Nine Novel Germline Gene Variants in the *RET* Proto-Oncogene Identified in Twelve Unrelated Cases

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We report nine novel DNA alterations in the *RET* proto-oncogene in 12 unrelated cases identified by DNA sequencing of exons 10 and 11 of the gene. The novel variants K666E, IVS9-11G→A, D631V in *cis* with H665Q, D631E (with C634Y), E623K (in *trans* with C618S), 616delGAG (in *trans* with C609Y), Y606C, C630R, and R635-T636insELCR;T636P were detected in patients with various clinical presentations ranging from thyroid goiter, medullary thyroid carcinoma, and pheochromocytoma to classic multiple endocrine neoplasia type 2A. When novel DNA alterations are found, extended family studies can be helpful in determining the clinical significance of such findings. Segregation within families suggests that K666E and T636insELCR;T636P are likely to be disease-causing mutations. However, the mechanism by which they affect the normal activity of the *RET* receptor is unclear. Absence of segregation with disease was observed for E623K and 616delGAG. For the remainder of the DNA alterations, family studies were not possible, and the clinical significance of these novel variants needs further assessment. Additional case reports, animal models, and/or functional studies are needed to determine the clinical significance of these newly identified variants. (*J Mol Diagn* 2005, 7:283–288)

Multiple endocrine neoplasia type 2A (MEN2A) is associated with a high risk for medullary thyroid cancer (MTC), pheochromocytoma, and parathyroid adenoma, whereas familial MTC (FMTC) families present with MTC alone.^{1–3} These autosomal dominant diseases are caused by germline mutations in *RET*, a tyrosine kinase receptor gene expressed in tissues and tumors derived from neural crest. The receptor ligands of *RET* are growth factors of the glial cell line-derived neurotrophic factor (GDNF)₃ family. Mutations in the conserved cysteine res-

idues at codons 609, 611, 618, 620, or 634 of exons 10 and 11 occur in 95% of MEN2A and 85% of FMTC.^{3–8} In each case, single base pair substitutions result in replacement of a critical cysteine residue by another amino acid.^{5,9,10} Evidence from *in vitro* studies suggests that mutations lead to ligand-free *RET* activation by the formation of covalent disulfide-bonded homodimers.^{9–11} For MEN2A and medullary thyroid cancer cases, in addition to common mutations at these cysteine residues, at least 10 other missense/nonsense mutations, two duplications, two small indels, and three small insertions in the *RET* are documented in the Human Gene Mutation Database (<http://archive.uwcm.ac.uk/uwcm/mg/hgmd0.html>).¹² For example, mutations in codons 768 and 804 are associated with a small percentage of FMTC pedigrees.^{8,13–18} The mechanism of *RET* activation by these mutations is uncertain.^{19,20} Furthermore, because there are very few reports of noncysteine residue mutations, genotype-phenotype correlation is unclear.

We present 12 unrelated cases with novel DNA alterations in *RET* proto-oncogene that have not been previously reported. Clinical findings are presented. For nine of the 12 cases, we had an opportunity to investigate multiple family members.

Materials and Methods

Patients

The described cases were referred to the Molecular Genetics Laboratory at the Mayo Clinic and Quest Diagnostic Laboratories from April 1993 to March 2004. During that period, approximately 6700 cases were analyzed for mutations in *RET* proto-oncogene by sequencing exons 10 and 11. Indications for testing included suspected clinical diagnosis of MEN2A or FMTC, or a family history of MEN2A or FMTC.

DNA Extraction and PCR Conditions

Genomic DNA was extracted from peripheral blood leukocytes using the Puregene DNA isolation kit (Gentra Systems, Inc., Minneapolis, MN). Analyses of exons 10 and 11 of *RET* used previously described conditions.^{4,7,21} Briefly, the template for sequencing was generated using

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Table 1. Summary of Novel Variants/Mutations in the RET Proto-Oncogene Detected in 12 Unrelated Cases

