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Cys611Ser mutation in *RET* proto-oncogene in a kindred with medullary thyroid carcinoma and Hirschsprung's disease

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Germline mutations in the *RET* proto-oncogene are responsible for the development of human hereditary diseases, including multiple endocrine neoplasia (MEN) type 2A and 2B, familial medullary thyroid carcinoma (FMTC), and Hirschsprung's disease (HSCR). It has been reported that some families developed both MEN 2A/FMTC and HSCR, in which a mutation in a cysteine residue at codon 609, 618, or 620 in the *RET* gene was present. Here we report a novel *RET* mutation detected in a Japanese family with medullary thyroid carcinoma and HSCR. A germline mutation in cysteine 611 of the *RET* gene was identified in this family, which introduced an amino-acid change from cysteine to serine. By biological and biochemical analyses of mutant RET proteins, we previously predicted the potentiality that amino-acid substitution for cysteine 611 as well as cysteines 609, 618, and 620 would promote the development of MEN 2A/FMTC and HSCR. This clinical case substantiates our suggestion for the mechanism of the development of both the diseases.

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Introduction

The *RET* proto-oncogene encodes a transmembrane receptor with a tyrosine kinase domain that is expressed in cells derived from neural crest.^{1–3} It is known that its germline mutations develop several human hereditary disorders, including multiple endocrine neoplasia type 2 (MEN 2) and Hirschsprung's disease (HSCR).^{4,5} MEN 2 is an autosomal dominant neoplastic syndrome that is classified into three clinical subtypes, MEN 2A, MEN 2B, and familial medullary thyroid carcinoma (FMTC). MEN 2A is character-

ized by the clinical feature of medullary thyroid carcinoma (MTC), pheochromocytoma, and parathyroid hyperplasia. MEN 2B patients are affected by a more complex phenotype including MTC, pheochromocytoma, skeletal abnormalities, and mucosal neuroma, whereas FMTC is defined by the sole phenotype of MTC without any other abnormalities. HSCR is a neurocristopathy characterized by the absence of intrinsic ganglion cells in the distal gastrointestinal tract, and is inherited in a non-Mendelian fashion.⁶

The mechanisms of development of MEN 2 and HSCR by *RET* mutations are quite different. MEN 2 and HSCR are caused by its 'gain of function' and 'loss of function' mutations, respectively.⁴ In MEN 2A patients, mutations are mostly detected in one of the six cysteine residues at codons 609, 611, 618, 620, 630, and 634 in the RET extracellular cysteine-rich domain. These cysteine mutations

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constitutively activate RET by inducing its disulfide-linked homodimerization.⁷⁻⁹ On the other hand, *MEN 2B* mutations are detected at codon 883 or 918 in the RET intracellular kinase domain, leading to RET activation without dimer formation.⁹⁻¹¹ In FMTC patients, most *RET* mutations are identified in cysteine residues like the *MEN 2A* mutations, and some are at codon 768, 804, or 891 in the intracellular kinase domain. *HSCR* mutations including missense, nonsense, deletion, insertion, and frameshift mutations were reported throughout the whole coding region of *RET* gene without any hot spots, and impair RET function by various mechanisms. We and others demonstrated that *HSCR* mutations identified in the RET extracellular domain markedly impair the RET cell surface expression, whereas the mutations in the kinase domain impair the RET kinase activity or downstream signaling.¹²⁻¹⁶

Interestingly, the development of both *MEN 2A*/FMTC and *HSCR* has been reported in some families.¹⁷⁻²⁹ The screening for germline mutations of the *RET* proto-oncogene revealed that a single amino-acid substitution in one of the three cysteine residues at codons 609, 618, and 620 is involved in the development of both diseases. In this study, we report the first clinical case to prove the pathogenesis of a cysteine 611 mutation for the development of both MTC and HSCR.

Patients and methods

Patients

Figure 1 shows the pedigree of the family described in this paper. The proband (II-2), a 47-year-old male, underwent left hemithyroidectomy for preoperative diagnosis as papillary thyroid carcinoma of the left thyroid gland in Aichi Cancer Center Hospital (Nagoya, Japan). It was diagnosed as an MTC by the postoperative histopathological examination of his surgical specimens. His family history showed that the proband's father (I-1) died at the age of 56 years from an advanced MTC in Nagoya University Hospital (Nagoya, Japan). In addition, the proband's son (III-3) underwent an operation for HSCR (short segment type) at the age of 1 year in the Central Hospital, Aichi Prefectural Colony (Kasugai, Japan).

