

Mutations of the *ret* Protooncogene in German Multiple Endocrine Neoplasia Families: Relation between Genotype and Phenotype*

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ABSTRACT

It has been suggested that not only the position but also the nature of the mutations of the *ret* protooncogene strongly correlate with the clinical manifestation of the multiple endocrine neoplasm type 2 (MEN 2) syndrome. In particular, individuals with a Cys⁶³⁴-Arg substitution should have a greater risk of developing parathyroid disease. We, therefore, analyzed 94 unrelated families from Germany with inherited medullary thyroid carcinoma (MTC) for mutation of the *ret* protooncogene. In all but 1 of 59 families with MEN 2A, germline mutations in the extracellular domain of the *ret* protein were found. Some 81% of the MEN 2A mutations affected codon 634. Phenotype-genotype correlations suggested that the prevalence of pheochromocytoma and hyperparathyroidism is significantly higher in families

with codon 634 mutations, but there was no correlation with the nature of the mutation. In all but 1 of 27 familial MTC (FMTC) families, mutations were detected in 1 of 4 cysteines in the extracellular domain of the *ret* protooncogene. Half of the FMTC mutations affected codon 634. Mutations outside of codon 634 occurred more often in FMTC families than in MEN 2A families. In all but 1 of 8 MEN 2B patients, *de novo* mutations in codon 918 were found. These data confirm the preferential localization of MEN 2-associated mutations and the correlation between disease phenotype and the position of the *ret* mutation, but there was no correlation between the occurrence of hyperparathyroidism or pheochromocytoma and the nature of the mutation. (*J Clin Endocrinol Metab* 81: 1780–1783, 1996)

MEDULLARY THYROID carcinoma (MTC) may occur as part of the autosomal dominant, inherited, multiple endocrine neoplasia type 2 (MEN 2) syndromes. Three hereditary forms of MEN 2 are known: MEN 2A is characterized by MTC in virtually 100% of patients, pheochromocytoma (pheo) in about 50% of patients, and/or primary hyperparathyroidism (pHpt) in about 20% of patients. The MEN 2B syndrome consists of MTC, pheo, mucosal neuromas, ganglioneuromatosis of the gut, and marfanoid habitus. The MTC only syndrome [also known as familial MTC

(FMTC)] is not associated with other endocrinopathies, and inherited predisposition to MTC is the only disease feature (1).

Germline mutations of the *ret* protooncogene have been identified as the underlying cause of the MEN 2 and FMTC syndromes (2–6). *ret* is a member of the family of receptor tyrosine kinases and is expressed in tissues derived from neural crest. Mutations at one of five codons for cysteine in the *ret* extracellular domain occur in MEN 2A and FMTC; MEN 2B is associated with a mutation of codon 918 in the intracellular tyrosine kinase domain of *ret* (7–10). These mutations convert *ret* into a dominant transforming gene (11). Identification of mutated gene carriers by DNA analysis allows earlier identification of subjects at risk in this familial cancer syndrome and provides the basis for preventative thyroidectomy (12, 13).

Recently, two studies (3, 5) showed an association between disease phenotype and the nature and position of the *ret* mutation. Although there is some overlap, mutations in cysteine 634 are more strongly associated with MEN 2A than are mutations of cysteines farther from the cell membrane, which tend to occur in families with FMTC. Moreover, it has been suggested that patients with a Cys⁶³⁴-Arg substitution have

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a greater risk of developing parathyroid disease (3), but this could not be confirmed by others (5).

In the present study, we analyzed 94 unrelated German families with inherited MTC for *ret* mutations in exons 10, 11, and 16. We also examined the frequency, position, and nature of the mutations and the relation between genotype and phenotype.

