study of the *BRAF* gene showed the presence of a missense thymine to adenine transversion at nucleotide 1796, resulting in the V599E substitution, in 24 of 60 PTCs (40%), none of six follicular adenomas, and none of five follicular carcinomas or one anaplastic carcinoma. Moreover, nine of 60 PTCs (15%) presented RET/PTC expression. A genetico-clinical association analysis showed a statistically significant correlation between *BRAF* mutation and development of PTCs of the classic papillary histotype ($P = 0.038$). On the contrary, no link could be detected between expression of $BRAF^{V599E}$ and age at diagnosis, gender, dimension, and local invasiveness of the primary cancer, presence of lymph node metastases, tumor stage, and multifocality of the disease.

These data clearly confirm that $BRAF^{V599E}$ is the more common genetic alteration found to date in adult sporadic PTCs, that it is unique for this thyroid cancer histotype, and that it might drive the development of PTCs of the classic papillary subtype. (*J Clin Endocrinol Metab* 89: 2414–2420, 2004)

THYROID CARCINOMAS REPRESENT about 1% of all human malignancies, with papillary thyroid carcinoma (PTC) being the most common histotype (1). *RET/PTC* rearrangements arise as a consequence of chromosomal recombinations, which drive the fusion of *RET* proto-oncogene tyrosine kinase domain to the 5′ sequences of unrelated and heterologous genes, creating chimeric oncoproteins with a constitutive active tyrosine kinase function, which were considered a unique genetic hallmark of PTCs (2). They comprise three main forms, *i.e. RET/PTC1*, *2*, and *3*, of which *RET/PTC1* and *3* are definitely more frequent, and several other rare variants recently described mainly in thyroid cancers from areas contaminated by the Chernobyl nuclear accident (2). Several lines of evidence point to their role as initiator factors in follicular cell transformation (3–11). RET/PTCs have been shown to be highly expressed in sporadic pediatric PTCs (48–65%) (12–14) and in PTCs from children exposed to neck irradiation for medical or incidental reasons, such as after the Chernobyl nuclear accident (67–87%) (13–17). On the contrary, in adult sporadic PTCs, their prevalence is low, less than 30% in many studies (2, 6, 18, 19), with some exceptions due to differences in the genetic or environmental background of the studied populations or to methodological differences applied in their search. In addition to *RET/PTCs*, also activating point mutations of the *RAS* oncogene are considered to account for the development of a small subset (10–20%) of adult sporadic PTCs (20). Thus, altogether, until a few months ago, it was possible to find a genetic event potentially involved in tumor initiation in less than 30–40% of sporadic PTCs. However, very recently, after the report of *BRAF* mutations in different human epithelial cancers, most notably melanomas (66% of cases) but also colorectal and ovarian carcinomas (21), several groups sought for *BRAF* mutations in thyroid neoplasms, demonstrating a high prevalence of the same missense thymine (T) to adenine (A) transversion at nucleotide 1796, resulting in the substitution of a valine to glutamate at residue 599, exclusively in PTCs (29–69%) (22–27). Thus, *BRAF* mutations are the most frequent genetic aberration found in sporadic PTCs, and the mutation of one of the intermediates of the RET/PTC-RAS-BRAF pathway accounts for a genetic event, potentially involved in tumor initiation, in two thirds of PTCs (22, 24).

Two very recent reports (27, 28) analyzed also the relationship existing between *BRAF* mutations and the clinico-

Abbreviations: A, Adenine; AC, anaplastic carcinoma; FA, follicular adenoma; FTC, follicular carcinoma; MEK, MAPK kinase; PTC, papillary thyroid carcinoma; SSCP, single-stranded conformational polymorphism; T, thymine.

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pathological features of the cancers. Both studies showed the association of the genetic event with a more advanced stage of the disease (27, 28).

BRAF is one of the three isoforms of RAF, a serine-threonine kinase involved in the phosphorylation of MAPK kinase (MEK) in the Shc-RAS-ERK signaling pathway. The valine-to-glutamate substitution at residue 599, found in all the PTCs and in 98% of the melanomas, 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 (21). Indeed, $BRAF^{V599E}$ has been demonstrated to transform NIH3T3 cells with higher efficiency than the wild-type form of the kinase, consistent with its functioning as an oncogene (21).

