In vitro transforming potential, intracellular signaling properties, and sensitivity to a kinase inhibitor (sorafenib) of RET proto-oncogene variants Glu511Lys, Ser649Leu, and Arg886Trp

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Abstract

Multiple endocrine neoplasia type 2 and a subset of apparently sporadic medullary thyroid carcinoma (AS-MTC) are caused by germ line activating point mutations of the rearranged during transfection (RET) proto-oncogene. RET encodes a receptor with tyrosine kinase activity that targets several intracellular signaling cascades, such as RAS–RAF–ERK1/2, PIK3–AKT, and STAT transcription factors. The objective of this study was to assess the function of three germ line RET variants Arg886Trp, Ser649Leu, and Glu511Lys of undetermined pathogenic significance, which were found in three kindreds of isolated AS-MTC. For this purpose, we employed vectors expressing each of the RET variants and measured the number of NIH3T3 transformation foci and soft agar colonies, the degree of activation of known RET intracellular signaling targets (ERK1/2, STAT1, STAT3, and TCF4), and the extent of ERK1/2 inhibition on sorafenib treatment. We found that RET variants Arg886Trp and Glu511Lys have shown increased in vitro transforming potential in a glial-derived neurotrophic factor-dependent manner. In contrast, the Ser649Leu variant did not significantly increased the number of foci and agar colonies relative to wild-type RET (RET-WT). The variants Glu511Lys and Arg886Trp showed 10- and 12.5-fold ERK1/2 activation respectively, that was significantly higher than that observed for RET-WT (fivefold). Increased levels of STAT1 and TCF4 activation were only observed for RETArg886Trp (2.5- and 3-fold versus 1.2- and 2-fold in RET-WT respectively). The three RET variants analyzed here were sensitive to treatment with sorafenib. In conclusion, our results allow to classify previously uncharacterized RET genotypes, which may be of use to define follow-up and therapeutic regimens.

Endocrine-Related Cancer (2011) 18 401–412

Introduction

Inherited germ line activating mutations of the rearranged during transfection (RET) proto-oncogene (Donis-Keller et al. 1993, Mulligan et al. 1993) underlie the predisposition to develop the autosomal dominantly inherited multiple endocrine neoplasia type 2 cancer syndromes (MEN2A, MEN2B, and familial medullary thyroid carcinoma (FMTC; Eng et al. 1996,
Kouvaraki et al. 2005, Raue & Frank-Raue 2007)).

