Activation of the RAS/RAF/ERK Signaling Pathway Contributes to Resistance to Sunitinib in Thyroid Carcinoma Cell Lines

Annamaria Piscazzi, Eleonora Costantino, Francesca Maddalena, Maria Iole Natalicchio, Assunta Maria Teresa Gerardi, Raffaele Antonetti, Mauro Cignarelli, and Matteo Landriscina

Clinical Oncology Unit (A.P., E.C., A.M.T.G., M.L.), Department of Medical Sciences, and Endocrinology Unit (M.C.), Department of Medical Sciences, University of Foggia, 71122 Foggia, Italy; Laboratory of Pre-clinical and Translational Research, Instituto di Ricovero e Cura a Carattere Scientifico-Centro di Riferimento Oncologico Regionale della Basilicata (F.M.), 85028 Rionero in Vulture, Italy; and Molecular Biology Laboratory (M.I.N., R.A.), Azienda Ospedali Riuniti, Foggia, Italy

Context: Sunitinib is currently being evaluated in advanced human thyroid carcinomas, based on the rationale that the vascular endothelial growth factor and platelet-derived growth factor receptors and the RET/PTC rearrangement are valuable targets for the treatment of this malignancy. However, criteria for selecting thyroid tumors that may benefit from sunitinib are lacking.

Design: The effect of activating somatic mutations in the *KRAS* and *BRAF* genes on the responsiveness to sunitinib was evaluated in a panel of thyroid cancer cell lines harboring wild-type *KRAS* and *BRAF* genes, the RET/PTC1 rearrangement, the G12R KRAS, or the V600E BRAF mutation.

Results: Sunitinib was found to selectively inhibit cell proliferation, induce cell accumulation in the G0-G1 phase, and inhibit the phosphorylation of ERK1/2 in both KRAS/BRAF wild-type thyroid cancer cells and in tumor cells harboring the RET/PTC rearrangement, whereas it was completely ineffective in KRAS- or BRAF-mutated thyroid carcinoma cells. This differential antitumor activity of sunitinib did not correlate with the expression profile of the vascular endothelial growth factor receptors 1, 2, and 3, platelet-derived growth factor receptor-α and *cKIT* genes. Of note, the constitutive activation of RAS/RAF/ERK signaling in KRAS/BRAF wild-type cells by transfection of the R12 HRAS or V600E BRAF mutants or stimulation with epithelial growth factor resulted in the loss of responsiveness to sunitinib, whereas pharmacological inhibition of MAPK kinase activity resulted in the resensitization of KRAS- or BRAF-mutated cells to the multikinase inhibitor.

Conclusions: The constitutive activation of the RAS/RAF/ERK pathway may favor resistance to sunitinib in thyroid carcinoma cells. **(*J Clin Endocrinol Metab* 97: E898–E906, 2012)**

The vast majority of human thyroid carcinomas (TC) are well-differentiated malignancies with an excellent prognosis. Indeed, distant metastases occur in less than 10% of patients, and their treatment is based on radioiodine therapy when metastatic uptake of radioiodine has been demonstrated. However, current therapeutic strategies induce complete remission of disease in about 30% of patients with metastatic disease. The other two thirds of patients are deemed refractory to radioiodine therapy, and among them, the rate of progression is extremely variable (1). Some patients have stable disease for years, even in the absence of systemic treatments, whereas others have rapidly progressing diseases, poorly responsive to traditional antiblastic agents (2). Thus, there is a need for new therapeutic strategies for patients with iodine-refractory progressive advanced TC and for predic-

ISSN Print 0021-972X ISSN Online 1945-7197
Printed in U.S.A.

doi: 10.1210/jc.2011-3269 Received December 1, 2011. Accepted February 28, 2012.
First Published Online March 22, 2012

Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; EGF, epithelial growth factor; EGFR, EGF receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEK, MAPK kinase; MTT, dimethylthiazoldiphenyltetra-zoliumbromide; PDGFR, platelet-derived growth factor receptor; PI, propidium iodate; TC, thyroid carcinoma; TK, tyrosine kinase; VEGFR, vascular endothelial growth factor receptor.

tive criteria to help select tumors which may benefit from these treatments.

In recent years, a variety of molecular-targeted agents that inhibit tyrosine kinases (TK) related to tumor growth and/or angiogenesis have been developed, and some of them (*i.e.* axitinib, motesanib, sorafenib, sunitinib, and pazopanib) have been evaluated in advanced TC in humans with promising results (3, 4). The rationale behind these studies is provided by the evidence that most papillary TC are driven in part by single activating somatic mutations of the *BRAF* and *RAS* oncogenes, and/or by translocations producing RET/PTC oncogenes (5).

Sunitinib is a multitargeted agent, with antiangiogenic and antitumor properties, acting as a selective inhibitor of vascular endothelial growth factor receptors (VEGFR) 1, 2, and 3, platelet-derived growth factor receptor (PDGFR)-α, cKIT, and RET/PTC subtypes 1 and 3. Sunitinib is currently used as standard treatment for clear-cell renal carcinoma and gastrointestinal stromal tumor and is under investigation in several other human malignancies (6). The interest in sunitinib for the treatment of TC arises from its activity against tumor cells bearing the RET/PTC rearrangement (7). Several preclinical studies have evaluated sunitinib in RET/PTC TC cell lines demonstrating, indeed, that this agent is able to inhibit cell proliferation and cause cell cycle arrest by inhibiting the RET/PTC kinase (8, 9). Interestingly, sunitinib appears to target the cytosolic MAPK kinase (MEK)/ERK and stress-activated protein kinase/c-Jun N-terminal kinase pathways in RET/PTC cell lines, suggesting that blocking these pathways is at least part of the mechanism by which sunitinib exerts its direct antiproliferative activity (9). Sunitinib has been tested in phase II clinical trials in metastatic iodine-refractory TC, showing promising results (10, 11). However, under sunitinib therapy, the majority of TC showed disease stability, whereas few of them exhibited major tumor regression (10, 11), suggesting that the clinical activity of sunitinib may be restricted to a specific subgroup of human thyroid malignancies (4). In fact, given the indolent nature of most TC, a report highlighting tumor stability may be of limited value. Thus, taken all together, these observations suggest that criteria for identifying tumors that may benefit from sunitinib therapy are lacking.

Because of several factors [including 1) only 20% of human papillary TC harbor the RET/PTC rearrangement (12), 2) the molecular characterization of human thyroid cancers revealed a great heterogeneity with genotypes showing driving mutations in other oncogenes (12), and 3) little information is available on the antiproliferative activity of sunitinib in non-RET/PTC TC cell lines], we sought to evaluate sunitinib activity in a panel of TC cell lines at different degrees of cell differentiation and characterized by different molecular genotypes, in an attempt to find the molecular determinants responsible for sensitivity/resistance.

