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A Family of Multiple Endocrine Neoplasia Type 2A: Genetic Analysis and Clinical Features

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Abstract. Since a heterozygous missense mutation of the *RET* proto-oncogene in the germline was found to cause multiple endocrine neoplasia type 2A (MEN 2A) in 1993, some 20 different mutations of this gene have been identified in MEN 2A kindreds. We report an MEN 2A family in which serine (AGC) substitutes for cysteine (TGC) at codon 618 in exon 10 of the *RET* proto-oncogene. The mutation was identified by sequencing PCR products of exons 10 and 11 in the proband. Since this mutation results in creation of a new cleavage site for *Alu* I restriction enzyme, most of the other members of the family were screened by digestion of the PCR product of exon 10 with this enzyme. Eleven of 20 subjects across four generations examined have the mutation of the *RET* proto-oncogene, and all of the adult gene carriers except one woman had MTC. Characteristics of this family are 1) pheochromocytoma has been found in only the proband, 2) no obvious hyperparathyroidism has been observed, and 3) the prognosis is favorable, with nobody dying of MEN 2A itself. Genetic analysis of MEN 2A is definitely useful and essential for screening of a MEN 2A family. It is very important to accumulate cases with MEN 2A and investigate the phenotype and the prognosis in each mutation.

Key words: Multiple endocrine neoplasia type 2A, *RET* proto-oncogene

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MULTIPLE endocrine neoplasia type 2A (MEN 2A) is an autosomal dominant cancer syndrome which is characterized by medullary thyroid carcinoma (MTC) and pheochromocytoma and is occasionally associated with parathyroid hyperplasia or adenoma. In 1993, germline missense mutations in exon 10 or 11 of the *RET* proto-oncogene located at the pericentromeric region of the long arm of chromosome 10 (10q11.2) were shown in MEN 2A and familial MTC (FMTC) [1, 2], suggesting that mutations in the *RET* proto-oncogene may be responsible for the development of the syndrome. Almost all of the mutations found so far in MEN 2A and FMTC involve one of five cysteine residues in the extracellular cysteine-rich region of the *RET* proto-oncogene [1–4]. Here, we report a large family of MEN 2A with a mutation in the *RET* proto-oncogene with clinical analysis.

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Case Report

The proband (I-3 in Table 1 and Fig. 1) is an 84-year-old woman who was admitted to Toyokawa City Hospital because of dyspnea on exertion. One of her daughters, the third child (II-3 in Table 1 and Fig. 1), had undertaken hemithyroidectomy of

Table 1. Clinical features of patients at risk for MEN 2A who had inherited the RET mutation

Subject/ Gender	Age (yrs)	Serum Calcitonin (ng/L)	Serum CEA (μg/L)	Serum Intact PTH (ng/L)	24-h urinary excretion Metanephrine (μmol/day)	24-h urinary excretion Normetanephrine (μmol/day)	Diameter of MTCs (mm)
I-3 / F	84	4300	484	53	1.3	2.8	25 and 6 by US
II-2 / F	57	3900	170	49	1.3	1.0	20, 16, 15and 10
II-3 / F	54	71	n.e.	45	1.4	1.4	18 and 12
II-4 / M	50	320	13.2	33	0.9	0.9	13, 6 and 5 by US
II-5 / M	47	230	8.3	35	0.9	1.3	10, 8 and 7 by US
III-4 / F	30	650	32.4	49	0.8	0.9	12 and 10
III-5 / F	35	110	n.e.	30	1.0	1.3	5 by US
III-7 / F	26	83	n.e.	36	n.e.	n.e.	5 by US
III-9 / F	27	35	n.e.	38	n.e.	n.e.	not detected
IV-8 / M	10	45	n.e.	25	n.e.	n.e.	n.e.
Normal range		M, 35–89 F, 29–69	<5.0	15–55	0.3–1.3	0.4–1.4	

CEA, carcinoembryonic antigen; MTC, medullary thyroid cancer; M, male; F, female; US, ultrasonography. n.e., not examined.

