The oncogene BRAF^{V600E} is associated with a high risk of recurrence and less differentiated papillary thyroid carcinoma due to the impairment of Na⁺/I[−] targeting to the membrane

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Abstract

The oncogene BRAF^{V600E} is the most frequent genetic event in papillary thyroid carcinoma (PTC) but its prognostic impact still remains to be elucidated. We evaluated a representative series of 67 individuals with PTC who underwent total thyroidectomy. BRAF-positive tumours correlated with early recurrences (32% vs 7.6%; $P=0.02$) during a median postoperative follow-up period of 3 years. Interestingly, within the recurrences, a significant majority had negative radioiodine (¹³¹I) total body scans, predicting a poorer outcome as treatment with ¹³¹I is not effective. This last observation led us to investigate the role of BRAF^{V600E} and the MEK-ERK pathway in thyroid dedifferentiation, particularly in Na⁺/I[−] symporter (NIS) impairment, as this thyroid-specific plasma membrane glycoprotein mediates active transport of I[−] into the thyroid follicular cells. A subset of 60 PTC samples was evaluated for NIS immunoreactivity and, accordingly, we confirmed a significant low NIS expression and impaired targeting to membranes in BRAF-positive samples (3.5% vs 30%; $P=0.005$). Furthermore, experiments with differentiated PCCl3 thyroid cells demonstrated that transient expression of BRAF^{V600E} sharply impaired both NIS expression and targeting to membrane and, surprisingly, this impairment was not totally dependent on the MEK-ERK pathway. We have concluded that BRAF^{V600E} is a new prognostic factor in PTC that correlates with a high risk of recurrences and less differentiated tumours due to the loss of NIS-mediated ¹³¹I uptake.

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Introduction

Papillary thyroid carcinoma (PTC) is the most frequent malignancy of the thyroid, accounting for 1% of all human malignancies. In general terms, it has a good prognosis and high cure rates are achieved after initial treatment. However, it has been estimated that about 20–30% of patients with PTC will develop a local or distant recurrence but only about 1% will die (Schlumberger & Pacini 2003a). Identifying these high-risk patients at the time of diagnosis through

well-established prognostic factors can help to ascertain the most appropriate treatment and follow-up for these patients. Several prognostic scoring systems, such as the tumour node metastasis (TNM) classification (American Joint Committee on Cancer (AJCC) 2002), have been developed for thyroid cancer and they are based on multiple regression analysis of combined prognostic factors. All of them include extrathyroidal extension and distant metastasis, and practically all are based on age at diagnosis, tumour size and histological type. Despite all the former epidemiologic data, there is

still a debate on the definition and best management of low-risk versus high-risk patients, and each centre has its own protocols (Schlumberger & Pacini 2003a).

To date, no biological marker has yet been included as a prognostic factor for thyroid cancer. BRAF is a serine-threonine kinase that mediates signal transduction through the MEK-ERK pathway, and the T transversion to A at 1796 creates a constitutive active molecule (BRAF^{V600E}) that has been proved to be an oncogene in human cancer (Davies *et al.* 2002, Garnett & Marais 2004). Since the oncogene BRAF^{V600E} has become the most common genetic event associated with PTC, some studies, but not all, have recently found BRAF to be associated with aggressive clinicopathological features such as advanced clinical stages and extrathyroidal extension (Namba *et al.* 2003, Nikiforova *et al.* 2003). BRAF has also been proved to be present in poorly differentiated and anaplastic carcinomas that arise from a PTC (Nikiforova *et al.* 2003, Soares *et al.* 2004, Quiros *et al.* 2005). Finally, targeted expression of BRAF^{V600E} in thyroid cells of transgenic mice resulted in invasive PTCs that underwent dedifferentiation (Knauf *et al.* 2005). Thus, the biological behaviour of BRAF-positive tumours seems to be more aggressive than its partnerships in thyroid tumourigenesis, RET and RAS, in which no clear evidence of aggressiveness has been found. Nevertheless, the prognostic impact of BRAF remains to be elucidated, since at least four studies have not found any association with aggressive clinicopathological features (Xu *et al.* 2003, Fugazzola *et al.* 2004, Puxeddu *et al.* 2004, Trovisco *et al.* 2005) and the risk of recurrence or mortality have not been studied. In this retrospective study, we have demonstrated that BRAF^{V600E} is associated with a high recurrence rate during the early follow-up of a representative series of 67 patients with PTC. Interestingly, the majority of these recurrences had no avidity for radioiodine (¹³¹I), being ineffective by ¹³¹I treatment and pointing to a less differentiated state.