On the basis of these episodes, an inherited disorder was speculated in this family. The proband was admitted to our hospital for further examinations and treatments. The molecular examinations for germline *RET* mutations in the affected family members were performed under informed consent and permission of ethical committee of Nagoya University School of Medicine. The examination of the proband's father was performed by agreement from his family.

DNA preparation

Genomic DNA of the proband was extracted from peripheral blood leukocytes according to the standard protocols.

Figure 1 The pedigree of the family with MTC and HSCR. Individual II-2 is the proband.

Genomic DNA of the proband's son was extracted from the paraffin-embedded tissue sections of his ileum that was resected at the operation for adhesive ileus. Three pieces of 10- μ m-thick paraffin-embedded tissue sections were incubated in xylene for 30 min in an Eppendorf tube to remove paraffin from the tissues. Xylene was discarded, and the tissues were washed with ethanol and incubated in proteinase K buffer (10 mM Tris-HCl, pH 8.3, 2.5 mM MgCl₂, 0.45% Tween 20, 0.45% NP-40, 10 mg/ml proteinase K) overnight. The DNAs were purified by phenol/chloroform DNA extraction method. Genomic DNA of the proband's father was also isolated from the paraffin-embedded tissue sections of his autopsied liver.

Mutation analysis

Sets of oligonucleotide primers to amplify the protein coding sequences of the *RET* gene were designed based on the published genomic sequences.³⁰ PCR reactions were performed using the genomic DNAs of the proband, proband's son, and proband's father as templates, and the PCR products were subjected to sequencing directly, or they were cloned into pGEM-T plasmids (Promega, Madison, WI, USA) and sequenced. Double-strand sequencing of the PCR products and the inserts in pGEM-T plasmids was performed by a cycle sequencing program using the BigDyeTM Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were determined by an automated Applied Biosystems sequencer model 310 (Applied Biosystems, Foster City, CA, USA).

Results

A 47-year-old male (II-2 in Figure 1) underwent a completion thyroidectomy with bilateral lymph node dissection.

Small lesions of the remnant thyroid were diagnosed as MTC, but no pathological changes such as adenoma or hyperplasia were detected in the biopsy specimens of the parathyroid glands. It was also pointed out that he had a small tumor (2 cm in diameter) in the left adrenal gland by a screening computed tomography (CT) examination. His serum and urinary catecholamine levels and other adrenal hormones were within normal range, and he has not undergone an operation to resect the adrenal tumor to date. However, it seems likely that the left adrenal tumor is pheochromocytoma, thus, the possibility that he may be a MEN 2A patient is not excluded.

The sequences of *RET* exons 10 and 11, where mutations are usually present in MEN 2A/FMTC patients, were examined, and a TGC to TCT heterozygous germline mutation was detected in codon 611 of the *RET* gene, resulting in the amino-acid change from cysteine to serine (C611S) (Figure 2). The proband's son developed HSCR and was also screened for *RET* mutations. The same heterozygous mutation in codon 611 was detected. No other nucleotide alterations were found in the whole coding sequence of the *RET* gene of the proband's son. The same C611S mutation was present in the proband's father who died of MTC.

Discussion

A total of 22 families with *RET* mutations that developed both MEN 2A/FMTC and HSCR were previously reported.^{18–29} The *RET* mutations identified in these families include two C609Y, five C618R, three C618S, eight C620R, three C620S, and one C620W mutations. In the present report, we described a new family affected with both MTC and HSCR. The mutation analyses of the *RET* gene revealed that a substitution of serine for cysteine at codon 611 was detected in affected members of the family. This family did not fulfill the definition of MEN 2A or FMTC, because MTC developed in only two members so far. Despite this fact, this is the first case of a family that suggests the pathogenesis of *RET* codon 611 mutation for the development of MTC and HSCR that are caused by gain-of-function and loss-of-function mutations of *RET*, respectively.

