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Mutation analysis of the RET proto-oncogene in Dutch families with MEN 2A, MEN 2B and FMTC: two novel mutations and one de novo mutation for MEN 2A

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Abstract Hereditary C-cell carcinoma is encountered in multiple endocrine neoplasia type 2A (MEN 2A), MEN 2B, and familial medullary thyroid carcinoma (FMTC). Mutations of the RET proto-oncogene are associated with all three diseases. To obtain an insight into the molecular heterogeneity of MEN 2 syndromes and FMTC in the Netherlands, probands of 20 MEN 2A families, two FMTC families, and seven MEN 2B families were analyzed by the polymerase chain reaction (PCR), DNA sequencing, and restriction enzyme digestion for abnormalities in the RET proto-oncogene. RET mutations were found in all cases. All MEN 2A families had a mutation involving one of five cysteine codons in exons 10 and 11 of RET. Two novel dinucleotide mutations and a de novo mutation were found. Both FMTC families had a mutation of the Cys at codon 618. All MEN 2B probands carried a Met to Thr mutation in exon 16. All mutations could be confirmed by restriction enzyme digestion of PCR amplicons. Identification of the RET mutation in the Dutch population with hereditary C-cell carcinoma facilitates genetic testing for families or individuals at risk for MEN 2A, FMTC, and MEN 2B.

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Introduction

Thyroid C-cell carcinoma is encountered in three different hereditary diseases. Multiple endocrine neoplasia type 2A (MEN 2A) is a syndrome characterized by medullary thyroid carcinoma (MTC) in almost all gene carriers, pheochromocytoma in about 50% of the gene carriers, and hyperparathyroidism in about 20% of gene carriers (Sipple 1961). Only tumors derived from thyroid C-cells develop in familial medullary thyroid carcinoma (FMTC) families (Farndon et al. 1986). Multiple endocrine neoplasia type 2B (MEN 2B) is also characterized by MTC and pheochromocytoma, but the age of onset of disease is earlier. In addition, MEN 2B gene carriers develop mucosal neuromas, intestinal ganglioneuromas, and a marfanoid habitus. Parathyroid glands are generally not involved (Schimke et al. 1968).

In 1993, the RET proto-oncogene was identified as the gene associated with MEN 2A, FMTC, and MEN 2B (Donis-Keller et al. 1993; Mulligan et al. 1993; Carlson et al. 1994; Eng et al. 1994; Hofstra et al. 1994). MEN 2A and FMTC are associated with single missense mutations that involve one of five cysteine residues that are encoded by exons 10 and 11 of the RET gene. The mutations lead to amino acid substitutions in the extracellular part of the protein (Donis-Keller et al. 1993; Mulligan et al. 1993). One FMTC family has been shown to have a mutation in codon 768 for glutamine in the tyrosine kinase domain of RET (Eng et al. 1995). A single RET mutation is found in all familial and de novo MEN 2B patients. The T2948C mutation in codon 918, in exon 16, leads to the substitution of a threonine for a methionine in the tyrosine kinase domain of the protein (Hofstra et al. 1994).

To obtain insights into the molecular heterogeneity of MEN 2 syndromes and FMTC in the Netherlands, and to facilitate genetic testing for families or individuals at risk, probands of 20 MEN 2A families, two FMTC families, and seven MEN 2B families were analyzed by the polymerase chain reaction (PCR). DNA sequencing and restriction enzyme digestion for abnormalities in the RET proto-oncogene were also used.

