A Single Missense Mutation in Codon 918 of the *RET* Proto-Oncogene in Sporadic Medullary Thyroid Carcinomas

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Abstract. The *RET* proto-oncogene is expressed in human medullary thyroid carcinoma and pheochromocytoma. Recently germline mutations of the *RET* proto-oncogene were reported in four syndromes (MEN 2A, MEN 2B, familial medullary thyroid carcinoma and Hirschprung's disease) and somatic mutation was also found in sporadic medullary thyroid carcinoma. To determine the incidence of *RET* mutations in medullary thyroid carcinoma in Japan, we investigated 14 medullary thyroid carcinomas (comprising 1 case of MEN 2A, 1 case of MEN 2B, 2 cases of familial medullary thyroid carcinoma and 10 cases of sporadic). Tumors from all cases were screened by PCR-SSCP on exons 10 and 11. DNA sequencing on these exons was performed for the hereditary medullary thyroid carcinoma cases. The PCR products of exon 16 from tumor DNA were analyzed by means of Fok1 restriction enzyme digestion analysis and mutations confirmed by DNA sequencing. We found no structural abnormalities in either exon 10 or exon 11 in any of the cases examined, but in four of 10 sporadic cases we detected a common point mutation at codon 918 (ATG to ACG) in exon 16, where methionine was replaced with threonine. Our results support the theory that a point mutation of exon 16 of the *RET* proto-oncogene may be related to the oncogenesis of sporadic medullary thyroid carcinomas. However, further studies on the entire *RET* proto-oncogene are needed to clarify the relationship between its expression and thyroid tumorigenesis.

Key words: *RET* proto-oncogene, Medullary thyroid carcinoma, Point mutation

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MEDULLARY thyroid carcinoma (MTC) arises from the C cells, which embryologically emerge from the neural crest, and have the ability to synthesize and secrete calcitonin and other peptides [1]. MTC may occur as either sporadic or heredi-

tary tumors, the latter constituting a dominantly inherited syndrome characterized by bilateral, often multifocal thyroid tumors and diffuse C-cell hyperplasia throughout the thyroid gland, and is accompanied to varying degrees by hyperplasia or tumor formation in the adrenal medulla and parathyroid glands. The autosomal dominant syndromes with MTC are classified into three types: multiple endocrine neoplasia type 2A (MEN 2A), MEN 2B and familial MTC (FMTC) [2]. MEN 2A is characterized by predisposition to adrenal pheo-

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chromocytomas and to parathyroid hyperplasia. MEN 2B is the most severe form of the MEN 2 syndrome and is associated with MTC, pheochromocytoma in 90% of cases, mucosal neuromas, ganglioneuromas of the intestinal tract, and skeletal and ophthalmic abnormalities. FMTC is characterized by an inherited predisposition to MTC without evidence of pheochromocytoma or parathyroid disease. The sporadic form has been found in about 20% of the total MTC. The causes of these phenotypic differences are still unknown. The locus of the defective gene for these syndromes was localized in a region on chromosome 10q 11.2 by means of genetic linkage analysis [3, 4]. Recently, the germ-line missense mutations of the *RET* proto-oncogene were identified in MTC associated with MEN 2A, 2B and FMTC [5–10]. These mutations were restricted to the extracellular cysteine rich domain (exon 10 and 11) and intracellular tyrosine kinase catalytic domain (exon 16). To investigate the genetic basis for MTC, we analyzed the exons 10, 11 and 16 of the *RET* proto-oncogene for mutations in sporadic and familial MTC.

Materials and Methods

Materials

Human medullary thyroid carcinoma tissues were obtained from surgical specimens at the Nagasaki University Hospital, Noguchi Hospital, Yamaguchi Prefectural Hospital and Ishigaki Clinic. Fourteen medullary thyroid cancers (MEN 2A 1 case MEN 2B 1 case, familial medullary thyroid carcinoma 2 cases and sporadic medullary thyroid carcinoma 10 cases) were investigated. These tissues were fixed with formalin and embedded in paraffin blocks. Fifty- μ m-thick sections from each block were deparaffinized (1 ml of xylene twice, followed by 100% ethanol twice) and used for DNA extraction. Deparaffinized samples were treated in 100 μ l of digestion buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.5% Tween 20) with 100 μ g of proteinase K at 37 °C for 48 h. After phenol-chloroform extraction, genomic DNA was precipitated with ethanol.

