High prevalence of the C634Y mutation in the RET proto-oncogene in MEN 2A families in Spain

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Abstract
The RET proto-oncogene encodes a receptor tyrosine kinase expressed in neural crest derived tissues. Germline mutations in the RET proto-oncogene are responsible for three different dominantly inherited cancer syndromes: multiple endocrine neoplasia type 2A (MEN 2A), type 2B (MEN 2B), and familial medullary thyroid carcinoma (FMTC). MTC can also occur sporadically. Molecular characterisation of the RET proto-oncogene has been performed by PCR-SSCP analysis, direct DNA sequencing, and restriction enzyme analysis in 49 unrelated, Spanish, MEN 2 families: 30 MEN 2A families, six FMTC families, and 13 families classified as "other". Germline mutations in one of six highly conserved cysteine codons (609, 611, 618, and 620 in exon 10, and codons 630 and 634 in exon 11), which encode part of the extracellular cysteine rich domain of RET, have been detected in the majority of these families: 100% of MEN 2A families, 67% of FMTC families, and 54% of families classified as "other". No RET mutations in exons 10, 11, 13, 14, 15, or 16 were detected in the remaining families. The most frequent RET mutation in MEN 2A Spanish families is C634Y, occurring in 73% of cases. Haplotype analysis does not exclude the possibility of founder effects in Spanish MEN 2A families with the C634Y mutation.

Keywords: medullary thyroid carcinoma; RET proto-oncogene; molecular analysis

Medullary thyroid carcinoma (MTC) is a tumour of the thyroid C cells which may occur sporadically or as part of the autosomal dominantly inherited cancer syndrome multiple endocrine neoplasia type 2 (MEN 2). Depending on the tissues involved, MEN 2 is divided into MEN 2A, MEN 2B, and familial medullary thyroid carcinoma (FMTC). MEN 2A is characterised by hyperplasia of the parafollicular C cells of the thyroid, with subsequent neoplastic progression to MTC in 95% of cases, phaeochromocytoma in 50% of cases, and parathyroid hyperplasia (HPT) in 15-30% of cases. The MEN 2B syndrome consists of MTC, phaeochromocytoma, and a variety of developmental abnormalities including mucosal neuromas, marfanoid habitus, and ganglioneuromatosis of the intestinal tract. In FMTC, the presence of MTC is the only disease phenotype. Germline mutations in the RET proto-oncogene are responsible for the MEN 2 syndromes.\(^1\) The RET proto-oncogene encodes a member of the receptor tyrosine kinase (RTK) family. Unlike most members of the RTK super family, RET activation requires the formation of a multimeric receptor complex that includes glial cell line derived neurotrophic factor (GDNF) as ligand, and a cell surface associated accessory protein designated GFR\(\alpha\) (GDNFR-\(\alpha\), RETL1, or TrnR1).\(^2,3\) Recently, a related ligand, neurturin (NTN), and an adaptor molecule GFR\(\alpha\)-2 (GDNFR-\(\beta\), RETL2, TrnR2, NTNR-\(\alpha\)) have been described.\(^4-11\) The majority of MEN 2A and FMTC families have germline missense mutations in one of six highly conserved cysteine codons (609, 611, 618, and 620 in exon 10, and codons 630 and 634 in exon 11), which encode part of the extracellular cysteine rich domain of RET.\(^1,3\) The RET proto-oncogene is a member of the RET proto-oncogene family, which includes glial cell line derived neurotrophic factor (GDNF) as ligand, and a cell surface associated accessory protein designated GFR\(\alpha\) (GDNFR-\(\alpha\), RETL1, or TrnR1).\(^1,2\) In FMTC families, other less frequent missense RET mutations in codons 768 (exon 13) and 804 (exon 14), within the intracellular tyrosine kinase domain, have been detected.\(^1,15-18\)

Recently, the results of a study carried out by the International RET Mutation Consortium showed that 85% of MEN 2A families had a mutation at codon 634 and the most frequent mutation at this codon was C634R (52%) followed by C634Y (26%). On the other hand, in FMTC families, the most frequent mutation at codon 634 was C634Y and there was no C634R mutation among these families.\(^7\) In this study, we analysed the presence, nature, and position of germline RET mutations in 49 Spanish MEN 2 families. Of these families, 30 were diagnosed with MEN 2A, six with FMTC, and 13 families were classified as “other”, according to the criteria of the International RET Mutation Consortium.\(^3\) Germline DNA was extracted from blood leukocytes by standard procedures.\(^19\) DNA from a representative member of each family was PCR amplified for RET exons 10, 11, 13, 14, 15, and 16 using primers previously described.\(^20-23\) Initially, we screened for the RET mutations more frequently identified in MEN 2 by restriction enzyme analysis. When a RET mutation was detected, the result was confirmed by direct sequencing of the corresponding exon in both sense and antisense directions by the dideoxynucleotide terminator cycle sequencing method (fmol\textsuperscript{TM} DNA Sequencing System,
Table 1 Distribution of amino acid substitutions in Spanish MEN 2 families

