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A New Hot Spot for Mutations in the *ret* Protooncogene Causing Familial Medullary Thyroid Carcinoma and Multiple Endocrine Neoplasia Type 2A*

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ABSTRACT

One hundred and eighty-one families with multiple endocrine neoplasia type 2A (MEN-2A) or familial medullary thyroid carcinoma (FMTC) have been investigated for mutations in the *ret* protooncogene in Germany. In 8 families with FMTC or MEN-2A, no mutation could be detected in the cysteine-rich domain encoded in exons 10 and 11 of the *ret* protooncogene. DNA sequencing of additional exons (no. 13–15) revealed rare noncysteine mutations in 3 families (codons 631, 768, and 844). In contrast to these rare events, heterozygous missense mutations in exon 13, codons 790 and 791, were found in 5 families (4 with MTC only; 1 family with MTC and pheochromocytoma) and 11 patients with apparently sporadic tumors.

Two different mutations in codon 790 (TTG→TTT, TTG→TTC; $Leu^{790}Phe$) and one mutation in codon 791 (TAT→TTT; $Tyr^{791}Phe$) created a phenylalanine residue.

We conclude that codons 790 and 791 of the *ret* protooncogene represent a new hot spot for FMTC/MEN-2A causing mutations. With the discovery of these considerably common mutations in codons 790 and 791 and the identification of some rare mutations, 100% of the German FMTC/MEN-2A families could be characterized by a mutation in the *ret* protooncogene. (*J Clin Endocrinol Metab* **83:** 770–774, 1998)

MEDULLARY thyroid carcinoma (MTC), pheochromocytoma (pheo) and primary hyperparathyroidism (pHPT) may occur as part of the autosomal dominant, inherited multiple endocrine neoplasia type 2 (MEN-2A). Missense mutations in the *ret* protooncogene have been found in five cysteine codons within exon 10 (codons 609, 611, 618, and 620) and exon 11 (codon 634) in about 92% of families with MTC only (FMTC) or MEN-2A (MTC and pheo and/or pHPT) (1–3). In addition to the common mutations, which affect cysteine residues, some rare noncysteine mutations have been described in FMTC patients (4, 5) located in exon 13, 14, and 15. These mutations do not represent more than 1% of the cases.

The *ret* protooncogene encodes a receptor tyrosine kinase that is involved in the normal development of neural crest cell lineages (6–8). Glial cell line-derived neurotropic factor (GDNF), a member of the transforming growth factor-β superfamily has been demonstrated to be the ligand for the c-Ret protein (9). The c-Ret protein forms a dimer that is associated with the glial cell-derived neurotropic factor α receptor (GDNFR-α), another membrane-bound receptor for GDNF (10, 11).

Presymptomatic identification of gene carriers by mutation analysis in the *ret* protooncogene and the option of prophylactic thyroidectomy have had a great impact on the diagnosis and management of FMTC and MEN-2A patients. In all families with an identified mutation in the *ret* protooncogene, it is possible today to perform a thyroidectomy before C cell carcinoma occurs. Therefore, genetic screening has become a routine procedure for these patients.

In addition, for patients with (apparently) sporadic MTC, the screening for common mutations in the *ret* protooncogene is used to exclude the hereditary forms of this tumor. Some patients with apparently sporadic MTC turned out to be index cases of new FMTC/MEN-2A families due to *de novo* mutations or unknown family history.

Genetic analysis is highly reliable in FMTC and MEN-2A if the specific mutation in the *ret* protooncogene of the family is known (92%). In the remaining 8% of the FMTC and MEN-2A families worldwide, no mutation has been found to date (3). To answer the question of which genetic defect causes the disease in these families, other loci in the *ret* protooncogene as well as the GDNF gene and the GDNFR-α gene are currently under investigation.

In Germany, among 181 FMTC/MEN-2A families, 8 did not have one of the common mutations in exon 10 or 11 of the *ret* protooncogene. In the present study we report the results of the molecular analysis of 5 families with an unidentified mutation and 11 patients of 305 with apparently sporadic tumors.

