Four novel RET germline variants in exons 8 and 11 display an oncogenic potential in vitro

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(M Muzza and D Cordella contributed equally to this work)

Abstract

Context: Most germline-activating mutations of the RET proto-oncogene associated with inherited medullary thyroid cancer (MTC) are localized in exons 10, 11 and 13–15. Four novel RET variants, located in the extracellular domain (p.A510V, p.E511K and p.C531R) coded by exon 8 and in the intracellular juxtamembrane region (p.K666N) coded by exon 11, were identified on the leukocyte DNA from apparently sporadic cases.

Methods: Plasmids carrying Ret9-wild-type (Ret9-WT), Ret9-C634R and all Ret9 variants were transfected, and the phosphorylation levels of RET and ERK were evaluated by western blot analyses. The transforming potentials were assessed by the focus formation assay.

Results: The p.A510V, p.E511K and p.C531R variants were found to generate RET and ERK phosphorylation levels and to have a transforming activity higher than that of Ret9-WT variant, but lower than that of Ret9-C634R variant. Differently, the p.K666N variant, located immediately downstream of the transmembrane domain, and involving a conserved residue, displayed high kinase and transforming activities. Computational analysis predicted non-conservative alterations in the mutant proteins consistent with putative modifications of the receptor conformation.

Conclusions: The molecular analyses revealed an oncogenic potential for all the novel germline RET variants. Therefore, the prevalence of exon 8 genomic variations with an oncogenic potential may be higher than previously thought, and the analysis of this exon should be considered after the exclusion of mutations in the classical hotspots. In addition, on the basis of these functional data, it is advisable to extend the genetic screening to all the first-degree relatives of the MTC patients, and to perform a strict follow-up of familial carriers.

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Introduction

The RET proto-oncogene (OMIM#164771) is constituted by 21 exons, and it encodes a tyrosine kinase receptor involved in growth and differentiation of neural crest-derived tissues (1). The Ret receptor consists in an extracellular region, where cadherin-like repeats and a cysteine-rich domain reside, of a transmembrane domain and two intracellular tyrosine kinase domains. Ret endogenous ligands, belonging to the glial-derived neurotrophic factor, induce the receptor dimerization, which results in the autophosphorylation of the intracellular tyrosines and activation of the downstream pathway.

Gain-of-function germline mutations of the RET gene are responsible for most MEN2A, MEN2B and familial medullary thyroid carcinoma (FMTC) syndromes, whereas somatic mutations including missense changes, insertions and deletions in both the extracellular and intracellular parts of the receptor (2) have been identified in about 50% of sporadic medullary thyroid cancers (MTCs) (3, 4). It is worth noting that 5–10% of apparently sporadic MTCs harbour a germline RET mutation, indicating that the disease is indeed hereditary (5). Most MEN2A (OMIM*171400) cases are associated with mutations in one of the six extracellular conserved cysteines located at codons 609, 611, 618, 620, 630 and 634, with these substitutions inducing a ligand-independent Ret dimerization by the formation of covalent disulphide-bonded homodimers (6). On the other hand, a single point mutation in exon 16, leading to the substitution of the methionine at codon 918 with a threonine, is responsible for most MEN2B (OMIM*162300) cases. The p.M918T mutation has

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been shown to alter the substrate specificity of the Ret intracellular kinase domain and to increase its enzymatic activity (7). FMTC (OMIM*155240) is associated with mutations either in the extracellular or in the intracellular domain of RET, mostly affecting exons 10, 11, 13, 14 and 15 and never involving exon 16. Mutations in exons 5 and 8 have been reported respectively in one Czech family (8) and in five families originating from Brazil (9), Italy (10) and Greece (11–13). Nevertheless, the genetic analysis of these exons is routinely performed only in few centres, consistent with the very recent ATA guidelines for the management of MTCs (14), which include among the relevant exons of RET to be screened only exons 10, 11, 13, 14, 15 and 16.