*RET* encodes a membrane receptor tyrosine kinase composed of four extracellular cadherin-like motifs and a cysteine-rich region, a transmembrane portion, and an intracellular domain with tyrosine kinase activity (Takahashi & Cooper 1987, Takahashi et al. 1988) that signals through a ligand/co-receptor/RET complex. To date, RET ligands of the glial-derived neurotrophic factor (GDNF) family, which include GDNF, ARTN, NRTN, and PSPN, and a family of GPI-linked RET co-receptors (GFR1–4) have been identified (Airaksinen et al. 1999). The formation of ligand/co-receptor and RET complexes results in RET dimerization and triggers autophosphorylation at intracellular tyrosine residues. Tyrosine phosphorylation of intracellular target proteins activates several downstream pathways, which include ERK1/2 mitogen-activated protein kinase, phosphatidylinositol 3-kinase, c-Jun N-terminal kinase, p38, STAT3 and STAT1, ERK5, and cAMP-responsive element-binding protein (Airighi et al. 2005). Recently, also the WNT pathway was shown to be activated by RET, through RET-mediated direct tyrosine phosphorylation of β-catenin (Gujral et al. 2008). In MTC and MEN2, *RET* mutations occur in a specific spectrum of codons and result in gain of function, increased kinase activity, and receptor activation (Asai et al. 1995, Borrello et al. 1995, Santoro et al. 1995). Mutational hotspots are located in the cysteine-rich region of the extracellular domain and in the intracellular tyrosine kinase domain (Eng et al. 1996, Kouvaraki et al. 2005, Raue & Frank-Raue 2007). A comprehensive description of all known germ line *RET* variations can be found at the MEN2 RET database (http://www.arup.utah.edu/database/MEN2/MEN2_welcome). In clinical terms, three disease phenotypes can be recognized: MEN2A, MEN2B, and a FMTC. MEN2A is found to be associated with substitutions at one of six specific cysteine residues in exons 10 (609, 611, 618, and 620) and 11 (630 and 634). MEN2A cysteine mutations result in the development of MTC, along with variable expression of pheochromocytoma (50%) and hyperparathyroidism (15–30%) (Eng et al. 1996, Kouvaraki et al. 2005, Raue & Frank-Raue 2007). On the other hand, MEN2B is mainly caused by a specific missense mutation located in the RET tyrosine kinase domain (Met918Thr), which accounts for 95% of cases (Carlson et al. 1994, Eng et al. 1994, Hofstra et al. 1994). A second tyrosine kinase domain substitution (Ala883Phe) has been detected in a small proportion of MEN2B patients (Gimm et al. 1997, Smith et al. 1997). In addition, double mutations affecting codons 804 and 805 and 804 and 806 were described in individual MEN2B cases (Miyaura et al. 1999, Cranston et al. 2006). MEN2B kinase domain mutations give rise to a more complex clinical phenotype characterized by the early onset of a very aggressive form of MTC, concomitant with pheochromocytoma in 50% of cases, and accompanied by other non-neoplastic features (Morrison & Nevin 1996). In FMTC, the only disease manifestation is MTC. *RET* mutations with low clinical expression, involving codons 321, 533, 768, 790, 791, 804, and 891, are usually found in these families (Eng et al. 1996, Hofstra et al. 1997, Berndt et al. 1998, Fattoruso et al. 1998, Pigny et al. 1999, Antinolo et al. 2002, Da Silva et al. 2003, Dvorakova et al. 2005, Kouvaraki et al. 2005, Raue & Frank-Raue 2007, Castellone et al. 2009). Occasionally, patients with these mutations may also develop the MEN2A phenotype (Berndt et al. 1998, Jimenez et al. 2004, Bethanis et al. 2007, Pinna et al. 2007). In addition, up to 10% of patients presenting with apparently sporadic MTC (AS-MTC) reveal occult *RET* germ line mutations (Prazeres et al. 2006). With generalized *RET* screening now being performed, rare mutations of undetermined significance are found in isolated families. These variants require further functional studies to ascertain their pathogenic potential and to define follow-up strategies accordingly. Furthermore, the finding of various compounds capable of inhibiting oncogenic RET (mutated or rearranged), such as PP1 and PP2 (Carломagno et al. 2002b), ZD6474 (Vandetanib) (Carломagno et al. 2002a), RPI1 (Lanzi et al. 2000), CEP-701 and CEP-751 (Strock et al. 2003), Imatinib (Cohen et al. 2002), Sunitinib (SU5416 and SU11248) (Kim et al. 2006), Gefitinib (Croyle et al. 2008), Sorafenib (BAY 43-9006) (Carломagno et al. 2006), Motesanib (AMG706) (Sherman et al. 2008), Axitinib (AG013736) (Cohen et al. 2008), and XL 184, has brought further clinical relevance to the classification of the pharmacological sensitivity of RET mutants, as metastatic MTC is the most common cause of death in patients with MEN2 (Skinner et al. 2005).

In this study, we report the in vitro transforming potential, intracellular signaling properties, and kinase inhibitor sensitivity of three distinct *RET* variants located in the extracellular domain (Glu511Lys), the transmembrane region (Ser649Leu), and the tyrosine kinase domain (Arg886Trp).

### Materials and methods

#### Subjects

We have previously reported the *RET* mutation spectrum in a study of MEN2 and AS-MTC patients...
originating from the Central Region of Portugal (Prazeres et al. 2006). The current study was specifically focused in three kindreds, in whom the index case has been diagnosed with MTC and, in which genetic variants of undetermined significance have been found. These variants consist of changes Glu511Lys, Ser649Leu, and Arg886Trp (first reported in our previous study (Prazeres et al. 2006), although functional analysis was not performed at the time).

**Population controls**

A population of 100 regional controls, consisting of a random series of patients admitted to our institution for conditions unrelated to endocrine pathology, was used to determine whether these variants could be considered as polymorphisms (allele frequency > 1%).

