RAPID COMMUNICATION Jpn. J. Cancer Res. 85, 879–882, September 1994

Germ Line Mutations of the *ret* Proto-oncogene in Japanese Patients with Multiple Endocrine Neoplasia Type 2A and Type 2B

Shoichi Maruyama,[1,2] Toshihide Iwashita,[1] Tsuneo Imai,[3] Hiroomi Funahashi,[3] Isabella Ceccherini,[4] Yin Luo,[4] Giovanni Romeo,[4] Seiichi Matsuo,[2] Mutsushi Matsuyama[1] and Masahide Takahashi[1,5]

[1]*Department of Pathology,* [2]*The Third Department of Internal Medicine,* [3]*The Second Department of Surgery, Nagoya University School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466 and* [4]*Laboratorio di Genetica Molecolare, Istituto G. Gaslini, 16148 Genova, Italy*

We investigated mutations of the *ret* proto-oncogene in Japanese patients with multiple endocrine neoplasia (MEN) type 2A and type 2B. DNAs from pheochromocytomas and/or medullary thyroid carcinomas (MTCs) of five MEN 2A and three MEN 2B patients were amplified by a polymerase chain reaction (PCR) and analyzed. Tumors of four MEN 2A patients had missense mutations in Cys 634 in the extracellular domain of the *ret* proto-oncogene. The same mutations were detected in normal tissues of the patients, indicating that the mutations had arisen in the germ line. Using a reverse transcriptase(RT)-PCR, both normal and mutant transcripts of the *ret* proto-oncogene were detected in a tumor of one patient with MEN 2A mutation. In addition, three MEN 2B patients examined had the same point mutation (ATG→ACG) at codon 918 in the tyrosine kinase domain of the *ret* proto-oncogene. Since all mutations identified in this study generated new restriction enzyme sites or eliminated a restriction site, the mutant alleles of affected family members could be readily detected without sequencing.

Key words: MEN 2A — MEN 2B — *ret* proto-oncogene — Germ line mutation

Multiple endocrine neoplasia (MEN) type 2A and type 2B are autosomal dominant cancer syndromes involving medullary thyroid carcinoma (MTC) and pheochromocytoma. In addition to these tumors, about 20–30% of MEN 2A patients develop parathyroid hyperplasia. MEN 2B is characterized by a more complex phenotype including mucosal neuroma, hyperganglionosis of the gastrointestinal tract and marfanoid habitus. Since the *MEN2A* and *MEN2B* genes have been mapped to 10q11.2 near the locus of the *ret* proto-oncogene[1,2] which is expressed at a high level in MTC and pheochromocytoma, it has been a candidate gene for *MEN2A* and *MEN2B* genes. Recently, germ line mutations of the *ret* proto-oncogene were identified in patients of MEN 2A, MEN 2B and familial MTC (FMTC).[3–7] Mutations identified in MEN 2A and FMTC affected one of five cysteine residues (codons 609, 611, 618 and 620 in exon 10 and codon 634 in exon 11) present in the extracellular domain of the *ret* proto-oncogene. On the other hand, all cases of MEN 2B contained the same point mutation which resulted in the substitution of threonine for methionine (codon 918 in exon 16) in the tyrosine kinase domain. These results strongly suggest that the *ret* proto-oncogene and the *MEN2A* and *MEN2B* genes are allelic. Here we report mutations of the *ret* proto-oncogene in Japanese MEN 2A and MEN 2B.

DNAs were extracted from paraffin sections of pheochromocytomas and/or MTCs of five MEN 2A and three MEN 2B patients. We also isolated DNAs from paraffin sections of normal tissues (spleen, kidney, colon or thyroid gland) or peripheral blood of the same patients. Using a polymerase chain reaction (PCR), we amplified DNA fragments corresponding to exons 10, 11 and 16[8] of the *ret* proto-oncogene in which germ line mutations in MEN 2A and MEN 2B families were reported. The PCR products were subcloned into the pGEM-T vector (Promega, USA) and the plasmid DNAs with the appropriate inserts extracted from approximately 20 independent colonies were mixed and sequenced. Four of five MEN 2A cases contained missense mutations in codon 634 of exon 11, causing cysteine to be replaced by tyrosine, arginine or glycine (Table I and Fig. 1a). DNAs from normal tissues of these patients also contained the same mutations, indicating that the mutations were present in the germ line. One case (designated A5 in Table I) showed no mutation in the amplified sequences of exons 10, 11 and 16.