Materials and Methods

Chemicals, cell cultures, and constructs

Reagents were purchased from Sigma-Aldrich (Milan, Italy) unless otherwise specified. The MEK inhibitor PD98059 was purchased from Cell Signaling Technology (Milan, Italy). The following unique human TC cell lines were used: papillary TPC-1; follicular ML-1; poorly differentiated WRO; and anaplastic CAL-62, FRO, and BHT-101 cells (13). TPC-1 cells were kindly provided by Professor Pontecorvi (Endocrinology Unit, Catholic University, Rome, Italy), whereas the other cell lines were purchased from DSMZ (Braunschweig, Germany). Cells were cultured in DMEM containing 10% (vol/vol) fetal bovine serum (20% for BHT-101), 1.5 mM glutamine, and 100 U/ml penicillin and streptomycin. Cell lines were routinely monitored by microscopic morphology.

Sunitinib was kindly provided by Pfizer (New York, NY) and dissolved in dimethylsulfoxide at 10 mM. Sunitinib or the same dimethylsulfoxide volume was added to the cultures at the concentrations specified in *Results*. To evaluate the growth rate, cells were cultured in six-well plates in the presence and absence of the reported concentrations of sunitinib, harvested after 4 d, and counted in a Bürker chamber (three countings per sample). Incubation with drugs was carried out continuously, and drug-containing fresh medium was changed at 48-h intervals. Cell viability was evaluated using the dimethylthiazoldiphenyltetrazoliumbromide (MTT) dye assay as previously described (14). Results represent the average of three experiments. Apoptosis was measured by cytofluorimetric evaluation of 7-aminoactinomycin D incorporation in nonpermeabilized cells.

The R12 HRAS construct was a kind gift of Dr. Alfonso Bellacosa (Fox Chase Cancer Center, Philadelphia, PA), and the wild-type BRAF and BRAF V600E constructs were kindly provided by Professor Franca Esposito (University of Naples Federico II, Naples, Italy). All the constructs were cloned in pcDNA3.1 vector (Invitrogen, San Giuliano Milanese, Italy). Transient transfection of DNA plasmids was performed with Polyfect Transfection reagent (QIAGEN, Milan, Italy), according to the manufacturer's protocol.

Cell cycle analysis

Cells were grown in the presence and the absence of 0.5 and 1 μM sunitinib for 48 h, incubated in the same medium supplemented with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 15 min, and harvested. Cell pellets were resuspended in a solution containing 10 N HCl for 30 min at room temperature to obtain DNA denaturation and subsequently incubated in the presence of anti-BrdU monoclonal antibodies (Becton Dickinson and Co., Milan, Italy) for 1 h at room temperature in the dark. After washing with PBS, cells were incubated with 1 μg/ml propidium iodate (PI) for 20 min and evaluated using the EPICS XL flow cytometer (Beckman Coulter, Cassina De' Pecchi-Milan, Italy). In specific experiments, cells were transiently cotransfected with pcDNA3.1 (scramble), R12 HRAS, wild-type BRAF or V600E BRAF constructs, and pEGFP-F (CLONTECH, Mountain View, CA) and 24 h later treated with sunitinib. The transfection vector

pEGFP-F, which encodes for a farnesylated enhanced green fluorescent protein, was used as a reporter vector both to monitor transfection efficiency and as a cotransfection marker. Cell cycle was evaluated by PI labeling in green fluorescent protein-positive cells. Experiments were performed at least three times using three replicates for each drug concentration.

Immunoblot analysis

Immunoblot analysis was performed as previously reported (15). Specific proteins were detected by using a rabbit polyclonal antiphosphophosphorylated ERK1/2 (Upstate Cell Signaling Technology), a mouse monoclonal antiphosphophosphorylated ERK1/2 (Thr202/Tyr204-E10; Cell Signaling Technology), a rabbit polyclonal anti-ERK1/2 (Calbiochem, Rome, Italy), a mouse monoclonal anti-BRAF (F-7, sc-5284; Santa Cruz Biotechnology, Segrate, Italy), a rabbit polyclonal anti-HRAS (C-20, sc-520; Santa Cruz Biotechnology), and a mouse monoclonal anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH; sc-47724; Santa Cruz Biotechnology) antibodies.

RNA extraction and real-time RT-PCR analysis

Total RNA from cell pellets was extracted using the TRIzol reagent (Invitrogen). For the first-strand synthesis of cDNA, 3 μg of RNA was used in a 20-μl reaction mixture using a cDNA Superscript II (Invitrogen). For real-time PCR analysis, 1 μl of cDNA sample was amplified using the Platinum SYBR Green qPCR Supermix UDG (Invitrogen) in an iCycler iQ real-time detection system (Bio-Rad Laboratories GmbH, Segrate, Italy). The following primers were used: VEGFR1, forward, *5′-CAAGTGGCCAGAGGCATGGAGTT-3′* reverse,*5′-GATGTAGTCTTTACCATCCTGTTG-3′* (PCR product 417 bp); VEGFR2, forward, *5′-TAAGGGCATGGAGTTCTTGG-3′*, reverse, *5′-AGGAAACAGGTGAGGTAGGC-3′* (PCR product 562 bp); PDGFRα, forward, *5′-TTTGACGGTCCCCGAGGCCA-3′*, reverse, *5′-TGGCGGGCAGCACATTCGTA-3′* (PCR product 60 bp); and GAPDH, forward, *5′-CAAGGCTGAGAACGGGAA-3′*, reverse, *5′-GCATCGCCCCACTTGATTTT-3′* (PCR product 90 bp). Primers were designed to be intron spanning. Reaction conditions were 50 C for 2 min, 95 C for 2 min, followed by 45 cycles of 15 sec at 95 C, 30 sec at 60 C (VEGFR2, 58 C; PDGFRα, 62 C), and 30 sec at 72 C. GAPDH was chosen as an internal control. Primers specific for VEGFR3 and cKIT mRNA were purchased from QIAGEN (catalog no. QT00063637 and QT01679993) and used according to the manufacturer's protocol.

Mutational analysis

DNA was extracted from TC cells using the QIAamp DNA minikit (QIAGEN), according to the manufacturer's protocol. Assays of samples for KRAS and BRAF mutations were performed by using, respectively, the anti-epithelial growth factor receptor (EGFR) monoclonal antibodies response-KRAS status and the anti-EGFR monoclonal antibodies response-BRAF status kits (Diatech Pharmacogenetics, Ancona, Italy), according to the manufacturer's instructions. Codons 12, 13, 61, and 146 (exon 2) of the *KRAS* gene and codons 464, 466, and 469 (exon 11), and 600 (exon 15) of the *BRAF* gene were amplified by PCR reactions on the Rotor-Gene Q 6000 (QIAGEN), single-stranded DNA templates were prepared using the PyroMark Vacuum Prep Workstation (Biotage, Uppsala, Sweden) and, lastly, pyrosequencing analysis was performed on the PyroMark Q96 ID (Biotage, Uppsala, Sweden).

