CLINICAL STUDY

BRAF<sup>V600E</sup> mutation and expression of proangiogenic molecular markers in papillary thyroid carcinomas

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Abstract

Objective: Tyrosine kinase inhibitors (TKIs) are evaluated for treatment of radioiodine refractory thyroid cancer. Their effects in this setting are based on blockade of proangiogenic signaling mediated by receptors for vascular endothelial growth factors (VEGFs) and platelet-derived growth factors (PDGFs). Most TKIs also block other cancer-relevant kinases, such as B-type Raf kinase (BRAF), which are constitutively activated in approximately half of papillary thyroid carcinomas (PTCs), but the impact of these effects is not clear.

Design: The aim of our study was to investigate the impact of BRAF<sup>V600E</sup> on proangiogenic gene expression and microvascular features of PTCs.

Methods: mRNA levels for VEGFA, VEGF<sub>A</sub>, VEGF<sub>B</sub> receptors, and coreceptors (VEGFRs 1, 2, and 3, neuropilin-1, and PDGFR receptor β (PDGFRβ or PDGFRB)) were measured with real-time PCR in BRAF<sup>V600E</sup> (n = 55) and wild-type BRAF (BRAF-wt; n = 35) PTCs. VEGF and VEGFR protein expression and microvessel densities (MVD) and lymphatic vessel densities (LVDs) were assessed by immuno-histochemistry in 22 of the 90 PTCs (including 11 BRAF<sup>V600E</sup> cases). Angiogenic gene expression was also studied <i>in vitro</i> after induction/silencing of the BRAF<sup>V600E</sup> mutation in thyrocyte lines.

Results: Transcript levels of proangiogenic factors were significantly lower in BRAF<sup>V600E</sup> PTCs versus BRAF-wt PTCs (P < 0.0001), but MVD and LVDs were not significantly different. VEGFA mRNA levels in thyroid cell lines decreased when BRAF<sup>V600E</sup> mutation was induced (P = 0.01) and increased when it was silenced (P = 0.01).

Conclusions: Compared with BRAF-wt PTCs, those harboring BRAF<sup>V600E</sup> exhibit downregulated VEGFA, VEGFR, and PDGFRβ expression, suggesting that the presence of BRAF mutation does not imply a stronger prediction of response to drugs targeting VEGF and PDGFβ signaling pathways.

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Introduction

Approximately 90% of differentiated thyroid cancers (DTCs) can be successfully managed with surgical resection and radioactive iodine (¹¹³I) ablation (1). However, when distant metastases occur and the cancer becomes refractory to radioiodine therapy mainly for the loss of the sodium/iodide symporter expression (2), survival rates plummet, because effective alternatives to radioiodine are lacking (3, 4). Hopes have been raised by the development of novel, molecularly targeted cancer therapies, in particular tyrosine kinase inhibitors (TKIs) or agents targeting epigenetic alterations, which can block critical oncogenic signal transduction pathways (5). Two first-generation TKIs, sorafenib and sunitinib, have been approved for treatment of several solid tumors and are now being tested in patients with thyroid cancer. Promising results have already emerged from phase II studies of sorafenib for the treatment of metastatic, radioiodine-resistant thyroid cancer (6, 7), and the 2009 American Thyroid Association (ATA) guidelines for the management of DTCs strongly recommend TKIs for progressive or symptomatic metastatic disease (1). However, responses to these drugs vary and they can have serious adverse effects, so they need to be used where they are most likely to produce benefits.

The TKIs undergoing clinical testing for DTCs all have antiangiogenic effects that are mediated in large part by...
blocking the activation of receptors for vascular endothelial growth factors (VEGFs) and platelet-derived growth factors (PDGF) expressed on endothelial cells. Interaction between VEGF receptors (VEGFRs) 1, 2, and 3 and their ligands (VEGFA in particular) gives rise to intracellular signaling that plays major roles in producing new blood vessels to sustain tumor growth. VEGFR2 is the major mediator of angiogenesis, and its signal transduction capacity is enhanced by neuropilin 1 (NRP1), which acts as a facilitative coreceptor during VEGFA–VEGFR2 binding. VEGFR1 can also bind NRP1, rendering it less available for interaction with VEGFR2. The maturation and stability of the new vessels also depend on another signaling pathway, which is activated by binding between PDGF and their receptors (PDGFRα or PDGFRα and PDGFRβ or PDGFRβ) (8). Studies of microvascular density have generated conflicting pictures of the vascular phenotypes of DTCs (10, 11), but increased VEGF and PDGFRβ expression has been extensively documented in tumors (12–19) and serum (20) from patients with papillary thyroid cancers (PTCs). VEGF- and PDGF-mediated angiogenesis thus appears to be a reasonable target in the treatment of PTCs.

