BRAFV600E mutation, TIMP-1 upregulation, and NF-κB activation: closing the loop on the papillary thyroid cancer trilogy

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Abstract

BRAFV600E is the most common mutation found in papillary thyroid carcinoma (PTC). Tissue inhibitor of metalloproteinases (TIMP-1) and nuclear factor (NF)-κB have been shown to play an important role in thyroid cancer. In particular, TIMP-1 binds its receptor CD63 on cell surface membrane and activates Akt signaling pathway, which is eventually responsible for its anti-apoptotic activity. The aim of our study was to evaluate whether interplay among these three factors exists and exerts a functional role in PTCs. To this purpose, 56 PTC specimens were analyzed for BRAFV600E mutation, TIMP-1 expression, and NF-κB activation. We found that BRAFV600E mutation occurs selectively in PTC nodules and is associated with hyperactivation of NF-κB and upregulation of both TIMP-1 and its receptor CD63. To assess the functional relationship among these factors, we first silenced BRAF gene in BCPAP cells, harboring BRAFV600E mutation. We found that silencing causes a marked decrease in TIMP-1 expression and NF-κB binding activity, as well as decreased invasiveness. After treatment with specific inhibitors of MAPK pathway, we found that only sorafenib was able to increase IkB-α and reduce both TIMP-1 expression and Akt phosphorylation in BCPAP cells, indicating that BRAFV600E activates NF-κB and this pathway is MEK-independent. Taken together, our findings demonstrate that BRAFV600E causes upregulation of TIMP-1 via NF-κB. TIMP-1 binds then its surface receptor CD63, leading eventually to Akt activation, which in turn confers antiapoptotic behavior and promotion of cell invasion. The recognition of this functional trilogy provides insight on how BRAFV600E determines cancer initiation, progression, and invasiveness in PTC, also identifying new therapeutic targets for the treatment of highly aggressive forms.

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Introduction

Thyroid cancer accounts for <1% of all new malignancies, although the occult form can be found in 5–36% of well-differentiated tumors of the thyroid gland (Schlumberger 1998, Davies & Welch 2006). Differentiated thyroid tumors include papillary thyroid carcinoma (PTC) and follicular thyroid carcinoma (FTC), which globally account for more than 80% of all forms (Leenhardt et al. 2004, Fontaine et al. 2009). The biological behavior of PTC varies widely, from indolent microcarcinomas, growing slowly with little or no invasion, to invasive tumors that metastasize and cause death. Nevertheless, the small size of the primary tumor is not necessarily related to lower malignant
potential, about 10% of PTC patients die from distant metastases, which may appear several years after diagnosis (Hundahl et al. 1998, Cooper et al. 2006, Xing 2007).

The molecular mechanisms regulating initiation, progression, and invasiveness of PTC have not been fully elucidated. The RET/PTC-RAS-BRAF-MEK-ERK pathway (mitogen-activated protein kinase pathway or MAPK), which plays a fundamental role in several cell functions such as proliferation, differentiation, apoptosis, and survival, has been indicated as a key intracellular signaling pathway involved in thyroid carcinogenesis (Xing 2007). In particular, BRAF T1799A transversion has been described as the most frequent genetic alteration (Garnett & Marais 2004, Dhomen & Marais 2007). This mutation characterized by a V600E amino acidic change in the BRAF protein (BRAFV600E), results in constitutive activation of BRAF (Davies et al. 2002, Wan et al. 2004) and has been associated with aggressive PTC subtypes (Xing 2007). BRAFV600E has been reported to be responsible for both the initiation of tumorigenesis and its progression. PTC metastatic lesions in lymph nodes harboring BRAFV600E have been described as larger in size than those harboring wild-type alleles (BRAFWT; Rodolico et al. 2007).

Nuclear factor (NF)-κB has been also reported to play an important role in thyroid cancer (Visconti et al. 1997, Pacifico & Leonardi 2010). Recently, it has been hypothesized that BRAFV600E promotes invasiveness of thyroid cancer cells via NF-κB activation (Karim 2006, Palona et al. 2006), leading to acquisition of apoptotic resistance, although the mechanism has not been fully defined.

Metalloproteinases (MMPs) play a central role in tissue invasion, as they degrade extracellular matrices. MMPs are tightly regulated by specific inhibitors (tissue inhibitor of metalloproteinases, TIMPs), which control their activation in both physiological and pathological conditions (Sounni & Noel 2005, Deryugina & Quigley 2006). The TIMP-1 gene has been reported to be upregulated in thyroid tumors (Griffith et al. 2006). Specifically, increased expression of TIMP-1 has been identified as a candidate molecular biomarker for PTC (Hawthorn et al. 2004). Notably, in other tumors TIMP-1 is thought to exert functions that are independent of MMP inhibition, such as stimulation of proliferation and inhibition of apoptosis (Stetler-Stevenson 2008). Recently, TWIST1 was identified as an important regulation factor responsible for the expression of TIMP-1 (Okamura et al. 2009).

The aim of our study was therefore to evaluate whether interplay among BRAFV600E, NF-κB, and TIMP-1 exists and exerts a functional role in PTCs. In this study, we demonstrate that BRAFV600E mutation, NF-κB activation, and TIMP-1 upregulation act as a trilogy which might explain the initiation, progression, and invasiveness of PTC.

Materials and methods

Tissue specimens

Fifty-six PTC specimens collected after patients’ informed consent for the Sicilian Registry of Thyroid Tumors were retrospectively selected and used for this study. For each case, tissue from the tumoral nodule and the contralateral healthy lobe was analyzed for BRAFV600E mutation. We then selected 28 cases harboring BRAFV600E mutation and 28 BRAFWT based on matched age, gender distribution, and macroscopic characteristics. Clinical and histological characteristics with pTNM staging of selected PTCs are shown in Table 1. BRAFV600E was found in 22/28 classical variant (78.6%), 2/16 follicular variant (12.5%), 2/4 tall cell variant (50%), and 2/8 sclerosant variant (25%). BRAFV600E mutation was never detected in the contralateral healthy lobe. Correlation with prognosis was not available because the follow-up was <2 years. The IRB of the University of Palermo, Italy, approved the study.

