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BRAF^{V600E} mutation influences hypoxia-inducible factor-1α expression levels in papillary thyroid cancer

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Hypoxia-inducible factor-1α is found frequently overexpressed in solid tumors cells, exerting an important role in angiogenesis, glucose metabolism, cell proliferation, survival and invasion. In thyroid carcinomas, hypoxia-inducible factor-1α expression was found increased in differentiated, poorly differentiated, medullary and anaplastic variants. Hypoxia represents the principal stimulus responsible for hypoxia-inducible factor-1α induction. Other nonhypoxic stimuli increase hypoxia-inducible factor-1α synthesis through the activation of phosphatidylinositol 3-kinase and mitogen-activated protein kinase pathways in a cell-type-specific manner. We have previously shown the role of *BRAF*^{V600E} mutation in papillary thyroid cancer cells as a factor that facilitates tumor cell growth and progression. In this study, we tested the hypothesis that *BRAF*^{V600E} mutation influences hypoxia-inducible factor-1α expression in papillary thyroid carcinoma cells. We analyzed 27 papillary thyroid carcinomas, 13 of which presented *BRAF*^{V600E} mutation. In tumor tissues, immunoreactivity for hypoxia-inducible factor-1α was detected in the majority of analyzed *BRAF*^{V600E} mutated cases. Transcriptional analyses revealed elevated hypoxia-inducible factor-1α levels with significant differences between wild-type and mutated group. A *BRAF* wild-type papillary thyroid carcinoma cell line and a *BRAF*^{V600E} mutated papillary thyroid carcinoma cell line were selected to study the effects of *BRAF* mutation on hypoxia-inducible factor-1α expression *in vitro*. Knockdown of mutant *BRAF*^{V600E} or both the wild type and the *BRAF*^{V600E} by RNA interference induced a significant reduction of hypoxia-inducible factor-1α expression at mRNA and protein levels. Pharmacological inhibition of *BRAF* significantly reduces hypoxia-inducible factor-1α expression levels in papillary thyroid carcinoma cell line harboring *BRAF*^{V600E} mutation. Our results suggest that hypoxia-inducible factor-1α is expressed in papillary thyroid carcinomas and is regulated not only by hypoxia but also by *BRAF*^{V600E}-mediated signaling pathway.
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Genetic alteration leading to aberrant activation of the RAS/RAF mitogen-activated protein kinase (MAPK)/extracellular signal-regulated kinase (ERK) signal transduction pathway has long been seen in many human cancers. Several studies report B-type Raf kinase (BRAF) mutation as a potent activator of MAPK pathway.[1] *BRAF*^{V600E} mutation is the most common mutation identified in the *BRAF* gene and represents a novel indicator of poorer prognosis in a broad range of human cancers especially in thyroid cancers.[2–5]

Papillary thyroid carcinoma is the most frequent thyroid cancer accounting for 85–90% of all thyroid malignancies.[6]

BRAF^{V600E} mutation was found exclusively in papillary thyroid carcinoma and in papillary thyroid carcinoma-derived anaplastic thyroid carcinoma; in papillary thyroid carcinoma the overall prevalence of *BRAF*^{V600E} mutation is relatively high, around 45% on average, influencing the expression levels of

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several molecular markers through constitutive activation of the MAPK/ERK pathway, providing a potent promitogenic force that drives malignant transformation.[7,8]

Recent evidence has implicated the BRAF/MAPK MEK/ERK pathways as regulators of the transcriptional enhancer hypoxia-inducible factor-1 (HIF-1).[9] HIF-1 consists of a heterodimer of two proteins (HIF-1α and HIF-1β), which mediates the transcription of several genes involved in angiogenesis, glucose metabolism, cell proliferation, survival and tumor metastases.[10]

Intratumoral hypoxia and genetic alterations selectively regulate HIF-1α subunit through different mechanisms. Hypoxia ubiquitous induces HIF-1α in all cells tested, decreasing its degradation by the ubiquitin–proteasome system.[11] Other non-hypoxic stimuli, such as growth factors, cytokines and oncogenic signals, increase HIF-1α synthesis through the activation of Akt/phosphatidylinositol 3-kinase and MAPK pathways in a cell-type-specific manner.[12] HIF-1α is generally overexpressed in solid tumors including lung, prostate, breast and colon carcinoma;[13] *BRAF*^{V600E} mutation increases HIF-1α expression in melanoma cells, exerting an important role in melanoma cell survival under hypoxic conditions.[14]

HIF-1α expression was found increased at transcripts and protein levels in differentiated, poorly differentiated, medullary and anaplastic carcinoma of the thyroid.[15–17]

In this study, we tested the hypothesis that *BRAF*^{V600E} mutation influences HIF-1α expression levels in papillary thyroid carcinoma cells, using RNA interference targeting *BRAF* and the RAF-1 kinase inhibitor (sorafenib), which target both the wild-type and the V600E mutant *BRAF in vitro*.[1,18–20]

Materials and methods

Case Selection

In our previous work, *BRAF*^{V600E} mutation was evaluated in a series consecutive of patients by a real-time, allele-specific amplification essentially as described by Jarry *et al*.[21,22] Here we randomly selected 27 patients from the described group. Age of the 27 patients who underwent surgery for the classical variant of papillary thyroid carcinoma ranged from 17 to 76 years (median age was 48 years), and there was a strong predominance of female patients (81%). For each case, all histological slides were reviewed by two pathologists (AM and VR), and histological diagnoses were reassessed according to the *World Health Organization Classification of Tumours*.[23] All primary tumors included in this study were classified as classical variants of papillary thyroid carcinoma; tumors with morphological features of the follicular or others variants of papillary carcinoma were not included.

