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ORIGINAL ARTICLE

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Mutational analysis of the *RET* proto-oncogene in 71 Japanese patients with medullary thyroid carcinoma

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Abstract Multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B) and familial medullary thyroid carcinomas (FMTC) are caused by germline mutations in the *RET* proto-oncogene. To investigate the spectrum of *RET* mutations among Japanese patients, we screened the *RET* gene in 71 patients with thyroid carcinomas. The panel included representatives of 44 families carrying FMTC or MEN2, 22 sporadic medullary thyroid carcinomas (MTCs), and five MTCs without familial information. Mutations in nucleotide sequences encoding one of three specific cysteine residues in the extracellular domain of the *RET* protein were found in 33 of the 34 MEN2A patients and in five of the six FMTC patients examined. A mutation at codon 918, causing the substitution of threonine for methionine in the tyrosine kinase domain of the protein, was found in germline DNAs of all four patients with MEN2B and in two of the 22 patients with sporadic MTCs; codon 918 was mutated somatically in tumor DNAs from three other sporadic cases. Germline mutations of codon 768, GAG to GAC (Glu to Asp), were detected in one FMTC, in one patient with sporadic MTC, and in one of the patients without familial information. Two somatic mutations, an Asp to Gly substitution at codon 631 and a Cys to Arg substitution at codon 634, had not been reported previously. Of five germline mutations found among the 22 sporadic cases, four were confirmed as de novo mutations since in each case neither parent carried the mutation. As nearly one-fourth of the patients with sporadic MTCs carried germline mutations and 50% of their children are expected to develop MTC and other endocrine tumors, these results indicated the importance of careful clinical surveillance of family members of any patient with MTC.

Key words Multiple endocrine neoplasia type 2 (MEN2) · Familial medullary thyroid carcinoma (FMTC) · Sporadic medullary thyroid carcinoma (MTC) · *RET* proto-oncogene

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Introduction

Multiple endocrine neoplasia types 2A and 2B (MEN2A and MEN2B), and familial medullary thyroid carcinoma (FMTC) are autosomal, dominantly inherited syndromes involving endocrine tumors (Schimke 1984; Farnden et al. 1986). MEN2A is characterized by medullary thyroid carcinoma (MTC), pheochromocytoma (pheo), and parathyroid hyperplasia; MEN2B is characterized by MTC, pheo, mucosal ganglioneuroma, and marfanoid habitus. Affected individuals in FMTC families develop MTC without any other abnormalities. The gene(s) responsible for MEN2A, MEN2B, and FMTC were mapped to chromosome 10q11.2 by genetic linkage analyses involving a large number of affected families (Mathew et al. 1987; Simpson et al. 1987). Subsequent mutational screening of the *RET* gene, located within the candidate region, identified point mutations resulting in substitution of Cys residues at codons 609, 611, 618, 620, or 634 in the extracellular domains of the *RET* protein (Donis-Keller et al. 1993; Mulligan et al. 1994; Shirahama et al. 1995) in patients with MEN2A and FMTC. A T-to-C transition at codon 918 in the tyrosine kinase domain, causing substitution of threonine for methionine, was found in patients carrying MEN2B. The same mutations have been reported as somatic events in sporadic

MTC tumors (Hofstra et al. 1994; Eng et al. 1994). Mutations at codon 768, GAG to GAC (Glu to Asp), in the region (exon 13) that encodes part of the intracellular tyrosine kinase domain of RET, have also been detected in some patients with FMTC or sporadic MTC (Eng et al. 1995a).

The *RET* proto-oncogene encodes a receptor tyrosine kinase which is involved in normal development of the neural crest lineage. Glial cell-derived neurotrophic factor (GDNF), a member of the transforming growth factor (TGF)-β superfamily, is a ligand for the *c-RET* proto-oncogene product (Trupp et al. 1996; Durbec et al. 1996). However, the physiological relevance of GDNF and the existence of other ligands is an open question.