Immunohistochemistry

All 56 primary lesions and the respective contralateral healthy lobes were analyzed. Briefly, 5 μm paraffin-embedded sections were deparaffinized, rehydrated, and microwave-heated in 10 mM sodium citrate buffer for antigen retrieval. Sections were then incubated with 3% hydrogen peroxide/PBS for 5 min, and blocked with 3% PBS/BSA. Incubation with mouse anti-human TIMP-1 antibody (MP Biomedical, DBA Italia, Milan, Italy, cat. 631661), mouse anti-human CD63 (Santa Cruz Biotechnology, DBA Italia, Milan, Italy, sc-5275), mouse anti-Bcl-2 IgG1 (Santa Cruz Biotechnology, sc-7382), and rabbit anti-Bcl-xL (Santa Cruz Biotechnology, sc-1041) was performed at room temperature (RT) for 1 h. To detect specific expression, secondary biotinylated antibodies, streptavidin/HRP, and chromogen 3-amino-9-ethylcarbazole substrate were used. Counterstaining of cells and tissue sections was performed by aqueous hematoxylin. Images were acquired on a Zeiss Imager Z1 microscope with a Zeiss Axiocam driven by Zeiss Axiovision Rel.4.7 software. For quantification analysis, cells were counted using the Cell Counter Plugin of ImageJ software (http://rsb.info.nih.gov/ij/plugins/cell-counter.html).
Positive and negative stained nuclei were marked placing different color marks by mouse clicking directly from the screen. For each case a minimum of 10³ cells was counted, and the percentage of tumor cells stained with TIMP-1, CD63, Bcl-2, and Bcl-xL antibodies was regarded as labeling index (LI; Hsu et al., 2003). Only epithelial cells were counted regardless of intensity of staining. Twenty random cases were evaluated separately by two different observers (V R and M Z); since the variation was <5%, the first pathologist’s data were used.

RNA extraction, RT-PCR, and qRT-PCR

All 56 primary lesions and the respective contralateral healthy lobes were analyzed. RNA was extracted using RNeasy Mini Kit (Qiagen, Milan, Italy) and was reverse transcribed with Oligo-dT primers (Applied Biosystems, Monza, Italy) and StrataScript RT (Stratagene, Amsterdam, The Netherlands), according to the manufacturer’s protocol. Gene expression was analyzed by real-time quantitative PCR (qRT-PCR) with Quantitect SYBR Green PCR Kit (Qiagen) using a LightCycler 1.5 Instrument (Roche Diagnostics). Reactions were performed at least in triplicate. The specificity of the amplified products was determined by melting peak analysis. Quantification for each gene of interest was performed in relation to the appropriate standard curve. Gene expression was normalized against the housekeeping gene ß-actin, which was stable among all the samples. PCR primers for TIMP-1, TWIST1, and BRAF were purchased from Qiagen (Quantitect Primer Assay, Hs_TIMP-1_SG, QT00084168; Hs_TWIST1_SG, QT00011956; Hs_BRAF_1_SG, QT00078176) while ß-actin primers were purchased from Realtimeprimers.com. The PCR thermal cycler conditions were 95 °C for 15 min followed by 45 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 15 s.

Oligonucleotide microarray analysis

The expression profile of 113 genes was analyzed by the OligoGEArray Human Cancer PathwayFinderT microarray (Superarray Bioscience Corporation, Frederick, MD, USA) in 12 primary lesions and the respective contralateral healthy lobes. Briefly, 3 mg total RNA was used as a template, the cRNA probes were hybridized to Cancer GEArray DNA Microarray membrane overnight at 60 °C, and membranes were analyzed using GEArray Expression Analysis Suite.

DNA extraction and detection of \textit{BRAF}^{V600E} mutation

\textit{BRAF}^{V600E} mutation was detected in all 56 primary lesions and the respective contralateral healthy lobes by real-time allele-specific amplification as described previously (Pizzolanti et al., 2007). DNA was extracted and purified using Qiagen DNAeasy Tissue Kit, according to the manufacturer’s protocol (Qiagen). DNA quantity and quality was assessed by u.v. spectrophotometry. Sequencing and Mutector assay (TrimGen, Milan, Italy) confirmed the allele-specificity of \textit{BRAF}^{V600E} mutation (Pizzolanti et al., 2007).

Protein extraction and western blot analysis

Proteins were extracted from 32 samples (16 nodules and 16 healthy counterparts) and thyroid tumor cell lines using RadioImmunoPrecipitation Assay buffer.
(50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P40), supplemented with protease inhibitor cocktail (Complete mini, Roche) and phosphatase inhibitors. For cytosolic and nuclear protein extraction, two buffers were used: lysis buffer (NaCl 150 mM, Tris–HCl, pH 7.8, 0.01 M, MgCl₂ 1.15 mM, NP-40 0.65%, supplemented with protease inhibitor cocktail and phosphatase inhibitors) and nuclear extraction buffer (NaCl 0.15 M, Tris–HCl, pH 8, 50 mM, NP-40 1%, DOC 0.5%, SDS 0.1%, supplemented with protease inhibitor cocktail and phosphatase inhibitors). Protein content was determined according to Bradford’s method.