Most recently developed TKIs also affect other kinase-dependent signaling pathways involved in tumor cell proliferation. The actual significance of these other forms of signal repression is unclear, although they are expected to increase efficacy and/or broaden the drugs' range of action. Sorafenib, for example, inhibits B-type Raf kinase (BRAF), an important component of the mitogen-activated protein kinase pathway. This property should be particularly useful in the treatment of PTCs because approximately half of these tumors harbor activating BRAF mutations, in most cases involving a glutamine for valine substitution at residue 600 (BRAFV600E). Apart from its proproliferation effects, BRAFV600E has also been linked to reduced expression of several iodine metabolism genes, a phenomenon with potential negative effects on tumor responsiveness to radioiodine ablation (21). It has also been associated with various proangiogenic phenomena, including significantly increased VEGF (17) and PDGFRβ (18) expression in PTCs, as determined by immunohistochemistry.

In light of these findings, BRAF-mutant PTCs look like excellent candidates for antiangiogenic therapy based on multiple kinase inhibition. This hypothesis has been marginally explored in two phase II clinical trials only, but in both cases the number of patients whose BRAF status known was too small to allow any meaningful conclusions (7, 22). We attempted to clarify this issue with a multilevel study of thyroid cancer angiogenesis that included molecular and histological studies of a large study of human PTCs as well as in vitro experiments involving the induced expression and silencing of BRAFV600E. The questions we tried to answer were as follows: i) are PTCs indeed characterized by increased vascularization? what effect (if any) does BRAFV600E have on tumor angiogenesis and on the expression of components of VEGF- and PDGF-mediated signaling? ii) how do angiogenic gene expression and BRAF mutation status correlate with clinicopathological markers of increased risk PTCs?

Materials and methods

The study followed protocols approved by the Review Board, and written informed consent was obtained from all patients whose tumors were analyzed. Unless specified, all commercial products mentioned below were used according to manufacturers' instructions.

Patients and tissues

Thyroid cancer tissues were obtained at thyroidectomy from 90 patients operated for sporadic PTCs at the University Hospitals of Rome and Perugia (these cases have been the subject of other analyses in previous reports by our group (21)). The samples were snap-frozen in liquid nitrogen and stored at −80 °C. Tumors harboring the ret/PTC1 or 3 rearrangement were excluded from this analysis. All samples were reviewed by a single pathologist, who confirmed the diagnosis of PTC, identified the histological variant of the tumor, and excluded the presence of normal tissue in the specimen. Clinical information on the study population was collected by retrospective review of hospital charts, and tumors were staged according to the criteria of the American Joint Committee on Cancer (23). Details on BRAF mutation analysis have been provided elsewhere (21). Routinely processed, formalin-fixed sections from 22 of the 90 PTCs were used for immunohistochemical studies (described below).

The 90 cases were risk-stratified on the basis of clinical and histological data in accordance with ATA recommendations (1). Low risk was defined as a primary tumor stage of T1–T2, no evidence of local or distant metastases at the time of primary treatment or during follow-up, and the absence of aggressive histotypes (e.g. tall cell variant). Intermediate risk was manifested by stage T3 primary tumors, and/or locoregional metastases, and/or an aggressive histotype, and high risk included stage T4 primary tumors and/or those associated with distant metastases.