To characterize germline and somatic mutations of the *RET* oncogene in Japanese patients, we performed polymerase chain reaction — single-strand conformation polymorphism (PCR-SSCP); PCR coupled with digestion with restriction enzymes; and/or DNA sequencing of exons 10, 11, 13, and 16 of the *RET* gene in DNAs from representatives of 44 families carrying hereditary MTCs (34 MEN2A, 4 MEN2B, and 6 FMTC), on 22 patients with sporadic MTCs and on five MTC patients without familial information. We report here the mutational spectrum of *RET* in MEN2 and its related syndromes in this panel of patients.

Materials and methods

Patients and families

The panel of 71 patients gathered for our study consisted of representatives of 44 unrelated families carrying MEN2A (34 patients, including the 8 families reported by Shirahama et al. 1995), MEN2B (four patients), or FMTC (six patients) as well as 22 patients with sporadic MTC and 5 without available family histories. Diagnoses of MTC, pheochromocytoma, and parathyroid hyperplasia were confirmed by histopathological examination of the tumors. In some cases, measurements of blood pressure, urinary or plasma catecholamines, and calcitonin contributed to the diagnosis.

DNA extraction

Genomic DNA was extracted from peripheral leukocytes or frozen MTC tissues using a DNA extraction kit (TALENT srl., Italy), according to methods described in the manufacturer's instructions. Some tissues had been embedded in paraffin; in those cases sections were deparaffinized twice with xylene at 50°C and washed in absolute ethanol. Dried samples were treated with 1 mg/ml proteinase K and 1% SDS in 2 ml TEN buffer (0.5 M Tris-HCl, 16 mM ethylenediaminetetraacetic acid (EDTA), 10 mM NaCl, pH9.0) overnight at 50°C; DNA was then purified by phenol-chloroform extraction and precipitated by ethanol. We obtained DNAs of 12 healthy individuals as normal controls.

SSCP analysis

The oligonucleotide primer sequences required for amplifying exons 10 and 11 of *RET* by the PCR were 10FM (5′-CAGCATTGTTGGGGGACACGA-3′) and 10RL (5′-TTGTTGGACCTCAGATGTGC-3′) for exon 10, and 11FJ (5′-GCAG CCTGTACCCAGTGGTG-3′) and 11RH (5′-ACCGGAAGAGGAGTAGCTG-3′) for exon 11 (Shirahama et al. 1995). Each 5-μl reaction mixture contained 50 ng of genomic DNA, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.75 mM $MgCl_2$, 5 mM NH_4Cl, 200 mM dNTPs, 0.5 U Taq polymerase (AmpliTaq, Perkin Elmer-Cetus, Norwalk, CT, USA), 1.5 μCi α[^{32}P]dCTP, and 10 μM each of primers 10FM and 10RL, or 11FJ and 11RH. PCR conditions consisted of 30 s at 94°C, 60 s at 60°C, and 60 s at 72°C for 35 cycles. The expected sizes of the PCR products were 193 bp for exon 10 and 279 bp for exon 11. Reaction mixtures were diluted 1:10 with 45 μl of reaction-stop buffer (95% formamide, 20 mM ethylenediaminetetraacetic acid (EDTA), 0.05% bromophenol blue, 0.05% xylene cyanol). SSCP analysis was done as described by Orita et al. (1989).

DNA sequencing

Exons 10 and 11 of the *RET* gene were amplified separately from genomic DNAs of affected individuals. 100 ng of each DNA was used as a template in 25 μl of the same reaction mixture and the same primers described for SSCP, except for the absence of α[^{32}P]dCTP. PCR conditions were also the same as for SSCP. As before, the sizes of expected PCR products were 193 bp for exon 10 (116-bp exonic sequence and flanking intronic sequences) and 279 bp for exon 11 (194-bp exonic sequence and flanking intronic sequences). Direct DNA sequencing was performed with primers 10RK (5′-GTGCTGTTGAGACCTCTGTG-3′) for exon 10 or 11RG (5′-CCTCCGGAAGG TCATCTC-3′) for exon 11, using a T7 sequencing kit (Pharmacia Biotech, Uppsala, Sweden) according to the manufacturer's instructions.