Proteins were separated by NuPAGE 4–12% Bis-Tris Gel (Invitrogen), electrophoresed to nitrocellulose membranes, and blotted with the following primary antibodies: mouse antihuman TIMP-1 (MP Biomedical, cat. 631661), mouse anti-Raf-B F-7 IgG2a (Santa Cruz Biotechnology, sc-5284), rabbit anti-p-Akt1/2/3 Thr 308 (Santa Cruz Biotechnology, sc-16646-R), rabbit anti-MEK-1/2 (Ser 218/Ser 222) (Santa Cruz Biotechnology, sc-7382), rabbit anti-Bcl-xL (Santa Cruz Biotechnology, sc-7995), rabbit anti-MEK-1/2 (12-B) (Santa Cruz Biotechnology, sc-436), mouse anti-NF-κB p65 (F-6) (Santa Cruz Biotechnology, sc-8008), mouse anti-IκB-α (6A920) (Santa Cruz Biotechnology, sc-56710), mouse anti-histone H2A (Upstate Millipore, Milan, Italy, cat. 07-146), mouse anti-histone H2A (Upstate Millipore, Milan, Italy, cat. 07-146), mouse anti-Bcl-2 IgG₁ (Santa Cruz Biotechnology, sc-7382), rabbit anti-Bcl-xL (Santa Cruz Biotechnology, sc-1041), and mouse anti-β-actin IgG₁ (Sigma–Aldrich, A5441). Secondary antibodies: goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, sc-2030) goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, sc-2031) and donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, sc-2033). Antigen–antibody complexes were visualized using SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific, Milan, Italy) on a CCD camera (Chemidoc, Bio-Rad). Western blot bands were quantified with Quantity One software (Bio-Rad).

**Gelatin zymography for MMP-9**

Electrophoresis of 50 μg proteins was performed on 7.5% polyacrylamide gels containing gelatine (2 mg/ml; Difco Lab, Milan, Italy) in 32 primary lesions and their healthy counterparts tissues as described previously (La Rocca et al. 2004). Gels were rinsed twice in 2.5% Triton-X100, and incubated at 37 °C for 20 h in a buffer containing 0.2 M NaCl, 5 mM CaCl₂, 50 mM Tris–HCl (pH 7.6), and 0.02% Briiji-35 (Sigma–Aldrich). Gels were stained with 0.25% Coomassie blue and de-stained in 25% methanol and 10% acetic acid.

**Electrophoretic mobility shift assay**

Twenty microgram of total proteins from 32 samples (16 nodules and 16 respective contralateral healthy lobes) were incubated with a double-stranded 32P-labeled oligonucleotid probe containing the specific recognition sequence for NF-κB (5’-AGTTGAGGGACTTCCAGGCK-3’), as described previously (Kitchener et al. 2004). In addition, an unlabeled oligonucleotide was added in excess (100:1) to the labeled NF-κB probe, when appropriate for specific detection.

**Cell cultures**

Well-recognized human thyroid cell lines (Schweppe et al. 2008) harboring or not harboring BRAFV600E mutation were used for in vitro experiments. BCPAP (BRAFV600E/V600E) and TPC-1 (BRAFWT/WT) cell lines were cultured in RPMI 1640 medium supplemented with 10% FBS, 5% penicillin–streptomycin, and 5% glutamine. KTC-1 (BRAFV600E/WT) cell line was cultured in DMEM supplemented with 5% FBS, 5% penicillin–streptomycin, and 5% glutamine. Cultures were maintained in 5% CO₂ at 37 °C in a humidified incubator.

**Small interfering RNA transfection**

Small interfering RNAs (siRNAs) were introduced into BCPAP cells using Interferin transfection agent (EuroClone, Milan, Italy), according to the manufacturer’s instructions. Briefly, cells were seeded into six-well plates at a density of 300 000 cells/well or 96-well plates at a density of 7000 cells/well. Transfection agent and siRNA complex were added to cells and incubated for 48 and 72 h. Final concentration of siRNA was 100 nM. Each assay was performed in triplicate in at least five independent experiments. TIMP-1 was silenced using On target Plus Smart Pool Timp-1 siRNA (L-011792-00, Dharmacon, Lafayette, CO, USA). BRAF was silenced using a chemically synthesized siRNA targeting BRAFV600E mutation (MU-A), as described previously (Hingorani et al. 2003). siCONTROL, non-targeting siRNA pool was used as a control (D-001206-13, Dharmacon).

**Treatment with MAPK inhibitors**

BCPAP cells were treated with 5 μM sorafenib (LKT Laboratories, Alexis Corporation, Lausen, Switzerland) and 3 μM U0126 (662005, Calbiochem, DBA Italia, Milan, Italy) up to 4 h. p-MEK, MEK, p-AKT, AKT, p65, IκB-α, and histone H2A expression was evaluated by western blot as described above. TIMP-1 expression was evaluated by real-time PCR after 24 h of treatment.
Flow cytometry

Cells were incubated at RT for 30 min with mouse anti-human CD63 (Santa Cruz Biotechnology, sc-5275). After two washing steps with PBS, cells were incubated with goat anti-mouse IgG F(ab)2 FITC (Santa Cruz Biotechnology, sc-3699). Cells were fixed with 4% paraformaldehyde for 10 min at RT and then resuspended in permeabilization buffer containing 0.1% (w/v) saponin, 0.05% (w/v) NaN3 in Hanks’ balanced salt solution (HBSS). Cells were then incubated at RT for 30 min with PE-labeled monoclonal antibody for TIMP-1 (R&D Systems, Space Import-Export srl, Milan, Italy, IC970P, clone 635115). A negative control with an isotype-matched antibody was included. Cells were analyzed on a FACSCalibur flow cytometer (BD Biosciences, Milan, Italy), using CellQuest software (Becton and Dickinson, Milan, Italy).

Inhibition of NF-κB

BCPAP cells were plated in a six-well plate in 2 ml RPMI, stimulated for 24 h with 5 μM MG-132, 50 nM dexamethasone 21-phosphate (Sigma–Aldrich) and 10 μM parthenolide (sc-3523, Santa Cruz Biotechnology). Cells were then cultured for another 24 h with 30 μM monensin.

