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Two Germline Missense Mutations at Codons 804 and 806 of the *RET* Proto-oncogene in the Same Allele in a Patient with Multiple Endocrine Neoplasia Type 2B without Codon 918 Mutation

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Multiple endocrine neoplasia (MEN) type 2B is a clinically distinct entity among the autosomal dominant MEN 2 syndromes. Most patients with MEN 2B carry a germline mutation (M918T) of the *RET* proto-oncogene, while a few carry A883F. We examined a patient with MEN 2B, but without M918T or A883F, and her relatives. Here, we report the presence in this patient of 2 germline mutations, V804M and Y806C in the same allele. While the novel Y806C was inherited from her father, its carriers (her father and brother) was not affected by MEN 2. In contrast, V804M was a *de novo* mutation, that has been reported in patients with familial medullary thyroid carcinoma. Combinations of mutations of the *RET* proto-oncogene may cause oncogenic activities different from those of single mutations.

Key words: Multiple endocrine neoplasia 2B — *RET* proto-oncogene — Germline mutation — V804M — Y806C

Multiple endocrine neoplasia type 2 syndromes (MEN 2) are autosomal dominant cancer syndromes, which comprise 3 clinically distinct entities: MEN 2A, MEN 2B and familial medullary thyroid carcinoma (FMTC).[1)] The presence of medullary thyroid carcinoma is a common clinical feature. In patients with FMTC, only the thyroid gland is affected, while patients with MEN 2A may develop pheochromocytoma and primary hyperparathyroidism. MEN 2B is characterized by mucosal neuromatosis, ganglioneuromas of the intestinal tract and a marfanoid habitus.[2,3)]

RET is the susceptibility gene for MEN 2 syndromes.[4)] The *RET* proto-oncogene encodes a receptor tyrosine kinase, which contains extracellular, transmembrane and intracellular domains. Mutations in patients with MEN 2A are exclusively found in 1 of the 6 cysteine residues in exon 10 or 11 of the extracellular domain.[4)] Patients with FMTC may carry the same mutations as those of MEN 2A or mutations in the intracellular domain[4)]: E768D in exon 13,[5,6)] V804L[6)] or V804M[7)] in exon 14, or S891A in exon 15.[8)] More than 95% of MEN 2B cases are caused by germline mutation at codon 918 (M918T) in exon 16 of the *RET* proto-oncogene.[1,9)] Very recently, a germline codon 883 mutation (A883F) in exon 15 was reported in 4 cases of MEN 2B without M918T.[10,11)]

Here, we report the presence of 2 germline mutations, V804M and Y806C in the same allele, in a patient with MEN 2B, but without M918T, and we present the results of *RET* mutation analyses in members of her family. The Y806C mutation has not been previously reported.

A 23-year-old woman was referred to Kuma Hospital for evaluation of a right anterior neck mass which she had noticed 2 months previously. On physical examination, 2 firm thyroid tumors were felt, measuring 4.2 cm in the right lobe and 0.8 cm in the left. She was 170 cm in height, and weighed 52 kg. She had had bumpy lips since her childhood, and multiple nodules on her tongue. She had no other symptoms except for constipation. Fine needle aspiration cytology of the tumors strongly suggested medullary thyroid carcinoma. The diagnosis was supported by high levels of serum calcitonin (7000 pg/ml) and carcinoembryonic antigen (97 ng/ml). Her serum calcium was normal. Her urinary excretion of catecholamines and their metabolites per day were; 30.2 μg of epinephrine, 116 μg of norepinephrine, 1115 μg of dopamine, 0.29 mg of metanephrine, and 0.16 mg of normetanephrine. The values of epinephrine and metanephrine

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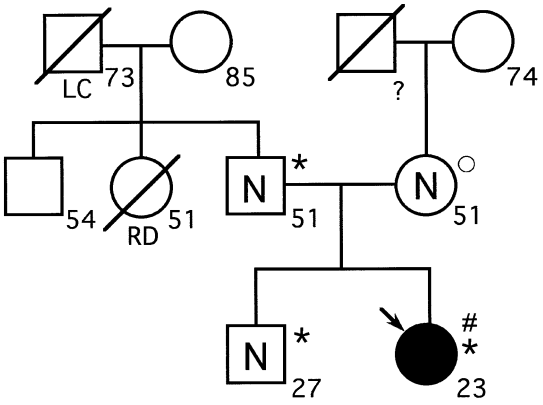

Fig. 1. Pedigree of the present family. The arrow indicates the patient. None of her relatives had multiple endocrine neoplasia type 2 syndrome, although three had died, one of lung cancer (LC), one of renal disease (RD), and one of an unknown cause (?). The number to the lower right of the subject symbol indicates the age in years. N, normal serum calcitonin; #, V804M; ∗, Y806C; ○, no mutation.