PCR-restriction fragment length polymorphism (RFLP) analysis of exons 13 and 16

Genomic DNAs corresponding to exons 13 or 16 with their flanking intronic sequences were amplified using the primers CRT4E (5′-GGAGAACAGGGCTG CATGGA-3′) and CRT4F (5′-GCAGGCCTCTCTGTCTGAACTT-3′) for exon 13 (Eng et al. 1995a), or fRET16 (5′-AGGGATAGGGCCTGGGCTTC-3′) and rRET (5′-TAACCTCCACCCCAAGAGAG-3′) for exon 16 (Hofstra et al. 1994) under the same PCR conditions as just described. The products were digested for 3 h with 2 U of enzyme (AluI for the amplified sequences corresponding to exon 13, or FokI for exon 16) in the restriction buffer recommended by the manufacturer (Boehringer-Mannheim, Mannheim, Germany). Samples were separated on 3% agarose gels.

Results

By means of SSCP analysis and genomic sequencing of exons 10 and 11 of the *RET* proto-oncogene in DNAs from the 71 patients in our panel, we found 12 different patterns of SSCP variation. Seven of these patterns were found among PCR products corresponding to exon 10; five corresponded to exon 11. PCR products from the 12 types of variant were analyzed by means of asymmetric PCR followed by DNA sequencing. In accordance with the SSCP results for exon 10, we found seven different missense point mutations involving codons 618 or 620, both of which encode cysteine. For codon 618, TGC (Cys) was substituted to GGC (Gly), AGC (Ser), CGC (Arg), or TCC (Ser); for codon 620, TGC (Cys) was changed to GGC (Gly), CGC (Arg), or TCC (Ser). The five variants found for exon 11 were also identified as missense mutations: at codon 631, GAC (Asp) was substituted to GGC (Gly); at codon 634, TGC (Cys) was altered to CGC (Arg), TGG (Trp), TAC (Tyr), or TCC (Ser). Two of the codon-634 mutations had arisen de novo in germlines of patients with sporadic MTC (Fig. 1). In total, we found that 33 of the 34 MEN2A patients and five of the six FMTC patients carried point mutations at one of three specific codons for cysteine in exons 10 and 11 (Table 1).

Subsequently, the four families with MEN2B, as well as the one family with FMTC, the 18 patients with clinically

Table 1 Distribution of *RET* mutations in endocrine neoplasias

Disease	*n*	Exon 10				11	13	16	Other	Total
		Codon 609	611	618	620	634	768	918		
Germline mutation										
MEN2A	34[a]	0	0	3	7	23	0	0	0	33
FMTC	6	0	0	2	2	1	1	0	0	6
MEN2B	4	0	0	0	0	0	0	4	0	4
Sporadic MTC	22	0	0	0	0	2	1	2	0	5
FH unknown	5[b]	0	0	1	0	3	1	0	0	5
Somatic mutation										
Sporadic MTC	22	0	0	0	0	1	0	3	1[c]	5

FMTC, familial medullory thyroid carcinoma.
[a] Includes the eight families reported by Shirahama et al. (1995).
[b] Family history not confirmed.
[c] This somatic mutation is an alteration of GAC to GGC (Asp to Gly) at codon 631.