Immunofluorescence

Cells were cultured in culture-slides (BD Biosciences), and treated with 10 μM parthenolide for 24 h and subsequently for another 4 h with 30 μM monensin. Cells were fixed for 15 min at RT in 2% (wt/vol) paraformaldehyde, permeabilized, washed and blocked for 30 min with buffer containing 0.05% (w/v) saponin, and PBS/5% BSA (Sigma–Aldrich). Mouse monoclonal CD63 (Santa Cruz Biotechnology, sc-5275) was incubated for 1 h at RT and rabbit polyclonal TIMP-1 (H-150; Santa Cruz Biotechnology, sc-5538) for 24 h at 4 °C. Secondary antibodies (goat anti-rabbit IgG (H+L), Alexa Fluor 488, cat. A11008; chicken anti-mouse IgG (H+L), Alexa Fluor 594, cat. A21201, Invitrogen) were incubated for 1 h at RT. Images were acquired with TCS SP5 confocal microscope (Leica Microsystems).

Treatment with recombinant TIMP-1

BCPAP cells were plated in six-well plates in 2 ml serum-free medium/well for 24 h and then incubated with 250 ng/ml recombinant TIMP-1 protein (Calbiochem, PR019) for 30 and 60 min. Western blot for Akt, p-Akt, Bcl-2, and Bcl-xL was performed as described in the previous section.

BCPAP cells were plated in a 96-well plate in 100 μl serum-free medium/well and incubated with recombinant TIMP-1 protein up to 24 h at different concentrations (5–250 ng/ml). Cell proliferation was assessed by colorimetric assay using BrdU kit (Roche Diagnostics). BCPAP cells were also treated with 250 ng/ml of recombinant TIMP-1 protein (Calbiochem, PR019) up to 24 h to perform an invasion assay using BD BioCoat Matrigel invasion chambers (BD Biosciences).

Treatment with Akt inhibitor

BCPAP cells were treated with 10 μM Akt inhibitor VIII, Isozyme-Selective, Akt-1/2 (Calbiochem, 124018) for 4 h and p-Akt and Akt were evaluated by western blot. In addition, for evaluation of cell proliferation after Akt inhibition, BCPAP cells were incubated at 37 °C for 24 h with 10 μM Akt inhibitor. Cell proliferation was assessed by colorimetric assay using BrdU kit (Roche Diagnostics). BCPAP cells were also treated with 10 μM Akt inhibitor to perform an invasion assay using BD BioCoat Matrigel invasion chambers (BD Biosciences).

Invasion assay

BD BioCoat Matrigel invasion chambers (BD Biosciences) were rehydrated just before the assay using FBS-free RPMI according to the manufacturer’s instructions. Cells were then resuspended in RPMI containing 0.1% BSA and 5×10^5 cells/0.5 ml cells were added to the invasion chamber. Inserts were transferred into the wells of a 24-well plate with 750 μl culture medium containing 5% FBS as a chemo-attractant. The assembled chambers were incubated for 24 h at 37 °C. Non-migrating cells, which remained on the upper surface of the filter, were completely removed by wiping with a cotton swab. Cells on the underside of the insert were stained with 2.5 μg/ml of Hoechst33342 (Sigma–Aldrich) for 30 min at 37 °C. Then inserts were observed using an Inverted Microscope (Leica DM IRB) and acquired with Leica Qfluoro software. Quantification of invading cells was performed by counting Hoechst33342-stained nuclei in five random fields. Analysis was performed with ImageJ software. A minimum of three inserts was used for each sample to assess invasion. Invasion assay was performed on BCPAP cells silenced with MU-A, Timp-1 and siControl siRNAs for 48 h, and treated with rTIMP-1 and Akt inhibitor as described above.
Effects of TIMP-1 addition on BCPAP cell doxorubicin chemoresistance

BCPAP cells were plated in a 96-well plate in 100 μl serum-free medium/well. Cells were treated with: 100 or 250 ng/ml rTIMP-1; 100 nM TIMP-1 siRNA; 100 nM BRAF siRNA; TIMP-1 and BRAF siRNA combined; 10 μM Akt inhibitor. Cells were cultured with 2 μM doxorubicin up to 48 h. Cell proliferation was assessed by colorimetric assay using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Absorbance was read at 550 nm in a Multiskan FC microplate reader (Thermo Fisher Scientific). Apoptosis was evaluated by caspase-3 assay. Briefly, cells were fixed and permeabilized with Cytofix–Cytoperm kit (BD Pharmingen, Milan, Italy) according to the manufacturer’s instructions. Data were analyzed with CELLLQuest Pro software (Becton Dickinson). Gating was implemented based on negative control staining profiles.

Statistical analysis

Continuous variables are represented as mean ± s.d. Rates and proportions were calculated for dichotomic data and differences were analyzed by χ² test and Fisher exact test when appropriate. For continuous variables, nonparametric tests were used, and differences were analyzed by Mann–Whitney U test. Differences between paired continuous variables (mRNA expression of TIMP-1, in tumoral nodule vs contralateral healthy tissue) were analyzed by Wilcoxon test. P < 0.05 was considered statistically significant. All analyses were performed with Statistical Package for Social Science (SPSS for Windows, v. 11.0, SPSS Italia, Bologna, Italy).

Results

**NF-κB and MMP-9 are hyperactivated in BRAFV600E PTC nodules compared with BRAFWT nodules and their healthy contralateral lobes**

NF-κB signaling pathway has been described as being upregulated in BRAFV600E tumors (Ikenoue et al. 2004, Palona et al. 2006), including PTCs (71%; Mitsiades et al. 2006). The mutation has been associated with more aggressive histology/behavior. Concerning the underlying mechanism, BRAFV600E apparently promotes activation of NF-κB independently of downstream MAPK signaling (Palona et al. 2006, Liu & Xing 2008). However, no precise correlation has been found between increased NF-κB activation and degree of malignant phenotype. Hence, we investigated NF-κB activation in BRAFV600E mutated and BRAFWT PTCs by electrophoretic mobility shift assay (EMSA; Kitchener et al. 2004). We found that NF-κB binding activity is increased in BRAFV600E nodules in comparison to both BRAFWT nodules and healthy counterparts (Fig. 1A). Thus, our findings confirmed that the NF-κB system is an important pathway involved in BRAFV600E PTCs.