**RET germ line mutation screening**

RET mutation screening was performed in DNA obtained from peripheral blood leucocytes, by PCR amplification and direct Sanger sequencing of exons 8, 10, 11, and 13–16.

**Site-directed mutagenesis**

A pRcCMV vector expressing RET isoform 51 (i51) was mutated to generate the Glu511Lys, Ser649Leu, and Arg886Trp variants. We have also employed the Cys634Arg, a MEN2A mutation, and Met918Thr, a MEN2B mutation, as controls. Site-directed mutagenesis was performed by a PCR approach employing primers with mismatches for the intended nucleotide change. All plasmids were re-sequenced to confirm that the desired mutation was introduced without changes to the vector backbone (Supplementary Figure 1, see section on supplementary data given at the end of this article).

**Cell lines**

HEK293 and NIH3T3 cell lines were maintained in DMEM medium supplemented with 10% FBS, 100 µg/ml streptomycin, and 100 U/ml penicillin (PENST) in a humified atmosphere with 5% CO₂ at 37 °C. For transient transfections, NIH3T3 cell was transfected using the Lipofectamine Reagent (Invitrogen) according to manufacturer’s instructions. HEK293 cells were transfected by the calcium phosphate method.

**NIH3T3 focus formation assays**

For focus formation assays, NIH3T3 cells were transfected with 1 µg expression vector in 6-well plates. After 3 days of transfection, 1 × 10⁵ cells were plated in 100 mm plates and grown in DMEM, 5% FBS, and 1% PENST. In parallel, the same number of cells was put in selection medium (DMEM, 5% FBS, 1% PENST, and 400 µg/µl G418). Medium was renewed every 3 days and cells were allowed to grow for a total time of 3 weeks. After this period, plates were stained with crystal violet and foci were counted. Transfection efficiency was assessed by counting the number of stable clones arising in the selection plates. Results were expressed as the number of foci normalized to the corresponding transfection efficiency.

**Soft agar assays**

For soft agar assays, we prepared plates containing a bottom layer of 0.7% agar in DMEM, 10% FBS, and 1% PENST. NIH3T3 cells were transfected with 1 µg expression vector, and after 1 day, 5000 cells were diluted in 2 × DMEM, 20% FBS, and 2% PENSTP, mixed with equal volume of 1% agar at 40 °C, and plated on the agar plates. All the mutations were analyzed in parallel and for the same period of time, allowing a minimum of 2 weeks for the colonies to grow. After this period wells were stained with crystal violet and colonies were counted. For each well, ten low-power fields were photographed and images were counted using the colony-counting tool of the Quantity One software (Bio-Rad).

**Western-blot analysis**

Cells were lysed for 5 min at 4 °C using RIPA buffer (1% NP-40 in 150 mM NaCl, 50 mM Tris (pH 7.5), and 2 mM EDTA), containing phosphatase and protease inhibitors. Proteins were quantified using a modified Bradford assay (Bio-Rad). Protein samples (25 mg) were separated in 8, 10, or 12% SDS/PAGE gels depending on the molecule to be analyzed and electroblotted to Hybond ECL membrane (Amersham Biosciences). The primary antibodies used were purchased from the following sources: anti-phospho-p44/42 MAPK (Thr202/Tyr204) and anti-p44/42 MAPK total were from Cell Signaling Technology Inc (Danvers, MA, USA) and anti-RET C-20 (isoforms 51) and anti-actin were from Santa Cruz Biotechnology. Secondary antibodies (goat anti-rabbit and goat anti-mouse) were conjugated with peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and visualized by the ECL detection system (Amersham). Quantization of the specific signal was performed by Quantity One software from Bio-Rad.
Luciferase reporter assays