We previously proposed a possible mechanism for the development of both diseases by a single cysteine mutation in *RET*.^{4,31,32} In NIH3T3 cells, the transforming activities of the RET proteins harboring the mutations in cysteine 609, 611, 618, or 620 were significantly lower than those harboring the mutations in cysteine 634.^{31,33} The low transforming activities of the former mutant proteins were caused by impairment of their cell surface expression. Cysteine 609, 611, 618 or 620 mutations may alter the correct folding of RET as observed with the HSCR mutations identified in the RET extracellular domain,^{13,14} thereby interfering with RET maturation, intracellular

Figure 2 C611S mutation detected in the family. Sequences of *RET* exon 10 of the proband were determined by direct sequencing of the PCR products (a). *RET* exon 10 was also sequenced after subcloning of the PCR products into the pGEM-T plasmid (b, c). A TGC to TCT heterozygous mutation was detected at codon 611. (b) wild-type allele, (c) mutant allele.

trafficking or stable expression on the cell surface. The signal through the reduced RET proteins on the cell surface would not be sufficient for the differentiation of the intestinal ganglion cells, resulting in the development of HSCR.

In addition, cysteine 609, 611, 618, or 620 mutations can induce ligand-independent RET dimerization at low levels responsible for the transforming activity in NIH3T3 cells.^{31,33} Since these mutations were also identified in many MEN 2A/FMTC families, the low transforming activity appears to be sufficient to trigger the development of MTC. Based on these data, we predicted that cysteine 611 mutations have the potential to develop both HSCR and MTC.³¹ Mutation analysis of the family reported here supported our prediction.

Another interesting point of the present case is that two nucleotides of *RET* codon 611 were substituted (from TGC to TCT). No case that shows two-nucleotide alteration in one codon of the *RET* gene was previously reported, although some cases that have two codons with one-nucleotide alteration in the *RET* gene were reported.²⁷ In addition, the present case showed no nucleotide alterations at polymorphic codons in the *RET* gene that may affect the development of HSCR.^{34–38}

Germline mutations in several genes including GDNF, neurturin, endothelin receptor B, endothelin-3, SOX10, endothelin-converting enzyme 1, and ZFH1B were reported in HSCR patients. However, the mutation frequencies of these genes in HSCR patients are significantly low compared with that of the *RET* gene.^{39–47} Furthermore, some other loci were proposed to be associated with the risk of HSCR.⁴⁸ Although the possibility that the mutations of these genes are present and affect the development of HSCR in this case is not excluded, it is not practical to screen all the genes. Among the genes described above, the *RET* status is the most important for follow-up of HSCR patients because the patients who have a mutation at codon 609, 611, 618, or 620 in the *RET* gene are at risk for the development of MTC. Thus, the screening for these codons in the *RET* gene should be performed in all cases of HSCR routinely.