Subjects and Methods

Cases of sporadic MTC as well as FMTC/MEN-2A families were analyzed for mutations in the *ret* protooncogene (12). Clinical data for

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the FMTC/MEN-2A families and apparently sporadic cases with mutations in exon 13 of the *ret* protooncogene with respect to manifestation, age of diagnosis, and family history were collected from the referring physicians by a questionnaire. Patients with MTC and a negative family history were assigned as apparently sporadic.

Extraction of genomic DNA and amplification of exons 10, 11, 13, 14, 15, and 16 from the ret protooncogene

Genomic DNA was isolated from peripheral blood lymphocytes using the QIAMP blood kit (Qiagen, Hilden, Germany). PCR amplifications were carried out with the following oligonucleotide primers: exon 10, Ret10F (5′-GCAGCATTGTTGGGGGACA-3′) and Ret10R (5′-GTCCCGGCCACCCACT-3′; size of amplified fragment, 140 bp); exon 11, Ret11F (5′-CATGAGGCAGAGCATACGCA-3′) and Ret11R (5′-GACAGCAGCACCGAGACGAT-3′; size of amplified fragment, 156 bp); exon 13, Ret 13F (5′-AACTTGGGCAAGGCCATCA-3′) and Ret13R (5′-AGAACAGGGCTGTATGGAGC-3′; size of amplified fragment, 108 bp); exon 14, Ret14F (5′-AAGACCCAAGCTGCCTGAC-3′) and Ret14F (5′-GCTGGGTGCAGAGCCATAT-3′; size of amplified fragment, 294 bp); exon 15, Ret15F (5′-GTGACCGCTGCCTGGCCATGG-3′) and Ret15R (5′-CCTAGGCTTCCCAAGGACTGCCTGC-3′; size of amplified fragment, 349 bp); and exon 16, Ret16F (5′-TAACCTCCACCCCAAGAGAG-3′) and Ret16R (5′-AGGGATAGGGCCTGGGCTTC-3′; size of amplified fragment, 192 bp). One hundred nanograms of DNA were amplified in a Perkin-Elmer 9600 thermocycler in a volume of 25 μL containing 1 μmol/L of each oligonucleotide primer, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L $MgCl_2$, and 1 U *Taq* polymerase (Roche Molecular Systems, Inc., Brauchburg, NJ). The PCR was started with 1 min of denaturation at 95 C, followed by 35 cycles of 1 min each at 65, 72, and 95 C, and completed with 5 min at 72 C. The amplified DNA was analyzed on a 2% agarose gel and purified with the Qiagen Quickspin kit.

For single strand conformational polymorphism screening (exons 10, 11, 13, 14, and 15), the amplified DNA fragments were denatured in formamide-50 μmol/L ethylenediamine tetraacetate and cooled on ice before loading on the gel. Separation was carried out in a vertical gel electrophoresis apparatus in an MDE-gel (AT Biochem, Malvern, PA) at 4 C (exon 10) and 12% polyacrylamide 0.8% bis acrylamide at 45 C (exon 11), 30 C (exon 13), or room temperature (exons 14 and 15) at 240–300 mV for 10–16 h. DNA bands were visualized by silver staining according to standard procedures.

PCR-amplified DNA was sequenced by direct cycle sequencing using the fluorescent-labeled dideoxy terminators (dRhodamine Terminator Cycle Sequencing Ready Reaction Kit, Applied Biosystems, Foster City, CA) and run on the automated sequencer 377 from Applied Biosystems.

The mutation in codon 918 in exon 16 was detected by digestion with the restriction enzyme *fokI* as described by Hofstra *et al.* (13).

Results

One hundred and eighty-one FMTC/MEN-2A families have been investigated for germ-line mutations in the *ret* protooncogene since 1993 (updated from Ref. 14). In all but eight families, one of the common mutations in exon 11, codon 634 (66%), or in exon 10, codons 609, 611, 618, and 620 (23%), has been identified.