In order to confirm this less differentiated state associated with BRAF-positive tumours, we studied the specific role of BRAF^{V600E} in thyroid dedifferentiation, particularly Na⁺/I⁻ symporter (NIS) impairment. NIS is an integral plasma membrane glycoprotein that mediates active I⁻ transport into the thyroid follicular cells, the first step in thyroid hormone biosynthesis, and provides the basis for the effective diagnosis and therapeutic management of thyroid cancer and its metastases (Dohan *et al.* 2003). Assessment of NIS expression by immunohistochemistry (IHC) in human tumour samples and transfection experiments in rat thyroid cells demonstrated that

BRAF^{V600E} sharply impairs both NIS expression and trafficking to membrane. Surprisingly, this impairment is not dependent on MEK-ERK pathway activation. This last observation may have special significance as pharmacological therapies targeted to inhibit the MEK-ERK pathway will not be sufficient to re-differentiate tumours with constitutive activation of BRAF.

Materials and Methods

Subjects

A representative series of 67 patients with PTC who underwent surgical resection during the period 2000–2003 was selected from the Hospital Universitario La Paz (Madrid, Spain). The study protocol was approved by the Hospital Human Ethics Review Committee. The mean age of the patients was 42.8 ± 14 years and the female to male ratio was 3:1. No differences between patients existed as regards initial treatment or follow-up. Initial treatment in every patient consisted of total thyroidectomy, ¹³¹I ablation and thyrotrophin (TSH) suppression. When suspicious neck lymph nodes were noted preoperatively or at the time of surgery they were removed. Follow-up was carried out periodically with ¹³¹I total body scan (TBS) and serum thyroglobulin (Tg) (Delphia thyroglobulin kit; Perkin-Elmer, Wellesley, MA, USA), and in some selective cases cervical ultrasonography (US) was performed. We defined a low Tg as being less than 2 ng/ml when TSH was suppressed. Remission after thyroid surgery was considered when (i) no ¹³¹I uptake was found outside the thyroid bed on the post-ablation TBS, (ii) serum Tg levels remained undetectable following TSH stimulation and (iii) eventually no ¹³¹I uptake was seen on control ¹³¹I TBS. After thyroid ablation by surgery and ¹³¹I treatment, low uptake in the thyroid bed was not considered evidence of recurrent disease and did not warrant further treatment. Preoperative serum TSH levels were assessed in almost every patient, who were euthyroid at the time of surgery in all cases except one.

Clinical outcome

Clinical outcome was carefully reviewed, looking for any locoregional or distant recurrence. Preferentially, histology or cytology was needed to confirm recurrent disease. However, as this is not always possible, any elevation of serum Tg associated with positive ¹³¹I TBS and/or any other abnormal imaging study, including US, computerized tomography (CT) or

8F-deoxyglucose positron emission tomography (FDG-PET), were considered as showing recurrent disease. In all cases except two, Tg levels were measured during thyroxine withdrawal and significant elevations of TSH levels ($>30\mu\text{UI/dl}$) were obtained. In the other two cases, the levels of Tg were measured during TSH suppressive therapy, but they were sufficiently high to consider them positive.

Tumour samples

Paraffin-embedded tissues were obtained from the Department of Pathology of the aforementioned hospital. Clinical and pathological staging were carried out according to the TNM classification of the AJCC (2002). After the initial review and selection, glass slides from all carcinomas were re-examined by two independent pathologists who were blinded as to BRAF status and all other patient characteristics, and were subclassified as classic papillary carcinoma or as distinct histological variants based on the histopathological typing of the World Health Organization (2004).

DNA isolation, single-strand conformational polymorphism (SSCP) and sequencing

Genomic DNA was isolated using proteinase K digestion, phenolchloroform extraction and ethanol precipitation as previously described (Nikiforov *et al.* 1996). BRAF exon 15 was amplified by PCR. The following exon-based PCR primers were designed to amplify exon 15: forward, CAT AAT GCT TGC TCT GAT AGG and reverse, GTA ACT CAG CAG CAT CTC A. PCR conditions were as follows: amplifications were carried out for 40 cycles with an annealing temperature of 58°C . Fifty microlitre PCRs were performed on 200–300 ng genomic DNA, 7.5 pmol of each primer, 100 μM dNTPs, 5 μCi [$\alpha^{32}\text{P}$]dCTP, 1.5 mM MgCl_2 , TaqDNA polymerase high fidelity (Biotools, Madrid, Spain) and buffer. The amplified products were screened for mutation by SSCP (polyacrylamide 10%, without glycerol). Subsequently, amplified products from aberrant SSCP bands were purified using a PCR purification kit (Qiagen) and were sequenced using an automatic sequencer (ABI PRISM 3100; Applied Biosystems, Foster City, CA, USA).

