

## mTOR Pathway Overactivation in BRAF Mutated Papillary Thyroid Carcinoma

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**Context:** There are several genetic and molecular evidences suggesting dysregulation of the mammalian target of rapamycin (mTOR) pathway in thyroid neoplasia. Activation of the phosphatidylinositol-3-kinase/AKT pathway by *RET/PTC* and mutant *RAS* has already been demonstrated, but no data have been reported for the *BRAF*<sup>V600E</sup> mutation.

**Objective:** The aim of this study was to evaluate the activation pattern of the mTOR pathway in malignant thyroid lesions and whether it may be correlated with known genetic alterations, as well as to explore the mechanisms underlying mTOR pathway activation in these neoplasias.

**Results:** We observed, by immunohistochemical evaluation, an up-regulation/activation of the mTOR pathway proteins in thyroid cancer, particularly in conventional papillary thyroid carcinoma (cPTC). Overactivation of the mTOR signaling was particularly evident in cPTC samples harboring the *BRAF*<sup>V600E</sup> mutation. Transfection assays with *BRAF* expression vectors as well as *BRAF* knock-down by small interfering RNA revealed a positive association between *BRAF* expression and mTOR pathway activation, which appears to be mediated by pLKB1 Ser428, and emerged as a possible mechanism contributing to the association between *BRAF* mutation and mTOR pathway up-regulation. When we evaluated the rapamycin in the growth of thyroid cancer cell lines, we detected that cell lines with activating mutations in the MAPK pathway show a higher sensitivity to this drug.

**Conclusions:** We determined that the AKT/mTOR pathway is particularly overactivated in human cPTC harboring the *BRAF*<sup>V600E</sup> mutation. Moreover, our results suggest that the mTOR pathway could be a good target to enhance therapy effects in certain types of thyroid carcinoma, namely in those harboring the *BRAF*<sup>V600E</sup> mutation. (*J Clin Endocrinol Metab* 97: E1139–E1149, 2012)

**A**bnormalities in the mammalian target of rapamycin (mTOR) signaling pathway have emerged as common events observed in different types of cancer (1). mTOR is a serine/threonine kinase that functions as a central regulator of cell growth-related processes (2) and can

be found in two distinct complexes: mTORC1 and mTORC2. mTOR and its associated proteins mLST8/GβL, PRAS40, and raptor constitute the mTORC1, whose kinase activity is sensitive to rapamycin. S6 kinase 1 and eIF4E-binding protein (4EBP1) are the main

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Abbreviations: AMPK, AMP kinase; cPTC, conventional PTC; 4EBP1, eIF4E-binding protein; FBS, fetal bovine serum; FCT, Portuguese Foundation for Science and Technology; FFPE, formalin-fixed paraffin-embedded; FTC, follicular thyroid carcinoma; FVPTC, follicular variant of PTC; HEK293, human embryonic kidney (cells); LKB1, liver kinase B1; mTOR, mammalian target of rapamycin; NIS, Na<sup>+</sup>/I<sup>−</sup> symporter; PI3K, phosphatidylinositol-3-kinase; pmTOR, phosphorylated mTOR; PTC, papillary thyroid carcinoma; PTEN, phosphatase and tensin homolog; siRNA, small interfering RNA; SRB, sulforhodamine B.

mTORC1 effectors (3). mTORC2, composed of mTOR, mLST8/G $\beta$ L, mSIN1, protor, and rictor, is insensitive to acute rapamycin treatment and is involved in the regulation of cell proliferation and survival through phosphorylation of AKT (at Ser473) (4).

mTOR regulates cell growth by integrating signals derived from different inputs (2). Regulation of mTOR by growth factors classically occurs through the phosphatidylinositol-3-kinase (PI3K)/AKT pathway, which is counteracted by the phosphatase and tensin homolog (PTEN). The cellular energy status is connected to mTORC1 through AMP-activated protein kinase (AMPK), which is activated in response to energy stress (5). The tumor suppressor liver kinase B1 (LKB1) has been identified as the main upstream kinase of AMPK (6). LKB1 binds and phosphorylates AMPK on its Thr172, activating this kinase in case of energy deprivation, which in turn inhibits mTORC1 signaling (7). Recent data reported that *BRAF* mutant melanoma cells have a dysfunctional LKB1-AMPK-mTOR energy stress sensor (8, 9). In *BRAF*<sup>V600E</sup>-transformed melanoma cells, LKB1 is phosphorylated by the *BRAF*<sup>V600E</sup> downstream kinases ERK and p90RSK, compromising its ability to bind and activate AMPK (8).

Mutations or rearrangement of genes codifying for components of the MAPK pathway seem to be crucial for the transformation process in thyroid (10), and thyroid tumors show a strong genotype-phenotype relationship (11). Papillary thyroid carcinoma (PTC) is the most common thyroid malignancy [85–90% (10)], being genetically characterized by a high incidence of mutations in the *BRAF* gene (particularly *BRAF*<sup>V600E</sup>) and by *RET/PTC* rearrangements (12, 13).

In thyroid lesions, information on mTOR expression is not available, to the best of our knowledge. Despite this, PI3K/AKT pathway genetic alterations have been reported in follicular thyroid carcinoma (FTC) and PTC, namely *PTEN* promoter hypermethylation (14), *PTEN* loss of heterozygosity (15, 16), and *PI3KCA* amplification (17, 18), suggesting a role for mTOR signaling in thyroid neoplasia. Furthermore, previous data demonstrated that *RET/PTC* rearrangements and *RAS* mutations can lead to the activation of the PI3K/AKT pathway (19–23), whereas no data have been reported for the *BRAF*<sup>V600E</sup> mutation.

The aims of this study were to evaluate the activation pattern of the mTOR signaling pathway in malignant thyroid lesions, to verify whether it may be correlated with known genetic alterations, and to explore the mechanisms underlying mTOR pathway activation in these neoplasias.

## Materials and Methods

### Thyroid samples and tissue microarray construction

Thyroid tissue specimens [formalin-fixed paraffin-embedded (FFPE)] of 133 patients were collected from the files of the Hospital São João/Medical Faculty of the University of Porto, Portugal; the Institute of Molecular Pathology and Immunology of the University of Porto (IPATIMUP), Portugal; Clinical University Hospital (CHUS), University of Santiago de Compostela, Spain; and Hospital S Marcos, Portugal. Cases were histologically classified by experienced pathologists (J.M.C.-T., F.P., and M.S.-S.) in FTC (n = 20), follicular variant of PTC (FVPTC; n = 22), conventional PTC (cPTC; n = 60), and cPTC metastases (n = 21), and representative areas were selected. Areas of adjacent normal thyroid (n = 34) were also selected. Duplicate tissue cores 2.0 mm in diameter were extracted from the selected areas and arrayed on a recipient paraffin block. All procedures with samples were in accordance with the institutional and national ethical rules.

