Correlation between *B-RAF*^{V600E} mutation and clinico–pathologic parameters in papillary thyroid carcinoma: data from a multicentric Italian study and review of the literature

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Abstract

Recently, a somatic point mutation of the *B-RAF* gene (V600E) has been identified as the most common genetic event in papillary thyroid carcinoma (PTC), with a prevalence variable among different series. Since discordant data on the clinico-pathologic features of *B-RAF* mutated PTC are present in the literature, the aim of the present co-operative study was to establish the prevalence of this genetic alteration and to perform a genotype–phenotype correlation in a large cohort of patients with PTC. To this purpose, a series of 260 sporadic PTCs with different histological variants were included in the study. The mutational analysis of the *B-RAF* gene was performed either by RT-PCR followed by single-stranded conformational polymorphism or by PCR and direct sequencing. Statistical analyses were obtained by means of χ^2/Fisher's exact test and *t*-test. Overall, a heterozygous T > A transversion at nucleotide 1799 (V600E) was found in 99 out of 260 PTCs (38%). According to the histological type of the tumor, the *B-RAF*^{V600E} mutation was present in 48.3% of cases of classic PTCs (85 out of 176), in 17.6% (nine out of 51) of follicular variants of PTCs, in 21.7% (five out of 23) in other PTC variants and in none of the ten poorly differentiated tumors. *B-RAF*^{V600E} was significantly associated with the classic variant of PTC ($P = 0.0001$) and with an older age at diagnosis ($P = 0.01$). No statistically significant correlation was found among the presence of *B-RAF*^{V600E} and gender, tumor node metastasis (TNM), multicentricity of the tumor, stage at diagnosis and outcome. In conclusion, the present study reports the prevalence of *B-RAF*^{V600E} (38%) in the largest series of sporadic PTCs, including 260 cases from three different Italian referring centers. This prevalence is similar to that calculated by pooling together all data previously reported, 39.6% (759 out of 1914 cases), thus indicating that the prevalence of this genetic event lies around 38–40%. Furthermore, *B-RAF*^{V600E} was confirmed to be associated with the papillary growth pattern, but not with poorer differentiated PTC variants. A significant association of *B-RAF* mutation was also found with an older age at diagnosis, the mutation being very rare in childhood and adolescent PTCs. Finally, no correlation was found with a poorer prognosis and a worse outcome after a median follow-up of 72 months.

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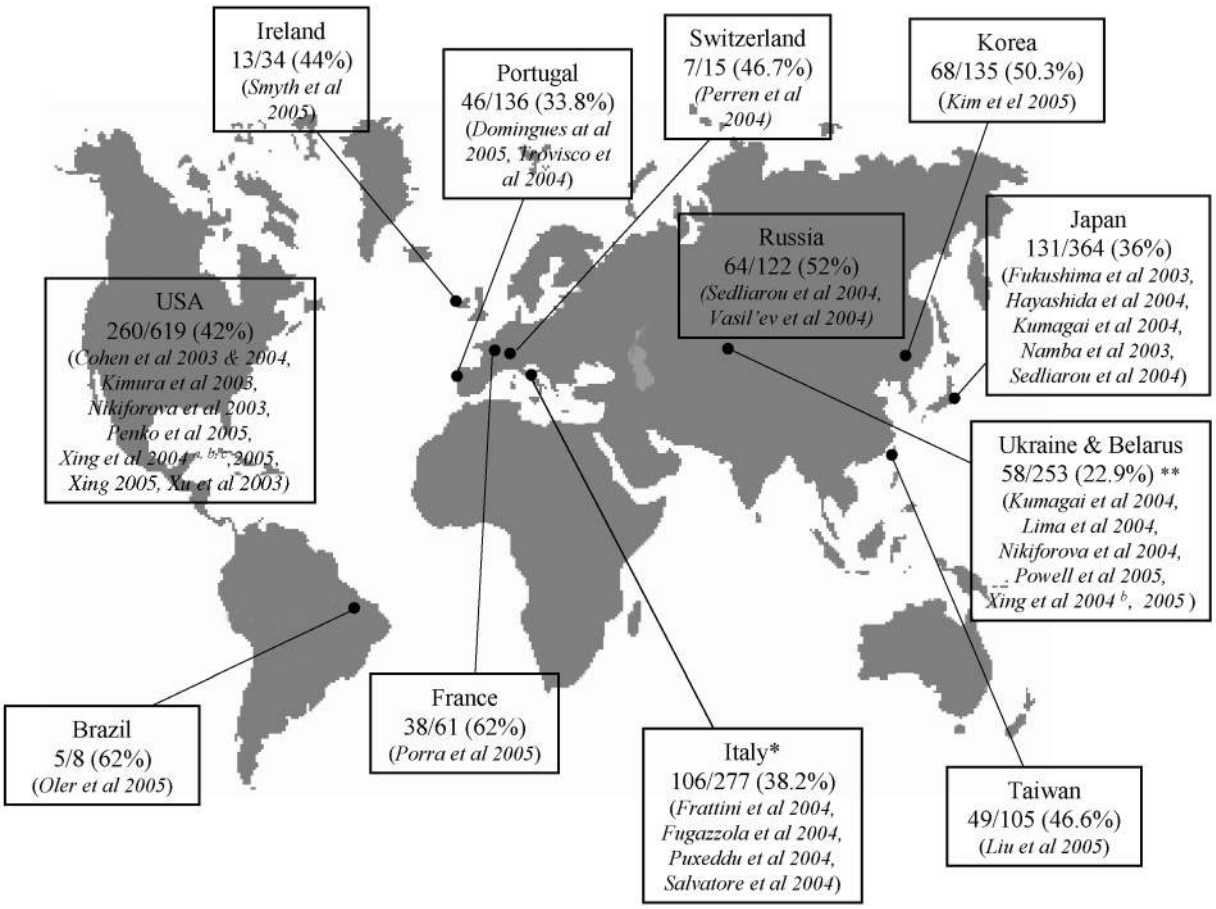

Figure 1 The worldwide prevalence of *B-RAF* mutations in PTC is reported. Samples originating from the same country have been grouped and the total prevalence of *B-RAF* positive cases calculated. References are in parentheses. *The data reported for Italy do not include the present paper; **most of the patients examined were children.