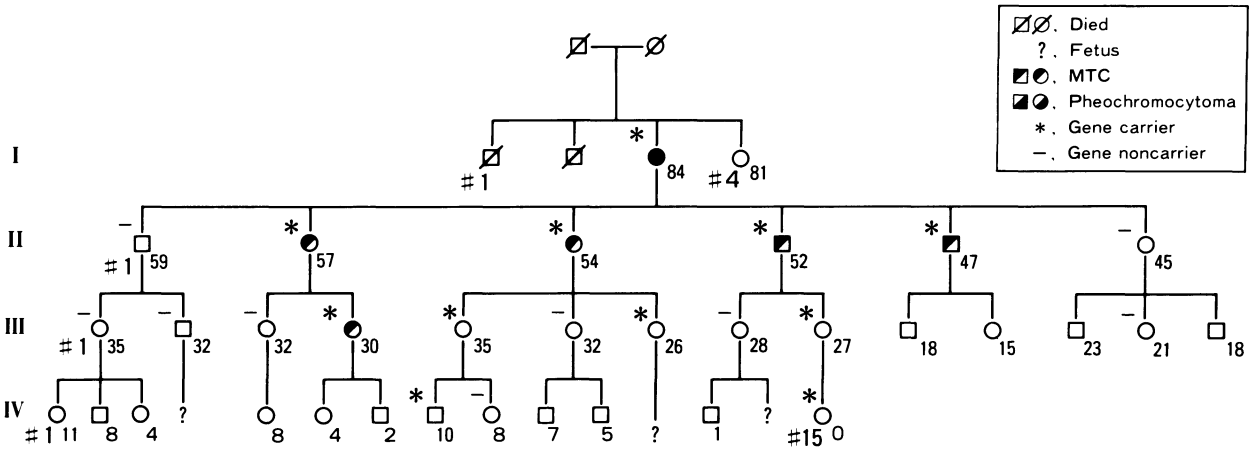

Fig. 1. Pedigree: showing results of genetic analysis of the family. Numerals at bottom right and left for the individuals indicate the age and the sequence numbers within a generation in which only the first (left side) and last (right side) numbers are given. Each square represents a male and each circle represents a female.

the right lobe and external radiation to the neck for a MTC at the age of 32 because of a rapidly growing thyroid mass. At the time her disease was thought to be a solitary case, so further analysis for her family was not done. The proband was 1.38 m tall and weighed 42.5 kg. Her blood pressure was 136/50 mmHg and pulse rate was 60/min. Several hard masses with an irregular surface were palpated on the right lobe of the thyroid. A systolic heart murmur (Grade 2/6) was audible. No abdominal mass was palpated. The face and upper extremities were edematous.

Hepatic and renal functions were not remarkable. Serum electrolytes were as follows: Na, 140 mEq/L; K, 4.9 mEq/L; Cl, 102 mEq/L; Ca, 3.8 mEq/L; P, 3.9 mEq/L. Serum carcinoembryonic antigen (CEA) and calcitonin levels were noticeably high (Subject I-3 in Table 1 and Fig. 1). Serum intact PTH was normal and urinary normetanephrine excretion was increased. Computed tomography (CT) of the neck revealed bilateral thyroid masses with a diameter of 30 mm with coarse calcifications and no enlarged parathyroid glands or cervical lymphnodes. CT of the abdomen

showed a right adrenal mass with a diameter of 25 mm. ^{123}I-MIBG scintigraphy showed abnormal accumulations at the thyroid and the right adrenal gland as well as the middle mediastinum. The chest CT demonstrated a mass 45 mm in diameter with some calcifications located in the middle mediastinum, and it was considered to be a metastasis of the thyroid mass based on similar CT findings. She was diagnosed as having MEN 2A with a pheochromocytoma from the right adrenal gland and MTC with a metastasis to the middle of the mediastinum. She has not received surgery because of her advanced age.