### Immunohistochemistry

The following antibodies were used: rabbit monoclonal antibodies specific for PTEN (1:75), phospho-AKT Ser473 (1:50), phospho-AKT Thr308 (1:50), mTOR (1:50), phospho-S6 Ser235/236 (1:75), phospho-4EBP1 Thr37/46 (1:200), raptor (1:75), and phospho-LKB1 Ser428 (1:50), all from Cell Signaling Technology (Danvers, MA); rabbit polyclonal antibody specific for phospho-mTOR Ser2448 (1:50; Cell Signaling Technology); and antirictor mouse monoclonal antibody (1:500; Abnova, Jhongli City, Taiwan). The antibodies were validated in previous work of our group for immunohistochemical analysis in FFPE samples (24, 25).

Sections were subjected to heat-induced antigen retrieval in 1 mM EDTA (pH 8.0) for the anti-phospho-S6 Ser235/236 antibody or in 10 mM sodium citrate buffer (pH 6.0) for the remaining antibodies, followed by blocking of endogenous peroxidase activity (3% of H<sub>2</sub>O<sub>2</sub>) and of nonspecific binding with Large Volume Ultra V Block reagent (Thermo Scientific/Lab Vision, Fremont, CA). Sections were incubated with the primary antibody overnight at 4 C. Negative controls were carried out by omitting the primary antibody.

The detection was performed with a labeled streptavidin-biotin immunoperoxidase detection system (Thermo Scientific/Lab Vision) followed by 3,3'-diaminobenzidine incubation. For the anti-phospho-AKT Thr308 antibody, the immunostaining was performed with an alkaline phosphatase kit (Dako, Glostrup, Denmark), and the samples were developed with a permanent red chromogen.

The immunostaining was blindly semiquantitatively evaluated in terms of staining intensity (0, negative; 1, weak; 2, intermediate; 3, strong) and percentage of stained cells (0, <5%; 1, 5 to 25%; 2, 25 to 50%; 3, 50 to 75%; 4, >75%). An immunohistochemical score was calculated by multiplying the proportion of positive cells by the intensity of the staining, with 12 being the maximum score. The cellular localization was also evaluated as membranar and/or cytoplasmic and/or nuclear.

### DNA extraction and mutational analysis of *BRAF*

Selected representative areas were manually dissected from 10- $\mu$ m sections of FFPE cases of cPTC. DNA extraction was

based on salting-out technology (CitogeneDNA Purification Kits; Citomed, Lisbon, Portugal).

Screening for *BRAF* mutations was restricted to exon 15, which was amplified by PCR and sequenced on an ABI Prism 3130 *xl* Automatic sequencer (Perkin-Elmer, Foster City, CA).

### Cell lines, *BRAF* vectors, and transfection assays

T243, TPC1, K1, 8505C, C643, T241, and XTC-1 thyroid cancer cell lines used in this study were already characterized at the molecular and genotypic level (Supplemental Table 5) (26, 27). All cell lines were maintained in RPMI supplemented with 10% (vol/vol) fetal bovine serum (FBS) and antibiotics [1% (vol/vol) Pen Strep and 0.5% (vol/vol) fungizone; all from GIBCO, Invitrogen, Carlsbad, CA], except XTC-1, which was cultured in DMEM/F12 (GIBCO, Invitrogen) supplemented with 10% (vol/vol) FBS, insulin at 10  $\mu$ g/ml, TSH at 10 mU/ml (Sigma-Aldrich, St. Louis, MO), and antibiotics. Human embryonic kidney (HEK293) cells used in the transfection assays were cultured in DMEM supplemented with 10% (vol/vol) FBS and antibiotics. All cells were grown in a humidified incubator with 5% CO<sub>2</sub> at 37 C.

The expression vectors for *BRAF* were constructed in-house by insertion of the coding sequences of *BRAF*<sup>WT</sup> and *BRAF*<sup>V600E</sup> into the multiple cloning site of pCMV empty vector (pCMV-*BRAF*<sup>WT</sup> and pCMV-*BRAF*<sup>V600E</sup>). After transformation, the clones were subjected to automated sequencing to verify the integrity of the *BRAF* sequence.

For transfection assays, HEK293 cells were transiently transfected by the calcium phosphate coprecipitation method with 5  $\mu$ g of DNA, including 100 ng of expression plasmid (pCMV-empty vector, pCMV-*BRAF*<sup>WT</sup>, or pCMV-*BRAF*<sup>V600E</sup>), 100 ng of pEGFP-C1 (Clontech, Mountain View, CA) to monitor transfection efficiency, and 4.8  $\mu$ g of “carrier DNA”- pUC18. Confirmation of *BRAF* expression as well as of pERK 1/2 (as a read-out of *BRAF* activity) was done by Western blotting.

The small interfering RNA (siRNA) assays were performed as previously reported (28), using 50 nM of oligo *BRAF* (*BRAF*-C2) and 25 nM of LKB1 siRNA (Smartpool from Dharmacon; Thermo Scientific). Cells were seeded and transfected with Lipofectamine 2000, and cell lysates were obtained after 48 h. *BRAF* and LKB1 down-regulation was confirmed by Western blotting.

### Growth inhibition assay

The growth of thyroid cancer cell lines ( $3 \times 10^3$  C643 and K1 cells;  $5 \times 10^3$  8505C and TPC1 cells;  $6 \times 10^3$  XTC-1 and T243 cells; and  $8 \times 10^3$  T241 cells) was assessed after 48 h of rapamycin treatment (range, 0.1 to 1000 nM) by the sulforhodamine B (SRB) assay. Cells were fixed with 50% (wt/vol) trichloroacetic acid and stained with a 0.1% (wt/vol) SRB solution. SRB was solubilized in a 10 mM Tris-base buffer, and the absorbance was read at 560 nm.

### Western blotting

Cells were lysed in RIPA buffer supplemented with phosphatase and protease inhibitors. Proteins were resolved by SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare, Little Chalfont, UK). The primary antibodies were referred in the immunohistochemistry section and used at a 1:1000 dilution. Anti-phospho-44/42 MAPK (pERK 1/2) (Thr202/Tyr204) (1:1000; Cell Signaling Technology) and anti-

*BRAF* (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) primary antibodies were also used.

Protein was detected using a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) and a luminescence system (Perkin-Elmer). For protein-loading control, membranes were reprobed with an anti- $\alpha$ -Tubulin (Sigma-Aldrich) or an anti-actin (Santa Cruz Biotechnology) antibody. Protein expression was quantified using the Bio-Rad Quantity One 1-D Analysis software (Bio-Rad Laboratories, Inc., Hercules, CA) and normalized by the levels of actin or  $\alpha$ -Tubulin.

### Statistical analysis

Statistical analysis was done using StatView version 5.0 software (SAS Institute Inc., Cary, NC). All data are expressed as mean  $\pm$  SEM. Differences between groups were examined by the unpaired Student's *t* test and the Mann-Whitney *U* test. *P* values <0.05 were considered statistically significant.

