

# BRAF<sup>V600E</sup> mutation, but not RET/PTC rearrangements, is correlated with a lower expression of both thyroperoxidase and sodium iodide symporter genes in papillary thyroid cancer

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## Abstract

A low sodium iodide symporter (NIS) expression has been shown in papillary thyroid carcinomas (PTCs) harboring the BRAF<sup>V600E</sup> mutation. In the present study, we analyzed the mRNA expression of thyroid differentiation genes, glucose transporter (GLUT)-1 and GLUT-3, in 78 PTCs according to the presence of BRAF<sup>V600E</sup> or RET/PTC rearrangements. We found BRAF<sup>V600E</sup> and RET/PTC rearrangements in 35.8 and 19.4% of PTCs respectively. The mRNA expression of NIS and thyroperoxidase (TPO) genes were significantly lower ( $P < 0.0001$  and  $P = 0.004$  respectively) in BRAF<sup>V600E</sup>-positive PTC with respect to non-mutated samples. In support of this result, immunohistochemistry showed that the percentage of NIS-positive cells was significantly lower ( $P = 0.005$ ) in BRAF<sup>V600E</sup>-mutated PTC (mean 53.5%) than in negative cases (mean 72.6%). In contrast, no difference either in NIS or in any other thyroid differentiation genes' mRNA expression was found in PTC with or without RET/PTC rearrangements. When GLUT-1 and GLUT-3 mRNA expression was considered, no correlation was found either in BRAF<sup>V600E</sup>- nor in RET/PTC-mutated cases. In conclusion, this study confirmed the presence of a genetic alteration of BRAF and/or RET oncogenes in 64% of PTC cases and revealed a significant correlation of BRAF<sup>V600E</sup> mutation with a lower expression of both NIS and TPO. This latter finding could indicate that an early dedifferentiation process is present at the molecular level in BRAF<sup>V600E</sup>-mutated PTC, thus suggesting that the previously demonstrated poor prognostic significance of BRAF<sup>V600E</sup> mutation could be related to the dedifferentiation process more than to a more advanced stage at diagnosis.

*Endocrine-Related Cancer* (2008) 15 511–520

## Introduction

Papillary thyroid carcinomas (PTCs) are the most frequent malignant disease of the thyroid gland and are usually well-differentiated, as demonstrated by their ability to take up iodine, to secrete thyroglobulin (Tg), and to be responsive to thyrotropin-stimulating hormone (TSH; Braverman & Utiger 2000). In 15–20% of cases, they lose these features as a possible consequence of a

decreased expression of the thyroid differentiation genes (i.e., thyroperoxidase (TPO), Tg, TSH receptor (TSH-R), sodium iodide symporter gene (NIS), thyroid transcription factor-1 (TTF-1), and PAX-8; Elisei *et al.* 1994, Arturi *et al.* 1998). The dedifferentiation process is responsible for a worse prognosis in these PTCs as they are usually not responsive to conventional 131-Iodine radiotherapy (Haugen 1999).

RET gene rearrangements, BRAF and RAS oncogene point mutations, which can activate the mitogen-activated protein (MAP) kinase signaling pathway, have been highly studied in PTC and found to be present in 75–80% of PTCs, and are usually mutually exclusive (Soares *et al.* 2003, Melillo *et al.* 2005, Ciampi & Nikiforov 2007). RET/PTC rearrangements, mainly RET/PTC1 and RET/PTC3, are present in about 20–40% of PTCs and, according to the literature, they are more frequent in pediatric thyroid cancer and in cancer patients with a previous history of radiation exposure (Nikiforov *et al.* 1997, Elisei *et al.* 2001). Although it is a fact that RET/PTC3 is usually associated with the more aggressive phenotype, no clear correlation between RET/PTC rearrangements and a better or worse prognosis has been documented (Basolo *et al.* 2001).

The V600E mutation is the only BRAF genetic alteration (BRAF<sup>V600E</sup>) consistently found in PTC with a median prevalence of 35–40% (Xing 2005b, Fugazzola *et al.* 2006). The association of BRAF<sup>V600E</sup> mutation with clinical features of PTC is controversial; although some studies have recently shown that BRAF<sup>V600E</sup> mutation is associated with an advanced stage of the disease at diagnosis (Namba *et al.* 2003, Nikiforova *et al.* 2003), this correlation has not been confirmed by other groups (Xu *et al.* 2003, Puxeddu *et al.* 2004, Trovisco *et al.* 2005, Fugazzola *et al.* 2006).