Immunohistochemistry

A monoclonal antibody against the carboxy-terminal portion of human (hNIS) was used (Pohlenz *et al.*

2000). Thyroid tissue sections were studied using the catalyzed signal amplification protocol (DAKO Corp., Barcelona, Spain). Sections ($4\mu\text{m}$) were mounted on charged slides. All sections were baked at 60°C for 30 min. Slides were washed with three changes of xylene and hydrated through alcohol to distilled water. Antigen retrieval was performed using 10% citrate buffer in a steamer for 40 min, and rapid cooling was achieved with distilled water. Tissues were incubated in 3% peroxide for 15 min to quench endogenous peroxidase. Sections were blocked with serum-free protein, and endogenous biotin and avidin activity was blocked with the biotin blocking system (DAKO Corp.) All washes were performed with TBST (0.3 M NaCl, Tween 20 and 0.05 M Tris-HCl, pH 7.6) three times for 5 min each time. Slides were incubated for 30 min with human anti-NIS antibody diluted (1/60) in serum-free protein block. The streptavidin–biotin method as specified by the supplier (Dako) was followed. Peroxidase activity was detected with diaminobenzidine-hydrogen peroxide and was observed as a brown product.

Interpretation and grading of NIS staining was carried out by two independent pathologists. Immunoreactivity was characterized as negative (score = 0), absent or not interpretable (score = 1), weak positive (score = 2) or strong positive (score = 3) as described by Wapnir *et al.* (2003). Briefly, positive samples, either weak or strong, had to encompass at least 20% of cells to receive this overall score. When plasma membrane immunoreactivity was noticed, it was always scored as strong if 10% or more of cells demonstrated this feature either alone or in the presence of intracellular immunoreactivity. The main criterion to score NIS immunoreactivity as strong was the presence of plasma membrane immunoreactivity, as it is essential for NIS to be functional.

Cell culture

PCC13 thyroid cells were cultured in Coon's modified Ham's F-12 medium supplemented with 5% donor calf serum and a six hormone mixture necessary for the growth of the thyroid cells (1 nM TSH, 10 $\mu\text{g/ml}$ insulin, 10 ng/ml somatostatin, 5 $\mu\text{g/ml}$ transferrin, 10 nM hydrocortisone and 10 ng/ml glycyl-L-histidyl-L-lysine acetate; complete medium). To study the effect of TSH on NIS regulation, cells were also cultured in the same medium without TSH for different periods of time, as indicated in each experiment.

Plasmids

The following promoters fused to luciferase were used: pNIS-2.8 (Garcia & Santisteban 2002), p420 (thyroperoxidase) TPO–(luciferase) LUC (Aza-Blanc *et al.* 1993), minimal pTSH receptor (TSHR) (Civitareale *et al.* 1993) and pTg (Garcia-Jimenez *et al.* 2005). The expression vector pMCEF, harbouring the myc-tagged BRAF^{V600E} and BRAF wild type (wt), is described by Marais *et al.* (1996). PRL-TK, which contains a cDNA encoding Renilla (Promega), was used to monitor transfection efficiency.

Transfection assays

PCCI3 cells were plated at 6×10^5 cells per 60 mm diameter tissue culture dish 48 h before transfection. Transfection assays were performed with calcium phosphate coprecipitation and in some cases the Eugene lipid reagent (Roche) was used. Cells were collected for a LUC and Renilla activity assay using the dual-luciferase reporter assay system (Promega). In cotransfection experiments, the amount of DNA was normalized using the corresponding insertless expression vector as the carrier. The experiments were performed in triplicate.