## Results

### Overexpression of mTOR pathway proteins in malignant thyroid lesions

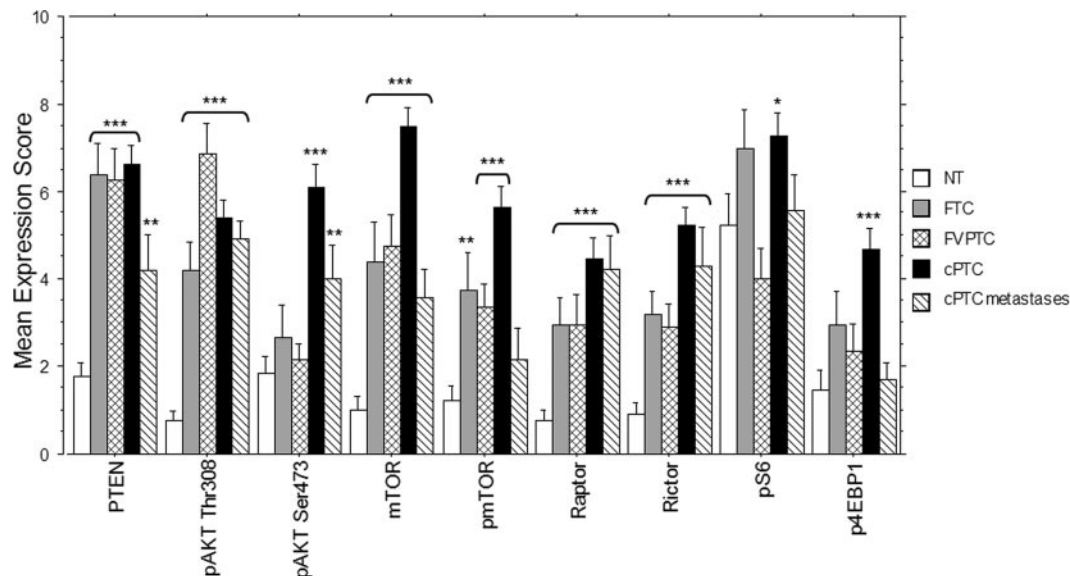
The proportion of immunopositive cases observed in the different histological types and the respective immunohistochemical scores are summarized in Fig. 1 and in Supplemental Tables 1 and 2 (published on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>). Information relative to the cellular localization of the staining is provided in Supplemental Table 3.

PTEN expression was observed in all malignant lesions with significantly higher staining scores than in normal thyroid ( $P \leq 0.0001$  to 0.0026; Fig. 1). Additionally, an increase in cytoplasmic PTEN and a decrease in the proportion of cells with nuclear PTEN were observed, particularly in cPTC (Supplemental Table 3).

A statistically significant increased expression of pAKT Ser473, relative to normal tissue, was observed in cPTC and cPTC metastases ( $P < 0.0001$  and  $P = 0.0082$ , respectively; Fig. 1). The pAKT Thr308 protein was observed in almost all the cases of thyroid carcinoma with a significantly elevated expression when compared with normal thyroid tissue ( $P < 0.0001$ ; Fig. 1).

Enhanced expression of mTOR and phosphorylated mTOR (pmTOR) Ser2448 was detected in FTC, FVPTC, and cPTC, when compared with normal thyroid tissue ( $P \leq 0.0001$  to 0.0022; Fig. 1). Total mTOR expression was significantly higher in cPTC, relative to the other carcinoma histotypes ( $P \leq 0.0001$  to 0.0022). The levels of pmTOR in cPTC were significantly higher than in FVPTC and cPTC metastases ( $P = 0.0108$  and  $P = 0.0005$ , respectively) and were borderline higher than in FTC ( $P = 0.0589$ ).

Raptor and rictor were overexpressed ( $P \leq 0.0001$  to 0.0007; Fig. 1) in thyroid lesions in comparison to normal



**FIG. 1.** Expression of mTOR pathway proteins in normal thyroid and malignant thyroid lesions. The immunostaining of PTEN, pAKT Thr308, pAKT Ser473, mTOR, pmTOR Ser2448, raptor, rictor, pS6 Ser235/236, and p4EBP1 Thr37/46 was evaluated in normal thyroid (NT), FTC, FVPTC, cPTC, and cPTC metastases. The antigen expression was represented by a score calculated through multiplication of the value attributed to the staining intensity (0 to 3) by the value attributed to the percentage of stained cells (0 to 4) (maximum value is therefore  $3 \times 4 = 12$ ). Results are shown as mean score  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (unpaired Student's *t* test and Mann-Whitney *U* test).

tissue. In cPTC, rictor score was significantly higher than in FTC and FVPTC ( $P = 0.0094$  and  $P = 0.0023$ , respectively).

A significant increase in the expression of mTORC1 downstream targets, pS6 Ser235/236 and p4EBP1 Thr37/46, relative to normal thyroid tissue, was observed only in cPTC samples ( $P = 0.0180$  and  $P < 0.0001$ , respectively; Fig. 1).

In primary cPTC, we found significantly higher expression of PTEN ( $P = 0.0086$ ), pAKT Ser473 ( $P = 0.0477$ ), mTOR ( $P < 0.0001$ ), pmTOR ( $P = 0.0005$ ), and p4EBP1 Thr37/46 ( $P = 0.0011$ ) than in cPTC metastases.

These results show that, in thyroid cancer, particularly in cPTC, mTOR pathway proteins are overexpressed.

### Overexpression of mTOR pathway proteins in cPTC with *BRAF*<sup>V600E</sup> mutation

Because we observed distinct expression of mTOR pathway proteins across the different histotypes, with particular overactivation in cPTC, and knowing the close genotype-phenotype correlation in thyroid tumors, we verified whether the expression of mTOR pathway proteins in cPTC was correlated with the presence of the *BRAF* mutation. *BRAF*<sup>V600E</sup> mutation was present in 23 of the 60 (38.3%) cPTC cases.

We observed a significantly higher expression of pAKT Ser473 ( $P < 0.0001$ ), mTOR ( $P < 0.0001$ ), pmTOR Ser2448 ( $P < 0.0001$ ), raptor ( $P = 0.0037$ ), rictor ( $P = 0.0323$ ), and pS6 Ser235/236 ( $P = 0.0084$ ) in cPTC-*BRAF*<sup>V600E</sup> than in cPTC-*BRAF*<sup>wt</sup> (Fig. 2, A and B, and Supplemental Table 4).

These observations suggest that *BRAF*<sup>V600E</sup>-mutated cPTC is associated to a higher activation of the mTOR pathway.