Three MEN 2B cases contained the same missense mutation in Met 918 in the tyrosine kinase domain, resulting in the substitution of threonine for methionine (Table I and Fig. 1b). Although these three patients had

[5] To whom correspondence should be addressed.

Table I. *ret* Mutations in MEN 2A and MEN 2B

Case	Codon[a]	Nucleotide change	Amino acid change	Family history	Method used for detection of the mutant allele[b] (materials[c])	
					Tumor	Normal
MEN 2A						
A1	634	TGC→TAC	Cys→Tyr	+	Seq/Enz (PH)	Seq/Enz (SP)
A2	634	TGC→CGC	Cys→Arg	?[d]	Seq/Enz (PH)	Seq/Enz (KD)
A3	634	TGC→TAC	Cys→Tyr	+	Seq/Enz (PH)	Seq/Enz (TH)
A4	634	TGC→GGC	Cys→Gly	+	Seq/Enz (PH)	Seq/Enz (PB)
A5		undetectable		+	Seq/Enz (MTC)	not examined
MEN 2B						
B1	918	ATG→ACG	Met→Thr	−	Seq/Enz (PH)	not examined
B2	918	ATG→ACG	Met→Thr	−	Seq/Enz (PH, MTC)	Seq/Enz (TH)
B3	918	ATG→ACG	Met→Thr	−	Seq/Enz (PH, MTC)	Enz (CO)

a) Codon was numbered according to the published sequence of the *ret* proto-oncogene.[9, 12]
b) Seq: DNA sequencing, Enz: restriction enzyme digestion assay.
c) PH: pheochromocytoma, MTC: medullary thyroid carcinoma, SP: spleen, KD: kidney, TH: normal portion of the thyroid gland, PB: peripheral blood, CO: colon.
d) ? denotes lack of information.