Here we report the results of a *BRAF* mutation and *RET/PTC* rearrangement screening in an Italian thyroid tumor cohort collected at our institutions. Moreover, we report the results of an association analysis between the clinical and pathological features of the PTCs in the cohort and the presence or absence of *BRAF* mutations.

Subjects and Methods

Patient population

Seventy-two thyroid tumors, including 60 PTCs, six follicular adenomas (FAs), five follicular carcinomas (FTCs), and one anaplastic carcinoma (AC), were studied. All the analyzed specimens were sampled from primary tumors treated surgically at the Department of Surgery of the University of Perugia, at the Service of Endocrine Surgery of the Catholic University in Rome, and at the Department of Surgery of the University of Pisa, with the exception of three, which were collected in patients submitted to second surgery, after the thyrodectomy, for PTC cervical lymph node metastases. In every case, samples were collected only from the principal tumor lesion or a metastatic lymph node. In the case of multiple lesions or doubts on the lesion nature, the more suspected nodule was examined with frozen sections; and after confirmation of its tumoral nature, samples were collected. All the specimens were snap-frozen after collection and stored at −80 C until use. Before the surgical procedure, all patients signed an informed consent form in which they approved the collection of fresh thyroid samples to be used for genetic studies.

The medical records relative to the hospitalization for the surgery of each patient were reviewed to obtain data about possible radiation exposure. Moreover, for the patients followed after surgery at the Dipartimento di Medicina Interna of the University of Perugia and at the Dipartimento di Endocrinologia e Metabolismo of the University of Pisa, a tumor stage could be defined according to the sixth edition of the "American Joint Committee on Cancer" Cancer Staging Manual (29).

Histology

In 69 of 72 cases, histological slides from the thyroid tumors stained by hematoxylin and eosin stain were reviewed by at least two of the three pathologists in the study group (G.F., A.C., and R.R.), blinded to each other's results and to the molecular findings, to confirm the diagnoses, and in the case of PTCs, to define the pathologic T and N stage and subclassify the histological variants. In detail, the cancers of papillary histology were subdivided in the following categories: classic papillary PTCs, solid/follicular PTCs, other variants (including the tall cell and the diffuse sclerosing), and PTC with loss of differentiation (meaning tumors in which the papillary histotype was still recognizable but characterized by the coexistence of poorly or undifferentiated cancer areas).

Genetic analysis

RNA extraction was performed using Trizol reagent (Invitrogen Corp., Carlsbad, CA), according to the manufacturer's instructions. Five micrograms of total RNA were used for the reverse transcription reactions. In detail, the RNA was incubated at 42 C with Superscript reverse transcriptase II (Invitrogen Corp.) using a random hexamer mixture as primers in a total vol of 20 μl. For each set of reactions, a negative control tube containing double-distilled water and no nucleic acids was coincubated.