Several *in vitro* and animal models studies (De Vita *et al.* 1998, Knauf *et al.* 2005, Mitsutake *et al.* 2006) showed a relationship between the oncogene activation of the MAP kinase pathway and a lower mRNA expression level of some thyroid differentiation genes (i.e., TPO, Tg, TSH-R, NIS, TTF-1). Both a decreased expression and impaired NIS protein cell membrane targeting has been demonstrated recently in a series of PTCs harboring the BRAF<sup>V600E</sup> mutation (Riesco-Eizaguirre *et al.* 2006, Durante *et al.* 2007).

In the present study, we analyzed the mRNA expression levels of TPO, Tg, NIS, TSH-R, and TTF-1 in 78 PTCs according to the presence of BRAF<sup>V600E</sup> mutation or RET/PTC1 and RET/PTC3 rearrangements. Based on the observation that PTCs that have lost the capability to take up iodine are more prone to take up 18-fluorodeoxyglucose (18-FDG; Feine *et al.* 1996), we also investigated the relationship between BRAF<sup>V600E</sup> mutation and the expression of two facilitative glucose transporter isoforms (GLUT-1 and GLUT-3) that have been recently demonstrated to be involved in the uptake of 18-FDG in thyroid tumors (Matsuzu *et al.* 2004, 2005).

## Materials and methods

### Tumoral tissues

A total of 78 thyroid primary tumoral tissues belonging to 78 patients affected by PTC (mean and median age 35.4 and 32 years respectively; F/M ratio 58/20) were collected at surgery from January to June 2005, immediately frozen in liquid nitrogen, and stored at –80 °C. In 44 out of 78 cases, the contralateral normal thyroid tissue was collected simultaneously and similarly stored. No history of radiation exposure was present in the clinical history of these patients.

The present study was approved by the institutional review board. All the patients were informed about the study and signed an informed consent for the collection of their tumor samples.

### Histology

Histological diagnosis and description of variants of each tumor were performed according to the histopathological typing of the World Health Organization. Clinical and pathological staging were carried out according to the tumor-node-metastasis (TNM) classification of the AJCC (Greene 2002).

### RNA extraction and RT-PCR

Total RNA was extracted from 30 to 100 mg tissue using a commercial kit based on the guanidinium isothiocyanate method (RNAzol B Tel-Test, Friendswood, TX, USA). Total RNA was reverse-transcribed into cDNA using Avian Myeloblastosis Virus reverse transcriptase (Promega Corp.) and random hexamers (Promega Corp). In particular, 1 µg RNA was used for the production of cDNA in a final volume of 20 µl for 1 h at 42 °C.

### PCR amplification, Southern blot, and sequence analysis

The good quality and the thyroid follicular origin of the samples were verified by the amplification of PAX-8 gene (364 bp). cDNA was used as the template for the amplification of RET/PTC1-3 rearrangements and BRAF gene (exon 15). For the analysis of the RET/PTC rearrangements, 10 µl PCR product were electrophoresed in 1.5% agarose gel and blotted onto a nylon membrane. Each filter was then hybridized with internal probes specific for each amplified fragment and labeled using a chemoluminescent method (Gene Images 3'-Oligolabelling and CDP-Star Detection System; Amersham Pharmacia Biotech). For the sequencing analysis of BRAF, the PCR products were purified with a commercial kit (QIAquick PCR Purification Kit,

Qiagen) and sequenced using an automated system employing fluorescent dye terminators (ABI Prism 310, Perkin–Elmer, Foster City, CA, USA). The primer sequences for BRAF and RET/PTC amplification have been reported previously (Elisei *et al.* 2001, Fugazzola *et al.* 2006).

