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A Novel Somatic Mutation in the RET Proto-oncogene in Familial Medullary Thyroid Carcinoma with a Germline Codon 768 Mutation

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In individuals who carry germline mutations in tumor suppressor genes predisposing them to inherited cancer syndromes, occurrence of somatic mutations in the same genes contributes to tumorigenesis. Germline mutations in the RET proto-oncogene predispose individuals to multiple endocrine neoplasia (MEN) type 2 syndromes. Since these mutations are oncogenic by themselves, somatic mutations in the same gene had been thought unnecessary. Recently, a somatic mutation at codon 918 of RET was reported in medullary thyroid carcinoma (MTC) and C-cell hyperplasia in patients with MEN 2A or familial MTC (FMTC), suggesting its possible contribution to tumorigenesis. We describe here a novel somatic mutation at codon 919 in a patient with FMTC carrying a germline mutation at codon 768 that may also be related to tumor progression.

Key words: Familial medullary carcinoma — Germline mutation — Somatic mutation — RET proto-oncogene

In most inherited cancer syndromes, the individuals predisposed to the syndromes carry mutations in tumor suppressor genes on one allele in their germline. Somatic mutations on the other allele of the same genes, resulting in loss of function, contribute to tumorigenesis.[1)] Multiple endocrine neoplasia (MEN) 2 syndromes, MEN types 2A and 2B and familial medullary thyroid carcinoma (FMTC), are exceptional among inherited cancer syndromes, being caused by oncogenic mutations.[2)] MTC is a characteristic component of the syndromes. Germline mutations in the RET proto-oncogene encoding a receptor tyrosine kinase are responsible for these syndromes.[3–5)] Previous studies have demonstrated that mutations in RET codon 634 or 918 increase receptor tyrosine kinase activity.[2, 6)] Since a single activating mutation in RET was shown to transform cells,[2)] somatic mutation was thought unnecessary in MEN 2 syndromes. However, Marsh *et al.*[7)] reported a somatic missense mutation at codon 918 of RET in 3 MTCs and a sample of C-cell hyperplasia in cases with a germline codon 618, 620, or 634 mutation, and suggested the possible contribution of the somatic mutation to tumorigenesis. We report a novel somatic missense mutation at codon 919 of RET in MTC with a rare germline mutation at RET codon 768 that may also stimulate tumor progression.

The index patient was a 76-year-old woman with a history of elevated serum carcinoembryonic antigen levels (up to 42 ng/ml) for 4 years. She was otherwise healthy. After a thorough examination for possible origin of malignancy, a thyroid tumor was found, and she was referred to Kuma Hospital. Her family history was not specific, except for a younger brother being operated for rectal cancer (Fig. 1). An ultrasound examination showed bilateral thyroid tumors, 2.4×1.8 cm in the left lobe and 0.5×0.5 cm in the right lobe, and enlarged left jugular lymph nodes. Aspiration biopsy cytology of the main tumor was consistent with medullary thyroid carcinoma. Her serum calcitonin level was 2800 pg/ml. There was no evidence of hyperparathyroidism or pheochromocytoma on laboratory or imaging studies. A total thyroidectomy and left modified radical neck dissection were performed. The diagnosis of bilateral medullary thyroid carcinoma with lymph node metastases was confirmed histologically. After surgery, her basal calcitonin level decreased to a normal value of 82 pg/ml with an equivocal response (maximum of 140 pg/ml) to a combined calcium and gastrin loading test.[8)] Four of her relatives were screened for medullary thyroid carcinoma with the combined provocative test and ultrasound scan. Two of them showed inconclusive minor responses in serum calcitonin levels (Table I). None of the examinees had thyroid tumors demonstrable on an ultrasound scan.

DNA was extracted from blood samples and paraffin-embedded tumor tissues using QIAamp blood and tissue kits (QIAGEN, Chatsworth, CA). DNAs were subjected to polymerase chain reaction (PCR) using a thermal cycler (Robocycler gradient, Stratagene, La Jolla, CA). The primer sequences and the PCR conditions are de-

scribed elsewhere.[9] The primers were labeled with [γ-^{32}P]ATP (>5,000 Ci/mmol, Amersham, Tokyo) and T4 polynucleotide kinase (10 units, Nippon Gene Ltd., Toyama). PCR products for all 20 exons were screened for the presence of mutations by single strand conformation polymorphism (SSCP) analysis on a polyacrylamide gel containing 10% glycerol.[10] The electrophoreses were performed under strict temperature control at 16°C and 18°C on an electrophoresis apparatus (ATTO, Tokyo) connected to a circulating water bath. The PCR products showing conformational variants were subjected to direct sequencing using a T7 polymerase PCR product sequencing kit (United States Biochemical, Cleveland, OH). The mutations at codon 768 were confirmed by Alu I restriction endonuclease digestion.