The *BRAF* mutations characterized so far are localized in two critical segments of the gene, namely exon 11 and exon 15, with a hot spot at nucleotide 1796 of exon 15. Thus, all the cDNAs were submitted, in the first place, to two PCRs using sets of primers designed to flank exons 11 and 15 of *BRAF* (Table 1). For each PCR, 1 μl of reverse-transcribed mixture was amplified with 50 pmol of each primer, 200 μM deoxynucleotide triphosphates, 10 mM Tris-HCl (pH 9.0 at 25 C), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, and 2.5 U of Taq DNA polymerase (Promega Corp., Madison, WI) in a final vol of 50 μl for exon 15, and 25 μl for exon 11. Thirty-five cycles of denaturation (95 C for 1 min), annealing (60–68 C for 1 min), and extension (72 C for 1 min) were conducted on an automated heat block (iCycler, Bio-Rad, Hercules, CA). Mutational analysis was then performed on the obtained PCR products by single-stranded conformational polymorphism (SSCP) screening. SSCP analysis was conducted using a modification of a method previously reported (30). In detail, 5 μl PCR product was mixed with 25 μl DNA gel-loading buffer (95% formamide, 20 mM NaOH, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol), denatured by incubating at 95 C for 5 min, placed on ice, and loaded onto a 0.5× mutation detection enhancement gel (MDE gel solution, Cambrex, Rockland, ME) containing 10% glycerol. Gels were run in 0.6× Tris-Borate-EDTA buffer at 4 W overnight using a D-Code Universal Mutation Detection System (Bio-Rad) at room temperature. Staining of the separated DNA bands on the gels was performed using the Gelstar Nucleic Acid Gel Stain (Cambrex), according to manufacturer's instruction, and the gels analyzed on a UV transilluminator. Direct forward and reverse sequencing of the PCR products showing a gel shift at SSCP analysis was conducted after purification with the Wizard SV Gel and PCR Clean-up System purification kit (Promega Corp.) on a 16-capillary DNA sequencer (ABI PRISM 3100 Genetic Analyzer, Applied Biosystem, Foster City, CA) at GeneLab (ENEA Casaccia, S. M.di Galeria, Roma) using the same primers of the PCR amplification (Table 1). The results of the mutation screening for BRAF of each sample were confirmed in at least two independent experiments.

Second, the cDNAs of the PTCs were amplified using sets of primers spanning the breakpoints of the *RET/PTC1* and *RET/PTC3* rearrangements (Table 1) (14). For each PCR, 2 μl reverse-transcribed mixture were amplified with 50 pmol of each primer, 200 μM deoxynucleotide triphosphates, 10 mM Tris-HCl (pH 9.0 at 25 C), 1.5 mM $MgCl_2$, 50 mM KCl, 0.1% Triton X-100, and 2.5 U of Taq DNA polymerase (Promega Corp.) in a final vol of 50 μl. After a 5-min hot-start at 99 C, four cycles of touchdown amplification were performed (progressively lowering the annealing temperature from 61 C to 57 C), followed by 40 cycles of regular amplification (95 C for 1 min, 57 C for 1 min, and 72 C for 1 min). Ten microliters of each PCR product were then electrophoresed on an ethidium bromide-stained 1.8% agarose gel, and all samples with a visible band were sent for sequencing confirmation of the amplicon specificity. All the samples were amplified at least three times, and a tumor was considered *RET/PTC1*- or *3*-positive when all three PCRs resulted in the amplification of a specific amplicon.

Southern blotting

Genomic DNA extraction from six selected PTC samples and their Southern blot analysis were conducted as previously described (31). In

TABLE 1. Primers used for the PCR amplifications

	Primer sequences (5′–3′)
BRAF exon 15	CATTGCACGACAGACTGCAC
	TCTGACTGAAAGCTGTATGG
BRAF exon 11	GTCATCTTCATCCTCAGAAGACAGG
	TAACTGCTGAGGTGTAGGTGCTGTC
RET/PTC1	GCTGGAGACCTACAAACTGA
	GTTGCCTTGACCACTTTTC
RET/PTC3	AAGCAAACCTGCCAGTGG
	CTTTCAGCATCTTCACGG

The sense primer is listed first and the antisense primer second.

detail, tumor tissue fragments, snap-frozen after collection, were digested with proteinase K (100 μg/ml) in 0.5 ml of 10-mM Tris, pH 8.0; 100-mM EDTA; 0.5% SDS at 50 C overnight. After phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0) extraction, the high molecular mass DNA was precipitated in isopropanol, washed in 70% ethanol, dissolved in double-distilled H_2O, and stored at 4 C until use. Ten micrograms of genomic DNA were digested with EcoRI (Invitrogen Corp.) at 37 C overnight, fractionated on a 1% agarose gel, transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech Italia, Milan, Italy), and hybridized with a 1.8-kb DNA probe specific for the 5′ terminal region of the *RET* proto-oncogene (kindly provided by Dr. Massimo Santoro, University of Naples Federico II) (32) labeled with [^{32}P]deoxy-CTP by random priming (Prime-It II kit; Stratagene Inc., La Jolla, CA). Hybridization and washing were conducted under stringent conditions (31) and images acquired using a phosphoimager system (Instant Imager, Camberra-Packard, Meriden, CT).