BRAF^{V600E} mutation was present in 13 (48%) of the 27 tumors examined. This retrospective study was performed in accordance with the rules of the institutional review board at the Faculty of Medicine (University of Palermo).

Immunohistochemistry

Tissue samples were fixed in 10% buffered formalin, dehydrated in ethanol and paraffin-embedded according to the routine technique. Immunohistochemical analyses were performed on 3-μm-thick paraffin-embedded sections of thyroid tumors. Dewaxed sections were heated for antigen unmasking in Tris/EDTA solution (pH 9) in a water bath for 30 min at 98°C. After rinsing in distilled H_2O, we inhibited endogenous peroxidase with 3% H_2O_2 for 5 min. Unspecific staining was blocked with protein block (Novolink Polymer Detection System; Novocastra Laboratories, Newcastle, UK). Sections were subsequently exposed to rabbit polyclonal anti-HIF-1α (1:100) (Novus Biologicals, Littleton, CO, USA) or unconjugated rabbit immunoglobulins (negative control) overnight at 4°C. Staining was detected using Novolink Polymer Detection System, (Novocastra Laboratories) according to manufacturer's instructions and counterstained with aqueous hematoxylin. Human melanoma tissues from our archives were used as HIF-1α antibody-positive control (data not shown).

The non-neoplastic tissue present on the same slides was considered as a healthy control.

Immunohistochemical Evaluation

For the evaluation of HIF-1α immunoreactions, we examined tissues for evidence of staining. For each case a minimum of 10^3 cells was counted under high-powered fields (final magnification $\times 400$) and reported as the percentage of expressing cells among the total number of counted cancer cells and regarded as a labeling index. Cases were evaluated separately by two different pathologists (AM and VR); because the variation was less than 5%, the first pathologist's data were used.

Cell Lines and Culture Conditions

Human papillary thyroid cell lines, BCPAP and FB2, were kindly provided by Dr F Frasca, University of Catania, Italy. BCPAP cell line, harboring the *BRAF*^{V600E} mutation in hemizygous,[24] was cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum in 5% CO_2. FB2 cell line was cultured in DMEM medium containing 10% fetal bovine serum in 5% CO_2. $CoCl_2$ is a hypoxia mimic and is known to stabilize HIF-1α in the cells. For $CoCl_2$ treatment, thyroid cells were subjected to 100 μmol/l $CoCl_2$ for 24 h in a CO_2 incubator.

Thyroid cancer cell lines were cultured, respectively, in the presence of sorafenib (LKT Laboratories, St Paul, MN, USA) 5 μmol/l in dimethyl sulfoxide (DMSO) or DMSO as control for 24 h; after exposures, cells were detached with trypsin/EDTA for HIF-1α mRNA analyses.

Immunofluorescence

Cells were cultured on chamber slides (Labtek, Nunc, Naperville, IL, USA) in RPMI 1640 medium containing 10% fetal bovine serum fixed in 2% paraformaldehyde/phosphate-buffered saline, permeabilized in phosphate-buffered saline containing Triton X-100 for 3 min at room temperature, and stained with rabbit polyclonal antibody anti-HIF-1α (Upstate, Millipore, Billerica, MA, USA) antibody overnight at 4°C. Cells were then incubated with Alexa Fluor 488 goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA) antibody for 1 h in the dark. Slides were mounted with UltraCruz mounting medium containing 4′,6-diamidino-2-phenylindole used to counterstain nuclei (Santa Cruz Biotechnology, Heidelberg, Germany). Images were captured with a fluorescence microscope (DM4000B; Leica Microsystems, Wetzlar, Germany).

Generation of siRNA for Transfection

Small-interference duplex RNA (siRNA) targeting human *BRAF* were purchased (synthesized) from MWG as described by Wellbrock *et al.*[25] These siRNAs target both the sequence outside and inside the V600E mutation site and thus abrogates the expression of $BRAF^{V600E}$ and *BRAF* wild-type specifically. A nonspecific siRNA (scrambled) was also synthesized. BCPAP cells were plated in six-well plates at 3×10^5 per well. After 24 h of plating, we transfected cells with scrambled, $BRAF^{V600E}$ siRNA (Mu-A) or *BRAF* wild-type siRNA (Com-4) using Interferin siRNA transfection reagent (Polyplus-transfection SA, Illkirch, France) according to the manufacturer's instructions. After 72 h of transfection, we detached cells with trypsin/EDTA, and harvested them. Knockdown efficiency was evaluated by qRT-PCR and western blot analysis.

