A rare RET gene exon 8 mutation is found in two Greek kindreds with familial medullary thyroid carcinoma: implications for screening

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Summary

Objective Familial medullary thyroid carcinoma (FMTC) is caused by germ-line mutations in the RET proto-oncogene. These mutations concern mainly cysteine residues in exons 10 and 11, whereas noncysteine mutations in exons 13–16 are rare. Mutations in other exons have been reported only in isolated families. In this study we have analysed the RET gene in two FMTC families negative for mutations in the above exons.

Design We have analysed exons 7–19 and 21 in one index patient from each family using DNA sequencing.

Patients Twenty-eight subjects from both families were clinically assessed and subsequently molecularly analysed for the presence of RET gene mutations.

Results We have found the mutation c.1597G→T (Gly533Cys) in two Greek families with FMTC. The mutation was detected in all seven MTC patients of both families as well as in 13 asymptomatic relatives in the heterozygote state, although one of the patients was also a homozygote due to consanguinity. The mutation shows a wide clinical heterogeneity, as there are carrier patients with age of diagnosis ranging from 23 to 88 years.

Conclusions It is likely that this mutation causes FMTC, as no other mutation was found in the RET gene, the mutation co-segregates with FMTC, and family members without the mutation are clinically unaffected. As the same point mutation was previously found in a large Brazilian family, it may be present in other populations as well. Therefore, exon 8 of RET should be screened in FMTC families with no identified common RET mutations.

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Introduction

Hereditary MTC is the most frequent clinical manifestation of the multiple endocrine neoplasia type 2 (MEN2) syndromes. This syndrome is caused by gain-of-function mutations in the RET proto-oncogene. MEN2A is caused by point mutations in the cysteine-rich domain and the tyrosine-kinase domain of the RET gene and is characterized by medullary thyroid carcinoma (MTC), pheochromocytoma and primary hyperparathyroidism and rarely cutaneous lichen amyloidosis.1,6 Familial MTC (FMTC) is a heritable form of MTC without the other clinical features of MEN2A.7

Most of the RET mutations related to FMTC are in the same amino acids as those in MEN2A; for example, six cysteine residues in a small 25-amino-acid domain in the extracellular region in codons 609, 611, 618 and 620 (exon 10) and in codons 630, 634 (exon 11). Other mutations seemingly specific to FMTC are in amino acids 768, 790, 791, 804, 844, 891 and 912 of the cytoplasmic domain and two rare distinct mutations in exon 8, reported only once in single families.1,6 The first exon 8 mutation described is a 9-bp duplication8 while the second is the c.1597G→T9 both create an additional cysteine residue in the extracellular cysteine-rich domain of RET.

In this paper we describe the identification of the c.1597G→T mutation in two Greek families with FMTC. These findings suggest that in certain populations, c.1597G→T may be more frequent than previously thought, with implications for diagnostic testing.

Subjects and methods

Informed consent was obtained from all individuals, from both families, before genetic analysis was performed and patients were counselled about the implications of genetic testing. A careful and detailed personal and family history was obtained from all patients and most of the screened relatives. The study was approved by the ethics and research committee of Metaxa Hospital (Piraeus, Greece) and was in agreement with the 1975 Helsinki statement, revised in 1983.

The pedigrees of the families A and B are shown in Fig. 1. Clinical, laboratory and histological findings of the patients are presented in Table 1.
All mutation carriers from both families were assessed for metanephrines and PTH with no laboratory or clinical evidence of phaeochromocytoma or primary hyperparathyroidism.

**Family A**

A total of 12 individuals from three generations were studied. There was a history of MTC in four members of the family (II1, II3, II6, III1). The index case was a 54-year-old man (II1) who underwent total thyroidectomy in another hospital at the age of 52, for multinodular goitre diagnosed 15 years earlier. The patients were referred to us for follow-up and genetic counselling. Genetic screening was performed in all patients’ first- and second-degree relatives.

Patients II1 and III1 presented residual disease, as evidenced by the increased basal calcitonin (CT) levels, more than 2 years postoperatively. No obvious lesions on standard imaging studies were found, whereas sites of selective increased radioisotope uptake were detected in the neck and the mediastinum on 111-In-octreotide scan. To date, they are scheduled for surgical treatment. Patient I1 presented with high calcitonin (CT) levels but refused any further evaluation and treatment. Patient III5 was advised to undergo total thyroidectomy plus dissection of lymph nodes of the central compartment, as she exhibited high CT levels during a pentagastrin (Pg) stimulation test.