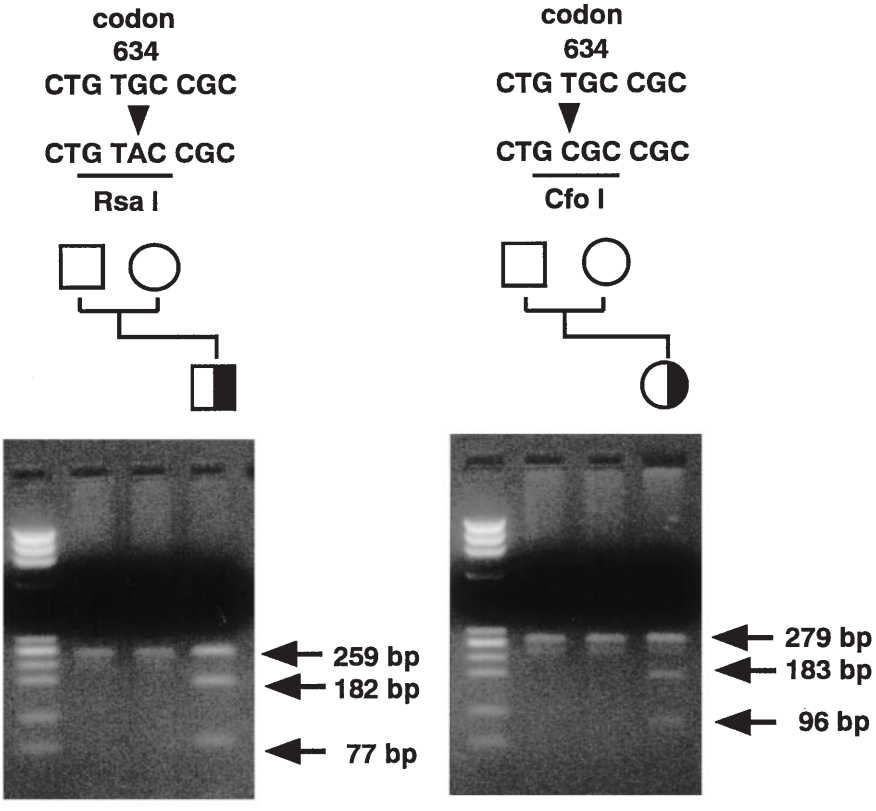

Fig. 1 Analysis of de novo mutations at codon 634. *Half-blackened symbols* represent affected individuals. *Open symbols* represent healthy individuals. The two medullary thyroid carcinoma (MTC) kindreds are characterized by a single affected sibling. By genomic sequencing, two MTCs revealed mutations at codon 634 resulting in a change to TAC (Tyr) or CGC (Arg) from TGC (Cys). These mutations created new restriction sites for RsaI and CfoI, respectively. Polymerase chain reaction (PCR) products of DNAs from members of each family were digested with RsaI or CfoI. *Lane numbers* correspond to individuals indicated in the pedigrees above the stained panels. The affected individual of the family on the *left* revealed 182-bp and 77-bp fragments in addition to a 259-bp fragment. The affected individual of the family on the *right* revealed 183-bp and 96-bp fragments in addition to the normal 279-bp fragment

sporadic MTC, and the single MTC patient with unknown family history in whom no mutations had been detected in exons 10 or 11, were screened for mutations at codons 768 or 918 of the *RET* gene. Mutations at codon 768 in exon 13 or codon 918 in exon 16 would eliminate restriction enzyme sites for AluI or FokI, respectively. Using the AluI enzyme we found germline alterations in the FMTC family, in one sporadic MTC patient, and in the MTC patient with unknown family history. DNA sequencing revealed that all of them were alterations from GAG to GAC (Glu to Asp). We also detected a substitution from CTG to CTT at codon 769 during these experiments; however, as this variation would not alter an amino acid and was also found in 4 of the 12 normal controls, it did not appear to be disease-related. Using the FokI enzyme we detected mutations at codon 918 in all four families with MEN2B and in five of the sporadic MTCs. Subsequent sequence analysis of the PCR products involving this codon revealed a missense mutation (ATG to ACG) causing a substitution of threonine for methionine, in all nine cases. Three of the five codon-918 mutations in clinically sporadic MTCs were confirmed to be somatic events. The remaining two sporadic MTC patients carried this mutation in their germline DNAs; however, as none of the four parents carried the same mutation, it is clear that those mutations had arisen de novo. In all, five patients among the 22 with sporadic MTCs were found to carry germline mutations in exons 11, 13, or 16 of the *RET* proto-oncogene, and all five MTC patients whose family histories were not available carried germline mutations in exons 10, 11, or 13. Since five of the missense mutations in exons 11 or 16 were found in MTC tumor DNAs but not in corresponding constitutional DNAs, those mutations were considered to have been somatic events (Fig. 2; see also Table 1).

