Identification of soluble candidate biomarkers of therapeutic response to sunitinib in Medullary Thyroid Carcinoma in preclinical models.

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Statement of translational relevance:

Sunitinib, a multi-tyrosine kinase inhibitor, has been reported as clinically effective in some patients with locally advanced or metastatic Medullary Thyroid Carcinoma (MTC). However, molecular mechanisms of action of sunitinib remain still poorly understood in this disease. Discovery of biomarkers that can predict for early therapeutic response is highly required in order to improve patient care. Here, we investigated molecular mechanisms of action of sunitinib and identified a collection of putative biomarkers predictive of response to sunitinib in MTC using a kinetic microarray approach in preclinical models. These biomarkers include IL-8, TGF-β2, TN-C, and CD44. Furthermore, we demonstrated that metastatic MTC patients presented increased serum levels of IL-8 and TGF-β2. We suggest that these soluble candidate biomarkers may be of value for monitoring sunitinib response in metastatic MTC patients and should be evaluated in clinical trials.
Abstract

Purpose: Medullary thyroid carcinoma (MTC), an aggressive rare tumor due to activating mutations in the proto-oncogene RET, requires new therapeutic strategies. Sunitinib, a potent inhibitor of RET, VEGFR-1, VEGFR-2, VEGFR-3, and PDGFRα/β, has been reported as clinically effective in some patients with advanced MTC. In this study, we examine molecular mechanisms of action of sunitinib and identify candidate soluble biomarkers of response.

Experimental Design: Both in vitro and in vivo assays, using the human TT RET<sup>C634W</sup> MTC cell line, were done to assess the activity of sunitinib. Kinetic microarray studies were used to analyze molecular pathways modified by sunitinib and to identify candidate biomarkers that were subsequently investigated in the serum of patients.

Results: Sunitinib displayed antiproliferative and antiangiogenic activities and inhibited RET auto-phosphorylation and activation of downstream signaling pathways. We showed that sunitinib treatment induced major changes in the expression of genes involved in tissue invasion and metastasis, including VIM, PLAU, TN-C, SPARC, and CD44. Analyzing down-regulated genes, we identified those encoding secreted proteins and, among them, IL-8 was found to be modulated in the serum of xenografted mice under sunitinib treatment. Furthermore, we demonstrated that metastatic MTC patients presented increased serum levels of IL-8 and TGF-β2.

Conclusions: Experimental models confirm the clinical efficacy of sunitinib observed in a few studies. Molecular pathways revealed by genomic signatures underline the impact of sunitinib on tissue invasion. Selected soluble candidate biomarkers could be of value for monitoring sunitinib response in metastatic MTC patients.
Introduction

Medullary thyroid carcinoma (MTC) arises from calcitonin (CT)-producing parafollicular C cells and accounts for 5-8% of all thyroid cancers (1). MTC occurs as either a sporadic form or in a familial context (25% of cases). Activating mutations of the RET proto-oncogene, encoding a tyrosine-kinase receptor (TKR), are responsible for familial forms and are also detected in one-third of sporadic tumors (2). Constitutive activated RET receptor stimulates downstream signaling pathways (PI3K/AKT, Ras/ERK, PLCγ, ...) resulting in C cell growth and differentiation (2, 3).

MTC patients with local disease may be cured after initial surgery, but persistent or recurrent disease occurs in half of cases, and distant metastases are the major cause of tumor related death. Cytotoxic chemotherapy or external radiation therapy are poorly effective and new therapeutic strategies are needed for locally advanced or metastatic MTC patients (4, 5).

The constitutive activation of RET is crucial in MTC pathogenesis and led to the development of small compounds targeting its tyrosine-kinase activity, some of them being currently used in clinical trials (6-9). Among these compounds, sunitinib (SU11248, Sutent®, Pfizer, New York, NY) is a multikinase inhibitor with both antiangiogenic and antitumoral activities, that inhibits VEGFR1, VEGFR2 (KDR), VEGFR3, PDGFRα, PDGFRβ, C-KIT, FLT3, CSF1R, and also displays inhibition of RET in vitro (10, 11). Sunitinib has been effective in some patients with advanced MTC (12-15). Although its effects at the TKR target level are well-documented in GIST and renal cell carcinoma models, the molecular mechanisms of action remain poorly understood in MTC (16, 17). Furthermore, in parallel to the development of molecular targeted therapies, identification of surrogate biomarkers able to early determine those patients most likely to achieve clinical benefits, will be useful for clinical management of such therapies (18, 19).