Immunoblot analysis

Cells were harvested with RIPA buffer (PBS, 1% Nonidet, 0.5% sodium deoxycholate, 0.1% SDS) and proteinase inhibitors. In the next step, 40 µg of whole cell lysates were separated by electrophoresis in 10% SDS-PAGE, and then blotted onto nitrocellulose membrane (Protran; Schleicher & Schuell, Dassel, Germany). To quantitate the levels of ERK and phospho (P)-ERK, the blots were incubated for 60 min with the respective antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The antigen–antibody complexes were visualized with horseradish peroxidase-conjugated anti-rabbit IgG antibody by the enhanced chemiluminescence system (Amersham Pharmacia Biotech). A polyclonal rat NIS antibody (Wapnir *et al.* 2003) was used for NIS detection.

Immunofluorescence

Cells grown on coverslips were fixed in paraformaldehyde at room temperature for 15 min and stained for anti-NIS and anti-myc (Santa Cruz Biotechnology). The secondary antibodies used were anti-rabbit Alexa 594, anti-rabbit Alexa 488 (Molecular Probes, Paisley, UK). The cells were mounted with medium

Table 1 Relationships between the status of BRAF mutations and clinicopathological features of papillary carcinomas

	BRAF positive (%) n=28	BRAF negative (%) n=39	P
Age > 45 years	15 (53.5)	16 (41)	0.33
Male	4 (14.2)	8 (20.5)	0.74
Histologic variants of PTC			
Classic	18 (64.2)	17 (43.5)	0.2
Follicular variant	5 (17.8)	20 (51.2)	0.01
Tall cells	4 (14.2)	1 (2.5)	0.09
Columnar cells	1 (3.5)	1 (2.5)	
Oncocytic	1 (3.5)	0 (0)	
Multicentric	4 (14.2)	12 (30)	0.15
Extrathyroidal extension	18 (64)	11 (28)	0.003
Node metastases	9 (32)	9 (23)	0.42
Distant metastases	1 (3.5)	1 (2.5)	
Stage			
I–II	5 (17.8)	23 (60)	0.01
III–IV	23 (82)	16 (41)	

containing DAPI (Vectashield; Vector Laboratories, Peterborough, UK) and preparations were visualized with a Leica confocal TCS SP2 microscope.

Statistical analysis

Data were stored and analyzed using the SPSS software (version 12.0). Association between recurrent disease, BRAF^{V600E} and the histological variants or clinicopathological parameters of the thyroid tumours was determined by a χ^2 test. Statistical significance was based on $P < 0.05$.

Results

BRAF^{V600E} is associated with some aggressive clinicopathological features

The BRAF mutation was detected in 41.7% (28 of 67) of PTCs. Relationships between BRAF^{V600E} and clinicopathological variables are shown in Table 1. Significant association was seen between BRAF^{V600E} and both extrathyroidal extension and advanced AJCC stages. There was no significant relationship between BRAF mutation and gender, age >45 years and nodal or distant metastases at the time of diagnosis. Tumours lacking BRAF mutation were significantly associated with the follicular variant and, conversely, BRAF^{V600E} was more frequent in the classic variant, although not statistically significant. Also, BRAF mutation was significantly associated with the tall cell variant.

Table 2 Univariate relationships with recurrence rate

	Recurrence rate (%)	P
Age at time of diagnosis		
>45	19.4	
<45	16.7	NS
Gender		
Male	25	
Female	16.4	NS
PTC variants		
Classic	25	
Follicular	4	
Tall cells	40	NS
Multicentric		
Yes	25	
No	15	NS
Extrathyroidal extensión		
Yes	35	
No	5.3	0.003
Lymph nodes		
Yes	44.4	
No	8.2	0.002
Stage		
I–II	3.6	
III–IV	28.2	0.01
BRAF		
wt	7.6	
Mutant	32.1	0.02

NS, not significant.

BRAF^{V600E} is associated with a high recurrence rate, particularly with negative ¹³¹I TBS

Locoregional or distant recurrences occurred in 12 PTC patients (18%) at a median postoperative follow-up period of 3 years. Table 2 shows the univariate relationship between several clinicopathological features and the recurrence rate. Extrathyroidal extension, advanced AJCC stages and lymph node metastases were associated with a high recurrence rate, whereas a lower but not significant recurrence rate was observed in the follicular variant. Interestingly, there was a significant association between BRAF mutation and cancer recurrence (32% vs 7.6%; $P = 0.02$). Table 3 shows the main characteristics of the 12 patients who developed recurrences after a median postoperative follow-up period of 3 years. As mentioned before, patients with any elevation of Tg associated with positive ¹³¹I TBS and/or any abnormal imaging study were considered to have recurrent disease. Seven patients had persistent elevation of serum Tg but were negative for ¹³¹I TBS and therefore additional imaging studies were needed. FDG-PET was performed in four of them, finding anatomical substrate in three.