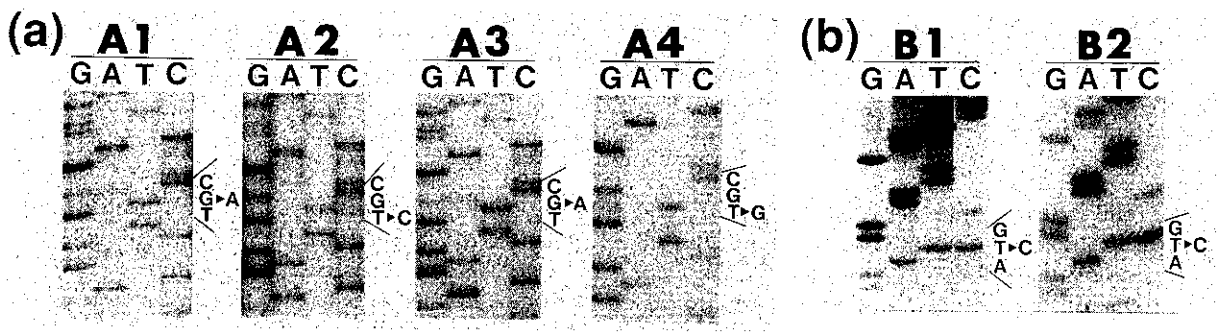

Fig. 1. Mutations of the *ret* proto-oncogene in MEN 2A and MEN 2B. (a) Mutations in Cys 634 in four MEN 2A patients (designated A1 to A4). A fragment corresponding to a portion of exon 11 of the *ret* proto-oncogene was amplified from tumor DNA of each patient and inserted into the pGEM-T vector (Promega). Plasmid DNAs with the PCR products extracted from approximately 20 independent colonies were mixed and sequenced by the dideoxy-termination method. PCR primers used are RET11F (5′-GAGAGAAAGCTTGGTGCCAAGCCTCACACCA-3′) and RET11R (5′-GAGAGAGAATTCGAAGGTCATCTCAGCTGAGGA-3′). Underlined sequences with a restriction enzyme site (*Eco*RI or *Hin*dIII) in the primers are not derived from the sequence of the *ret* proto-oncogene. These sequences were designed to insert the PCR products into the pUC vector but not used for the present study. PCR amplification was carried out at 94°C (1 min), 58°C (75 s) and 72°C (1 min) for 35 cycles. Closed triangles indicate base changes in codon 634. (b) A mutation in Met 918 in two MEN 2B patients (designated B1 and B2). A fragment corresponding to a portion of exon 16 was amplified as described above. Primers used are RET16F (5′-GAGAGAGAATTCGTCTTTATTCCATCTTCTC-3′) and RET 16R (5′-GAGAGAAGCTTATCACTTTGCGTGGTGTAGA-3′). Closed triangles indicate a base change in codon 918.

no family history, we confirmed that the mutations in two patients were present in the germ line (Table I).

The three different mutations identified in MEN 2A patients created new restriction enzyme sites including *Rsa*I, *Hha*I and *Hae*III sites (Fig. 2). For example, in the case of the A1 patient, the PCR product with the mutation digested by *Rsa*I generated two bands of 140 and 67 bp while no digestion of the product derived from the normal allele was observed. Similarly, both normal and mutant alleles in A2 and A4 patients were detected by digestion with *Hha*I and *Hae*III, respectively. On the other hand, the MEN 2B mutation eliminated a *Fok*I enzyme site, resulting in no digestion of the mutant fragment of 117 bp (Fig. 2). Thus, these results could facilitate the detection of the mutant alleles in affected members of the MEN 2A and MEN 2B families.