Introduction

Papillary thyroid carcinoma (PTC) is the most common type of endocrine tumor, with an annual incidence of two to four per 100 000 individuals. Among several oncogenes, *RET* (Santoro *et al*. 1992, Bongarzone *et al*. 1998) and, more recently, *B-RAF* (Cohen *et al*. 2003, Namba *et al*. 2003, Nikiforova *et al*. 2003, Soares *et al*. 2003) have been found frequently and alternatively activated in PTCs. Both genes act through the MAP kinase pathway, which seems to play a very central role in the pathogenesis of this tumor.

B-RAF mutations have been described at high frequency in melanoma (70%) and, with a lower prevalence (10–20%), also in colon and ovarian cancer, in lung and stomach cancer and in sarcomas (Davies *et al*. 2002). The prevalence of *B-RAF* mutated thyroid cancers varies between 23 and 62% among different series (reviewed in Fig. 1), this variability being related to the heterogeneity of the histological types of PTC, to epidemiological factors or to the age group analysed. With the exception of the mutation K601E found in one follicular adenoma and four PTC follicular variants (Trovisco *et al*. 2004, 2005), the gene rearrangement *AKAP9-BRAF* described in one sporadic and three radiation-associated PTCs (Ciampi *et al*. 2005), the three nucleotide deletion (K601del) found in three metastatic lymph nodes and in a solid variant of PTC (Oler *et al*. 2005, Trovisco *et al*. 2005) and the three nucleotide insertion (V599Ins) found in a classic variant of PTC (Carta *et al*. 2006), the amino acid change *B-RAF*^{V600E} is the only mutation consistently found in PTC. This mutation is of great importance because the V600 residue significantly contributes to the stabilization of the inactive conformation of the *B-Raf* kinase domain.

The substitution of the valine with a glutamic acid (V600E) leads to the destabilization of the inactive conformation, promoting an active state and enhancing *B-Raf* kinase activity toward MEK (Wan *et al*. 2004, Wellbrock *et al*. 2004).

B-RAF mutations and *ret*/PTC rearrangements, which are present in about 20–40% of PTCs (Santoro *et al*. 1992, Bongarzone *et al*. 1998), have been shown to be mutually exclusive (Kimura *et al*. 2003, Soares *et al*. 2003, Frattini *et al*. 2004, Puxeddu *et al*. 2004). Moreover, compared with *ret*/PTCs, which are more frequently associated with post-Chernobyl tumors (Fugazzola *et al*. 1996, Elisei *et al*. 2001), *B-RAF* mutation is not a major event in irradiated PTCs (Lima *et al*. 2004, Nikiforova *et al*. 2004, Powell *et al*. 2005).

B-RAF mutations are present not only in papillary histotype, but also in anaplastic (Namba *et al*. 2003, Begum *et al*. 2004, Soares *et al*. 2004) and in poorly differentiated thyroid carcinomas, when a well-differentiated PTC component is present (Nikiforova *et al*. 2003). In the papillary histotype, *B-RAF* mutations are significantly associated with tumors with a papillary or mixed follicular–papillary growth pattern (Nikiforova *et al*. 2003, Fugazzola *et al*. 2004, Trovisco *et al*. 2004, 2005).

A correlation between older age at diagnosis and *B-RAF*^{V600E} has been described (Nikiforova *et al*. 2003, Trovisco *et al*. 2005) and recently some authors have reported a low prevalence of *B-RAF*^{V600E} in PTCs diagnosed in childhood (Kumagai *et al*. 2004, Lima *et al*. 2004, Penko *et al*. 2005, Powell *et al*. 2005, Rosenbaum *et al*. 2005). At present, no other unequivocal correlations between genotype and clinico–pathologic features of PTC patients have been reported both in previous reports from the present authors (Fugazzola *et al*. 2004, Puxeddu *et al*. 2004) and in other more recent studies (Sedliarou *et al*. 2004, Kim *et al*. 2005, Liu *et al*. 2005, Trovisco *et al*. 2005). A few studies (Namba *et al*. 2003, Nikiforova *et al*. 2003, Xing 2005, Xing *et al*. 2005) have shown a correlation of *B-RAF*^{V600E} with more advanced stage, nodal/distant metastases at diagnosis or tumor recurrence. Correlation with the male gender has been reported in a single study (Xu *et al*. 2003). These discrepancies might be due to the heterogeneity of the histological variants of PTCs, to epidemiological factors, to the age group analyzed or to the small number of cases studied.

The aim of the present co-operative study was to correlate *B-RAF*^{V600E} with several clinical and pathological features in a large and homogeneous cohort of 260 Italian PTC patients in order to better define the prevalence of the mutation and the statistically significant correlations.