Materials and Methods

DNA sequence analysis

Genomic DNAs in the family of MEN 2A were isolated from the peripheral blood leukocytes with a DNA Extractor WB kit (Wako, Tokyo). Exons 10 and 11 of the *RET* proto-oncogene in the proband were amplified by polymerase chain reactions (PCR) with the respective oligonucleotide primers. The primer sequences were derived from the *RET* proto-oncogene sequence [2, 4]. Primers for exon 10 were 5′-GAATTCGCTGAGTGGGCTACGTCT-3′ and 5′-CTGCAGACCCACTCACCCTGGATG-3′. *Eco*R I and *Pst* I restriction sites were added at the 5′-end of the primers, respectively. Primers for exon 11 were 5′-TAGGAATTCCTCTGCGGTGC-CAAGC-3′ and 5′-CTCAAGCTTCACCGGAAG-AGGAGTAGC-3′. *Eco*R I and *Hind* III restriction sites were added at the 5′-end of the primers, respectively. The PCR amplifications for both exons were done for 35 cycles with a Takara PCR kit (Takara, Tokyo). Each cycle was consisted of 1 min at 94 °C, 2 min at 55 °C and 1.5 min at 72 °C. The PCR fragments (exon 10; 174 bp, exon 11; 234 bp) were digested with restriction enzymes (exon 10; *Eco*R I and *Pst* I, exon 11; *Eco*R I and *Hind* III) and were purified from the primers and free nucleotides with a GENECLEAN II kit (BIO 101 Inc., CA). The fragments were cloned into a plasmid vector, pUC118 (Takara, Tokyo). The plasmid inserts obtained from 10 single clones were sequenced with a Sequenase version 2.0 kit (USB, Ohio).

Familial analysis for RET mutation

Written informed consent for analysis of the mutation was obtained from all the adult members of the family, and from the parents of those less than 20 years old. The mutation of the *RET* proto-oncogene in the proband which exists in exon 10 creates an additional cleavage site (AG/CT) in the mutant allele for a restriction enzyme, *Alu* I (Toyobo, Tokyo). After the PCR fragment of exon 10 in the family was digested with *Alu* I, the samples were size fractionated by polyacrylamide gel electrophoresis (15% polyacrylamide, 1 X TBE buffer at 200 V for 1 h). The restriction fragments were visualized by ethidium bromide staining.

Results

Germline mutation of the RET proto-oncogene in the proband

As a result of sequence analysis, we found a heterozygous missense mutation of the *RET* proto-oncogene in which AGC (serine) is substituted for TGC (cysteine) at codon 618 in exon 10 as shown in Fig. 2. Five of the clones were mutant

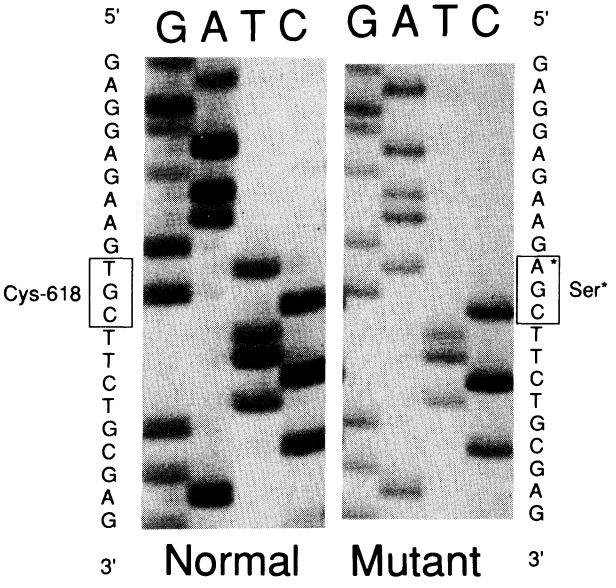

Fig. 2. Germline mutation in the *RET* proto-oncogene in the proband of the MEN 2A family.

and 5 were wild types. No mutation was found in the other codon in exon 10 or 11.

Genetic analysis of the family

The 174 bp PCR product in exon 10 was digested with *Alu* I. The normal allele gives two fragments of 88 and 86 bp. But the mutation creates an additional *Alu* I site in the 88 bp fragment, which further produces 45 and 43 bp fragments. Of the proband, 6 of 6 children, 10 of 14 grandchildren and 3 of 12 great-grandchildren were examined by the above method. An example of the analysis is shown in Fig. 3 and the results of the analysis of the family are shown in Fig. 1. Four of the children, 4 of the grandchildren and 2 of the great-grandchildren of the proband were diagnosed as gene carriers.