Tumor invasion is mediated by the action of MMPs, which are expressed more in tumor thyroid cells (Baldini et al. 2004, Yeh et al. 2006, Frasca et al. 2008). Metastasis formation is more common in BRAFV600E PTCs (Xing 2007). Indeed, MMP overexpression has been induced upon expression of BRAFV600E in several thyroid cell lines (Melillo et al. 2005, Mesa et al. 2006, Palona et al. 2006). We investigated the expression of MMPs in our PTCs by zymography. We observed higher proteolytic activity of MMP-9 in BRAFV600E nodules in comparison to both BRAFWT nodules and their healthy counterparts (Fig. 1B). Therefore, our findings support the role of the MMP system in mediating BRAFV600E mutation-promoted progression of PTC.

**TIMP-1 and its receptor CD63 are upregulated in BRAFV600E PTC nodules compared with BRAFWT nodules and their healthy contralateral lobes**

TIMP-1 has been proposed as a good candidate gene involved in thyroid carcinogenesis in PTC (Griffith et al. 2006). We investigated TIMP-1 expression in BRAFV600E and BRAFWT PTC nodules and their respective healthy contralateral lobes. Microarray performed on 12 PTCs showed that BRAFV600E PTC nodules are characterized by TIMP-1 upregulation (4.03 ± 0.46-fold increase) in comparison to BRAFWT nodules (0.98 ± 0.5-fold increase; P < 0.001; Supplementary Figure 1A and B, see section on supplementary data given at the end of this article). Results were then validated by qRT-PCR in all 56 thyroid samples. PCR also showed that TIMP-1 was significantly upregulated in BRAFV600E nodules compared with their healthy contralateral lobes (P < 0.001). By contrast, no difference was found between BRAFWT nodules and the healthy tissue (P = 0.405; Fig. 1C).

It is well known that TIMP-1 is negatively regulated by TWIST1 (Okamura et al. 2009). When we investigated TWIST1 expression, as expected we found it downregulated in BRAFV600E nodules in comparison to healthy contralateral lobes (P = 0.0014). No difference was found between
**Figure 1** NF-κB, MMP-9, TIMP-1, and CD63 expression in BRAF<sup>V600E</sup> and BRAF<sup>WT</sup> PTCs. (A) EMSA analysis shows that NF-κB binding activity is increased in BRAF<sup>V600E</sup> nodules (no. 16) in comparison to both BRAF<sup>WT</sup> nodules (no. 16) and the respective contralateral healthy lobes (no. 16). Figure is representative of four independent experiments. (B) Zymography shows higher proteolytic activity of MMP-9 in BRAF<sup>V600E</sup> nodules (no. 16) in comparison to both BRAF<sup>WT</sup> nodules (no. 16) and the respective contralateral healthy lobes (no. 16). Figure is representative of five experiments. (C) qRT-PCR performed on 28 BRAF<sup>V600E</sup> and 28 BRAF<sup>WT</sup> PTCs shows that TIMP-1 is upregulated in BRAF<sup>V600E</sup> nodules in comparison to their respective contralateral healthy lobes (no. 16). Figure is representative of five experiments. (D) Western blot analysis confirms TIMP-1 hyper-expression in PTCs harboring TIMP-1 receptor CD63 (cell membrane) is hyper-expressed in BRAF<sup>V600E</sup> PTC (left) in comparison to both BRAF<sup>WT</sup> PTC (central) and matched healthy tissue (right). Scale bar 100 μm. Inset shows higher magnification (40×). Arrows indicate positive cells.

BRAF<sup>WT</sup> nodules and healthy tissues (P = 0.99; Supplementary Figure 1C, see section on supplementary data given at the end of this article).

TIMP-1 expression was further confirmed by western blot analysis (densitometric analysis expressed as optical density units (OD): BRAF<sup>V600E</sup> nodule 122.7 ± 6.7 vs BRAF<sup>WT</sup> nodule 40.9 ± 25.9; P = 0.003; Fig. 1D) and immunohistochemistry (TIMP-1 LI: BRAF<sup>V600E</sup> nodule 71.48 ± 15.67% vs both BRAF<sup>WT</sup> nodule 22.9 ± 10.27% and contralateral matched healthy tissue 12.7 ± 6.4; P < 0.001; Fig. 1E).

CD63, a member of the tetraspanin family, has been indicated as TIMP-1 receptor in MCF10A human mammary epithelial cells (Jung et al. 2006). CD63 acts as a regulator of PI3K, FAK, Src, and Akt signaling pathways, which are implicated in the anti-apoptotic activity of TIMP-1 (Berdichevski & Odintsova 1999). Immunohistochemistry analysis confirmed higher expression of CD63 in BRAF<sup>V600E</sup> in comparison to BRAF<sup>WT</sup> nodules (CD63 LI: BRAF<sup>V600E</sup> nodule 57.16 ± 11.41% vs BRAF<sup>WT</sup> nodule 13.32 ± 8.74%; P < 0.001; Fig. 1F); contralateral matched healthy tissue resulted negative for CD63.

**Proof of the principle**

We hypothesized that a relationship exists between BRAF<sup>V600E</sup>, NF-κB activation, and TIMP-1 hyper-expression. To validate this hypothesis we used well-established thyroid cell lines harboring or not BRAF<sup>V600E</sup> mutation (Schweppe et al. 2008). We found that TIMP-1 mRNA expression was significantly higher in BCPAP cell line (BRAF<sup>V600E/V600E</sup>) in comparison to KTC-1 (BRAF<sup>V600E/WT</sup>), TPC-1 (BRAF<sup>WT/WT</sup>) cell lines, and normal thyroid mRNA (Fig. 2A). Flow cytometry analysis confirmed that BCPAP cells co-express TIMP-1 and CD63 (75.6 ± 3.2%; Fig. 2B). Thus, BCPAP cell line was considered a suitable and reproducible in vitro system for testing our hypothesis.