Isolation of Total RNA and qRT-PCR

Total RNA was extracted from formalin-fixed paraffin-embedded 10 μm sections of cancer thyroid tissues using High Pure RNA Paraffin Kit (Roche Diagnostic, Mannheim, Germany). Total RNA was extracted and purified from BCPAP and FB2 cell lines using the RNeasy Mini kit (Qiagen Italia, Milan, Italy), including a digestion step with DNase I set. RNA quantity and quality were assessed by UV spectrophotometry.

Total RNA (2 μg) was reverse transcribed in a volume of 20 μl with Oligo dT primers (Applied Biosystems, Darmstadt, Germany) and ImpromII RT (Promega Italia, Milan, Italy), according to the manufacturer's protocol.

BRAF and *HIF-1α* expression was analyzed by real-time quantitative PCR (qRT-PCR) in individual samples. Two microgram of total RNA was used to measure mRNA levels relative to *β-actin* mRNA expression. PCR primers and probes were purchased from Qiagen (Quantitect Primer *BRAF* and *HIF* α). All reactions were performed in a final volume of 20 μl with 2 μl cDNA template using a LightCycler (Roche Diagnostics). Data analysis was performed with qBASE Browser that uses a Δ-Ct relative quantification model with PCR efficiency correction and single reference gene normalization (β-actin: 5′-GGACTTCGAGCAAGAGATGG-3′ and 5′-AGCAC TGTGTTGGCGTACAG-3′).[26]

Western Blotting

Human papillary thyroid cell lines BCPAP and FB2 were resuspended in ice-cold NP-40 lysis buffer (50 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% SDS, 0.5 sodium deoxycolate, 2 mM NaF, 2 mM sodium orthovanadate (Na_3VO_4)) and protease inhibitors (Complete Roche Diagnostic). Cells were incubated for 30 min on ice and lysates were cleared at 14 000 r.p.m. for 30 min. A 30 μg aliquot of each sample was mixed with reduce Laemmli sample buffer and electrophoresed on a 4–12% SDS–PAGE (NuPage Novex 4–12% Bis-Tris Gel; Invitrogen) before transfer to PVDF membrane. Membrane staining was assessed using specific antibodies for β-actin (1:5000) (monoclonal mouse anti-human β-actin, clone AC-15; Sigma-Aldrich), HIF-1α (1:1000) (Novus Biologicals) and BRAF (1:1000) (monoclonal mouse anti-human B-raf, clone F-7; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Santa Cruz Biotechnology) and SuperSignal (Pierce, Rockford, IL, USA) were used for detection with a Bio-Rad Chemidoc XRS Imager.

Statistical Analyses

Continuous variables were analyzed as median and interquartile range. As continuous variables were without normal distribution, we used nonparametric tests and analyzed differences by the Mann–Whitney *U*-test. $P < 0.05$ was considered statistically significant. All analyses were performed with Statistical Package for Social Science (SPSS for Windows, version 11.0; ©SPSS, Chicago, IL, USA).

Results

The clinicopathological characteristics of the 27 patients who underwent surgery for the classic variant of papillary thyroid carcinomas (PTCs) are shown in Table 1.

Table 1 Clinicopathological characteristics of *BRAF* wild-type and *BRAF*^{V600E}-mutated papillary thyroid carcinomas

Parameters	BRAF *wild-type (%)*	n = *14*	BRAF^{V600E} *mutant (%)*	n = *13*
Clinical signs				
Non-incidental	57	8	85	11
Incidental	43	6	15	2
Age (years)				
<45	43	6	38	5
≥45	57	8	62	8
Sex				
Female	86	12	77	10
Male	14	2	23	3
Maximum diameter of primary lesion grading (mm)				
<5	36	5	23	3
≥5	64	9	77	10
Intraglandular multifocality				
Present	29	4	54	7
Bilaterality				
Present	0	0	23	3
Extrathyroidal extension				
Present	14	2	31	4
Vascular invasion				
Present	0	0	8	1
Grade of sclerosi				
Sclerosing	0	0	23	3
Non-sclerosing	100	14	77	10
Degree of encapsulation				
Encapsulated	57	8	54	7
Nonencapsulated	43	6	46	6
Lymph nodes				
Negative	86	12	69	9
Positive	14	2	31	4

HIF-1α Expression Is Increased in PTCs with *BRAF*^{V600E} Mutation

On the basis of the presence of *BRAF*^{V600E} mutation, we divided human tumor specimens in two subgroups, 13 cases with *BRAF*^{V600E} mutation and 14 without *BRAF*^{V600E} mutation. Immunohistochemical staining was performed to identify the presence of the HIF-1α protein; immunoreaction was revealed in all cases but two of the wild-type group.

