BRAF$^{(V600E)}$ mutation and the biology of papillary thyroid cancer

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Abstract

BRAF$^{(V600E)}$ mutation is the most frequent genetic alteration in papillary thyroid carcinomas (PTCs) that are 80–90% of all thyroid cancers. We evaluated the relationship between *BRAF*$^{(V600E)}$ and tumor, host, and environmental factors in PTCs from all geographical areas of Sicily. By PCR, *BRAF*$^{(V600E)}$ was investigated in a series of 323 PTCs diagnosed in 2002–2005. The correlation between clinicopathological tumor, host, and environmental characteristics and the presence of *BRAF*$^{(V600E)}$ were evaluated by both univariate and multivariate analyses. *BRAF*$^{(V600E)}$ was found in 38.6% PTCs, with a 52% frequency in the classical PTCs and 26.4% in the tall cell variant. Univariate analysis indicated that *BRAF*$^{(V600E)}$ was associated with greater tumor size ($P=0.0048$), extra-thyroid invasion ($P<0.0001$), and cervical lymph nodal metastases ($P=0.0001$). Multivariate logistic regression analysis confirmed that *BRAF*$^{(V600E)}$ was an independent predictor of extra-thyroid invasion ($P=0.0001$) and cervical lymph nodal metastasis ($P=0.0005$). The association between *BRAF*$^{(V600E)}$ and extra-thyroid invasion was also found in micro-PTCs ($P=0.006$). In 60 classical PTCs, *BRAF*$^{(V600E)}$ was positively correlated with matrix metalloproteinase-9 expression ($P=0.0047$), suggesting a possible mechanism for BRAF$^{(V600E)}$ effect on PTC invasiveness. No association was found between *BRAF*$^{(V600E)}$ and patient age, gender, or iodine intake. In contrast, a strong association was found with residency in Eastern Sicily ($P<0.0001$ compared with Western Sicily). These results indicate that *BRAF*$^{(V600E)}$ mutation is a marker of aggressive disease in both micro- and macro-PTCs. Moreover, for the first time, a possible link between *BRAF*$^{(V600E)}$ mutation and environmental carcinogens is suggested.

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Introduction

Thyroid cancer is the 8th most common malignancy in women (3% of all cancers in women) and this value doubles the prevalence of 15 years ago, when it was 1.7% of all cancers, ranking 14th in the list. Indeed, thyroid cancer incidence has increased faster than any other malignancy (3.8% per year in the period 1992–2001). At present, the incidence of thyroid cancer is estimated between 5 and 8 cases per 10^5 inhabitants per year in developed countries (Akslen *et al*. 1993, Colonna *et al*.

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2002, Gomez Segovia *et al*. 2004). Furthermore, among men, age-adjusted mortality for thyroid cancer has increased faster than any other cancer (2.3% per year in the period 1992–2001; Howe *et al*. 2001).

Increased diagnostic activity and accuracy, with micro-papillary thyroid cancer (micro-PTCs; tumors up to 1 cm in diameter) increasing from 15–30 to 45–50% (Leenhardt *et al*. 2004), and changes in the histological WHO diagnostic criteria (Pathology and Genetics of Tumors of Endocrine Organs edited by R A DeLellis, R V Lloyd, P U Heitz and C Eng. IARC Press, Lyon 2004) have certainly contributed to the very high prevalence of the papillary histotype among thyroid cancer. However, a real increase of PTC incidence cannot be excluded and has been attributed to either iodine supplementation (Farahati *et al*. 2004) or exposure to radiation or other environmental endocrine disruptors, especially during childhood (Tronko *et al*. 2006).

Many genetic alterations have been implicated in PTCs, and mostly involve the aberrant activation of the RAS–RAF–MEK–MAP kinase pathway, due to either *RET/PTC* rearrangement (10–50% of PTCs; Nikiforov 2002, Santoro *et al*. 2002*a*), or *RAS* mutations (1–10% of PTCs; Giordano *et al*. 2005) or *BRAF* mutations (28–83% of PTCs; Xing 2005). BRAF is a serine–threonine kinase abundantly expressed in thyroid follicular cells (Daum *et al*. 1994), which activates MEK1 and MEK2 (Peyssonnaux & Eychene 2001). The most common *BRAF* mutation (*BRAF*$^{(V600E)}$) accounts for over 90% of all *BRAF* mutations and consists of a thymine-to-adenine transversion at position 1799 in exon 15 of *BRAF*, leading to a valine-to-glutamate transversion at residue 600 and thus facilitating ATP binding (Garnett & Marais 2004). Analysis of *BRAF* exon 15 in large series of thyroid carcinomas indicated a high frequency of *BRAF*$^{(V600E)}$ in classical and tall cell variants of PTCs (Nikiforova *et al*. 2003, Nakamura *et al*. 2005, Xing *et al*. 2005). *BRAF*$^{(V600E)}$, but not RET/PTC rearrangements (Santoro *et al*. 1992), is often found in anaplastic thyroid cancer histotype (Begum *et al*. 2004), suggesting that this mutation may be involved in thyroid cancer progression to poorly differentiated and aggressive phenotypes (Nikiforova *et al*. 2003, Nikiforov 2004, Quiros *et al*. 2005). Studies evaluating *RET/PTC* rearrangement, *BRAF*$^{(V600E)}$, and *RAS* mutations indicated that in PTCs these molecular alterations are mutually exclusive and suggest that one activating mutation along the RAS–MAPK pathway is sufficient for thyroid cell malignant transformation (Melillo *et al*. 2005, Xing *et al*. 2005). However, different mechanisms and different tumor biology may characterize cancers arising from different oncogenes. At variance with RET/PTC, *BRAF*$^{(V600E)}$ positive PTCs were not found to be in relationship to previous radiation exposure (Nikiforova *et al*. 2004); no other environmental factors has been identified that favors *BRAF*$^{(V600E)}$ mutation.

Several studies tried to establish a correlation between *BRAF*$^{(V600E)}$ and the clinical features of PTCs, but results have been controversial (Lee *et al*. 2007, Xing 2007): in some studies, *BRAF*$^{(V600E)}$ was associated with a more advanced tumor or a more aggressive phenotype (Namba *et al*. 2003, Nikiforova *et al*. 2003, Kim *et al*. 2004, 2006*a*,*b*, Oler *et al*. 2005, Powell *et al*. 2005, Vasko *et al*. 2005, Xing *et al*. 2005, Jin *et al*. 2006, Lee *et al*. 2006, Riesco-Eizaguirre *et al*. 2006, Giannini *et al*. 2007, Kebebew *et al*. 2007, Lupi *et al*. 2007, Rodolico *et al*. 2007), while other studies did not find this association (Fugazzola *et al*. 2004, 2006, Puxeddu *et al*. 2004, Kim *et al*. 2005, Liu *et al*. 2005, Trovisco *et al*. 2005, Jo *et al*. 2006, Park *et al*. 2006, Sapio *et al*. 2006, Abrosimov *et al*. 2007, Durante *et al*. 2007, Mitsiades *et al*. 2007; see Table 1).