**Family B**

A total of 13 individuals from two generations were studied. In this family the index case was a 26-year-old female (IV3) who underwent total thyroidectomy plus dissection of lymph nodes of the central compartment, as she exhibited high CT levels during a pentagastrin (Pg) stimulation test.

Fig. 1 Pedigrees of FMTC families A and B with exon 8 c.1597G→T mutation. Genotyping of nine subjects (AII1, AIII1, AIII2, BIII2, BIII3, BIII4, BIV3, BIV4, BIV5) using informative RET gene polymorphisms. Consanguinity of subjects III1 and II2 in family B is indicated by a thick black line.
Table 1. Clinical, biochemical and histological features of family members carriers of c.1597G→T mutation

<table>
<thead>
<tr>
<th>Patients</th>
<th>Sex/age</th>
<th>Age at surgery</th>
<th>Preop. basal CT/Pg-CT (ng/l, NV &lt; 11.5 ng/l)</th>
<th>Preoperative thyroid ultrasound</th>
<th>Histology</th>
<th>Lymph node dissection</th>
<th>Post-surgery/to date CT (ng/l, NV &lt; 11.5 ng/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Family A</em>&lt;br&gt; I-I</td>
<td>M/88</td>
<td>345</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II-1</td>
<td>M/54</td>
<td>52</td>
<td>NA</td>
<td>Two nodules (RL 1.9 cm, LL 2.1 cm)</td>
<td></td>
<td></td>
<td>RL 2 cm, LL 1.8 cm; MTC</td>
</tr>
<tr>
<td>II-3</td>
<td>M/57</td>
<td>55</td>
<td>244</td>
<td>Two nodules (RL 1.1 cm, IS 0.9 cm)</td>
<td></td>
<td></td>
<td>RL 1 cm, IS 1 cm; MTC</td>
</tr>
<tr>
<td>II-6</td>
<td>F/49</td>
<td>47</td>
<td>2900</td>
<td>One nodule (RL 2.7 cm)</td>
<td></td>
<td></td>
<td>RL 2.5 cm, LL 0.5 cm; MTC</td>
</tr>
<tr>
<td>III-1</td>
<td>F/25</td>
<td>23</td>
<td>80</td>
<td>One nodule (RL 1 cm)</td>
<td></td>
<td></td>
<td>RL 0.8 cm, LL 0.2 cm; MTC</td>
</tr>
<tr>
<td>III-3</td>
<td>M/21</td>
<td>(scheduled) 4</td>
<td>NA</td>
<td>NED</td>
<td></td>
<td></td>
<td>No (scheduled)</td>
</tr>
<tr>
<td>III-4</td>
<td>M/18</td>
<td>(scheduled) 4-8</td>
<td>NA</td>
<td>NED</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III-5</td>
<td>F/25</td>
<td>(scheduled) 1-1/52</td>
<td>One nodule (IS 0.4 cm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Family B</em>&lt;br&gt; III2</td>
<td>F/59</td>
<td>26</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td>NA</td>
</tr>
<tr>
<td>III3</td>
<td>M/52</td>
<td>10.3</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III6</td>
<td>F/54</td>
<td>193</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III7</td>
<td>M/48</td>
<td>279</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>III11</td>
<td>M/55</td>
<td>19.2</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV1</td>
<td>F/33</td>
<td>191</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>IV2</td>
<td>F/27</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV3</td>
<td>F/26</td>
<td>26</td>
<td>191</td>
<td>Three nodules (two RL 0.66 + 0.52 cm, LL 0.65 cm)</td>
<td>RL 0.7 cm, LL 0.8 cm; MTC</td>
<td>Yes (2'/24)</td>
<td>1.2/1.3</td>
</tr>
<tr>
<td>IV4</td>
<td>M/24</td>
<td>24</td>
<td>16/0/24.9</td>
<td>NED</td>
<td></td>
<td></td>
<td>RL 0.2 cm, 0.4 cm; MTC</td>
</tr>
<tr>
<td>IV5</td>
<td>M/28</td>
<td>(scheduled) 41-4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Yes (0'/22) 1.22</td>
</tr>
<tr>
<td>IV6</td>
<td>M/32</td>
<td>19-0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IV7</td>
<td>M/30</td>
<td>12-1</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

CT, calcitonin; Pg, pentagastrin; NV, normal value; M, male; F, female; RL, right lobe; LL, left lobe; IS, isthmus; NED, no evidence of disease; NA, not available; MTC, medullary thyroid carcinoma.
with a history of micronodular goitre since the age of 24. In her regular visits to the endocrinologist, CT was found to be elevated. Consequently, she underwent total thyroidectomy plus functional dissection of central and bilateral cervical lymph-node compartments. Histological analysis revealed bifocal, bilateral MTC. Two out of 24 dissected cervical lymph nodes presented neoplastic infiltration. Post-operatively calcitonin was normalized. Her brother (IV4) underwent total thyroidectomy due to elevated CT levels; histological analysis revealed a bifocal microscopic MTC with no lymph-node metastasis.

