Functional characterization of the MTC-associated germline RET-K666E mutation: evidence of oncogenic potential enhanced by the G691S polymorphism

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Abstract

Activating mutations of RET, a gene encoding two isoforms of a tyrosine kinase receptor physiologically expressed in several neural crest-derived cell lineages, are associated with the inherited forms of medullary thyroid carcinoma (MTC). The identification and characterization of novel RET mutations involved in MTC is valuable, as RET gene testing plays a crucial role in the management of these patients. In an MTC patient, we have identified a germline c.1996A>G transition in heterozygosis leading to K666E substitution. In addition, the conservative S904S (c.2712C>G) and the non-conservative functional G691S (c.2071G>A) polymorphisms have been identified. Through functional studies, we demonstrate for the first time that K666E is a gain-of-function mutation with oncogenic potential, based on its ability to transform NIH3T3 cells. It was not possible to define whether K666E is a de novo or inherited RET variant in the patient, as the family history was negative for MTC, and the carrier status of family members could not be tested. Our results, together with a recent report of co-segregation of the mutation in three MTC families, suggest that K666E is a causative MTC mutation. As we have shown that the same patient allele carries both K666E and G691S variants, the latter known to increase downstream RET signaling, a possible role for the G691S polymorphism has also been investigated. We have demonstrated that, although RET-G691S is not oncogenic per se, it enhances the transforming activity of the RET-K666E mutant, thus suggesting a modifier role for this functional polymorphism.

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Introduction

The RET (rearranged during transfection) gene encodes a tyrosine kinase receptor with a crucial role in development. RET comprises 21 exons and generates a transcript subjected to alternative splicing leading to two main isoforms: a protein of 1114 residues displaying 51 C-terminal-specific amino acids (RET51) and a shorter protein of 1072 residues displaying nine unrelated C-terminal-specific amino acids (RET9), as shown in Fig. 1A. The RET receptor system also includes the GFRα1–4 alternative co-receptors and GDNF family ligands, including GDNF, neurturin, persephin, and artemin (reviewed in Arighi et al. (2005)).
RET is involved in thyroid neoplasias through different gain-of-function alterations. Somatic rearrangements of RET are present in about one-third of papillary thyroid carcinomas (reviewed in Greco et al. (2009)), whereas specific germline activating mutations of RET are causally involved in hereditary medullary thyroid carcinoma (MTC), including multiple endocrine neoplasia type 2A (MEN2A, OMIM 171400), type 2B (MEN2B, OMIM 162300), and familial MTC (FMTC; OMIM 155240) syndromes (Lodish & Stratakis 2008). Activating RET mutations involved in hereditary MTC forms are also present in a fraction of sporadic MTCs.

MTC, which accounts for around 5% of thyroid tumors, is a neuroendocrine neoplasia arising from the thyroid parafollicular C cells. Physiologically, RET, GFRα4, and persephin appear to be necessary for migration of neural crest calcitonin producing cells into the developing thyroid gland: markedly diminished parafollicular C cells are found in RET-K666E mice (Lindahl et al. 2000, Lindfors et al. 2006).

MTC is relatively unresponsive to radiotherapy and conventional chemotherapy; thyroidectomy is the standard treatment and the only curative therapy (Wells & Santoro 2009). RET mutation analysis is largely exploited to optimize the diagnostic and clinical management of hereditary MTC (Chiefari et al. 1998, Marx 2005, Gagel & Marx 2008). The presence of a known MEN2-associated RET mutation in the germline of an MTC patient identifies hereditary MTC disease, thus allowing preclinical identification of family members at risk of developing MTC, as well as providing information about the risk of the proband developing other tumors associated with MEN2 syndromes. Moreover, the RET mutation type, assessed by RET genetic testing, guides clinical decisions, as different RET mutants have been associated with different risk profiles. Consequently, prophylactic thyroidectomy for asymptomatic mutation carriers is recommended at early ages (from the first months of life to 5 years) for higher risk mutations (codons 883/918 and 634 respectively), whereas for milder mutations surgery in carriers may be delayed (Frank-Raue et al. 2010). Moreover, the identification and functional characterization of novel activating RET mutations responsible for the disease contributes to the knowledge of genetic–phenotypic relationships in hereditary MTC.