Table 1 Studies indicating or not a positive correlation of *BRAF*$^{(V600E)}$ with increased papillary thyroid carcinomas (PTC) aggressiveness

Studies	No. of PTCs	Country
Positive correlation		
Namba *et al*. (2003)	170	Japan
Nikiforova *et al*. (2003)	119	USA (OH) and Italy
Kim *et al*. (2004)	70	Korea
Oler *et al*. (2005)	13	Brazil
Powell *et al*. (2005)	67	Ukraine
Vasko *et al*. (2005)	33	Ukraine
Xing *et al*. (2005)	219	Multicentric
Jin *et al*. (2006)	58	USA (MI)
Kim *et al*. (2006*a*)	203	Korea
Kim *et al*. (2006*b*)	103	USA (CA)
Lee *et al*. (2006)	100	Korea
Riesco-Eizaguirre *et al*. (2006)	67	Spain
Giannini *et al*. (2007)	69	Italy
Kebebew *et al*. (2007)	274	USA (CA)
Lupi *et al*. (2007)	500	Italy
Rodolico *et al*. (2007)	214	Italy
Total (16)	2279	
No correlation		
Fugazzola *et al*. (2004)	53	Italy
Puxeddu *et al*. (2004)	60	Italy
Kim *et al*. (2005)	60	Korea
Liu *et al*. (2005)	105	Taiwan
Trovisco *et al*. (2005)	126	Portugal
Jo *et al*. (2006)	163	Korea
Park *et al*. (2006)	140	Korea
Sapio *et al*. (2006)	43	Italy
Fugazzola *et al*. (2006)	260	Italy (multicentric)
Abrosimov *et al*. (2007)	44	Russia
Durante *et al*. (2007)	53	Italy
Mitsiades *et al*. (2007)	58	Greece
Total (12)	1165	

Discrepancies between those studies, however, may depend on heterogeneous series of tumors, including different PTC variants and tumors from different geographical areas (Lee *et al*. 2007, Xing 2007).

BRAF$^{(V600E)}$ is also the most common genetic alteration occurring in micro-PTCs (~30–50% of cases; Sedliarou *et al*. 2004, Barbaro *et al*. 2005, Trovisco *et al*. 2005). Micro-PTCs are thyroid tumors up to 10 mm in diameter, nearly always with papillary histotype and generally considered at a very low risk of progression and/or recurrence. Although predictors of persisting/relapsing disease have been established for PTCs over 10 mm in diameter and include lymph nodal metastases, extra-thyroid invasion, sclerosant variant, and bilateral foci, data on micro-PTCs are scanty (Lin *et al*. 2005, Ito *et al*. 2006). The possible predictive value of *BRAF*$^{(V600E)}$ in micro-PTCs is unknown.

We have now examined the prevalence of *BRAF*$^{(V600E)}$ in a large series of PTCs, occurring in Sicilian residents, and report that *BRAF*$^{(V600E)}$ is a common genetic alteration in PTCs. *BRAF*$^{(V600E)}$ was significantly related to classical PTCs and also to greater tumor size, extra-thyroid invasion, lymph nodal metastases, and advanced stage. Moreover, *BRAF*$^{(V600E)}$ was independently associated with extra-thyroid invasion and with patient residency in certain geographical areas (Eastern Sicily), suggesting a possible link between environmental factors, *BRAF* mutation, and thyroid tumorigenesis.

Patients and methods

Study design, specimen, and information collection

The study was designed as a *BRAF*$^{(V600E)}$ prevalence survey in PTC occurring in residents of the island of Sicily (a large island in the Mediterranean sea, 5.1 million inhabitants) and also an association study between *BRAF*$^{(V600E)}$ positivity in PTCs and tumor, host, and environmental variables. All patients were diagnosed and underwent surgery of the thyroid in the years 2002–2005. The study was approved by our institution's Ethics and Research Committees and was in agreement with the World Medical Association's 1975 Declaration of Helsinki, revised in 1983. Paraffin-embedded specimens were obtained from Pathological Anatomy Institutes from all geographical areas of Sicily. Histological slides, after hematoxylin and eosin staining, were reviewed by two independent pathologists to confirm the diagnosis and to classify the papillary tumors for the different PTC histotype variants, based on the histopathological typing of the World Health Organization Classification of Tumors (Pathology and Genetics of Tumors of Endocrine Organs; R A DeLellis, R V Lloyd, P U Heitz and C Eng. Eds; IARC Press, Lyon, 2004). Classical papillary thyroid carcinomas were classified all carcinomas containing true papillae with a central fibrovascular core and a lining of cuboidal cells with ground glass nuclei, nuclear pseudoinclusions, and nuclear grooves. We considered as follicular variant papillary thyroid carcinomas (FV-PTCs) tumors composed entirely or almost entirely of follicles, with cells displaying nuclear features of PTCs. Tall cell variant papillary carcinomas (TCV-PTCs) were characterized by papillae lined by a single layer of tall cells (height at least twice the width), with nuclei usually lacking the features of classical papillary thyroid carcinomas and present in more than 50% of each slide. Finally, PTCs measuring 10 mm or less in maximum diameter were considered micro-PTCs.

As a total, 393 archival specimens were collected: 323 were PTCs and are the object of the present study. Additional 70 specimens that included normal (peritumoral or controlateral) thyroid tissue and colloid goiters, follicular adenomas and carcinomas, and anaplastic carcinomas (ATCs) were also examined at the same time.

Surgery medical records provided the information regarding patients (age, sex, and residence) and tumors (volume, multifocality, bilaterality, lymph nodal involvement, extra-thyroid invasion, distant metastases, and grading). Clinical staging of thyroid cancer was classified according to the Tumor-node-metastasis (TNM) classification (VI Edition; Dobert *et al*. 2004). Persistent disease was evaluated in 118 patients, with a 2- to 4-year follow-up.

Information regarding environmental goitrogens (iodine deficiency and cyanate) were obtained by previous scientific publications regarding the presence of these goitrogens in different areas of Sicily (Delange *et al*. 1978, Belfiore *et al*. 1987, 1992, Vigneri 1988).