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References

- Takahashi M, Buma Y, Iwamoto T, Inaguma Y, Ikeda H, Hiai H: Cloning and expression of the *ret* proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. *Oncogene* 1988; **3**: 571–578.
- Pachnis V, Mankoo B, Costantini F: Expression of the *c-ret* proto-oncogene during mouse embryogenesis. *Development* 1993; **119**: 1005–1017.
- Tsuzuki T, Takahashi M, Asai N, Iwashita T, Matsuyama M, Asai J: Spatial and temporal expression of the *ret* proto-oncogene product in embryonic, infant and adult rat tissues. *Oncogene* 1995; **10**: 191–198.
- Takahashi M: The GDNF/*RET* signaling pathway and human diseases. *Cytokine Growth Factor Rev* 2001; **12**: 361–373.
- Manié S, Santoro M, Fusco A, Billaud M: The *RET* receptor: function in development and dysfunction in congenital malformation. *Trends Genet* 2001; **17**: 580–589.
- Amiel J, Lyonnet S: Hirschsprung disease, associated syndromes, and genetics: a review. *J Med Genet* 2001; **38**: 729–739.
- Asai N, Iwashita T, Matsuyama M, Takahashi M: Mechanism of activation of the *ret* proto-oncogene by multiple endocrine neoplasia 2A mutations. *Mol Cell Biol* 1995; **15**: 1613–1619.
- Borrello MG, Smith DP, Pasini B *et al*: *RET* activation by germline *MEN2A* and *MEN2B* mutations. *Oncogene* 1995; **11**: 2419–2427.
- Santoro M, Carlomagno F, Romano A *et al*: Activation of *RET* as a dominant transforming gene by germline mutations of *MEN2A* and *MEN2B*. *Science* 1995; **267**: 381–383.
- Iwashita T, Asai N, Murakami H, Matsuyama M, Takahashi M: Identification of tyrosine residues that are essential for transforming activity of the *ret* proto-oncogene with *MEN2A* or *MEN2B* mutation. *Oncogene* 1996; **12**: 481–487.
- Iwashita T, Kato M, Murakami H *et al*: Biological and biochemical properties of *Ret* with kinase domain mutations identified in multiple endocrine neoplasia type 2B and familial medullary thyroid carcinoma. *Oncogene* 1999; **18**: 3919–3922.
- Pasini B, Borrello MG, Greco A *et al*: Loss of function effect of *RET* mutations causing Hirschsprung disease. *Nat Genet* 1995; **10**: 35–40.
- Carlomagno F, De Vita G, Berlingieri MT *et al*: Molecular heterogeneity of *RET* loss of function in Hirschsprung's disease. *EMBO J* 1996; **15**: 2717–2725.
- Iwashita T, Murakami H, Asai N, Takahashi M: Mechanism of *Ret* dysfunction by Hirschsprung mutations affecting its extracellular domain. *Hum Mol Genet* 1996; **5**: 1577–1580.
- Iwashita T, Kurokawa K, Qiao S *et al*: Functional analysis of *RET* with Hirschsprung mutations affecting its kinase domain. *Gastroenterology* 2001; **121**: 24–33.
- Bordeaux MC, Forcet C, Granger L *et al*: The *RET* proto-oncogene induces apoptosis: a novel mechanism for Hirschsprung disease. *EMBO J* 2000; **19**: 4056–4063.
- Verdy M, Weber AM, Roy CC, Morin CL, Cadotte M, Brochu P: Hirschsprung's disease in a family with multiple endocrine neoplasia type 2. *J Pediatr Gastroenterol Nutr* 1982; **1**: 603–607.
- Chretien P, Blanchard L, Gaboury L, Lacroix A, Verdy M, Villeneuve L: Cosegregation of Hirschsprung's disease (HSCR) and a *RET* mutation in a French Canadian family with *MEN 2A*. *Am J Hum Genet* 1994; **55**: A357.
- Mulligan LM, Eng C, Attie T *et al*: Diverse phenotypes associated with exon 10 mutations of the *RET* proto-oncogene. *Hum Mol Genet* 1994; **3**: 2163–2167.
- Borst MJ, VanCamp JM, Peacock ML, Decker RA: Mutational analysis of multiple endocrine neoplasia type 2A associated with Hirschsprung's disease. *Surgery* 1995; **117**: 386–391.
- Blank RD, Sklar CA, Dimich AB, LaQuaglia MP, Brennan MF: Clinical presentations and *RET* protooncogene mutations in seven multiple endocrine neoplasia type 2 kindreds. *Cancer* 1996; **78**: 1996–2003.
- Caron P, Attie T, David D *et al*: C618R mutation in exon 10 of the *RET* proto-oncogene in a kindred with multiple endocrine neoplasia type 2A and Hirschsprung's disease. *J Clin Endocrinol Metab* 1996; **81**: 2731–2733.
- Peretz H, Luboshitsky R, Baron E *et al*: Cys 618 Arg mutation in the *RET* proto-oncogene associated with familial medullary thyroid carcinoma and maternally transmitted Hirschsprung's disease suggesting a role for imprinting. *Hum Mutat* 1997; **10**: 155–159.
- Borrego S, Eng C, Sanchez B, Saez ME, Navarro E, Antinolo G: Molecular analysis of the *ret* and GDNF genes in a family with multiple endocrine neoplasia type 2A and Hirschsprung disease. *J Clin Endocrinol Metab* 1998; **83**: 3361–3364.
- Decker RA, Peacock ML, Watson P: Hirschsprung disease in *MEN 2A*: increased spectrum of *RET* exon 10 genotypes and strong genotype–phenotype correlation. *Hum Mol Genet* 1998; **7**: 129–134.
- Romeo G, Ceccherini I, Celli J *et al*: Association of multiple endocrine neoplasia type 2 and Hirschsprung disease. *J Intern Med* 1998; **243**: 515–520.
- Sijmons RH, Hofstra RM, Wijburg FA *et al*: Oncological implications of *RET* gene mutations in Hirschsprung's disease. *Gut* 1998; **43**: 542–547.
- Inoue K, Shimotake T, Inoue K, Tokiwa K, Iwai N: Mutational analysis of the *RET* proto-oncogene in a kindred with multiple endocrine neoplasia type 2A and Hirschsprung's disease. *J Pediatr Surg* 1999; **34**: 1552–1554.