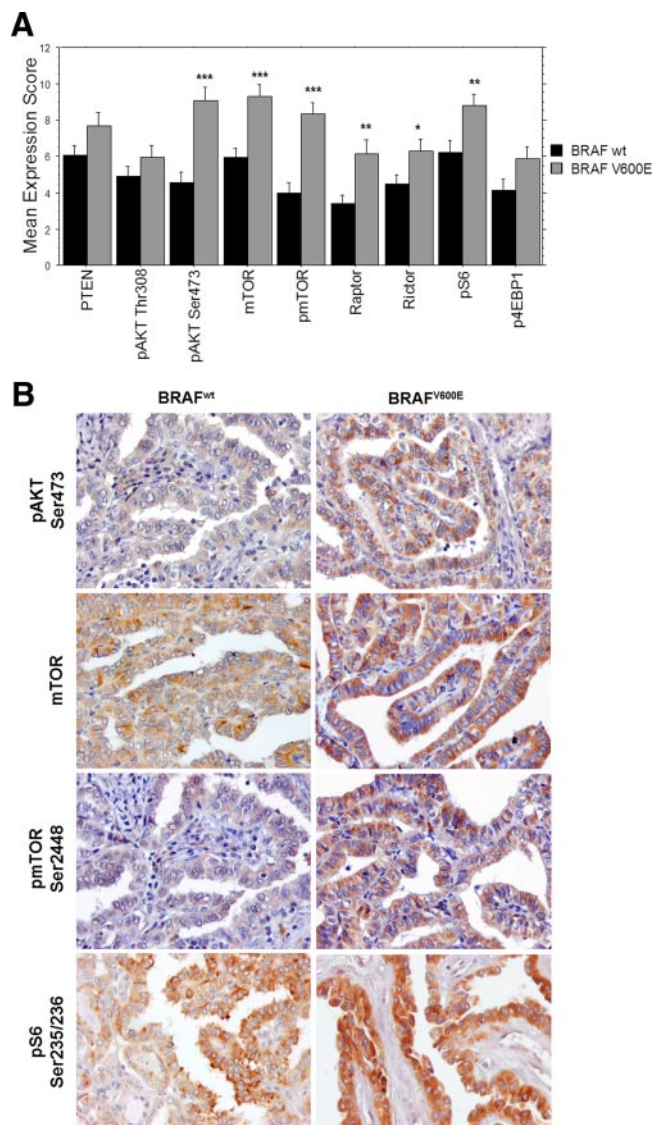
### Regulation of mTOR pathway activation by *BRAF*

To verify the possible effects of *BRAF*<sup>V600E</sup> in the activation of the mTOR pathway, we transiently transfected HEK293 cells with control pCMV, pCMV-*BRAF*<sup>wt</sup>, or pCMV-*BRAF*<sup>V600E</sup> vectors. Additionally, we down-regulated *BRAF* by siRNA in thyroid cancer cell lines and analyzed the alterations on the levels of mTOR pathway effectors.

As observed in Fig. 3A, after transfection with pCMV-*BRAF*<sup>wt</sup> and pCMV-*BRAF*<sup>V600E</sup>, an increase in the mean fold expression of mTOR ( $2.8 \pm 0.9$  and  $3.3 \pm 1.1$ , respectively), pmTOR Ser2448 ( $2.5 \pm 0.6$  and  $2.2 \pm 0.2$ , respectively), raptor ( $1.8 \pm 0.4$  and  $2.1 \pm 0.7$ , respectively), rictor ( $1.9 \pm 0.6$  and  $2.4 \pm 1.1$ , respectively), and pS6 Ser235/236 ( $1.9 \pm 0.5$  and  $2.0 \pm 0.3$ , respectively) was observed relative to pCMV-empty vector. The pCMV-*BRAF*<sup>V600E</sup> expression led to significant enhanced pmTOR Ser2448 and pS6 Ser235/236 levels ( $P = 0.0023$  and  $P = 0.0284$ , respectively; Fig. 3, A and B).

The levels of mTOR pathway effectors were evaluated in K1, 8505C, TPC1, C643, and T241 thyroid cancer cell lines after *BRAF* down-regulation by siRNA. As observed in Fig. 4A, *BRAF*-C2 siRNA led to down-regulation of *BRAF* expression in all cell lines, although less efficiently in TPC1. Decrease of pERK1/2 was observed in all the cell lines, except in C643. In K1, such a difference was statistically significant ( $P = 0.0002$ ). In K1, 8505C, and C643,

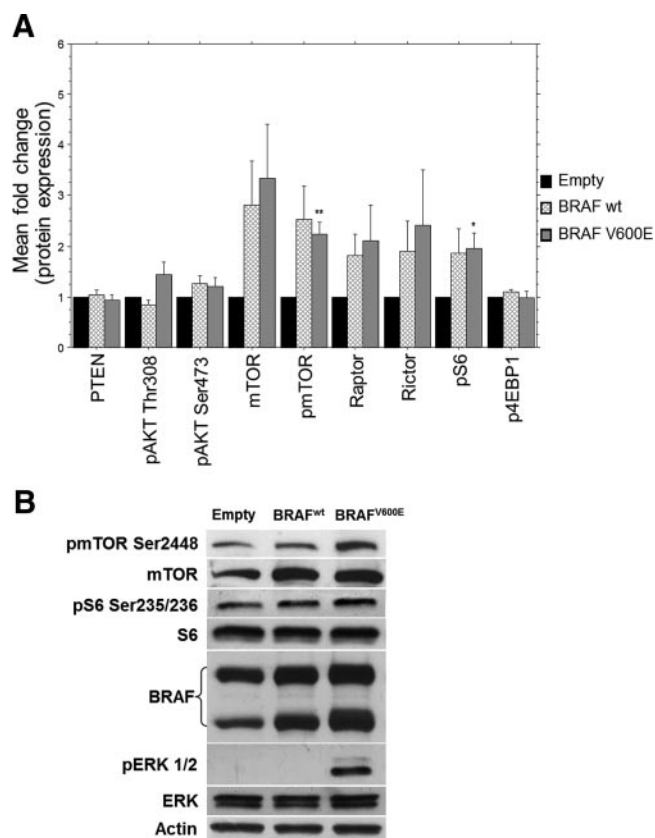




**FIG. 2.** Expression of mTOR pathway proteins in cPTC with and without *BRAF*<sup>V600E</sup> mutation. A, The immunostaining of PTEN, pAKT Thr308, pAKT Ser473, mTOR, pmTOR Ser2448, raptor, rictor, pS6 Ser235/236 and p4EBP1 Thr37/46 was evaluated. The expression score was obtained by multiplying the value attributed to the staining intensity by the value attributed to the percentage of stained cells. Results are shown as mean  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (unpaired Student's *t* test and Mann-Whitney *U* test). B, Microphotographs representative of the immunohistochemical staining of pAKT Ser473, mTOR, pmTOR Ser2448, and pS6 Ser235/236 in cPTC-BRAF<sup>wt</sup> and cPTC-BRAF<sup>V600E</sup> cases. For all the evaluated proteins, the immunohistochemical staining was performed with 3,3'-diaminobenzidine. Microphotographs were obtained at a 400× magnification.

a significant decrease of pmTOR Ser2448 was observed upon BRAF silencing ( $P = 0.0039$  to  $0.0127$ ; Fig. 4B), whereas this treatment led to significant reduced expression of pS6 Ser235/236 in the five cell lines ( $P = 0.0004$  to  $0.0252$ ; Fig. 4B).