In the present report, we demonstrate that sunitinib displays antiproliferative and antiangiogenic activities in TT cells, a human RET<sup>C634W</sup> MTC model. Sunitinib inhibits RET auto-phosphorylation and activation of downstream signaling pathways. Furthermore, sunitinib inhibits calcitonin production at both the gene and protein expression levels. Using an original approach based on in vitro kinetic microarray studies (16), we identified during sunitinib treatment major changes in tissue invasion and metastasis gene expression. Among down-regulated genes, some encode secreted proteins that were found to be highly expressed...
in the serum of metastatic MTC patients and, thus may constitute putative biomarkers for sunitinib response.

**Materials and Methods**

**Compounds.** Sunitinib or SU11248, \((N\text{-}[2-\text{(diethylamino)ethyl}]\text{-}5\text{-}[\text{(Z)}\text{-}(5\text{-fluoro-1,2-dihydro-2-oxo-3H-indol-3-ylidine)methyl}]\text{-}2,4\text{-dimethyl-1H-pyrrole-3-carboxamide})\) was synthesized as previously described (20). For *in vitro* experiments sunitinib stock solutions were made in 100% dimethylsulfoxide (DMSO). Equivalent DMSO concentration (0.1%) served as vehicle control. For *in vivo* experiments sunitinib was dissolved in a citrate buffered (pH 3.5) solution.

**Serum from MTC patients and healthy donors.** Serum samples were obtained according to ethical rules from 10 healthy donors and 27 MTC patients with distant metastases who were treated at Institut Gustave Roussy (Supplementary data 1).

**Cell Culture.** The TT cell line (CRL-1803™), derived from a human MTC harboring the RET/C634W mutation, was purchased from ATCC (Manassas, VA). TT cells were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Pasching, Austria), 2 mM L-glutamine (Glutamax®, Invitrogen), 100 units/ml penicillin-streptomycin and 0.25 µg/ml amphotericine (Antibiotic-Antimyototic®, Invitrogen).

**Cell proliferation assay.** TT cells (4000/well) were seeded in 96-well plates and treated with vehicle or sunitinib over 12 days. Culture media were changed every three days. Cell proliferation was quantified by means of a colorimetric assay based on the reduction of the tetrazolium salt 4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate (WST-1®, Roche Applied Science) to formazan, according to manufacturer’s instructions.

**Western blot analysis.** TT cells were treated with vehicle or sunitinib over 6 days. Briefly, TT cells were lysed in a RIPA solution containing 50 mM HEPES (pH 7.5), 1% (vol/vol) Triton X-100, 150 mM NaCl, 5 mM EGTA, 50 mM NaF, 20 mM sodium pyrophosphate, 1 mM sodium vanadate, 2 mM phenylmethylsulfonylfluoride, and 1X protease inhibitor cocktail set III (Calbiochem, Merck Chemicals Ltd., Nottingham, United-Kingdom). Lysates were clarified by centrifugation at 10,000×g for 20 minutes.
Immunoblotting experiments were performed according to standard procedures. Extracted proteins (50 µg/lane) were separated by 4-12% SDS-PAGE (Invitrogen) and subjected to immunoblots onto nitrocellulose membrane (Protran BA 83, 0.2µm, Schleicher & Schuell). A rabbit polyclonal anti-RET antibody was purchased from Santa Cruz Biotechnology (sc-167, 1:500) (Santa Cruz, CA). Rabbit polyclonal antibodies directed against phospho-RET (3221, 1:750), anti-p44/42 MAPK (9102, 1:1000), phospho-p44/42 MAPK (9106, 1:1000), AKT (4691, 1:1000) and phospho-AKT (9271, 1:750) were purchased from Cell Signaling (Beverly, MA). Anti-GAPD (1:1250) was a mouse polyclonal antibody from Millipore (Billerica, MA). Blots were incubated with primary antibodies overnight at +4°C. Immune complexes were detected with the West Dura Signal kit (Pierce Biotechnology, Rockford, IL).