Laser capture microdissection (LCM), DNA isolation, and sequencing

Ten micrometer sections of paraffin-embedded thyroid tissue samples (micro-PTCs and TCV-PTCs) were deparaffinized with xylene, washed with ethanol, rehydrated in deionized water, and stained with hematoxylin and eosin. Normal thyroid follicular cells and a subset of micro-PTCs and TCV-PTCs with prominent stromal component were microdissected by Prof. M Loda at the Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, using the Veritas LCM and Laser Cutting System (model LCC1704) from Arcturus Engineering

(Mountain View, CA, USA). The presence of the tumor tissue was confirmed in the first and the last section for each section series. Microdissected tissues were collected on CapSure Macro LCM Caps (Arcturus Bioscience, Basel, Switzerland). Genomic DNA was extracted using Pico Pure DNA extraction kit (Arcturus Bioscience), according to the manufacturer's recommendations. The samples were immediately placed into 50 µl proteinase K DNA extraction solution and incubated at 65 °C for 16 h. Samples were subsequently heated at 95 °C for 10 min to inactivate the proteinase K, centrifuged, and the supernatant collected. DNA was measured with NanoDrop ND-1000 spectrophotometer (Wilmington, DE, USA) and used as template for PCR amplification and sequencing analysis. The following intron-based PCR primers were designed to amplify the *BRAF* exon 15: forward TCATAATGCTTGCTCTGATAGGA and reverse GGCCAAAAATTTAATCAGTGGA. PCRs were performed using standard PCR conditions (95 °C for 5 min, 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, for 40 cycles; 70 °C for 10 min). p53 mutations were evaluated by PCR using primers amplifying p53 exons 5–8, in accordance with Fagin *et al*. (1993). H-, N-, and K-Ras families (codons 12/13 and 59/61) were evaluated by PCR using primers in accordance with Vasko *et al*. (2003). The amplified products were purified by MinElute PCR Purification kit (Qiagen) and sequenced on an ABI PRISM 3730xl automated capillary DNA Sequencer using the BigDye 3.1 terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City CA, USA).

RT-PCR

Frozen thyroid tissues were available in 50 cases of papillary thyroid carcinomas and 10 normal thyroid tissues near tumor. Total RNA was extracted from 30 to 100 mg tissue using a commercial kit (Trizol, Invitrogen-Life Technologies), according to the manufacturer's protocol.

Total RNA was reverse transcribed into cDNA using ThermoScript RT-PCR system (Invitrogen-Life Technologies). In particular, 1 µg RNA recovered from fresh tissues was used for the production of cDNA in a mixture containing 10 mM dNTP mix, 1 µl random hexameres (50 ng/µl), 5× cDNA synthesis buffer (250 mM Tris acetate pH 8.4, 375 mM potassium acetate, and 40 nM magnesium acetate), 0.1 M dithiothreitol, 15 U ThermoScript transcriptase, and 40 U RNaseOUT (Invitrogen-Life Technologies) in a final volume of 20 µl using the following RT-PCR conditions: denature RNA by incubating at 65 °C for 5 min and then the samples were incubated at 25 °C for 10 min, followed by 50 min at 55 °C. To verify the integrity of mRNA of all samples, primers specific for the ELE-1 gene (forward ATTGAAGAAATTG-CAGGCTC and reverse TGGAGAAGAGGAGCTG-TATCT) were used as a control. Moreover, cDNA was also used for the detection of *AKAP9–BRAF* rearrangement by PCR analysis and using primers located in exon 8 of *AKAP9* (Ex8A, 5′-AGCAAGAACAGTT-GATTTTGGA-3′) and exon 10 of *BRAF* (Ex10B, 5′-GCAGACAAACCTGTGGTTGA-3′, with the expected product of 181 bp, according to Ciampi *et al*. (2005).

The activation of tyrosine kinase (TK) domain of the RET was screened according to Santoro *et al*. (2002*b*) (sense primer 5′-TGGGAATTCCCTCGGAAGAA-3′, nucleotide position 2149–2168; antisense primer 5′-TGCAGGCCCCATACAATTTG-3′, nucleotide position 2364–2383; expected fragment size 235 bp). RET/PTC1 and RET/PTC3 rearrangement expression were performed through PCR analysis using reaction conditions according to Puxeddu *et al*. (2003). RET/PTC-1 was amplified using sense primer 5′-GCTGGAGACCTACAAACTGA-3′ and antisense primer 5′-GTTGCCTTGACCACTTTTC-3′ (expected fragment size 165 bp); RET/PTC-3 was amplified using sense primer 5′-AAGCAAACCTGCCAG-TGG-3′ and antisense primer 5′-CTTTCAGCATCTT-CACGG-3′ (expected fragment size 242 bp). Positive controls included cDNA samples from a thyroid medullary carcinoma with constitutive expression of wild-type RET (for amplification of RET-TK), cDNA samples of TPC-1 cell line for amplification of RET/PTC1, and cDNA samples of a classical papillary carcinoma (PTC) case (kindly provided by Dr E Puxedddu, University of Perugia) for amplification of RET/PTC3. Amplification in the absence of RNA was used as a negative control. Experiments to investigate RET-TK activation, RET/PTC1 and RET/PTC3 rearrangement expression were carried out for five times. Finally, the specificity of rearrangements was verified by direct sequencing analysis.

Immunohistochemistry (IHC)

Thyroid tissue sections were immunohistochemically stained for matrix metalloproteinase-2 and -9 (MMP-2 and MMP-9), using the standard avidin–biotin peroxidase complex (ABC) method. In brief, the sections were processed to unmask antigens by means of microwave oven heating in 10 nM citric acid buffer (pH 6.0) and subsequent detergent treatment using polyoxyethylene sorbitan monolaurate (Tween 20) for

30 min. The sections were then treated with normal serum for 20 min, followed by the application (4 °C overnight) of primary monoclonal antibodies against either MMP-2 (clone A-Gel-VC2, dilution 1:1000) or MMP-9 (polyclonal, dilution 1:500; Thermo Scientific, Freemont, CA, USA). The sections were then treated with a biotinylated antibody (Vector Lab, Burlingame, CA, USA), and then with the ABC (Vectastein ABC kit, Vector Lab) for 1 h each. The reaction products were developed in 3.3-diaminobenzidine tetrahydrochloride solution containing 0.03% H_2O_2. Nuclei were lightly counterstained with hematoxylin. No staining was obtained when nonimmune serum or PBS was used instead of the primary antibodies. Finally, we have used the following staining intensity in order to evaluate MMP-2 and MMP-9 immunohistochemical expression: 3+ (strong), 2+ (moderate), 1+ (weak), and 0 (absent). The average score in our series was 2 to 3+. The threshold of positivity for MMP staining was 1+ in more than 50% of each tumor slide.