First, we aimed to verify whether BRAF<sup>V600E</sup> activates NF-κB independently via MEK pathway, as previously suggested (Palona et al. 2006). Because BRAF is an upstream activator of MAPK kinase (MEK)/ERK pathway, we assessed the activation of MAPK pathway in BCPAP cell line by treating cells with U0126, a specific MEK inhibitor, and sorafenib, a multikinase inhibitor that targets several serine/threonine and receptor tyrosine kinases interacting with multiple intracellular (CRAF, BRAF, and mutant Braf) and cell surface (KIT, FLT-3, VEGFR-2, VEGFR-3, and PDGFR-β) kinases (Xing 2007).

Treatment with both sorafenib and U0126 induced significant reduction of p-MEK assessed by WB (OD: sorafenib-treated BCPAP: 3.66 ± 1.52; U0126-treated BCPAP: 28.16 ± 2.02 vs untreated: 78.17 ± 2.02; P < 0.001). In our experiments, only sorafenib was
able to increase IκB-α expression (OD: sorafenib-treated BCPAP vs untreated cells: 92.44 ± 2.64 vs 69.2 ± 2.15; P = 0.013; U0126-treated BCPAP vs untreated cells: 65.5 ± 2.1 vs 69.2 ± 2.15; P = NS; Fig. 2C). These results indicate that, in BCPAP cells, BRAFV600E activates NF-κB and this pathway is MEK-independent.

As NF-κB is kept inactive in the cytoplasm by association with its specific inhibitor IκB-α, we evaluated the presence of the NF-κB subunit p65 in both cytoplasm and nucleus after treatment with both sorafenib and U0126. We found that sorafenib induced selective hyper-expression of p65 in the cytoplasm (OD: sorafenib-treated cells vs untreated cells: 97.12 ± 2.2 vs 65.7 ± 3.1; P = 0.007), while the nuclear fraction remained unchanged (26.33 ± 3.2 vs 65.7 ± 3.1; P = NS). By contrast, U0126 determined nuclear p65 hyper-expression (treated cells vs untreated cells: 90.2 ± 3.2 vs 24.56 ± 3.5; P < 0.001) with no significant concomitant cytoplasmatic reduction (treated cells vs untreated cells: 51.1 ± 3.5 vs 65.7 ± 3.1; P = NS; Fig. 2D).

To further verify the relationship between BRAFV600E, NF-κB activation, and TIMP-1 hyper-expression, we silenced the BRAF gene in BCPAP cell line using a specific siRNA for BRAFV600E (MU-A). MU-A is incorporated into the RNA-induced silencing complex, promoting target mRNA degradation and/or translation. Silencing efficacy is shown in Fig. 3A and B. EMSA for NF-κB activation showed marked...
reduction in NF-κB binding activity at 72 h post-silencing, thus confirming that NF-κB is regulated by BRAFV600E (Fig. 3C). At the same time points, qRT-PCR showed significantly decreased TIMP-1 expression in MU-A-treated cells (P=0.028; Fig. 3D).

As expected, BRAFV600E silencing in BCPAP cells also determined significant reduction in p-MEK protein expression with respect to untreated cells (OD: 26.23 ± 3.02 vs 45.21 ± 4.7; P=0.017; Fig. 3E), demonstrating the efficiency of silencing. MU-A caused also reduction of Akt phosphorylation (OD: 32.5 ± 2.5 vs 62.8 ± 2.2; P<0.001), suggesting its involvement in

**Figure 3** BRAFV600E regulates NF-κB activity, TIMP-1 expression, and tumor invasion in BCPAP cell line. Silencing with a chemically synthesized siRNA targeting BRAFV600E mutation (MU-A) decreases BRAF mRNA and protein expression, resulting in reduction of NF-κB binding activity, TIMP-1 expression, and cell invasion. (A) qRT-PCR shows that MU-A reduces BRAF expression in comparison to untreated cells (P=0.002). Data are normalized against β-actin gene expression. (B) Western blot analysis shows that MU-A decreases BRAF expression in comparison to scrambled siRNA (siCONTROL) and untreated cells. (C) EMSA shows that MU-A decreases NF-κB binding activity in comparison to both siCONTROL and untreated cells. (D) qRT-PCR shows that MU-A reduces TIMP-1 expression in comparison to siCONTROL and untreated cells (P=0.028). (A and D) Data are representative of at least three independent experiments. (E) Western blot analysis of p-MEK, MEK, p-Akt, and Akt after silencing of BRAF with MU-A siRNA. (F) Invasiveness of BCPAP cells is reduced after BRAFV600E silencing with MU-A (left panel) in comparison to siCONTROL (central panel), and untreated cells (right panel). Scale bar 200 μm. (G) Quantification of cell invasiveness. Percentages of cell migration after BRAFV600E silencing considering control as equal to 100%. (H) qRT-PCR shows that TIMP-1 siRNA reduces TIMP-1 expression in comparison to untreated cells (P<0.001). Data are normalized against β-actin gene expression. (I) Invasiveness of BCPAP cells is reduced after TIMP-1 silencing (left panel) in comparison to siCONTROL (central panel), and untreated cells (right panel). Scale bar 200 μm. (J) Quantification of cell invasiveness. Percentages of cell migration after TIMP-1 silencing considering control as equal to 100%. Data are representative of three independent experiments. Bars indicate mean ± s.d. of the data collected in triplicate.
the regulation of cell proliferation. Furthermore, we evaluated whether BRAFV600E inhibition can affect invasion in BCPAP cells after treatment with MU-A. After 24 h in a BioCoated Matrigel chamber, MU-A-treated cells significantly decreased their ability to invade \( (P=0.009; \text{Fig. 3F and G}) \), thus proving BRAFV600E mutation also exerts a pivotal role in regulating tumor invasion. The same decrease in invasion ability was found after TIMP-1 silencing \( (P=0.003; \text{Fig. 3H, I, and L}) \).