Cell lines

PC-BRAF9.6 cells, which are characterized by doxycycline-inducible expression of BRAFV600E mutation, were derived from the PCCCL3 line (a clonal rat thyroid line requiring TSH for growth), as described elsewhere (24). PC-BRAF9.6 cells were grown to near confluence in 10 cm dishes and then transferred to TSH-free medium for 3 days before the addition of 1 mg/ml doxycycline for 48 h.
We obtained 8505C cells from Dr C Nucera (Harvard Medical School, Boston, MA, USA), whose team established this cell line from undifferentiated human thyroid carcinoma cells expressing the BRAFV600E mutation and more recently verified its follicular cell origins (25). Cells were grown in DMEM supplemented with 10% fetal bovine serum (Gibco). BRAF silencing was performed using stealth RNAi technology (Invitrogen Corp.) and the 8505 cells were transiently transfected with Lipofectamine 2000 reagent according to the manufacturer’s instructions (Invitrogen Corp.). In order to find the more efficient BRAF silencing conditions, transfections were repeated in the presence of increasing BRAF’s concentrations, varying from 160 to 320 nM, and cells were grown for 24 to 96 h, 200 nM appeared as the most efficient concentration.

In detail, 24 h after seeding (density: 1.5×10⁶/100 mm dish), cells were transfected in Optimem with 200 nM of BRAF-specific Stealth RNAi (5’-UGGA-GAUGGUGUACACAAGCUGGAC-3’) or negative control RNAi (Invitrogen Corp.). After 72 h, the cells were harvested and lysed in radioimmunoprecipitation assay buffer. To evaluate the degree of BRAF silencing, lysates (20 μg) were analyzed with 10% SDS gel electrophoresis and western blotting with commercial anti-BRAF antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

**Gene expression analysis of angiogenic factors**

These analyses were based on the use of real-time quantitative RT-PCR (RT-qPCR). Details of the method (including RNA isolation and reverse transcription) have been described elsewhere (26). Assays-on-Demand Gene Expression Products (Applied Biosystems, Foster City, CA, USA) were used to measure transcription levels of mRNA for VEGFA, VEGFRs 1, 2, and 3, NRP1, and PDGFRβ in i) each of the 90 PTC samples and a control group consisting of 31 follicular adenomas (the latter have been employed in a previous study from our group (26)); a commercial pool of RNA from 64 normal thyroids (obtained from male and female Caucasians aged 15–61 years, who had succumbed to sudden death; Clontech) was used as calibrator; ii) in PC-BRAF 9.6 cells incubated with or (as controls) without doxycycline; and iii) in 8505C cells transfected with BRAF-specific siRNA or (as controls) with negative control RNAi. The β-actin gene was used as an endogenous reference (pre-developed TaqMan assay reagents; P/N 4326315E; Applied Biosystems). Results (determined with the 2−ΔΔCt method (27)) were normalized to corresponding calibrator samples (specified above), and the coefficient of interassay variation (calculated on Ct values) was consistently ≤1.40% (VEGFA: 0.40%; VEGFR1: 0.53%; VEGFR2: 0.36%; VEGFR3: 1.40%; NRP1: 0.75%; PDGFRβ: 0.31%).

**Immunohistochemistry and analysis of microvessel and lymphatic vessel densities**

Immunohistochemical studies included assessments of angiogenic protein expression and microvessel densities (MVD; blood and lymphatic). They were performed according to standard procedures (28) on routinely processed, formalin-fixed sections from 12 to 22 of the 90 PTCs analyzed by RT-qPCR. All of these sections came from the archives of the Pathology Department of the University of Perugia, and equal numbers of tumors were randomly selected from subgroups of tumors with VEGF (BRAFwt) and wild-type BRAF (BRAF-mut) genotypes.

Sections were immunostained with antibodies against the following angiogenic proteins: VEGF (mouse MAB, Zymed Laboratories, San Francisco, CA, USA; dilution 1:500); VEGFR1 (Flt1 receptor; mouse MAB, Diagnostic Biosystem, Pleasanton, CA, USA; dilution 1:50); VEGFR2 (rabbit polyclonal antibody, R&D Systems, Minneapolis, MN, USA; dilution 1:100); and VEGFR3 (rabbit polyclonal antibody, Zymed Laboratories; dilution 1:300) (Immunohistochemistry for PDGFRs was not performed due to the limited amounts of tissue that were available.). Labeling in tumors and adjacent non-neoplastic thyroid tissue was revealed with a biotin-free HRP polymer detection method (Ultravision LP, LabVision Corporation, Fremont, CA, USA) using pretreatments, prediluted antibodies, and a biotin-free detection method.