### Quantitative analysis of mRNA expression by real-time RT-PCR

To perform quantitative RT-PCR, we used the Real-Time Sequence Detection System 7700 (PE Applied Biosystems, Foster City, CA, USA). The primers and probes specific for analyzed genes, such as GAPDH, NIS, Tg, TPO, TSH-R, TTF-1, GLUT-1, and GLUT-3 were from Applied Biosystems (PDAR and Assay-on-Demand Gene Expression, Monza, Italy). Samples omitting either reverse transcriptase or cDNA were included in each run as the controls of potential laboratory and/or assay contamination. Each sample was assayed in triplicate according to the conditions recommended by Applied Biosystems; 10 ng cDNA were added to a mixture of 1× Universal Master Mix, 900 nM each primer, and 200 nM probe in a final volume of 25 µl. The reaction mixtures were incubated for 2 min at 50 °C, denatured for 10 min at 95 °C, and subjected to 40 cycles of a two-step PCR consisting of a 15 s denaturation at 95 °C and 1 min annealing/extension at 60 °C. The mRNA gene expression was determined by applying the  $C_t$  method ( $C_t = C_t$  (analyzed gene) –  $C_t$  (GAPDH, ubiquitous referral gene)) in which  $C_t$  is the threshold cycle for the quantitative real-time RT-PCR.

### Immunohistochemical detection

Thyroid tissue sections (5 µm), obtained from 40 PTCs, were deparaffinized and rehydrated. All slides were subjected to antigen retrieval using 10% citrate buffer. Washes were done with PBS for 5 min. The endogenous peroxidase activity was blocked with 5% H<sub>2</sub>O<sub>2</sub> for 15 min.

Tissue sections were incubated with the purified NIS antibody (kind gift of Brahms Diagnostica GmbH, Berlin, Germany) at a dilution of 1:1000 at room temperature for 1 h, subjected to avidin and biotin block for 20 min each, streptavidin peroxidase for 10 min, and DAB substrate chromogen for 5 min. These sections were then counterstained with hematoxylin. The immunohistochemistry for TPO antibody at a dilution of 1:100 (Mab hTPO 47, Alexis Biochemicals, Lausen, Switzerland) was also performed following the same protocol.

For each sample, the percentage of NIS-positive and -negative and TPO-positive and -negative cells was calculated and an arbitrary score indicating the degree

of positivity was also applied (from the lowest (+) to the highest (+++)).

The slides were independently scored by two pathologists (P F and F B) and the mean score was considered for the analysis. Both pathologists were blinded to the BRAF mutation status of the tumors.

### TSH measurement

Since it has been demonstrated that TSH regulates the NIS symporter at both transcriptional and post-transcriptional levels (Dohan *et al.* 2003), serum TSH values at the time of surgery were retrieved from clinical database and analyzed according to the molecular data. Serum TSH was measured using an ultrasensitive commercial IMA method (DPC, Los Angeles, CA, USA) with a normal range of 0.4–3.4 µU/ml.

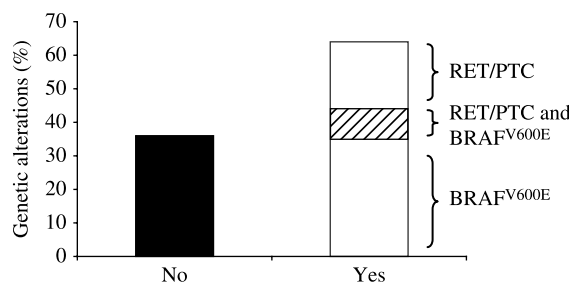
### Statistical analysis of the results

Statistical analysis was performed using the  $\chi^2$ , Mann–Whitney and *t*-test for the unpaired data, according to the data to be analyzed. Statistical significance was defined as  $P < 0.05$ .

## Results

### Genetic alterations and clinical–pathological features

As shown in Fig. 1, 50 out of 78 PTCs (64.1%) harbored a genetic alteration. In 7 out of 78 (9%) PTCs, two different mutations were present simultaneously (three cases showed both RET/PTC1 and RET/PTC3 rearrangement, four cases both BRAF<sup>V600E</sup> point mutation and RET/PTC rearrangement (one PTC1 and three PTC3)). BRAF<sup>V600E</sup> mutation alone was present in 28 out of 78 (35.8%) PTCs. RET/PTC1 or RET/PTC3 rearrangement was found in 15 out of 78 (19.4%) PTCs.



**Figure 1** Prevalence of BRAF and RET/PTC genetic alterations in papillary thyroid carcinoma. It is worth noting that in 7 out of 78 (9%) cases two different mutations were present simultaneously (3 cases showed both RET/PTC1 and RET/PTC3 rearrangement, 4 cases both BRAF point mutation and RET/PTC rearrangement (1 PTC1 and 3 PTC3)).