Statistical analysis

Differences in frequency of single variables were tested by the χ^2 test, with Yate's correction whenever appropriate, or by the Fisher exact test. Differences in median and range were tested by the nonparametric Mann-Whitney rank sum test. Moreover, the dependence of BRAF mutations on several variables, such as age, gender, tumor size, extracapsular growth, lymph node metastases, and papillary or solid/follicular histology or loss of differentiation, was assessed by forward stepwise conditional logistic regression analysis. P values < 0.05 were considered significant. All statistical tests were performed using the Statistical Package for Social Sciences for Windows for personal computers (SPSS, Inc., Chicago, IL).

Fifty-seven of 60 PTC cases were included in the genetico-clinical association analysis because a revision of the histological slide could be performed. However, complete clinical information was not always obtainable. Thus, search of differences in the BRAF-mutated and BRAF wild-type tumor subgroups were conducted using the available data as specified for each variable.

TABLE 2. Results of the molecular genetic analysis

	$BRAF^{V599E}$	RET/PTC1	RET/PTC3
Papillary carcinomas	24 /60 (40%)[a]	4/60 (6.5%)	5/60 (8.5%)
Follicular adenomas	0/6	nt	nt
Follicular carcinomas	0/5	nt	nt
Anaplastic carcinomas	0/1	nt	nt

nt, Not tested.
[a] $P = 0.006$ *vs.* not papillary tumors.

Results

Molecular genetic analysis

Search of *BRAF* mutations in the thyroid tumors showed the presence of a mutation of exon 15 in 24 of 60 (24/60) PTCs (40%) (Table 2). All the mutations resulted to be the same missense T to A transversion at nucleotide 1796, resulting in the substitution of a valine to glutamate at residue 599 (Fig. 1). None of the 12 non-PTC neoplasms, including six FAs, five FTCs, and one AC, showed any exon 15 mutation. Moreover, no mutation was detected at the level of exon 11 in any of the 72 thyroid tumors examined.

Search of RET/PTC expression by RT-PCR showed the presence of a reproducible *RET/PTC* rearrangement in 9/60 PTCs (15%) (Table 2). In detail, four PTCs tested positive for RET/PTC1 and five for RET/PTC3. Moreover, four samples showed nonreproducible amplification of the rearrangements, two in the BRAF mutation positive subgroup and two in the BRAF wild-type one (Table 3). The former two cases displayed one RET/PTC3 amplification out of five PCR runs for this rearrangement, whereas the latter two displayed both a RET/PTC1 and a RET/PTC3 amplification out of five PCR runs for each rearrangement. Both the BRAF mutation positive tumors resulted monofocal.

Search of *RET* rearrangements by Southern blotting in six selected PTC samples, *i.e.* one positive for RET/PTC1, one positive for RET/PTC3, two negative for RET/PTC, and two with nonreproducible RET/PTC amplification (one in the BRAF mutation positive subgroup and one in the BRAF wild-type subgroup) at RT-PCR, showed the presence of an aberrant band only in the cases with reproducible RET/PTC1 or 3 amplification (Fig. 2).

As displayed in Table 3, none of the tumors showed an overlap between a *BRAF* mutation and a reproducible *RET/PTC* rearrangement.

BRAF genetico-clinical association analysis

Table 4 displays the clinical and pathological characteristics of the PTCs and the results of the comparisons of the prevalence of the examined variables in the BRAF-mutated ($BRAF^{V599E}$) and BRAF wild-type ($BRAF^{WT}$) subgroups. The