Patients and Methods

Patients and tumor specimens

A series of 260 PTCs tissues were included in this study. In particular, the following histologic PTC variants were studied: classic ($n = 176$), follicular ($n = 51$), others (including tall cell, oncocytic and solid variants, $n = 23$) and poorly differentiated carcinomas, without well-differentiated components ($n = 10$). Samples were obtained consecutively at the time of surgery from the endocrine surgery units of the Universities of Milan, Perugia and Pisa, Italy. All tissues were snap frozen and archived at -80°C. In the three centers, histological diagnoses were carried out following the same criteria (LiVolsi 1990, LiVolsi *et al*. 2004, Rosai 2004) (Fig. 2) and cancers were staged according to the last tumor node metastasis (TNM) staging system from American Joint Committee on Cancer 2002 and International Classification of Diseases for Oncology (ICD-O C73). In order to limit the variability of different case sets subjected to diverse histopathological interpretations, 230 out of 260 glass slides were histologically re-examined, in a blind manner, by experienced pathologists at the participating centers. All tumors were sporadic and excised from patients not exposed to external radiation or radioactive fall-out. The remission, persistence or recurrence of the disease was evaluated for each patient by radioiodine total body scan, thyroglobulin and anti-thyroglobulin autoantibody measurement either when off L-thyroxine treatment or after recombinant human thyrotropin stimulus (thyrogen, thyrotropin α; Genzyme Corporation, Cambridge, MA, USA), neck ultrasound and fine needle aspiration cytology on suspicious masses. Because of the slow progression of most PTCs, for the statistical analysis, only patients ($n = 81$) with a follow-up longer than 24 months (median follow-up 72 months) were considered.

In order to evaluate possible differences in *B-RAF*^{V600E} prevalence according to the origin of the samples, patients were sub-classified as originating from the north, center or south of Italy.

Data from 104 out of 260 cases (40%) included in the present study have been reported previously

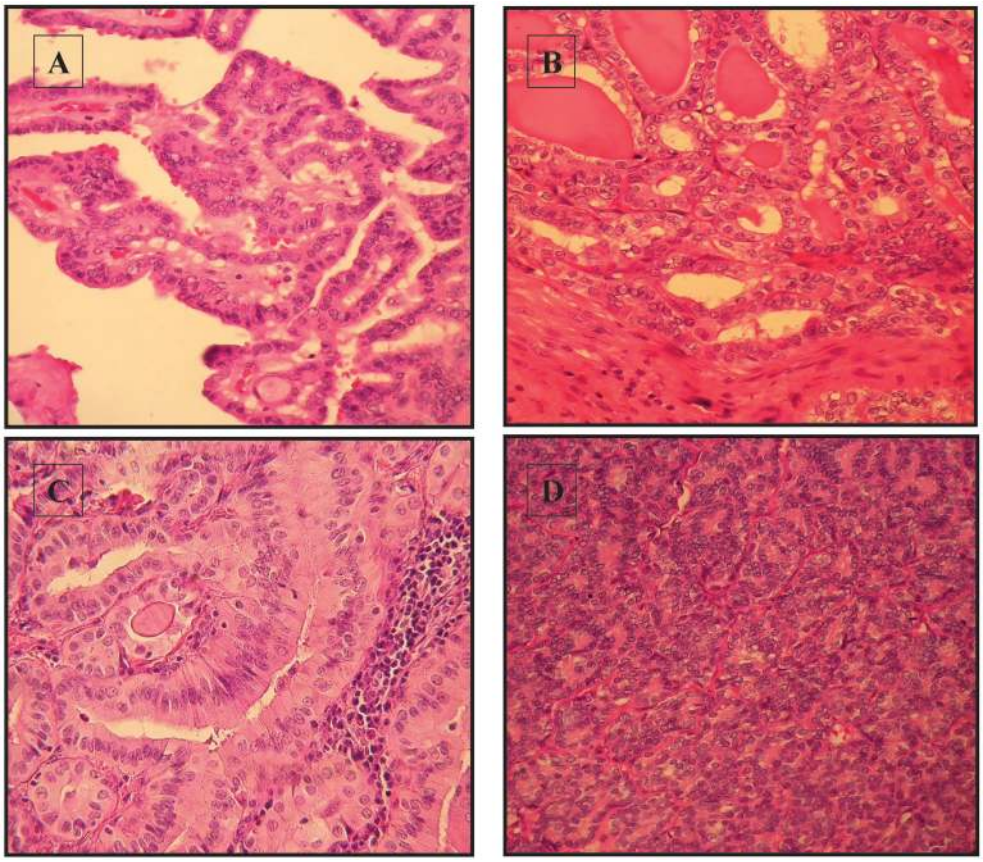

Figure 2 Examples of cases of different variants of PTC, according to the criteria followed by the pathologists of the three participating centers (LiVolsi 1990, LiVolsi *et al.* 2004, Rosai 2004). (A) Classic variant: the tumor growth consists of papillary structures delimited by cuboidal cells with the characteristic nuclear changes (hematoxylin and eosin; ×300). (B) Follicular variant: at high magnification the tumor consists of follicular structures delimited by cuboidal cells showing prominent nuclear grooves and pseudoinclusions (hematoxylin and eosin; ×400). (C) Tall cell variant: the tumor consists of follicular and papillary structures; the tumor cells have a columnar shape and an eosinophylic cytoplasm; stratification of nuclei is also observed (hematoxylin and eosin; ×300). (D) Poorly differentiated: the tumor consists of a solid proliferation of medium sized cells with scanty cytoplasm; a focally trabecular pattern of growth is seen (hematoxylin and eosin; ×400).

($n = 51$ by Puxeddu *et al.* (2004) and $n = 53$ by Fugazzola *et al.* (2004)).

Methods

Two different techniques were used for *B-RAF* analysis on the tumoral specimens. About half of the samples were analyzed using tumor DNA while the others were studied using tumor RNA. All procedures for handling the tissues were approved by each hospital ethics committee. Informed consent was obtained from all screened subjects.