MVD was assessed with a commercial antibody (Cell Marque, Rocklin, CA, USA) that recognizes the CD31 marker expressed by endothelial cells of blood and lymphatic vessels (29). Microscopic assessment of microvessel density (MVD) was done with Cell Marque’s mouse MAB clone D2–40, which recognizes podoplanin, a marker specific for lymphatic endothelial cells (30). Immunostaining for CD31 and D2–40 was performed with a BenchMark XT automated platform (Ventana Medical Systems, Tucson, AZ, USA) using pretreatments, prediluted antibodies, and a biotin-free detection method.

MVD and LVD were measured in intra- and peri-tumoral tissues and in non-neoplastic thyroid tissue by two independent observers using the Weidner protocol (31) with minor modifications. Briefly, intra- and peri-tumoral microvascular hot spots and representative areas of non-neoplastic thyroid tissue were identified during low-power (×40 and ×100, total magnification) scans. In each of these areas, the three zones with the highest vessel density were then selected, individual vessels were counted at high power (×200) in each, and the three counts were averaged. The mean of the average values determined from three such readings from each of the intratumoral, peritumoral, and non-neoplastic areas of the sample determined the final scores. Vessels located within 2 mm of the tumor

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front were considered peritumoral. When a well-defined capsule was present, the 2 mm margin was calculated from the outer surface of the fibrous wall. To assess MVD in non-neoplastic thyroid parenchyma tissue, we examined tissue located beyond the peritumoral zone (often in a different paraffin block), avoiding areas of lymphocyte infiltration or fibrosis.

Statistical analysis

RT-qPCR results are expressed as mean ± S.D. Intergroup differences were assessed with the one-sample t-test (PTC versus normal thyroid tissues) and the Mann–Whitney nonparametric test (PTC versus follicular adenomas and BRAF-mut tumors versus BRAF-wt tumors). Associations between risk category (low versus intermediate/high) and BRAF mutation status or level of mRNA for proangiogenic factors were assessed with the Fisher exact test and Mann–Whitney nonparametric test respectively. This analysis was performed with StatView 5.0.1 Software (SAS Institute, Inc., Cary, NC, USA). Immunohistochemical data were subjected to one-way ANOVA with the Bonferroni multiple comparison post-test or to analysis with the unpaired t-test when appropriate. Both analyses were performed with GraphPad Prism Software (GraphPad, San Diego, CA, USA). P < 0.05 was used as the cutoff for statistical significance.

Results

Gene expression analysis of proangiogenic factors in PTCs

As shown in Table 1, RT-qPCR was used to evaluate the transcription of six proangiogenic genes in the 90 PTCs. Compared with the normal thyroid tissue pool, the PTCs displayed significantly lower mean mRNA levels for VEGFRs 1, 2, and 3 and for PDGFRβ, and a similar mean levels of VEGFA, and NRP1. When compared with follicular adenomas, the PTCs displayed higher mRNA transcript levels in all but one (i.e. VEGFR3) proangiogenic factors. The latter differences reached the significance only for VEGFR3, NRP1, and PDGFRβ. As shown in Table 1, this trend can be almost entirely ascribed to the BRAF-wt PTCs. Subgroup analysis of the 90 PTCs showed that transcription of all six proangiogenic genes was significantly reduced in the BRAF-mut tumors.

Immunohistochemical analysis of proangiogenic protein expression and vessel densities