The PCR-SSCP analysis of RET proto-oncogene in blood samples detected a conformational variant in exon 13 in the index patient and three of her relatives (data not shown). The direct sequencing analysis revealed a missense mutation, from GCG (Glu) to GAC (Asp), at codon 768 (Fig. 2). A reported DNA polymorphism at codon 769 leucine (CTT/CTG) in exon 13 was also observed (Fig. 2). Since the G-to-C conversion at codon 768 destroys the restriction site of Alu I, this mutation was confirmed by digestion with Alu I (data not shown). After a complete explanation, two of the carriers chose to be followed annually, and a 51-year-old woman with an equivocal response in calcitonin level underwent a total thyroidectomy and central node dissection. Histological examinations showed multiple medullary cancer foci up to 1 mm in maximum diameter in both lobes. Lymph nodes were not involved. After surgery, the results of the combined loading test were normal, with basal and maximum values of 31 pg/ml and 36 pg/ml, respectively.

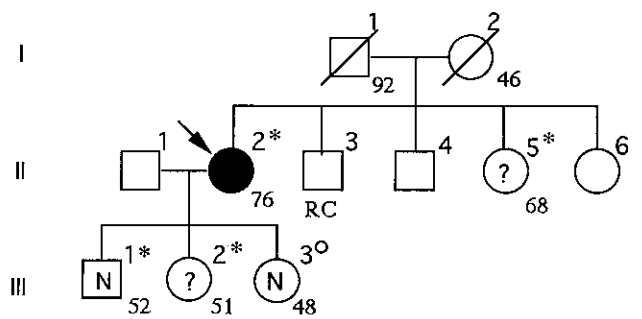

Fig. 1. Pedigree of the present family. The arrow indicates the index patient. The results of combined calcium and gastrin loading tests are shown in the subject symbols: ?, equivocal; N, normal response. The number to the lower right of the subject symbols indicates age in years. RC, rectal cancer; *, mutant carrier; ○, non-carrier.

The PCR-SSCP and direct sequencing analyses of the samples from the large MTC of the left lobe and metastatic lymph node of the index patient detected a novel missense mutation substituting CCA (Pro) for GCA (Ala) at codon 919 in exon 16 of the RET proto-oncogene (Fig. 3, PCR-SSCP data not shown). This mutation was not detected in her blood or in the small MTC in the right lobe (Fig. 3).

This is the first report of a novel somatic mutation at codon 919 within exon 16 of the RET proto-oncogene in MTC of a patient with FMTC carrying a rare codon 768 mutation in the germline. The somatic mutation was present in the large tumor of the left lobe and in metastatic lymph nodes, while it was not detected in either the small tumor of the right lobe or her blood sample. This may suggest that the second mutation in the RET proto-oncogene is related to the tumor progression.

In more than 90% of patients with MEN 2A, 1 of the 5 cysteines (609, 611, 618, 620, and 634) of the RET exons 10 and 11 is mutated.[4, 11] The mutations in FMTC cover the same region[4, 11] with additional rare mutations in non-cysteine codons 768 and 804 coding for the intracellular tyrosine kinase domain.[12, 13] Mulligan *et al.*[4] proposed that families with a minimum of 4 members with MTC and without evidence of pheochromocytoma or parathyroid disease in any of the members be classified

Table I. Subjects' Clinicopathological Data and Germline RET Mutation

Pt.	Age	Sex	Tumor status	Plasma calcitonin (pg/ml)		RET mutation	Pathology
				Basal	Maximum[a]		
II-2	76	F	Bilat. Tumors N(+)	2800	—	Yes	Bilat. MTCs, N(+)
II-5	68	F	ND	42	430	Yes	
III-1	52	M	ND	26	84	Yes	
III-2	51	F	ND	31	340	Yes	Microscopic MTCs, N(−)
III-3	48	F	ND	15	18	No	

a) Maximum values after combined calcium and gastrin loading.
N(+): lymph nodes involved, N(−): not involved.
ND: no detectable tumor in ultrasound examinations.