These *in vitro* results reveal a positive association between BRAF expression and mTOR pathway activation and support the correlation between *BRAF*<sup>V600E</sup> and



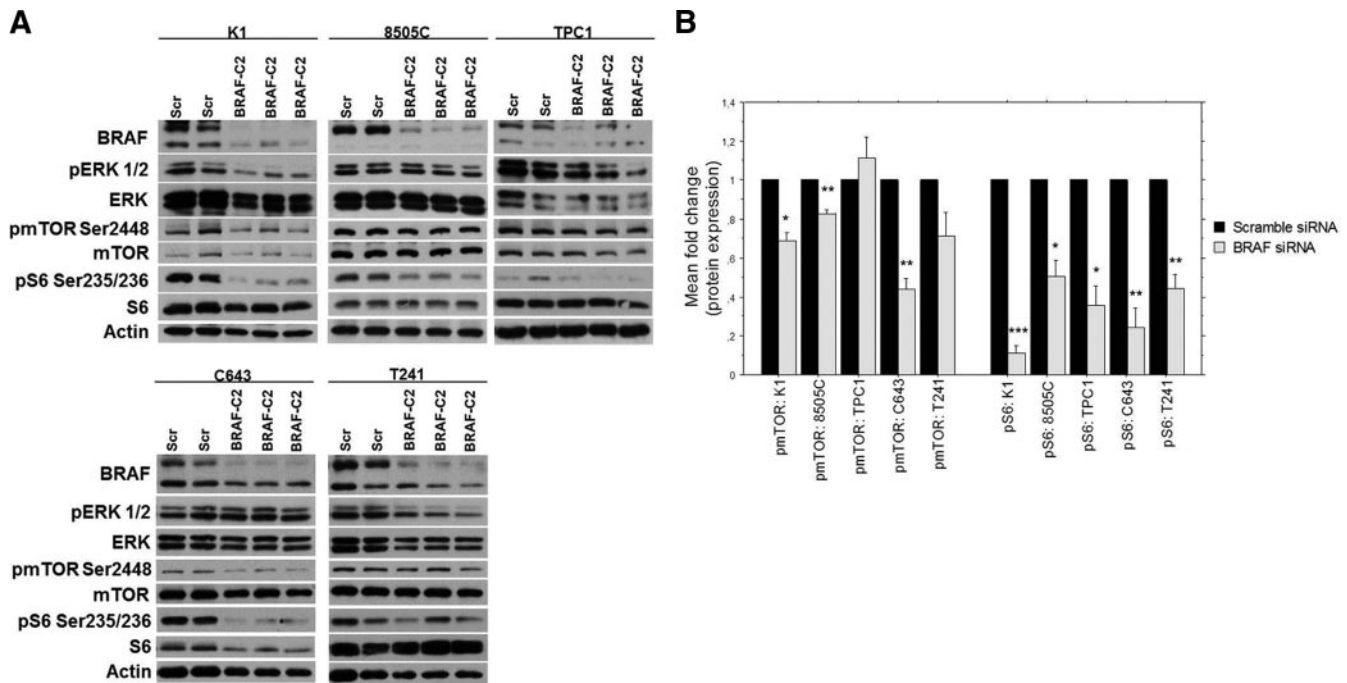
**FIG. 3.** mTOR pathway activation in transfected cells expressing exogenous *BRAF*<sup>wt</sup> or *BRAF*<sup>V600E</sup>. A, Mean fold change of protein expression observed in HEK293 cells transfected with the expressing vectors pCMV-BRAF<sup>wt</sup> and pCMV-BRAF<sup>V600E</sup> in comparison to cells transfected with a pCMV-empty vector. The levels of PTEN, pAKT Thr308, pAKT Ser473, mTOR, pmTOR Ser2448, raptor, rictor, pS6 Ser235/236, and p4EBP1 Thr37/46 were quantified and normalized by the levels of control protein (actin or  $\alpha$ -Tubulin). Results are shown as mean expression value  $\pm$  SEM of at least three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (unpaired Student's *t* test). B, Representative Western blot analysis of pmTOR Ser2448, mTOR, pS6 Ser235/236, and S6 expression levels in protein extracts of HEK293 cells transiently transfected with pCMV-empty vector and with the expressing plasmids pCMV-BRAF<sup>wt</sup> or pCMV-BRAF<sup>V600E</sup>. The levels of BRAF were analyzed for control of transfection efficiency, and the levels of pERK 1/2 were used as readout of BRAF activity. Representative actin expression pattern is shown.

overactivation of the mTOR pathway observed in cPTC cases, particularly the overexpression of pmTOR Ser2448 and pS6 Ser235/236.

### Oncogenic alterations in the ERK/MAPK pathway predict higher sensitivity of thyroid cancer cell lines to rapamycin

Basal activation of mTOR pathway was observed in the seven studied cell lines (Supplemental Fig. 1).

The sensitivity of thyroid cancer cell lines to rapamycin was compared. As observed in Fig. 5, A and B, rapamycin inhibited the growth of the seven cell lines, which was particularly evident at 10 and 100 nM concentrations (Supplemental Table 5). 8505C, K1, T243,



**FIG. 4.** mTOR pathway expression in thyroid cancer cell lines with BRAF silencing. A, Western blot analysis of pmTOR Ser2448, mTOR, pS6 Ser235/236, and S6 expression levels in protein extracts of K1, 8505C, TPC1, C643, and T241 thyroid cancer cell lines treated with BRAF-C2 siRNA. The levels of BRAF were analyzed for control of silencing efficiency, and the levels of pERK 1/2 as readout of BRAF activity. Representative actin expression pattern is shown. Protein level, in scramble siRNA-treated cell lines, was evaluated in duplicate (Scr), whereas in BRAF siRNA-treated cell lines, it was analyzed in triplicate. B, Mean fold change of protein expression observed in K1, 8505C, TPC1, C643, and T241 thyroid cancer cell lines treated with BRAF-C2 siRNA in comparison to cells treated with scramble siRNA. The levels of pmTOR Ser2448 and pS6 Ser235/236 were quantified and normalized by the levels of control protein (actin or  $\alpha$ -Tubulin). Results are shown as mean expression value  $\pm$  SEM. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (unpaired Student's *t* test).

TPC1, and C643 showed significantly higher growth inhibition than XTC1 and T241. Altogether, at 1 to 1000 nM doses, a lower sensitivity to rapamycin was observed in cell lines without MAPK alterations, compared with the ones harboring MAPK oncogenic alterations ( $P < 0.0001$ ; Fig. 5B).