Clinical analysis of the gene carriers of the family

Thyroid and abdominal ultrasonography (US) and/or CT were performed, and serum calcitonin and intact PTH concentrations and/or 24-h urinary catecholamine excretions were measured for the gene carriers above the age of 10 (Table 1). Thyroid masses with calcifications and increased serum calcitonin concentrations were detected in all the adult gene carriers except Subject III-9 (27 yrs old). She had a normal serum calcitonin concentration and no thyroid mass detected by US. Calcitonin stimulation test was not performed because she was pregnant at the time. Subject II-3 had undergone hemithyroidectomy of the right lobe at 32 years because of MTC. And in the present study, 2 masses were detected in the residual thyroid. In Subjects III-5 and III-7, US demonstrated a single calcified thyroid mass with a diameter of 5 mm. No pheochromocytoma or hyperparathyroidism was detected radiographically or biochemically in the family except for the pheochromocytoma in the proband. A total thyroidectomy was performed in Subjects II-2 and III-4. MTCs with (Subject III-4) and without (Subjects II-2) metastasis to the cervical lymphnodes were pathologically verified. Other gene carriers with

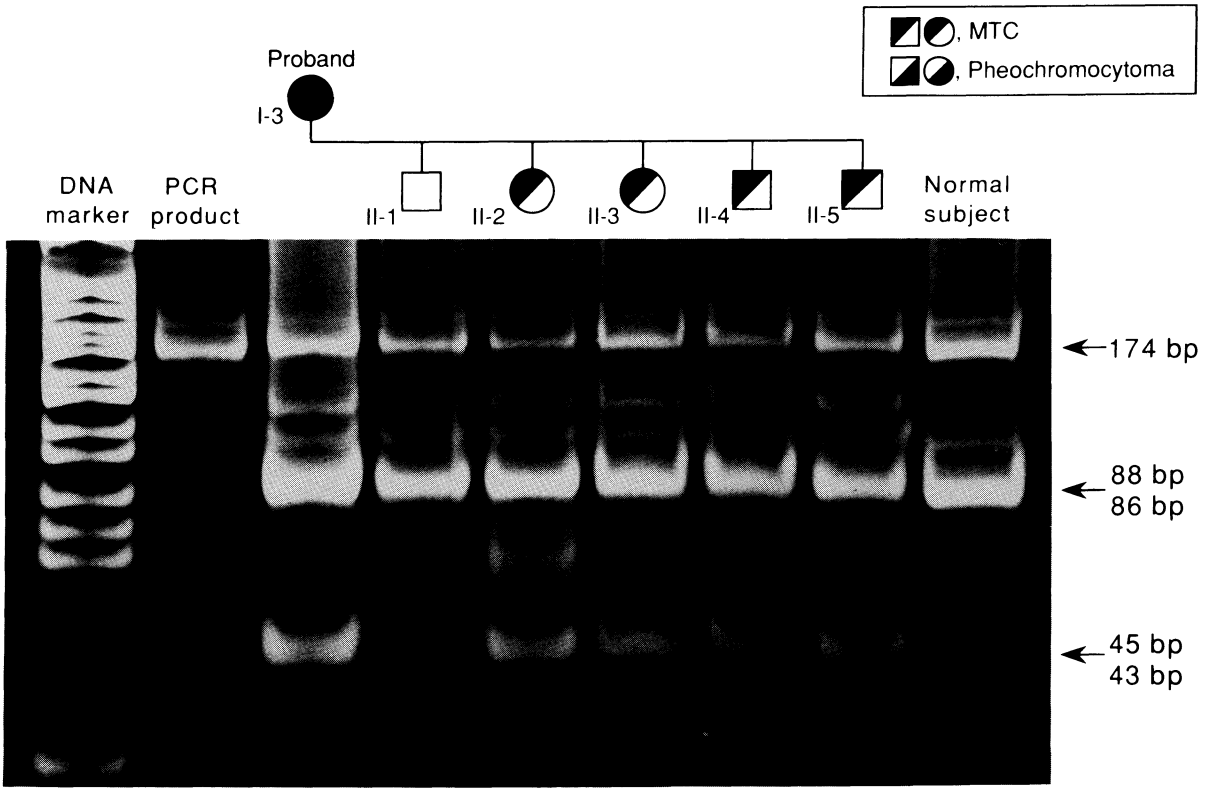

Fig. 3. An example of genetic analysis of the family by PCR fragments digested with *Alu* I.

thyroid masses and increased serum calcitonin concentrations will be totally thyroidectomized. On the other hand, all of the gene noncarriers examined have normal serum calcitonin concentrations (Data not shown).