**Table 1** Correlation of BRAF<sup>V600E</sup> mutation and RET/PTC rearrangements with clinical and pathological features of papillary thyroid cancer at diagnosis

	BRAF+	BRAF-	P	RET/PTC1+	RET/PTC1-	P	RET/PTC3+	RET/PTC3-	P
Age (years)									
Mean	36.5	34.2		26.9	36.4		32.5	35.1	
Median	32.5	32	NS	26	33	NS	28	32	NS
Range	19–79	18–62		18–38	14–79		20–62	14–79	
Diameter									
Mean	1.65	1.13	NS	1.45	1.43	NS	2.3	1.25	NS
Median	1.75	1.2		1.45	1.4		1.4	1.4	
Female (58)	24	34		11	43		9	45	
Male (20)	8	12	NS	2	14	NS	3	13	NS
Tumor size	<i>n</i> (%)	<i>n</i> (%)		<i>n</i> (%)	<i>n</i> (%)		<i>n</i> (%)	<i>n</i> (%)	
T1 (33)	14 (42)	19 (58)		6 (18)	27 (82)		5 (15)	28 (85)	
T2 (13)	6 (46)	7 (54)	NS	2 (15)	11 (85)	NS	3 (23)	10 (77)	<i>P</i> =0.01
T3 (19)	5 (26)	14 (74)		3 (16)	16 (84)		2 (10)	17 (90)	
T4 (2)	0	2 (100)		0	2 (100)		2 (100)	0	
Multicentricity	7	18	NS	4	21	NS	3	22	NS
Node metastases	5	16	NS	6	16	NS	6	15	NS
Class	<i>n</i> (%)	<i>n</i> (%)		<i>n</i> (%)	<i>n</i> (%)		<i>n</i> (%)	<i>n</i> (%)	
I (46)	20 (43)	26 (57)		6 (13)	40 (87)		5 (11)	41 (89)	
II (19)	5 (26)	14 (74)	NS	5 (26)	14 (74)	NS	5 (26)	14 (74)	<i>P</i> =0.003
III (2)	0	2 (100)		0	2 (100)		2 (100)	0	
Variant	<i>n</i> (%)	<i>n</i> (%)		<i>n</i> (%)	<i>n</i> (%)		<i>n</i> (%)	<i>n</i> (%)	
Classic (37)	19 (51)	18 (49)	<i>P</i> =0.014 <sup>a</sup>	7 (19)	30 (81)		5 (13)	32 (87)	
Aggressive <sup>b</sup> (14)	4 (28)	10 (72)		0	14 (100)	NS	1 (7)	13 (93)	NS
Foll (13)	1 (7)	12 (93)		3 (23)	10 (77)		3 (23)	10 (77)	

<sup>a</sup>The *P* value was even lower (*P*=0.0076) when comparing BRAF<sup>V600E</sup>-positive cases versus cases negative for any mutation.

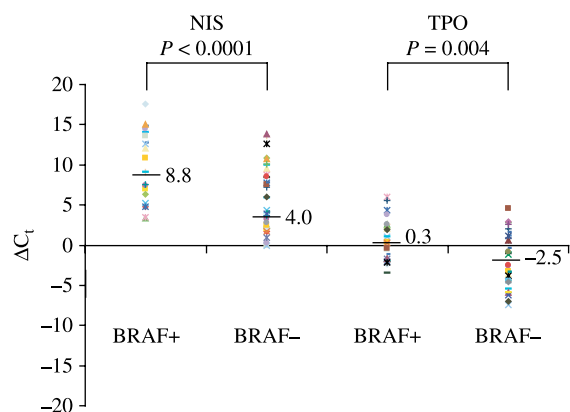
<sup>b</sup>Including solid, tall cell, insular, sclerosing, and Hurthle cell variants.

As shown in Table 1, the correlation of BRAF<sup>V600E</sup> mutation with clinical–pathological features showed a significantly higher prevalence of the mutation in the classical rather than in the follicular variant group (*P*=0.014), while no difference was found between the prevalence of BRAF<sup>V600E</sup> mutation in the classical variant group and the group including the most aggressive variants (i.e. tall cells, solid, insular, sclerosing, and Hurthle variants). No correlation of the BRAF<sup>V600E</sup> mutation with any other clinical–pathological features analyzed was revealed. Regarding the correlation of RET/PTC rearrangements and the above-mentioned parameters, our data showed that the RET/PTC3 rearrangement was significantly more frequent in PTCs with a bigger size (*P*=0.01) and a more advanced class at diagnosis (*P*=0.003). In contrast, RET/PTC1 rearrangements did not correlate with any clinical–pathological characteristics of PTC (Table 1).