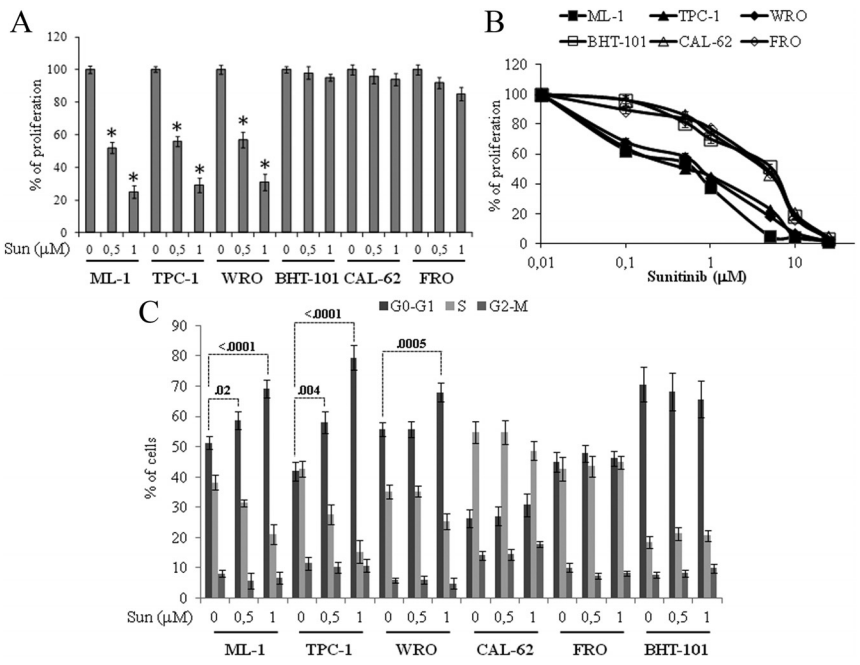

FIG. 1. Differential activity of sunitinib in thyroid carcinoma cell lines. Papillary TPC-1, follicular ML-1, poorly differentiated WRO, and undifferentiated CAL-62, FRO, and BHT-101 thyroid carcinoma cells were cultured in the presence and absence of 0.5 and 1 μM sunitinib (Sun) for 96 h (A) or in the presence and absence of 0.01–25 μM sunitinib for 48 h (B) or in the presence and absence of 0.5 and 1 μM sunitinib for 12 h (C). Cell proliferation/viability was evaluated by cell count (A) and MTT incorporation (B), whereas cell cycle distribution was evaluated by BrdU and PI incorporation (C). Results are reported as percentage of proliferation relative to the respective untreated controls (A), as percentage of MTT incorporation relative to the respective untreated control (B), and as the percentage of cells in each phase of the cell cycle (C). *Error bars*, $\pm$SD *P* values indicate the statistical significance between sunitinib- and vehicle-treated cells, *, $P <$ 0.0001.

Statistical analysis

The paired Student's *t* test was used to establish the statistical significance between different levels of growth rate and cell cycle distribution. Statistically significant values ($P < 0.05$) are reported in the figure legends.

Results

Sunitinib antiproliferative activity is selective for specific TC cell lines

To explore the antiproliferative activity of sunitinib in TC cells, a panel of six TC cell lines at distinct degrees of cell differentiation was evaluated for growth rate (Fig. 1A) and cell viability by MTT incorporation (Fig. 1B) in response to increasing concentrations of sunitinib. Thus, in addition to papillary TPC-1 cells harboring the RET/PTC translocation

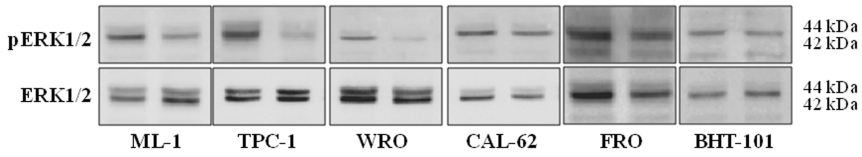

FIG. 2. Inhibition of ERK1/2 phosphorylation in thyroid carcinoma cell lines upon sunitinib treatment. Total cell lysates from papillary TPC-1, follicular ML-1, poorly differentiated WRO, and undifferentiated CAL-62, FRO, and BHT-101 thyroid carcinoma cells, treated with 0.5 μM sunitinib for 4 h, were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

(16), follicular ML-1, poorly differentiated WRO, and anaplastic FRO, BHT-101, and CAL-62 cell lines were evaluated. Sunitinib induced a similar inhibition of cell proliferation in TPC-1, ML-1, and WRO cells, whereas it was ineffective in CAL-62, FRO, and BHT-101 cells (Fig. 1, A and B). Of note, this sunitinib-dependent differential antiproliferative activity was not dependent on a selective induction of apoptosis, as demonstrated by cytofluorimetric evaluation of 7-aminoactinomycin D incorporation in nonpermeabilized cells (data not shown) but correlated with the accumulation of cells in the G0-G1 phase of the cell cycle and the parallel inhibition of the S phase. Consistently, sunitinib failed to induce similar effects in FRO, BHT-101, and CAL-62 cells (Fig. 1C).

Because it is well established that the direct antitumor activity of sunitinib depends mostly on its ability to inhibit ERK signaling (17), the ability of the drug to inhibit ERK phosphorylation was evaluated in TC cell lines (Fig. 2). The exposure of ML-1, TPC-1, and WRO cells to 0.5 μM sunitinib for 4 h resulted in a significant inhibition of ERK phosphorylation, whereas the same drug concentration failed to inhibit ERK signaling in FRO, CAL-62, and BHT-101 cells.

The resistance to sunitinib correlates with the mutational status of KRAS and BRAF genes in TC cell lines

A major aim of this study is to find molecular determinants of sunitinib sensitivity/resistance in TC cell lines. Thus,