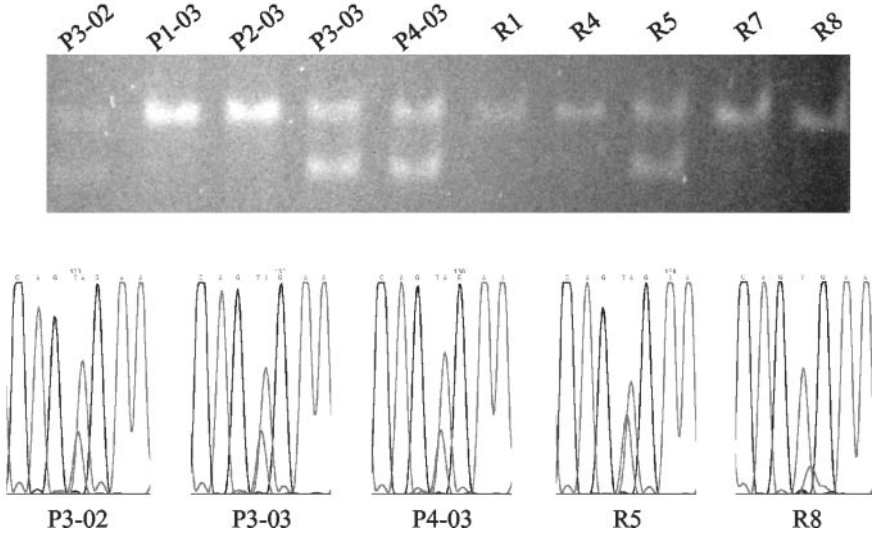

FIG. 1. Examples of *BRAF* mutations in exon 15. *Upper panel*, Results of exon 15 SSCP screening in 10 of the 60 PTC samples. Cases P3–02, P3–03, P4–03, and R5 showed gel-shifted bands, in addition to bands migrating in the expected position, suggestive of the presence of an exon 15 mutation in one of the BRAF alleles. *Lower panel*, Sequence chromatograms of PCR products of five of the PTC samples. Sequence analysis confirmed the presence of a T to A transition at base 1796 in one of the BRAF alleles in samples P3–02, P3–03, P4–03, and R5. The mutation appeared as a *double peak* at the specific position, in contrast to the *single peak* of wild-type samples, such as that of sample R8. The T to A transition introduces a substitution of amino acid valine to glutamic acid at codon 599 (V599E).

TABLE 3. Distribution of the *BRAF* mutations and the *RET/PTC* rearrangements in the PTC cohort

Case	$BRAF^{V599E}$	RET/PTC	Case	$BRAF^{V599E}$	RET/PTC
P1-97	+	−	P8-98	−	−
P2-97	+	−	P10-98	−	−
P4-98	+	−	P14-98	−	−
P7-98	+	−	P1-99	−	−[a]
P11-98	+	−	P4-99	−	−
P15-98	+	−	P5-99	−	−
P17-98	+	−[b]	P6-99	−	+
P18-98	+	−	P1-01	−	−
P19-98	+	−	P1-03	−	−
P2-99	+	−	P2-03	−	−
P8-99	+	−	R1	−	−
P9-99	+	−	R4	−	−
P1-00	+	−	R7	−	−
FA-01	+	−	R8	−	−
P1-02	+	−	R10	−	−
P3-02	+	−	44	−	−
P3-03	+	−	P5-03	−	+
P4-03	+	−	P6-03	−	+
R5	+	−	953	−	+
201	+	−[b]	40	−	−
463	+	−	111	−	−
P8-03	+	−	47	−	+
P9-03	+	−	82	−	−
P10-03	+	−	595	−	−
P3-97	−	−	69	−	−
P4-97	−	+	976	−	−[a]
P1-98	−	−	981	−	+
MP1-98	−	+	946	−	−
P3-98	−	−	77	−	−
P6-98	−	−	48	−	+

[a] Cases P1-99 and 976 displayed in 1/5 PCRs for RET/PTC1 and 1/5 PCRs for RET/PTC3 a specific amplicon.