TT cells xenografts in Nude Mice. All animal experiments were carried out according to the French Laws for animal welfare under the supervision of authorized investigators. TT cells (6.10⁶) in 200 µl of serum-free medium containing 50% Matrigel Matrix® (BD Biosciences, Bedford, MA) were injected s.c. into the flank of 18 six-week-old athymic nu/nu female mice. Tumor volume was measured using an Aplio® ultrasound scanner (Toshiba, France), equipped with a 14 MHz-probe (PZT, PLT-1204AT, Toshiba). Two weeks after cell injection, tumors reached a mean volume of 247 mm³ (± 62) and animals were randomized into 2 groups of 9 mice each: mice then received (per os, once daily (5d/7)) either sunitinib (50 mg/kg/day) or vehicle (citrate buffered - pH 3.5 solution) during 6 weeks. Tumor volume and body weights were monitored at weekly intervals.

Immunohistochemistry. Xenografts were formalin-fixed and paraffin-embedded for preparation of 4 µm sections used for histological examination after standard Hematoxylin and Eosin (H&E) staining or immunohistochemistry labeling with Ki-67 (SP6, 1:150, Neomarkers), and CD34 (MEC 14.7, 1:50, Tebu-bio) antibodies. Microvessel density was quantified by counting the number of CD34-positive cells in three representative areas of 0.3 mm² (x 200). The percentage of Ki-67-positive cells was estimated in one representative area (x 630). The percentages of necrosis and fibrosis were evaluated on the whole section of all the xenografts (x 25) and a mean score was reached. Negative controls were performed by omitting the primary antibody. Pictures were acquired on a Leica DM 2000 video camera.

Biomarker analysis. TT cell culture media were collected simultaneously to cell lysates, as described above. Mice blood samples were collected by orbital puncture (day -3 and day 18) or by cardiac puncture (day 38) from TT cells xenografted mice treated with sunitinib or placebo as described above. Human calcitonin (Biomerica, Newport Beach, CA),
IL-8, TGF-β2 (R&D Systems, Minneapolis, MN), CD44 (Tebu-bio, Le-Perray-en-Yvelines, France) and TN-C (IBL International, Hamburg, Germany) levels were quantified by ELISA methods according to manufacturers’ instructions in TT cell culture media and/or xenografted mice sera and/or MTC patients’ sera and/or healthy donors’ sera. Immunoenzymatic assay of human Carcino Embryonic Antigen (CEA) was performed on the UniCel™ DxI 800 Access® device (BeckmanCoulter, Brea, CA, USA). All measurements were done in duplicate.

**RNA extraction and purification.** Frozen xenografts and cell samples were lysed in Trizol® Reagent (Invitrogen), total RNAs were isolated according to manufacturer recommendations then purified using the RNeasy microkit® (QIAGEN, Courtaboeuf, France). RNA quality (28S/18S ratio > 1.5) was assessed using the RNA 6000 Nano Lab-On-Chip® technology (Agilent Technologies, Palo Alto, CA).