In all seven cell lines, rapamycin remarkably reduced the levels of pS6 Ser235/236 (Fig. 5C), at 10 and 100 nM. Upon drug treatment, a decrease of pmTOR Ser2448 was also observed, except for the XTC-1 cell line.

These results indicate that oncogenic mutations in the MAPK pathway may confer higher sensitivity of thyroid cancer cell lines to rapamycin treatment.

#### BRAF<sup>V600E</sup> expression regulates the levels of pLKB1 Ser428

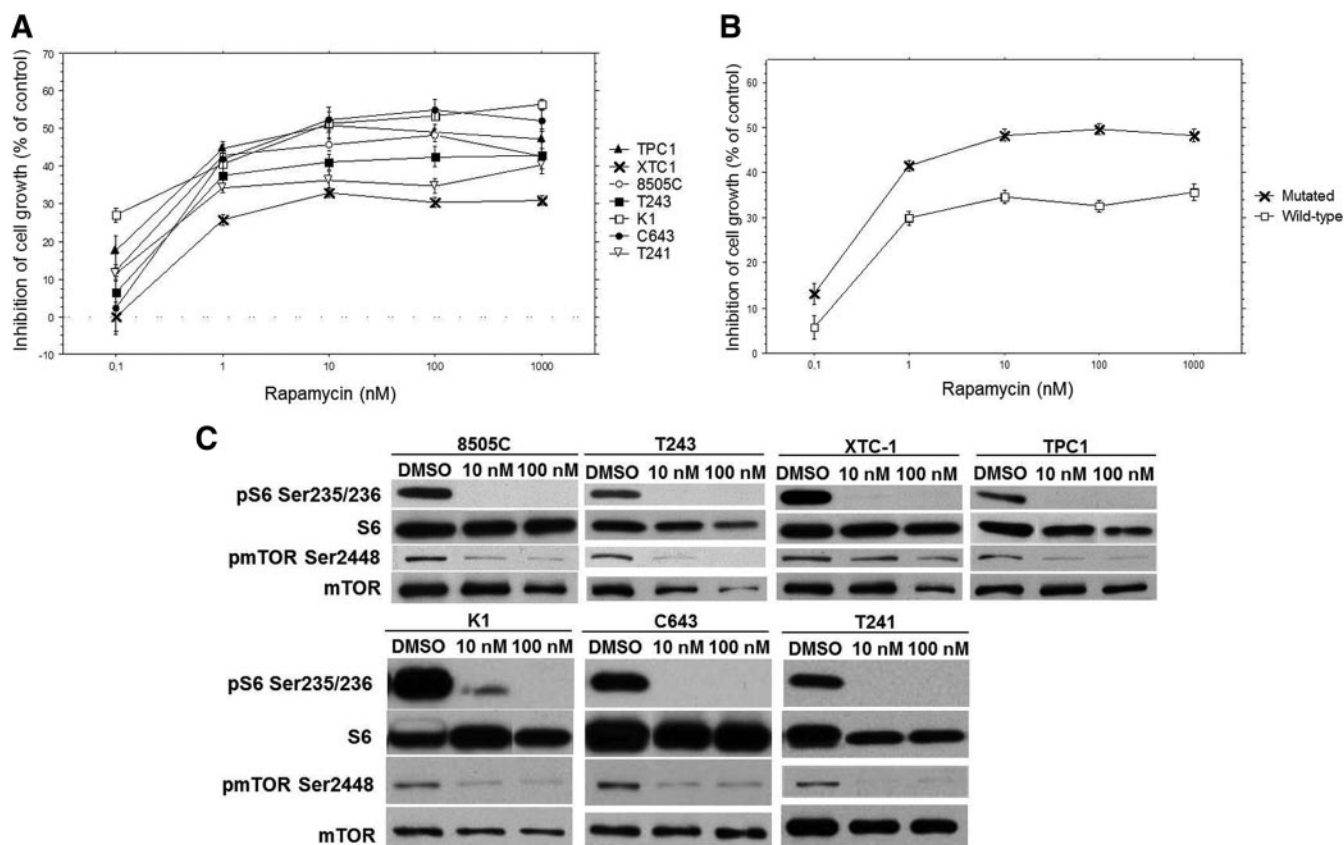
To assess a possible mechanism underlying the link between BRAF<sup>V600E</sup> mutation and mTOR pathway activation observed in cPTC, we evaluated the levels of pLKB1 Ser428 in HEK293 cells transfected with pCMV-empty vector, pCMV-BRAF<sup>wt</sup>, or pCMV-BRAF<sup>V600E</sup> as well as in thyroid cancer cell lines with BRAF knockdown by siRNA.

Increased expression of pLKB1 Ser428 was observed upon transfection with both BRAF expression vectors

compared to cells transfected with the empty vector:  $2.2 \pm 0.8$ -fold for pCMV-BRAF<sup>wt</sup>, and  $6.0 \pm 1.5$ -fold for pCMV-BRAF<sup>V600E</sup> (Fig. 6A); the 6-fold increase of pLKB1 Ser428 expression observed in pCMV-BRAF<sup>V600E</sup> transfected cells was statistically significant ( $P = 0.0313$ ; Fig. 6A).

At basal levels, pLKB1 Ser428 was barely detectable in C643 and T241. A decrease in pLKB1 Ser428 expression was observed in K1 ( $6.0 \pm 0.06$ -fold), 8505C ( $2.3 \pm 0.03$ -fold), and TPC1 ( $2.5 \pm 0.2$ -fold) upon BRAF-C2 siRNA (Fig. 6B), which was only significant in the BRAF<sup>V600E</sup> cell lines, K1 and 8505C ( $P = 0.0019$  and  $P = 0.0009$ , respectively). We down-regulated LKB1 in 8505C, TPC1, and K1 by siRNA. pLKB1 Ser 428 expression was confirmed to be decreased in 8505C and TPC1 (Fig. 6C), whereas it failed to be efficiently down-regulated in K1 (data not shown). In parallel, a significant up-regulation of pS6 Ser235/236 was detected in 8505C and TPC1 cell lines ( $P = 0.03$  and  $0.02$ , respectively; Fig. 6C).

We analyzed the expression of phospho- LKB1 in our series of primary PTC characterized for BRAF<sup>V600E</sup> mutation. We observed that BRAF<sup>V600E</sup> positive cases had higher phospho- LKB1 levels compared with BRAF<sup>wt</sup> cases (mean scores, 7.1 vs. 6.4, respectively), although the difference did not reach statistical significance (*t* test,  $P = 0.33$ ).