[b] Cases P17-98 and 201, positive for BRAF, displayed in 1/5 PCR for RET/PTC3 a specific amplicon.

tumor cohort included PTCs obtained from 39 women and 18 men. The median age at surgery was 39 yr (range, 20–77), whereas the median tumor size was 2 cm (range, 0.7–6). No patient had an apparent history of neck irradiation. At the time of diagnosis, 20 of 51 cases presented with any kind of extrathyroidal extension of the tumor [classified as pT3 when minimal and pT4 in the other cases (29)] and 24 of 56 had cervical lymph node metastases (pN1). A tumor stage was defined for 38 patients. In detail, 19 patients were in stage I, two in stage II, one in stage III, and 16 in stage IV (including stages IVA, IVB, and IVC). Moreover, 20 patients of 55 had multifocal disease. Morphological diagnosis revealed 35 of 57 classic papillary PTCs, 14 of 57 solid/follicular variants, two of 57 tall cell variants, one of 57 diffuse sclerosing variant, and five of 57 tumors showing loss of differentiation. In three cases, the pathologists agreed on the diagnosis of poorly differentiated PTCs, based on the presence of areas of poorer differentiation. Moreover, in two cases, the agreement was for the diagnosis of anaplastic dedifferentiation, which was related to the finding of anaplastic cellular features in lymph node metastases generated by primary papillary carcinomas.

To disclose whether *BRAF* mutations have any relevance in driving biological behavior of PTC, we compared the prevalence of several critical clinical and pathological features of the tumors (age, sex, tumor size, extracapsular extension, pN, tumor stage, number of tumor foci, histologic

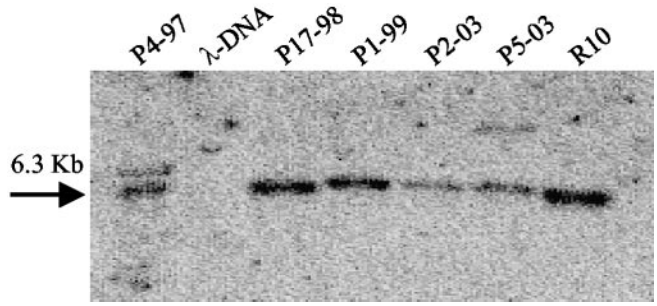

FIG. 2. Search of *RET/PTC* rearrangements in six papillary carcinomas by Southern blot. Ten micrograms of genomic DNA were digested with EcoRI, run on a 1% agarose gel, blotted onto a nylon membrane, and hybridized with a 1.8-kb DNA probe specific for the 5′ terminal region of the *RET* proto-oncogene. The *arrow* indicates the size of the normal fragment. Samples P4–97 and P5–03, characterized by RT-PCR as positive, respectively, for RET/PTC1 and RET/PTC3, showed an aberrant band. Samples P2–03 and R10, both characterized as RET/PTC negative, and samples P17–99 and P1–99, both showing nonreproducible RET/PTC amplicons, displayed only the wild-type restriction digestion pattern. λ-DNA/*Hin*dIII fragments (Invitrogen) were run in the gel as molecular weight marker control.

subtype such as classic papillary variant of PTC, solid-follicular variant of PTC, and loss of differentiation) in the BRAF-mutated ($BRAF^{V599E}$) and BRAF wild-type ($BRAF^{WT}$) subgroup. A statistical significant association was found between *BRAF* mutation and development of PTCs of the classic papillary histotype ($P = 0.038$).

Moreover, a forward stepwise conditional logistic regression analysis showed that only classic papillary histotype was significantly and independently associated with the response variable (presence of $BRAF^{V599E}$) ($P = 0.046$) (Table 5).

Discussion

BRAF mutations have been recently described to occur in several human cancer types, most notably melanomas (21), but to a lesser extent also in colorectal (21, 33), ovarian (21, 34), and lung carcinomas (33, 35) and in cholangiocarcinomas (36). Moreover, a high mutation rate of this new oncogene was reported in PTCs, with a frequency ranging from 29–69% in several thyroid tumor cohorts (22–27). In 100% of the PTCs (22–27) and in 98% of melanomas (21, 33), a unique missense T to A transversion at nucleotide 1796, resulting in the V599E substitution, has been described. The high frequency of *BRAF* mutations in PTCs and melanomas was correlated (22) to the important function played by cAMP in the regulation of thyroid cells and melanocyte growth (37, 38) and to the critical role of BRAF as intermediate in the cAMP-induced activation of MEK1 and the extracellular signal-regulated kinases in those cells (38, 39). $BRAF^{V599E}$ mutation was shown to induce transformation in NIH3T3 cells, proving its potential function as an oncogene (21).