We employed reporter constructs containing the firefly luciferase encoding gene under the control of DNA motifs recognized by STAT1 (GAS-LUC), STAT3 (interleukin response element-LUC), and TCF4 (LEF/TCF response element-LUC; Supplementary Figure 2A, see section on supplementary data given at the end of this article). We further employed a system that reports the activation of ERK1/2 in trans (Supplementary Figure 2B, see section on supplementary data given at the end of this article). In reporter assays, DNA mixtures consist of 500 ng expression plasmid, 1 µg reporter plasmid, 1 µg PDM2-LacZ (expressing β-galactosidase), and pUC18 to complete 5 µg total DNA. Cells were incubated with calcium phosphate–DNA complexes for 24 h and cultured in fresh medium for additional 24 h. Cells were harvested, lysed in 150 µl Reporter Lysis Buffer, and assays for luciferase and β-galactosidase were performed. Lysates (15 µl) were mixed with 50 µl SteadyLite HTS substrate and luciferase activity was measured using luminometer. For the β-galactosidase assay, 50 µl lysate were mixed with 250 µl β-galactosidase buffer (200 mM NaPO₄, 2 mM MgCl₂, 1.33 mg/ml ortho-nitrophenyl-β-D-galactopyranoside, and 100 mM β-mercaptoethanol) and measured in a colorimetric reaction at 405 nm. The luciferase activity was normalized with the corresponding β-galactosidase activity (measures transfection efficiency). All experiments were performed at least two times in triplicates.

Sensitivity to sorafenib

To assess the effect of sorafenib in different RET mutants, we have evaluated the degree of suppression of ERK1/2 in the presence of sorafenib. After 24 h of transfection of the ERK1/2 reporter vectors (Supplementary Figure 2B, see section on supplementary data given at the end of this article), cells were incubated with fresh medium containing a final concentration of 4 µM sorafenib for additional 48 h, after which β-galactosidase and luciferase activity were measured. Mutants Cys634Arg and Met918Thr were used as controls.

In addition, we have ascertained the effect of sorafenib in RET-mediated transforming activity. For this purpose, we have performed soft agar assays in the same procedure as mentioned above, with the exception that sorafenib, to a final concentration of 4 µM, was added to the top agar layer containing the cells transfected with different RET mutants.

Results

As the starting point of this work, we first asked whether RET variants Glu511Lys, Ser649Leu, and Arg886Trp occurred amongst individuals of the general population, so as to exclude the possibility that these constituted RET polymorphisms. For this purpose, we screened RET exons 8, 11, and 15 in a group comprising 100 regional controls (200 alleles) by means of single-strand conformation polymorphism analysis. We only found wild-type alleles in the control group, making it highly unlikely that these RET variants constitute polymorphisms.

Then, we examined whether co-segregation between carrier status and disease phenotype would be observed in these families. For this purpose, we performed clinical examination, biochemical measurements of calcitonin (CALC), vanillyl mandelic acid (VMA), urinary metanephrines (MET), and parathormone (PTH), as well as RET genetic analysis of the first-degree relatives of mutation carriers in these kindreds. Family members were available for testing only in Family A (Arg886Trp) and Family B (Glu511Lys). The results of clinical, biochemical, and carrier status are summarized in Fig. 1. In Family A, one of the two sons of the index, currently aged 27 years, was shown to have inherited the Arg886Trp mutation (Fig. 1A). However, clinically, this sibling had normal basal and pentagastrin-induced CALC levels and VMA, MET, and PTH levels were also within normal ranges (Fig. 1A). In Family B, we were able to study four offspring of the index case, three of which have shown to harbor the RET Glu511Lys variant. Clinically, no signs of MTC were found and all siblings showed normal values of CALC, VMA, MET, and PTH (Fig. 1B).