In this study, we report the identification, in a 48-year-old woman carrying unicentric MTC with...
C-cell hyperplasia, of the RET-K666E germline variant as well as two polymorphisms: the G691S non-synonymous variant and the conservative S904S (TCC→TCG) variant. K666E and G691S RET mutations occur in the juxtamembrane domain, a region not frequently affected by mutations. In this study, the co-segregation of the K666E mutation with MTC in our patient’s family was not verifiable. However, K666E has previously been reported in three unrelated families and it is thought to be a pathogenic mutation (Ahmed et al. 2005). In this study, we have demonstrated the in vitro oncogenic potential associated with the RET-K666E mutant, based on its ability to transform NIH3T3 cells, thus suggesting that this RET variant is indeed responsible for hereditary MTC. Moreover, our results, showing that the oncogenic activity of RET-K666E is enhanced by the presence of the polymorphism G691S, suggest a possible modifier role for this known non-synonymous RET polymorphism.

Materials and methods

Patient presentation and analysis

The patient, a 48-year-old woman, underwent neck ultrasound after clinical demonstration of cervical lymphadenomegaly. At the inferior region of the left thyroid lobe, a 6×5×5 mm nodule with irregular margins was detected. A fine needle aspiration biopsy gave a cytological diagnosis of follicular proliferation with oxyphilic metaplasia, and for this reason, a left hemithyroidectomy was performed. The histological examination, however, showed that the patient displayed a unicentric MTC of 5 mm, associated with C-cell hyperplasia, and vascular invasion. Before completing the thyroidecomy, abdomen computed tomography (CT) and 24 h urinary catecholamine determination were performed in order to exclude the presence of pheochromocytoma. In addition, C-cell hyperplasia and lymphocytic chronic thyroiditis were histologically detected in the right lobe. The patient is currently in active follow-up, which includes laboratory tests (e.g. s-calcitonin; carcinoembryonic antigen (s-CEA), s-chromogranin A; s-calcium; s-parathyroid hormone (s-PTH), s-25(OH)D, u-catecholamines) and instrumental examination (e.g. neck and abdomen ultrasound; chest and abdomen CT). At present, 3 years after surgery, these diagnostic procedures provide no evidence of clinical recurrence or metastases.

The patient attended genetic counseling and family history data were collected. The clinical investigation did not reveal any history of MTC, thyroid tumors/disorders, or pheochromocytomas in her family. Her father died at the age of 66 years from liver cirrhosis. Her mother was healthy at the age of 82 years and her brother at the age of 52 years. Her sister developed breast cancer at the age of 50 years. The patient had no offspring. The patient and her sister provided a written informed consent for RET mutation analysis and for research purposes approved by the local ethical committee. To date, no other family members have consented to testing.

Mutation analysis

DNA was extracted from peripheral venous blood using a commercial kit (Qiagen Blood Extraction Kit) as recommended by the manufacturer. The screening for germline RET gene mutations was carried out in exons 8, 10, 11, 13, 14, 15, and 16, where most known mutations take place. RET exons 10, 11, 13, and 14 were analyzed by double-gradient denaturing gradient gel electrophoresis (DG-DGGE) with primers and conditions previously described (Hofstra et al. 1996). RET exons 8, 15, and 16 (together with exon 11 that showed a DG-DGGE migration pattern suggestive of base substitution) were amplified with exon flanking primers and analyzed by automated sequencing (ABIprism 377; Applied Biosystems, Foster City, CA, USA). The amplified exon 11 PCR product was cloned in pCRII-TOPO vector (Invitrogen) according to the manufacturer’s instructions, and single clones were sequenced.