Table 1 Sequences of oligonucleotides used for PCR, ACRS-PCR, and DNA sequencing. The *underlined* nucleotides represent a mismatch with the normal RET sequence

Name	Sequence oligonucleotide
10F	5′-GAGGCTGAGTGGGCTACGTC-3′
10R	5′-AGACCTCTGTGGGGCTGGGA-3′
11F	5′-CCTCTGCGGTGCCAAGCCTC-3′
11R	5′-TCCGGAAGGTCATCTCAGCT-3′
16F	5′-AGGGATAGGGCCTGGGCTTC-3′
16R	5′-TAACCTCCACCCCAAGAGAG-3′
ACRS-C611Y	5′-TGGCTATGGCACCTGCACCT-3′
ACRS-C618S	5′-CTTCCCTGAGGAGGAGGAGT-3′

Materials and methods

Families were categorized as MEN 2A when several members had both MTC and pheochromocytoma. Families were classified as FMTC when affected members developed only MTC without evidence of pheochromocytoma or parathyroid disease. All 29 MEN 2A, FMTC, and MEN 2B probands were of Dutch-Caucasian origin. This study was reviewed by the ethics committee of our hospital, and all persons included in the study gave their informed consent.

High-molecular-weight DNA was isolated from white blood cells according to established procedures. Exons 10, 11, and 16 of the RET gene were amplified by PCR using the primers mentioned in Table 1. The amplification was performed for 33 cycles of 1 min at 94°C, 1 min at 60°C, and 1 min at 72°C. The reaction mixture contained 400 ng DNA, 400 ng of each primer, 50 µg/ml bovine serum albumin, 10% dimethyl sulfoxide, 0.2 m*M* of each dNTP, 16.6 m*M* $(NH_4)_2SO_4$, 67 m*M* TRIS/HCl pH 8.8, 6.7 m*M* $MgCl_2$, 6.7 µ*M* EDTA pH 8.0, 10 m*M* β-mercaptoethanol, 1 U AmpliTaq polymerase (Perkin Elmer Cetus, USA). The amplified DNA was purified by gel electrophoresis on 1% ultra-low gelling-temperature agarose (Sigma, St. Louis, USA). The excised fragments were directly sequenced by the dideoxy chain termination reaction with a pUC sequencing kit (Boehringer Mannheim, Germany), using the PCR oligonucleotides as sequencing primers.

The detected family-specific point mutations in RET were confirmed by analysis of the PCR products by restriction enzyme digestion. All mutations, with the exception of Cys611Tyr and Cys618Ser, resulted in the creation or disappearance of a cleavage site for a restriction enzyme. For the mutations Cys611Tyr and Cys618Ser, an amplification-created restriction site (ACRS)-PCR was applied to identify the mutation. The Cys611Tyr mutation was identified by PCR with the forward primer ACRS-C611Y, as it leads to the loss of a *Pvu*II restriction site when a G2027C mutation is present. The forward oligonucleotide primer ACRS-C618S creates a novel *Hin*fI site after PCR when a G2048C mutation is present. Restriction enzyme digests were analyzed on ethidium-bromide-stained 3% agarose gels.

Results and discussion

All 22 probands from MEN 2A and FMTC families showed heterozygosity for missense mutations of the RET gene in which one of the codons for cysteine was involved. The seven probands with MEN 2B showed heterozygosity for the missense mutation in codon 918. The results of the genetic testing are summarized in Table 2. For MEN 2A, seven different mutations, including two novel mutations, were found at the codons for Cys611 and Cys620 in exon 10, and Cys634 in exon 11. A Cys634Arg mutation was found in 12 out of 20 probands investigated. One of these 12 probands presented with an apparently sporadic MTC. However, a Cys634Arg mutation was found both in the tumor DNA and in DNA from peripheral blood lymphocytes. MTC was not observed in other family members. It was possible to analyze DNA from both parents of this subject. Since the Cys634Arg mutation could not be demonstrated in either parent, it was concluded that this subject had a de novo MEN 2A mutation. The Cys611Tyr and Cys634Tyr mutations were each found twice. Mutations Cys634Trp, Cys634Gly, and Cys620Arg were found once. The proband with the Cys620Arg substitution showed a dinucleotide change, TTC TGC to TTT CGC, involving codons 619 and 620. The C-T transition in codon 619 does not result in an amino acid substitution. In the family with the Cys620Arg mutation, MEN 2A is associated with Hirschsprung's disease.