Among 360 MTC index cases routinely submitted to RET genetic analysis at the Institute Gustave Roussy, Villejuif (France) between 1993 and 2008, 61 were found to harbour germline variations: 53 were deleterious mutations (1, codon 611; 5, codon 618; 4, codon 620; 15, codon 634; 1, codon 649; 3, codon 768; 1, codon 781; 4, codon 790; 9, codon 804; 2, codon 891; 7, codon 918; 1, codon 982) and 4 were novel missense variants (located at codons 510, 511, 531 and 666), while 3 were silent ones (located at codons 513, 696 and 699). The four MTC carriers of novel missense RET variants were apparently sporadic; therefore, functional studies have been performed in order to test their oncogenic potential.

Materials and methods

Patients

The four patients were referred to surgery based on the presence of at least one thyroid nodule associated with elevated basal and/or stimulated calcitonin (Ct) levels. The presence of hyperparathyroidism or pheochromocytoma was excluded preoperatively by appropriate testing (urinary catecholamines, serum ionized calcium and PTH). All patients were submitted to total thyroidectomy and central neck dissection, and the histological examination revealed the presence of MTC with lymph node metastases in patients 1 and 2 (Table 1). Patients were followed up annually with clinical examination, basal/stimulated Ct determination, and urinary catecholamines, serum ionized calcium and PTH measurements. The clinical investigations did not reveal any history of MTC or thyroid tumours in the four families. The carrier status could not be tested in any of the first-degree relatives.

Genomic variants analyses

An informed consent for DNA analysis was obtained for all screened subjects. DNA was extracted from peripheral leukocytes by standard methods. PCR amplifications of exons 8, 10, 11, 13, 14, 15 and 16 of the RET gene were performed using appropriate primers flanking each exon, and PCR products were directly sequenced.

Functional studies

Construction of the RET mutants

Plasmids carrying Ret9-wild-type (Ret9-WT; RefSeq NM_020630, the short isoform of protoRet gene) and Ret9-C634R (the short isoform of protoRet gene containing a MEN2A-causing mutation) were kindly donated by Dr Bongarzone, and have been described in detail previously (15). For each genomic variant, site-directed mutagenesis was performed on Ret9-WT construct using an in vitro oligonucleotide mutagenesis system (QuikChange XL site-directed mutagenesis; Stratagene, La Jolla, CA, USA). The mutant clones were identified by direct sequence. The full-length cDNAs carrying the variants were entirely sequenced and cloned into a eukaryotic expression vector, pRC-CMV (Invitrogen). Plasmid DNA was extracted using the MAXI PREP kit (Qiagen) as suggested by the supplier.

Cell cultures and transfection

Transient transfection experiments were performed in human HEK 293T cells. One million cells were seeded into 60-mm plates and were grown overnight in DMEM containing 10% FCS.

Table 1  Clinical features of RET mutated patients (RefSeq NM_020630).

<table>
<thead>
<tr>
<th>Number/ gender</th>
<th>RET nt mutation</th>
<th>RET AA mutation</th>
<th>Age at diagnosis (years)</th>
<th>Ct pre (pg/ml)</th>
<th>Ct post (pg/ml)</th>
<th>CEA pre (ng/ml)</th>
<th>CEA post (ng/ml)</th>
<th>pTNM</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M 1529C&gt;T A510V</td>
<td>55</td>
<td>1100</td>
<td>600</td>
<td>NA</td>
<td>NA</td>
<td>T3N1bM0</td>
<td>Ten years after surgery: Cc = 302 pg/ml; CEA = 65 pg/ml; no radiological evidences of disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2/M 1531G&gt;A E511K</td>
<td>66</td>
<td>NA</td>
<td>29</td>
<td>31</td>
<td>8</td>
<td>T2N1bM1</td>
<td>Lung metastases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3/F 1591T&gt;C C531R</td>
<td>53</td>
<td>1680</td>
<td>&lt;3</td>
<td>14</td>
<td>1</td>
<td>T2N0M0</td>
<td>Remission</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4/F 1998G&gt;T K666N</td>
<td>65</td>
<td>797</td>
<td>&lt;4</td>
<td>47</td>
<td>1</td>
<td>T2N0M0</td>
<td>Remission</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Cc, calcitonin; CEA, carcinoembryonic antigen; NA, not available.
5000 units/ml penicillin and 5 mg/ml streptomycin at 37 °C with 5% CO₂. The mutant plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Twenty-four hours after transfection, the medium was replaced with fresh medium containing 1% FCS, and the cells were grown for 24 h.