Construction of the RET mutants

Recombinant plasmids carrying RET9-WT (the short isoform of the proto-RET gene), RET51-WT (the long isoform of the proto-RET gene), RET9-C634R (containing an MEN2A causing mutation), and RET9-M918T (containing the main MEN2B causing mutation) are described elsewhere (Arighi et al. 2004). Site-directed mutagenesis was performed, using an in vitro oligonucleotide mutagenesis system (QuickChange XL site-directed mutagenesis; Stratagene, La Jolla, CA, USA), on RET9-WT and RET51-WT cDNAs cloned in the eukaryotic expression vector pCDNA3 (Invitrogen), to obtain RET9-K666E, RET51-K666E, RET9-G691S and RET51-G691S constructs. The K666E single mutants were subjected to a second cycle of site-directed mutagenesis to obtain the double mutants RET9-K666E–G691S and RET51-K666E–G691S. The mutant clones were identified by direct sequencing and then entirely sequenced to exclude possible additional mutations. Plasmid DNA was extracted using the MAXI PREP Kit (Qiagen) as suggested by the supplier.
Cell culture, transfections, focus formation assay, and anchorage-independent assay

Human HEK293T cells were maintained in DMEM with 10% FCS. The recombinant plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions.

NIH3T3 cells were cultured in DMEM supplemented with 10% FCS. Stable transfection of NIH3T3 cells was performed using the CaPO4 method (Bongarzone et al. 1989) and using 250 ng plasmid DNA together with 30 µg NIH3T3-derived DNA carrier. Transfected cells were grown in DMEM with 10% FCS and selected in the presence of G418 antibiotic (650 µg/ml) to determine the transfection efficiency; transforming activity was determined in medium containing 5% serum in the presence or absence of 10 ng/ml GDNF (AlomoneLabs, Jerusalem, Israel). Both G418-resistant colonies and transformed foci were fixed and counted.

For anchorage-independent assay, cell lines NIH3T3 expressing RET mutants were suspended in DMEM + 10% FCS and plated at 1000–10 000 cells per 60 mm dish in the same medium 0.33% final agar. After 2 weeks, the colonies were stained with p-iodonitrotetrazolium violet, photographed, and counted.

Western blot analysis

Cells were serum-starved for 24 h, then lysed in ice-cold RIPA buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 2 mM EDTA, pH 8.0) supplemented with protease/phosphatase inhibitors. Protein samples were boiled in NuPAGE LDS sample buffer (Invitrogen), separated by 7% NuPAGE Novex Bis–Tris Gels with the appropriate running buffer (Invitrogen), then transferred onto nitrocellulose filters, and immunoblotted with the indicated antibodies. For densitometric analyses, blots were analyzed using the Image Quant 5.2 Software (Molecular Dynamics, GE Healthcare, CA, USA). The following antibodies were used in blotting experiments: anti-RET (C-19) from Santa Cruz Biotechnology (Santa Cruz, CA, USA), anti-phospho-RET (Tyr905) from Cell Signaling Technologies (Beverly, MA, USA), anti-phospho-Tyr 4G10 from Upstate Biotechnology (Lake Placid, NY, USA) anti-MAP kinase (ERK1/2), and anti-MAP kinase activated (pERK1/2) from Sigma–Aldrich.

Statistical analysis

Excel software was used to analyze all the data. Significance of differences was determined by two-tailed Student’s t-test with unequal variance.

Results

Patient’s and family’s RET mutation analysis

DG-DGGE analysis and/or direct DNA sequencing after PCR amplification of RET exons 8, 10, 11, 13, 14, 15, and 16, from the patient’s peripheral blood, has shown three variations in the RET gene sequence: a c.1996A>G transition in heterozygosis, leading to the missense K666E mutation, a c.2071G>A transversion in heterozygosis, leading to a missense mutation corresponding to the known non-conservative functional G691S polymorphism (SNIP rs1799939), both in exon 11 (Fig. 1B), and a c.2712C>G transversion, a silent mutation leading to the conservative S904S polymorphic variant (SNIP rs1800863) in exon 15 (not shown). The patient’s sister, the only family member who gave informed consent, carried the G691S polymorphism but not the K666E genetic variant (not shown).

Most mutations associated with hereditary MTC map in the Cys-rich region, such as the classical MEN2A-associated C634R mutation, or in the kinase domain, such as the prevalent MEN2B-associated mutation M918T (Fig. 1A); at variance, the RET-K666E variant maps in the juxtamembrane region of the receptor.

To assess whether the putative MTC-associated K666E mutation and the polymorphic G691S mutation carried by the proband were on the same allele, the amplified exon 11 PCR product was cloned in pCR2.1-TOPO vector. A total of 11 clones were sequenced: six presented both wt codons and five presented both mutated codons, thus indicating that the two mutations are on the same allele.