Subjects and Methods

The members of the 94 families were classified clinically and biochemically and placed in 1 of 3 groups according to cancer syndrome, as defined in Table 1. The number of affected family members in each family with MEN 2A or FMTC is given in Table 2. The patient population for DNA analysis included 158 affected and 100 nonaffected individuals from 59 apparently independent kindreds with MEN 2A. There were 62 affected and 32 nonaffected individuals who belonged to 27 kindreds with FMTC. We examined 8 MEN 2B patients; in 7 there was no family history of MEN 2B in other family members. Clinical classification of affected individuals was based on 1) an abnormal baseline or stimulated calcitonin level and/or pathological findings of C cell hyperplasia (clusters of C cells or 50 or more C cells/visual field at a magnification of $\times 100$) or MTC at the time of thyroidectomy; 2) biochemical, morphological, or histological evidence of pheo; and/or 3) elevated serum calcium and PTH levels and/or parathyroid hyperplasia or adenoma at the time of parathyroidectomy. Screening for pheo and hyperparathyroidism was performed annually in affected and at risk individuals, using excretion of catecholamines and serum calcium measurements.

Extraction of genomic DNA and amplification of exons 10, 11, and 16 from the *ret* protooncogene

Genomic DNA was isolated using the QIAMP blood kit (Qiagen, Hilden, Germany). PCR amplifications were carried out with the oligonucleotide primer Ret19S (5'-GCAG-CATTGTTGGGGGACA-3') and Ret10Rb (5'-GTCCCGGCCACCCACT-3') for exon 10 (size of amplified fragment 140 bp), Ret20S (5'-CATGAGGCAGAGCATAACGCA-3') and Ret2C (5'-GACAGCAGCACCGAGACGAT-3') for exon 11 (156 bp), and rRet16 (TAACCTCCACCCCAAGAGAG-3') and fRet16 (5'-AGGGAT-AGGG-CCTGGGCTTC-3') for exon 16 (192 bp). A total of 100 ng DNA were amplified in a Hybaid Omnigene apparatus (Hybaid, Teddington, UK) in a volume of 25 μ L containing 1 μ mol/L of each oligonucleotide primer, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L MgCl₂, and 1 U *Taq* polymerase (Appligen, Heidelberg, Germany). 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, finally, 5 min at 72 C. The amplified DNA was analyzed on a 2% agarose gel and purified with the Qiagen Quickspin kit (Qiagen, Chatsworth, MA).

Exons 10 and 11 were screened for mutations by single strand conformational polymorphism analysis. The amplified DNA fragments were denatured in formamide and 50 μ mol/L ethylenediamine tetraacetate and cooled on ice before loading onto the gel. Separation was carried out in a vertical gel electrophoresis apparatus in 1 \times mutation detection enhancement (MDE[®]) gels (AT Biochem, Malvern, PA; exon 10) or 12% polyacrylamide at 45 C and 300 millivolts (mV) for 6 h. The amplified and denatured DNA fragment representing exon 10 was separated at 4 C and 6 V for 18 h. The fragment from exon 11 was separated at 45 C and 300 mV for 7 h. DNA bands were visualized by silver staining according to standard procedures.

PCR-amplified DNA was sequenced using either fluorescent-labeled dideoxy terminators (Prism Ready Reaction, Applied Biosystems kit, Foster City, CA) or one fluorescently labeled PCR primer.

The mutation in codon 918 in exon 16 was detected by digest with the restriction enzyme FokI, as described by Carlson *et al.* (7). If a MTC-

TABLE 2. Numbers of affected family members in each family with MEN 2A/FMTC

Affected/family	1	2	3	4	5	6	>7
No. of families with MEN 2A	10	9	11	6	5	4	14
No. of families with FMTC	9	5	5	5	1		2

causing mutation creating a new restriction site had been identified in one family member, the amplified DNA fragments from other members of this family were usually analyzed solely by restriction enzyme analysis. Restriction enzyme digests were carried out according to the instructions of the supplier. Analysis of the DNA fragments was performed by PAGE with subsequent silver staining.