In this article, we confirm the frequent occurrence of *BRAF* mutations in sporadic PTCs and its exclusive association with this thyroid cancer histotype. A single type of mutation was found, namely $BRAF^{V599E}$, as previously reported in other cancer types (21) and in PTCs (22–27). Moreover, also in our study, 12 of 12 non-PTC thyroid tumors, including six FAs, five FTCs, and one AC, did not exhibit mutations either in *BRAF*'s exon 15 or in *BRAF*'s exon

TABLE 4. Summary of the clinicopathological features of the PTCs and comparison of the prevalence of the examined variables in the BRAF-mutated ($BRAF^{V599E}$) and BRAF wild-type ($BRAF^{WT}$) subgroups

	Total	$BRAF^{V599E}$	$BRAF^{WT}$	*P*
Gender (male/female)	18/39	8/16	10/23	0.848
Median age (yr) (range)	39 (20–77)	39 (22–77)	38 (20–64)	0.352
Median diameter (cm) (range)	2 (0.7–6.0)	2.3 (0.7–4.0)	1.8 (1.0–6.0)	0.884
Extracapsular extension	20/51 (39.2%)	10/22 (45.4%)	10/29 (34.5%)	0.655
pN1	24/56 (42.9%)	8/23 (34.8%)	16/33 (48.5%)	0.328
Stage				
I	19/38 (50%)	8/16 (50%)	11/22 (50%)	0.929
II	2/38 (5.3%)	0/16	2/22 (9.1%)	
III	1/38 (2.6%)	1/16 (6.3%)	0/22	
IV[a]	16/38 (42.1%)	7/16 (43.7%)	9/22 (40.9%)	
Multifocality	20/52 (38.5%)	9/21 (42.9%)	11/31 (35.5%)	0.975
Classic papillary	35/57 (61.4%)	19/24 (79.2%)	16/33 (48.5%)	0.038
Solid/follicular	14/57 (24.6%)	4/24 (16.7%)	10/33 (30.3%)	0.385
Loss of differentiation	5/57 (8.8%)	0/24	5/33 (15.2%)	0.067
Other histology	3/57 (5.2%)	1/24[b] (4.1%)	2/33[c] (5.1%)	1

[a] Including stages IVA, IVB, and IVC.
[b] One tall cell variant PTC.
[c] One tall cell variant and one diffuse sclerosing variant PTC.

TABLE 5. Distribution of the histological subtypes between the BRAF-mutated and the RET/PTC-positive tumors

	$BRAF^{V599E}$	RET/PTC
Classic papillary	19/35 (54%)[a]	4/35 (11%)
Solid/follicular	4/14 (29%)	2/14 (14%)
Loss of differentiation	0/5	0/5
Other	1/3 (33%)	1/3 (33%)

[a] $P = 0.046$ by forward stepwise conditional logistic regression analysis.

11, despite the common origin of those tumors and PTCs from the follicular cell lineage. This finding confirmed the importance of the activation of the BRAF-MAPK pathway, specifically in PTC development.

A low rate of *RET/PTC* rearrangement occurrence is shown in this Italian cohort of PTCs. This finding may be related to a peculiar environmental background of the studied population. Indeed, none of the subjects had an apparent history of radiation exposure. A low sensitivity of the RT-PCR approach can be excluded, because, as already reported previously (19), this technique enables the detection of minimal amounts of both RET/PTC1 and RET/PTC3 at levels as low as 10^{-4} pg of a positive control plasmid.