Their father (III3) was recently diagnosed with final stage non-small-cell lung cancer (NSCLC). Genetic testing and a basal CT measurement were performed without any further evaluation for MTC.

Patient III2 underwent total thyroidectomy for palpable multinodular goitre and MTC was diagnosed histologically 33 years ago. Consequently she underwent external radiotherapy of the neck and upper mediastinum.

**Pg test and CT assay**

The Pg stimulation test consisted of a pulse administration of 0.5 µg/kg of Pg (Pentagastrin Injection BP, POM, Cambridge Laboratories Ltd, UK). Blood was collected before and at 2, 5, 10 and 15 min after the injection. Serum CT levels were determined by a chemiluminescence CT immunoassay (Advantage, Nichols Institute, San Juan Capistrano, CA, USA). CT levels were considered normal with reference to the data provided (normal values < 11.5 µg/l).

**DNA analysis**

Genomic DNA was prepared from peripheral blood samples collected on ethylenediaminetetraacetic acid (EDTA) tubes according to standard protocols. The sequences of primers used for exons 7–19 and 21 and polymerase chain reaction (PCR) protocols were obtained from previously published sources. Sequencing was performed in an ABI3100 genetic analyser.

**Results**

Index patients (AI11, BIV3) of both families were initially screened for RET mutations in exons 10, 11, 13, 14, 15 and 16. As no molecular abnormalities were observed, analysis was extended to exons 7, 8, 9, 12, 17, 18, 19 and 21 of the RET gene, according to guidelines for the diagnosis and therapy of MEN2 syndrome. Prevalently reported polymorphisms A432A, G691S, L769L, S904S and IVS14-24 G→A were detected in exons 7, 11, 13, and 15 and at intervening sequence 14.

Direct sequencing of exon 8 PCR products from genomic DNA of all index patients revealed a G→T transversion in the heterozygote state at position 1597, which leads to a Gly to Cys amino-acid change at codon 533. As the above nucleotide change has been reported previously as the cause of FMTC in a large kindred, it was assumed that it was also the cause of the disease in these families.

To confirm this, all remaining members of both families were screened for this mutation. The mutation was detected in the heterozygote state in patients AI11, AI13, AI16, AI1II1, BIV3 and BIV4 and in 13 asymptomatic relatives. Clinical features of the mutation carriers are shown in Table 1. In addition, the mutation was also detected once in the homozygote state in patient BIV3. All family members without the mutation were clinically unaffected.

Patients AI1, AI11, AI1II1, AI1II2, BIV3, BIV4 and BIV5 were sequenced for polymorphisms A432A, G691S, L769L, S904S and IVS14-24 G→A and their genotypes are shown in Fig. 1. The haplotype inferred was the same for all mutation carriers genotyped.

Fifty unrelated controls were sequenced for exon 8 and no base changes were revealed at position 1597 or elsewhere.

**Discussion**

We report the finding of the c.1597G→T mutation (Gly533Cys) in exon 8 of the RET gene in two Greek families with familial distribution of MTC. According to the consensus statement on MEN2, family B can be categorized as FMTC because it contains three MTC patients in a total of 12 mutation carriers, five of whom are above the age of 50. Although family A contains eight carriers, that is slightly less than the consensus threshold of 10, we consider it safe to characterize it as FMTC as four of them are above the age of 50, including an unaffected 88-year-old patient. Notably, none of the patients of either family showed any evidence of hyperparathyroidism or phaeochromocytoma.

As the sequence involved in this mutation is not known to be a mutational hotspot and has only been reported once in the literature, its double occurrence in the Greek population suggested the possibility of a common origin. To investigate this, intragenic polymorphic markers flanking the mutation located at exons 7, 11, 13, IVS14 and 15 were screened. The haplotypes were deduced based on patient and relative genotypes and a common haplotype was found to be shared between all mutation carriers screened from both families, suggesting a possible common origin for the two families.