Results

We identified point mutations including two insertions in all but one MEN 2A families. In 48 of 59 families (81%), mutations were detected at codon 634 (exon 11; Table 3). In 10 (17%) of the 59 MEN 2A families, a mutation at exon 10 was found. At codon 634, the most frequent base change found in MEN 2A was TGC to CGC, which altered the amino acid sequence from cysteine to arginine. This mutation was found in 22 of 59 (37%) families (Table 4).

By contrast, mutations in exon 11 were detected in 14 of 27 (52%) FMTC families and in exon 10 in 12 of 27 (44%) FMTC families (Table 3). Thus, the prevalence of mutations at codon 634 is higher in MEN 2A families (81%) than in FMTC families (52%; $P < 0.008$, by Fisher's exact test, two-tail).

In FMTC, the position and type of mutation were more heterogeneous, suggesting a correlation between the position and the type of mutation and the spectrum of tissues involved. To test this, we correlated the presence of pheo or pHpt and mutations at codon 634 in all MEN 2A and FMTC families. The data in Table 5 show a strong association ($P < 0.004$) between any mutation at codon 634 and the presence of pheo, but no association between the occurrence of pheo and any specific mutation at codon 634. The data in Table 6 show an association between a mutation in codon 634 and the presence of parathyroid disease, but no association between pHpt and any specific mutation at codon 634.

Discussion

Mutations associated with MEN 2A or FMTC in German families

Some 98% of German MEN 2A families have germline missense mutations in the extracellular domain of the *ret* protooncogene compared to 93–97% reported previously (2, 3, 5, 6, 14–16). These mutations converted the cysteines at codons 609, 618, 620 (all in exon 10), or 634 (in exon 11) to other amino acids. In our MEN 2A series, codon 611 was not affected. The types of mutations were very similar to those described in previous studies (3, 5). The most frequent mutation in MEN 2A is located at codon 634, representing 83%

TABLE 1. Classification of MEN 2 and FMTC

MEN 2A:	Families with MTC and at least 1 family member with either pheo or parathyroid disease or both
MEN 2B:	Families with MTC, with or without pheo or parathyroid disease and the characteristic phenotype
FMTC:	Families with MTC only and at least 2 family members with MTC or 1 family member with MTC and a mutation in the RET gene in which pheo and pHpt have been excluded by biochemical screening

TABLE 3. Distribution of *ret* mutations in 94 German families

Codon	No. of mutations						Total	
	Exon 10			11 Insertion		16 918		
	609	611	618	620	634			
MEN 2A	1		3	6	46	2	1	59
FMTC		1	5	6	14		1	27
MEN 2B							7	8

TABLE 4. Distribution of amino acid sequence changes in MEN 2A and FMTC

Mutation	MTC pheo pHpt (n = 18)	MEN 2A MTC pheo (n = 36)	MTC pHpt (n = 5)	FMTC MTC (n = 27)
634				
Arg	10	10	2	5
Tyr	2	9	1	5
Trp	1			1
Gly	1	1		
Phe		4	1	2
Ser	2	2		1
Insert		1	1	
620				
Arg		4		4
Tyr				1
Ser	1	1		1
618				
Phe				2
Ser	1	1		1
Arg		1		1
Gly				1
611				
Phe				1
609				
Gly		1		
None		1		1

TABLE 5. Mutations at codon 634 and pheo

	Disease phenotype		P value ^a
	MTC and pheo	MTC no pheo	
No mutation at 634	10	12	0.004
Any mutation at 634	43	19	
Specific mutation 634 CGC (Arg)	20	7	0.588
Other	23	12	

^a Determined by Fisher's exact test (two-tail).

of all disease mutations in our series and 84–94% in recent studies (3, 5). The substitution of arginine at codon 634 represents the most frequent event of all changes at this codon: 38% in our series and 46–54% in recent work (3, 5).