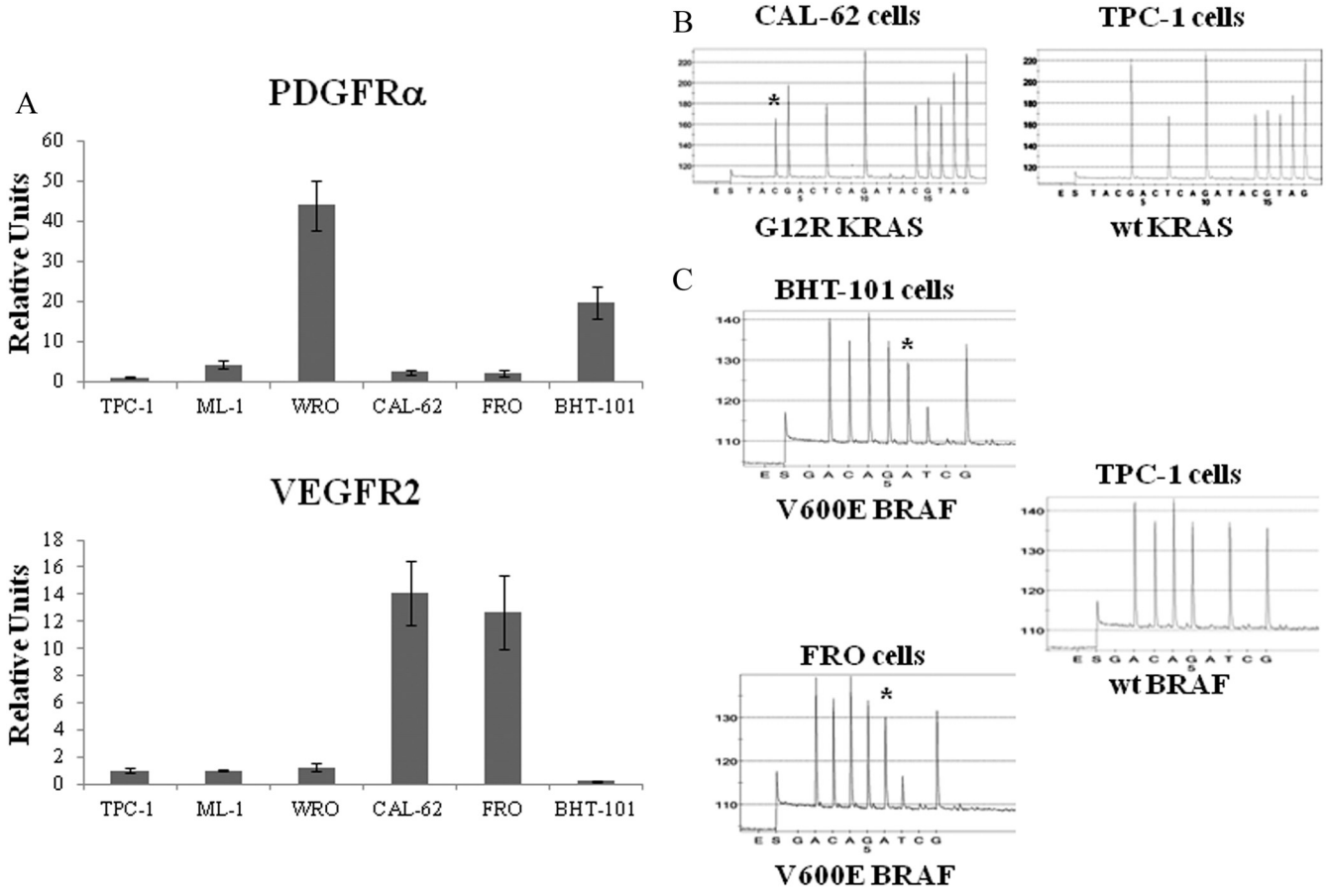

FIG. 3. Gene expression and mutational profiling of thyroid carcinoma cells. A, Real-time PCR analysis of PDGFRα and VEGFR2 gene expression in papillary TPC-1, follicular ML-1, poorly differentiated WRO, and undifferentiated CAL-62, FRO, and BHT-101 thyroid carcinoma cells. *Error bars*, $\pm$SD. B, Pyrograms of codons 12 and 13 (exon 2) of the KRAS gene showing the G12R KRAS mutation in CAL-62 thyroid carcinoma cells and a wild-type genotype in thyroid carcinoma TPC-1 cells. C, Pyrograms of codon 600 (exon 15) of the BRAF gene, showing the V600E BRAF mutation in FRO and BHT-101 thyroid carcinoma cells and a wild-type genotype in thyroid carcinoma TPC-1 cells. *Asterisks* indicate the substituted nucleotides in mutated cell lines.

the panel of TC cells was evaluated, by real-time PCR, for the expression profile of VEGFR1, VEGFR2, and VEGFR3, PDGFRα, and cKIT, all well-established targets of sunitinib (17). Of note, the expression of none of these genes correlated with the antiproliferative activity of sunitinib. Indeed, VEGFR1 and VEGFR3, as well as cKIT mRNA levels were below the detection limit of a quantitative RT-PCR assay (data not shown). The expression of the VEGFR2 gene was up-regulated in FRO and CAL-62 cells that are nonresponsive to sunitinib, whereas it was poorly expressed in both sunitinib-insensitive BHT-101 cells and the three sunitinib-sensitive TC cell lines (Fig. 3A). Furthermore, PDGFRα was up-regulated in both sunitinib-sensitive WRO and sunitinib-insensitive BHT-101 cells (Fig. 3A).

Because the inhibition of the RAS/RAF/ERK signaling downstream of the TK receptors is a critical step for the direct antitumor activity of sunitinib (17) and taking into account that mutations of *KRAS* or *BRAF* genes have been proven to drive oncogenic events in thyroid carcinogenesis (12), we further evaluated the relationship between the mutational status of *BRAF* and *KRAS* genes and the responsiveness to sunitinib. As reported in Fig. 3B, sunitinib-resistant CAL-62 cells were shown to be heterozygous for the KRAS G12R mutation, whereas FRO and BHT-101 cell lines were revealed to harbor the BRAF V600E heterozygous mutation. By contrast, all sunitinib-sensitive TC cell lines exhibited wild-type KRAS and BRAF genotypes.

The activation of RAS/RAF/ERK pathway favors the resistance to sunitinib in TC cells

To establish whether the activation of the RAS/RAF/ERK signaling pathway may be responsible for resistance