**RET and ERK phosphorylation analyses** The biological effect of the Ret9 variants was tested by comparing their Ret and ERK phosphorylation levels with those of Ret9-WT variant, and with the construct Ret9-C634R which was used as a positive control. All experiments were repeated in triplicate as reported already (16). Briefly, for Ret analysis, total proteins were immunoprecipitated with 0.5 μg of a short isoform-specific rabbit polyclonal anti-Ret (C-19) antibody sc-167 (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Immunoprecipitates were submitted to western blot (WB) analysis, and the filter reacted with 1:2000 anti-phosphotyrosine MAB (antiphosphotyrosine, clone 4G10; Upstate Biotechnology, Inc., Lake Placid, NY, USA) or 1:1000 anti-Ret antiserum. Immunoreactive bands were visualized by ECL (Amersham).

For ERK phosphorylation analysis, lysates from HEK 293T cells transfected with all constructs were submitted to WB analysis and probed with 1:1000 anti-phospho ERK1/2 (α-P-ERK1/2) or anti-ERK1/2 antibodies (zERK1/2; Cell Signaling Technology, Inc., Danvers, MA, USA).

**In vitro transformation assays** NIH3T3 cells (2×10⁵) were seeded into 100-mm plastic dishes, transfected with 1 μg of plasmid DNA for each expression construct and grown in DMEM containing 10% Colorado Serum (Denver, Colorado, USA). 5000 units/ml penicillin and 5 mg/ml streptomycin at 37 °C with 5% CO₂. Three days after transfection, the growth medium was replaced with DMEM/5% CS. After 3 weeks, cells were fixed in methanol, and foci were counted after Giemsa staining. The in vitro transforming activity of RET mutants was tested by comparison with Ret9-WT, while the Ret9-C634R was used as a positive control. The assay was performed in triplicate for each construct, and the mean number of foci obtained with each construct was calculated.

**Structure prediction and modelling**

The PredictProtein server was used to assess the effect of the genomic variants on the predicted secondary structure of Ret protein (www.predictprotein.org/). META-PP provides a single-page interface to various online web services for sequence analysis, homologue retrieval and prediction of protein structure.

**Results**

**Patients**

Clinical features of the four patients are reported in Table 1. Three months after surgery, basal C1 levels were normal in patients 3 and 4, and they remained elevated in patients 1 and 2. Patient 1 was submitted to a further lymph node dissection and to external radiotherapy to the neck 1 year later. After 10 years of follow-up, basal C1 and carcinoembryonic antigen levels remained elevated, though imaging modalities revealed no abnormal findings. Patient 2 was submitted to lymph node dissection 1, 3 and 4 years after surgery, and to external radiotherapy to the neck. At 9 years of follow-up, the patient had documented lung metastases. Finally, patients 3 and 4 are in remission after 3 years and 1 year of follow-up. No MEN2A features, such as hyperparathyroidism or pheochromocytoma, were found in any patient during the follow-up.