DNA sequencing of exons 10, 11, 13, 14, and 15 and mutation-specific restriction enzyme analysis of the mutation in codon 918 (exon 16 of the *ret* protooncogene) were employed to try to identify mutations in the remaining eight families.

The pedigree of a family with features of multiple endocrine neoplasia 2A, but no mutations in exon 10 or 11 of the *ret* protooncogene, is shown in Fig. 1 (pedigree A). The index patient of this family presented with pheo diagnosed at the age of 31 yr. Elevated serum calcitonin levels were detected 10 yr later, with subsequent thyroidectomy and histological diagnosis of MTC. Upon family screening, three members were identified as having pheo and MTC, and two other

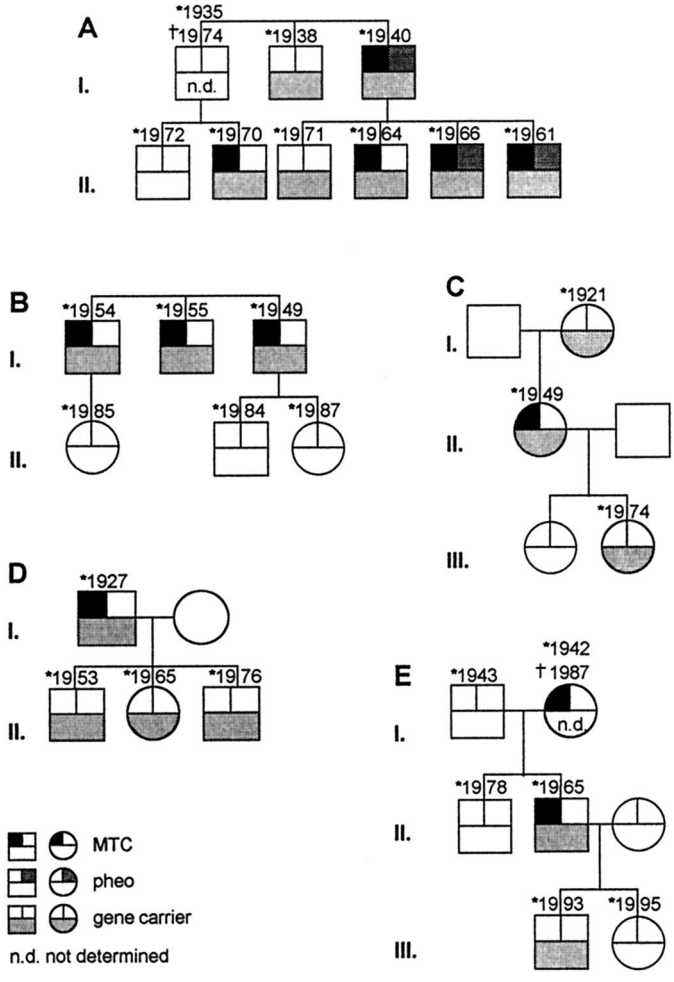

FIG. 1. Pedigrees of MTC/MEN-2A families with mutations in exon 13 of the *ret* protooncogene (families A, B, and C in codon 790; families D and E in codon 791).

members had MTC. Mutation screening in exons 10 and 11 revealed no mutation. However, sequencing of exon 13 demonstrated a heterozygous mutation in codon 790 (TTG→TTT) converting a leucine to phenylalanine (Fig. 2A). In this family, seven members carried the mutation ($Leu^{790}Phe$), and five of them were clinically affected. The unaffected gene carriers are 26 and 58 yr of age. One member of this family, who does not carry this mutation, was biochemically and clinically screened for symptoms of C cell hyperplasia and pheo with a negative outcome.

In a second family (pedigree B, Fig. 1) with three cases of MTC, DNA analysis revealed no mutation in exon 10 or 11. In this family the same heterozygous mutation in codon 790 was detected by DNA sequencing (TTG→TTC, Leu→Phe), converting leucine to phenylalanine. All three affected members carry this mutation, whereas three relatives without this mutation did not display any clinical or biochemical features of MEN-2A.