Figure 1 summarizes the immunohistochemical findings for VEGF and VEGFR1, 2, and 3 expression. In tumor cells and non-neoplastic thyroid cells adjacent to the tumor, VEGF expression was predominantly cytoplasmic (Fig. 2a) and in most cases similar in intensity, but VEGF immunostaining was clearly increased in two tumors (one classic PTC that was BRAF-wt and in one tall cell variant that was BRAF-mut; Fig. 1). VEGF expression intensity was not significantly different in BRAF-mut and BRAF-wt subgroups. VEGFR1, 2, and 3 expression (assessed in only 12 cases) was localized to the cell membrane and cytoplasm in both neoplastic and non-neoplastic thyroid cells (Fig. 2b–f), and VEGFR3 labeling was often less intense than that for VEGFR1 or 2. Compared with non-neoplastic thyrocytes, tumor cells usually exhibited reduced VEGFR1 expression (Figs 1 and 2c and d); some PTCs also displayed diminished expression of VEGFRs 2 and 3 (Figs 1 and 2e and f). No clear relation could be discerned between expression of the three VEGFRs and the BRAF mutation status of the tumors, although VEGFR1 was reduced in all eight BRAF-mut PTCs (P = 0.090; Fig. 1). On the whole,

Table 1 Relative mRNA expression versus that observed in normal thyroid pool (reference value, 1.0)) for 90 PTCs, 31 follicular adenomas (FA) and for mutant and wild-type BRAF PTC subgroups (BRAF-mut and BRAF-wt respectively. All values are mean ± s.d. For the normal control, s.d. indicates variation between duplicate assays; for FAs, PTCs, BRAF-mut, and BRAF-wt, s.d. reflects variation among the number of samples included in each group. Intergroup differences were assessed with the one-sample t-test (PTC vs normal thyroid (NT) tissues) and the Mann–Whitney nonparametric test (PTC vs FA and BRAF-mut tumors versus BRAF-wt tumors).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Normal tissue (n = 31)</th>
<th>Follicular adenoma (n = 31)</th>
<th>PTC (n = 90)</th>
<th>Wt (n = 35)</th>
<th>Mut (n = 55)</th>
<th>PTC vs NT</th>
<th>PTC vs FA</th>
<th>Mut vs Wt</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGFA</td>
<td>1 ± 0.08</td>
<td>0.80 ± 0.75</td>
<td>1.09 ± 0.98</td>
<td>1.82 ± 1.04</td>
<td>0.64 ± 0.62</td>
<td>0.41b</td>
<td>0.26c</td>
<td>&lt;0.0001d</td>
</tr>
<tr>
<td>VEGFR1</td>
<td>1 ± 0.09</td>
<td>0.56 ± 0.62</td>
<td>0.81 ± 0.90</td>
<td>1.47 ± 1.06</td>
<td>0.39 ± 0.40</td>
<td>0.049c</td>
<td>0.34d</td>
<td>&lt;0.0001d</td>
</tr>
<tr>
<td>VEGFR2</td>
<td>1 ± 0.06</td>
<td>0.37 ± 0.37</td>
<td>0.58 ± 0.65</td>
<td>1.04 ± 0.82</td>
<td>0.29 ± 0.26</td>
<td>&lt;0.0001b</td>
<td>0.16c</td>
<td>&lt;0.0001d</td>
</tr>
<tr>
<td>VEGFR3</td>
<td>1 ± 0.29</td>
<td>0.61 ± 0.33</td>
<td>0.56 ± 0.60</td>
<td>0.94 ± 0.68</td>
<td>0.31 ± 0.36</td>
<td>&lt;0.0001b</td>
<td>&lt;0.0001c</td>
<td>&lt;0.0001d</td>
</tr>
<tr>
<td>NRP1</td>
<td>1 ± 0.12</td>
<td>0.32 ± 0.32</td>
<td>1.07 ± 0.72</td>
<td>1.53 ± 0.84</td>
<td>0.79 ± 0.46</td>
<td>0.37b</td>
<td>&lt;0.0001c</td>
<td>&lt;0.0001d</td>
</tr>
<tr>
<td>PDGFRβ</td>
<td>1 ± 0.05</td>
<td>0.25 ± 0.21</td>
<td>0.69 ± 0.65</td>
<td>1.07 ± 0.82</td>
<td>0.44 ± 0.35</td>
<td>&lt;0.0001b</td>
<td>0.039d</td>
<td>&lt;0.0001d</td>
</tr>
</tbody>
</table>

* aMRNA pooled from 64 normal human thyroids (BD Sciences Clontech).
* bPTC vs normal thyroid tissues.
* cPTC vs follicular adenomas.
* dBRAF-mut vs BRAF-wt.
VEGF or VEGFR immunoreactivity was similar in tumoral, peritumoral, and non-neoplastic tissues and displayed no clear relation with microvascular hot spots in the sections.