Because co-segregation studies were not conclusive, we performed functional in vitro analyses to better address the transforming potential of the uncharacterized RET variants. RET displays the classical features of an oncogene, and RET activating mutations induce transformation of NIH3T3 fibroblasts, leading to the formation of foci. We generated vectors expressing RET mutants (Supplementary Figure 1, see section on supplementary data given at the end of this article) and we evaluated the ability of these RET variants to transform NIH3T3 fibroblasts, relative to wild-type RET (RET-WT) and to known RET activating mutations (Cys634Arg), by performing NIH3T3 focus formation assays. By counting the number of foci induced by different RET variants, we found that RET Glu511Lys (in the presence of GDNF) and Arg886Trp showed increased transforming potential in vitro. This was demonstrated by increased number of foci relative
We ascertain whether intracellular signaling pathways known to be targeted by RET were increasingly activated as a result of transient expression of the different RET variants. By performing western blot analysis employing a phosphor-specific ERK1/2 antibody, we could observe that all mutants induced higher ERK1/2 phosphorylation relative to RET-WT, although this occurred in different extents, among the distinct RET genotypes (Fig. 3A and B). Variants Cys634Arg (a known MEN2A mutation), Arg886Trp, Glu511Lys, and Leu649Ser showed decreasing levels of ERK1/2 phosphorylation (Fig. 3A and B). To further characterize RET signaling properties, we employed luciferase pathway reporting assays (Supplementary Figure 2A and B, see section on supplementary data given at the end of this article). We found that variants Glu511Lys and Arg886Trp showed a 10- and 12.5-fold ERK1/2 activation, which is higher than RET-WT (fivefold), yet lower than RET Cys634Arg (Fig. 3C). These results were in accordance with previous western-blot analysis. Increased levels of STAT3, STAT1, and TCF4 activation were only observed for RET Arg886Trp (2.0-, 2.5-, and 3-fold versus 1.3-, 1.2-, and 2-fold in RET-WT respectively; Fig. 3D–F). Overall, distinct RET variants seemed to display different signaling properties. We have further performed a hierarchical clustering according to the signaling profile of the distinct RET genotypes (Fig. 3G). This classification showed that variant Ser649Leu clustered close together with RET WT, indicating a similar signaling behavior (Fig. 3G). Variant Glu511Lys grouped in a different subcluster because it displayed a higher signaling activity relative to RET-WT, which nonetheless is moderate (Fig. 3G). Variant Arg886Trp induced a higher degree of activation and clustered closer to Cys634Arg (a mutation known to be highly activating; Fig. 3G).

In addition, we have performed experiments designed to evaluate the sensitivity of the RET genotypes to treatment with the kinase inhibitor sorafenib. Although sorafenib (BAY 43-9006) was originally designed as a RAF inhibitor (Lyons et al. 2001), preclinical studies have shown that sorafenib could inhibit the kinase activity and signaling of wild-type and oncogenic RET (Carlomagno et al. 2006, Gupta-Abramson et al. 2008, Henderson et al. 2008). Because ERK1/2 was the most prominently induced pathway by the RET mutants (as we have observed in previous experiments), we used the ERK1/2 reporting system to address the degree of inhibition of RET intracellular signaling activity on treatment with 4 μM sorafenib for 48 h. Treatment with sorafenib suppressed ERK1/2 activation to different extents among
**H Prazeres et al.: Signaling by RET E511K, S649L, and R886W**

**Figure 2** Evaluation of the transforming capacity of RET variants in NIH3T3 focus formation assays and anchorage-independent soft agar assays. Representative photographs of plates obtained after focus formation experiments performed in the absence (A) or in the presence of 10 ng/ml GDNF (B). (C) The transformation capacity is expressed by the number of transformation foci generated by each RET mutant plasmid, normalized to the transfection efficiency estimated by counting the number of stable clones obtained under antibiotic selection conditions. Direct comparisons were made to wild-type RET under the corresponding condition (with or without GDNF) and significance of the observed differences is expressed by the $P$ value calculated by the Student’s $t$-test (on top of each bar). Significance, $^*P<0.05$, $^{**}P<0.01$; NS, not significant. The inset shows photographs of NIH3T3 focus formed as a result of RET-induced transformation. (D) Transforming activity of RET mutants was further addressed by soft agar assays with and without addition of GDNF. The graph presents values obtained from counting 10 low-power fields for each RET mutant. Again, direct comparisons were made to wild-type RET under the corresponding condition (with or without GDNF) and significance of the observed differences is expressed by the $P$ value calculated by the Student’s $t$-test (on top of each bar). The inset shows photographs of NIH3T3 soft agar colonies formed as a result of RET-induced transformation. Full colour version of this figure available via [http://dx.doi.org/10.1530/ERC-10-0258](http://dx.doi.org/10.1530/ERC-10-0258).

RET genotypes, nonetheless all three variants under study have shown some degree of response (Fig. 4A). In addition, we have ascertained the effect of sorafenib on RET transforming activity, by performing soft agar assays, with or without the addition of 4 μM sorafenib to the top agar layer containing transfected cells. We found that the transforming activity mediated by the distinct genotypes was inhibited by sorafenib, because a reduction of RET-transformed colonies was observable (Fig. 4B).