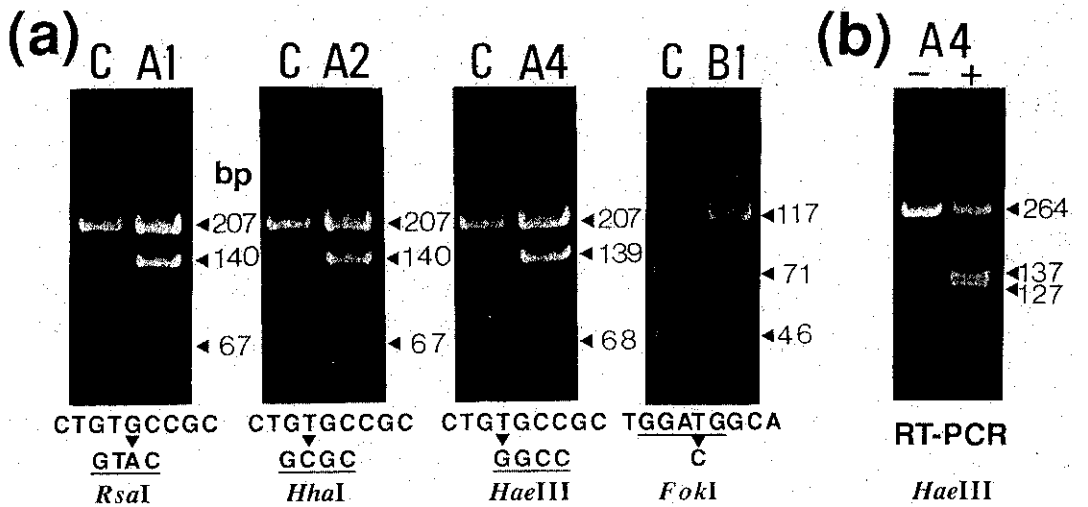

Fig. 2. Restriction enzyme digestion of PCR products with MEN 2A or MEN 2B mutations. (a) The PCR products corresponding to a portion of exon 11 of the *ret* proto-oncogene were digested with *Rsa*I, *Hha*I or *Hae*III. Similarly, the product corresponding to a portion of exon 16 was digested with *Fok*I. C: The PCR product amplified from peripheral blood DNA of a normal individual; A1, A2 and A4: The PCR products from tumor DNAs of the A1, A2 and A4 patients with MEN 2A; B1: The PCR product from tumor DNA of the B1 patient with MEN 2B. PCR was performed as described in Fig. 1a. (b) A fragment including part of exons 10 and 11 (nucleotides 1696 to 1960[9]) was amplified from tumor RNA of the A4 patient by RT-PCR. Primers used were RET10F (5′-AGCATTGTTGGGGGACAC-3′) and RET11R described in the legend to Fig. 1a. Each lane indicates before (−) or after (+) digestion with *Hae*III.

Since a fresh tumor of the A4 patient was available, we purified tumor RNA and investigated the expression of the mutant allele of the *ret* proto-oncogene by using reverse transcriptase (RT)-PCR. The contamination of non-tumor cells in this tumor was less than 10%. We designed PCR primers to amplify a 264 bp DNA fragment including both exons 10 and 11 (corresponding to nucleotides 1696 and 1960[9]) to exclude the possibility of amplification from genomic DNA contaminating the RNA sample. As shown in Fig. 2b, two digested fragments (127 and 137 bp) derived from mRNA with the MEN 2A mutation as well as a fragment (264 bp) from the normal allele were detected after digestion with *Hae*III. These results indicated that the tumor expressed both normal and mutant alleles of the *ret* proto-oncogene.

In the present study, we found germ line mutations of the *ret* proto-oncogene in four out of five MEN 2A patients examined, causing cysteine at codon 634 to be replaced by tyrosine, arginine or glycine. Mulligan *et al.*[5] reported that 97% of patients with MEN 2A had mutations of the *ret* proto-oncogene, all of which were detected at one of five cysteines (codons 609, 611, 618, 620 and 634) in exon 10 or 11, and 84% of the mutations affected codon 634. This is consistent with our finding that all four MEN 2A mutations involved Cys 634. In addition, they found that the most frequent mutation in MEN 2A was cysteine to arginine at codon 634 and that the second most frequent was cysteine to tyrosine at the same codon. These mutations account for 54 and 11% of all mutations, respectively.[5] Although we obtained only four positive cases, two of them showed cysteine to tyrosine change and one showed cysteine to arginine change, suggesting that these changes could also be more frequent than others in Japanese MEN 2A.

All three cases with MEN 2B examined had the same point mutation in Met 918. It is known that most Japanese MEN 2B patients do not have a contributory family history,[10] indicating that MEN 2B had developed *de novo*. The fact that we found germ line mutations in two MEN 2B patients suggests that the mutations had arisen at an early developmental stage or in germ cells of their parents.

At present we do not know whether MEN 2A and MEN 2B syndromes develop via a dominant or dominant-negative mechanism. However, the former seems more likely because a mouse strain in which the *ret* proto-oncogene was knocked out did not show a phenotype similar to these syndromes.[11] In addition, both the adrenal medulla and thyroid C cells differentiated normally in this mouse strain. Generation of transgenic mice with the MEN 2A or MEN 2B mutation could provide further information on the mechanism(s) of these syndromes.

We are grateful to Drs. N. Hirabayashi, H. Kuhara, Y. Fujimoto, Y. Ohiso, N. Nakashima and J. Watanabe for providing specimens from MEN 2A and MEN 2B patients. This work was supported in part by Grants-in-Aid for Scientific Research and for Cancer Research from the Ministry of Education, Science and Culture of Japan and by a grant from the Ishida Foundation.