In the mutated group, we found a median value of labeling index of 60.4% (53.45–70.25%), whereas in the wild-type group the median labeling index was 18.55% (11.36–26.10%); these differences proved to be significant ($P<0.001$). Generally, immunoreactivity for HIF-1α was detected with intense cytoplasmatic and nuclear positivity in tumor cells of cases with *BRAF*^{V600E} mutation, whereas a weak cytoplasmatic expression was found in tumor cells of the wild-type group (Figure 1a and b). In our specimens HIF-1α positivity was detected throughout the analyzed fields, not associated with areas of necrosis, and also observed in cells immediately adjacent to blood vessels. Adjacent non-neoplastic tissue, the stromal and follicular cells did not show immunoreactivity for HIF-1α (data not shown).

HIF-1α mRNA expression levels were analyzed in all samples considered. We found that in the mutated group HIF-1α mRNA expression showed the median value of 73.25 (45.18–91.09) relative levels, whereas in the wild-type group the median was 23.47 (16.23–31.67); these differences proved to be significant ($P<0.001$) (Figure 2).

PTC Cell Lines Express HIF-1α

To study the effects of *BRAF* mutation on HIF-1α expression, we selected a *BRAF* wild-type papillary thyroid carcinoma cell line (FB2) and a *BRAF*^{V600E} mutated papillary thyroid carcinoma cell line (BCPAP). To analyze the expression pattern and the intracellular localization of HIF-1α in thyroid cancer cell lines, we conducted an immunofluorescence staining with an antibody specific to HIF-1α (Figure 3, green). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (Figure 3, blue). The results showed that HIF-1α was barely expressed on the cytoplasm of FB2 cell line (Figure 3, upper panel, merge), whereas a strong nuclear localization was revealed on BCPAP cells (Figure 3, lower panel, merge).

HIF-1α gene expression evaluated by qRT-PCR was found 14-fold increased in papillary thyroid carcinoma cell line harboring *BRAF*^{V600E} mutation comparing with papillary thyroid carcinoma cell line with wild-type *BRAF* (Figure 4).

BRAF Knockdown Decreases HIF-1α Expression Levels

To evaluate the role of the *BRAF* mutation in the regulation of HIF-1α transcription and expression, we knocked down *BRAF* with siRNA duplexes. Two well-characterized RNA interference constructs were used that selectively knockdown the mutant V600E *BRAF* (Mu-A) or both the wild-type and the mutated form (Com-4). The effective block of *BRAF* was verified by evaluating *BRAF* expression at mRNA and protein levels. Exposure of BCPAP cells to Mu-A or to Com-4 siRNA constructs was associated with a marked downregulation of *BRAF* expression at RNA level (Figure 5, left panel). Remarkably, western blot analysis revealed a significant reduction of BRAF protein in silenced cells. In contrast, there was no detectable downregulation of *BRAF* in BCPAP cells exposed to unspecific siRNA (Figure 6).

Moreover, FB2 cells exposed only to Com-4 showed a significant reduction of *BRAF* expression at transcription level (Figure 5, right panel).

Therefore, analysis of *HIF-1α* expression levels in *BRAF* silenced cells by real-time PCR revealed a

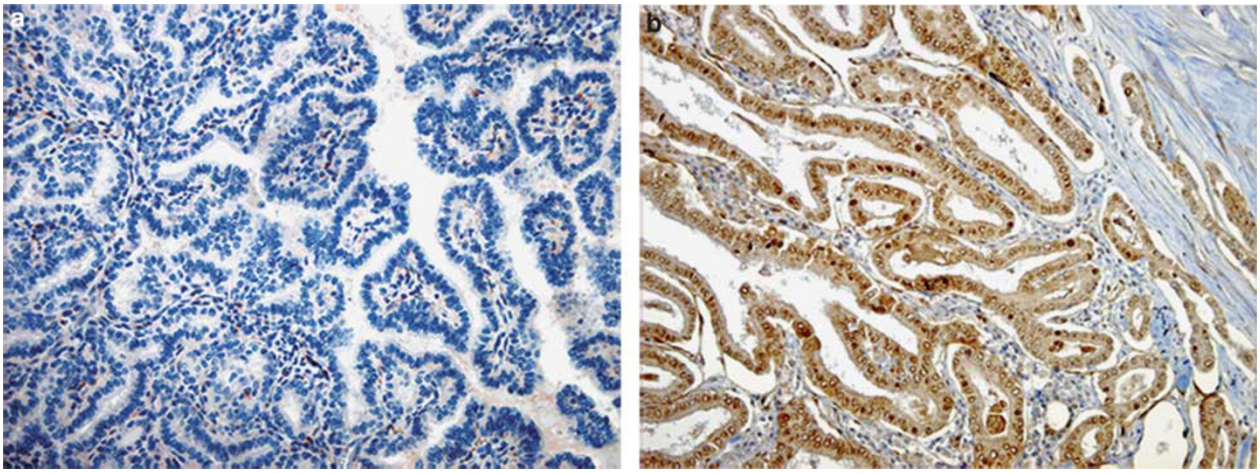

Figure 1 Representative images of HIF-1α immunostaining in PTCs; (**a**) weak cytoplasmic, no nuclear expression in BRAF wild-type PTC (magnification ×400); (**b**) distinct cytoplasmic and/or nuclear staining in $BRAF^{V600E}$ PTC (magnification ×400).