Table 2 Results of screening for mutations of the RET proto-oncogene in probands with MEN 2A, FMTC, and MEN 2B in The Netherlands

Mutation[a]	Amino acid	Probands[b]	Phenotype	Enzyme[c]
G2027A	C611Y	2	MEN 2A	ACRS *Pvu* II –
G2048A	C618Y	1	FMTC	*Rsa*I +
G2048C	C618S	1	FMTC	ACRS *Hin*fI +
CT2052TC	C620R	1	MEN 2A	*Nru*I +
T2095G	C634G	1	MEN 2A	*Sau*96I +
T2095C	C634R	12 (2)	MEN 2A	*Cfo*I +
G2096A	C634Y	2	MEN 2A	*Rsa*I +
C2097G	C634W	1 (1)	MEN 2A	*Cfo*I +
CC2097GG	C634WR635G	1 (1)	MEN 2A	*Bmy*I +
T2948C	M918T	7 (4)	MEN 2B	*Fok*I –

[a] The number denotes the position of the nucleotide substitution in the cDNA sequence, where nucleotide 1 is the start of transcription
[b] The number in parenthesis is the number of families reported in Hofstra et al. (1994) and Lips et al. (1994)
[c] + denotes the creation of a new restriction site, – denotes the disappearance of an existing restriction site by the mutation

One proband showed a dinucleotide change involving codons 634 and 635, TGC CGC to TGG GGC, causing a CysTrp to ArgGly substitution. This proband represents a family where MEN 2A is associated with cutaneous lichen amyloidosis. The two FMTC families both have a mutation of codon 618: one proband had the mutation Cys618Ser, and the other Cys618Tyr. All seven MEN 2B probands had a Met918Thr mutation.

The sequence data were confirmed by analysis of the PCR products by restriction enzyme digestion. This was possible because all mutations, with the exception of Cys611Tyr and Cys618Ser, result in the creation or disappearance of a cleavage site for a restriction enzyme. For the mutations Cys611Tyr and Cys618Ser, an ACRS-PCR was applied to identify the mutation. For identification of the Cys611Tyr mutation, PCR with the forward primer ACRS-C611Y (TGGCTATGGCACCTGCAGCT) leads to the loss of a *Pvu*II restriction site (CAGCTG) when the G2027C mutation is present. The forward oligonucleotide primer ACRS-C618S (CTTCCCTGAGGAGGAGGAGT)