Site-directed mutagenesis was performed on pCDNA3 eukaryotic expression vector carrying the two RET isoforms (RET9 or RET51) to obtain the following mutants: RET9-K666E, RET51-K666E, RET9-G691S, RET51-G691S, RET9-K666E–G691S, and RET51-K666E–G691S.

To assess the quality and efficiency of recombinant plasmids, all the constructs were transiently transfected into HEK293T cells to analyze RET protein expression, as well as their associated downstream signaling on ERK1/2, as read-out of RET activation, in comparison with RET-wt, and the MEN2A- and MEN2B-associated RET-C634R and RET-M918T mutants (Fig. 1C). All the constructs express proteins of the expected molecular weight. RET protein displaying K666E amino acid variant shows a stronger ERK phosphorylation than RET-wt. The RET protein
encoded by the double mutants, RET K666E–G691S, shows an even stronger ERK phosphorylation, similar to mutant proteins encoded by RET-C634R and RET-M918T controls. The respective long isoforms behave similarly (data not shown). This preliminary analysis prompted us to analyze the biological activity of either single- or double-mutated RET forms.

To define the oncogenic potential of the K666E mutation, the RET-K666E constructs, together with vectors expressing the short and the long isoforms of RET-wt and RET-C634R, were stably transfected in NIH3T3 cells for a focus formation assay in the presence or absence of chronic stimulation with the RET ligand GDNF. G418 selection was also performed to normalize the results. Both isoforms of RET were tested, as we have demonstrated that long isoforms of oncogenic RETs display a stronger transforming activity compared with the short ones (Borrello et al. 2002), thus enhancing the possibility of identifying mild activating mutations. The results of three transfections, performed in triplicate and normalized on the number of NIH3T3 G418-resistant colonies, are shown in Fig. 2. Both RET isoforms carrying the K666E mutation show transforming activity, although significantly lower than RET carrying the classical C634R mutation \( (P<0.01) \). At variance, no transforming activity was associated with the non-synonymous polymorphic variant G691S carried by either RET isoforms. Interestingly, RET gene isoforms carrying both variants RET9- and RET51-K666E–G691S, mimicking the mutated allele of the patient, proved to be significantly more efficient \( (P<0.01) \) in focus formation compared with the respective single mutants (Fig. 2A). Moreover, RET9-K666E–G691S generates foci larger than single mutant RET9-K666E and more similar to those obtained with C634R mutation, as shown in representative plates for each RET mutant (Fig. 2B). Similar data, but with lower foci numbers (about 1/4), were obtained without GDNF ligand stimulation (data not shown).

Several NIH3T3-transformed cell lines carrying RET-K666E or RET-K666E–G691S mutants were isolated and analyzed. At variance with RET-wt expressing cells, all the NIH3T3 cells expressing RET mutants are able to grow in soft agar. The RET-K666E–G691S agar clones were generally bigger than the RET-K666E ones and comparable to RET-C634R clones (Fig. 2C).

![Bar chart](chart.png)

**Figure 2** Transforming activity by focus formation assay analysis of the RET genes variant RET-K666E, RET-G691S, and RET-K666E–G691S. NIH3T3 cells were transfected with the indicated constructs and subjected to G418 or foci selection. Plates were fixed and stored after Giemsa staining. (A) Relative transforming activity of the indicated constructs. *Statistically significant results. (B) Representative plates transfected with RET short isoform and treated with GDNF. (C) Morphology of representative agar clones (magnification 1.25×).
The same NIH3T3-derived cell lines were analyzed for RET protein expression and Tyr905 phosphorylation, as well as for ERK1/2 phosphorylation, as read-out of RET receptor activation (Fig. 3). All the analyzed transformed cell lines, either expressing RET-K666E–G691S or RET-K666E, show different levels of RET protein and are able to elicit a stronger ERK1/2 phosphorylation than the mock-transfected NIH3T3 cells. The cell lines expressing RET-K666E–G691S receptor are significantly more efficient in ERK1/2 phosphorylation (P = 0.02) than those expressing RET-K666E receptor, in keeping with the described biological effect of the respective RET mutants. RET-K666E clones show a certain correlation between RET expression and phosphorylation (evaluated either as total Tyr- (data not shown) or as Tyr905-phosphorylation) and ERK1/2 activation levels. At variance, ERK1/2 activation is high in all the RET-K666E–G691S expressing clones, despite the different levels of RET protein and autophosphorylation, thus highlighting the capability of double mutant to strongly activate ERK1/2, even at low expression level, as shown by clone 5.