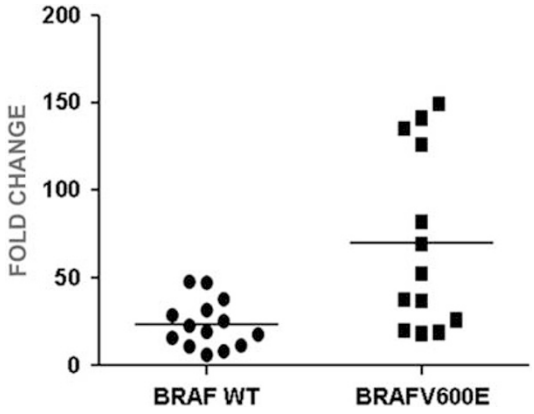

Figure 2 qRT-PCR of HIF-1α mRNA expression in PTC tissues (13 cases $BRAF^{V600E}$ and 14 *BRAF* wild type). Levels of mRNA expression were expressed after normalization with endogenous control β-actin. Results are mean values of three independent experiments.

significant reduction of HIF-1α transcription (Figure 7a). Knockdown of both wild-type and mutated V600E *BRAF* decreased HIF-1α with a comparable effect. No differences in HIF-1α mRNA expression level were detected in FB2 *BRAF* knockdown cells compared with untreated or scrambled siRNA treated cells (Figure 7b). Remarkably, as shown by western blot analysis and its relative densitometric analysis, *BRAF* knockdown has a significant effect in attenuating HIF-1α expression at protein level in BCPAP cells (Figure 8, upper and lower panels).

Pharmacological Inhibition of *BRAF* Reduces HIF-1α Expression

To confirm *BRAF* knockdown experiments, we next study the pharmacological inhibition of BRAF. Treatment with sorafenib induced a ~2-fold reduction of *HIF-1α* mRNA levels in BCPAP in comparison with untreated cells. No differences were seen in FB2-treated cells comparing with DMSO control (Figure 9).

Discussion

BRAF serine/threonine kinase regulates the expression levels of several molecular markers through the activation of the MAPK/ERK pathway. *BRAF* mutations are known to alter the biological status of cancer cells, promoting tumor progression in a range of cancers such as melanoma, colon carcinoma and thyroid carcinoma.[3,25,27] $BRAF^{V600E}$ is the most common mutation identified in thyroid papillary carcinoma, representing a novel indicator of poorer prognosis.[28] Several proteins have been shown to be targeted by $BRAF^{V600E}$-mediated activation. Recent studies indicate that in melanoma and in thyroid cancer cell lines, harboring $BRAF^{V600E}$ mutation, the activation of oncogenic pathways was observed, which have been reported to influence HIF-1α expression.[14,15,29]

In this study, we report that $BRAF^{V600E}$ mutation increased HIF-1α expression levels in papillary thyroid carcinoma cells. Moreover, molecular and pharmacological targeting of *BRAF* results in significant reduction of HIF-1α expression in thyroid tumor cells.

In several human cancers, HIF-1α is generally found overexpressed in solid tumors, including lung, prostate, breast and colon carcinoma, as a result of intratumoral hypoxia or genetic alteration due to the upregulation of oncogenes or downregulation of tumor suppressor genes.[13] HIF-1α overexpression has been positively associated with tumor aggressiveness and poorer prognosis.[9,30,31]

Burrows *et al*[15] reported that HIF-1α protein is regulated by a combination of tumor genotype and