**Genomic variants analyses**

Initial analysis of RET gene revealed a novel variant (c.1998G>T, p.K666N) in exon 11, corresponding to the intracellular portion of RET, eight residues downstream of the transmembrane domain in one case. In contrast, the analysis of exons 10, 11 and 13–15 was completely normal in the remaining three cases. More recently, the genetic analysis was extended to other RET exons, thus leading to the identification of novel germline variants. In particular, these three variants were located in exon 8 (c.1529C>T, p.A510V; c.1531G>A, p.E511K and c.1591T>C, p.C531R), within the extracellular cysteine-rich domain of the receptor. All these four variants have not been reported previously, and were not found in a series of 400 normal control alleles.

Differently from the cysteines commonly found to be mutated in hereditary MTC (i.e. those lying in exons 10 and 11), which are fully conserved among species, the presently discussed C531 and the already described C515 (both mapping in exon 8) are not fully conserved, being absent in fugu and zebrafish RET sequences (17). On the contrary, the K666 residue, together with the three amino acids downstream, displayed a full evolutionary conservation (Fig. 1).

**Functional studies**

To determine whether these DNA variants are able to convert RET into a transforming oncogene, HEK 293T cells were transiently transfected with expression vectors for the WT human Ret (Ret9-WT), for the mutant variants (Ret9-A510V, -E511K, -C531R and -K666N) and for the positive control Ret9-C634R. Thereafter, the activation status of the mutants was evaluated by measuring tyrosine autophosphorylation levels in Ret immunoprecipitates and ERK phosphorylation in lysates.
As shown in Fig. 2, all mutant proteins were more tyrosine phosphorylated than Ret9-WT, confirming that mutant receptors can be activated even in the absence of the ligand. The amount of phosphorylation found for p.A510V, p.E511K and p.C531R variants was significantly lower than that obtained with Ret9-C634R, while Ret9-K666N mutant displayed a high kinase activity.

In all cases, ERK phosphorylation resulted to be higher than that of the Ret9-WT and lower than that of Ret9-C634R, with Ret9-K666N being the variant with the highest kinase activity.

The transforming activity of RET mutants has been further assessed by transfecting the constructs into NIH3T3 cells using a well-known activating mutation (Ret9-C634R) as a positive control. The cells transfected with new mutant vectors displayed a morphology that was similar to that of those transfected with the MEN2A (C634R) mutation and different from that of the parental NIH3T3 cells, suggesting that all variants are capable of inducing foci of transformed NIH3T3 cells. The transformation potential of all novel mutants (measured as mean number of foci) was lower when compared with C634R, with the K666N construct being the one with the highest transforming activity (Fig. 3).

Possible effects on protein structure

To assess whether these amino acid changes could affect the secondary structure or protein folding, the whole WT and mutant Ret protein sequences were analyzed by means of several web-based tools. Most algorithms predicted significant changes to the protein folding as a result of each of these variants. In particular, proteins with variants lying in the extracellular cysteine-rich domain (p.A510V, p.E511K and p.C531R) display a different conformation with respect to the WT receptor as far as helices and β-strands are concerned, which could alter the function of the receptor. The mutant mapping in the intracellular juxtamembrane domain

Figure 1 Sequence alignment of RET in different species, according to the prediction and alignment of Ret protein orthologues reported by Kashuk et al. (10). The residues discussed in the present study are indicated by arrows. Conserved residues are reported in bold. TM, transmembrane domain; JM, juxtamembrane domain.

Table: Intracellular domain

<table>
<thead>
<tr>
<th>Mutant</th>
<th>Extracellular Domain</th>
<th>Intracellular Domain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>Tyr1151Cys</td>
<td>His1180Pro</td>
</tr>
<tr>
<td>Rat</td>
<td>Tyr1151Cys</td>
<td>His1180Pro</td>
</tr>
<tr>
<td>Human</td>
<td>Tyr1151Cys</td>
<td>His1180Pro</td>
</tr>
<tr>
<td>Chimpanzee</td>
<td>Tyr1151Cys</td>
<td>His1180Pro</td>
</tr>
<tr>
<td>Rabbit</td>
<td>Tyr1151Cys</td>
<td>His1180Pro</td>
</tr>
<tr>
<td>Cat</td>
<td>Tyr1151Cys</td>
<td>His1180Pro</td>
</tr>
<tr>
<td>Dog</td>
<td>Tyr1151Cys</td>
<td>His1180Pro</td>
</tr>
<tr>
<td>Pig</td>
<td>Tyr1151Cys</td>
<td>His1180Pro</td>
</tr>
<tr>
<td>Chicken</td>
<td>Tyr1151Cys</td>
<td>His1180Pro</td>
</tr>
<tr>
<td>Zebrafish</td>
<td>Asp1157Cys</td>
<td>Cys1192Arg</td>
</tr>
</tbody>
</table>