All PTCs exhibited numerous CD31-positive intra- and peritumoral microvessels (Fig. 3A and B). As shown in Fig. 3C, mean intratumoral MVD (±SD) were significantly higher than those observed in peritumoral tissues (36.41 ± 8.95 vs 24.62 ± 5.039, P < 0.001), and even more marked increases were found with respect to normal parenchymal values (8.25 ± 1.97, P < 0.001). Intra- and peri-tumoral MVD were even higher in BRAF-mut PTCs (38.94 ± 8.01 and 26.03 ± 4.69 vs 33.88 ± 9.49 and 23.21 ± 5.19 in BRAF-wt tumors), although neither of these differences was statistically significant (Fig. 3D).

In all PTCs, D2-40 immunostaining revealed significantly higher microscopic LVDs (±SD) in the peritumoral zone (13.15 ± 5.37) than inside the tumor (1.51 ± 3.02) or in the non-neoplastic parenchyma (4.50 ± 3.04; P < 0.001; Fig. 4A–C). In many tumors (17/22), intratumoral D2-40 immunoreactivity was completely absent. When microscopic LVD hot spots were identified within the tumor, the vessels were smaller than normal or peritumoral lymphatics, their lumens were often collapsed, and/or they were associated with areas of tumor fibrosis. Again, intra- and peritumoral LVDs displayed no significant variation related to BRAF status (Fig. 4D).

### Proangiogenic gene expression and clinicopathological characteristics

The median age of the study population was 46 years (range 15–85), and 65 (72%) of the 90 patients were women. The risk of persistent/recurrent disease based on ATA recommendations was estimated to be low in 43 patients, intermediate in 35, high in ten, and indeterminate in the remaining two. Risk levels showed no significant correlation with BRAF status or with transcript levels for any of the angiogenic factors (data not shown).

### Gene expression analysis of proangiogenic factors in thyroid cell lines

In PC-BRAF9.6 cells, doxycycline-induced BRAFV600E expression (Fig. 5A) decreased VEGFA mRNA levels compared with those observed in controls (0.78 ± 0.06 vs 1.00 ± 0.07; P = 0.01; Fig. 5B), whereas the VEGFRs were not expressed in PC-BRAF9.6 cells before or after BRAFV600E expression. Low levels of NRP1 and PDGFRβ transcripts were detected in control cells. Compared with these cells, those that expressed the BRAFV600E mutation had even lower levels of NRP1 mRNA (0.60 ± 0.10 vs 1.00 ± 0.01; P = 0.006) and higher levels of PDGFRβ transcript (3.85 ± 1.00 vs 1.00 ± 0.08; P = 0.01). RNAi-mediated silencing of the BRAFV600E mutation in human thyroid cancer cells (8505C line; Fig. 5C)
increased the transcription of VEGF A (1.37 ± 0.09 vs 1 ± 0.06; P = 0.004; Fig. 5D) and PDGFRβ (4.99 ± 0.64 vs 1 ± 0.04; P = 0.0004) and significantly reduced NRP1 mRNA levels (0.55 ± 0.08 vs 1 ± 0.13; P = 0.006). It had no significant effect on VEGFR1 or 2 expression. As for VEGFR3, it was not expressed in 8505C cells before or after BRAF silencing.

**Discussion**

Drugs that selectively inhibit tumor angiogenesis are a potentially important option for thyroid cancers that are unresponsive to radioiodine therapy because there are currently no effective alternative treatments for these tumors. Encouraging results have emerged from phase II clinical trials of antiangiogenic TKI therapy for these tumors, but the response is by no means uniform (6, 7, 22). We reasoned that BRAFV600E-positive PTCs might be good candidates for multi-kinase inhibitors like sorafenib, which target BRAF as well as the VEGF and PDGF pathways. Not only has the BRAFV600E mutation been associated with loss of differentiation in PTCs (21), which could reduce their radioiodine avidity, but previous reports also suggest that VEGF- and PDGF-mediated signaling – the major angiogenic target of all TKIs – may be even more active in BRAF-mut PTCs than it is in their BRAF-wt counterparts. **In vitro** studies have linked BRAFV600E mutation with various proangiogenic phenomena, including upregulated matrix metalloproteinase expression (32–34), methylation-mediated silencing of the tissue inhibitor of metalloprotease-3 gene (35), activation of the nuclear transcription factor-kB (34), and last but not least significantly increased VEGF and PDGFRβ protein expression (17, 18). Contrary to our expectations, the BRAF-mut tumors we analyzed exhibited significant underexpression