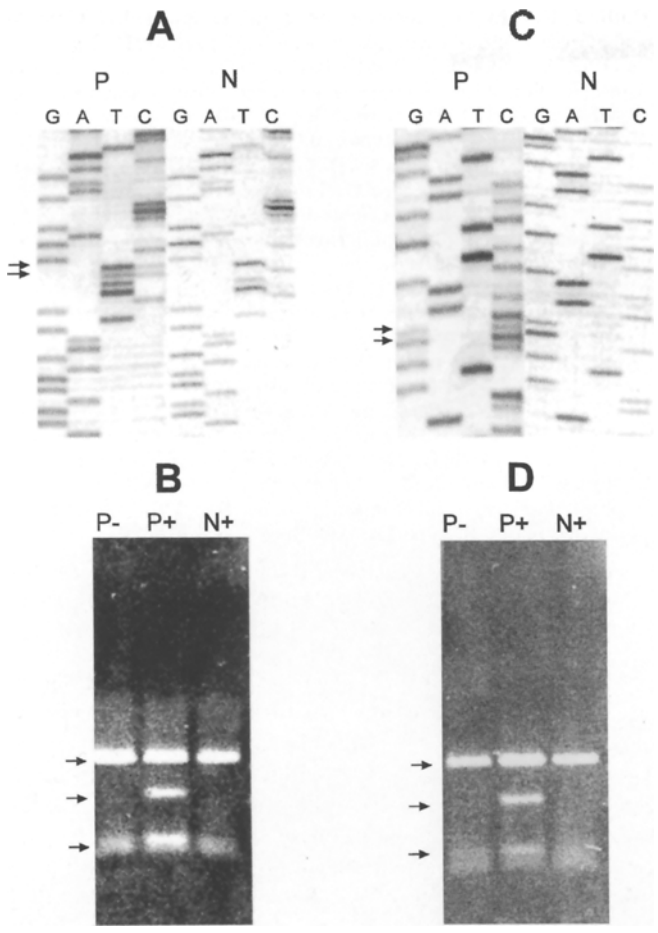

Fig. 1 **A** DNA sequence analysis of exon 10 of the RET gene in the proband with the CT2055TC mutation (*P*) and a normal control (*N*). Heterozygosity is indicated by *arrows*. The sequencing primer was 10F. **B** Analysis of the exon 10 amplicon by digestion with restriction enzyme *Nru*I in the proband with the CT2055TC mutation (*P*) and a normal control (*N*). + = with *Nru*I, – = without *Nru*I. **C** DNA sequence analysis of exon 11 of the RET gene in the proband with the CC2097GG mutation (*P*) and a normal control DNA (*N*). Heterozygosity is indicated by *arrows*. The sequencing primer was 11R. **D** Analysis of the exon 11 amplicon by digestion with restriction enzyme *Bmy*I in the proband with the CC2097GG mutation (*P*) and a normal control (*N*). + = with *Bmy*I, – = without *Bmy*I

creates a novel *Hin*fI site (GANTC) after PCR when the G2048C mutation is present (Table 2). The sequence analysis and analysis with restriction enzymes of amplicons of the probands with the Cys620Arg and the Cys634Trp/Arg635Gly mutation, which represent MEN 2A mutations not reported before, are shown in Fig. 1. Both probands show mutations of two nucleotides affecting two consecutive codons. The restriction enzymes *Nru*I (for Cys620Arg) and *Bmy*I (for Cys634Trp/Arg635Gly) specifically recognize these dinucleotide changes.

In earlier studies, haplotypes of the alleles harboring the MEN 2A or FMTC mutation could be established. Although some of the families have identical mutations of the RET gene, no inter-familial relationships could be demonstrated (data not shown). The nature and distribution of the mutations reported in this study are similar to those in previous reports (Mulligan et al. 1993, 1994; Quadro et al. 1994). Two novel mutations, both comprising dinucleotide changes, have been found. Donis-Keller et al. (1993) have reported a low incidence of Cys634 mutations for North American families. In our panel, 17 out of 20 MEN 2A families have a Cys634 mutation; in 12 of these 17 cases, the cysteine is replaced by an arginine. Interestingly, the two Dutch FMTC families have a mutation of Cys618. In other reports FMTC is not confined to codon 618 and FMTC mutations are found in all cysteine codons that are also involved in MEN 2A. However, no FMTC family had the Cys634Arg mutation that seems to predispose to a more severe course of the disease, including development of pheochromocytoma and hyperparathyroidism. As more mutation studies are published, it may become clear that the course of the disease in MEN 2A and FMTC families is related to specific mutations (Mulligan et al. 1994). The MEN 2B phenotype is clearly related to the Met918Thr mutation (Carlson et al. 1994; Eng et al. 1994; Hofstra et al. 1994).

The probands of all families were tested only for mutations of exons 10, 11, and 16 of the RET proto-oncogene. In the Netherlands, there were no families with hereditary MTC without a constitutional RET mutation of one of these exons. The identification of specific disease-related mutations in the RET gene enables a highly reliable, presymptomatic diagnosis for family members at risk for MEN 2A, FMTC, or MEN 2B. This is important because early diagnosis and appropriate treatment, e.g., presymptomatic thyroidectomy, significantly decrease the morbidity of the disease (Lips et al. 1994; Wells et al. 1994). Furthermore, it is now possible to identify gene carriers on an individual basis, once the family-specific RET mutation is known, without the mandatory involvement of other family members.