Statistical analysis

Group comparisons of categorical variables were performed using the Fisher's exact test for independence. Nonparametric statistics was used to compare the continuous variables. Multivariate logistic regression analyses were used to assess the independent association of *BRAF*$^{(V600E)}$ mutation with the different host and geographical characteristics. A P value <0.05 was considered statistically significant. Statistical analysis was performed with the StatView Software (SAS Institute, Cary, NC, USA).

Results

Prevalence of *BRAF*$^{(V600E)}$ mutation in PTCs

BRAF$^{(V600E)}$ mutation was evaluated in a series of 323 papillary carcinomas (PTCs) by direct DNA sequencing of the PCR-amplified exon 15. Overall, the prevalence of *BRAF*$^{(V600E)}$ in PTCs was 125 out of 323 (38.6%). When the prevalence of *BRAF*$^{(V600E)}$ was calculated for each PTC histological variant it was present in 116 out of 223 (52%) C-PTCs, 9 out of 34 (26.4%) TCV-PTCs, none of the 52 FV-PTCs, and none of the remaining 14 PTCs, including Warthin-like, diffuse sclerosant, and oncocytic variant PTCs. Additional 70 specimens including 16 goiters, 15 specimens of peritumoral normal thyroid tissue, 12 follicular adenomas, and 12 follicular carcinomas were all *BRAF*$^{(V600E)}$ negative. In contrast, *BRAF*$^{(V600E)}$ was found in 5 out of 15 (33.3%) ATCs.

An aliquot of the 323 PTC specimens was also examined by sequencing for other thyroid cancer common molecular abnormalities, including mutations of *BRAF* exon 11 ($n=207$ PTCs), K-, N-, and H-Ras (61 PTCs including 24 classical PTCs, 24 FV-PTCs, and 13 TCV-PTCs, all negative for *BRAF*$^{(V600E)}$), and of p53 ($n=14$ ATCs). With the exception of one mutation in codon 273 of *TPp53* in one ATC, no other mutation was found. *RET/PTC* and *AKAP9–BRAF* rearrangements were also evaluated in 50 PTCs, where frozen tissue was available. All samples were negative for *AKAP9–BRAF* rearrangement; one tumor was positive for *RET/PTC-1* and another for *RET/PTC-3* rearrangements (2 out of 50 cases=4%). Both these tumors were negative for *BRAF*$^{(V600E)}$.

These data are in accordance with previous reports (Nikiforova *et al*. 2003, Trovisco *et al*. 2004, Nakamura *et al*. 2005, Xing *et al*. 2005) and indicate that *BRAF*$^{(V600E)}$ is the most common molecular abnormality in PTCs. *BRAF*$^{(V600E)}$ mutation is strongly associated with the classical PTC histotype (C-PTC) and is also present in approximately one out of four TCV-PTCs and one out of three ATCs.

BRAF$^{(V600E)}$ and PTC patient characteristics

We first evaluated the correlation between the presence of *BRAF*$^{(V600E)}$ mutation and patient characteristics, including age, sex, and geographical residency of the patient in Sicily, by univariate analysis (Table 2). The presence of *BRAF*$^{(V600E)}$ was not associated with patient age or gender ($P=0.7709$ and 0.6887 respectively) in accordance with some (Puxeddu *et al*. 2004, Sedliarou *et al*. 2004) but not all previous reports (Penko *et al*. 2005, Powell *et al*. 2005, Rosenbaum *et al*. 2005).

In contrast, *BRAF*$^{(V600E)}$ frequency was significantly higher in PTCs occurring in the 222 patients resident of Eastern Sicily with respect to the 101 patients resident of Western Sicily (45.9% vs 22.7% respectively, $P<0.0001$; Table 2 and Fig. 1). To better clarify this association, a multivariate analysis adjusted for patient age, gender, tumor size, histotype, multifocality, and iodine intake was performed (Table 2, insert). The odd ratio for *BRAF*$^{(V600E)}$ positivity in PTCs from residents in Eastern Sicily was 3.5 (95% CI=1.9–6.4) with respect to Western Sicily. This correlation was maintained even when data were further adjusted for lymph nodal metastases and extra-thyroid invasion (Table 2, insert, $P<0.0001$ and $P=0.003$ respectively). Taken together, these results suggest that the

Table 2 *BRAF*$^{(V600E)}$ mutation and patient characteristics in 323 papillary thyroid carcinomas (PTCs)

	BRAF+	BRAF−	*P* value
Age (years)	46.3±15.4 (*n*=125)	45.8±14.1 (*n*=198)	0.7709
Sex			
Male (*n*=56)	23 (41.1%)	33 (58.9%)	0.6887
Female (*n*=267)	102 (38.2%)	165 (61.8%)	
Provenience			
Eastern Sicily (*n*=222)	102 (45.9%)	120 (54.1%)	0.0001 (see insert)
Western Sicily (*n*=101)	23 (22.8%)	78 (77.2%)	
Iodine deficiency			
Yes (*n*=53)	18 (34.0%)	35 (66.0%)	0.4394
No (*n*=270)	107 (39.6%)	163 (60.4%)	

	Odds ratio	95% CI	*P* value
Provenience from Eastern Sicily	3.5	1.9–6.4	<0.0001
(a) Adjusted also for lymph nodal metastasis	3.5	1.9–6.6	<0.0001
(b) Adjusted also for both lymph nodal metastasis and extra-thyroid invasion	3.2	1.7–6.0	0.0003

Patient residence in either Eastern or Western Sicily was also analyzed by multivariate logistic regression analysis adjusted for age, gender, multifocality, tumor size, histology, and iodine-deficient area of residence.

occurrence of *BRAF*$^{(V600E)}$ positive PTCs may be influenced by environmental factors active in certain geographical areas of Eastern Sicily and indicate, for the first time, a strong independent association between *BRAF*$^{(V600E)}$ mutation in PTCs and environmental factors.