DNA and RNA extraction from tissues

To ensure a pure tumor tissue isolation in tumors <1.5 cm, microdissection was performed. In larger tumors, the core of the tumoral nodule was dissected macroscopically and analyzed after histological confirmation of malignancy. DNA was extracted from tumor tissues by means of commercial kits (Puregene; Gentra Systems, Minneapolis, MN, USA). RNA extraction was performed using Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA), according to the manufacturer's instructions.

PCR amplification and direct sequencing analysis

For *B-RAF* analysis, the DNA was PCR amplified using specific intronic primers (Xu *et al.* 2003) according to the following protocol: 35 cycles of denaturation (94°C for 1 min), annealing (60°C for 1 min) and extension (72°C for 2 min) on a TouchDown thermal cycler (Hybaid, Basingstoke,

Middx, UK). After purification, PCR products were directly sequenced. An aliquot of 3–10 ng/100 bp purified DNA and 3.2 pmol of either the forward or reverse primer were used in standard cycle sequencing reactions with ABI PRISM big dye terminators and run on an ABI PRISM 310 genetic analyzer (PE Applied Biosystems, Foster City, CA, USA). The cycle-sequencing conditions consisted of 25 cycles of 96°C for 30 s, 50°C for 15 s and 60°C for 4 min. One sequence read from each direction across the entire coding region and including intron–exon boundaries was obtained for each sample.

Reverse transcription and single-stranded conformational polymorphism (SSCP) analysis

Total RNA was submitted to reverse transcription with either AMV reverse transcriptase (Promega Corp., Madison, WI, USA) or Superscript reverse transcriptase II (Invitrogen Corp.) using a random hexamer mixture as primers. For each set of reactions a negative control tube containing double-distilled water and no nucleic acids was co-incubated.

All the cDNAs were submitted to a PCR using sets of primers designed to flank exon 15 of *B-RAF* (forward: CATTGCACGACAGACTGCAC; reverse: TCTGACTGAAAGCTGTATGG) according to the following protocol: 35 cycles of denaturation (95°C for 1 min), annealing (60°C for 1 min) and extension (72°C for 1 min) on an automated heat block (iCycler; BioRad, Hercules, CA, USA). Mutational analysis of PCR products was performed by SSCP screening. SSCP analysis was conducted using a method reported previously (Puxeddu *et al.* 2004). Briefly, 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 and 0.05% xylene cyanol), denatured and loaded onto a 0.5 × mutation detection enhancement gel (MDE gel solution; Cambrex, Rockland, ME, USA) 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 (BioRad). Staining of the separated DNA bands on the gels was performed using the Gelstar nucleic acid gel stain (Cambrex). ARO and NPA human thyroid carcinoma cell lines were used as heterozygous and homozygous positive controls respectively, while WRO cells were used as negative control (Kimura *et al.* 2003). 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 Biosystems) at GeneLab (ENEA Casaccia, Rome, Italy) using the same primers as the PCR amplification. The results of the mutation screening for *B-RAF* of each sample were confirmed in at least two independent experiments.

Statistical analysis

Correlations between *B-RAF* mutation and various clinico–pathologic parameters were analyzed in all 260 patients. The statistical analyses were obtained by means of *t*-test and χ^2/Fisher's exact test. The difference between two values was considered significant when $P < 0.05$. All tests were performed using the Statistical Package for Social Sciences for Windows (SPSS Inc., Chicago, IL, USA).

Results

Genetic analysis of *B-RAF*

All mutations detected involved a T > A transversion at nucleotide 1799 (V600E) and were heterozygous. Overall, the *B-RAF*^{V600E} mutation was found in 99 out of 260 PTCs (38%). It is worthy to note that about 40% of the negative samples (60 out of 161) were tested with the two methods (starting from RNA or from DNA) and the results were comparable. According to the histological type of tumor, the *B-RAF*^{V600E} mutation was present in 48.3% of cases of classic PTC (85 out of 176), in 17.6% (nine out of 51) of follicular variants of PTC, in 21.7% (five out of 23) of other PTC variants and in none of the ten poorly differentiated tumors.

Correlation analysis between *B-RAF* mutation and clinical parameters

The correlation between *B-RAF*^{V600E} and various clinico–pathologic parameters were examined (Table 1). The *B-RAF*^{V600E} was significantly associated with the classic variant of PTC with respect to all the other variants ($P = 0.0001$). Moreover, *B-RAF*^{V600E} was correlated with an older age at diagnosis ($P = 0.01$). It is worthy of note that only one out of ten patients ≤18 years of age harbored the *B-RAF* mutation. No statistically significant correlation of *B-RAF*^{V600E} with gender, TNM, multicentricity of the tumor and stage of the

Table 1 Correlations between *B-RAF*^{V600E} (BRAF) and various clinico–pathological parameters in the 260 patients studied