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References

Carlson KM, Dou S, Chi D, Scavarda N, Toshima K, Jackson CE, Wells SA Jr, Goodfellow PJ, Donis-Keller H (1994) Single missense mutation in the tyrosine kinase catalytic domain of the RET proto-oncogene is associated with multiple endocrine neoplasia type 2B. Proc Natl Acad Sci USA 91: 1579–1583

Donis-Keller H, Dou S, Chi D, Carlson KM, Toshima K, Lairmore TC, Howe JR, Moley JF, Goodfellow PJ, Wells SA (1993) Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. Hum Mol Genet 2: 851–856

Eng C, Smith DP, Mulligan LM, Nagai MA, Healey CS, Ponder MA, Gardner E, Scheumann GFW, Jackson CE, Tunnacliffe A, Ponder BAJ (1994) Point mutation within the tyrosine kinase domain of the RET proto-oncogene in multiple endocrine neoplasia type 2B and related sporadic tumours. Hum Mol Genet 3: 237–241

Eng C, Smith DP, Mulligan LM, Healey CS, Zvelebil MJ, Stonehouse TJ, Ponder MA, Jackson CE, Waterfield MD, Ponder BAJ (1995) A novel point mutation in the tyrosine kinase domain of the RET proto-oncogene in sporadic medullary thyroid carcinoma and in a family with FMTC. Oncogene 10:509–513

Farndon JR, Leight GS, Dilley WG, Baylin SB, Smallridge RC, Harrison TS, Wells SA (1986) Familial medullary thyroid carcinoma without associated endocrinopathies: a distinct clinical entity. Br J Surg 73:278–281

Hofstra RMW, Landsvater RM, Ceccherini I, Stulp RP, Stelwagen T, Luo Y, Pasini B, Höppener JWM, Ploos van Amstel JK, Romeo G, Lips CJM, Buys CHCM (1994) A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. Nature 367:375–376

Lips CJM, Landsvater RM, Höppener JWM, Geerdink RA, Blijham G, Jansen-Schilhorn van Veen JM, Van Gils APG, De Wit MJ, Zewald RA, Berends MJH, Beemer FA, Brouwers-Smalbraak J, Jansen RPM, Ploos van Amstel JK, Van Vroonhoven TJMV, Vroom TM (1994) Clinical screening as compared with DNA analysis in families with multiple endocrine neoplasia type 2A. N Engl J Med 331:828–835

Mulligan LM, Kwok JBJ, Healy CS, Elsdon MJ, Eng C, Gardner E, Love E, Mole SE, Moore JK, Papi L, Ponder MA, Telenius H, Tunnacliffe A, Ponder BAJ (1993) Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia syndrome type 2A. Nature 363:458–460

Mulligan LM, Eng C, Healey CS, Clayton D, Kwok JBJ, Gardner E, Ponder MA, Frilling A, Jackson CE, Lehnert H, Neumann HPH, Thibodeau SN, Ponder BAJ (1994) Specific mutations of the RET proto-oncogene are related to disease phenotype in MEN 2A and FMTC. Nature Genet 6:70–74

Quadro L, Panariello L, Salvatore D, Carlomagno F, Del Prete M, Nunziata V, Colantuoni V, Di Giovanni G, Brandi ML, Mannelli M, Gheri R, Verga U, Libroia A, Berger N, Fusco A, Grieco M, Santoro M (1994) Frequent RET proto-oncogene mutations in multiple endocrine neoplasia type 2A. J Clin Endocrinol Metab 79:590–594

Schimke RN, Hartmann WH, Prout TE, Rimoin DL (1968) Syndrome of bilateral pheochromocytoma, medullary thyroid carcinoma and multiple neuromas: a possible regulatory defect in the differentiation of chromaffin tissue. N Engl J Med 279:1–7

Sipple JH (1961) The association of pheochromocytoma with carcinoma of the thyroid gland. Am J Med 31:163–166

Wells SA Jr, Chi DD, Toshima K, Dehner LP, Coffin CM, Dowton SB, Ivanovich JL, DeBenedetti MK, Dilley WG, Moley JF, Norton JA, Donis-Keller H (1994) Predictive DNA testing and prophylactic thyroidectomy in patients at risk for multiple endocrine neoplasia type 2A. Ann Surg 220:237–250