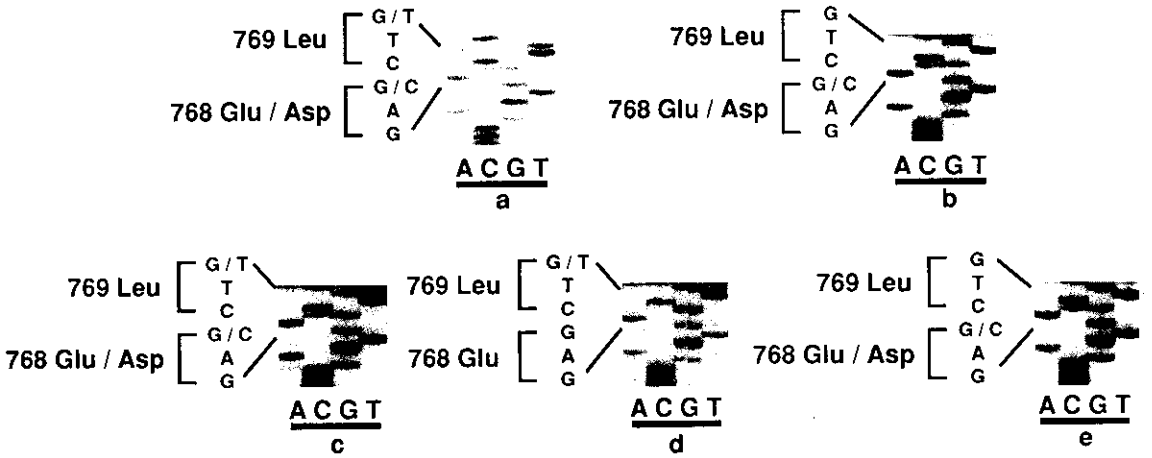

Fig. 2. Direct sequencing analysis for a germline mutation at codon 768. Codon 768 glutamic acid-to-aspartic acid (GAG→GAC) mutation is shown in a, b, c, and e. DNA polymorphism at codon 769 leucine (CTG/CTT) is displayed in a, c, and d. a, II-2; b, III-2; c, II-5; d, III-3; e, III-1.

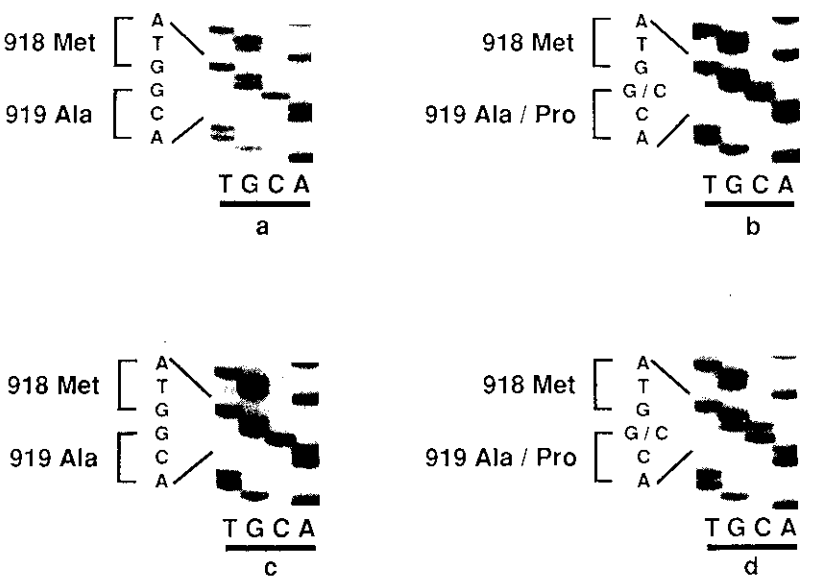

Fig. 3. A somatic point mutation at codon 919 in exon 16. Direct sequencing analysis identified the presence of a codon 919 alanine-to-proline (GCA→CCA) mutation only in the tumors of the left thyroid lobe (b) and metastasized lymph node (d). a, blood sample of the patient; c, tumor of the right lobe.

as FMTC. In the present family, there are at least 4 mutant gene carriers, and 2 of them had MTC only. The codon 768 mutation identified in the present family has been identified only in individuals with FMTC.[12, 13] As far as we know, the present family is the first reported case of FMTC with codon 768 mutation in Japan. MEN 2B is exclusively caused by a single mutation at codon 918[5, 11] that codes for part of the substrate binding pocket in the tyrosine kinase domain.[14] The most common codon 634 mutations in MEN 2A cause dimerization of the mutant receptor tyrosine kinase in the absence of ligand, leading to increased phosphorylation activity.[2, 6] The RET 918 mutation increases receptor autophosphorylation in the absence of receptor dimerization and changes the substrate specificity.[2] Both mutations have been shown to transform cells *in vitro*.[2]

Although every C cell of patients with MEN 2 syndromes obviously carries one of these oncogenic mutations from birth, the number of MTC tumor foci is small, and the time necessary for tumor development is usually very long. These facts imply the involvement of other factors in the development of MTC. Recently, Marsh *et al.*[7] reported the somatic codon 918 mutation in MEN 2A MTC and C-cell hyperplasia and suggested its possible contribution to tumorigenesis. They speculated that the combination of a dimer-inducing MEN2A mutation and a syntenic codon 918 mutation results in a receptor activity level surpassing that achieved by either mutation alone, or alternatively, that in the case of mutations being located on different alleles, every RET molecule is mutated.

Although the biological effect of the codon 919 missense mutation demonstrated in the present study has not been clarified, the mutation may also affect the activity of receptor tyrosine kinase or change the substrate specificity, since it covers part of the substrate binding pocket.[14] Although determining which of the already mutated allele or normal allele had the second somatic mutation is important, we could not clarify this issue with our PCR products from formalin-fixed and paraffin-embedded material. Because of the limited number of reported cases of FMTC with codon 768 mutation, the clinical features of patients with this rare mutation are not clear. However, FMTC is generally associated with a late onset and less aggressive nature of the disease.[15] In the present family with 4 mutant gene carriers aged more than 50 years, only the index patient aged 76 years had clinical disease, and one aged 51 years had microscopic tumors. This low penetrance and mild phenotype may indicate that the activity of codon 768 mutation in tumorigenesis is weak, and additional mutations are required for tumor development. The presence of the somatic codon 919 mutation only in the large tumor and metastatic foci may suggest its possible role in tumor progression.

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