Interestingly, the frequency of recurrences with negative ¹³¹I TBS was higher in the BRAF-positive group (66% vs 33%), predicting a worse outcome and indicating a less differentiated state. Statistical significance was not achieved, presumably because the number of recurrences needs to be higher.

Low NIS immunoreactivity in BRAF-positive tumours

As BRAF-positive recurrences seem to have no avidity for ¹³¹I uptake, we further studied NIS immunoreactivity in a subset of 60 tumour samples and eight correspondent lymph node metastases. Previous studies have revealed that up to 70–80% of thyroid cancer express or even overexpress NIS in IHC profiles (Wapnir *et al.* 2003). In our study, tumours harbouring the BRAF mutation expressed significantly less NIS immunoreactivity than those without the mutation. The NIS staining was negative or weak positive and not targeted to the membrane in a significant majority of the samples within the BRAF-positive group compared with the BRAF-negative group (2.5% vs 30%; $P = 0.005$). In addition, strong positive samples that had membrane staining in at least 10–20% of the cells were observed predominantly in the follicular variant, yet this was not statistically significant. When concomitant node metastases at initial diagnosis were analyzed ($n = 8$), NIS staining was concordant to its primary tumours in all cases. Figure 1 shows several tissue samples with different patterns of NIS immunoreactivity.

BRAF^{V600E} impairs NIS promoter transcriptional activity in PCCl3 thyroid cells

We have already observed that lack of ¹³¹I uptake and low NIS immunoreactivity are associated with BRAF^{V600E}. We therefore wanted to study if BRAF^{V600E} affects NIS transcriptional activity in differentiated PCCl3 thyroid cells. Indeed, BRAF^{V600E} dramatically decreased the NIS promoter transcriptional activity (>90% reduction). We also investigated the effect on other thyroid specific promoters. A smaller effect was observed on TPO promoter (60% reduction) and TSHR promoter (50% reduction), being almost undetectable on Tg promoter (10% reduction) (Fig. 2A). As BRAF mediates signal transduction through the MEK-ERK pathway, we used the MEK inhibitor U0126 to study whether this pathway was involved in NIS transcriptional impairment induced by BRAF^{V600E}. As shown in Fig. 2B, BRAF^{V600E} repression of NIS was partially reversed

Table 3 Main characteristics of the 12 patients with thyroid cancer recurrences

Patient no.	Age1	Age2	G	Surgery	Subtype histology	Stage	ExExt	TotDo	Serum Tg (ng/ml)	TSH (μIU/dl)	TBS	Additional findings	BRAF status
Case 1	63	67	F	T.T.	Tall cells	IV	Yes	400	105	100	LR	US: cervical nodes *Extirpation of cervical nodes	POS
Case 2	36	37	F	T.T.	Classic	I	Yes	250	4	182	LR	Partial response to ¹³¹ I ablation	POS
Case 3	27	28	F	T.T.	Classic	I	No	300	15.5	98	NEG	US: cervical nodes FDG-PET: negative	POS
Case 4	75	80	F	S.T.+LNS	Classic	III	No	250	4.6	1.75	NEG	TC: cervical nodes *Extirpation cervical nodes	POS
Case 5	40	41	F	T.T.	Classic	I	No	250	8.08	100	LR+Med	FDG-PET: pulmonic nodule	NEG
Case 6	40	41	M	T.T.+LNS	Follicular	I	Yes	100	4.3	0.01	NEG	US: cervical node	POS
Case 7	47	49	M	T.T.+LNS	Classic	III	Yes	150	11.6	>100	NEG	FDG-PET:cervical, clavicular and axilar *Extirpation cervical nodes	NEG
Case 8	67	68	F	T.T.	Classic	III	Yes	200	14.5	80	NEG	TC: cervical nodes *Extirpation cervical nodes	POS
Case 9	38	39	F	T.T.	Classic	I	No	215	2.5	63	LR	Good response to ¹³¹ I ablation	POS
Case 10	38	39	M	T.T.+LNS	Tall cells	IV	Yes	200	2.17	90	LR	FDG-PET: cervical and mediastinal nodes TC: diffuse pulmonary Bone scan: mts	NEG
Case 11	31	33	F	T.T.	Classic	I	Yes	100	62	85	NEG	FDG-PET: cervical nodes	POS
Case 12	61	62	M	T.T.	Classic	IV	Yes	100	32	95	NEG	TAC: cervical FNDA: positive *Extirpation cervical nodes	POS