Three other pedigrees demonstrating MTC and more than one gene carrier are shown in Fig. 1 (pedigrees C–E). In pedigree C (Fig. 1), the heterozygous mutation TTG→TTT (Leu→Phe) in codon 790 of the *ret* protooncogene was de-

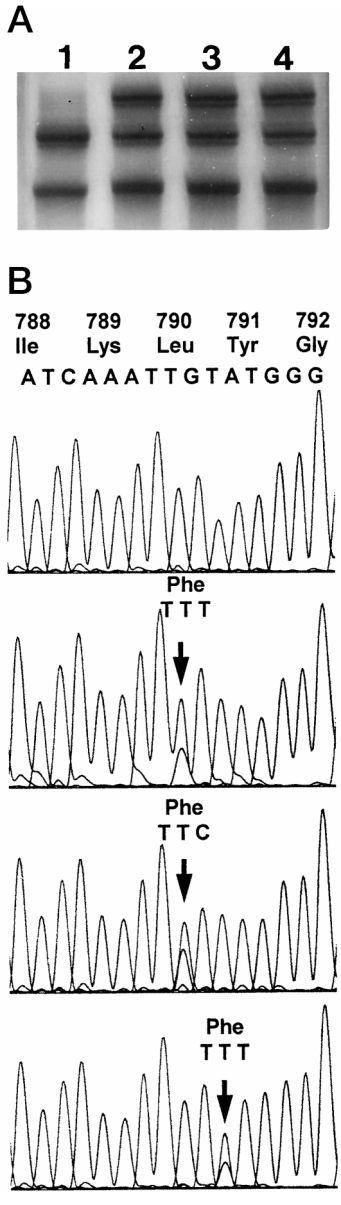

FIG. 2. Mutation analysis by single strand conformational polymorphism (SSCP) analysis (A) and DNA sequencing (B) in exon 13 of the *ret* protooncogene. A, SSCP analysis of heterozygous mutations in PCR-amplified DNA from exon 13 of the *ret* protooncogene in a 12% polyacrylamide gel. Lane 1, Healthy control; lanes 2–4, index patients; lane 2, codon 790, TTG→TTT; lane 3, codon 790, TTG→TTC; lane 4, codon 791, TAT→TTT. The DNA bands were visualized by silver staining. B, Sequence analysis of the amplified DNA from exon 13 of the *ret* protooncogene of the wild-type allele (*upper panel*) and the mutated alleles from index patients (*lower panels*). The positions of heterozygote mutations are indicated by *arrows*. The sequencing was performed directly from PCR-amplified DNA with the dRhodamine Terminator Cycle Sequencing Ready Reaction kit from Perkin-Elmer and was run on the automated sequencer 377 from Applied Biosystems.

tected. For the index patient, MTC was diagnosed at an age of 46 yr. The 71-yr-old mother of the index patient is a gene carrier, but showed no clinically symptoms of C cell hyperplasia, medullary thyroid carcinoma, or pheo. After it was discovered that she carried the mutation, a normal serum calcitonin was measured (2 pg/mL).

In pedigree D (Fig. 1) sequencing of exon 13 of the *ret* protooncogene revealed a mutation in codon 791 (TAT→TTT, Tyr→Phe) in four of five family members over two generations. One member has a clinically established MTC, and three gene carriers showed no symptoms of the MEN-2A syndrome (20, 31, and 43 yr of age).

In pedigree E (Fig. 1), MTC was diagnosed in one member at an age of 21 yr; he was subsequently cured by thyroidectomy. His father had died from MTC at the age of 45 yr. Mutation analysis revealed the mutation TAT→TTT (Tyr→Phe) in codon 791, exon 13 of the *ret* protooncogene of the index patient and his 4-yr-old son.

None of the gene carriers from the above-described families had biochemical evidence of parathyroid disease.