**Discussion**

With the generalization of RET screening, the finding of rare uncharacterized variants is increasingly common. This demands for functional studies to ascertain pathogenicity, given that the number of cases (and the size of the families) is usually not sufficient to establish co-segregation, age-dependent penetrance, and genotype–phenotype associations on the basis of clinical presentation alone. We have performed co-segregation studies and in vitro functional analyses to help ascertain the pathogenic potential of RET germ line variants Glu511Lys, Ser649Leu, and Arg886Trp that were found in kindreds presenting with isolated MTC. The index cases in these kindreds have been screened biochemically for pheochromocytoma and hyperparathyroidism (HPT) and values have remained in the normal range, suggesting an MTC-only phenotype. For a number of reasons, co-segregation studies were of limited value in the clarification of the pathogenicity of these variants in the kindreds that we have studied. On the one hand, in one of the families, siblings were not willing to be examined (as was the case for the Ser649Leu variant). On the other hand, even when siblings were found to have inherited the RET variant, they did not display clinical or biochemical symptoms of MTC (as was the case for Families A and B). In Family A, one can argue that the carrier was relatively young (27 at this time) to have developed hereditary MTC, leaving the question unresolved as to MTC will not present at older age. It is known from studies of families with MEN2A and FMTC that mutation carriers with normal CALC values can develop raised CALC levels later in life, with demonstration of C-cell hyperplasia when thyroidectomy is performed. In Family B, despite the three carrier siblings were older (between 49 and 56 years old), presumable low penetrance of the mutation may also explain lack of clinical manifestation at these ages.

In terms of their in vitro transformation ability, variants Glu511Lys and Arg886Trp induced significantly increased numbers of NIH3T3 transformation foci relative to RET-WT, suggesting that they somehow cause RET activation. However, the transformation efficiency of Glu511Lys and Arg886Trp is lower than that of the Cys634Arg mutation, suggesting that
moderate transformation potential was achieved by these variants. Variant Ser649Leu, however, displayed very little or no gains in transforming potential in comparison with RET-WT (Fig. 2C and D). These findings are consistent with other studies that have previously addressed and concordantly showed that these variants have a relatively modest transforming activity (Colombo-Benkmann et al. 2008, Muzza et al. 2010).

A noteworthy observation was that despite transformation induced by variant Glu511Lys was modest, it was highly augmented by GDNF treatment (Fig. 2C and D). This is in clear contrast with the behavior of another extracellular mutation Cys634Arg, which nonetheless was unresponsive to GDNF (Fig. 2C and D). These differences in GDNF dependence of the transforming potential may well reflect the existence of two distinct mechanisms of receptor activation associated with two different sorts of extracellular domain mutations. On the one hand, the most commonly detected extracellular mutations replacing the cysteine residue at codons 609, 611, 618, 620, 630, and 634, cause the loss of an intramolecular disulfide bond and, as a consequence, one cysteine residue becomes available for the formation of an intermolecular disulfide bond. This generates covalently bound receptors that are constitutively active because of receptor dimerization irrespective of ligand binding (Asai et al. 1995, Borrello et al. 1995, Santoro et al. 1995), leading to the experimental observation of GDNF-independent transformation potential. On the other hand, non-cysteine mutations, such as the variant Glu511Lys herein analyzed along with variants detected by others (Pigny et al. 1999, Dvorakova et al. 2005, Castellone et al. 2009), displays a GDNF-dependent transforming capacity. This suggests that these non-cysteine extracellular mutations mediate receptor activation through a different mechanism, presumably by interfering with structural motifs affecting ligand-binding properties of the receptor. Indeed, Glu511Lys involves change of a neutral amino acid with a positively charged basic residue, which may have strong structural implications for receptor-binding properties, as explored in a recent report addressing the transforming ability of this variant, that nevertheless did not employ GDNF treatment (Muzza et al. 2010).