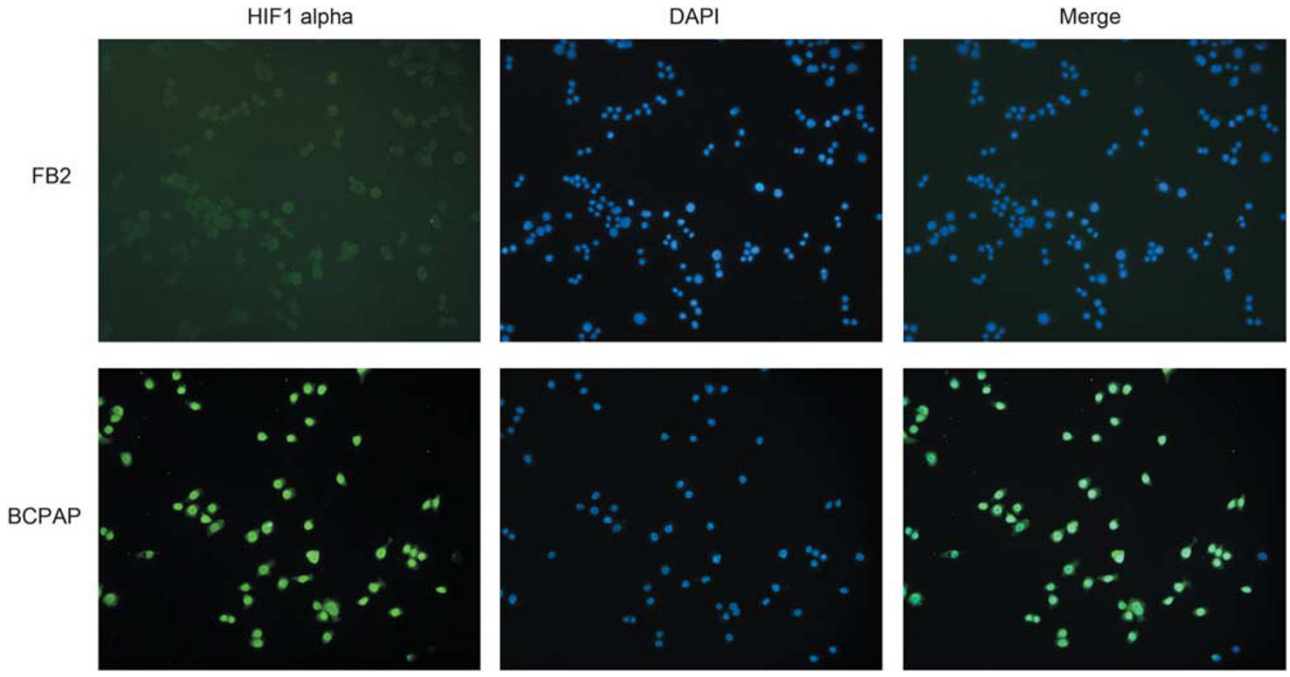

Figure 3 Immunofluorescence staining with anti-HIF-1α antibody (green). Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; blue). HIF-1α was barely expressed in FB2 cell line (upper panel), strong nuclear localization in BCPAP cells (merge, lower panel) (magnification × 200).

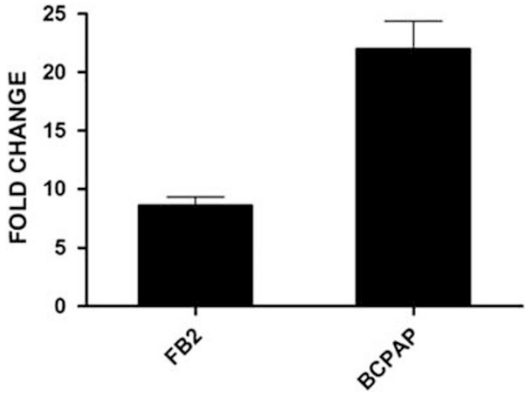

Figure 4 Expression of HIF-1α mRNA in cultured BCPAP and FB2 cell lines as assessed by quantitative PCR. mRNA expression in the mutated group is lower than that in the wild-type group. The results are shown relative to mRNA levels from the corresponding control cells, assigned the value 1. One representative of three experiments in total of each cell type is shown in the figure. β-Actin was used as internal control.

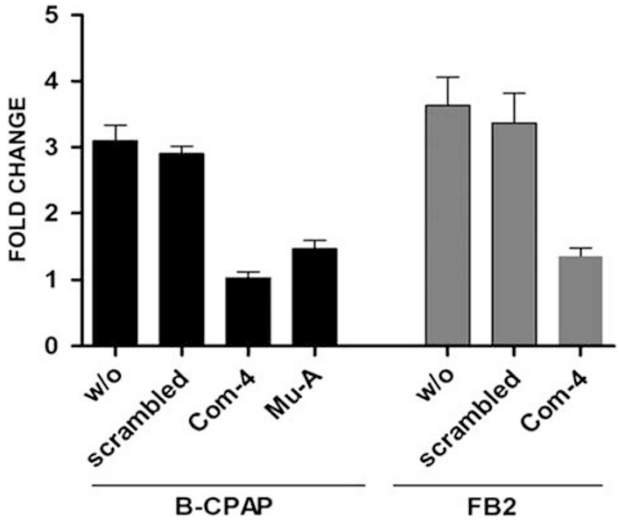

Figure 5 Transfection control assessed by qRT-PCR of BRAF mRNA in BCPAP and FB2 cells exposed to Mu-A and/or to Com-4 small-interference RNA.

microenvironment with a highest expression and a diffuse distribution of positivity in the aggressive dedifferentiated, anaplastic thyroid tumors and a focal expression in differentiated papillary and follicular tumors.

In our specimens, HIF-1α positivity was detected throughout the analyzed fields, not associated with areas of necrosis and also observed in cells immediately adjacent to blood vessels, suggesting an O_2-independent regulatory mechanism, such as genetic alteration, that could influence HIF-1α expression levels.