Oligonucleotide primers and PCR

The sequence of *RET* intronic and exonic prim-

ers blanketing exons 10, 11 and 16 were synthesized according to published reports [5, 7, 11] and information from Dr. M. Takahashi (Nagoya University, School of Medicine).

Oligonucleotide primer sequences in exons 10, 11 and 16 are as follows ;

fRET 10 5'-GCGCCCCAGGAGGCTGAGTG-3'

rRET 10 5'-CGTGGTGGTCCCGGCCGCC-3'.

fRET 11 5'-GGTGCCAAGCCTCACACCAC-3'

rRET 11 5'-ATCTTGAAGGCATCCACGGA-3'

fRET 16 5'-AGGGATAGGGCCTGGGCTTC-3'

rRET 16 5'-TAACCTCCACCCCAAGAGAG-3'

PCR was performed with 100 ng genomic DNA, 20 pmol of each primer, 200 μ M dNTPs 10 mM KCl, 10 mM Tris-HCl (pH 8.8), 1.5 mM MgCl₂, 1% TritonX-100, and 1.25 units of Taq DNA polymerase, in a final volume of 50 μ l. The PCR (35 cycles) was carried out in a Thermal Cycler under the following conditions;

for exon 10: 30 sec at 94 °C (denaturation); 30 sec at 68 °C (annealing); and 1 min at 72 °C (polymerization)

for exon 11: 1 min at 94 °C (denaturation), 30 sec at 55 °C (annealing); and 30 sec at 72 °C (polymerization)

for exon 16: 35 sec at 94 °C respectively (denaturation), 35 sec at 58 °C (annealing); and 35 sec at 72 °C (polymerization). Predicted DNA sizes were 185 bp, 272 bp and 192 bp, respectively.

Single-Strand Conformation Polymorphism (SSCP) analysis

In an effort to evaluate *RET* proto-oncogene as a candidate for medullary thyroid carcinoma, we applied single strand conformation polymorphism (SSCP) analysis of constitutional and tumor DNA, screening amplified products of exon 10 and 11. Formamide dye (88% formamide, 10 mM EDTA, 0.01% bromophenol blue, pH 8.0) was added to an aliquot (0.1–0.2 μ g) of PCR product to obtain a final concentration of 50% formamide. Samples were incubated at 96 °C for 5 min, and rapidly chilled on ice. The samples were electrophoresed in an 8 % polyacrylamide gel (0.4 $\times$ TBE) in 0.5 $\times$ TBE. Electrophoresis was performed 4 times for each product by changing the temperature and the concentration of the glycerol. DNA fragments were detected with a silver staining kit (Daiichi Chemical Co., Ltd., Tokyo) according to the manufacturer's instructions [12].

Restriction enzyme digestion

PCR products (0.5 μ g of each) of exon 16 were digested with 10 units of Fok 1 at 37 °C for 16 h (according to the manufacturers' recommendations) and fractionated by electrophoresis on the 10% polyacrylamide gel. Gels were stained with 0.5 μ g/ml ethidium bromide for 30 min.