The intracellular RET mutations such as the MEN2B-specific Met918Thr and other tyrosine kinase domain mutations affect receptor activation in a totally different way. By altering the conformation of the catalytic core of the tyrosine kinase domain, they result...
in increased catalytic activity and alter substrate specificity (Borrello et al. 1995, Santoro et al. 1995, Bocciardi et al. 1997). We suspect that RET Arg886Trp works in similar ways, because this change occurs in the catalytic core of the tyrosine kinase domain, in close proximity with the highly conserved region between tyrosine kinase subdomains VI and VII, which are believed to determine substrate specificity and ATP binding at the substrate-binding pocket (Hanks et al. 1988). This effect would be predicted due to the substitution of a positively charged amino acid with tryptophan, a large, polar, and hydrophobic aromatic residue, with the potential to sterically interfere with the structure of nearby regions. Experimentally, we have demonstrated that the Arg886Trp variant displays very different signaling properties, with increased activation of STAT1 and TCF4 pathways, relative to other RET variants and to RET-WT (Fig. 3). It is also of interest to emphasize the observation that RET-WT, Cys634Arg, and Arg886Trp variants were competent in activating the β-catenin/TCF4 pathway, providing confirmation of the observations first made by Gujral et al. (2008) that this pathway contributes to RET-mediated transformation. The overall differences in profile and degree of activation of intracellular signaling pathways further highlight that distinct RET mutants display somehow different signaling properties (Fig. 3E). This information may be important for the design of genotype-adjusted combinatorial therapies employing drugs targeting the most relevant pathways activated by distinct genotypes. A recent, rapidly evolving field is the use of kinase inhibitors to target specific oncogenes activated in thyroid cancer with small molecules that sterically block the ATP-binding pocket and result in impaired phosphorylation activity, inhibition of signal transduction, and reduction in the activation of intracellular signaling pathways relevant to tumor growth and angiogenesis (Couto et al. 2009). Most oncogenic MEN2-associated RET kinase mutants are highly susceptible to inhibitors such as PP1, PP2, and ZD6474 (now known as Vandetanib/Zactima) and phosphorylation of RET mutants at codons 768, 790, 883, 918, and 634 was shown to be inhibited by these compounds (Carlomagno et al. 2002b). In contrast, MEN2-associated swap of valine 804 for bulky hydrophobic leucine or methionine in the RET kinase domain causes primary resistance to the three compounds and valine 804 seems to be a structural determinant amino acid mediating resistance to pyrazolopyrimidines and 4-anilinoquinazolines (Carlomagno et al. 2004, Knowles et al. 2006). In contrast, sorafenib (BAY 43-9006) is efficient in inhibiting the growth of cells carrying RET Val804Leu or RET Val804Met with an IC50 around 100–150 nM, both mutants that are resistant to PP1, PP2, and Vandetanib (Carlomagno et al. 2006, Plaza-Menacho et al. 2007). Given the accumulating evidence that response to treatment with kinase inhibitors will be genotype dependent, it is increasingly important to classify RET genotypes in this respect. In this respect, our results show that all three RET genotypes focused in this study are sensitive to sorafenib inhibition in vitro, because an inhibition of ERK1/2 activation

**Figure 4** Sensitivity of RET genotypes to sorafenib. (A) To check for sorafenib-mediated suppression of ERK1/2 associated with each RET genotype, plasmids encoding variants Glu511Lys, Ser649Leu, and Arg886Trp, as well as wild-type RET, Cys634Arg, and Met918Thr (used as controls), were co-transfected along with ERK1/2 reporters after which cells were put either in medium with 4 µM sorafenib or in medium without sorafenib. Luminescence units were defined relative to the values obtained in wells in which cells were transfected with reporter vectors and the empty vector (pRcCMV). Comparisons were made between treated and non-treated conditions within each genotype. Statistical significance, determined by the Student’s t-test, *P<0.05, **P<0.001; NS, not significant. (B) The effect of sorafenib in the transforming activity of RET mutants was assessed by soft agar assays with and without addition of 4 µM sorafenib. The graph presents values obtained from counting 10 low-power fields for each RET mutant. Direct comparisons were made to wild-type RET under the corresponding condition (with or without sorafenib) and significance of the observed differences is expressed by the P value calculated by the Student’s t-test (on top of each bar).
and a reduction of RET-mediated transforming activity in soft agar assays could be observed. This may allow us to predict that patients found to harbor these genotypes will most probably respond favorably to sorafenib treatment. Curiously, no significant effect on ERK1/2 activation was observed in RET634Arg mutants. We believe this was not observed to the same extent as in other mutations because a very strong activation of this pathway is induced by this specific mutation, which counteracts the inhibitory effect of sorafenib, resulting only in a slight net reduction of ERK1/2 activation. However, a significant reduction in soft agar growth of cells transfected with this mutation was observed on sorafenib treatment.