Discussion

About 20 kinds of a missense mutation of the *RET* proto-oncogene have been reported in MEN 2A and FMTC [1–3, 6–11]. Almost all of the mutations were involved in a change in a cysteine to a different amino acid in the cysteine-rich extracellular domain of the *RET* protein. We identified a mutation of the *RET* proto-oncogene in which AGC (serine) is substituted for TGC (cysteine) at codon 618 in the proband and genetically screened the family by DNA restriction analysis with *Alu* I of the PCR product of exon 10. Although the mutation found in this family has already been reported, the present type of mutation in the *RET* proto-oncogene in MEN 2A is relatively rare.

Characteristics of this family include the finding of pheochromocytoma in only the proband, who was 84 years old, and a lack of obvious hyperparathyroidism. The present mutation (Cys 618 to Ser) has been found in not only MEN 2A families but also FMTC ones [2, 3]. This type of mutation may cause late onset of pheochromocytoma, and may not predispose to hyperparathyroidism. Alternatively, other genes or molecular events could modify the age of onset for MEN 2A and FMTC resulting in the absence of pheochromocytoma [2]. Several reports have analyzed the relationship between genotype and phenotype in MEN 2A. The presence of any mutation at codon 634 is strongly predictive of pheochromocytoma compared with that at codon 618 or 620 [3, 4, 11]. Furthermore, a TGC to CGC mutation at codon 634 (cysteine to arginine) predisposes strongly to hyperparathyroidism [4]. Subtle differences in the amino acid sequences of the *RET* extracellular domain may have different consequences for *RET* function or expression between organs. It is very important to accumulate further cases with MEN 2A and analyze in each mutation what kind of tumor is promoted and what the growth potency is.

The penetrance in MEN 2A has been considered to be high [12]. In order to find early stage MTCs, biochemical methods such as calcitonin stimulation test have been used. But this method sometimes produces false-negative cases [9]. Gene analysis has recently become available, and this is the most validated method for identifying carriers. The present study showed that all of the adult gene carriers in the family had MTCs or thyroid masses with increased serum calcitonin concentrations except a 27-year-old woman. She was pregnant when we investigated the family, so we could not do calcitonin stimulation, *e. g.* calcium or gastrin loading test on her. It is possible that she had a latent MCT. In general, biochemical manifestations of MTC appear between the ages of 5 and 25 yr (mean 15 yr) [9], and approach 100 percent by the age of 30 years [12]. These findings are consistent with the results in the present family. We should follow up the woman and the juvenile gene carriers at regular intervals by biochemical and radiological methods.

The prognosis of the MTC in the present family is quite favorable. The proband is still alive even with metastasis of MTC to the mediastinum and her present age of 84 years exceeds the mean life span of women in Japan. Although subject II-3 had had a part of the thyroid resected due to a MCT, she now has recurrent tumors in the residual lobe. No family member has died of MEN 2A so far. There are only two men among the gene carriers but serum calcitonin concentrations in the men seem to be lower and the MTC seem to be smaller than those in the women. Easton *et al.* also reported that MTC appears earlier in female gene carriers than in male ones [12]. Accumulation of cases with MEN 2A may also elucidate the relationship between the genotype and clinical prognosis.

The *RET* proto-oncogene encodes a transmembrane-receptor tyrosine kinase [5], but not even the ligand has been identified. Expression of the *RET* proto-oncogene was detected in most of human neuroblastomas, pheochromocytomas and MTCs, all of which originate in neural crest cells [13, 14]. It was recently reported that MEN 2A mutations induce dimerization of *RET* kinase, leading to activation of its instrinsic tyrosine kinase [15, 16]. This mechanism may be involved in the tumorigenesis. It is possible that we can prevent the occurrence of tumors in MEN families by further molecular investigations.

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