Age1, age at diagnosis; Age2, age at recurrence; G, gender; T.T, total thyroidectomy; LNS, lymph node surgery; S.T., subtotal thyroidectomy; LR, local recurrence; Med, mediastinum; NEG, negative; POS, positive, ExExt, extrathyroid extension; TotDo, total cumulative dose (mCi); TC, computerised tomography; FNAC, fine needle aspiration cytology; Mts, metastasis.

*The histological data provided by the pathologist confirmed the presence of thyroid cancer recurrence in these lesions.

Figure 1 NIS expression in several samples of PTC. Sections (4 μ m) were probed with anti-NIS antibody. Scoring system: 0=negative, 2=weakly positive and 3=strongly positive. Almost all strongly positive samples had membrane staining in at least 10% or more of the cells. (A) Normal thyroid tissue showing plasma membrane immunoreactivity in 10–20% of the follicular cells (score 3; original magnification 20 $\times$). (B) Graves' disease showing follicular hyperplasia and predominant plasma membrane staining in more than 90% of the cells, used as a positive control (score 3; original magnification 20 $\times$). (C) Classic PTC with intracellular immunoreactivity and distinct plasma membrane in >10% of the cells (score 3; original magnification 40 $\times$). (D) Follicular variant of PTC with intracellular immunoreactivity and distinct plasma membrane in >10% of the cells (score 3; original magnification 20 $\times$). (E) Several cells with distinct plasma membrane in a follicular variant of PTC (score 3; original magnification 40 $\times$). (F) Lymphatic node with metastases of PTC showing distinct plasma membrane immunoreactivity (score 3; original magnification 20 $\times$). Scale bars=50 μ M.

Figure 2 BRAF^{V600E} impairs NIS promoter transcriptional activity. (A) PCCl3 cells were co-transfected with expression vectors harbouring wt or BRAF^{V600E} mutation, together with the promoter constructs of NIS, TPO, TSHR or Tg genes respectively. (B) The MEK inhibitor UO126 was used to study the MEK-ERK pathway, in this case DMSO was used as a control. Forty-eight hours after transfection the cells were harvested and luciferase and Renilla were assayed. The promoter activity is expressed as relative luciferase activity over control cells transfected with empty vector.

when MEK was inhibited, proving a limited role of the MEK-ERK pathway.

BRAF^{V600E} impairs NIS trafficking to the membrane in PCCl3 thyroid cells

Active I⁻ transport into fully differentiated PCCl3 cells depends on NIS localization in the plasma

membrane. As previously described (Dohan & Carrasco 2003) and shown in Fig. 3A, this process is dependent on TSH. After TSH withdrawal, NIS targeting to membrane was impaired immediately (Fig. 3A, left panel) whereas NIS protein expression diminished progressively in 7 days and higher molecular bands became predominant (Fig. 3A, right panel), confirming the previous suggestions that

Figure 3 BRAF^{V600E} impairs NIS targeting to plasma membrane through pathways others than MEK-ERK. Immunofluorescence assays (left) and Western blot analysis (right) performed in differentiated PCCl3 or K-RAS-transformed cells to determine NIS subcellular distribution and protein levels. (A) Effect of TSH withdrawal, (B) effect of BRAF^{V600E} over-expression, (C) effect of a cAMP inducer (forskolin (Forsk)) and an MEK inhibitor UO126 and (D) effect of UO126 over K-RAS-transformed PCCl3 cells. Immunofluorescence with myc antibody was performed as the control of BRAF transfection efficiency. In all cases the Western blots were also hybridized with anti-P-ERK and ERK antibodies. The arrows in the Western blot indicate the high molecular weight bands of NIS protein. d, day.

postranslational mechanisms dependent on TSH are responsible for NIS targeting to the membrane (Dohan & Carrasco 2003). Interestingly, when PCCI3 cells were transiently transfected with BRAF^{V600E} a very similar pattern was observed: NIS targeting to membrane was impaired immediately (Fig. 3B, left panel), protein expression decreased progressively and higher molecular bands became predominant (Fig. 3B, right panel), suggesting that NIS impairment is due to BRAF interference of TSH-mediated responses. To study whether the TSHR by itself or its distal signalling was affected, we stimulated PCCI3 cells expressing BRAF^{V600E} with a cAMP inducer (forskolin). NIS expression was partially recovered but was not targeted to the membrane (Fig. 3C, left and right panels) suggesting that NIS impairment by BRAF^{V600E} occurs, at least in part, downstream of the TSHR/cAMP signalling.