were close to their normal upper limits. Scintigraphy with ^{131}I-*meta*-iodobenzylguanidine showed faint accumulations in the adrenal regions, clear accumulation in the right lobe of the thyroid and decreased accumulation in the heart. Computed tomography revealed thickening of the bilateral adrenal glands with no tumor formation and the digestive tract was dilated with gas. Barium enema demonstrated a dilated colon with many diverticles. Ophthalmologists confirmed the presence of thickening of corneal nerves by slit lamp examinations. Thus, a clinical diagnosis of MEN 2B with adrenal glands in medullary hyperplasia, a precursory state of pheochromocytoma, was made. There was no family history of MEN 2 (Fig. 1). We performed a total thyroidectomy and bilateral modified radical neck dissection. At surgery, her nerves were found to be thicker than those of usual cases. Histological examinations confirmed bilateral multiple foci of medullary carcinoma with metastasis in one of the dissected lymph nodes. Two excised parathyroid glands were normal. The combined calcium and gastrin loading test performed 7 days after surgery showed no response in serum calcitonin levels, suggesting no residual tumor.

Her parents and brother were evaluated and exhibited normal appearance and normal serum calcitonin levels. They and the patient gave informed consent for mutation analysis of the *RET* proto-oncogene and blood samples were drawn.

Genomic DNA was extracted from the blood samples with QIAamp blood kits (Qiagen, Hilden, Germany). The DNA sample of the patient was amplified for exons 10, 11, 13, 14, and 16 of the *RET* proto-oncogene by polymerase chain reaction (PCR) using a thermal cycler, and the PCR products were subsequently subjected to single-strand conformation polymorphism (SSCP) analysis to detect mutations, as described previously.[12] Since no single-strand conformational variant was detected, direct cycle sequencing of the PCR products was performed for exons 10 through 16 with DNA sequencing kits and an automated DNA sequencer (dRhodamine Terminator Cycle Sequencing Ready Reaction and ABI PRISM310, Perkin Elmer, Foster City, CA). PCR for the sequencing was performed in a volume of 50 μl containing 0.5 μM of each oligonucleotide primer, 50 ng of DNA, 1× PCR buffer (Takara, Shiga), 250 μM dNTP (Takara), and 2.5 U of *Taq* polymerase (Takara) using an automated thermal cycler (Robocycler, Stratagene, La Jolla, CA). Nucleotide sequences of the primers were as follows: exon 11, 5′-CCTCTGCGGTGCCAAGCCTC-3′ and 5′-GAAGAGGAGTAGCTGACCGG-3′; exon 14, 5′-TGGCTCCTGGAAGACCCAAG-3′ and 5′-TGGCTGGGTGCAGAGCCATA-3′. Primers for exon 10, 13, 15, and 16 were previously described by Ceccherini *et al*.[13] PCR was started with 1 min of denaturation at 95°C, followed by 31 cycles of 1 min at 95°C, 1 min at 68°C, 64°C, and 56°C for exons 10, 11, and 15, respectively, then 2 min at 72°C, or 35 cycles of 1 min at 95°C, 1 min at 66°C, 70°C, and 54°C for exons 13, 14, and 16, respectively, then 2 min at 72°C, and the procedure was completed with 5 min at 72°C. The PCR products were purified using QIAquick PCR purification kits (Quiagen) and subjected to direct cycle sequencing and restriction enzyme analysis.

The direct sequencing analysis detected 2 heterozygous missense mutations at codons 804 and 806 in exon 14 (Fig. 2A). No mutation was detected at codon 883 in exon 15 or at codon 918 in exon 16. The point mutation of GTG to ATG at codon 804 resulted in a change of the amino acid from valine to methionine (V804M), and point mutation of TAC to TGC at codon 806 substituted cysteine for tyrosine (Y806C). The presence of these mutations were confirmed by digestion analyses with restriction enzymes *Nla*III and *Hha*I, respectively (Fig. 3). The mutant (GTG to ATG) at codon 804 creates an additional *Nla*III site (CATG), and the variant (TAC to TGC) at codon 806 creates a *Hha*I site (GCGC). Digestions with *Nla*III and *Hha*I were performed according to the instructions of the supplier (Toyobo Co., Ltd., Osaka). Digested or non-digested PCR products were subjected to 8% polyacrylamide gel electrophoresis in TBE buffer and visualized by exposure to ethidium bromide. The PCR products for exon 14 of the family members' DNA samples were subjected to digestion analyses. Her mother was negative for both analyses. Her father and brother were positive only for *Hha*I digestion analysis (Fig. 3). The presence of TAC-to-TGC mutation at codon 806 was confirmed by cycle sequencing analysis (Fig. 2A).