Figure 2 (A) Tyrosine phosphorylation of Ret receptors. Lysates from HEK 293T cells transfected with Ret9-WT, Ret9-A510V, Ret9-E511K, Ret9-C531R, Ret9-K666N and Ret9-C634R constructs were immunoprecipitated (IP) with anti-Ret antiserum. Western blot (WB) filters were developed with anti-phosphotyrosine (αPtyr) or anti-Ret antiserum (αRet). The fully glycosylated mature form of Ret (175 kDa) and the immature form (155 kDa) are indicated by arrows. All mutant proteins displayed an amount of phosphorylation higher than that displayed by the Ret9-WT protein. Moreover, p.A510V, p.E511K and p.C531R mutations were significantly less phosphorylated than Ret9-C634R, while Ret9-K666N mutant displayed a high kinase activity. Signal corresponding to Ret phosphorylation/Ret protein, as shown in the upper panel, was quantified by densitometric scanning: results are expressed as intensity-fold increase over wild-type, and are representative of three independent experiments. (B) ERK phosphorylation. Total lysates from all constructs were probed with anti-ERK antiserum. Western blot (WB) filters were developed with anti-phospho ERK (αP-ERK) or anti-ERK antiserum (αERK). The ERK1 (44 kDa) and ERK2 (42 kDa) isoforms are indicated by arrows. All constructs displayed an amount of phosphorylation higher than that displayed by the Ret9-WT. The almost undetectable levels of phosphorylation observed in non-transfected cells (mock) are due to the endogenous ERK activity in HEK cells. RET variants p.A510V, p.E511K and p.C531R were significantly less phosphorylated than Ret9-C634R, while Ret9-K666N mutant displayed the highest kinase activity. Signal corresponding to ERK phosphorylation/ERK protein, as shown in the upper panel, was quantified by densitometric scanning, and is representative of three independent experiments.

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(p.K666N) leads to a significant alteration of the transmembrane α-helix, likely changing the secondary structure of the protein.

In addition, alterations in the proportion of solvent-exposed residues were found, likely leading to variations in the interactions with a non-aqueous environment.

Discussion

In the present study, novel germline RET variants found in apparently sporadic MTCs have been reported. In particular, three variants were located in exon 8 (c.1529C>T, p.A510V; c.1531G>A, p.E511K and c.1591T>C, p.C531R), within the extracellular cysteine-rich domain of RET, while one (c.1998G>T, p.K666N) was found in the juxtamembrane intracellular portion of the receptor. Unfortunately, the carrier status could not be tested in any of the first-degree relatives, and the history was silent for MTC in all families. Thus, the functional activity of these novel genomic variations was tested, and increased levels of RET and ERK phosphorylation associated with in vitro transforming activity were demonstrated for all variants. In particular, Ret9-C531R variant displayed an intermediate increase in both RET and ERK phosphorylation with respect to Ret9-WT, consistent with the mild phenotype observed in the patient, characterized by late onset and low aggressiveness.

Similar biological activity and clinical behaviour have been reported for another cysteine of exon 8, Cys 515, which has been found to be mutated (Cys515Ser) in an Italian woman with a late-onset and poorly aggressive isolated MTC (10). The putative mechanism leading to the increased constitutive activity of this variation is unknown. Nevertheless, other cysteine mutations in the extracellular cysteine-rich domain were shown to be involved in the creation of intermolecular disulphide bonds between unpaired partner cysteines, thereby generating a ligand-independent dimerization and a constitutive activation (6, 10, 18).