Nine PTCs showed a potential clonal expression of a *RET/PTC* rearrangement, either *RET/PTC1* (four cases) or *RET/PTC3* (five cases), whereas four other tumors displayed a nonreproducible appearance of RET/PTC amplicons. *RET* rearrangement search by Southern blotting in six selected PTC samples confirmed the RET/PTC occurrence only in cases with reproducible RET/PTC amplification by RT-PCR. The observation of cases with sporadic appearance of RET/PTC amplification at RT-PCR might be due to a low expression of the oncogene, eventually limited to focal areas of the tumor, or to the occurrence of sporadic contaminations of the specific PCRs. The consistent absence of contaminations in the RT and PCR negative control reactions is in favor of the first hypothesis. Moreover, nonclonal mutations have already been shown for other oncogenes, such as activated RET in sporadic medullary thyroid carcinomas (40), and a recently identified rearrangement between the *H4* and *PTEN* genes in normal thyroid and PTC tissues (41).

Two previous studies (22, 24) have reported the absence of an overlap of *BRAF*, *RAS*, and *RET/PTC* mutations in a total number of 117 PTCs, implying the sufficiency of any component in the RET-RAS-RAF-MAPK pathway for the initiation of sporadic PTCs, whereas a third study suggested the possibility of an overlap between *BRAF* mutations and *RET/PTC* rearrangements detected using an histochemical approach (25). Our data, which show no overlap between *BRAF* mutations and reproducible RET/PTC detection, seem to confirm the sufficiency of each of the two genetic events in follicular cell transformation.

Although *RET/PTC* rearrangements presented a low frequency, altogether *BRAF* mutations and *RET/PTC* rearrangements accounted for a genetic alteration, potentially involved in tumor initiation, in 55% of the cancers. *RET/PTC* rearrangements have been specifically associated above all with thyroid radiation exposure in childhood (13–17). Thus, it is possible to speculate that the finding of a high prevalence of *BRAF* mutations associated with a low rate of *RET/PTC* rearrangements in adult sporadic PTC reflects a difference in the etiologic factors involved in the development of the two conditions; in the former case, ionizing radiation being the leading factor; in the second, a yet nonidentified cause, if not simply spontaneous mutations of the *BRAF* gene.

Finally, our study allows us to outline a potential biological property of *BRAF* mutations. Indeed, the genetico-clinical association analysis showed the significant and independent association of $BRAF^{V599E}$ with the development of a classic papillary histotype. Moreover, none of the PTCs showing loss of differentiation tested positive for the BRAF mutation, even if, in this case, we could not reach a statistically significant association ($P = 0.067$). Interestingly, this latter observation is in agreement with a previous report that points out that another intermediate in the RET-RAS-RAF-MAPK signaling pathway, namely *RET/PTC* rearrangement, defines a subset of PTCs lacking evidence of progression to poorly differentiated or undifferentiated tumor phenotypes (42). However, though the finding of an association between *BRAF* mutation and the development of classic papillary PTCs has already been reported (28), the possibility that

BRAF mutations reduce the risk of a histological progression is in conflict with two previous studies (27, 28). Indeed, in one study, two of the six undifferentiated thyroid cancers examined showed a *BRAF* mutation (27); whereas, in the other, two of 16 poorly differentiated thyroid carcinomas and three of 29 ACs had the mutation (28). In the latter, the positive cases represented five of the 12 dedifferentiated cancers still showing a component of well-differentiated PTC, which itself displayed the same mutation (28). The reasons for these discrepancies is not clear, but might be related to differences in the cancer selection criteria and to the low number of cases composing each tumor cohort. By pulling together the poorly differentiated and undifferentiated thyroid cancers showing a papillary origin from our and the former studies, BRAF mutations are present in five of 17 cancers. This proportion might indicate that less than 30% of poorly or undifferentiated thyroid cancers, deriving from PTC, harbor a mutation of *BRAF*, implying that $BRAF^{V599E}$ may represent a mutation that is equal to others, or eventually protective, in driving tumor progression. Additional studies of *BRAF* mutation in poorly or undifferentiated thyroid cancers originating from well-differentiated PTCs are needed to reach more certain conclusions on this topic.

In summary, these data suggest that $BRAF^{V599E}$ mutation is the most frequent genetic alteration detected in adult sporadic PTCs and that its activation may drive the development of classic papillary variant PTCs.

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