There is generally a good correlation between functional data obtained in these experimental models and clinical response (Carlomagno et al. 2004, 2006, Knowles et al. 2006, Plaza-Menacho et al. 2007).

In terms of prophylactic therapy, age-dependent penetrance for MTC is codon specific and classification of the risk of developing MTC can be done on the basis of the RET genotype to define the most appropriate timing for prophylactic thyroidectomy (reviewed by Raue & Frank-Raue 2007)). Very high-risk, level 3 mutations are the most aggressive MEN2B-associated specific changes in codons 918 and 883, and in these cases, thyroidectomy should be performed under the age of three. High-risk, level 2 mutations affecting codons 609, 611, 618, 620, 630, and 634 are found in MEN2A patients and thyroidectomy is advised before the age of 5 years. For low-risk, level 1 mutation, comprising codons 609, 768, 790, 791, 804, and 891, thyroidectomy can be postponed until adult age and ideal timing can be decided on an individual basis (Brandi et al. 2001, Niccoli-Sire et al. 2003, Szinzai et al. 2003, Gimm et al. 2004, Kouvarakis et al. 2005, Frank-Raue et al. 2006). In terms of the follow-up strategies for carriers of the studied variants herein, our functional analyses demonstrate that Ser649Leu, Glu511Lys, and Arg886Trp were transforming in vitro and induced increased signaling activity relative to RET-WT. However, these variants display a lower transforming potential when compared with the Cys634Arg high-risk/level 2 mutation, which was assessed in parallel. Adding to the apparently incomplete penetrance in clinical presentation of the kindreds, our recommendations would be to follow up patients harboring Glu511Lys, Leu649Ser, and Arg886Trp in the same approach used for a level 1 mutation. Monitoring CALC levels (basal and stimulated) should be done on a regular basis and prophylactic thyroidectomy should be performed in adult life, when CALC levels rise.

In conclusion, this research provides insight into the function of rare and poorly characterized RET variants associated with isolated MTC. They attribute a relatively indolent nature to the Ser649Leu genotype and depict the extracellular Glu511Lys as a GDNF-dependent RET activating variant. The experimental data presented herein supports that the intracellular tyrosine kinase Arg886Trp variant activates the receptor by changing its intracellular signaling properties. Finally, we report all three variants to be sensitive to sorafenib inhibition in the in vitro setting used in our experiments. This information may be of value for the design of follow-up strategies and/or therapeutic regimens for patients.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-10-0258.

Declaration of interest
The authors declare that they have no proprietary, financial, professional, or other personal interest of any nature and kind in any product, service, and/or company that could be construed as influencing the position presented in this manuscript.

Funding
We would like to acknowledge funding from grants from the Portuguese Foundation for Science and Technology (SFRH/BD/30041/2006 and PTDC/SAU-OBD/101242/2008), the Portuguese Society of Endocrinology and Metabolism (Edward Limber Prize), and the Portuguese Ministry of Health (13/2007). IPATIMUP is an associated Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by the Portuguese Foundation for Science and Technology.

Author contribution statement
H Prazeres performed the experimental work, analyzed data, and wrote the manuscript; J P Couto gave technical support on western blot analyses and luciferase experiments; F Rodrigues performed clinical evaluation of patients; J Vinagre and V Trovisco gave technical support in site-directed mutagenesis; J Torres gave technical support in focus formation and soft agar assays; T C Martins and M Sobrinho-Simões revised the manuscript; and P Soares supervised the work and revised the manuscript.

Acknowledgements
We acknowledge Dr Plamen Naidenov (Endocrinology Service of the Portuguese Institute of Oncology of Coimbra, FG, EPE) and Dr Francisco Carrilho (Endocrinology Service of the University Hospital of Coimbra) for referring some of the patients involved in this study. We thank Dr Patricia
Oliveira for her help in the clustering analysis of signaling properties of RET variants.

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Received in final form 8 February 2011
Accepted 4 May 2011
Made available online as an Accepted Preprint 5 May 2011