Since iodine is an important environmental factor affecting thyroid cancer prevalence and histotype,

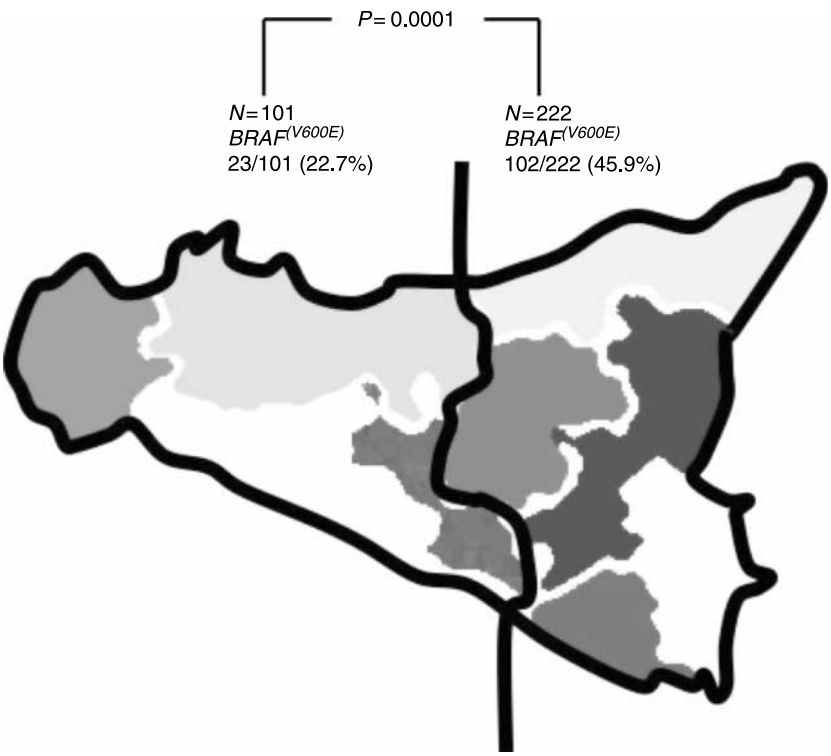

Figure 1 *BRAF*$^{(V600E)}$ frequency was calculated in PTCs occurring in patients resident of Eastern Sicily (right), including the districts of Catania, Messina, Enna, Ragusa, and Siracusa, and Western Sicily (left), including districts of Palermo, Caltanissetta, Agrigento, and Trapani.

we analyzed the relationship between iodine intake and $BRAF^{(V600E)}$ prevalence in PTCs (Table 2). Endemic goiter due to mild–moderate iodine deficiency has been previously described in Sicily in both the highlands and also in scattered areas in the mountains of both Eastern and Western Sicily (Delange *et al*. 1978, Belfiore *et al*. 1987, 1992, Vigneri 1988, Regalbuto *et al*. 1996). Approximately, 10% of the Sicilian population is exposed to the risk of iodine deficiency diseases (Delange *et al*. 1978, Belfiore *et al*. 1987, 1992, Vigneri 1988, Regalbuto *et al*. 1996). The 323 PTCs were therefore subdivided according to patient residency in either iodine-sufficient ($n=270$ cases) or iodine-deficient areas ($n=53$ cases). The prevalence of $BRAF^{(V600E)}$ positive PTCs was similar in the two groups: 107 out of 270 (39.6%) and 18 out of 53 (33.9%) in iodine-sufficient and iodine-deficient areas respectively ($P=0.4394$). These data suggest that iodine deficiency *per se* is not an environmental factor favoring $BRAF^{(V600E)}$ mutations in PTCs.

Correlation between $BRAF^{(V600E)}$ mutation and clinicopathological features of PTCs

A positive correlation between $BRAF^{(V600E)}$ and PTC aggressiveness has been reported in some studies but not in all studies (Table 1). These differences might be due to the heterogeneity of PTCs recruited, in terms of different histological subtypes, different populations studied (in many series patients were from different areas or even different Countries). Genetic and/or environmental differences, therefore, might have influenced the results obtained.

In our homogeneous series, we first analyzed the association between $BRAF^{(V600E)}$ positivity and histopathological parameters of tumor aggressiveness (Table 3). By univariate analysis, the presence of $BRAF^{(V600E)}$ in the 323 PTCs was strongly associated with greater tumor size ($P=0.0048$), extra-thyroid invasion ($P<0.0001$), and lymph nodal metastases ($P=0.0001$; Table 3, Top). A weak association was observed with stages III–IV ($P=0.051$; Table 3, Top). Moreover, in a subset of 118 PTCs, where follow-up longer than 2 years was already available, a weak association of $BRAF^{(V600E)}$ and persistent disease was also found ($P=0.0531$; Table 3, Top). No significant correlation was found with multiple foci ($P=0.1717$). Distant metastases and tumor recurrence were not evaluated because of the insufficient number for statistical analysis. Univariate analysis, therefore, suggested that $BRAF^{(V600E)}$ mutation in PTCs was strongly associated with at least three markers of aggressive and invasive disease.

When extra-thyroid invasion, lymph nodal metastases, multifocality, and tumor stages (I and II versus III and IV) were separately evaluated in the subset of 223 classical variant PTCs according to the presence or the absence of $BRAF^{(V600E)}$ mutation, a clear correlation was observed at univariate analysis between $BRAF^{(V600E)}$ positivity and indicators of tumor aggressiveness (not shown). Such a relationship was not found in the other PTC histological variants but the number of cases (9 $BRAF^{(V600E)}$ positive tumors in 100 PTCs) was insufficient to allow a reliable analysis. Hence, we then evaluated by multivariate analysis the $BRAF^{(V600E)}$ association with PTC aggressiveness (Table 3, bottom). In fact, after correcting for histology subtype, $BRAF^{(V600E)}$ was still associated with extra-thyroid invasion ($P<0.0001$), lymph nodal metastasis ($P=0.0004$), and advanced tumor stage ($P=0.0126$; Table 3, bottom). These data indicate that $BRAF^{(V600E)}$ is a predictor of tumor aggressiveness independent from PTC histotype variants.

Since the presence of $BRAF^{(V600E)}$ is more frequent in PTCs of larger size (over 10 mm), a parameter that may strongly influence tumor aggressiveness and staging, we then performed a multivariate logistic regression analysis by first adjusting for tumor size (Table 3, bottom). With this analysis, a significant correlation was still found between $BRAF^{(V600E)}$ presence and both extra-thyroid invasion ($P=0.0001$) and lymph nodal metastasis ($P=0.0005$) but not with more advanced tumor stage (a likely consequence of the fact that size is an important parameter for tumor staging). These results indicate that $BRAF^{(V600E)}$ is a good predictor of extra-thyroid invasion and lymph nodal metastases, independently from tumor size.

We then evaluated whether patient geographical residence could affect the relationship between $BRAF^{(V600E)}$positivity and tumor aggressiveness (Table 3, bottom). The correlation between $BRAF^{(V600E)}$ positivity and extra-thyroid invasion was significant both in patients living in Eastern Sicily and Western Sicily at univariate analysis (not shown). At multivariate analysis, after adjusting for patient provenience, $BRAF^{(V600E)}$ correlation with extra-thyroid invasion and lymph nodal metastases was also significant ($P=0.0002$ and 0.0004 respectively; Table 3 bottom).