**FIG. 5.** Rapamycin effect on thyroid cancer cell lines. A, TPC1, XTC1, 8505C, T243, K1, C643, and T241 were grown in 0 to 1000 nM of rapamycin during 48 h, and the induced growth inhibition was determined by the SRB assay. Results were normalized to growth of cells treated with dimethylsulfoxide (DMSO) and are represented as mean  $\pm$  SEM of six replicates. Differences between cell lines were evaluated by unpaired Student's *t* test. B, Growth inhibition observed in thyroid cancer cell lines, with (mutated) and without (wild-type) mutation in the MAPK pathway, upon treatment with 0 to 1000 nM of rapamycin during 48 h. Results were normalized to growth of cells treated with DMSO and are represented as mean  $\pm$  SEM of six replicates. Differences between the two groups were evaluated by unpaired Student's *t* test. C, Western blot analysis of rapamycin effect on the expression of AKT/mTOR pathway proteins. The levels of pS6 Ser235/236, S6, pmTOR Ser2448, and mTOR were detected in thyroid cancer cell lines after treatment with 10 or 100 nM of rapamycin during 48 h. For each antibody, the image represented is the result of grouping parts of the same gel, with the same exposure.

Taken together, these results suggest that increased phosphorylation of pLKB1 Ser428 can be a mechanism contributing to the mTOR pathway overactivation observed in *BRAF*<sup>V600E</sup> thyroid cancer cells.

## Discussion

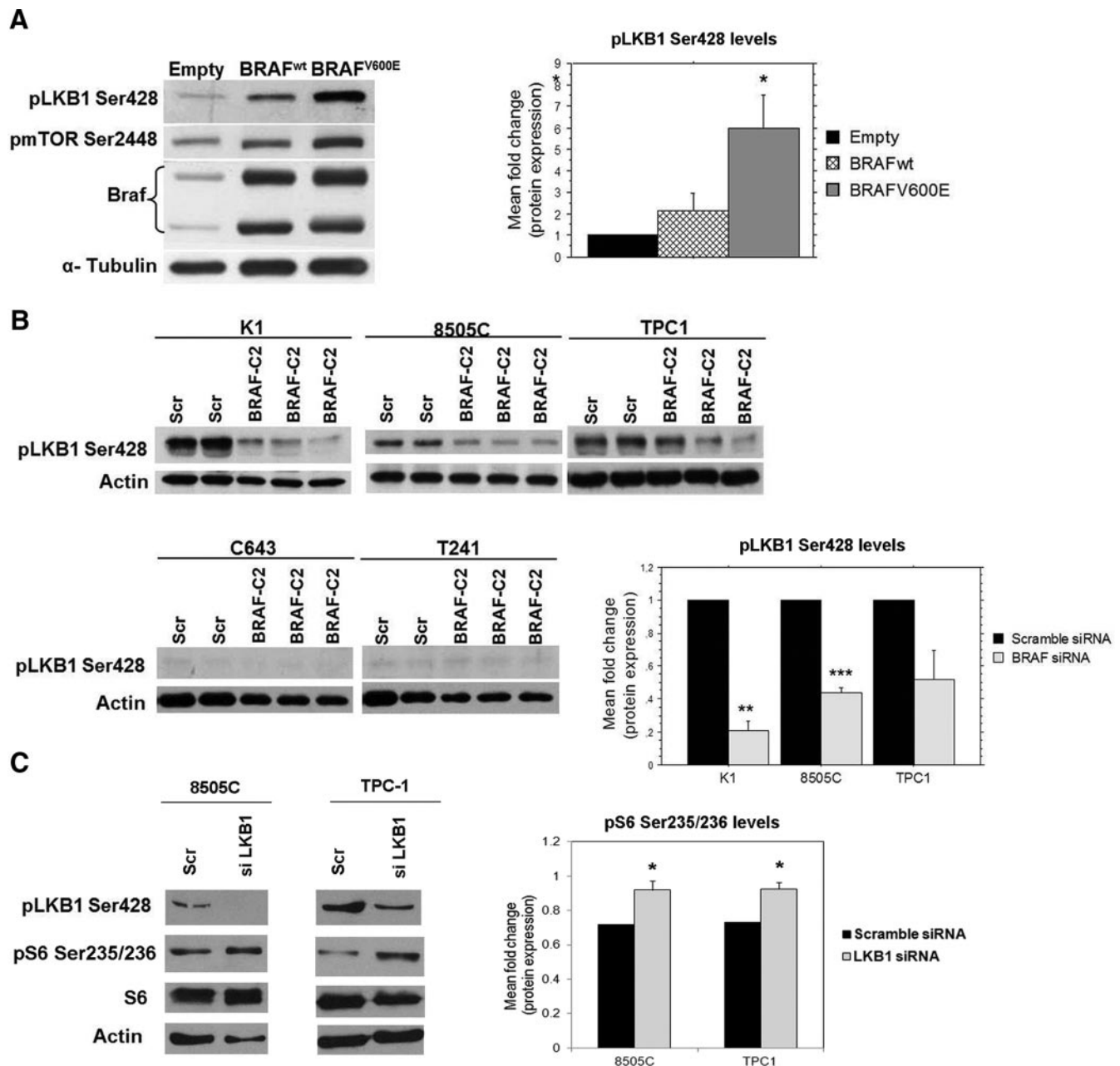
In the present study, we observed a particularly evident overactivation of AKT/mTOR pathway in cPTC. A significantly higher expression of mTOR pathway proteins was detected in cPTC compared with normal tissue and with the other types of differentiated thyroid carcinoma. Our immunohistochemical results fit with the previously reported increased expression of phospho-p70S6K and pAKT Ser473 in PTC tissues (29, 30). We observed a significantly higher up-regulation of the mTOR pathway in *BRAF*<sup>V600E</sup> mutated cPTC compared with *BRAF*<sup>wt</sup> cPTC. As far as we are aware, this is the first time that a corre-

lation between *BRAF*<sup>V600E</sup> mutation and overactivation of the mTOR pathway is demonstrated in cPTC. In a previous work of our group, *BRAF*<sup>V600E</sup>-mutated cutaneous melanomas also showed up-regulation of mTOR pathway (25), suggesting that this effect is not tumor-type specific.

Paradoxically, PTEN levels were also higher in cPTC, although with a preferential cytoplasmic localization. In fact, a decrease in PTEN nuclear localization was detected in these cases, which is in agreement with previous data showing reduced intensity of nuclear PTEN staining in PTC (16). Recent evidence showed that *PTEN* posttranslational regulation (e.g. ubiquitination, oxidation, or phosphorylation) can have a role in cancer susceptibility by leading to inactive cytoplasmic forms and/or to deficient PTEN nuclear import (31–33).