All MTC patients in both families were found to be carriers of the c.1597G→T mutation while all members without the mutation were unaffected, as CT levels and clinical findings suggest. In addition, 50 unrelated controls from the Greek population were sequenced in exon 8 but showed no base change at position 1597. All the above provide further evidence to previous work that the mutation c.1597G→T is the cause of the disease.

One patient (BII12) was found to be homozygous for the mutation c.1597G→T in exon 8. To accept the homozygosity detected in this patient, and considering that she could not be tested through her parental DNA (parents not alive), the possibility of germline loss of one allele of RET had to be excluded first. Unfortunately, as both of her parents were relatives, heterozygosity at any of A432A, G691S, L769L, S904S and IVS14-24 G→A positions, which would prove the presence of two alleles, would be unlikely. In fact, all the above loci were indeed homozygous, thus no conclusion could be drawn (Fig. 1). However, as both of her parents had died of cancer-related causes and both of her offspring (BIV1 and BIV2) were sequenced and found to carry the mutation (obligatory carriers), it was considered safe to accept this patient’s homozygosity.

The clinical course of MTC has been variable in these two families with 26-year-old patient BIV3 showing lymph-node metastasis while the 88-year-old carrier AI1 was hypercalcitoninaemic but asymptomatic.
(Table 1) and so the age at diagnosis of the disease ranged from 23 to 57. As the earliest age at diagnosis was 23 among our patients and 21 among those described in the literature, prophylactic surgery seems mandatory before the age of 20. This cut-off point could be set at a younger age, although the number of patients under 20 does not permit reliable conclusions.

The phenotypic variability seen in our patients is reported in FMTC kindreds with mutation in codon 80414,15 and even the same c.1597G→T mutation in the first and only existing report in the literature, in a Brazilian family with origin from Spain.16 The observation that a specific RET mutation behaves differently, even in the same genetic background, suggests that other genetic or epigenetic or environmental factors play a role.

For example, polymorphisms G691S and S904S, known to be in complete linkage disequilibrium, have been described to modify the RET mutation-associated disease phenotype as they are significantly over-represented among patients with sporadic MTC.16,17 These polymorphisms are not present in family A; however, in family B patients IV3, IV4 and carrier IV5 do carry these two polymorphisms on a chromosome not bearing c.1597G→T. Therefore, the early onset of MTC in patients IV3 and IV4 could be explained by the increased risk conferred by the above polymorphisms as described in Cebrian et al.16

Homozygous patient BIII2 developed clinical signs of disease at 26, an earlier age than other patients of her generation. In addition, at the time of her diagnosis (1972), neither ultrasound nor CT measurement was available. The fact that she was diagnosed by physical examination shows that the size of the tumour was much greater than that of patients of the following generations at the same age.

Therefore, there seems to be a difference between homozygote and heterozygote subjects in the age of diagnosis and the severity of phenotype. This could be explained by the fact that in heterozygotes the wild-type RET gene product dimerizes in the presence of two mutant alleles, the protective effect of wild-type protein is lost, leading to dimerization between the mutant monomers.

An alternative hypothesis of possible modulation of the expression of the disease would be that involving the allelic changes that have been described (somatic trisomy, loss or gain of one of the two alleles, and expression differences).18,19 In our patients, these possibilities could not be tested because of the lack of tissue. In the case of the homozygote mutant, amplification expression or any other gain of one of the two alleles would probably contribute to a more aggressive phenotype, by saturating the cell with mutant RET protein without the mitigating effect of the normal allele. Loss of one of the two alleles, without concurrent amplification of the other, would probably have the same effect as simple homozygosity.

Homozygosity for RET gene mutations is rare and has only been reported three times for codons 804 and 883 (V804M, V804L, A883T).16,20,21 We report the first biallelic c.1597G→T case, which, like the above cases, is due to parental consanguinity.

Specific exons (excluding exon 8) of the RET gene have been screened in families with MEN2 in earlier studies. However, molecular abnormalities were only detected in 0/3,22 3/522 and 4/1011 of FMTC families, whereas almost all mutations were identified in MEN2A and MEN2B families. This agrees with an earlier observation that FMTC mutations are more evenly distributed across the RET gene than other MEN2 mutations.5,6 The finding of c.1597G→T in more than one ethnic population suggests that it may be more frequent than previously considered and could account for some of the FMTC families with unidentified mutations in the commonly screened exons of RET. According to the consensus,13 these families should be screened in the remaining 15 exons; among them, exon 8 should probably be the first to be tested.

Acknowledgements
We are indebted to the patients for their collaboration in this study.

References