	BRAF+ (%)	**BRAF**− (%)	***P***
Age (years)	Median 42 (range 15–85)	Median 39 (range 8–78)	0.01
Diameter (mm)	Median 20 (range 6–50)	Median 18 (range 5–70)	0.9
Female gender ($n = 190$)	72 (37.9)	118 (62.1)	1
Tumor size and extension			0.1
T1 ($n = 92$)	33 (35.8)	59 (64.2)	0.7
T2 ($n = 63$)	32 (50.8)	31 (49.2)	0.02
T3 ($n = 86$)	27 (31.4)	59 (68.6)	0.1
T4 ($n = 19$)	7 (36.8)	12 (63.2)	1
Multicentricity ($n = 101$)	38 (37.6)	63 (62.4)	1
Nodal metastases (pN1) ($n = 110$)	41 (37.2)	69 (62.8)	0.4
Distant metastases (pM1) ($n = 10$)	3 (30)	7 (70)	0.7
Stage			0.2
I ($n = 184$)	65 (35.3)	119 (64.7)	0.1
II ($n = 13$)	8 (61.5)	5 (38.5)	0.1
III ($n = 38$)	18 (47.3)	20 (52.7)	0.3
IV ($n = 25$)	10 (40)	15 (60)	1
Histological variant			0.0001
Classic ($n = 176$)	85 (48.3)	91 (51.7)	0.0001
Follicular ($n = 51$)	9 (17.6)	42 (82.4)	0.0007
Dedifferentiated ($n = 10$)	0	10 (100)	0.01
Other ($n = 23$)	5 (21.7)	18 (78.3)	0.1
Remission ($n = 81$)	29 (35.8)	52 (64.2)	1

Statistical analyses were performed by means of Fisher's exact test, except for age and tumor diameter (*t*-test).

disease at diagnosis was found. It should be noted that a trend towards a greater proportion of patients with *B-RAF*^{WT} presenting at stage I was observed, but this was not statistically significant. As far as the outcome is concerned, this was analysed in the 81 patients with a follow-up longer than 24 months (median 72 months). Also for this parameter, no correlation with the presence/absence of *B-RAF* mutation was revealed.

No significant differences were noted in the prevalence of *B-RAF*^{V600E} positive tumors according to the origin of patients from the three different areas of Italy (north, center and south), with the exception of patients coming from Umbria, a small mountainous region in the center of Italy. In this area the prevalence of *B-RAF* mutated tumors was significantly higher (35 out of 51 (69%) positive samples vs 64 out of 209 (31%), $P < 0.0001$).

Discussion

In the present homogeneous series of sporadic PTCs, which is the largest reported to date and included 260 cases from three different Italian referring centers, *B-RAF*^{V600E} mutation was found in 99 out of 260 patients (38%). In the literature, the prevalence of *B-RAF* varies between 23 and 62% among different series probably due to the heterogeneity of the histological variants of PTC, to epidemiological factors or to the age group analyzed. In Fig. 1, all the reports on *B-RAF* analyses in PTC have been considered, and the mean frequency per country was calculated. Pooling together all the data from the various cohorts, a prevalence of 39.6% (845 out of 2129 cases) was obtained, which is similar to that found in the present study, indicating that the prevalence of this genetic event in PTCs is around 38–40%. According to previous data (Nikiforova *et al*. 2003, Fugazzola *et al*. 2004, Trovisco *et al*. 2004, 2005), *B-RAF*^{V600E} was detected mainly in the classic variant of PTC ($P = 0.0001$), confirming that this mutation is strongly associated with the papillary growth pattern. Tumors with histological variants generally considered more aggressive (tall cells, oncocytic and solid variants) harbored, as expected (Frattini *et al*. 2004, Xing *et al*. 2005), a relatively high percentage of the *B-RAF* mutations (five out of 23, 21.7%) which was, however, not statistically significant ($P = 0.1$). At variance, none of the ten poorly differentiated tumors displayed the *B-RAF* mutation ($P = 0.01$). This result, even though

obtained in a relatively small cohort, is in agreement with previous observations indicating the absence of *B-RAF* mutation in this histological type when not associated with a well-differentiated component (Nikiforova *et al*. 2003, Soares *et al*. 2004). On the basis of this evidence, it is conceivable that PTC with a poorer differentiation might be associated with other pathogenic events.

A significant correlation of *B-RAF*^{V600E} was found with an older age at presentation ($P = 0.01$), confirming the data obtained in the series reported by Nikiforova *et al*. (2003).Moreover, in accordance with the reported low prevalence of this genetic event in children (Kumagai *et al*. 2004, Lima *et al*. 2004, Penko *et al*. 2005, Powell *et al*. 2005, Rosenbaum *et al*. 2005), nine out of ten PTCs in patients $\leq$18 years of age were *B-RAF*^{WT}. On the contrary, *RET* activation has been demonstrated to be more frequent in PTCs originating during the first three decades of life (Bongarzone *et al*. 1996). On the basis of these observations, it is possible to postulate that childhood PTCs are genetically different from adulthood PTCs.

As far as other possible correlations between *B-RAF*^{V600E} and the clinical features of PTC patients are concerned, no statistically significant differences in gender, stage, TNM, multicentricity or recurrence between *B-RAF* mutated and non-mutated cases were found. These results are in agreement with those reported in other series (Kim *et al*. 2005, Liu *et al*. 2005, Trovisco *et al*. 2005). On the contrary, previous data on the association of *B-RAF* with a worse stage and/or local or distant metastases or recurrence (Namba *et al*. 2003, Nikiforova *et al*. 2003, Xing 2005, Xing *et al*. 2005) are not confirmed in the present large series. However, it is worth noting that the multivariate analysis, which was indeed performed only in one of these studies (Xing *et al*. 2005), clearly demonstrated that when the histological subtypes of the PTCs were included, the significance of *B-RAF* mutation association with all the other high-risk pathological features of the tumor was lost. With regard to the association between *B-RAF*^{V600E} and cancer recurrence described recently by Xing *et al*. (2005) and not found in the present series, a possible explanation could reside in the different median follow-up of the two cohorts (15 months in the paper by Xing *et al*. (2005) and 72 months in the present study).

The analysis of the prevalence of *B-RAF*^{V600E} according to the geographic origin of cases showed a significantly higher prevalence of *B-RAF*^{V600E} ($P < 0.0001$) in the cohort of patients coming from Umbria. Since this group was not significantly different from the others coming from north, center and south of Italy concerning sex, age, TNM, histology and including the variant of the PTC, we are tempted to speculate that a genetic shared background in this small area or the particularly high iodine deficiency of this region could be the explanation for this intriguing finding.

