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 (^{131}I) total body scans, predicting a poorer outcome as treatment with ^{131}I 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 PCC13 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 ^{131}I 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
BRAFV600E is associated with a high recurrence rate. In this retrospective study, we have demonstrated that recurrence or mortality have not been studied. In stages and extrathyroidal extension (Namba et al. 2003), BRAF has been found to be associated with aggressive clinicopathological features such as advanced clinical features (Xu et al. 2003, Fugazzola et al. 2004, Puxeddu et al. 2005, Soares et al. 2004, Quiros et al. 2005). Finally, targeted expression of BRAFV600E 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 BRAFV600E 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 BRAFV600E 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 BRAFV600E 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 μU/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, phenol-chloroform 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 [α32P]dCTP, 1.5 mM MgCl2, 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 μm) 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 strepavidin–biotin method as specified by the supplier (Dako) was followed. Peroxidase activity was detected with diamino-benzidine-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 μg/ml insulin, 10 ng/ml somatostatin, 5 μg/ml transferrin, 10 nM hydrocortisone and 10 ng/ml glycy1-L-histidyl-L-lysine actetate; 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 BRAFV600E 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
PCCl3 cells were plated at 6×10^5 cells per 60 mm diameter tissue culture dish 48h before transfection. Transfection assays were performed with calcium phosphate coprecipitation and in some cases the Fugene 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, 40µg 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 60min 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 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, BRAFV600E and the histological variants or clinicopathological parameters of the thyroid tumours was determined by a χ² test. Statistical significance was based on P<0.05.

Results
BRAFV600E is associated with some aggressive clinicopathological features
The BRAF mutation was detected in 41.7% (28 of 67) of PTCs. Relationships between BRAFV600E and the histological variants or clinicopathological parameters of the thyroid tumours was determined by a χ² test. Statistical significance was based on P<0.05.
BRAFV600E 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%; \( P = 0.02 \)). Statistical significance was not achieved, presumably because the number of recurrences needs to be higher.

**Table 2** Univariate relationships with recurrence rate

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

NS, not significant.

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.

**BRAFV600E 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 BRAFV600E. We therefore wanted to study if BRAFV600E affects NIS transcriptional activity in differentiated PCCl3 thyroid cells. Indeed, BRAFV600E 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 BRAFV600E. As shown in Fig. 2B, BRAFV600E repression of NIS was partially reversed.
Table 3  Main characteristics of the 12 patients with thyroid cancer recurrences

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

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 μm) 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×). (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×). (C) Classic PTC with intracellular immunoreactivity and distinct plasma membrane in >10% of the cells (score 3; original magnification 40×). (D) Follicular variant of PTC with intracellular immunoreactivity and distinct plasma membrane in >10% of the cells (score 3; original magnification 20×). (E) Several cells with distinct plasma membrane in a follicular variant of PTC (score 3; original magnification 40×). (F) Lymphatic node with metastases of PTC showing distinct plasma membrane immunoreactivity (score 3; original magnification 20×). Scale bars=50 μM.
when MEK was inhibited, proving a limited role of the MEK-ERK pathway.

**BRAF^V600E** impairs NIS trafficking to the membrane in PCC13 thyroid cells

Active I^- transport into fully differentiated PCC13 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

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Figure 3 BRAFV600E 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 BRAFV600E over-expression, (C) effect of a cAMP inductor (forskolin (Forsk)) and an MEK inhibitor U0126 and (D) effect of U0126 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.
posttranslational mechanisms dependent on TSH are responsible for NIS targeting to the membrane (Dohan & Carrasco 2003). Interestingly, when PCCl3 cells were transiently transfected with BRAF\textsuperscript{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 PCCl3 cells expressing BRAF\textsuperscript{V600E} with a cAMP inductor (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\textsuperscript{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\textsuperscript{V600E}**

We have already described the limited role of the MAP kinase (MAPK) pathway on the impairment of NIS promoter activity by BRAF\textsuperscript{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, PCCl3 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 (Santorò 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 \textsuperscript{131}I 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 \textsuperscript{131}I 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 \textsuperscript{131}I 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 \textsuperscript{131}I scans (Hoofit 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 cytoplasmic 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\textsuperscript{−} 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 \textsuperscript{131}I 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 (PCCI3), 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 PCCI3 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 posttranslational 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 posttranslational 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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