To investigate the relationship between NF-kB and TIMP-1, we treated BCPAP cells with dexamethasone, a generic inhibitor of the inflammatory pathway; parthenolide, a specific NF-kB inhibitor; and MG-132, a proteasome inhibitor blocking IkB-\(\alpha\) degradation (Revest et al. 2005, Domingo-Domènech et al. 2008, Zhang et al. 2009). We then evaluated TIMP-1 expression by qRT-PCR. Our results showed remarkable downregulation of TIMP-1 (Fig. 4A) which was confirmed by flow cytometry and IF. In particular, TIMP-1 expression was consistently reduced after parthenolide (Fig. 4B–E, Supplementary Figure 2, see section on supplementary data given at the end of this article). By contrast, TIMP-1 silencing did not affect either BRAFV600E expression or NF-kB activity (Supplementary Figure 3A and B, see section on supplementary data given at the end of this article).

**Figure 4** TIMP-1 expression is significantly reduced after treatment with NF-kB inhibitors. BCPAP cells were stimulated for 24 h with 5 \( \mu \)M MG-132 (proteasome inhibitor), 50 nM dexamethasone 21-phosphate (non-specific NF-kB inhibitor), and 10 \( \mu \)M parthenolide (specific NF-kB inhibitor) in the presence of monensin, and evaluated by qRT-PCR and flow cytometry. (A) qRT-PCR shows a major reduction in TIMP-1 expression after treatment with all three drugs. This reduction is higher with parthenolide, which is a specific NF-kB inhibitor. \( *P=0.014; **P=0.003; \) and ***\( P<0.001. \) (B–E) Quantification of TIMP-1 expression by flow cytometry confirms higher decrease after parthenolide (E), in comparison to MG-132 (D), dexamethasone (C) treated cells, and control cells (B). Cells were incubated for 30 min with PE-labeled TIMP-1 monoclonal antibody (gray lines). Solid black lines represent control cells stained with PE-secondary isotype-matched antibody. Percentages of TIMP-1-positive cells are indicated as a representative experiment. About 2 \( \times \) \( 10^4 \) cells per experimental condition of three independent experiments were analyzed in duplicates.

**BRAFV600E/NF-kB/TIMP-1/CD63 pathway exists in BCPAP cell line**

It has been demonstrated in colorectal and breast cancer that TIMP-1 binding with CD63 upregulates cell proliferation through Akt phosphorylation at Ser-473 (Stetler-Stevenson 2008). To clarify whether TIMP-1 activates Akt phosphorylation in PTC, we forced expression of TIMP-1 in BCPAP cell line by treatment with recombinant TIMP-1 at different concentrations (10–250 ng/ml) and time points (30 and 60 min). BrdU proliferation assay showed that cells treated with rTIMP-1 exhibit concentration-dependent proliferative activity compared with untreated cells, with higher values at 250 ng/ml (Fig. 5A). Indeed, treatment with 250 ng/ml rTIMP-1 already induced Akt phosphorylation at 30 min in comparison to untreated cells (OD: 30’=25.11±1.01 and 60’=20.22±1.34 vs 6.45±0.74; \( P=0.002 \) and \( P=0.004 \) respectively; Fig. 5B).

We then investigated the Akt-dependent proliferative activity of TIMP-1. To this end, we treated BCPAP cells with doxorubicin after addition of exogenous rTIMP-1, 10 \( \mu \)M Akt inhibitor and silencing of TIMP-1 and/or BRAF. As expected, cell viability was significantly increased in rTIMP-1-treated cells in a dose-dependent manner. By contrast, it was reduced after
silencing with single or combined BRAF and TIMP-1 siRNA and after treatment with Akt inhibitor (Fig. 5C). The decreased viability was not related to caspase-3 (data not shown). These data collectively confirm the faculty of TIMP-1 to confer resistance to doxorubicin-treated BCPAP cells. To further assess targets of Akt that are involved in regulation of cell proliferation and apoptosis, we also evaluated Bcl-2 and Bcl-xL expression in cells treated with 250 ng/ml recombinant TIMP-1. We found that the expression of both anti-apoptotic molecules increased significantly in comparison to untreated cells ((Bcl-2 OD: 30' = 26.3 ± 1.5 and 60' = 46 ± 1 vs 17 ± 2.6; P = 0.023 and P = 0.001, respectively); (Bcl-xL OD: 30' = 65.6 ± 3.5 and 60' = 76.6 ± 2.08 vs 36.3 ± 1.5; P = 0.004 and P < 0.001 respectively; Fig. 5D). As expected, both Bcl-2 and Bcl-xL proved to be hyper-expressed (Fig. 5E and F) in BRAF^{V600E} in comparison to BRAF^{WT} PTCs (Bcl-2 LI: BRAF^{V600E} nodule 69.38 ± 14.72% vs BRAF^{WT} nodule 11.84 ± 3.62%; P < 0.001. Bcl-xL LI: BRAF^{V600E} nodule 83.21 ± 17.46% vs BRAF^{WT} nodule 13.25 ± 4.18%; P < 0.001).

To further evaluate the role of TIMP-1 and Akt expression in tumor invasiveness we performed an invasion assay after treatment with rTIMP-1 or Akt inhibitors. We found that rTIMP-1 stimulated cell invasion (P = 0.003), while after Akt inhibition a significant reduction in cell invasion (> 50%) was observed (**P < 0.001; Fig. 6A and B). These results demonstrated that the role of TIMP-1 in cell invasion and proliferation is mediated by Akt phosphorylation. Indeed, when we considered BCPAP cell proliferation after specific Akt inhibition we found a significant decrease in cell proliferation in comparison to untreated cells (70–85%; P = 0.010; Fig. 6C). Since MEK inhibitors are currently used in clinical trials for the treatment of thyroid cancers, we further explored whether Akt phosphorylation was influenced by sorafenib and U0126 treatment by western blot. As expected, p-Akt protein expression in BCPAP was significantly reduced after sorafenib in comparison to untreated cells (OD: 33.15 ± 2.01 vs 45.2 ± 2.04, P < 0.001), but no difference was found after UO126 treatment (OD: 47 ± 1 vs 45.25 ± 2.04, P = NS; Fig. 6D). As shown in Fig. 6E, TIMP-1 mRNA expression in BCPAP proved to be strongly decreased, by more than 50%, after sorafenib treatment in comparison to the reduction observed in U0126-treated BCPAP cells (above 20%).