Overall, our in vitro analyses suggest that K666E is a gain-of-function alteration of RET receptor kinase with a weaker oncogenic potential than the MEN2A-associated C634R mutation; however, its oncogenic ability is enhanced by the presence of the G691S polymorphism.

**Discussion**

A complete classification and functional characterization of all RET mutations involved in inherited MTC is essential for an effective genetic analysis in clinical practice. Timely thyroidectomy in family members carrying an inherited mutated RET allele prevents MTC, the most common cause of death in MEN2 syndromes (Modigliani et al. 1998).

In this study, we report the identification of three RET variants in an MTC patient: the K666E mutation, the non-synonymous polymorphism G691S, and the conservative polymorphism S904S. Through functional analysis of the corresponding RET mutants, we have demonstrated the in vitro oncogenic potential associated with RET-K666E, based on its ability to transform NIH3T3 cells, thus suggesting that this RET variant is indeed responsible for hereditary MTC. Moreover, we have shown that the oncogenic activity of RET-K666E is enhanced by the presence of G691S, suggesting a possible modifier role of this non-synonymous RET polymorphism.

Sequence variations in the intracellular juxtamembrane domain of RET have been infrequently reported and affect codons 665 and 666 (Ahmed et al. 2005, Cordella et al. 2006, Muzza et al. 2010). Both K666N and the complex K666NinsS mutations have been shown to display oncogenic potential (Cordella et al. 2006, Muzza et al. 2010). In particular, K666N and K666E mutants show a comparable transforming activity; in fact, in both cases, their transforming activity is one half compared to that of the C634R mutant (Muzza et al. 2010). Moreover, biochemical analysis of K666E shows higher amounts of fully glycosylated mutant receptor compared to RET-wt and other mutants. This feature was also previously displayed by the K666NinsS mutant (Cordella et al. 2006).

The K666E variant has already been described in three unrelated families (Ahmed et al. 2005) and it is reported as a pathogenic mutation in the web-based ARUP online Scientific Resource RET database http://www.arup.utah.edu/database/MEN2/MEN2_search.php (Margraf et al. 2009). Pathogenicity was deduced from clinical and segregation analysis, although penetrance was incomplete and one of the MEN2A/FMTC families had one discordant member displaying no mutation and a positive pentagastrin stimulation. A functional analysis for the RET-K666E variant has not been performed earlier. We have now demonstrated for the first time that K666E is a gain-of-function mutation with oncogenic potential, as it displays transforming activity by NIH3T3 focus formation assay. It is not known whether K666E is a de novo or inherited RET.
variation in the presented patient, as the family history was negative for MTC, and the carrier status of family members could be tested only for the patient’s sister, who proved negative for the mutation.

However, the RET-K666E variant has a clear-cut transforming activity, displayed by short and long isoforms of RET and in the presence/absence of the RET ligand GDNF. This is consistent with the fact that the presence of lysine residue at codon 666 is highly conserved among species, and with a recent report showing the presence of the wt codon for Lys in a series of 400 normal control alleles (Muzza et al. 2010).

Our in vitro studies show a milder transforming activity of the K666E mutation compared with the classical MEN2A-associated C634R mutation. The clinical data obtained from three families (Ahmed et al. 2005), along with the patient described in this study, showed that among 15 carriers of the K666E variant, five developed MTC (35 to 64 years old), one presented pheochromocytoma and elevated calcitonin at 35 years old, two developed C cell hyperplasia at 35 and 71 years old, and seven remained asymptomatic individuals (2–66 years old). Altogether, this variability suggests that K666E could be a mild mutation with variable expressivity and penetrance. The presence of modifier genes or of an undetected segregating mutation could explain the segregation pattern observed in these families. RET polymorphic variants might be possible modifiers contributing to the variable penetrance and expressivity associated with K666E mutation (see below).