In conclusion, in this study we found that 38% of PTCs in this large series of Italian cases harbored a *B-RAF*^{V600E} mutation. This prevalence is highly comparable with the 'worldwide' mean prevalence. According to previous observations, this genetic event was found to be more frequently associated with the classic variant of PTC. Among all the other clinico–pathologic features that we analyzed *B-RAF*^{V600E} was found to be correlated only with an older age at diagnosis. In particular, no correlation was found with a poorer prognosis and a worst outcome after a median follow-up of 72 months.

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References

American Joint Committee on Cancer 2002 TNM classification of malignant tumours. In *AJCC Cancer Staging Handbook*, edn 6, ch. 8, pp 89–98. Eds FL Greene *et al*. New York: Springer-Verlag.

Begum S, Rosenbaum E, Henrique R, Cohen Y, Sidransky D & Westra WH 2004 BRAF mutations in anaplastic thyroid carcinoma: implications for tumor origin, diagnosis and treatment. *Modern Pathology* **17** 1359–1363.

Bongarzone I, Fugazzola L, Vigneri P, Mariani L, Mondellini P, Pacini F, Basolo F, Pinchera A, Pilotti S & Pierotti MA 1996 Age-related activation of the tyrosine kinase receptor protooncogenes RET and NTRK1 in papillary thyroid carcinoma. *Journal of Clinical Endocrinology and Metabolism* **81** 2006–2009.

Bongarzone I, Vigneri P, Mariani L, Collini P, Pilotti S & Pierotti MA 1998 RET/NTRK1 rearrangements in thyroid gland tumors of the papillary carcinoma family: correlation with clinicopathological features. *Clinical Cancer Research* **4** 223–228.

Carta C, Moretti S, Passeri L, Barbi F, Avenia N, Cavaliere A, Monacelli M, Macchiarlo A, Santeusanio F, Tartaglia M *et al*. 2006 Genotyping of an Italian papillary thyroid carcinoma cohort revealed high prevalence of *BRAF* mutations, absence of *RAS* mutations and allowed the detection of a new mutation of BRAF oncoprotein (BRAF^{V599Ins}). *Clinical Endocrinology* **64** 105–109.

Ciampi R, Knauf JA, Kerler R, Gandhi M, Zhu Z, Nikiforova MN, Rabes HM, Fagin JA & Nikiforov YE 2005 Oncogenic AKAP9-BRAF fusion is a novel mechanism of MAPK pathway activation in thyroid cancer. *Journal of Clinical Investigation* **115** 94–101.

Cohen Y, Xing M, Mambo E, Guo Z, Wu G, Trink B, Beller U, Westra WH, Ladenson PW & Sidransky D 2003 BRAF mutation in papillary thyroid carcinoma. *Journal of the National Cancer Institute* **95** 625–627.

Cohen Y, Rosenbaum E, Clark DP, Zeiger MA, Umbricht CB, Tufano RP, Sidransky D & Westra WH 2004 Mutational analysis of BRAF in fine needle aspiration biopsies of the thyroid: a potential application for the preoperative assessment of thyroid nodules. *Clinical Cancer Research* **10** 2761–2765.

Davies H, Bignell GR, Cox C, Stephens P, Edkins S, Clegg S, Teague J, Woffendin H, Garnett MJ, Bottomley W *et al*. 2002 Mutations of the BRAF gene in human cancer. *Nature* **417** 949–954.

Domingues R, Mendonca E, Sobrinho L & Bugalho MJ 2005 Searching for RET/PTC rearrangements and BRAF V599E mutation in thyroid aspirates might contribute to establish a preoperative diagnosis of papillary thyroid carcinoma. *Cytopathology* **16** 27–31.

Elisei R, Romei C, Vorontsova T, Cosci B, Veremeychik V, Kuchinskaya E, Basolo F, Demidchik EP, Miccoli P, Pinchera A & Pacini F 2001 RET/PTC rearrangements in thyroid nodules: studies in irradiated and not irradiated, malignant and benign thyroid lesions in children and adults. *Journal of Clinical Endocrinology and Metabolism* **86** 3211–3216.

Frattini M, Ferrario C, Bressan P, Balestra D, De Cecco L, Mondellini P, Bongarzone I, Collini P, Gariboldi M, Pilotti S *et al*. 2004 Alternative mutations of BRAF, RET and NTRK1 are associated with similar but distinct gene expression patterns in papillary thyroid cancer. *Oncogene* **23** 7436–7440.

Fugazzola L, Pilotti S, Pinchera A, Vorontsova TV, Mondellini P, Bongarzone I, Greco A, Astakhova L, Butti MG, Demidchik EP *et al*. 1996 Oncogenic rearrangements of the RET proto-oncogene in papillary thyroid carcinomas from children exposed to the Chernobyl nuclear accident. *Cancer Research* **55** 5617–5620.

Fugazzola L, Mannavola D, Cirello V, Vannucchi G, Muzza M, Vicentini L & Beck-Peccoz P 2004 BRAF mutations in an Italian cohort of thyroid cancers. *Clinical Endocrinology* **61** 239–243.

Fukushima T, Suzuki S, Mashiko M, Ohtake T, Endo Y, Takebayashi Y, Sekikawa K, Hagiwara K & Takenoshita S 2003 BRAF mutations in papillary carcinomas of the thyroid. *Oncogene* **22** 6455–6457.