Data were then adjusted for patient age, gender, and residence and also for tumor characteristics such as multifocality, size, and histology subtype. The association of $BRAF^{(V600E)}$ with extra-thyroid invasion, lymph nodal metastasis, and advanced stage was maintained ($P<0.0001$, $P=0.0074$, and $P=0.0374$ respectively; Table 3, bottom), confirming that

Table 3 Statistical analysis of *BRAF$^{(V600E)}$* mutation and clinicopathological features of tumor aggressiveness in 323 papillary thyroid carcinomas (PTCs)

Univariate analysis	**BRAF+**	**BRAF−**	***P* value**
Size	19.9±10.9 (n=125)	16.3±11.3 (n=198)	0.0048
Histology			
Classical (n=223)	116 (52%)	107 (48%)	<0.0001
Other variants (n=100)	9 (9%)	91 (91%)	
Extra-thyroid invasion			
Yes (n=87)	51 (58.6%)	36 (41.4%)	<0.0001
No (n=236)	74 (31.4%)	162 (68.6%)	
Lymph nodal metastasis			
No (n=256)	85 (33.2%)	171 (66.8%)	0.0001
Yes (n=67)	40 (59.7%)	27 (40.3%)	
Multifocality			
Yes (n=127)	55 (43.3%)	72 (56.7%)	0.1717
No (n=196)	70 (35.7%)	126 (64.3%)	
Tumor stage			
I–II (n=251)	90 (35.9%)	161 (64.1%)	0.051
III–IV (n=72)	35 (48.6%)	37 (51.4%)	
Persistent disease (n=118)			
Yes (n=29)	19 (65.5%)	10 (34.5%)	0.0531
No (n=89)	38 (42.7%)	51 (57.3%)	
Multivariate analysis	**Odds ratio**	**95% CI**	***P* value**
Adjusted for tumor histology			
Extra-thyroid invasion	5.5	2.9–10.5	<0.0001
Lymph nodal metastasis	3.1	1.7–5.8	0.0004
Multifocality	1.6	1.0–2.7	0.0564
Tumor stages III–IV	2.2	1.2–4.0	0.0126
Adjusted for tumor size			
Extra-thyroid invasion	2.8	1.7–4.7	0.0001
Lymph nodal metastasis	2.7	1.5–4.8	0.0005
Multifocality	1.2	0.8–2.0	0.3678
Tumor stages III–IV	1.5	0.9–2.6	0.1446
Adjusted for patient residence			
Extra-thyroid invasion	2.7	1.6–4.5	0.0002
Lymph nodal metastasis	2.8	1.6–4.9	0.0004
Multifocality	1.2	0.8–2.0	0.3865
Tumor stages III–IV	1.6	1.0–2.8	0.0778
Adjusted for age, gender, multifocality, histology, size, and residence			
Extra-thyroid invasion	4.1	2.0–8.2	<0.0001
Lymph nodal metastasis	2.5	1.3–5.0	0.0074
Tumor stages III–IV	2.2	1.0–4.6	0.0374
Extra-thyroid invasion[a]	3.6	1.8–7.3	0.0003
Lymph nodal metastasis[b]	1.9	0.9–3.8	0.089
Tumor stages III–IV[a]	1.8	0.8–3.9	0.1357
Tumor stages III–IV[b]	1.1	1.0–4.6	0.8746

[a]Adjusted also for lymph nodal metastasis.
[b]Adjusted also for extra-thyroid invasion.

BRAF$^{(V600E)}$ is a good independent predictor of tumor aggressiveness. A possible relationship between these three parameters, independent from *BRAF$^{(V600E)}$* cannot be excluded, especially for advanced stage, which is influenced by both extra-thyroid invasion and lymph nodal metastases. To test this hypothesis, extra-thyroid invasion was adjusted for lymph nodal metastases and vice versa (Table 3, bottom). At this additional analysis, the association of *BRAF$^{(V600E)}$* with extra-thyroid invasion was maintained (P=0.003), while the correlations with lymph nodal metastases (P=0.089) and advanced stage were lost (Table 3, bottom). These analyses support the possibility that the association between *BRAF$^{(V600E)}$* and extra-thyroid

invasion, but not lymph nodal metastases is an independent relationship. In contrast, the association between $BRAF^{(V600E)}$ and advanced stage is most likely influenced by the other two parameters (Table 3, bottom).

Taken together these results suggest that $BRAF^{(V600E)}$ is independently and strongly associated with extra-thyroid invasion and also with other indicators of tumor aggressiveness.

Association of $BRAF^{(V600E)}$ with the expression of MMPs

Recent reports indicated that of $BRAF^{(V600E)}$ is able to induce MMPs, which play an important role in tumor invasiveness and metastasis (Maeta *et al*. 2001, Mesa *et al*. 2006). We tested therefore the hypothesis that $BRAF^{(V600E)}$ may promote tumor invasiveness by up-regulating MMPs. Sixty classical PTCs were studied by IHC, staining with either anti-MMP-2 or MMP-9 antibodies. MMP-2 was detected in 32 specimens (53.3%), whereas MMP-9 in 52 out of 60 (86.7%; Table 4). At univariate analysis, a significant correlation was found between $BRAF^{(V600E)}$ positivity and IHC detection of either MMP-2 or MMP-9 or both ($P=0.028$). At the same time, the presence of both MMP-2 and MMP-9 was significantly correlated ($P=0.030$) with tumor extra-thyroid extension. These results confirm that MMP expression is related to the presence of $BRAF^{(V600E)}$ and to tumor invasiveness in man. They suggest, therefore, that MMPs are possible mediators of $BRAF^{(V600E)}$ effect on tumor invasiveness.

$BRAF^{(V600E)}$ and micro-PTCs

Since there is a strong correlation between $BRAF^{(V600E)}$ presence and tumor size, we separately examined tumors having a maximum diameter of 10 mm or less (micro-PTCs, $n=103/323$). In these small tumors, tissue sampling for $BRAF^{(V600E)}$ analysis was performed using microdissection (see Patients and methods).