The patient's PCR product of exon 14 was cloned into

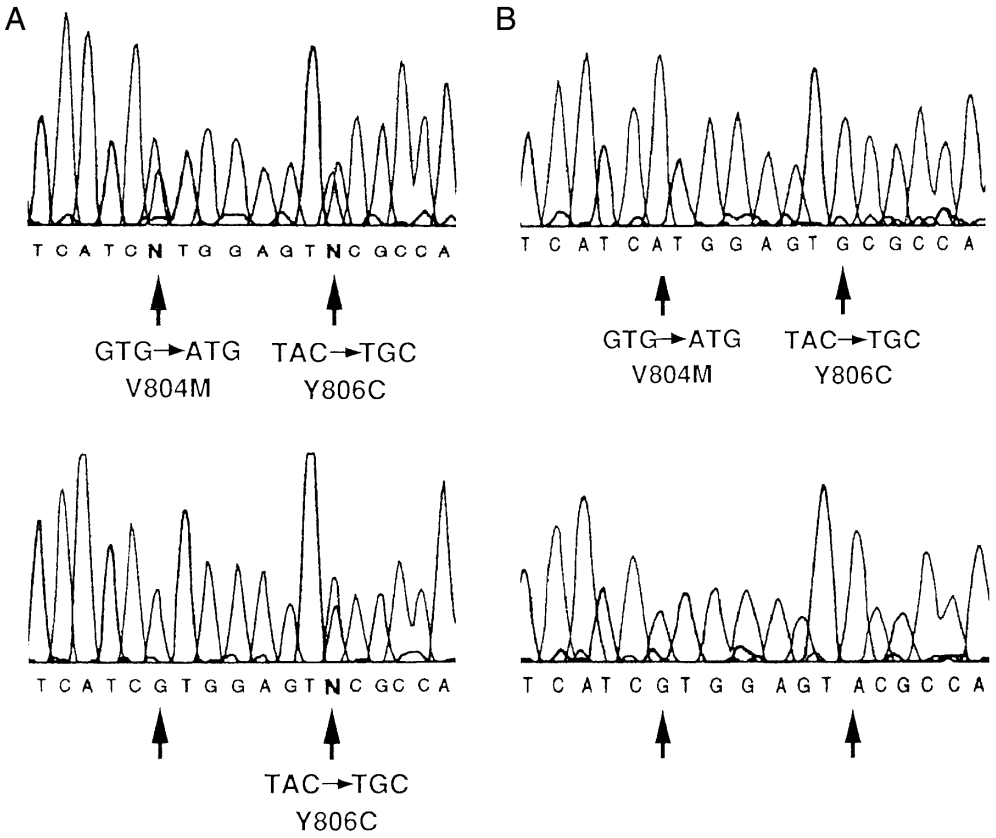

Fig. 2. Sequence analysis of the PCR products of exon 14. A) The patient exhibited 2 missense mutations; GTG to ATG at codon 804 resulting in an amino acid change from valine to methionine (V804M), and TAC to TGC at codon 806 substituting cysteine for tyrosine (Y806C) (upper). Her father carried Y806C only (lower). B) Sequence analysis of the plasmids in which the patient's PCR product was cloned. The clones exhibit both mutations (upper) or no mutation (lower), indicating the presence of the mutations on the same allele. Arrows indicate nucleotides involved in the mutations.

the pCRII vector and sequenced (Invitrogen, San Diego, CA). Sequence analysis of 6 clones revealed that 1 of the 6 clones carried the 2 mutations, GTG to ATG at codon 804 and TAC to TGC at codon 806 (Fig. 2B), whereas the remaining 5 had the wild-type sequence.

We further examined the presence or absence of the Y806C mutation in 195 healthy Japanese volunteers. No subject was found to carry this mutation by restriction enzyme analysis using *Hha*I on genomic DNA samples from peripheral blood.

MEN 2B is characterized by medullary thyroid carcinoma and mucosal neuromatosis or ganglioneuromas of the digestive tract.[1)] Thickening of the nerves can be observed noninvasively by slit lamp examination of the cornea. About 50% of patients with this syndrome develop pheochromocytoma, often in association with a marfanoid habitus, diverticles of the colon and dilated colon.[2, 3)] The clinical features of the present case fulfill the definition of MEN 2B by the International RET Consortium.[1)]

Although several mutations at several codons are associated with MEN 2A and FMTC, only germline M918T was found (in nearly 95% of the cases) in MEN 2B until recently.[1, 9)] Recently, Gimm *et al.*[10)] and Smith *et al.*[11)] examined 3 unrelated patients with MEN 2B, but without M918T, and each group found that 2 of their 3 patients had a germline dinucleotide mutation in codon 883 (GCT to TTT, A883F). They considered this mutation to be pathogenic due to the presence of somatic A883F mutation in 3.7% of sporadic medullary thyroid carcinoma (MTC) cases and the absence of reported polymorphisms at codon 883, although no functional analysis was performed.