The patient in this study presents the non-synonymous polymorphism RET-G691S in the same allele carrying the K666E mutation. RET-G691S is a common polymorphic variant with a reported allele frequency of 11–33% (ARUP database). It was shown that RET-G691S is able to increase downstream signaling compared with RET-wt (Sawai et al. 2005, Narita et al. 2009). However, this variant is not oncogenic in focus formation assays, as previously shown by Fugazzola et al. (2008) for the RET9 isoform and now confirmed in this paper for both RET isoforms. Even in the presence of GDNF ligand, no valuable transforming activity different from RET-wt background was appreciated. Regarding S904S polymorphism, also detected in the presented patient, strong co-segregation with G691S polymorphism has already been demonstrated (Gil et al. 2002, Robledo et al. 2003). Both polymorphisms have been shown to be under-represented in Hirschsprung’s disease (HSCR, OMIM 142623) patients compared with controls, thus suggesting that they might protect against the development of HSCR (Borrego et al. 1999).

The RET-G691S polymorphism has been suggested as a genetic modifier in MEN2A (Gil et al. 2002), but this issue is controversial (Lesueur et al. 2006). Conflicting results are also reported with regard to a possible role for this polymorphism in susceptibility to sporadic MTC ((Fugazzola et al. 2008) and references herein). Interestingly, the G691S polymorphism has been suggested to play a role in a specific mutation affecting K666 residue. In fact, a complex in frame insertion–deletion mutation (insTTGTdelG) at codon 666 (resulting in replacement of the lysine by asparagine and serine insertion) was detected in the germline of a 12-year-old boy displaying MTC, and in his unaffected mother and maternal grandfather. Interestingly, the MTC-affected boy, but not the unaffected family members, displayed the G691S polymorphic variant allele inherited from his father (Vandenbosch et al. 2005). Thus, the G691S polymorphism seems to enhance RET oncogenicity of distinct mutations affecting RET codon 666, by increasing its penetrance in the clinical onset (Vandenbosch et al. 2005), and its transforming activity in in vitro assay (our study). Overall, these data strengthen the potential role of the RET-G691S polymorphism in MTC. This is the first demonstration of an in vitro effect of the G691S variant on the transforming activity of a RET mutant. This effect may be related to the capacity of the polymorphic variant G691S to enhance ERK1/2 activation triggered by RET-K666E mutant receptor, demonstrated by our biochemical analysis of NIH3T3 cell lines expressing RET-K666E or RET-K666E–G691S, and previously shown for RET-wt (Sawai et al. 2005, Narita et al. 2009).

It remains to be investigated whether the effect of G691S on in vitro transforming activity is relevant for all the gain-of-function RET mutations causing hereditary MTC. Different mutations might be differentially affected by the polymorphism, depending on their ‘strength’. Alternatively, as the variation at 691 residue has been hypothesized to modify the protein structure (Lesueur et al. 2006), it might differentially synergize with specific gain-of-function mutations. Future research on this issue could contribute toward clarifying the controversial role of G691S as a genetic modifier. It is worth noting that strong modifying effects of different RET variations on the oncogenic RET-V804M mutant have been reported. In particular, the double mutants V804M–E805K, V804M–Y806C, and V804M–S940C, at variance with V804M causing FMTC, have been found in MEN2B patients (Miyauchi et al. 1999, Menko et al. 2002, Cranston et al. 2006). Thus, the risk level associated with the double mutants is significantly higher (Kloos et al. 2002, 2006).
The V804M–V778I double mutant has also been reported, and it has been classified at higher risk level than V804M (Kasprzak et al. 2001). However, at variance with our report, in these cases, the mutations in tandem with V804M are classified as ‘uncertain’ variants and not as polymorphic variants, and in some cases, they display in vitro transforming activity (ARUP database).

In conclusion, we have demonstrated the in vitro oncogenic potential of a mutation located in the juxtamembrane domain of RET detected in the germline of three families and in our patient. Moreover, we have demonstrated for the first time the possibility that the in vitro oncogenic potential of a given gain of function RET mutation might be enhanced by the presence of the functional non-oncogenic polymorphism G691S, thus suggesting a modifier role for this polymorphic RET variant. Our results also suggest that integration of patients/families genotyping with functional characterization of RET mutants can provide useful information to optimize the diagnostic and clinical management of hereditary MTC.

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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