Table 4 $BRAF^{(V600E)}$ mutation and matrix metalloproteinase-2/9 (MMP-2/9) expression in 60 papillary thyroid carcinomas (PTCs)

	BRAF− ($n=17$)	BRAF+ ($n=43$)	*P* value
MMP-2			
No ($n=32$)	11 (34.4%)	21 (65.6%)	0.6887
Yes ($n=28$)	6 (21.4%)	22 (78.6%)	
MMP-9			
No ($n=8$)	6 (75.0%)	2 (25.0%)	0.0047
Yes ($n=52$)	11 (21.1%)	41 (78.9%)	

We investigated whether $BRAF^{(V600E)}$ positivity was associated with increased tumor invasiveness also in micro-PTCs. First, $BRAF^{(V600E)}$ was present in 25 out of 103 micro-PTCs (24.3%, Table 4), significantly less than in PTCs (100 $BRAF^{(V600E)}$ positive out of 220=45.5%; $P=0.0017$; Table 5). These findings are compatible with the possibility that most tumors that are $BRAF^{(V600E)}$ positive progress to a larger size while, in contrast, most tumors that are $BRAF^{(V600E)}$ negative are stationary or progress slowly in volume. Second, at univariate analysis, a strong positive association ($P=0.006$) was found between $BRAF^{(V600E)}$ and extra-thyroid invasion also in micro-PTCs. Third, in $BRAF^{(V600E)}$ positive micro-PTCs, a significantly higher prevalence of stages III and IV tumor was observed (Fig. 2). When we compared micro-PTCs with PTCs, the percentage of tumors in stage III or IV was higher in PTCs, both in the $BRAF^{(V600E)}$ positive and in the $BRAF^{(V600E)}$ negative tumors. The differences between PTCs and micro-PTCs, however, were markedly smaller and nonsignificant in the

Table 5 Univariate analysis of $BRAF^{(V600E)}$ mutation and pathological features in macro and micro papillary thyroid carcinomas (PTCs)

PTCs over 10 mm ($n=220$)	**BRAF+** ($n=100$)	**BRAF−** ($n=120$)	***P* value**
Extra-thyroid invasion			
Yes ($n=78$)	45 (57.7%)	33 (42.3%)	0.0075
No ($n=142$)	55 (38.7%)	87 (61.3%)	
Lymph nodal metastasis			
Yes ($n=60$)	38 (63.3%)	22 (36.7%)	0.0014
No ($n=160$)	62 (38.7%)	98 (61.3%)	
Multifocality			
Yes ($n=104$)	50 (48.1%)	54 (51.9%)	0.4990
No ($n=116$)	50 (43.1%)	66 (56.9%)	
Tumor stage			
I–II ($n=159$)	70 (44.0%)	89 (56.0%)	0.5462
III–IV ($n=61$)	30 (49.2%)	31 (50.8%)	
Micro-PTCs ($n=103$)	**BRAF+** ($n=25$)	**BRAF−** ($n=78$)	***P* value**
Extra-thyroid invasion			
Yes ($n=9$)	6 (66.7%)	3 (33.3%)	0.0060
No ($n=94$)	19 (20.2%)	75 (79.8%)	
Lymph nodal metastasis			
Yes ($n=7$)	2 (28.6%)	5 (71.4%)	0.6759
No ($n=96$)	23 (24.0%)	73 (76.0%)	
Multifocality			
Yes ($n=23$)	5 (21.7%)	18 (78.3%)	0.7481
No ($n=80$)	20 (25.0%)	60 (75.0%)	
Tumor stage			
I–II ($n=92$)	20 (21.7%)	72 (78.3%)	0.0941
III–IV ($n=11$)	5 (45.5%)	6 (54.5%)	

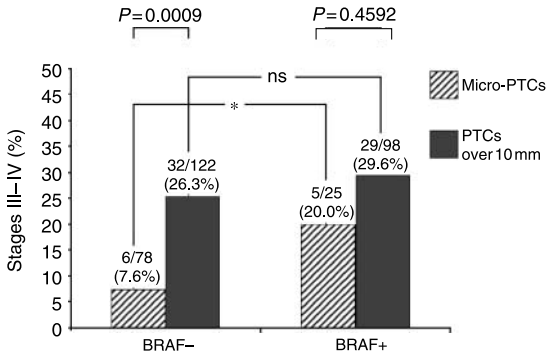

Figure 2 *BRAF*$^{(V600E)}$ in 103 micro-PTCs is associated with more advanced stage. Micro-PTCs harboring *BRAF*$^{(V600E)}$ (BRAF+) are in stages III–IV with a frequency similar to that of PTCs ($P=0.459$, NS), whereas BRAF-micro-PTCs are in advanced stage with a significantly lower frequency than BRAF-PTCs ($P=0.0009$).

BRAF$^{(V600E)}$ positive tumors while were highly significant in *BRAF*$^{(V600E)}$ negative tumors (Fig. 2). This observation is in concert with the multivariate analysis data indicating that the relationship between *BRAF*$^{(V600E)}$ positivity and tumor aggressiveness holds after adjusting for tumor size and is compatible with the possibility that *BRAF*$^{(V600E)}$ presence confers an increased aggressiveness to PTCs, whatever their size. *BRAF*$^{(V600E)}$ positivity, therefore, could be used as a molecular marker useful in order to identify micro-PTCs that require a more intensive treatment and follow-up.

Discussion

The present study concerns *BRAF*$^{(V600E)}$ mutation in a large series of 323 PTCs. This series is characterized by a homogeneous recruitment, diagnosis, follow-up, and treatment of patients.

It differs, therefore, from *BRAF*$^{(V600E)}$ investigations in other thyroid cancer series where patients were recruited at different times and/or from different populations with the possibility that a different genetic background and/or environmental characteristics, in addition to a significant heterogeneity in the medical procedures may have influenced the clinical results of the study.

In our series, *BRAF*$^{(V600E)}$ was positive in 52% classical variant PTCs and 26.4% tall cell PTCs, frequent also in anaplastic cancers (33.3%), while it was always absent in follicular variant PTCs and in follicular tumors, both benign (adenomas) and malignant. Abnormalities of RET/PTC, Ras were very rare in our series as well as *BRAF* mutations others than *BRAF*$^{(V600E)}$. It is confirmed, therefore, that *BRAF*$^{(V600E)}$ is the most common defined genetic abnormality in PTCs (overall 38.6% in our series) and that constitutive activation of the RAS–RAF–MEK pathway is specific for the papillary architecture of thyroid cancers. At variance with most studies (Nikiforova *et al*. 2003, Nakamura *et al*. 2005, Xing 2005, Xing *et al*. 2005), however, *BRAF*$^{(V600E)}$ was not frequent in our series of tall cell PTCs. This finding is in accordance with some previous reports (Trovisco *et al*. 2004, 2005, Fugazzola *et al*. 2006) and may depend on the absence of squamous cell and Hurthle cell carcinoma components, as previously observed (Baloch *et al*. 2001, Knudsen *et al*. 2002, Zwi & LiVolsi 2002). Moreover, some discrepancy may exist in PTC subgroup definitions by different pathologists. In our series, tall cell variant PTCs were identified by the presence of at least 50% tall cell component in each tumor sample.