In cPTC metastases, the levels of PTEN, pAKT Ser473, mTOR, pmTOR, and p4EBP1 were significantly lower relative to primary lesions. These results suggest that the





**FIG. 6.** Regulation of pLKB1 Ser428 levels by BRAF<sup>V600E</sup> expression. **A**, Western blot analysis of pLKB1 Ser428 expression levels in protein extracts of HEK293 cells transiently transfected with pCMV-empty vector and with the expressing plasmids pCMV-BRAF<sup>wt</sup> or pCMV-BRAF<sup>V600E</sup>. Representative  $\alpha$ -tubulin expression pattern is shown. The mean fold change of pLKB1 Ser428 levels in HEK293 cells transfected with the expressing vectors pCMV-BRAF<sup>wt</sup> and pCMV-BRAF<sup>V600E</sup> in comparison to cells transfected with a pCMV-empty vector is shown in the graph. Results are shown as mean expression value  $\pm$  SEM. \*,  $P < 0.05$  (unpaired Student's  $t$  test). **B**, Western blot analysis of pLKB1 Ser428 expression levels after treatment of K1, 8505C, TPC1, C643, and T241 thyroid cancer cell lines with BRAF-C2 siRNA. In scramble siRNA-treated cell lines (Scr), protein levels were evaluated in duplicate, whereas in BRAF siRNA-treated cell lines (BRAF-C2), it was analyzed in triplicate. The mean fold change of pLKB1 Ser428 levels in K1, 8505C, and TPC1 thyroid cancer cell lines treated with BRAF-C2 siRNA in comparison to cells transfected with scramble siRNA is shown in the graph. Results are shown as mean expression value  $\pm$  SEM. **C**, Western blot analysis of pLKB1 Ser428, pS6 Ser235/236 and total S6 protein levels after LKB1 knockdown by siRNA (LKB1 siRNA is shown in duplicate). Actin was used as the loading control. Quantification of pS6 levels is shown on the right. Results represent mean  $\pm$  SEM of three independent experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (unpaired Student's  $t$  test).

mTORC1 pathway might be less active in cPTC metastases than in the primary lesions. Further work and a larger cohort are needed to determine whether the discrepancy observed between primary lesions and cPTC metastases reflects true biological heterogeneity or biologically meaningless quantitative differences.

The connection observed in cPTC between BRAF<sup>V600E</sup> expression and mTOR pathway overactivation was further supported by our *in vitro* results: BRAF overexpression led to enhanced pmTOR and pS6 expression, whereas BRAF down-regulation led to reduced expression of these proteins of the mTOR pathway. The up-regulation of



mTOR signaling in *BRAF*-transformed HEK293 cells was not exclusively associated with the mutated form of *BRAF*, suggesting that overexpression of the wild-type *BRAF* protein likely mimics the situation created by the constitutional activation of *BRAF*<sup>V600E</sup>. Changes in mTOR pathway upon *BRAF* siRNA were also observed in cells harboring *RAS* mutation and *RET/PTC*. This finding, together with the reported activation of the PI3K/AKT/mTOR pathway in cells harboring those genetic alterations or *RET* point mutation (19–23, 34), led us to speculate that *BRAF* may (at least partially) modulate mTOR pathway activation in thyroid cells with genetic alterations in the MAPK pathway. In line with these observations, when we compared the sensitivity of thyroid cancer cell lines to rapamycin, a significantly higher growth inhibition was observed in thyroid cancer cell lines with oncogenic alterations in the MAPK pathway (*BRAF*<sup>V600E</sup>, *RAS*, and *RET/PTC1*).

Our results show for the first time that increased *BRAF* signaling leads to mTOR pathway activation and suggest that inhibition of both MAPK and mTOR pathways could be a good strategy in therapeutic targeting of *BRAF*<sup>V600E</sup>-positive tumors. In fact, previous data have shown an increased or synergistic activity of *BRAF* (RAF265) or MEK inhibitors (RDEA119, AZD6244) and mTOR inhibitors (BEZ-235, temsirolimus, or rapamycin) in the growth inhibition of thyroid cancer cell lines and xenograft tumors (35–37).

Additionally, we suggest a possible mechanism contributing to the effect of oncogenic *BRAF* on mTOR pathway activation. Specifically, we observed a positive correlation between *BRAF*<sup>V600E</sup> expression and pLKB1 levels *in vitro*. Moreover, LKB1 down-regulation by siRNA led to activation of the mTOR pathway, as seen by increased levels of pS6 in *BRAF*<sup>V600E</sup> as well as in *RET/PTC*-harboring thyroid cancer cell lines. As previously reported, LKB1 activity can be negatively regulated by Ser428 phosphorylation induced by the RAF-MEK-ERK signaling cascade as well as by p90-RSK (9). Therefore, it is plausible that *BRAF*<sup>V600E</sup> mutations and *RET/PTC* rearrangements, both activators of the ERK/MAPK pathway, can induce LKB1 Ser428 phosphorylation, compromising its ability to bind AMPK. In our series of human PTC, we observed a tendency for *BRAF*<sup>V600E</sup>-harboring cases to display increased pLKB1Ser428 levels, although it is unlikely that mutant *BRAF* is the only factor regulating LKB1 phosphorylation in these lesions *in vivo*.

Nonetheless, our results suggest that oncogenic *BRAF*-induced inactivation of LKB1 by Ser428 phosphorylation might underlie the mTOR pathway overactivation observed in cell lines and in human tumors harboring the *BRAF*<sup>V600E</sup> mutation.

These data further suggest that AMPK could be a good target in *BRAF*-mutated thyroid cancer, which is in accordance with a recent report showing that AICAR (AMPK activator) treatment inhibited the proliferation of *BRAF*<sup>V600E</sup> mutant thyroid cancer cell lines more strongly than of wild-type cell lines (38).

In PTC, *BRAF*<sup>V600E</sup> has been associated to a higher risk of recurrence and radioactive iodine treatment resistance, particularly due to decreased expression of Na<sup>+</sup>/I<sup>−</sup> symporter (NIS) and its impaired targeting to the membrane (39). A recent study demonstrated that rapamycin leads to an increase in NIS expression and iodine uptake in insulin- and TSH-stimulated thyroid PCCL3 cells (40). These previous data and our finding of *BRAF*<sup>V600E</sup> association with overactivation of mTOR pathway suggest that, in *BRAF*<sup>V600E</sup> cPTC cases, the up-regulation of the mTOR pathway may lead to a decrease in NIS expression, and therefore, the use of mTOR inhibitors could be a strategy to increase the radioiodine response of *BRAF*<sup>V600E</sup>-cPTC.

In conclusion, we demonstrated that the AKT/mTOR pathway is particularly overactivated in human cPTC harboring the *BRAF*<sup>V600E</sup> mutation. The association between *BRAF*<sup>V600E</sup> mutation and mTOR pathway activation was supported by *in vitro* results. *BRAF*-induced phosphorylation of LKB1Ser428 emerged as a possible mechanism contributing to such association, possibly through uncoupling of the LKB1-AMPK-mTOR energy stress sensor.

Our results suggest and reinforce previous observations showing that targeting the mTOR pathway (as well as in combination with MAPK pathway/AMPK inhibition) could be a good strategy to improve therapy effect in thyroid carcinomas harboring the *BRAF*<sup>V600E</sup> mutation.

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