DNA sequencing

Amplified PCR products were purified on 1% agarose gels by QIAx kit (QIAGEN Inc. Chatsworth, USA). Sequencing was performed with dsDNA Cycle Sequencing System (GIBCO BRL, Gaithersburg, USA) or a 373A DNA Sequencing System (Applied Biosystem Japan, Tokyo, Japan). For direct sequencing, primers were end-labeled with 1 μ l of 1 μ M primer, 1 μ l of 5 $\times$ polynucleotide kinase buffer, 1 μ l of γ -³²P-ATP and 1 μ l of T₄-PNKase. The reaction mixture was incubated at 37 °C for 40 min, and then heated at 65 °C for 20 min. PCR reactions were carried out under the same conditions as for the first PCR reaction. Autoradiography was performed on Kodak X-OMAT films at -70 °C for 1-7 days. For the sequencing, purified PCR products were ligated to the pCRTM II vector (TA cloning Kit; Invitrogen San Diego, USA) and chimeric vectors were transformed with competent *E. coli* cells. After small scale preparation of plasmid DNA, the products were purified by QIAGEN plasmid Kit (QIAGEN Inc.) and subjected to a fluorescence-based dye primer sequence reaction with a PRISMTM Ready Reaction Dye Primer Cycle Sequencing Kit (Applied Biosystem Japan, Tokyo, Japan) for analysis with a 373A DNA Sequencer.

Results

PCR-SSCP

In order to assess the presence of the structural change in the *RET* proto-oncogene in medullary thyroid carcinomas, we first performed PCR-SSCP analysis on exon 10 and exon 11 of the gene. Of 14 separate medullary thyroid carcinomas screened, we found no mobility shifts for exon 10 or 11 (data not shown).

Mutation analysis of *RET* exon 16 by means of Fok 1 digestion

Based on previous reports [7, 9], which disclosed that a mutation in exon 16 at codon 918 (ATG to ACG) resulted in the loss of a Fok 1 restriction site [GGATG(N)9/13], we analyzed the PCR products of exon 16 with this restriction enzyme (Fig. 1). Of 14 MTC patients, we detected the undigestion pattern in four cases (sporadic MTC 4 cases). No abnormal digestion was found in 5 amplified samples when using peripheral lymphocyte DNA of normal subjects.

DNA sequence

We performed direct sequence of *RET* exons 10 and 11 for hereditary medullary thyroid carcinoma (MEN 2A, MEN 2B, familial medullary thyroid carcinoma 2 cases). There was no evidence for any point mutations in either exon 10 or exon 11. To confirm the mutation in exon 16 in the four cases with sporadic MTC with the undigested Fok 1 restriction, we further examined the DNA sequence. Four of 10 sporadic MTCs had a common point mutation at the codon 918 from ATG to ACG mutation (Table 1). Table 2 shows the tumor size, serum calcitonin and CEA level of each patient examined.

Discussion

We have shown a specific mutation in the tyrosine kinase domain of the *RET* proto-oncogene in tumor DNA from 4 of 10 (40%) sporadic MTC. The *RET* proto-oncogene encodes a protein that is structurally related to the tyrosine kinase transmembrane receptors [13, 14]. Although the ligand is still unknown, the physiological expression of *RET* proto-oncogene is restricted to cells of neuroendocrine origin [15-17]. In cell lines of this type, such as medullary thyroid carcinoma or neuroblastoma cells, retinoic acid-induced differentiation is accompanied by enhancement in *RET* expression, suggesting a role in the transduction of differentiatonal signals [18].

Previous reports revealed point mutations in the extracellular domain of the *RET* proto-oncogene in germ line DNA from MEN 2A and FMTC [5, 6, 8]

Fig. 1. Restriction patterns of PCR products of exon 16 with FokI. PCR products of *RET* exon 16 were isolated and purified as described in Materials and Methods. After digestion with 10 U FokI for 16 h, the samples were electrophoresed on a 10% polyacrylamide gel and stained with ethidium bromide. Although the normal allele at codon 918 has a FokI restricted site, the mutated allele is not restricted. The predicted band sizes are 192 bp at undigestion and 119 and 73 bp at digestion. Lane 1, normal lymphocyte DNA; Lanes 2–7, tumor DNAs from sporadic MTC. Samples in lanes 2 and 7 have mutated patterns.