Hayashida N, Namba H, Kumagai A, Hayashi T, Ohtsuru A, Ito M, Saenko VA, Maeda S, Kanematsu T & Yamashita S 2004 A rapid and simple detection method for the BRAF(T1796A) mutation in fine-needle aspirated thyroid carcinoma cells. *Thyroid* **14** 910–915.

Kim KH, Suh KS, Kang DW & Kang DY 2005 Mutations of the BRAF gene in papillary thyroid carcinoma and in Hashimoto's thyroiditis. *Pathology International* **55** 540–545.

Kimura ET, Nikiforova MN, Zhu Z, Knauf JA, Nikiforov YE & Fagin JA 2003 High prevalence of BRAF mutations in thyroid cancer: genetic evidence for constitutive activation of the RET/PTC-RAS-BRAF signaling pathway in papillary thyroid carcinoma. *Cancer Research* **63** 1454–1457.

Kumagai A, Namba H, Saenko VA, Ashizawa K, Ohtsuru A, Ito M, Ishikawa N, Sugino K, Ito K, Jeremiah S *et al*. 2004 Low frequency of BRAFT1796A mutations in childhood thyroid carcinomas. *Journal of Clinical Endocrinology and Metabolism* **89** 4280–4284.

Lima J, Trovisco V, Soares P, Maximo V, Magalhaes J, Salvatore G, Santoro M, Bogdanova T, Tronko M, Abrosimov A *et al*. 2004 BRAF mutations are not a major event in post-Chernobyl childhood thyroid carcinomas. *Journal of Clinical Endocrinology and Metabolism* **89** 4267–4271.

Liu RT, Chen YJ, Chou FF, Wu WL, Tsai PC, Huang CC & Cheng JT 2005 No correlation between BRAF V600E mutation and clinicopathological features of papillary thyroid carcinomas in Taiwan. *Clinical Endocrinology* **63** 461–466.

LiVolsi VA 1990 Papillary lesions of the thyroid. In *Surgical Pathology of the Thyroid. Major Problems in Pathology*, pp 136–145. Ed. JL Bennington. Philadelphia: W.B. Saunders Company.

LiVolsi VA, Albores-Saavedra J, Asa SL, Baloch ZW, Sobrinho-Simoes M, Wenig B, DeLellis RA & Cady B 2004 Papillary carcinoma. In *Pathology and Genetics of Tumours of Endocrine Organs – World Health Organization Classification of Tumors*, pp 57–72. Eds RA DeLellis, RV Lloyd, PU Heitz & C Eng. Lyon: IARC Press.

Namba H, Nakashima M, Hayashi T, Hayashida N, Maeda S, Rogounovitch TI, Ohtsuru A, Saenko VA, Kanematsu T & Yamashita S 2003 Clinical implication of hot spot mutation, V599E, in papillary thyroid cancers. *Journal of Clinical Endocrinology and Metabolism* **88** 4393–4397.

Nikiforova MN, Kimuea ET, Gandhi M, Biddinger PW, Knauf JA, Basolo F, Zhu Z, Giannini R, Salvatore G, Fusco A *et al*. 2003 BRAF mutations in thyroid tumors are restricted to papillary carcinomas and anaplastic or poorly differentiated carcinomas arising from papillary carcinomas. *Journal of Clinical Endocrinology and Metabolism* **88** 5399–5404.

Nikiforova MN, Ciampi R, Salvatore G, Santoro M, Gandhi M, Knauf JA, Thomas GA, Jeremiah S, Bogdanova TI, Tronko MD *et al*. 2004 Low prevalence of BRAF mutations in radiation-induced thyroid tumors in contrast to sporadic papillary carcinomas. *Cancer Letters* **209** 1–6.

Oler G, Ebina KN, Michaluart P Jr, Kimura ET & Cerutti J 2005 Investigation of BRAF mutation in a series of papillary thyroid carcinoma and matched-lymph node metastasis reveals a new mutation in metastasis. *Clinical Endocrinol*ogy **62** 509–511.

Penko K, Livezey J, Fenton C, Patel A, Nicholson D, Flora M, Oakley K, Tuttle RM & Francis G 2005 BRAF Mutations are uncommon in papillary thyroid cancer of young patients. *Thyroid* **15** 320–325.

Perren A, Schmid S, Locher T, Saremaslani P, Bonvin C, Heitz PU & Komminoth P 2004 BRAF and endocrine tumors: mutations are frequent in papillary thyroid carcinomas, rare in endocrine tumors of the gastrointestinal tract and not detected in other endocrine tumors. *Endocrine-Related Cancer* **11** 855–860.

Porra V, Ferraro-Peyret C, Durand C, Selmi-Ruby S, Giroud H, Berger-Dutrieux N, Decaussin M, Peix JL, Bournaud C, Orgiazzi J *et al*. 2005 Silencing of the tumor suppressor gene SLC5A8 is associated with BRAF mutations in classical papillary thyroid carcinomas. *Journal of Clinical Endocrinology & Metabolism* **90** 3028–3035.

Powell N, Jeremiah S, Morishita M, Dudley E, Bethel J, Bogdanova T, Tronko M & Thomas G 2005 Frequency of BRAF T1796A mutation in papillary thyroid carcinoma relates to age of patient at diagnosis and not to radiation exposure. *Journal of Pathology* **205** 558–564.

Puxeddu E, Moretti S, Elisei R, Romei C, Pascucci R, Martinelli M, Marino C, Avenia N, Rossi ED, Fadda G *et al*. 2004 BRAF(V599E) mutation is the leading genetic event in adult sporadic papillary thyroid carcinomas. *Journal of Clinical Endocrinology and Metabolism* **89** 2414–2420.