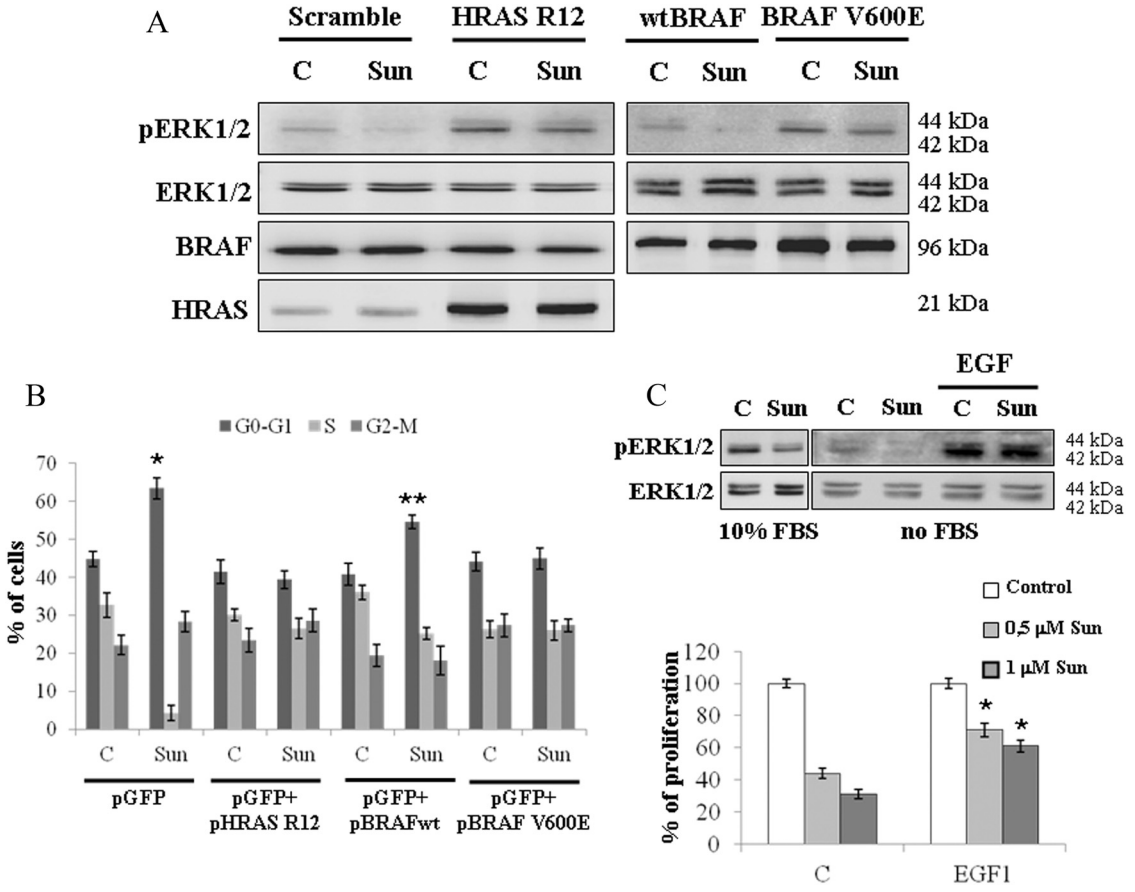

FIG. 4. The effect of the constitutive activation of ERK signaling on sunitinib activity. A, Thyroid carcinoma WRO cells were transfected with pcDNA 3.1 vector (Scramble) or with HRAS R12, wild-type BRAF or BRAF V600E constructs and subsequently cultured in the presence and absence of 0.5 μM sunitinib for 4 h. Total cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. B, Cell cycle distribution of thyroid carcinoma WRO cells cotransfected with phosphorylated EGFP-F vector and HRAS R12, wild-type BRAF or BRAF V600E constructs, and subsequently treated with 1 μM sunitinib for 12 h. Results are reported as percentages of cells in each phase. *Error bars*, $\pm$SD *P* values indicate the statistical significance between sunitinib- and vehicle-treated cells. *, $P < 0.0001$; **, $P = 0.002$. C, *Upper panel*, Serum-deprived thyroid carcinoma WRO cells were stimulated with 250 ng/ml EGF for 48 h and subsequently cultured in the presence and absence of 0.5 μM sunitinib for 4 h. WRO cells cultured in the presence of 10% Fetal Bovine Serum and treated with 0.5 μM sunitinib for 4 h were used as a control. Total cell lysates were separated by SDS-PAGE and immunoblotted with the indicated antibodies. *Lower panel*, Thyroid carcinoma WRO cells were preincubated in the presence of 250 ng/ml EGF for 48 h and subsequently cultured in the presence and absence of 0.5 and 1 μM sunitinib for an additional 48 h. Cell proliferation was evaluated by cell counts and reported as percentage of proliferation relative to the respective untreated controls. *Error bars*, $\pm$SD *P* values indicate the statistical significance between inhibition rates in EGF-stimulated and control cells. *, $P < 0.0001$.

to sunitinib in TC cells, we evaluated sunitinib activity on the constitutive activation of the ERK pathway in KRAS/BRAF wild-type WRO cells. This was achieved by transfecting a construct encoding for the HRAS R12 mutant, which is known to induce fibroblast transformation and the constitutive activation of ERK signaling (18), and a construct encoding for the BRAF V600E mutant, which is the most frequent BRAF mutation in human papillary TC and known to be responsible for the constitutive activation of ERK phosphorylation (19, 20). In these experimental conditions, sunitinib failed to inhibit ERK phosphorylation (Fig. 4A) and the S phase with accumulation of cells in the G0-G1 phase (Fig. 4B) in both WRO HRAS R12 and BRAF V600E transfectants. Furthermore, because the increased expression of alternative growth factors with proangiogenic potential and capable of directly stimulating tumor cells has been proposed as an escape mechanism from anti-vascular endothelial growth factor therapies (21), we hypothesized that epithelial growth factor (EGF), which is known to be involved in thyroid tumor progression (22), may favor the appearance of sunitinib resistance in TC cells. Thus, cell proliferation and ERK phosphorylation was evaluated in sunitinib-treated KRAS/BRAF wild-type WRO cells upon stimulation with high concentrations of EGF. Indeed, the EGF-dependent activation of ERK pathway reduced the ability of sunitinib to inhibit both ERK phosphorylation (Fig. 4C, *upper panel*) and the rate of cell proliferation (Fig. 4C, *lower panel*).

In a parallel experiment, we sought to inhibit ERK signaling in KRAS- or BRAF-mutated TC cells to reestablish sensitivity to sunitinib. This objective was achieved by pretreating CAL-62, BHT-101, and FRO cells with the MEK inhibitor, PD98059, before exposure to sunitinib (Fig. 5). Of note, inhibition of the ERK signaling pathway resulted in the partial reestablishment of sunitinib inhibitory activity on cell proliferation (Fig. 5A), cell cycle (Fig. 5B), and ERK phosphorylation (Fig. 5C).

Discussion

The mechanism of action of the multitargeted TK inhibitor, sunitinib, depends on both its antiangiogenic activity toward endothelial cells and its direct antiproliferative activity against tumor cells (17). The present study was specifically designed to evaluate the mechanisms responsible for sunitinib's direct antiproliferative activity in TC cell