**Pan genomic expression profile analysis during sunitinib treatment.** *In vitro* gene expression was analyzed by microarray experiments as previously described (21). Briefly, each experiment was performed three times. Samples analyzed were RNA pools of TT cells treated with 250 nM of sunitinib during 1, 3 or 6 days. References used were RNA pools of TT cells treated with vehicle at each time point. Reverse transcription, linear amplification, cRNA labeling, and purification were performed using the Agilent Linear amplification kit and labeling with Cyanine 3 (Cy3)-CTP or cyanine 5 (Cy5)-CTP (Agilent Technologies) (22). Dye-swap competitive hybridization procedure was performed with 1 µg of labeled sample’ cRNAs against 1µg of labeled time-matched references on 44K Whole Human Genome Oligo Microarray (Agilent Technologies) then scanned with an Agilent Technologies Scanner. Image analyses (quantification, normalization) were performed with Feature Extraction software (Agilent Technologies) and gene expression analysis was performed using Resolver software (Rosetta Inpharmatics, Kirkland, WA). Differential expressed genes compared to each time point matched references were retained for *Fold Change* over 1.75 and *p value* < 10⁻³ as previously published (16). Molecular and cellular functions of genes were investigated using the Ingenuity® software and considered as most regulated according to lowest *p value*. Top regulated genes using the Ingenuity® software refer to gene with highest fold change. All raw microarray data are available on Array Express at the European Bioinformatics Institute (http://www.ebi.ac.uk/arrayexpress ; accession number E-TABM-929).

**Real-time quantitative PCR (Q-PCR).** One microgram of total RNA from each sample was reverse transcribed by superscript II reverse transcriptase (Invitrogen) in the...
presence of random primers (Applied Biosystems). Q-PCR was performed on an equivalent amount of 12.5 ng total RNA per tube in a final volume of 25µL. Oligonucleotide primers and TaqMan probes were designed using the PrimerExpress computer software (Applied Biosystems) and purchased from MWG Biotech (Courtaboeuf, France) (G2M, GAPD, PPIA, TBP, CT, VIM and CD44) or obtained from Assays-On-demand (Applied Biosystems) (TN-C, SPARC, IL-8, PLAU, STMN4, SCEL, TGF-β2 and FST). The expression values were normalized using housekeeping genes (G2M, GAPD, PPIA, TBP) as previously described (21, 22).

**Small interfering RNA Cell transfection.** TT cells were transfected using the Nucleofector® transfection kit (Amaxa, Cologne, Germany), RET siRNA (Dharmacon, Lafayette, CO, USA), and non targeted Stealth siRNA used as control (Invitrogen), as previously published (21).

**Statistical analysis.** Data are reported as means ± SE or median. Statistical significance (p value<0.05) was evaluated by paired Student's t or Mann-Whitney’s w tests. Results were subjected to statistical analysis by using the GraphPad Prism software package, ver. 4.02 (Graph-Pad, San Diego, CA).

**Results**

**Sunitinib inhibits proliferation and angiogenesis in MTC models.** The effect of sunitinib on MTC was first evaluated in vitro on TT cells cultured over 12 days and treated with either sunitinib (50, 200, 350 and 500 nM) or vehicle as control. The cell proliferation was assessed by WST-1 assay. A significant antiproliferative effect occurred after 3 days of treatment for concentrations above 200 nM (p<0.05) and a complete growth arrest was obtained with 500 nM (p<0.001) (Fig. 1A). We then tested the effects of sunitinib on RET phosphorylation and signaling by western-blotting experiments on TT cell lysates. A dose dependent decrease of RET, p44/42 and AKT phosphorylation was observed after 4 hours of sunitinib treatment (Fig. 1B).

Then, sunitinib effect was explored in vivo on nude mice bearing TT cells xenografts which were treated once daily with either sunitinib (50 mg/kg/day) or placebo during 38 days. Treatment did not affect the body weight gain profile and no apparent toxicity was observed. Sunitinib significantly reduced tumor growth as soon as 11 days after initiation of treatment.
(p<0.001) (Fig. 2A) and after 38 days of treatment, a 70% decrease in tumor volume was observed in the sunitinib group. After sacrifice at day 38, sunitinib treated tumors displayed a reduced number of tumor cells (-30%), a decreased proliferation of tumor cells (-43%) determined using Ki67 immunostaining and also an increased necrosis (x 1.25) and fibrosis (x 2.2) (Fig. 2B).