As far as the two additional novel non-cysteine variants (p.A510V and p.E511K) lying in exon 8 are concerned, they were found to generate a significant increase in RET constitutive kinase activity and in ERK phosphorylation compared with that of Ret9-WT. The transforming potential was twofold and threefold that of Ret9-WT for A510V and E511K respectively. Regarding the variant E511K, a discrepancy can be noted between the mild increase in phosphorylation activity (similar to A510V and significantly lower than K666N) and the high transforming potential (significantly higher than A510V and similar to K666N). Similar discrepancies were reported by other authors (19, 20) for RET mutations, and these could be due to the different cell transformation and proliferation properties of distinct mutants, owing to the activation of different molecular pathways (21). It is also tempting to speculate that the high in vitro transforming potential found for this variant could be responsible for the aggressive behaviour of MTC with lung metastases found in patient 2. It is worth noting that another non-cysteine variant in RET exon 8 (Gly533Cys) has been described previously, but not functionally characterized, in five Greek families, three with MEN2A (11, 13) and two with FMTC (12), and in one large Brazilian multigenerational family with FMTC (9). On the basis of all these data, exon 8 appears to be an underestimated hotspot of RET variants with an oncogenic potential.

The novel missense variant found in exon 11, p.K666N, lies in a residue highly conserved among species. This variant has been found to harbour high phosphorylation activity of both RET and ERK, and a high transforming potential. This is consistent with the functional data obtained in a previously reported complex mutation c.2646delGinsTTCT, which leads to a lysine to asparagine change (p.K666N) followed...
by a serine insertion (16). These data suggest that alterations in the juxtamembrane region can strongly activate Ret in a ligand-independent manner. Consistently, a mutation in the same codon, but with a different amino acid change (p.K666E), has been detected in patients with MEN2A (22). Nevertheless, in the present patient harbouring p.K666N variant, MTC was discovered at the age of 65 years and surgery resulted in remission. This discrepancy is not surprising since phenotype variability, regarding the age of onset and clinical presentation of the disease, is well known for several RET mutations, being possibly due to genetic modifiers (such as RET polymorphisms).

Computational analysis performed by means of the PredictProtein server predicted various modifications induced in the mutant receptors. These include alterations in the number and localization of helices and β strands, including the profound modification/disappearance of the transmembrane α-helix for the p.K666N mutant. It is therefore likely that these mutants could affect the conformation and function of the receptor, leading to a ligand-independent increased kinase activity.

In conclusion, the molecular analysis of a large French cohort of apparently sporadic MTCs allowed us to identify four novel RET variants with an oncogenic potential demonstrated in vitro. We could not verify the co-segregation of these variants with the disease in the families as no first-degree relative was available for the study. Nevertheless, the negative familial history for MTC may argue in favour of a variable penetrance of the variants, as reported for other RET mutations, such as those lying in codon 804 or 891 (23, 24). However, on the basis of these functional data, it is advisable to extend the genetic screening to all the first-degree relatives of the MTC patients, and to perform a strict follow-up of eventual familial carriers by serial determinations of basal and stimulated C levels.

Indeed, the present data together with previous reports (9–13) indicate that exon 8 genomic variants with an oncogenic potential are more prevalent than previously thought, and that they can be associated with either FMTC or MEN2A. Therefore, in addition to the analysis of the classical RET exons indicated by the very recent ATA guidelines for the management of MTCs (14), it appears worthwhile to analyze exon 8 once that mutations in exons 10, 11 and 13–15 had been excluded.

Finally, the high constitutive activation and transforming potential exhibited by the fourth novel variation, lying in the juxtamembrane domain, lead us to suggest screening the family members and applying a strict follow-up starting at a young age for mutation carriers in this region.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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