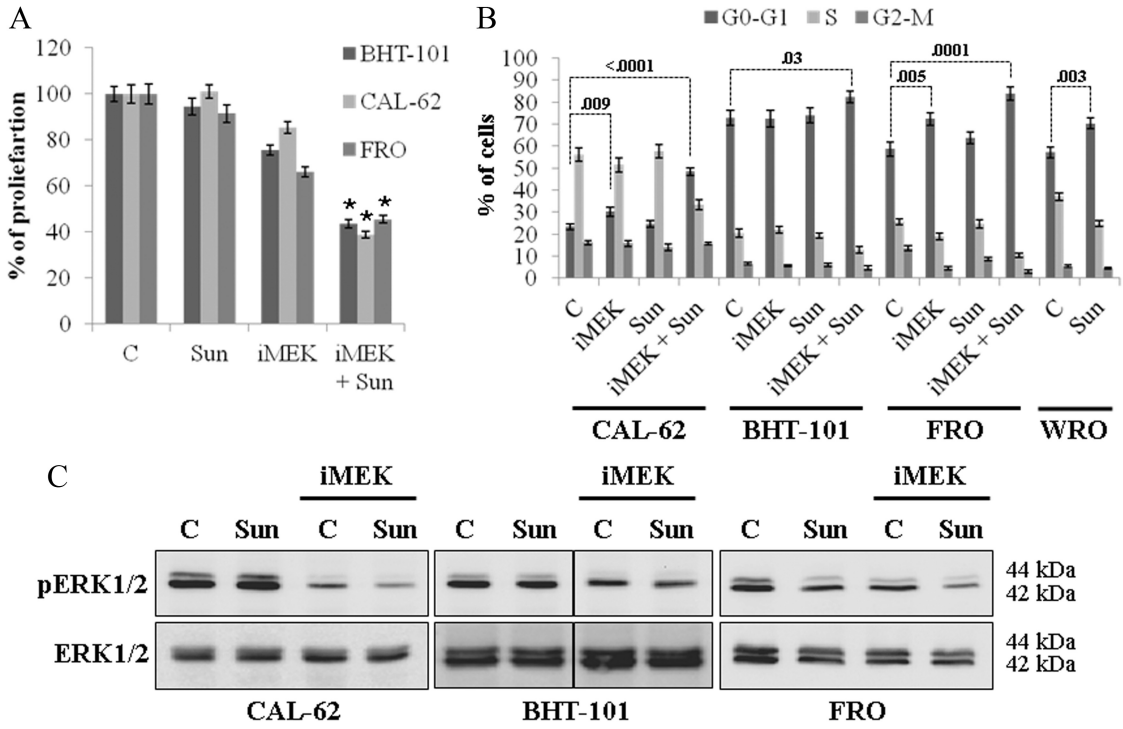

FIG. 5. Resensitization of KRAS- and BRAF-mutated thyroid carcinoma cells to sunitinib. A and B, Growth rate (A) and cell cycle distribution (B) of KRAS-mutated CAL-62 and BRAF-mutated BHT-101 and FRO thyroid carcinoma cells, pretreated with the MEK inhibitor PD98059 (20 μM) for 24 h and subsequently cultured in the presence and absence of 1 μM sunitinib for an additional 72 (A) or 24 h (B). Cell proliferation was evaluated by cell count and is reported as percentage of proliferation relative to the respective untreated controls (A); cell cycle distribution is reported as percentages of cells in each phase (B). *Error bars*, $\pm$SD *P* values indicate the statistical significance between sunitinib/iMEK- and sunitinib-treated or iMEK-treated and vehicle-treated cells. C, Control. C, Total cell lysates from CAL-62, BHT-101, and FRO thyroid carcinoma cells, pretreated with the MEK inhibitor PD98059 (20 μM) for 24 h, and subsequently cultured in the presence and the absence of 0.5 μM sunitinib for 48 h, were separated by SDS-PAGE and immunoblotted with the indicated antibodies.

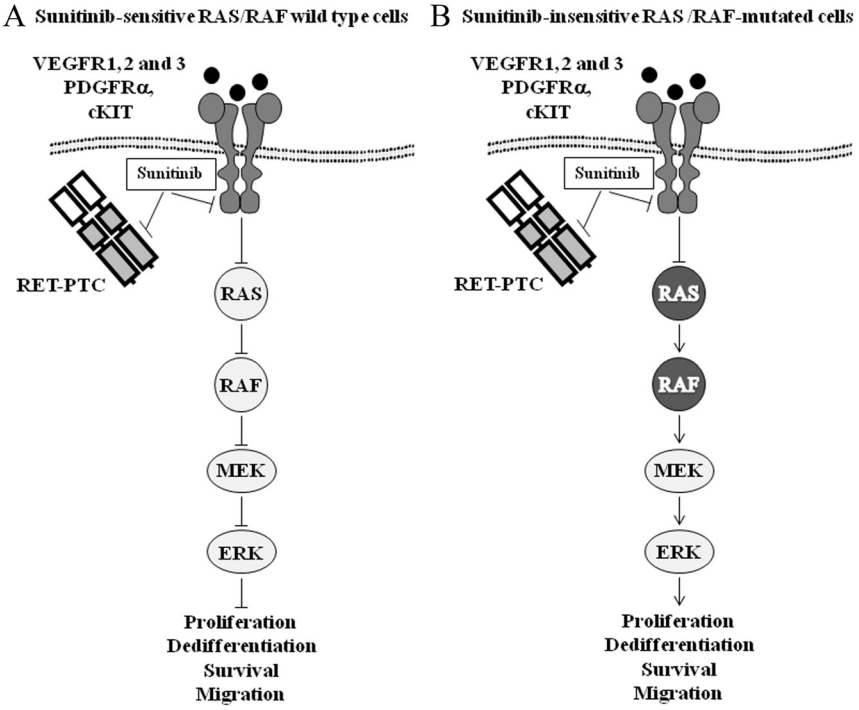

FIG. 6. Molecular pathways inhibited by sunitinb and mechanisms of resistance. Sunitinb is a selective inhibitor of VEGFR1, VEGFR2, VEGFR3, PDGFRα, and cKIT TKs as well as intracellular RET/PTC fusion protein. A, Tumor cells bearing wild-type RAS and RAF oncogenes (*clear circles*) are sensitive to sunitinib because the RAS/RAF/MEK/ERK signaling cascade is silenced upon upstream inhibition of receptor TK by sunitinib. B, By contrast, tumor cells bearing RAS- or RAF-activating mutations (*shaded circles*) are insensitive to sunitinib because the MEK/ERK signaling is constitutively active beside the still effective inhibitory activity of sunitinib on receptor TKs.

lines, with the aim of identifying determinants of resistance/sensitivity to the molecular-targeted agent. The results suggest that sunitinib does the following: 1) is equally active in both RET/PTC and non-RET/PTC cell lines, 2) induces arrest of the cell cycle in the G0-G1 phase with parallel inhibition of ERK signaling, and 3) exerts antiproliferative activity, likely independently of the expression of its molecular targets. Of note, sunitinib showed inefficacy in TC cells bearing activating somatic mutations of *KRAS* or *BRAF* oncogenes, which are known to be responsible for the constitutive activation of ERK signaling. Consistently, the constitutive activation of ERK signaling by the transfection of HRAS R12 or BRAF V600E mutants resulted in the loss of sunitinib's cytostatic activity and in its inability to suppress ERK phosphorylation. Finally, the attenuation of ERK activation, by the pharmacological inhibition of MEK, resulted in the partial resensitization of KRAS- or BRAF-mutated TC cells to sunitinib.

These results highlight the relevance of the inhibition of ERK signaling in the antiproliferative activity of sunitinib in TC cells. Indeed, the RAS/RAF/ERK pathway is a highly conserved system, responsible for transducing proliferative and survival signals from TK receptors to the nuclear transcriptional machinery (23). Thus, our results suggest that the inhibitory activity of sunitinib on multiple TK receptors is responsible for the inactivation of RAS/RAF/ERK signaling, this representing a major mechanism of its direct antitumor effect. Furthermore, the constitutive activation of this signaling pathway downstream of the TK receptors, due to either activating mutations of *KRAS* or *BRAF* oncogenes or the transfection of HRAS or BRAF constitutive active mutants, results in the loss of sunitinib antiproliferative activity (Fig. 6). Although this is, to our knowledge, the first demonstration that oncogenic mutations of *KRAS* or *BRAF* genes are predictive factors of sunitinib inefficacy *in vitro*, similar results have been reported for KRAS-activating mutations and for both the anti-EGFR1 monoclonal antibodies in human colorectal carcinomas (24) and the EGFR1 TK inhibitors in non-small cell lung carcinomas (25).