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Nucleotide change</th>
<th>MEN 2A (n=30)</th>
<th>FMTC (n=6)</th>
<th>Other (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C634Y</td>
<td>TGC→TAC</td>
<td>22</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>C634R</td>
<td>TGC→CGC</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>C634W</td>
<td>TGC→TGG</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>C634G</td>
<td>TGC→GGC</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C620S</td>
<td>TGC→TCC</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>C618R</td>
<td>TGC→CGC</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>C618F</td>
<td>TGC→TTC</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>C611F</td>
<td>TGC→TTT</td>
<td>0</td>
<td>2</td>
<td>6</td>
</tr>
</tbody>
</table>

Promega) using fluorescently labelled primers (synthesised 5' labelled with Cy5 Amidite) described elsewhere.20-23 Gel electrophoresis and analysis were carried out on an ALF Express™ Automatic DNA sequencer (Pharmacia Biotech). When no RET mutation was detected by restriction enzyme analysis, exons 10, 11, 13, 14, 15, and 16 were screened for mutations by single strand conformational polymorphism analysis (SSCP) using fluorescently labelled primers described elsewhere.20-23 Electrophoresis was carried out in an automated DNA sequencer (ALF Express™ Automatic DNA sequencer). When an aberrant SSCP pattern was observed, we searched for the mutation responsible by direct sequencing, as we have described previously. Haplotype analyses at the RET locus were performed using two extragenic flanking microsatellite polymorphisms, D10S141 in the centromeric position and ZNF22 in the telomeric position, and the highly polymorphic RET-INT5 intragenic CA repeat.24-26 Electrophoresis and analysis of these polymorphic loci were conducted using two extragenic flanking microsatellite polymorphisms, D10S141 in the centromeric position and ZNF22 in the telomeric position, and the highly polymorphic RET-INT5 intragenic CA repeat.24-26 Electrophoresis and analysis of these polymorphic loci were carried out in an ALF Express™ Automatic DNA sequencer.

Mutations of the RET proto-oncogene have been identified in the majority of Spanish MEN 2 families. The identification of these mutations has an important predictive value, allowing earlier diagnosis of at risk subjects and the exclusion of mutation negative subjects from further biochemical tests and prophylactic surgery. We have identified germline missense mutations in the extracellular domain of the RET proto-oncogene in all 30 MEN 2A families (100%). The most frequent mutations in MEN 2A families are at codon 634 in exon 11, occurring in 97% of cases (29/30 families). In 3% (1/30) of families, a mutation at codon 620 in exon 10 has been identified. In Spanish FMTC and “other” families, we have identified RET mutations in 67% (4/6) and 54% (7/13) of the cases, respectively. As described in previous studies, the position and nature of these mutations were more heterogeneous and they are distributed among the cysteine codons in exons 10 and 11. Mutations at codon 634 were found in 50% (3/6) of our FMTC families, in 17% (1/6) a mutation in exon 10 was identified, and no mutations in exons 10, 11, 13, 14, 15, or 16 were detected in 33% (2/6). In families classified as “other”, we have detected a mutation at codon 634 in exon 11 in 31% (4/13) of families, in 23% (3/13) a mutation in exon 10 was identified, and in 46% (6/13) of families, no mutation in exons 10, 11, 13, 14, 15, or 16 was detected. The distribution of amino acid changes in Spanish MEN 2 families are shown in table 1.

We have studied the relationship between the nature and position of the RET germline mutation and the disease phenotype. Our results also show the association of mutations at codon 634 and the presence of phaeochromocytoma or parathyroid disease observed in the Consortium series.27 An interesting result of our study is that of 19 families with MTC and phaeochromocytoma, 15 have the specific mutation C634Y. The other three MEN 2A families with the C634Y mutation have MTC and parathyroid disease only.

The results of the mutation analysis in Spanish MEN 2 families are consistent with the data of the large series studied by the International RET Mutation Consortium. An exception is the mutation C634R detected in one Spanish FMTC family, which was not found among the FMTC families included in the Consortium study. This FMTC family consists of seven affected family members over two generations with MTC confirmed by postsurgical histological study. The affected subjects range in age from 12 to 44 years. All affected subjects and six of their first degree relatives were screened for phaeochromocytoma and HPT and found to be negative. It is possible that this FMTC family is one where the other features of MEN 2A have yet to develop. Another interesting finding is the high frequency of the C634Y mutation in Spanish MEN 2A families (73%), in contrast to the large excess of C634R (52%) observed in the MEN 2A families of the Consortium study. Our finding is similar to that of Oriola et al.,27 in a smaller Spanish MEN 2A series, in which they found the C634Y mutation in five of seven families (71%). Our data, together with those reported by Oriola et al.,27 show the relative prevalence of the C634Y mutation in MEN 2A families in Spain.

To investigate whether the high frequency of this mutation in Spain might be the result of a common ancestor, we have performed haplotype analysis, using polymorphic markers within and flanking the RET gene, in MEN 2A families with the C634Y mutation.28 The different haplotypes observed associated with mutated chromosomes (table 2) seem to exclude a founder effect. However, table 2 shows that although we have detected seven haplotypes among 15 patients with this mutation, 14 subjects share one of two alleles at the
RET-INT5. Without having information on haplotype frequencies in the Spanish population, we cannot rule out two mutational events combined with founder effects and recombination. This hypothesis may explain the discrepancy between our findings in the Spanish population and the RET Mutation Consortium data with regard to the relative prevalence of the C634Y and C634R mutations.

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