(Received April 26, 1994/Accepted June 27, 1994)

REFERENCES

1) Gardner, E., Papi, L. Easton, D. F., Cummings, T., Jackson, C. E., Kaplan, M., Love, D. R., Mole, S. E., Moore, J. K., Mulligan, L. M., Norum, R. A., Ponder, M. A., Reichlin, S., Stall, G., Telenius, H., Telenius-Berg, M., Tunnacliffe, A. and Ponder, B. A. J. Genetic linkage studies map the multiple endocrine neoplasia type 2 loci to a small interval on chromosome 10q11.2. *Hum. Mol. Genet.*, **2**, 241–246 (1993).

2) Mole, S. E., Mulligan, L. M., Healey, C. S., Ponder, B. A. J. and Tunnacliffe, A. Localisation of the gene for multiple endocrine neoplasia type 2A to a 480 kb region in chromosome band 10q11.2. *Hum. Mol. Genet.*, **2**, 247–252 (1993).

3) Mulligan, L. M., Kwok, J. B. J., Healey, C. S., Elsdon, M. J., Eng, C., Gardner, E., Love, D. R., Mole, S. E., Moore, J. K., Papi, L., Ponder, M. A., Telenius, H., Tunnacliffe, A and Ponder, B. A. J. Germ-line mutations of the *RET* proto-oncogene in multiple endocrine neoplasia type 2A. *Nature*, **363**, 458–460 (1993).

4) Donis-Keller, H., Dou, S., Chi, D., Carlson, K. M., Toshima, K., Lairmore, T. C., Howe, J. R., Moley, J. F., Goodfellow, P. and Wells, S. A. Mutations in the RET proto-oncogene are associated with MEN 2A and FMTC. *Hum. Mol. Genet.*, **2**, 851–856 (1993).

5) Mulligan, L. M., Eng, C., Healey, C. S., Clayton, D., Kwok, J. B. J., Gardner, E., Ponder, M. A., Frilling, A., Jackson, C. E., Lehnert, H., Neumann, H. P. H., Thibodeau, S. N. and Ponder, B. A. J. Specific mutations of the *RET* proto-oncogene are related to disease phenotype in MEN 2A and FMTC. *Nature Genet.*, **6**, 70–74 (1994).

6) Hofstra, R. M. W., Landsvater, R. M., Ceccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Hoppener, J. W. M., van Amstel, H. K. P., Romeo, G., Lips, C. J. M. and Buys, C. H. C. M. A mutation in the *RET* proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature*, **367**, 375–376 (1994).

7) Carlson, K. M., Dou, S., Chi, D., Scavarda, N., Toshima, K., Jackson, C. E., Wells, S. A., Goodfellow, P. J. and Donis-Keller, H. Single missense mutation in the tyrosine kinase catalytic domain of the *RET* protooncogene is associated with multiple endocrine neoplasia type 2B. *Proc. Natl. Acad. Sci. USA*, **91**, 1579–1583 (1994).

8) Ceccherini, I., Bocciardi, R., Luo, Y., Pasini, B., Hofstra, R., Takahashi, M. and Romeo, G. Exon structure and flanking intronic sequences of the human RET proto-oncogene. *Biochem. Biophys. Res. Commun.*, **196**, 1288–1295 (1993).

9) Takahashi, M., Buma, Y., Iwamoto, T., Inaguma, Y., Ikeda, H. and Hiai, H. Cloning and expression of the *ret* proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. *Oncogene*, **3**, 571–578 (1988).

10) Yoshimoto, K., Iwahara, H. and Itakura, M. Relatively good prognosis of multiple endocrine neoplasia type 2B in Japanese: review of cases in Japan and analysis of genetic changes in tumors. *Endocr. J.*, **40**, 649–657 (1993).

11) Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Constantini, F. and Pachnis, V. Defect in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. *Nature*, **367**, 380–383 (1994).

12) Takahashi, M., Buma, Y. and Hiai, H. Isolation of *ret* proto-oncogene cDNA with an amino-terminal signal sequence. *Oncogene*, **4**, 805–806 (1989).