Limited role of the MEK-ERK pathway in NIS impairment induced by BRAF^{V600E}

We have already described the limited role of the MAP kinase (MAPK) pathway on the impairment of NIS promoter activity by BRAF^{V600E} (Fig. 2B). We further studied NIS protein expression and its subcellular localization. Although we observed partial reinduction of NIS protein expression using a MEK inhibitor, no relocalization to the membrane was observed, even when we concomitantly stimulated with forskolin (Fig. 3C, left and right panels). To further confirm these results, PCCI3 cells stably transfected with the oncogene Kirsten (K)-RAS were used. These fully transformed cells have constitutive activation of the MEK-ERK pathway and do not express NIS (Santoro *et al.* 1993). Again, the MEK inhibitor partially reinduced NIS protein expression but no targeting to membrane was observed (Fig. 3D, left and right panels). This indicates a very limited role of the MEK-ERK pathway in thyroid BRAF-induced dedifferentiation, especially in NIS impairment.

Discussion

The question as to whether BRAF confers a distinct biological behaviour to PTC that could have a prognostic value is still controversial. The inconsistent results described by several groups (Soares *et al.* 2003, Xu *et al.* 2003, Fugazzola *et al.* 2004, Trovisco *et al.* 2005) could be partially due to an insufficient number of patients and/or to the different combinations of various subtypes of PTC included in each study. Subtype stratification is likely to be important as

BRAF is present predominantly in the classic and tall cell variants of PTC and not in the follicular variant. In our study, with classic PTC accounting for more than 50% of the total, BRAF mutation predicted an increased risk of recurrence. The univariate analysis also revealed that BRAF was associated with extra-thyroidal extension and advanced clinical stage. Indeed, larger series including mortality rate and multivariate analysis with adjustment for various confounding factors will reveal the independent prognostic role of BRAF. We not only demonstrated that BRAF-positive tumours have more risk of recurrence, but also that they are likely to be less differentiated as ¹³¹I uptake is absent in the majority of the BRAF-positive recurrences. This is challenging for thyroid cancer management as anatomical localization of recurrences cannot be assessed and treatment with ablative doses of ¹³¹I is not effective, predicting a poorer outcome. As we can see in Table 2, six out of nine (66%) BRAF-positive recurrences were negative for ¹³¹I scans and residual disease was finally located performing FDG-PET in three of them. This is interesting, as the switch of iodide uptake into high FDG uptake due to enhanced glucose metabolism has been proposed as a model of tumoral dedifferentiation in thyroid cancer (Schlumberger & Pacini 2003b). Several studies have proved the utility of FDG-PET during the postoperative management of thyroid cancer, particularly in patients with elevated serum Tg and negative ¹³¹I scans (Hooft *et al.* 2001). Perhaps, in the future, FDG-PET may have an important role in the initial management of patients with BRAF-positive tumours because of their high risk of less differentiated recurrences.

Concordantly, we have also observed an association between BRAF-positive tumours and low NIS immunoreactivity in a subset of 60 paraffin-embedded samples. In previous studies (Saito *et al.* 1998, Dohan *et al.* 2001, Wapnir *et al.* 2003), assessment of NIS expression by IHC revealed that up to 70–80% of thyroid cancers expressed or even overexpressed NIS, yet this expression was mainly cytoplasmatic and not targeted to the basolateral membrane. It has been postulated that targeting to and retention in the plasma membrane is essential for NIS to be fully functional, explaining why I[−] uptake is diminished in thyroid cancer (Dohan & Carrasco 2003). Additionally, positive NIS immunoreactivity in primary tumours seems to be predictive of subsequent recurrences positive in ¹³¹I scans (Castro *et al.* 2001, Schmitz *et al.* 2005), whereas low NIS expression assessed by RT-PCR has been correlated with more aggressive tumours in another study (Ward *et al.* 2003). Overall,

NIS expression may have a role as a new biological marker in the postoperative management of patients with change to thyroid cancer (CDT). In our study, the tumours harbouring the mutation had significantly less NIS immunoreactivity and this is consistent with our previous data that suggest that BRAF-positive tumours have lost their ability to trap ^{131}I and, thus, they are less differentiated. Additional findings, such as the association of strong NIS staining with the follicular variant, suggest that cellular polarity may play an important role for NIS to be functional. In addition, we have observed that low NIS staining is associated with advanced stages in the TNM classification (data not shown). However, the main limitation to establish NIS as a biological marker is methodological. Highly sensitive visualization systems are required and NIS antibodies are still poorly developed.