### Genetic alterations and differentiation genes expression

The mRNA expression level of NIS and TPO genes (expressed as  $\Delta C_t$ ) were significantly lower (*P*<0.0001 and *P*=0.004 respectively, according to Mann–Whitney test) in PTCs harboring the BRAF<sup>V600E</sup> mutation than

in those without the mutation (Fig. 2). The NIS mRNA expression level was not different in the contralateral normal tissues of cases with or without BRAF<sup>V600E</sup> mutation ( $2.32 \pm 5.16$  and  $1.71 \pm 1.91$  respectively for NIS;  $-4.93 \pm 2.96$  and  $-5.69 \pm 0.84$  for TPO respectively). In contrast, neither the other differentiation genes (Tg, TPO, TSH-R, TTF-1) nor GLUT-1 and



**Figure 2** NIS and TPO mRNA expression in BRAF<sup>V600E</sup> mutated and non-mutated papillary thyroid carcinoma. It is worth noting that as the mRNA expression is indicated as  $\Delta C_t$ , the lowest is the value of  $\Delta C_t$  and the highest is the level of mRNA expression.

GLUT-3 mRNA expression was correlated with the BRAF<sup>V600E</sup> mutation (Table 2).

It is worth noting that this finding is based on the comparison of the mean value of NIS mRNA levels of expression in BRAF-mutated and -non-mutated cases. When individual tumors were considered, an overlap of the NIS levels of expression was observed (Fig. 2).

In the same type of analysis, PTCs that were positive for RET/PTC3 showed a slightly lower expression of TSH-R ( $P=0.05$ ) with respect to negative cases, while this difference was not observed when the contralateral normal tissues of positive and negative cases were compared. No significant differences in the levels of expression of NIS, TPO, TTF-1, and GLUT-1 and GLUT-3 mRNA were observed in relationship with the presence or absence of RET/PTC3 rearrangement (Table 2). RET/PTC1-positive cases did not show any difference in the mRNA levels of expression of any of the analyzed genes.

### NIS and TPO immunohistochemistry

NIS protein was expressed in all PTCs studied, with cytoplasmic ( $n=5$ ), membrane ( $n=19$ ), or both

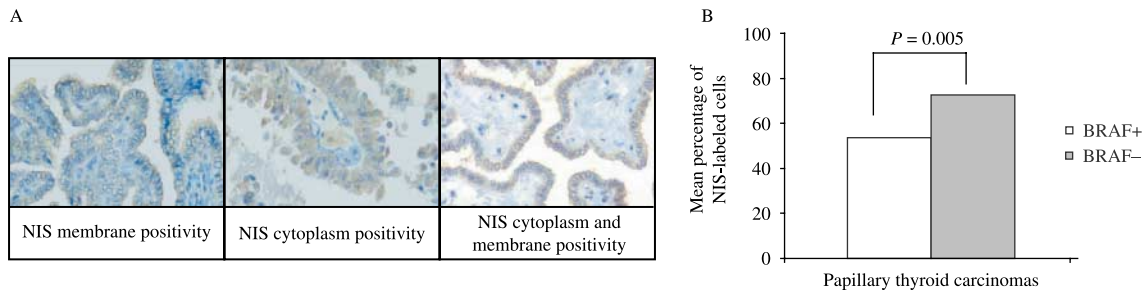
( $n=16$ ) localization (Fig. 3A). When the NIS-positive cells were counted, the percentage of positive cells was significantly lower ( $P=0.005$ ) in PTCs harboring the BRAF<sup>V600E</sup> mutation (percentage of positive cells: mean  $53.5 \pm 16.6\%$ , range 45–80%) than in negative cases (percentage of positive cells: mean  $72.6 \pm 13.8\%$ , range 40–90%; Fig. 3B).

The TPO protein expression was negligible (<10% of positive cells) in 25 out of 32 BRAF-positive PTCs and in 9 out of 46 BRAF-negative PTC ( $P<0.0001$ ). TPO-positive cases showed a different percentage of positive cells varying from >10 up to 90% (Fig. 4A). When the TPO-positive cells were counted, the percentage of positive cells was significantly lower ( $P<0.0001$ ) in PTCs harboring the BRAF<sup>V600E</sup> mutation (percentage of positive cells: mean  $16.8 \pm 15.3\%$ , range <10–70%) than in negative cases (percentage of positive cells: mean  $62.4 \pm 30.5\%$ , range <10–90%; Fig. 4B).