From the remaining three families reported to the German MTC registry with unidentified mutations in the *ret* protooncogene, one family had a heterozygous mutation in codon 768 in exon 13, a mutation previously described by others (13). In the second of the remaining families, we found a heterozygous mutation in codon 844, exon 14 of the *ret* protooncogene. For the last family a heterozygous mutation in codon 631 of exon 11 was identified. This mutation is a noncysteine mutation in the vicinity of two cysteines (630 and 634) in the conserved cysteine-rich domain of the Ret protein. Family screening is in progress.

Once our attention had been focussed on the mutations in exon 13, we also screened the sporadic cases of MTC for these mutations. Of 305 patients with apparently sporadic MTC, we discovered 24 patients (8%) with the rare mutations in exons 10 and 11 of the *ret* protooncogene and 11 (3.7%) additional patients with a mutation in exon 790 or 791 in exon 13 of the *ret* protooncogene.

The age at diagnosis ranged from 21–64 yr. Upon family screening, in three of these cases additional gene carriers were identified (families F-H). For the remaining eight families, screening is in progress. The index patient of family G presented with pheo and elevated calcitonin levels after pentagastrin stimulation. Histological examination after thyroidectomy revealed Hashimoto's thyroiditis and struma multinodosa, but no C cell carcinoma. C Cell hyperplasia cannot be ruled out by histological methods, as it is difficult to diagnose in Hashimoto's thyroiditis and struma multinodosa. The mutation analysis revealed a heterozygous germline mutation in codon 791.

To ensure that no other relevant mutations have been overlooked, we screened exons 14 and 15 as well as the MEN-2B-causing mutation in codon 918 of the *ret* protooncogene. No mutations were detectable in these exons beyond those mentioned above.

To verify that the mutations in codon 790 and 791 of the *ret* protooncogene are pathogenic mutations, we screened 200 healthy probands (blood donors from a local blood bank). The mutations were not detected in this group. Also, those members without the mutation are clinically unaffected in families with mutations in codon 790/791 of the *ret* protooncogene, which leads us to the conclusion that it is not a common variant of the *ret* protooncogene but, rather, a mutation relevant for the disease of these patients.

With the discovery of this new mutational hot spot and some rare noncysteine mutations in exons 11 and 14, 100%

of the families with FMTC/MEN-2A in the German MTC/MEN-2A registry can be characterized by a mutation in the *ret* protooncogene. Furthermore, we were able to detect mutations in 11.6% (8% exon 10 and 11; 3.6% exon 13) from a group of 305 MTC or pheo patients who were apparently sporadic cases and could now be classified as hereditary MTC.

Discussion

DNA testing for presymptomatic identification of gene carriers of affected families provides the most reliable indication for prophylactic thyroidectomy (14) if the mutation in the family can be identified. However, in approximately 8% of the families worldwide, no mutation is detectable in the *ret* protooncogene (4). For these families, clinicians have to rely on biochemical screening. The search for mutations in these families has been extended to other exons of the *ret* protooncogene by several groups and has led to the discovery of some rare mutations in individual families. Mutations have been reported in exons 13, 14, and 15 in the literature (15–17). Each of these mutations accounts for less than 1% of the families.