We also analyzed the specific effects of BRAF^{V600E} in a well-differentiated rat cell line (PCCl3), which expresses functional NIS in the membrane. In a previous study (Mitsutake *et al.* 2005), mRNA levels of NIS were decreased by conditional expression of BRAF^{V600E}. In our study, we observed a marked decrease of the transcriptional activity of the NIS promoter when PCCl3 cells were transfected with BRAF^{V600E} compared with wt. By contrast, TPO and TSHR decreased more moderately, and Tg even more slightly, which may reflect the fact that thyroid cancer is a multistep process where BRAF-positive tumours are still differentiated although to a lesser extent, and more steps are required in order to evolve to a poor or anaplastic thyroid cancer, where none of the thyroid specific genes are any longer expressed. Secondly, BRAF^{V600E} immediately impairs NIS targeting to the membrane and progressively decreases NIS protein expression in the same fashion as does TSH withdrawal. This suggests that the transcriptional and postranslational NIS modifications are due to BRAF interference of TSH-mediated responses. The fact that the impaired NIS protein has a distinct molecular weight, in this case higher, suggests that different patterns of glycosylation and especially phosphorylation are taking place. As previously described (Mitsutake *et al.* 2005), our study also shows that BRAF activation impairs cAMP-induced expression of NIS, although there is a partial recovery that it is not targeted to the membrane. This suggests that the effects of BRAF on NIS expression cannot be due only to a decreased abundance of TSHR, and other distal steps at a transcriptional level seem to be affected by BRAF^{V600E} in TSH-mediated responses.

Finally, the role of the MAPK pathway in thyroid dedifferentiation induced by BRAF^{V600E}, particularly

NIS, seems to be small and limited to a transcriptional level. It is worth noting that the MAPK pathway seems to play a central role in PTC tumourigenesis as RET/PTC, RAS and BRAF are mutually exclusive genetic events, all of which activate this pathway. A recent report has demonstrated that RET/PTC induces RAS- and BRAF-dependent ERK activation, pointing out a linear oncogenic signalling cascade that governs proliferation and invasion in transformed thyroid cells (Melillo *et al.* 2005). Moreover, Knauf *et al.* (2003) have reported that MEK inhibitors increase NIS mRNA levels in thyroid cells expressing RET/PTC or RAS^{V12} and they have suggested that inhibiting the MEK-ERK pathway may promote redifferentiation in tumours with constitutive activation of either RAS or RET/PTC (Knauf *et al.* 2003). In our study, although we found a partial reinduction in NIS expression with a MEK inhibitor in thyroid cells expressing BRAF^{V600E}, this NIS reinduction was low (see Figs 2 and 3) and, most importantly, was unable to target to the membrane, and consequently was not functional. Presumably BRAF acts either directly or through pathways others than MEK-ERK in inducing thyroid dedifferentiation, as constitutive activation of MEK-ERK does not fully explain the NIS modifications at transcriptional and postransductional levels. Therefore therapies that target inhibition of the MEK-ERK pathway may not be able to redifferentiate tumours with constitutive activation of BRAF in order to express NIS in the membrane. In addition, this also underscores that, although the three oncoproteins work together along a single cascade, they are able to trigger specific signals and therefore confer distinct biological behaviour.

Overall, BRAF confers more aggressiveness to the biological behaviour of PTC, as early recurrences are more frequent and tumours are less differentiated, predicting poorer outcomes as treatment with ^{131}I is not useful. This can help clinicians distinguish between high-risk and low-risk patients at the time of diagnosis. Although more studies, including larger series, longer follow-ups and mortality are needed, we believe that the preoperative assessment of BRAF status can improve the subsequent management of thyroid cancer, as more extensive surgery can be performed as well as a more exhaustive follow-up, including FDG-PET, can be considered (Soares *et al.* 2003).

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