The most frequent mutation found in our study occurred at codon 634, in 30 (52%) of the 58 germline or tumor DNAs in which *RET* mutations were identified; among them, a coding change from Cys to Tyr or to Arg accounted for nearly 50%. Furthermore, we confirmed de novo mutations in four of the five sporadic MTC patients whose constitutional DNA carried altered alleles. As no DNA samples were available from the parents of the remaining case, we cannot exclude a possibility that one parent carries the mutation without having developed any symptoms.

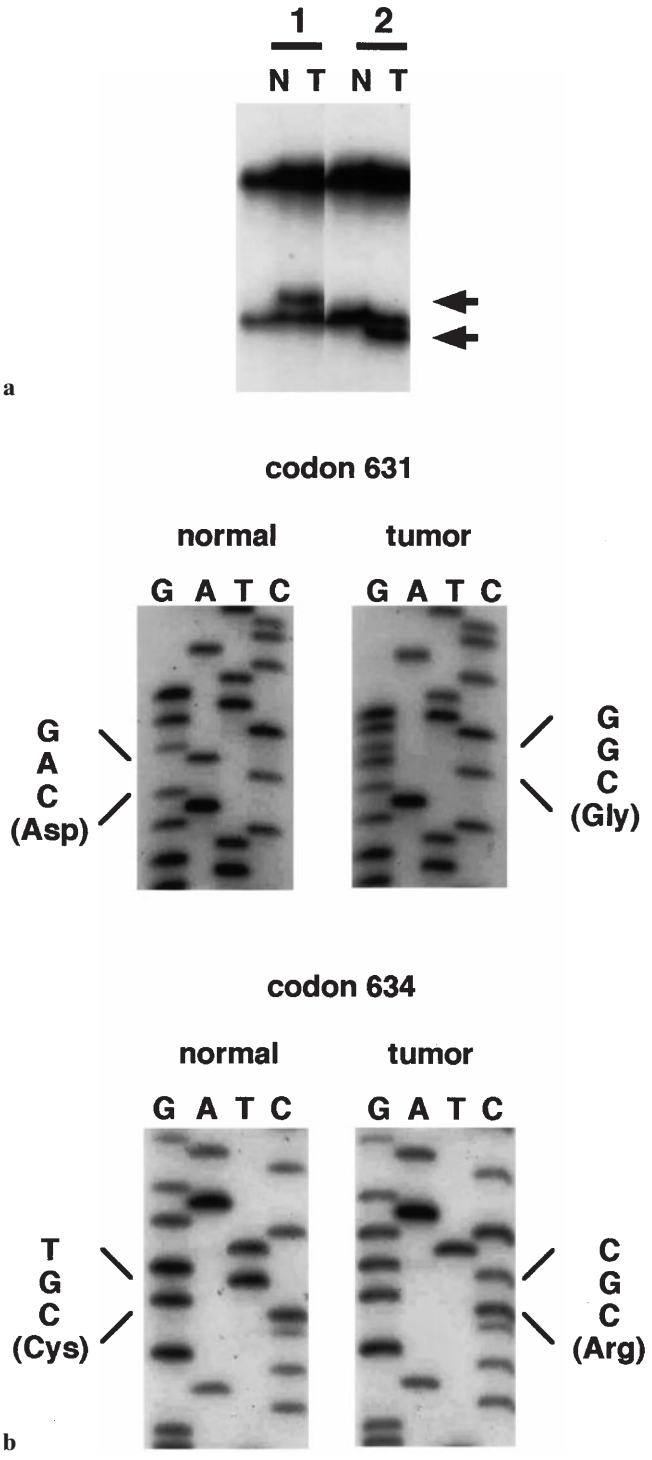

Fig. 2 SSCP and sequence analysis of exon 11 of the *RET* gene. **a** Variant SSCP band patterns are shown. *1, 2*, Genomic DNAs of two patients with sporadic MTC; *N* indicates DNA from peripheral leukocytes and *T* indicates DNA from tumor. *Arrows* indicate variant band patterns. **b** Genomic DNA sequence surrounding codon 631 (cloned exon-11 amplicons) from the patient represented by *lane 1* in **a**. The normal allele for codon 631 is GAC (Asp) and the mutant allele in the tumor is carrying GGC (Gly) (*upper panel*). The somatic mutation in patient 2 was TGC (Cys) to CGC (Arg) at codon 634 (*lower panel*)