No significant differences were observed in either NIS or TPO protein expression between RET/PTC rearrangements positive and negative PTC.

**Table 2** Correlation of BRAF<sup>V600E</sup> mutation and RET/PTC rearrangements with the expression level of thyroid differentiation genes and glucose transporter (GLUT) isoform mRNA expression

$\Delta C_t$	BRAF+	BRAF–	RET/PTC3+	RET/PTC3–	RET/PTC1+	RET/PTC1–
NIS						
Mean	9.5	4.8	5.3	6.8	6.4	6.6
Median	8.8	4.1	5.8	7.0	6.1	6.7
<i>P</i>	$P<0.0001$		$P=NS$		$P=NS$	
Tg						
Mean	–2.1	–3.1	–2.5	–2.7	–3.3	–2.6
Median	–2.2	–3.8	–1.1	–3.1	–1.7	–3.0
<i>P</i>	$P=NS$		$P=NS$		$P=NS$	
TPO						
Mean	0.5	–1.9	–0.9	–0.8	–1.8	–0.6
Median	0.3	–2.5	–0.1	–1.0	–2.0	–0.5
<i>P</i>	$P=0.004$		$P=NS$		$P=NS$	
TSH-R						
Mean	–0.6	–1.0	0.6	–0.8	–1.4	–0.5
Median	–0.5	–0.7	0.8	–0.6	–1.7	–0.5
<i>P</i>	$P=NS$		$P=0.05$		$P=NS$	
TTF-1						
Mean	0.3	–0.5	0.06	0.5	0.0	0.6
Median	0.2	0.5	0.5	0.0	0.3	0.2
<i>P</i>	$P=NS$		$P=NS$		$P=NS$	
GLUT-1						
Mean	2.5	2.6	3.2	2.4	2.2	2.7
Median	2.6	1.9	2.8	2.3	1.9	2.4
<i>P</i>	$P=NS$		$P=NS$		$P=NS$	
GLUT-3						
Mean	3.6	3.5	3.0	3.7	2.9	2.6
Median	3.9	4.4	3.7	3.8	3.6	3.9
<i>P</i>	$P=NS$		$P=NS$		$P=NS$	



**Figure 3** (A) PTC immunostaining for NIS antibody (1:1000; ×100) showing the labeling of the cytoplasm and basolateral membrane of the follicular cells. (B) A statistically significant lower expression of NIS protein was observed in BRAF<sup>V600E</sup>-mutated PTC when the percentage of labeled cells and degree of immunostaining were considered.

### Correlation between serum TSH and NIS expression

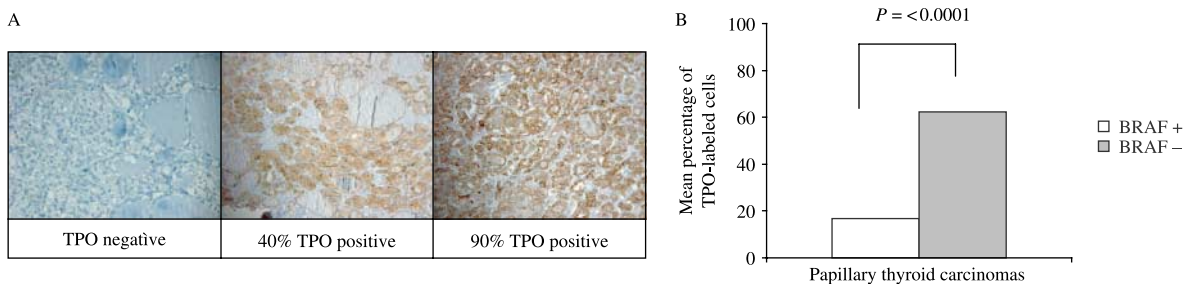
PTC patients showed different levels of serum TSH at the time of surgery varying from 0 (13 patients were on LT4 suppressive therapy at the time of surgery) to 4.2 μU/ml (mean, 1.2 ± 1.0 μU/ml, median, 1.0 μU/ml).

No relationship was found between serum TSH at the time of thyroidectomy and the levels of expression of NIS mRNA either in malignant ( $P=0.56$ ) or their contralateral normal tissues ( $P=0.94$ ; Fig. 5).