Table 1. Ret mutation in medullary thyroid carcinomas

Group	Total no.	Number of mutation in <i>RET</i> gene		
		exon 10	exon 11	exon 16
Control	5	0	0	0
MEN2A	1	0	0	0
MEN2B	1	0	0	0
Familial	2	0	0	0
Sporadic	10	0	0	4

and in the tyrosine kinase domain from MEN 2B and sporadic MTC [7, 9, 10]. The *RET* extracellular domain contains a ligand binding region characterized by highly conserved cysteine residues in *RET* exons 10 and 11. All of the mutations identified in cases of MEN 2A and FMTC are restricted to these cysteine rich regions and result in nonconservative substitutions for four different cysteines. Mulligan *et al.* [8] reported the mutations in this region were found in 97% of patients with MEN 2A and 86% with FMTC but not in MEN 2B patients. The most frequent mutation in MEN 2A

Table 2. Cases with sporadic medullary thyroid carcinoma

Case no.	Sex	Age	Mutation in <i>RET</i>	Tumor size (mm)	Calcitonin (pg/ml)	CEA (ng/ml)
1	F	52	+	40 × 30	n.m.	n.m.
2	F	61	+	rt; 35 × 25, lt; 22 × 13	2600	n.m.
3	F	38	+	rt; 60 × 44, lt; 43 × 30	11021	n.m.
4	F	46	+	rt; 19 × 11, lt; 40 × 27	7900	310
5	F	71	—	55 × 40	n.m.	n.m.
6	F	57	—	20 × 14	n.m.	n.m.
7	F	52	—	18 × 13	3100	n.m.
8	F	45	—	29 × 19	6100	30
9	F	42	—	rt; 18 × 16, lt; 28 × 23	8852	n.m.
10	F	43	—	rt; 25 × 14, lt; 15 × 10	1300	21

n.m., not measured.

was observed at codon 635 representing 84% of cases examined. In the cases which we examined (MEN 2A 1 case and FMTC 2 cases), no mutations was found in exon 10 or 11. As our MEN 2A case has unique clinical features characteristic of malignant pheochromocytoma that metastasized to the lungs and liver, *de novo* mutation site(s) might exist in other exon(s) of the *RET* gene or in other oncogenes. As only a small number of hereditary MTCs were examined in this study, a large scale study will help to find mutation sites in the *RET* gene in the hereditary MTCs in Japan.

The MEN 2B and sporadic MTCs examined to date all show the same heterozygous missense change at codon 918 (exon 16) in the *RET* tyrosine kinase domain [7, 9, 10]. The frequency of this mutation was approximately 60–100% in MEN 2B and 30–40% in sporadic MTC. The mutation (ATG → ACG) results in the replacement of methionine with threonine within the catalytic core region of the tyrosine kinase domain. Although we identified the missense mutation of the *RET* codon 918 in tumor cells of four sporadic MTC, no relationship was found between this mutation and clinical features. Further screening study may define the difference in the disease among tumors with and without the mutation. A previous report showed the mutations of the *RET* proto-oncogene are also associated with non-neuroendocrine tumors. Rearranged forms of *RET* were isolated from a human T-cell lymphoma [19] and subsequently from human colon DNA, by means of transfection onto NIH 3T3 fibroblasts [20,21] and thyroid papillary carcinoma *in vivo* [22]. In each of these cases, *RET* oncogenes arose through recombination events of the *RET* proto-oncogene with the 5'-terminal sequences of different genes [20–22]. A study with a chimeric expression vector, which encoded a mol-

ecule encompassing the extracellular domain of the epidermal growth factor receptor (EGFR) and the intracellular domain of the *RET* kinase, suggested that the *RET* kinase signal is transmitted through a *raf*-MAPK independent pathway [23]. These data suggest that activation of tyrosine kinase by *RET* rearrangement has a transforming activity in non-neuroendocrine cells. But it is not clear whether the point mutations of the *RET* proto-oncogene which exist in MTC continuously stimulate the activity of tyrosine kinase or diminish the signal transduction from the ligand in neuroendocrine cells. It is necessary to know the ligand and how functional alterations occur in cells due to missense mutations in the signal transduction system. Studies on transgenic mice carrying these mutations will be useful in elucidating how disease phenotypes are conferred by each mutation of the *RET* proto-oncogene.

To detect the mutation of codon 918 in *RET* proto-oncogene might be useful in assisting the early or definite diagnosis of MTC. Furthermore, ultimately these syndromes would be good targets for gene therapy.

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