Rosai J 2004 Papillary carcinoma. In *Rosai Ackerman's Surgical Pathology*, edn 9, pp 552–555. Edinburgh: Mosby.

Rosenbaum E, Hosler G, Zahurak M, Cohen Y, Sidransky D & Westra WH 2005 Mutational activity of BRAF is not a major event in sporadic childhood papillary thyroid carcinoma. *Modern Pathology* **18** 898–902.

Salvatore G, Giannini R, Faviana P, Caleo A, Migliaccio I, Fagin JA, Nikiforov YE, Troncone G, Palombini L, Basolo F *et al*. 2004 Analysis of BRAF point mutation and RET/PTC rearrangement refines the fine-needle aspiration diagnosis of papillary thyroid carcinoma. *Journal of Clinical Endocrinology and Metabolism* **89** 5175–5180.

Santoro M, Carlomagno F, Hay ID, Herrmann MA, Grieco M, Melillo R, Pierotti MA, Bongarzone I, Della Porta G, Berger N *et al*. 1992 Ret oncogene activation in human thyroid neoplasms is restricted to the papillary cancer subtype. *Journal of Clinical Investigation* **89** 1517–1522.

Sedliarou I, Saenko V, Lantsov D, Rogounovitch T, Namba H, Abrosimov A, Lushnikov E, Kumagai A, Nakashima M, Meirmanov S *et al*. 2004 The BRAFT1796A transversion is a prevalent mutational event in human thyroid microcarcinoma. *International Journal of Oncology* **25** 1729–1735.

Smyth P, Finn S, Cahill S, O'Regan E, Flavin R, O'Leary JJ & Sheils O 2005 ret/PTC and BRAF act as distinct, molecular, time-dependant triggers in a sporadic Irish cohort of papillary thyroid carcinoma. *International Journal of Surgical Pathology* **13** 1–8.

Soares P, Trovisco V, Rocha AS, Lima J, Castro P, Preto A, Maximo V, Botelho T, Seruca R & Sobrinho-Simoes M 2003 BRAF mutations and RET/PTC rearrangements are alternative events in the etiopathogenesis of PTC. *Oncogene* **22** 4578–4580.

Soares P, Trovisco V, Rocha AS, Feijao T, Rebocho AP, Fonseca E, Vieira de Castro I, Cameselle-Teijeiro J, Cardoso-Oliveira M & Sobrinho-Simoes M 2004 BRAF mutations typical of papillary thyroid carcinoma are more frequently detected in undifferentiated than in insular and insular-like poorly differentiated carcinomas. *Virchows Archives* **444** 572–576.

Trovisco V, Vieira de Castro I, Soares P, Maximo V, Silva P, Magalhaes J, Abrosimov A, Guiu XM & Sobrinho-Simoes M 2004 BRAF mutations are associated with some histological types of papillary thyroid carcinoma. *Journal of Pathology* **202** 247–251.

Trovisco V, Soares P, Preto A, de Castro IV, Lima J, Castro P, Maximo V, Botelho T, Moreira S, Meireles AM *et al*. 2005 Type and prevalence of BRAF mutations are closely associated with papillary thyroid carcinoma histotype and patients' age but not with tumour aggressiveness. *Virchows Archives* **446** 589–595.

Vasil'ev EV, Rumiantsev PO, Saenko VA, Il'in AA, Poliakova EIu, Nemtsova MV, Zaletaev DV 2004 Molecular analysis of structural abnormalities in papillary thyroid carcinoma gene. *Molecular Biology* (Moscow) **38** 642–653.

Wan PTC, Garnett MJ, Roe SM, Lee S, Niculescu-Duvaz D, Good VM, Cancer Genome project, Jones CM, Marshall CJ, Springer CJ *et al*. 2004 Mechanism of activation of the RAF-ERK signaling pathway by oncogenic mutations of B-RAF. *Cell* **116** 855–867.

Wellbrock C, Karasarides M & Marais R 2004 The Raf proteins take centre stage. *Nature Reviews Molecular Cell Biology* **5** 875–885.

Xing M, Cohen Y, Mambo E, Tallini G, Udelsman R, Ladenson PW & Sidransky D 2004*a* Early occurrence of RASSF1A hypermethylation and its mutual exclusion with BRAF mutation in thyroid tumorigenesis. *Cancer Research* **64** 1664–1668.

Xing M, Vasko V, Tallini G, Larin A, Wu G, Udelsman R, Ringel MD, Ladenson PW & Sidransky D 2004*b* BRAF T1796A transversion mutation in various thyroid neoplasms. *Journal of Clinical Endocrinology and Metabolism* **89** 1365–1368.

Xing M, Tufano RP, Tufaro AP, Basaria S, Ewertz M, Rosenbaum E, Byrne PJ, Wang J, Sidransky D & Ladenson PW 2004*c* Detection of BRAF mutation on fine needle aspiration biopsy specimens: a new diagnostic tool for papillary thyroid cancer. *Journal of Clinical Endocrinology and Metabolism* **89** 2867–2872.

Xing M 2005 BRAF mutation in thyroid cancer. *Endocrine-Related Cancer* **12** 245–262.

Xing M, Westra W, Tufano RP, Cohen Y, Rosenbaum E, Thoden KJ, Carson KA, Vasko V, Larin A, Tallini G *et al.* 2005 BRAF mutation predicts a poorer clinical prognosis for papillary thyroid cancer. *Journal of Clinical Endocrinology and Metabolism* **90** 6373–6379.

Xu X, Quiros RM, Gattuso P, Ain KB & Prinz RA 2003 High prevalence of BRAF gene mutation in papillary thyroid carcinomas and thyroid tumor cell lines. *Cancer Research* **63** 4561–4567.