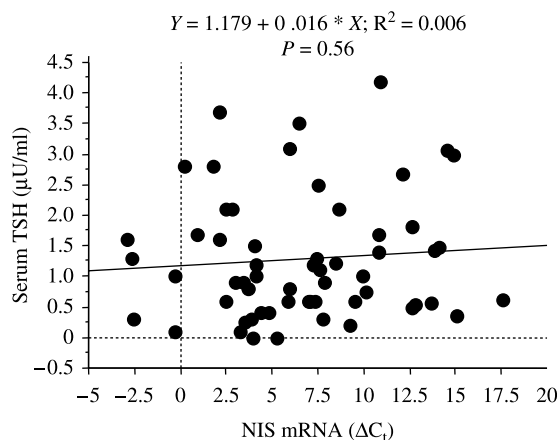
### Discussion

BRAF<sup>V600E</sup> point mutations and RET/PTC rearrangements are recognized as the two most common genetic alterations in thyroid carcinogenesis, their prevalences varying from 23 to 62% for BRAF<sup>V600E</sup> (Xing 2005b, Fugazzola et al. 2006) and from 2.5 to 78% for RET/PTC (Zou et al. 1994, Nikiforov et al. 1997). In the present series, the overall prevalence of genetic alterations was 64.1% (50/78). In particular, BRAF<sup>V600E</sup> mutation alone was present in 35.8% of the cases and RET/PTC1 or RET/PTC3 in 19.4%. The prevalence of BRAF<sup>V600E</sup> mutation was very

similar to that found by us in a large series of Italian subjects analyzed in a multicentric study (Fugazzola et al. 2006). In contrast, the prevalence of RET/PTC rearrangements was lower than that reported previously (Elisei et al. 2001) but, taking into account that these are not irradiated adult subjects, this low prevalence is not unexpected (Smida et al. 1999). An interesting finding was of the simultaneous presence of different gene alterations in the same tumor (9% of cases). To date, the description of the simultaneous presence of BRAF<sup>V600E</sup> and RET/PTC in the same PTC has been reported sporadically (Smyth et al. 2005), while the presence of two or even three different RET/PTC rearrangements have been described in several series (Chua et al. 2000, Elisei et al. 2001, Smyth et al. 2005). Although genetic alterations involving the MAP kinase pathway, such as BRAF<sup>V600E</sup>, RET/PTC, and RAS, have been considered as mutually exclusive (Soares et al. 2003, Melillo et al. 2005, Ciampi & Nikiforov 2007), it cannot be excluded that different gene mutations are present in different cells in the same tumoral mass. This possibility has been recently demonstrated by showing that the same tumor can harbor different RET/PTC rearrangements and BRAF mutations in different cells,



**Figure 4** (A) PTC immunostaining for TPO antibody (1:100; ×100) showing different patterns of TPO staining. (B) A statistically significant lower expression of TPO protein was observed in BRAF<sup>V600E</sup>-mutated PTC when the percentage of labeled cells and degree of immunostaining were considered.



**Figure 5** The regression linear plot showed that serum TSH values of papillary thyroid cancer patients at the time of surgery did not correlate with the level of expression of NIS mRNA in the tumoral tissues.

thus supporting the concept that tumors, although monoclonal, are heterogeneous (Zhu *et al.* 2006).

The presence of BRAF<sup>V600E</sup> mutation in the tumoral tissue has been recently demonstrated to predict a poorer clinical outcome for PTC patients (Xing 2005a, Kim *et al.* 2006). Since controversial data has been reported regarding the correlation of BRAF<sup>V600E</sup> mutation and a more advanced stage of PTC at diagnosis (Namba *et al.* 2003, Nikiforova *et al.* 2003, Puxeddu *et al.* 2004, Trovisco *et al.* 2005, Xing 2005a, Fugazzola *et al.* 2006), other reasons to explain the relationship between BRAF<sup>V600E</sup> mutation and poor prognosis should be found. Indeed, it is known that there is no correlation between the degree of invasion and the degree of differentiation and these two parameters must be kept separate for their prognostic significance. In the present study, we confirmed our previous observation (Fugazzola *et al.* 2006) that BRAF<sup>V600E</sup> mutation was significantly correlated only with the classical variant of PTC and not with a more advanced stage or a bigger tumor size. However, our data demonstrated a significantly lower expression of both NIS and TPO mRNA in PTCs with BRAF<sup>V600E</sup> mutation with respect to negative cases. According to this finding and in agreement with the previously reported data (Di Cristofaro *et al.* 2006, Riesco-Eizaguirre *et al.* 2006, Durante *et al.* 2007), we also observed a significantly lower degree of both NIS and TPO protein expression in BRAF<sup>V600E</sup>-positive cases. This finding was independent of serum TSH levels which could affect NIS mRNA and protein expression (Dohan *et al.* 2003). It is known that about 40% of metastatic PTCs show a progression from a