Discussion

By mutational screening of the *RET* proto-oncogene in 71 patients with MTCs, we found germline mutations in 33 of 34 patients with MEN2A and in five of six FMTC families at one of three codons (618, 620, 634), all of which encode cysteine residues in the transmembrane domain of the *RET* protein. The mutations at codon 634, known as a mutational "hot spot" of the *RET* gene in Caucasians (Donis-Keller et al. 1993; Mulligan et al. 1994), accounted for 70% (23/33) of all mutations found in Japanese patients with MEN2A. Among three different types of base substitution found in

this codon, changes from Cys to Tyr (12/23) were the most common, followed by Cys to Arg (10/23); a Cys to Trp change was found in only one patient. A comparison of our data with those in Caucasians, among whom the majority of the mutations at codon 634 are Cys to Arg substitutions (Mulligan et al. 1994), implies that the common alteration from Cys to Tyr among Japanese patients may represent a founder effect. Mulligan et al. (1994) reported that Cys to Arg mutation at this codon is strongly predictive of parathyroid diseases. We noted parathyroid hyperplasia in 6 of the 10 Japanese families carrying the Cys to Arg mutation at codon 634, but also in 5 of the 12 with Cys to Tyr. Hence, the relationship between the type of alteration at codon 634 and the presence or absence of parathyroid disease is not as clear in Japanese patients as it is in Caucasians. In any event, since nearly one-fourth of the patients with sporadic MTCs in our panel carried germline mutations and 50% of their children are expected to develop MTC and other endocrine tumors, the results suggest that family members of any patient with MTC ought to be monitored for risk of these diseases.

We detected two novel somatic mutations: one was a substitution from Asp to Gly at codon 631 and the other a change from Cys to Arg at codon 634. These mutations occurred within a cysteine-rich domain that includes three cysteine codons known to be mutational hot-spots (codons 618, 620, and 634). This domain is located immediately adjacent to the transmembrane domain of the RET protein that is considered to form a ligand-binding pocket, and to play an important role in preserving the stable structure (Carlson et al. 1994). Therefore, mutations resulting in the replacement of Cys residues in this region by other amino acids could, by diminishing or abolishing the regulatory effect of ligand-binding on the activity of the tyrosine kinase moiety of RET, lead to constitutive autophosphorylation and result in a transformed phenotype.

We detected point mutations in 10 of the 22 sporadic MTCs examined; half of them were confirmed to have occurred as somatic events and the other five were also found in corresponding germline DNAs. The frequency (18%) of de novo mutations in 22 sporadic MTCs in Japanese was similar to that in Caucasians (Eng et al. 1994; Schuffenecker et al. 1997). Among the somatic mutations of the *RET* gene in sporadic MTC cases reported by others, 23%–40% have revealed mutations at codon 918 (Hofstra et al. 1994; Blaugrund et al. 1994; Eng et al. 1995b). However, we found mutations at codon 918 in only 14% (3/22) of our Japanese patients with sporadic MTCs. Mutation at this codon, whose normally encoded methionine residue lies within the substrate-recognition pocket of the catalytic core of the intracellular tyrosine kinase domain, would affect substrate specificity of the kinase.

A germline mutation at codon 768 was detected in one FMTC family, one sporadic MTC patient, and one MTC patient with unknown family history in our Japanese panel. As codon 768 is located in subdomain III, a region implicated in ATP-binding within the tyrosine kinase domain, mutation at this codon would affect both substrate interactions and binding of ATP to receptors (Eng et al. 1995a).

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