More than 60 putative direct HIF-1α target genes have been identified involved in angiogenesis, glucose metabolism, cell proliferation, survival and invasion, including vascular endothelial growth factor, erythropoietin, nitric oxide synthase, heme oxygenase 1 and glucose transporters.[10]

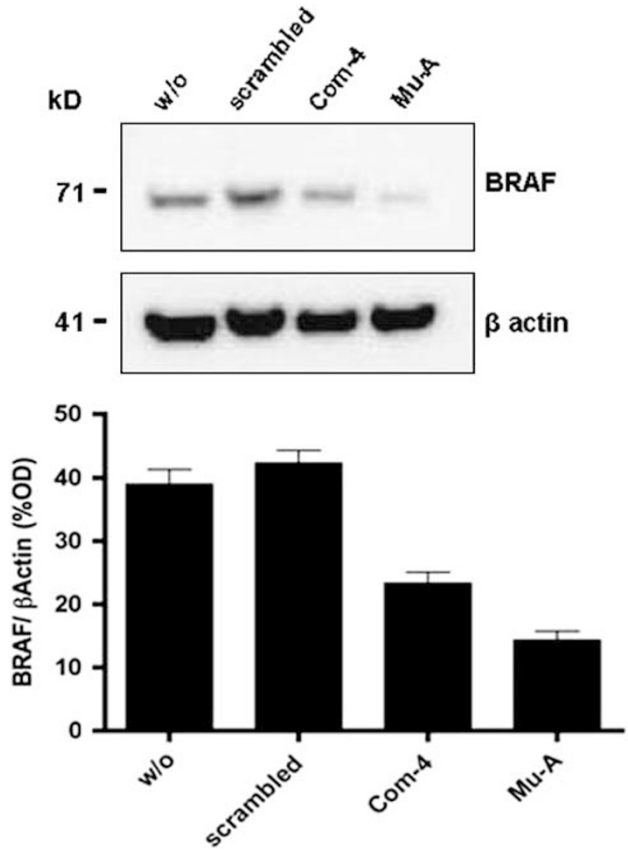

Figure 6 *BRAF* expression in thyroid cancer cell lines after silencing. Western blot analysis revealed a significant reduction of BRAF protein in silenced cells. No detectable downregulation of BRAF in BCPAP cells exposed to scrambled (upper panel). One representative of three experiments is showed. Densitometric analysis relative to BRAF protein levels *vs* *β*-actin expressed as percentage of optical density (OD) (lower panel).

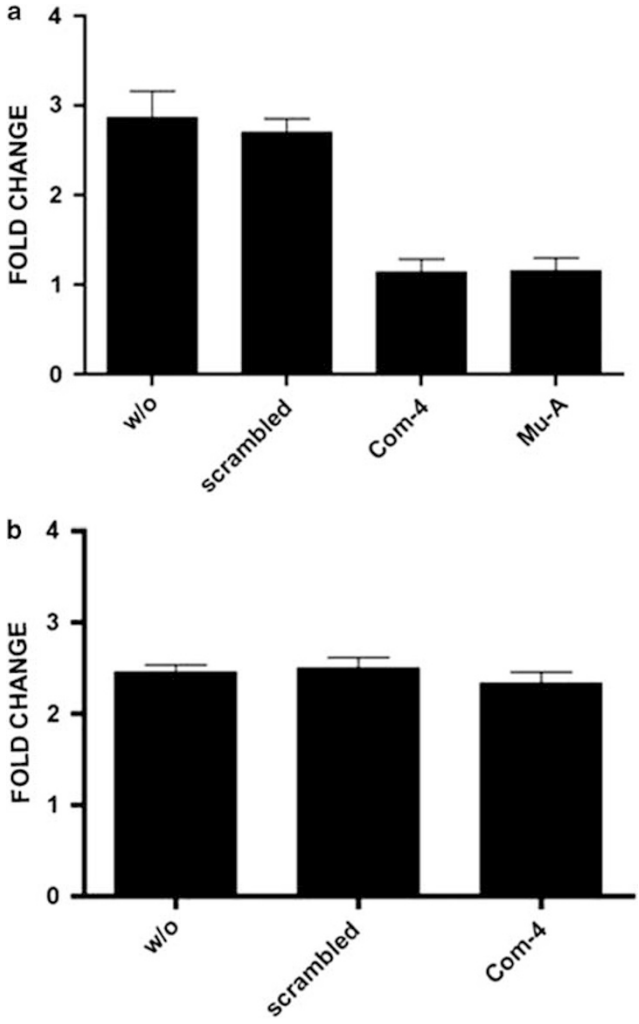

Figure 7 (**a**) Expression of HIF-1α mRNA in BRAF silenced BCPAP cell lines assessed by quantitative PCR. (**b**) Expression of HIF-1α mRNA in BRAF silenced FB2 cell lines assessed by quantitative PCR. The results are shown relative to mRNA levels from the corresponding control cells, assigned the value 1. One representative of three experiments in total of each cell type is shown in the figure. Normalization was carried out by using the gene *β-actin* as housekeeping control.

It is well known that vascular endothelial growth factor exerts a key role in tumor aggressiveness, becoming a target in cancer therapy.[32] Jo *et al*[7] reported that vascular endothelial growth factor was significantly higher in PTCs harboring $BRAF^{V600E}$ mutation, thus suggesting that HIF-1α could increase the expression of vascular endothelial growth factor in $BRAF^{V600E}$-mutated PTCs.