well-differentiated status at the time of diagnosis to a poorly differentiated or undifferentiated status during the follow-up (Schlumberger *et al.* 2007). These tumors progressively lose the typical features of thyroid follicular cells, and at first, the ability to take up and organify iodine, then to produce and secrete Tg and finally to respond to TSH (Elisei *et al.* 1994, Arturi *et al.* 1998). As a consequence of the dedifferentiation process, this subgroup of PTC is not responsive to conventional therapies, shows a poor prognosis and a worse outcome. On the basis of these considerations, our results suggest that BRAF<sup>V600E</sup>-mutated PTCs, although still well differentiated, are losing the typical features of follicular cells such as iodine uptake and organification, at least at the molecular level.

A correlation with a more aggressive phenotype and a more advanced stage has been also reported for RET/PTC rearrangements, especially RET/PTC3 (Nikiforov *et al.* 1997, Powell *et al.* 1998). In this series of sporadic and not irradiated PTCs, we confirmed a correlation between the presence of RET/PTC3 rearrangement and the bigger size of the tumor and a more advanced class at diagnosis. However, the few studies on the prognostic significance of RET/PTC rearrangements indicate the absence of a correlation between the presence of these genetic alterations and a bad prognosis (Tallini *et al.* 1998, Basolo *et al.* 2001, Puxeddu *et al.* 2003). In the present study, the expression of thyroid differentiation genes was not significantly different in PTCs with or without RET/PTC rearrangements, thus suggesting that these genetic alterations should not play a major role in the dedifferentiation process.

Clinical evidence indicates that whenever a differentiated primary or metastatic thyroid tumor loses the ability to take up iodine, it is more prone to take up 18-FDG (Feine *et al.* 1996). This ability is mediated by the activity of GLUT isoforms whose mRNA expression has been reported to be increased in more highly dedifferentiated thyroid tumors (Matsuzu *et al.* 2004, 2005). On the basis of these observations, we expected to find an inverse correlation between the expression levels of GLUT, either one or three isoforms, and NIS mRNA. The results of this correlation were surprisingly negative and in particular no correlation was found between GLUT expression and the presence or absence of BRAF<sup>V600E</sup> mutation. While we were preparing this manuscript, a paper has been published demonstrating a statistically significant higher expression of GLUT-1 mRNA in BRAF<sup>V600E</sup>-positive thyroid tumors (Durante *et al.* 2007). It is conceivable that, when compared with other studies, our results and those recently published can be

different because they are obtained by studying different series in different laboratories. However, taking into account that the majority of our PTCs were well differentiated at histology, our results are in keeping with the evidence of a late increase in GLUT mRNA expression during the dedifferentiation process of thyroid tumors (Matsuzu et al. 2004, 2005). In this regard, it is worth noting that human thyroid tumors positive for 18-FDG uptake have a worse prognosis because they are poorly differentiated and are usually very advanced (Wang et al. 2000, Robbins et al. 2006).

In conclusion, this study confirmed the presence of a genetic alteration of BRAF and/or RET oncogenes in 64% of PTC cases and revealed a significant correlation of BRAF<sup>V600E</sup> mutation with a lower expression of both NIS and TPO mRNA and protein. This latter finding could indicate that an early dedifferentiation process is present at the molecular level in BRAF<sup>V600E</sup>-mutated PTC, thus suggesting that the previously demonstrated poor prognostic significance of BRAF<sup>V600E</sup> mutation could be related to the dedifferentiation process more than to a more advanced stage at diagnosis.

## Acknowledgements

We thank Prof. Sebastiano Filetti from Rome, for his suggestions and criticisms. We also thank Dr J Froehlich (from Brahms) who kindly gifted the NIS antibody. This study was supported in part by grants from 'Associazione Italiana per la Ricerca sul Cancro'. The University of Pisa is a WHO collaborating centre. The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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