The second finding highlighted by this study is the observation that the stimulation of thyroid cancer cells with high concentrations of EGF results in loss of sensitivity to sunitinib and in its inability to inhibit ERK phosphorylation. Indeed, it has been previously suggested that, during anti-vascular endothelial growth factor therapy, the up-regulation of alternative growth factors may be responsible for secondary drug resistance. This is due either to the recruitment of alternative angiogenic circuits or to a direct tumor stimulating activity (21). Thus, it is likely that the ligand-dependent activation of TK receptors that are not specific targets of sunitinib may favor the activation of ERK signaling, and this in turn may be responsible for the loss of sunitinib activity, despite its still effective inhibitory activity on target receptors. In such a perspective, EGF signaling is critical for driving survival signals (22) and thus may be relevant for the escape of TC cells from the inhibitory activity of sunitinib. We have recently observed that the activation of EGFR1 signaling may represent one of the mechanisms responsible for the loss of TSH dependency in TC cells and for their transition toward a mesenchymal-like, angiogenic, and drug-resistant phenotype (14, 26, 27). Thus, it is likely that the activation of multiple/alternative TK-dependent pathways, in additon to the TSH receptor signaling, may be responsible for either the progression of thyroid cancer cells toward a TSH-in-

dependent phenotype or the escape from the antitumor activity of chemotherapeutics and TK-specific agents.

These observations may provide useful insights for the development of novel molecular-targeted therapies in human TC. In the last few years, several clinical trials evaluated novel agents for the treatment of radioiodine refractory progressive thyroid cancer (3, 4), all of them with antiangiogenic and antitumoral activity. Because most of these studies demonstrated promising but conflicting results (3, 4), it is likely that a selective approach to the use of these agents, based on the molecular profiling of thyroid tumors, may provide clearer evidence. In such a light, predictive criteria to drive the selection of the appropriate inhibitor are needed in human TC. This issue is even more critical in the light of recent studies demonstrating that mutations of *BRAF* and *KRAS* oncogenes and the RET/PTC translocations are the most common driving events responsible for papillary thyroid carcinogenesis (28, 29). Indeed, the RET/PTC translocation and the BRAF V600E mutation have been demonstrated in, respectively, about 20 and 30–40% of human papillary TC (12), whereas activating mutation of *RAS* gene represents the predominant molecular alteration of poorly differentiated thyroid cancers (30). Furthermore, it has been suggested that papillary TC bearing the BRAF V600E mutation are characterized by a worse prognosis, due to increased nodal metastases, high risk to multicentricity and extrathyroidal invasion, and reduced responsiveness to radioiodine therapy (31). Thus, in the light of this molecular heterogeneity, a differential sensitivity toward agents that inhibit different targets is expected. In such a perspective, based on the observation that sunitinib is active irrespective of the RET/PTC translocation but fails to inhibit TC cells bearing BRAF or KRAS mutations, we suggest that activating mutations of both these oncogenes need to be prospectively evaluated as predictive biomarkers in thyroid cancer clinical trials. In fact, even though KRAS- or BRAF-mutated human TC may still respond to sunitinib due to its antiangiogenic activity, it is intriguing to speculate that the constitutive activation of ERK signaling may result in insensitivity to sunitinib in human TC. Indeed, this hypothesis would be consistent with recent observations suggesting that sunitinib fails to inhibit BRAF-mutated TC cells (32) and would explain the sometimes conflicting results provided by sunitinib in clinical trials (10, 11). Indeed, although sorafenib, which is known to inhibit BRAF, has been suggested to be more effective in BRAF-mutated papillary TC (33), other agents, such as the recently developed BRAF inhibitors (34), may provide interesting clinical activity in BRAF- or KRAS-mutated thyroid cancers. Thus, future studies will aim to answer the question as to whether KRAS or BRAF wild-type tumors are the only subgroup of thyroid cancers responsive to sunitinib therapy, BRAF- or KRAS-mutated TC likely being more responsive to agents that target ERK signaling downstream of the TK receptors.

Acknowledgments

We thank Anthony Green for proofreading the manuscript and suggesting stylistic improvements.

Address all correspondence and requests for reprints to: Dr. Matteo Landriscina, Dipartimento di Scienze Mediche e del Lavoro, Università degli Studi di Foggia, Viale Pinto, 1-71100 Foggia, Italy. E-mail: m.landriscina@unifg.it; or Professor Mauro Cignarelli, Dipartimento di Scienze Mediche e del Lavoro, Università degli Studi di Foggia, Viale Pinto, 1-71100 Foggia, Italy. E-mail: m.cignarelli@unifg.it.

This work was supported by Grant 26/08 from the Lega Italiana Lotta Tumori (to M.C.), Grant IG8780 from the Associazione Italiana per la Ricerca sul Cancro (to M.L.), and Grant PRIN 2008 from the Ministero dell'Istruzione dell'Università e della Ricerca (to M.L.).

Disclosure Summary: No competing financial interests exist.

References

1. **Baudin E, Schlumberger M** 2007 New therapeutic approaches for metastatic thyroid carcinoma. Lancet Oncol 8:148–156
2. **Burns WR, Zeiger MA** 2010 Differentiated thyroid cancer. Semin Oncol 37:557–566
3. **Kapiteijn E, Schneider TC, Morreau H, Gelderblom H, Nortier JW, Smit JW** 2012 New treatment modalities in advanced thyroid cancer. Ann Oncol 23:10–18
4. **Sherman SI** 2010 Targeted therapy of thyroid cancer. Biochem Pharmacol 80:592–601
5. **Fagin JA** 2004 How thyroid tumors start and why it matters: kinase mutants as targets for solid cancer pharmacotherapy. J Endocrinol 183:249–256
6. **Rini BI** 2007 Sunitinib. Expert Opin Pharmacother 8:2359–2369
7. **Lanzi C, Cassinelli G, Nicolini V, Zunino F** 2009 Targeting RET for thyroid cancer therapy. Biochem Pharmacol 77:297–309
8. **Kim DW, Jo YS, Jung HS, Chung HK, Song JH, Park KC, Park SH, Hwang JH, Rha SY, Kweon GR, Lee SJ, Jo KW, Shong M** 2006 An orally administered multitarget tyrosine kinase inhibitor, SU11248, is a novel potent inhibitor of thyroid oncogenic RET/papillary thyroid cancer kinases. J Clin Endocrinol Metab 91:4070–4076
9. **Fenton MS, Marion KM, Salem AK, Hogen R, Naeim F, Hershman JM** 2010 Sunitinib inhibits MEK/ERK and SAPK/JNK pathways and increases sodium/iodide symporter expression in papillary thyroid cancer. Thyroid 20:965–974
10. **Ravaud A, de la Fouchardière C, Asselineau J, Delord JP, Do Cao C, Niccoli P, Rodien P, Klein M, Catargi B** 2010 Efficacy of sunitinib in advanced medullary thyroid carcinoma: intermediate results of phase II THYSU. Oncologist 15:212–213; author reply 214
11. **Carr LL, Mankoff DA, Goulart BH, Eaton KD, Capell PT, Kell EM, Bauman JE, Martins RG** 2010 Phase II study of daily sunitinib in FDG-PET-positive, iodine-refractory differentiated thyroid cancer and metastatic medullary carcinoma of the thyroid with functional imaging correlation. Clin Cancer Res 16:5260–5268
12. **Nikiforov YE** 2008 Thyroid carcinoma: molecular pathways and therapeutic targets. Mod Pathol 21(Suppl 2):S37–S43