Recent literature indicated that modulation of HIF-1α expression influences the sensitivity to chemotherapy and more importantly for thyroid tumors, to radiotherapy.[15,33] Here we have shown that silencing *BRAF* by the two well-characterized RNA interference constructs used, which selectively knockdown the mutant *V600E BRAF* (Mu-A) or both the wild-type and the mutated form (Com-4), leads to a significant reduction of HIF-1α expression in papillary thyroid cell line harboring $BRAF^{V600E}$ mutation even in hypoxia-mimetic conditions, indicating a predominant role $BRAF^{V600E}$ oncogenic activation.

Recent studies reported that sorafenib exerts a significant antitumor activity in advanced thyroid cancer, particularly in thyroid tumors harboring *BRAF* mutation, inhibiting cancer cell proliferation and inducing apoptosis.[19,34] Accordingly, our results showed that sorafenib significantly reduces HIF-1α expression levels in papillary thyroid cancer cells line only with V600E-mutated *BRAF*, suggesting a $BRAF^{V600E}$-dependent mechanism for sorafenib in the regulation of HIF-1α expression.

In summary, our results showed that HIF-1α is expressed in PTCs and in the considered cell lines, and that it is also regulated by $BRAF^{V600E}$ oncogenic activation.

This work aims to give new insights into mechanisms responsible for upregulation of HIF-1α in the classical variant of papillary thyroid cancers.

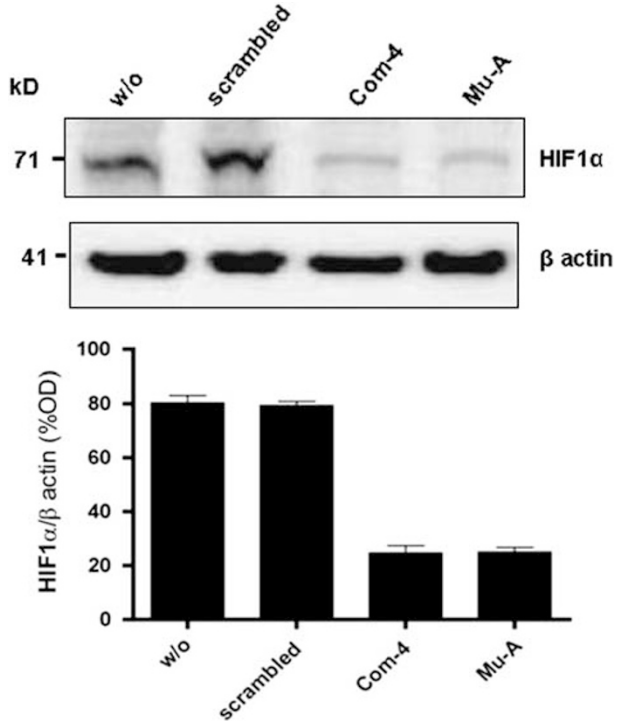

Figure 8 Western blot analysis revealed a strong reduction of HIF-1α protein in BCPAP silenced cells. No detectable downregulation of HIF-1α in BCPAP cells exposed to scrambled (upper panel). One representative of three experiments is showed. Densitometric analysis relative to BRAF protein levels *vs* β-actin expressed as percentage of optical density (OD; lower panel).

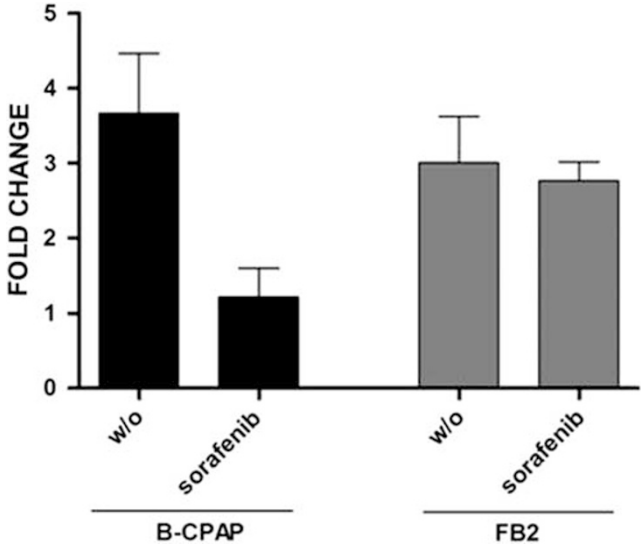

Figure 9 Pharmacological inhibition of *BRAF*. HIF-1α mRNA expression by real-time PCR in BCPAP and FB2 cell lines cultured with sorafenib 5 μmol/l in DMSO or DMSO as control for 24 h. Columns are mean of three independent experiments.

Therefore, we believe that HIF-1α downregulation could be considered to increase the effectiveness of conventional treatment of thyroid cancer.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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