In this paper we describe a new hot spot for mutations in the *ret* protooncogene, leading to FMTC and pheo. Three different mutations affect two adjacent codons in exon 13 converting either leucine 790 or tyrosine 791 to phenylalanine. These mutations have not yet been reported for FMTC/MEN-2A families in other countries, where mutation screening for the *ret* protooncogene is routinely performed. It may be that these mutations are specific for the German population, possibly due to a founder effect. Two reasons argue against this hypothesis. 1) There are three different base exchanges leading to a phenylalanine codon in either 790 or 791; this means that three founders have to be postulated. 2) One of the families with this mutation has immigrated from the former Yugoslavia and has no family connections to the German population. The fact that the mutations have not been detected in other countries may reflect differences in performing mutation screening. Most of the index patients of the families with mutations in codons 790 and 791 presented initially as sporadic cases. In Germany, the MEN-2 study group has recommended mutation screening for all sporadic MTCs. The mutation analysis for exon 13 is performed by direct sequencing. In other countries, some groups only test the mutation in codon 768 by SSCP and restriction digest (18). These discrepancies, however, reflect the necessity of international guidelines for the diagnostic procedures for MTC and FMTC/MEN-2 patients as well as a quality assurance program. The mutations in codons 790 and 791 differ from the common mutations in exons 10 and 11 of the *ret* protooncogene, as they affect not the extracellular domain of the protein but, rather, the intracellular TK1 domain. The molecular pathological mechanism leading to the transformation of cells must be different. It is conceivable that the presence of a phenylalanine in codon 790 or 791 leads to activation of the receptor protein. According to the results reported by Liu *et al.* (19), at least six tyrosine residues are autophosphorylated in the Ret protein. Phosphorylation of tyrosine 791 could not be demonstrated, but also could not be excluded. One could speculate that autophosphorylation of tyrosine 791 is necessary for the appropriate ligand-dependent activation of the receptor tyrosine kinase. Phosphorylation of tyrosine 791 may be prevented either by converting it to phenylalanine or by a structural change due to a mutation of the neighboring residue (Leu→Phe) resulting in constitutive activation or inappropriate binding to substrates of the intracellular signaling pathway. Asai *et al.* reported recently that binding of the Shc adaptor depends on the autophosphorylated tyrosine 1062 in the Ret protein harboring MEN-2A or MEN-2B mutations (20). In this case the transforming activity is lost when tyrosine 1062 is mutated to phenylalanine. In contrast to these findings, we consider that the mutation of Tyr^{791} to Phe is associated with a gain of transforming activity. Transfection studies of mutated Ret protein have to be performed to characterize the precise pathological mechanism and transformational activity conferred by mutations in codons 790 and 791.

The first family discovered with a mutation in exon 13 of the *ret* protooncogene had several members who were affected either with MTC or with MTC and pheo. In this family the carrier status for the mutation correlated with the clinical symptoms, and no mutations in exon 14, 15, or 16 were present. This is also true for four other small MTC families with a hitherto unidentified mutation in the *ret* protooncogene. We, therefore, are confident that this mutation causes the disease. This conclusion is further confirmed by the finding that none of these mutations was detectable in a control group of 200 normal probands.

The phenotype of these mutations is variable. In one family, MTC and pheo occur. In one patient of this family, pheo presented as the first manifestation of the syndrome, and two members have both features. Whereas in one family, the only affected member suffers from pheo. In all of the other families and individual patients, only MTC was diagnosed. In none of the patients was parathyroid disease detected. In addition to the different expression of symptoms of MEN-2A, the age of onset can differ remarkably. It ranges from 21–64 yr. Thirteen patients of 23 (56%) developed the first manifestation of the disease between 30–50 yr of age. On the other hand, the oldest gene carrier without clinical or biochemical manifestation is aged 71 yr.

Investigation of apparently sporadic MTC for *ret* mutations led to the identification of a large number of individuals with germ-line mutations (11.6%). The newly discovered mutations in exon 13 represent one third of these cases (3.6%). Compared to the established FMTC/MEN-2A families, in which exon 13 mutations represent less than 10%, exon 13 mutations are overrepresented in the group of apparently sporadic MTC.

Due to the small number of families with exon 13 mutations and the variable expression, it is not possible to calculate the penetrance of these mutations. A low penetrance could be the reason why most of these cases were initially not recognized as familial forms and why these mutations are overrepresented in apparently sporadic cases.

The frequency of mutations in exon 13 of the *ret* protooncogene found in German FMTC/MEN-2A makes it necessary to include mutation screening for codons 790 and 791 in exon 13 into the routine protocol for DNA testing. We expect

that the mutations described in this paper will cover a significant proportion of the 8% of the FMTC/MEN-2A families with a hitherto unidentified mutation in the *ret* protooncogene.

Furthermore, we propose DNA testing for all sporadic MTC patients, as in up to 10% of the cases, they will be hereditary. Testing for the mutations in codon 790/791 also needs to be included, as these mutations are even more frequent in this group than in the already known FMTC/MEN-2A families.

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