13. **Schweppe RE, Klopper JP, Korch C, Pugazhenthi U, Benezra M, Knauf JA, Fagin JA, Marlow LA, Copland JA, Smallridge RC, Haugen BR** 2008 Deoxyribonucleic acid profiling analysis of 40 human thyroid cancer cell lines reveals cross-contamination resulting in cell line redundancy and misidentification. J Clin Endocrinol Metab 93: 4331–4341
14. **Landriscina M, Piscazzi A, Fabiano A, Maddalena F, Costantino E, Farese A, Bufo P, Cignarelli M** 2009 Targeting epidermal growth factor receptor 1 signaling in human thyroid-stimulating hormone-independent thyroid carcinoma FRO cells results in a more chemosensitive and less angiogenic phenotype. Thyroid 19:629–637
15. **Landriscina M, Laudiero G, Maddalena F, Amoroso MR, Piscazzi A, Cozzolino F, Monti M, Garbi C, Fersini A, Pucci P, Esposito F** 2010 Mitochondrial chaperone Trap1 and the calcium binding protein Sorcin interact and protect cells against apoptosis induced by antiblastic agents. Cancer Res 70:6577–6586
16. **Meireles AM, Preto A, Rocha AS, Rebocho AP, Máximo V, Pereira-Castro I, Moreira S, Feijão T, Botelho T, Marques R, Trovisco V, Cirnes L, Alves C, Velho S, Soares P, Sobrinho-Simões M** 2007 Molecular and genotypic characterization of human thyroid follicular cell carcinoma-derived cell lines. Thyroid 17:707–715
17. **Faivre S, Demetri G, Sargent W, Raymond E** 2007 Molecular basis for sunitinib efficacy and future clinical development. Nat Rev Drug Discov 6:734–745
18. **Osada M, Tolkacheva T, Li W, Chan TO, Tsichlis PN, Saez R, Kimmelman AC, Chan AM** 1999 Differential roles of Akt, Rac, and Ral in R-Ras-mediated cellular transformation, adhesion, and survival. Mol Cell Biol 19:6333–6344
19. **Nucera C, Lawler J, Parangi S** 2011 BRAF(V600E) and microenvironment in thyroid cancer: a functional link to drive cancer progression. Cancer Res 71:2417–2422
20. **Cantwell-Dorris ER, O'Leary JJ, Sheils OM** 2011 BRAFV600E: implications for carcinogenesis and molecular therapy. Mol Cancer Ther 10:385–394
21. **Bergers G, Hanahan D** 2008 Modes of resistance to anti-angiogenic therapy. Nat Rev Cancer 8:592–603
22. **Vasko VV, Saji M** 2007 Molecular mechanisms involved in differentiated thyroid cancer invasion and metastasis. Curr Opin Oncol 19:11–17
23. **Chappell WH, Steelman LS, Long JM, Kempf RC, Abrams SL, Franklin RA, Bäsecke J, Stivala F, Donia M, Fagone P, Malaponte G, Mazzarino MC, Nicoletti F, Libra M, Maksimovic-Ivanic D, Mijatovic S, Montalto G, Cervello M, Laidler P, Milella M, Tafuri A, Bonati A, Evangelisti C, Cocco L, Martelli AM, McCubrey JA** 2011 Ras/Raf/MEK/ERK and PI3K/PTEN/Akt/mTOR inhibitors: rationale and importance to inhibiting these pathways in human health. Oncotarget 2:135–164
24. **Wheeler DL, Dunn EF, Harari PM** 2010 Understanding resistance to EGFR inhibitors—impact on future treatment strategies. Nat Rev Clin Oncol 7:493–507
25. **Hammerman PS, Jänne PA, Johnson BE** 2009 Resistance to epidermal growth factor receptor tyrosine kinase inhibitors in non-small cell lung cancer. Clin Cancer Res 15:7502–7509
26. **Landriscina M, Maddalena F, Fabiano A, Piscazzi A, La Macchia O, Cignarelli M** 2010 Erlotinib enhances the proapoptotic activity of cytotoxic agents and synergizes with paclitaxel in poorly differentiated thyroid carcinoma cells. Anticancer Res 30:473–480
27. **Landriscina M, Pannone G, Piscazzi A, Toti P, Fabiano A, Tortorella S, Occhini R, Ambrosi A, Bufo P, Cignarelli M** 2011 EGFR1 expression is upregulated in human undifferentiated thyroid carcinomas. Thyroid 21:1227–1234
28. **Giordano TJ, Kuick R, Thomas DG, Misek DE, Vinco M, Sanders D, Zhu Z, Ciampi R, Roh M, Shedden K, Gauger P, Doherty G, Thompson NW, Hanash S, Koenig RJ, Nikiforov YE** 2005 Molecular classification of papillary thyroid carcinoma: distinct BRAF, RAS, and RET/PTC mutation-specific gene expression profiles discovered by DNA microarray analysis. Oncogene 24:6646–6656
29. **Phay JE, Shah MH** 2010 Targeting RET receptor tyrosine kinase activation in cancer. Clin Cancer Res 16:5936–5941
30. **Volante M, Rapa I, Gandhi M, Bussolati G, Giachino D, Papotti M, Nikiforov YE** 2009 RAS mutations are the predominant molecular alteration in poorly differentiated thyroid carcinomas and bear prognostic impact. J Clin Endocrinol Metab 94:4735–4741
31. **Elisei R, Ugolini C, Viola D, Lupi C, Biagini A, Giannini R, Romei C, Miccoli P, Pinchera A, Basolo F** 2008 BRAF(V600E) mutation and outcome of patients with papillary thyroid carcinoma: a 15-year median follow-up study. J Clin Endocrinol Metab 93:3943–3949
32. **Jeong WJ, Mo JH, Park MW, Choi IJ, An SY, Jeon EH, Ahn SH** 2011 Sunitinib inhibits papillary thyroid carcinoma with RET/PTC rearrangement but not BRAF mutation. Cancer Biol Ther 12:458–465
33. **Takami HE** 2011 Current status of molecularly targeted drugs for the treatment of advanced thyroid cancer. Endocr J 58:151–153
34. **McCubrey JA, Steelman LS, Abrams SL, Chappell WH, Russo S, Ove R, Milella M, Tafuri A, Lunghi P, Bonati A, Stivala F, Nicoletti F, Libra M, Martelli AM, Montalto G, Cervello M** 2009 Emerging Raf inhibitors. Expert Opin Emerg Drugs 14:633–648