- 29 Pasini B, Rossi R, Ambrosio MR *et al*: *RET* mutation profile and variable clinical manifestations in a family with multiple endocrine neoplasia type 2A and Hirschsprung's disease. *Surgery* 2002; **131**: 373–381.
- 30 Ceccherini I, Hofstra RM, Luo Y *et al*: DNA polymorphisms and conditions for SSCP analysis of the 20 exons of the *ret* proto-oncogene. *Oncogene* 1994; **9**: 3025–3029.
- 31 Ito S, Iwashita T, Asai N *et al*: Biological properties of *Ret* with cysteine mutations correlate with multiple endocrine neoplasia type 2A, familial medullary thyroid carcinoma, and Hirschsprung's disease phenotype. *Cancer Res* 1997; **57**: 2870–2872.
- 32 Takahashi M, Iwashita T, Santoro M, Lyonnet S, Lenoir GM, Billaud M: Co-segregation of MEN2 and Hirschsprung's disease: the same mutation of *RET* with both gain and loss-of-function?. *Hum Mutat* 1999; **13**: 331–336.
- 33 Chappuis-Flament S, Pasini A, De Vita G *et al*: Dual effect on the *RET* receptor of MEN 2 mutations affecting specific extracytoplasmic cysteines. *Oncogene* 1998; **17**: 2851–2861.
- 34 Fitze G, Schreiber M, Kuhlisch E, Schackert HK, Roesner D: Association of *RET* protooncogene codon 45 polymorphism with Hirschsprung disease. *Am J Hum Genet* 1999; **65**: 1469–1473.
- 35 Fitze G, Cramer J, Ziegler A *et al*: Association between c135G/A genotype and *RET* proto-oncogene germline mutations and phenotype of Hirschsprung's disease. *Lancet* 2002; **359**: 1200–1205.
- 36 Borrego S, Saez ME, Ruiz A *et al*: Specific polymorphisms in the *RET* proto-oncogene are over-represented in patients with Hirschsprung disease and may represent loci modifying phenotypic expression. *J Med Genet* 1999; **36**: 771–774.
- 37 Borrego S, Ruiz A, Saez ME *et al*: *RET* genotypes comprising specific haplotypes of polymorphic variants predispose to isolated Hirschsprung disease. *J Med Genet* 2000; **37**: 572–578.
- 38 Sancandi M, Ceccherini I, Costa M: Incidence of *RET* mutations in patients with Hirschsprung's disease. *J Pediatr Surg* 2000; **35**: 139–142.
- 39 Puffenberger EG, Hosoda K, Washington SS *et al*: A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. *Cell* 1994; **79**: 1257–1266.
- 40 Angrist M, Bolk S, Halushka M, Lapchak PA, Chakravarti A: Germline mutations in glial cell line-derived neurotrophic factor (*GDNF*) and *RET* in a Hirschsprung disease patient. *Nat Genet* 1996; **14**: 341–344.
- 41 Edery P, Attie T, Amiel J *et al*: Mutation of the endothelin-3 gene in the Waardenburg–Hirschsprung disease (Shah–Waardenburg syndrome). *Nat Genet* 1996; **12**: 442–444.
- 42 Salomon R, Attie T, Pelet A *et al*: Germline mutations of the *RET* ligand *GDNF* are not sufficient to cause Hirschsprung disease. *Nat Genet* 1996; **14**: 345–347.
- 43 Doray B, Salomon R, Amiel J *et al*: Mutation of the *RET* ligand, neurturin, supports multigenic inheritance in Hirschsprung disease. *Hum Mol Genet* 1998; **7**: 1449–1452.
- 44 Pingault V, Bondurand N, Kuhlbrodt K *et al*: *SOX10* mutations in patients with Waardenburg–Hirschsprung disease. *Nat Genet* 1998; **18**: 171–173.
- 45 Hofstra RM, Valdenaire O, Arch E *et al*: A loss-of-function mutation in the endothelin-converting enzyme 1 (*ECE-1*) associated with Hirschsprung disease, cardiac defects, and autonomic dysfunction. *Am J Hum Genet* 1999; **64**: 304–308.
- 46 Wakamatsu N, Yamada Y, Yamada K *et al*: Mutations in *SIP1*, encoding Smad interacting protein-1, cause a form of Hirschsprung disease. *Nat Genet* 2001; **27**: 369–370.
- 47 Nagaya M, Kato J, Niimi N, Tanaka S, Wakamatsu N: Clinical features of a form of Hirschsprung's disease caused by a novel genetic abnormality. *J Pediatr Surg* 2002; **37**: 1117–1122.
- 48 Gabriel SB, Salomon R, Pelet A: Segregation at three loci explains familial and population risk in Hirschsprung disease. *Nat Genet* 2002; **31**: 89–93.