Then, the antiangiogenic properties of sunitinib were investigated in vivo in the MTC model. After 38 days of treatment, vascularization of the skin and of the xenograft surfaces were macroscopically less developed in sunitinib-treated animals. At a microscopic level, sunitinib treatment reduced the number of vessels (-43%) (5 ±1 vs. 9 ±2), as shown by CD34 immunostaining (Fig. 2C). This effect may explain the increased necrosis observed during sunitinib treatment.

**Sunitinib treatment induces major changes in tissue invasion and metastasis processes.** To identify biological pathways modified during sunitinib treatment with antiproliferative effects, we analyzed gene expression changes in TT cells treated with 250 nM of sunitinib for 1, 3 and 6 days. After one day of treatment, 1107 transcripts were differentially expressed, including 419 that were up-regulated and 688 that were down-regulated; at day 3, 292 transcripts (69 up-regulated / 223 down-regulated) and at day 6, 313 transcripts (95 up-regulated / 218 down-regulated) were modulated. Together these results showed that the number of genes modified in their expression was more important after a short exposure to sunitinib and, that a higher number of genes were down-regulated than up-regulated. At day one, about half of the genes were related to cell division and proliferation, in particular those encoding transcription factors (Supplementary data 2 & 3). Finally, 269 genes were shared for at least two time points and 115 genes for all 3 time points.

Analysis of molecular and cellular functions of genes, whose expression was modified during sunitinib treatment, was performed using Ingenuity® software. At any time of treatment, the major modified function, as defined in material and methods, was cellular growth and proliferation. After a one day-sunitinib treatment, genes involved in cell cycle and DNA replication, recombination and repair functions were changed in their expression. At day-3 and -6, modified gene functions were similar and included cell-to-cell signaling and interaction, cell death and cellular movement (Supplementary data 4).

Regarding biological functions, top regulated genes, in terms of fold change levels, mainly referred to tissue invasion and metastasis capabilities of tumor cells. Among these,
several genes were up-regulated during sunitinib treatment, including CDH4, FGF7, and MMP26 (Supplementary data 5). Most genes were down-regulated and encoded proteins that are mainly involved in the extracellular matrix composition and remodeling (TN-C, PLAU, PAI-1, LAMA4, LAMB3, SPARC), cytoskeleton and cell mobility (JUB, VIM, STMN4, LUM, SCEL) and cell-to-cell interactions (CD44, PCDH7, CDH6) (Table 1).

Gene expression profiling identifies potential soluble biomarkers of sunitinib response. We then focused transcriptome analysis on downregulated genes, looking for potential biomarkers of sunitinib response. To confirm microarray observations, real-time quantitative PCR (Q-PCR) was carried out for 9 selected genes, among those mostly downregulated during sunitinib treatment. In in vitro sunitinib-treated TT cells, comparable results were observed between microarray and Q-PCR experiments (Table 2). Moreover five of these nine genes (TN-C, PLAU, SCEL, FST, IL-8) displayed similar changes in expression during sunitinib treatment, both in vitro and in vivo (Table 2). Interestingly TN-C, PLAU, FST and IL-8 encode secreted proteins that may constitute soluble biomarkers of sunitinib response.