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**Figure 2** Immunohistochemical analysis of proangiogenic protein expression in PTCs. VEGF is expressed in the cytoplasm of PTC cells (arrows) in a BRAF-wt case (a). VEGFR1 immunoreactivity is predominantly localized to the membrane (arrows) in a positive control (follicular adenoma) (b). Papillary thyroid carcinoma BRAF-mut (T) and adjacent non-neoplastic thyroid tissue (N; H&E, X600) (c). Parallel section immunostained with anti-VEGFR1 antibody showing downregulated VEGFR1 expression in neoplastic papillary carcinoma cells (asterisk) compared with the adjacent non-neoplastic thyrocytes (T, tumor; N, non-neoplastic thyroid) (d). Low and heterogeneous immunohistochemical expression of VEGFR2 in a BRAF-wt papillary thyroid carcinoma. Arrows show a positive endothelial cell and areas of weak immunoreactivity in neoplastic thyroid cells (e). Low and heterogeneous immunohistochemical expression of VEGFR3 in a BRAF-wt PTC. Arrows show immunoreactivity in neoplastic thyroid cells (f). Full colour version of this figure available via http://dx.doi.org/10.1530/EJE-11-0283.

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**Figure 3** Microvessel density (MVD) in PTCs. Hematoxylin and eosin-stained section of a BRAF-wt papillary thyroid carcinoma at scanning magnification (A). Corresponding section immunostained with the anti-CD31 antibody that shows a marked increase in MVD in intratumoral and peritumoral tissues compared with non-neoplastic normal thyroid; the insets show that MVD is high in the tumor (top left, high magnification X400) and low in the normal thyroid (bottom right, high magnification X400) (B). MVD in intra- (T), peritumoral (T/N), and non-neoplastic thyroid tissues (N) (C). MVD in intra- (T), peritumoral (T/N), and non-neoplastic thyroid tissues (N) with respect to BRAF mutational status; intra- and peritumoral MVD tend to be higher in BRAF-mut cases but the difference is not statistically significant (D). All values are mean ± s.d. Intergroup differences were assessed with unpaired t-test. Full colour version of this figure available via http://dx.doi.org/10.1530/EJE-11-0283.
of VEGFA, VEGFRs/coreceptors, and PDGFRβ at the transcriptional level (compared with their BRAF-wt counterparts). These data are also supported by our in vitro studies of cultured thyroid cell lines. The induction of \( \text{BRAF}^{V600E} \) in PCCL3 cells significantly diminished VEGFA mRNA levels, and the opposite effect was achieved when the mutation was silenced in the 8505C cell line. Moreover, no relationship was found between risk categories attributed to the patients and angiogenic gene expression or BRAF mutation status in this series of PTCs. This is consistent with our previous findings regarding these tumors (21), which revealed no correlation between BRAF status and PTC histology or stage at diagnosis, and similar conclusions have been reached by other authors (36), although most studies examining the prognostic impact of \( \text{BRAF}^{V600E} \) on PTC phenotypes indicate that it is associated with more aggressive PTCs (37).

The findings regarding VEGF expression are at variance with the results of two previous studies in which immunohistochemistry revealed markedly increased VEGF (17) and PDGFRβ (18) expression in BRAF-mut PTCs. However, (strept) avidin-based detection systems like the ones used in these two studies are often associated with false-positive labeling – especially in PTCs (38) – which is related to the presence in many human tissues of endogenous molecules with high affinity for avidin (e.g. biotin) and/or endogenous peroxidase activity (39). As for our immunohistochemical findings, they were obtained with a biotin-free detection method. Nevertheless, no clear relation could be discerned between expression of these proangiogenic factors and the BRAF mutation status. The limited number of cases available for the analysis precludes any firm conclusion.