Figure 7 summarizes the proposed molecular mechanism for the interaction of BRAF^{V600E}, NF-κB, and TIMP-1.
Discussion

BRAF, a serine/threonine kinase known to activate the MAPK pathway, has been found mutated in several tumors, namely melanoma, colorectal carcinoma, and thyroid carcinoma (Karasarides et al. 2004, Liu et al. 2007, Preto et al. 2008). In thyroid carcinomas the molecules and pathways associated with the effect of BRAF inhibition in cellular proliferation and survival are not fully understood. Our study demonstrates the existence of a trilogy involving BRAFV600E, NF-κB, and TIMP-1 in ex vivo PTCs. We provided proof of the principle by using an in vitro model that confirmed that BRAFV600E activates NF-κB and subsequently increases TIMP-1 expression, which in turn binds CD63 leading to Akt phosphorylation. This pathway eventually results in increased proliferation and invasiveness, inhibition of apoptosis and resistance to doxorubicin.

We found that BRAFV600E-mutated PTC nodules hyper-express TIMP-1. TIMP-1 is known to be a specific inhibitor of MMP-9. Conditional expression of BRAF was associated with markedly increased invasion of Matrigel compared with cells expressing RET/PTC3 (Mesa et al. 2006); this mechanisms may be involved in extrathyroidal invasion and metastasis of PTCs harboring BRAFV600E. In this study, we confirm increased MMP-9 expression and activity in PTC nodules, along with TIMP-1 hyper-expression. The role played by BRAFV600E in cell invasion was proved on BCPAP cells after silencing along with remarkable inhibition of cell invasiveness.

We also found hyperactivation of the NF-κB system, which we propose could be the link between BRAFV600E mutation and TIMP-1 hyper-expression. Silencing of BRAFV600E resulted in decreased NF-κB and TIMP-1 expression. This observation suggests that BRAFV600E mutation is an upstream event. CRAF, which is another isoform of RAF family proteins, has been shown to activate NF-κB in NIH3T3 cells (Vale et al. 2001). On the other hand, NF-κB is...
advocated as playing an important role in a variety of thyroid cancers. In the present work, silencing of BRAF with MAPK inhibitors clearly acts on IκB-α, a cytoplasmic inhibitor of NF-κB, controlling the translocation of p65 into the nucleus (Karin 2006). In our study, we confirm that NF-κB activation is independent of MEK pathway. Furthermore, our findings suggest that the mutation in our series of BRAFV600E PTCs could influence NF-κB activation through IκB-α phosphorylation. Activated NF-κB translocates into the nucleus inducing the transcription of several known genes (Baud & Karin 2009). In fact, in our study NF-κB activation results in increased TIMP-1 expression.

Increased NF-κB activity is associated with thyroid carcinogenesis and tumor progression. In particular, it has been reported that activation of MAPK pathway by BRAFV600E is involved in the pathogenesis of PTC (Fusco et al. 1987, Kimura et al. 2003). Conversely, MAPK has also been showed to induce activation of NF-κB signaling (Tuyt et al. 1999). Degradation of IκB-α takes place shortly after ectopic accumulation of the BRAFV600E protein, resulting in the activation of NF-κB signaling via MEK-ERK-independent pathway (Palona et al. 2006). In line with these observations, a previous study demonstrated that oncogenic Ret-induced NF-κB activity depends on IKK-mediated IκB-α degradation and requires functional Ras, Raf, and MEKK1 and that NF-κB activation is not accomplished by MEK/ERK activation (Ludwig et al. 2001).

Silencing of TIMP-1 did not affect either BRAFV600E or NF-κB, confirming that TIMP-1 is the downstream factor of the trilogy. The link between these two players is NF-κB, as demonstrated by the decrease in TIMP-1 expression when NF-κB inhibitors were used.
We also identified one of the possible MMP-9-independent molecular mechanisms by which TIMP-1 exerts its biological activity on cell proliferation. TIMP-1 binds its receptor CD63 on cell membrane and activates Akt signaling pathway responsible for its anti-apoptotic activity (Stetler-Stevenson 2008). Akt exerts its effects in the cell by phosphorylating a variety of downstream substrates, all resulting in anti-apoptotic, or pro-proliferative effects (Shinohara et al. 2007). CD63 was found to be expressed in PTC specimens and in BCPAP cells. Addition of recombinant TIMP-1 protein in our in vitro model resulted in enhanced Akt phosphorylation, eventually leading to increased proliferation. These results are consistent with the anti-apoptotic activity of TIMP-1 mediated by CD63-binding (Stetler-Stevenson 2008). In addition, TIMP-1 increases Bcl-xL and Bcl-2 expression via Akt, confirming the crucial involvement of the PI3K/AKT pathway in TIMP-1 signaling (Guedez et al. 1998). Interestingly, in human breast epithelial cells, overexpression of Bcl-2 also proved to be associated with increased levels of TIMP-1 (Li et al. 1999). Subsequent studies showed that TIMP-1 is a potent inhibitor of apoptosis induced by a variety of intrinsic apoptotic stimuli. In fact, TIMP-1 causes phosphorylation of FAK, which in turn activates PI3-kinase and subsequently Akt, resulting in Bcl-2 and Bcl-xL activation (Liu et al. 2005).

Currently, promising therapeutic approaches targeting BRAF are being tested in clinical trials, particularly in more aggressive PTC (Lassalle et al. 2010). In this perspective, our findings suggest that targeting the described trilogy might be a more effective strategy for aggressive forms of PTC.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-11-0076.

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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