In this context, we also investigated sunitinib effect on calcitonin (CT) production, a seminal protein biomarker routinely used for follow-up of MTC patients. We first examined calcitonin gene and protein expressions using Q-PCR and ELISA assays, respectively, in both in vitro and in vivo models. In vitro, CT gene expression was inhibited up to 40% after a 6-day treatment with 250 nM of sunitinib (Fig. 3A left). This observation is in agreement with the dramatic decrease of CT concentration in culture medium that remained significant when normalized for total protein amounts (Fig. 3A right). During sunitinib treatment, CT decrease is thus likely to be due to both the decrease in the number of TT cells and the inhibition of CT expression in viable tumor cells. Similar results were observed in vivo, with a reduction (-40%) of CT gene expression in xenografts after a 38-day sunitinib treatment (Fig. 3B left). Moreover, serum CT concentration decreases up to 80% during sunitinib treatment as compared to that in control animals (Fig. 3B right). No linear relationship was found between serum CT values and xenograft volumes. Indeed, after 38 days of treatment, both placebo and sunitinib treated xenografts displayed necrosis, and tumor volume is not strictly representative of the number of viable tumor cells. No changes were observed both on Carcino Embryonic Antigen (CEA) gene and protein expressions (data not shown).
We then investigated the effects of sunitinib on IL-8, one of the putative biomarkers identified during the gene expression profiling analysis. As determined by ELISA, sunitinib strongly reduced IL-8 secretion in TT cell culture media, up to 50% after 6 days of sunitinib treatment (250 nM) (Fig. 3C left). Moreover, in vivo, IL-8 serum level was dramatically decreased after 38 days of sunitinib treatment (Fig. 3C right). To assess the link between RET inhibition and IL-8 gene expression, siRNA RET transfection was performed on the TT cell line. Non targeted siRNA, used as control, did not reduced RET and IL-8 gene expression levels as analyzed by Q-PCR (data not shown). After siRNA RET transfection, the expression of RET mRNA was strongly reduced as of 24h of exposure. In the same experimental conditions, IL-8 gene expression was decreased in a time-dependent manner, from 17% at 24h to 88% at 72h post-transfection. IL-8 half life was calculated to be about 43h (Fig. 3D).

Finally, we examined the presence of several identified potential biomarkers, including IL-8 and TGF-β2, in the serum of 27 MTC patients with metastatic disease and ten healthy donors. Basal levels in the healthy donor population were in accordance with already published data. In MTC patients, significantly higher serum concentrations of IL-8 (w<0.001) and TGF-β2 (w<0.05) were observed. In addition, serum concentrations of TN-C, and CD44 also appeared to be slightly higher in MTC patients but with a large range of variations (Fig. 4).

Discussion

Our results demonstrate that sunitinib has antiproliferative, antitumoral and antiangiogenic properties in in vitro as well as in vivo MTC models based on TT cell line. In vitro, sunitinib strongly reduces proliferation of TT cells in a dose and time-dependent manner. This observation is in line with previous results showing a growth-inhibitory effect of sunitinib on a thyroid carcinoma cell line (TPC-1) harboring a RET/PTC rearrangement (11). Moreover, doses used are in agreement with already published preclinical data showing effectiveness of sunitinib in different cancer types (23, 24). At the molecular level, RET kinase inactivation was associated with an inhibition of AKT and p44/42 pathways in TT cells. These findings are consistent with previous data involving PI3K/AKT and Ras/MAPK as major downstream RET signaling pathways. Indeed, other signaling cascades that have
been involved in MTC, such as PLC-γ, STAT3, NFκB, p38MAPK, c-Jun N-terminal kinase should also be explored in this model (2, 3, 14, 15).

A strong antitumor effect was also observed in vivo, at a well-tolerated dose of sunitinib (50mg/kg/d), as substantiated by the inhibition of TT cell xenografted tumor growth and the decrease of Ki67 immunostaining. Other multikinase inhibitors, such as vandetanib or sorafenib, have shown similar antitumor activity in this model (25-29). Interestingly, tumor volume was not reduced by sunitinib but tumor growth was limited. Moreover, sunitinib increased fibrosis in the xenografts stroma, indicating that it acts not only on tumor cells but also on their environment, and also decreased angiogenesis, as shown by the significant reduction in microvessel density. This effect is likely to be due to the potent inhibition of angiogenesis TKR, including KDR, PDGFRα and PDGFRβ. Recent evidence suggest that sunitinib inhibits renal cancer growth primarily through an antiangiogenic mechanism rather than through direct targeting of tumor cells (30). Indeed, the potential inhibition of additional TKR and their contribution to the therapeutic efficacy of sunitinib cannot be ruled out. Furthermore, in sporadic RET wild-type MTC, other molecular events are thought to contribute to malignant transformation and to the metastatic process (6, 21). These data may explain the clinical efficacy of sunitinib in sporadic advanced MTC patients without RET mutation (12).