Although \( \text{BRAF}^{V600E} \) seems to downregulate the expression in PTCs of major proangiogenic receptor tyrosine kinases, this deficit appears to have very little effect on the hypervascularity of the tumor itself. Immunohistochemical assessment of MVD showed active, tumor-associated neovascularization in and around all PTCs. The peritumoral zones were also characterized by increased lymphangiogenesis (which is mediated by VEGFR3 binding), a feature compatible with PTCs’ propensity for lymphatic spread (40). However, BRAF-mut PTCs were not significantly

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**Figure 4** Lymphatic vessel density (LVD) in PTCs. Hematoxylin and eosin-stained section of a BRAF-wt papillary thyroid carcinoma at scanning magnification (A). Corresponding section immunostained with the MAB D2-40 that recognizes Podoplanin, a specific lymphatic endothelial cell marker; there is a marked increase in LVD in the peritumoral tissue (arrows) compared with normal thyroid (bottom right inset, high magnification ×400), whereas no intratumoral lymphatics are identified in the case (top left inset, high magnification X400) (B). Lymphatic vessel density (LVD) in intra- (T), peritumoral (T/N), and non-neoplastic thyroid tissues (N) (C). LVD in intra- (T), peritumoral (T/N), and non-neoplastic thyroid tissues (N) with respect to BRAF mutational status; there is no significant variation with the BRAF mutation status of the tumor (D). All values are mean ± s.d. Intergroup differences were assessed with unpaired t-test. Full colour version of this figure available via http://dx.doi.org/10.1530/EJE-11-0283.

**Figure 5** Proangiogenic gene transcription in thyroid cell lines. Western blot analysis of PC-BRAF 9.6 cells: incubation with 1 mg/ml doxycycline for the indicated times induces ERK phosphorylation, a downstream marker of B-Raf \( ^{V600E} \) kinase activity. Total cell lysates (40 μg) were subjected to western blotting using the indicated primary antibodies (A). Real-time PCR was used to measure VEGFA mRNA levels in PC-BRAF9.6 cells before and 48 h after doxycycline-induced expression of \( \text{BRAF}^{V600E} \) (PC-BRAF9.6 dox– and dox+ respectively) (B). Western blot analysis of 8505C thyroid cancer cells demonstrates absence of B-Raf protein expression after transfection with BRAF-specific Stealth RNAi. CTRL, 8505C cell line; Si CTRL, cells transfected with negative control Stealth RNAi; Si-BRAF, cells transfected with BRAF-specific Stealth RNAi (C). Real-time PCR was used to measure VEGFA mRNA levels in 8505C cells in which \( \text{BRAF}^{V600E} \) expression has been silenced by transfection with BRAF-specific Stealth RNAi (Si BRAF) (D). The mRNA levels (mean ± s.d.) found in control cells (dox– in panel B, Si CTRL in panel D) are used as reference values (represented on the graph as 1). Intergroup differences were assessed with unpaired t-test.
different from BRAF-wt PTCs in terms of intratumoral and peritumoral MVD (blood and lymphatic). This suggests that angiogenesis in BRAF-mut PTCs might be largely sustained by factors other than VEGF and PDGF and their receptors, but this may not be the only explanation. It is important to recall that angiogenesis is mediated exclusively by endothelial cell VEGFRs, but these receptors (and their ligand, VEGFA) are also expressed by the neoplastic cells themselves, which supports the view that VEGF-related autocrine loops play roles in these cells’ growth (8). Our analyses provide no indication of how proangiogenic gene expression was distributed between these two cell compartments, but the neoplastic cell compartment is by far the larger. Therefore, we cannot exclude the possibility that the downregulated VEGFR expression we documented in PTCs is mainly confined to neoplastic cells and that VEGF expression in the endothelial compartment is still sufficient to drive tumor vascularization. In both of the scenarios hypothesized above, the BRAF-mut PTCs do not appear as preferential targets for drugs blocking VEGF and PDGFβ signaling pathways. A greater understanding of the molecular and cellular mechanisms that govern tumor angiogenesis remains a fundamental step in efforts to develop more effective multitarget kinase inhibitors.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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