Our study clearly indicates that the presence of *BRAF*$^{(V600E)}$ mutation in PTCs is associated with an aggressive tumor behavior. Both univariate and multivariate analyses indicated that *BRAF*$^{(V600E)}$ was a good predictor of extra-thyroid invasion and lymph nodal metastases, even after adjusting for tumor size, multifocality, histology subtype and also for patient characteristics such as age and gender.

The association between *BRAF*$^{(V600E)}$ mutation and clinicopathological factors of tumor aggressiveness has already been addressed in many studies and the conclusions have been controversial (Lee *et al*. 2007, Xing 2007). This relationship, in fact, has been observed in some studies but not in other studies (Table 1). However, study size (see Table 1), different recruitment, and follow-up procedures and also factors such as tumor classification may have made data in many of those studies less consistent and reliable (Lee *et al*. 2007, Xing 2007).

We believe that the present data add convincing evidence that *BRAF*$^{(V600E)}$ is an important marker of tumor invasiveness and, moreover, that this relationship is independent from tumor size. Although *BRAF*$^{(V600E)}$ mutation was found more frequent in PTCs (45.5%) with respect to micro-PTCs (<10 mm in diameter, 24.3%, $P<0.0017$), also in micro-PTCs *BRAF*$^{(V600E)}$ positivity was significantly related to extra-thyroid invasion and more advanced stage suggesting that, although small, *BRAF*$^{(V600E)}$ positive tumors carry a higher risk of progression and invasiveness than tumors of the same size but *BRAF*$^{(V600E)}$ negative. In fact, micro-PTCs, although having an excellent prognosis in most cases, may include a consistent aliquot that will progress to more

advanced stage and less favorable outcome in terms of disease persistence and/or recurrence (Pelizzo *et al*. 2004, Pellegriti *et al*. 2004, Barbaro *et al*. 2005, Ito *et al*. 2005, Lo *et al*. 2006).

BRAF$^{(V600E)}$ positive micro-PTCs, therefore, may faster progress to larger size, whereas *BRAF*$^{(V600E)}$ negative micro-PTCs may predominantly have a slower and more indolent growth. This would explain the different prevalence of *BRAF*$^{(V600E)}$ positivity in larger versus smaller PTCs.

Although the predictive power of *BRAF*$^{(V600E)}$ for identifying micro-PTCs at higher risk of progression will require prospective studies with systematic BRAF measurement and long-term follow-up, it may be important to consider the possibility that *BRAF*$^{(V600E)}$ positivity might provide an 'invasiveness marker' and permit a better risk stratification in small thyroid tumors that are nearly always classified T1 N0 M0 at the TNM classification, with no appreciable difference for those micro-PTCs that, although small, may have a less favorable outcome.

The present study provides also some evidences regarding the possible mechanism linking *BRAF*$^{(V600E)}$ positivity to tumor invasiveness. Recent data indicated that MMP (secreted proteinases that cause extracellular matrix degradation favoring tumor invasion) are preferentially induced by BRAF (Mesa *et al*. 2006, Palona *et al*. 2006). MMP expression is associated with markedly increased invasion of the extracellular matrix in cultured thyroid cells and with lymph nodal metastases, intra-thyroid, and vascular invasion in human PTCs (Maeta *et al*. 2001). In concert with this hypothesis in our nested study of 60 CV-PTCs, MMPs were expressed more frequently in *BRAF*$^{(V600E)}$ positive than *BRAF*$^{(V600E)}$ negative tumors. At the same time, PTCs expressing both MMP-2 and MMP-9 had significantly more frequent signs of extra-thyroid extension ($P=0.030$) with respect to those MMP-negative thyroid tumors. Although the complexity of the MMP system (which includes activators and inhibitors in addition to enzyme expression) was not investigated in our study, the association between *BRAF*$^{(V600E)}$, MMP expression, and tumor invasiveness is in concert with the possibility that this is one mechanism through which *BRAF*$^{(V600E)}$ promotes cancer spread and invasiveness.

An additional point that deserves attention is the important difference of *BRAF*$^{(V600E)}$ positivity in PTCs occurring in patients from different geographical areas of the same region. *BRAF*$^{(V600E)}$ positive PTCs were 45.9% in Eastern Sicily versus 22.7% in Western Sicily ($P=0.0001$). Populations from either Eastern or Western Sicily must be considered ethnically and genetically homogeneous because of the frequent exchanges and communications between the two areas in addition they display a similar risk for overall malignancies. The most likely explanation is that one or more environmental carcinogens, active in Eastern Sicily, contribute to the higher prevalence of *BRAF*$^{(V600E)}$ positive thyroid tumors in that area. Iodine deficiency is not the cause of this difference. Although ~1 out of 10 of the Sicilian population is exposed to mild-moderate iodine deficiency and related diseases (Belfiore *et al*. 1987, 1992, Vigneri 1988, Regalbuto *et al*. 1996), the frequency of *BRAF*$^{(V600E)}$ positive PTCs was not different in tumors occurring in patients from iodine-deficient areas (which are scattered all over Sicily) or iodine-sufficient areas.

A recent survey on thyroid tumors in Sicily, collecting data on tumor incidence for 3 consecutive years, indicated that thyroid cancer incidence is much higher in North-Eastern Sicily (unpublished data). This area includes the volcano Etna, the highest and most active volcano in Europe. Data from the literature indicate a high frequency of thyroid tumors also in other volcanic areas (such as Hawaii, Iceland, Philipines; Kolonel 1985, Arnbjornsson *et al*. 1986, Hernandez 2003). It is reasonable, therefore, to hypothesize that some thyroid carcinogens, not identified up to now, might be present in the volcanic soil, water, or atmosphere (Cimino & Toscano 1998). This hypothesis is supported by the observation of an increased incidence of other tumors (mesothelioma) in the same volcanic area (town of Biancavilla) due to an increased exposure to asbestos amphibole fluoro-edenite contained in quarry extracted materials (Comba *et al*. 2003). The novel information from the present study is that *BRAF*$^{(V600E)}$ mutation may be a specific genetic abnormality induced also by environmental carcinogens. This hypothesis requires, of course, confirmation. If confirmed by *in vivo* and *in vitro* studies, it may provide relevant information both in terms of preventive medicine and also for a better understanding of the biology of PTC.

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