Serial time microarray analysis allowed identifying changes in gene expression induced by sunitinib treatment that were confirmed by Q-PCR. Interestingly, sunitinib treatment induced major changes on the expression of genes with putative function in tissue invasion and metastasis suggesting a potential key role of the encoded proteins in MTC malignancy (31, 32). Thereby, urokinase plasminogen (PLAU) and endogenous protease inhibitor (PAI-1) that promote cancer metastasis were found to be inhibited by sunitinib (33). In addition, differential effects were observed on members of the cadherin and metalloprotease family genes. Expression of cadherins in MTC is poorly documented (28) and sunitinib treatment induced up- and down-regulation of CDH4 or R-cadherin and CDH6, respectively. R-cadherin acts as a critical regulator of the normal phenotype and its loss contributes to epithelial suppression and metastatic progression (34). Aberrant expression of cadherin-6 is associated with a poor prognosis. Interestingly, CDH11, that is expressed in the follicular histotype of differentiated thyroid carcinomas, has been reported to be modulated by sunitinib but does not appear to be a target in MTC (16). During sunitinib treatment, metalloprotease gene expression was up-, MMP26, or down-regulated, MMP16. The precise
role of these MMP as well as their differential regulation by sunitinib remains to be investigated. Finally, sunitinib also decreased vimentin, a key regulator of epithelial-mesenchymal transition whose expression is associated to invasiveness and metastasis (35) and CD44 that likely contributes to tumor progression (36).

Discovery of novel biomarkers of targeted therapies remains challenging for identifying patients likely to benefit from treatment (37). In this study, we showed that calcitonin (CT) gene expression, the current seminal biomarker of MTC, was inhibited by sunitinib in vitro and in vivo and consequently its protein secretion was decreased. This effect appears to be dissociated from that on tumor burden, as observed with other TKR inhibitors (25). Thus, serum CT may not indicate changes in tumor burden in MTC patients treated with TKR inhibitors and new early predictive biomarkers of therapeutic response would be of value.

Using gene expression profiling (16), we identified genes that were down-regulated by sunitinib, and that encoded secreted proteins, and we selected interleukin-8 (IL-8), tenascin-C (TN-C), TGF-β2, and CD44 as candidate biomarkers. IL-8, a cytokine with pro-angiogenic and mitogenic effects, was selected among genes displaying major changes. Its production is known to be regulated by the RET receptor (38), and the inhibitory effect observed during sunitinib exposure is likely to be due, at least partly, to the inhibition of RET phosphorylation, as shown in western-blotting and SiRNA experiments. Decreased tumor secretion of IL-8 during sunitinib treatment, observed both in vitro and in vivo, has been described in other carcinomas and can be due to other mechanisms, including inhibition of VEGF regulatory loops (39). Furthermore, recent results indicated that IL-8 is highly expressed in some carcinomas (40) and could mediate sunitinib resistance in renal carcinoma (41). This observation is in line with our clinical results showing significantly higher concentrations of IL-8 in the serum of metastatic MTC patients than in healthy donors. Therefore, IL-8 can be considered both as a potential therapeutic target and clinical biomarker for follow-up.

Transforming growth factor beta 2 (TGF-β2) was also found to be increased in the serum of metastatic MTC patients and down-regulated after sunitinib treatment in our preclinical models. TGF-β2 controls cell growth and differentiation, may promote angiogenesis, wound healing and also acts as a factor for remodeling the extracellular matrix (42). Interestingly, elevated serum levels of TGF-β2 have also been described in various cancer types and it has been considered as a potential biomarker of cancer (43, 44). TN-C, an extracellular matrix glycoprotein involved in tissue interactions, was also one of the most
down-regulated genes both \textit{in vitro} and \textit{in vivo}. TN-C has been reported to inhibit cell adhesion to fibronectin and to promote cell migration, tumor proliferation and metastasis (45, 46). A recent study showed that, if not expressed in normal tissue, TN-C is detectable in both stroma and tumor cells from human MTC samples, suggesting that it may be an early indicator of MTC carcinogenesis (47). Our results also indicate that sunitinib may control stroma remodelling induced by TN-C.