The present patient with MEN 2B, but without M918T or A883F, was found to bear 2 germline mutations, V804M and Y806C, both in exon 14 encoding the intra-

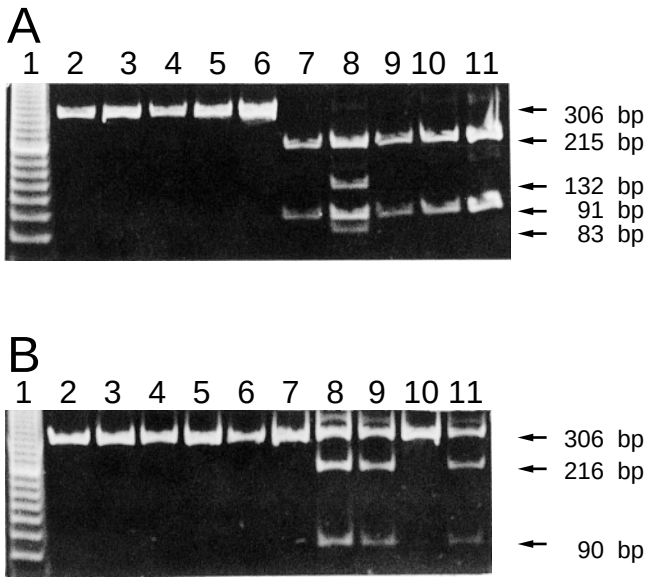

Fig. 3. Restriction enzyme analysis of the PCR products of exon 14 of the patient and her family members. Lane 1, markers; lanes 2 and 7, normal volunteer; lanes 3 and 8, the patient; lanes 4 and 9, the patient's father; lanes 5 and 10, the patient's mother; lanes 6 and 11, the patient's brother. Lanes 2 through 6 were not digested, and lanes 7 through 11 were digested. A) Digestion with *Nla*III. The wild-type amplicon (306 bp) is digested to 215 bp and 91 bp fragments (lanes 7–11). The mutant (GTG to ATG) at codon 804 creates an additional *Nla*III site, yielding 132 bp and 83 bp products (lane 8). B) Digestion with *Hha*I. The variant (TAC to TGC) at codon 806 creates a *Hha*I site. The amplicon from the mutant allele is digested to 216 bp and 90 bp fragments (lanes 8, 9, and 11).

cellular tyrosine kinase domain. The Y806C mutation has not been reported in patients with MEN 2 syndromes or in normal subjects. The absence of this mutation in 390 alleles of our healthy volunteers may suggest that this is unlikely to be a DNA polymorphism, although a rare polymorphism can not be completely excluded. This novel Y806C mutation, however, probably does not play an important role in the pathogenesis of MEN 2, since the 2 carriers, who were older than the patient, had normal serum calcitonin levels. At codon 804, 2 missense mutations, V804L and V804M, have been reported in FMTC families.[6, 7] These mutations have been associated with MTC of late onset, with a more favorable course. The present patient, however, was young, and a lymph node was already involved. Germline V804M has also been reported in patients with apparently sporadic MTC.[14, 15]

MEN 2A type mutations are typically clustered in the extracellular cysteine-rich domain and result in constitutive activation of the tyrosine kinase through the formation of disulfide-bonded *RET* homodimers.[16] M918T mutation of MEN 2B, located in the catalytic core of the tyrosine kinase domain, produces oncogenic activity through changes in catalytic activity and substrate specificity.[16] Although the functional change of V804M has not been clarified, the product of *RET* V804L was reported to show transforming activity and to induce neuronal differentiation of rat pheochromocytoma PC12 cells, though its effects were several-fold less potent than those of C634R of MEN 2A or M918T of MEN 2B.[17]

The present patient exhibited V804M and Y806C on the allele from her father, who carried Y806C. Thus, V804M is a *de novo* mutation, a common phenomenon found in about 50% of MEN 2B cases.[18] Although V804M is pathogenic for MTC or FMTC, no cases of the MEN 2B phenotype with this mutation have been reported. The significance of Y806C is unknown. The presence of non-pathogenic mutation of Y806C on the same allele might have caused a structural change in the RET protein, resulting in more potent activities than those of *RET* with V804M only. Alternatively, Y806C may actually exhibit weak oncogenic activity, and the 2 carriers of this mutation were not affected at the time of examination due to low penetrance, as reported in FMTC families. If this is the case, a *de novo* V804M mutation in Y806C carriers may have an additive effect. Marsh *et al.*[19] and we[20] reported somatic mutations in tumors of patients with germline *RET* mutations and suggested additional effects of the second mutations on tumorigenesis.

Although the functions of the products of *RET* with Y806C and *RET* with V804M and Y806C have not been clarified, the present case suggests that combinations of mutations of the *RET* proto-oncogene cause oncogenic activities different from those of single mutations.

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