Some 96% of German FMTC families have mutations compared to 67–86% reported previously (3, 5, 17); 52% of mutations affect exon 11, and 44% affect exon 10. There is a strong correlation between the position of the mutation and disease phenotype; mutations in exon 11 (codon 634) are more strongly associated with MEN 2A than are mutations farther from the cell membrane (exon 10), which tend to occur in families with FMTC. Mutations elsewhere in *ret* may be responsible for the remaining few MEN 2A and FMTC families who do not have exon 10 or 11 mutations. For example, a mutation in *ret* exon 13 (codon 768), which codes for

TABLE 6. Mutations at codon 634 and primary hyperparathyroidism

	Disease phenotype		P value ^a
	MTC and pHpt	MTC no pHpt	
No mutation at 634	2	24	0.016
Any mutation at 634	21	41	
Specific mutation 634 CGC (Arg)	12	15	0.117
Other	9	26	

^a Determined by Fisher's exact test (two-tail).

part of the intracellular kinase domain, was recently found in a FMTC family (18).

Nonetheless, there is some overlap, as identical mutations (codon 634), but distinct phenotypic features, in families with MEN 2A and FMTC are observed. Beside misclassification of MEN 2A as FMTC families, *i.e.* if there are no cases of pheo or pHpt, this overlap may be explained by the influence of a modifier gene. Alternatively, MEN 2A and FMTC families may represent two, not clearly separable, subgroups of MEN 2, in which the penetrance of pheo is high (MEN 2A) or low (FMTC) (19). The same is true for parathyroid disease, as, despite identical mutations, MTC can be associated in some families with either parathyroid disease or pheo and in others with both diseases.

A codon 634 cysteine to arginine mutation does not predict parathyroid disease

Any mutation at codon 634 was more likely to be associated with pheo or parathyroid disease. This is identical to other reports (3, 5), in which there was a high prevalence of pheo (87% and 58%, respectively) in families with codon 634 mutations. German results do not support the earlier report of Mulligan *et al.* (9), who found a codon 634 cysteine to arginine mutation to be predictive of parathyroid disease. Only 32% of German families with codon 634 cysteine to arginine mutations had parathyroid disease [88% in Mulligan's series (3) and 41% in Schuffenecker's (5)]. Therefore, German results mirror those of Schuffenecker *et al.* (5). The discordance between our results and those of Mulligan might be explained by 1) the low number of families with pHpt in both studies; 2) the exclusion of one third of all families for statistical analysis because of a lack of documented screening for parathyroid disease in the Mulligan study, and/or 3) the result of a founder effect leading to a relatively high prevalence of a codon 634 CGC mutation in a particular population.

In all but one of our seven patients with MEN 2B, there was a single identical point mutation at codon 918, a substitution of methionine by threonine in the catalytic core region of the tyrosine kinase domain. In only one familial case displaying the typical clinical features of MEN 2B was no mutation in codon 918 found; in this case no mutation in exons 10 and 11 was identified.

This suggests that a mutation at codon 918 is crucial for *ret* gene function. Recent results show that the MEN 2B mutation altered *ret* catalytic properties both quantitatively and qualitatively, thus altering the substrate specificity of *ret* (11). Therefore, a mutation at codon 918 of the *ret* gene is the most

prevalent genetic defect causing MEN 2B, but rare MEN 2B cases are associated with different mutations that have yet to be defined.

Conclusion

Three clinically distinct, autosomal dominant, inherited conditions involving MTC have been associated with germline mutations in *ret*. These three syndromes, MEN 2A, MEN 2B, and FMTC, involve different tissues in different combinations. We found a significant correlation between a particular syndrome, and thus the spectrum of tissues involved, and the site of the mutation. The *ret* 918 mutation is exclusively seen in MEN 2B syndrome; the *ret* 634 mutation is preferentially associated with MEN 2A and is strongly predictive of pheo and parathyroid disease.

Addendum

Meanwhile in the genetically unclassified MEN 2A family, a missense germline mutation in exon 13, and in the FMTC family in exon 14 could be detected, though the number of families with unidentified mutations decreased to one MEN 2B family.

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