Together, our observations demonstrated that sunitinib displays both antiproliferative and antiangiogenic properties on MTC models bearing a RET$^{C634W}$ activating mutation through direct inhibition of tumor cells and tumoral environment. Whether these effects are also observed for other RET mutations and/or resistance mutants remain to be investigated. Furthermore, mechanisms of action underlying its efficacy reveal major changes in tissue invasion and metastasis processes. Among modified genes, some encoded potential surrogate biomarkers of sunitinib response that would deserve further investigation. Finally, further clinical investigations of IL-8 and other proteins identified as a single or a panel of biomarkers of sunitinib response is warranted.
FIGURE LEGENDS

**Figure 1.** Sunitinib displays an antiproliferative activity. A) Sunitinib blocks proliferation of TT cells in a dose- and time-dependent manner. TT cells were treated with vehicle or sunitinib over 12 days. Cell growth was measured by WST-1 assay. Bars, ±SD. Unpaired t Test (*p<0.05, **p<0.01, ***p<0.001). B) Sunitinib inhibits RET, AKT and p44/42 activation in TT cells in a dose-dependent manner. TT cells were treated with vehicle or sunitinib for 4 hours. Lysates were immunoblotted with anti-RET, anti-phospho-RET/Y905 (P-RET), anti p44/42, anti-phospho-p44/42 (P-p44/42), anti-AKT, anti-phospho-AKT (P-AKT), anti-GAPD as described in Methods.

**Figure 2.** Sunitinib inhibits TT cells tumor growth and angiogenesis in nude mice. TT xenografted nude mice (9/group) received sunitinib (50 mg/kg/day) or vehicle for 5 days/week for 6 weeks by oral gavages (day 1 is the treatment starting day). A) Tumors diameters were determined by ultrasonographic measures. Data represent the mean of tumor volume. Bars, ±SD. Unpaired t Test (***p<0.001). Mouse weights were comparable. B) After 38 days, tumors were examined by conventional H&E (left & middle) and Ki67 immunostaining (right). Sunitinib reduced the number of tumor cells (-30%), increased necrosis (x1.25) and fibrosis (x 2.2) and decreased tumor cells proliferation (-43%). C) Sunitinib treatment reduced macro-vascularisation of xenografts and the number of vessels by 43% after 38 days as shown by CD34 immunostaining. Arrows indicate CD34-positive cells (vessel elements). Representative micrographs are shown.

**Figure 3.** Sunitinib inhibits biomarkers expression. TT cells were treated with vehicle or sunitinib (250 nM). TT xenografted nude mice received sunitinib (50 mg/kg/day) or vehicle for 5 days/week for 6 weeks. Biomarkers levels were assessed by ELISA. Gene expression was determined by Q-PCR. A) In vitro calcitonin (CT) gene (left) and protein (right) expression. B) In vivo CT gene (left) and protein (right) expression. Bars, median. C) In vitro (left) and in vivo (right) Interleukin-8 (IL-8) protein expression. D) Levels of RET and IL-8 gene expression in TT cells siRNA RET transfected as compared to TT cells transfected with a non targeted siRNA used as control.
**Figure 4.** Serum levels of potential biomarkers of sunitinib response in MTC patients (n=27) and healthy donors (control group) (n=10). Interleukin-8 (IL-8), Tenascin-C (TN-C), TGF-beta 2 and CD44 levels were assessed by ELISA. *Bars*, median. *w*, Mann-Whitney test.

**Table 1.** Summary of top down-regulated genes modulated by sunitinib in TT cells obtained by microarray technology as described in Methods. *p* value <0.05. *ND*: non determined.

**Table 2.** Gene expression of invasiveness markers modulated by sunitinib treatment (Quantitative PCR).
Bibliography


Figure 1_Broutin.
Figure 2_Brutin.
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**Table1_Broutin**
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**Table 2_Broutin**
Figure 3_Broutin.
Figure 4_Broutin.
Identification of soluble candidate biomarkers of therapeutic response to sunitinib in Medullary Thyroid Carcinoma in preclinical models.

Sophie Broutin, Nabout Ameur, Ludovic Lacroix, et al.

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