Case no.	No. of unrelated families	Genotype	Codon/nucleotide no.	Exon/intron	Nucleotide change	Amino acid change	Type of change	Segregates with disease	Clinical phenotype
Variants associated with MEN2A or MTC									
1, 2, and 3	3	K666E	666	Exon 11	AAG to GAG	Lys to Glu	Missense	Yes	MEN2A (MTC and pheochromocytoma)
12	1	R635-T636insELCR;T636P	635-636	Exon 11	13-bp insertion/ 1-bp deletion	Insertion Glu, Leu, Cys, and Arg/ Missense Pro to Thr	Indel	Yes	MTC
Variants NOT associated with MEN2 or MTC									
8	1	E623K (C618S)	623 (618)	Exon 10 (Exon 10)	GAA to AAA (TGC to AGC)	Gln to Lys (Cys to Ser)	Missense (Missense)	No (Known mutation)	MTC
9	1	616delGAG (C609Y)	616 (609)	Exon 10 (Exon 10)	Del GAG (TGC to TAC)	Glutamic acid deleted (Cys to Tyr)	Deletion (Missense)	No (Known mutation)	MEN2A/FMTC
Variants of undetermined significance to MEN2 or MTC									
4	1	D631V	631	Exon 11	GAC to GTC	Asp to Val	Missense	NA	Pheochromocytoma
		H665Q	665	Exon 11	CAC to CAG	His to Gln	Missense	NA	
5 and 6	2	D631E (C634Y)	631 (634)	Exon 11 (Exon 11)	GAC to GAA (TGC to TAC)	Asp to Glu (Cys to Tyr)	Missense (Missense)	NA (Known mutation)	MTC
7	1	IVS9(-11) G>A	1955-11	Intron 9	G to A	-	Splice site mutation	NA	Aggressive MTC
10	1	Y606C	606	Exon 10	TAT to TGT	Tyr to Cys	Missense	NA	MTC
11	1	C630R	630	Exon 11	TGC to CGC	Cys to Arg	Missense	NA	Thyroid Goiter

NA, family members were not available for testing.

the following primers that flank exons 10 and 11 were used to generate a 1785-bp amplicon (GenBank Accession no. AJ243297). Sense primer: RET A2, 5' CAA CAT TTG CCC TCA GGA CTG 3'; antisense primer: CRT 19-A, 5' CTT GAA GGC ATC CAC GGA GA 3' (all of the primers were purchased from Integrated DNA Technologies, Inc., Coralville, IA). The PCR was carried out in a 25- μ L mixture containing 250 ng of genomic DNA, 1.5 mmol/L MgCl₂, 200 μ mol/L dNTPs, 10 pmol each of sense and antisense primers, and 1 U of *Taq* polymerase (AmpliTaq DNA Polymerase; PE Biosystems, Inc., Foster City, CA). After initial denaturation at 95°C for 2 minutes, PCR was carried out in 30 cycles, each consisting of denaturation for 30 seconds at 94°C, annealing for 30 seconds at 65°C, and a polymerization for 1 minute at 72°C, followed by 10 minutes of final extension at 72°C.

Sequencing Analysis

The amplicon was sequenced from both forward and reverse directions using a radiolabeled terminator cycle sequencing kit (Thermo Sequenase no. US79770; Amersham Life Science, Piscataway, NJ), following the manufacturer's instructions. Sequencing primers used were RET A2 and RET D (5' TTG GGA CCT CAG ATG TGC TGT T 3') for exon 10 and CRT 19-B (5' GCA TAC GCA GCC TGT ACC C 3') and CRT 19-A for exon 11. Sequencing products were separated by 8% denaturing polyacrylamide gel electrophoresis, and signals were vi-

sualized using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

Results

Previously unreported DNA alterations were identified in 12 unrelated cases (Table 1). These cases represent 0.18% of a total of approximately 6700 tested cases.

Exon 11 Codon 666 AAG→GAG (K666E) in Cases 1, 2, and 3

This alteration occurred in three unrelated cases. In case 1, a 35-year-old male had unilateral pheochromocytoma and elevated calcitonin (122 pg/ml; normal value for males: 19 pg/ml). On testing the family, the K666E variant was detected in his 62-year-old hypertensive mother who did not have a pheochromocytoma. His 28-year-old brother and two sons aged 2 and 4, who were asymptomatic at the time of testing, were also found to carry the same alteration.

In case 2, a total of 12 members of the family were tested for this change in exon 11 (see Figure 1). The K666E alteration was observed in eight family members; three had MTC, two had C-cell hyperplasia, one had a positive pentagastrin stimulation test, and two did not have clinical findings of either MEN2A or FMTC. Of four other tested family members who did not have the K666E

variant, three of them are clinically normal individuals, and one had a positive pentagastrin stimulation test.

In case 3, this alteration was detected in a 64-year-old man who was diagnosed with an aggressive medullary thyroid carcinoma. His only son, who is 39 years old, was also found to have the same variant. To date, he is asymptomatic, and his unstimulated calcitonin levels are normal. He chose to not have a thyroidectomy.

Exon 11 Codon 631 GAC→GTC (D631V) and 665 CAC→CAG (H665Q) in Case 4

These two alterations were first identified in a male patient with pheochromocytoma whose maternal great-grandfather was also reported to have a pheochromocytoma. Subsequently, the same two variants were found in the asymptomatic mother and the healthy maternal grandmother of the patient.

Exon 11 Codon 634 TGC→TAC (C634Y) and 631 GAC→GAA (D631E) in Cases 5 and 6

In case 5, a 37-year-old female with MTC has a family history of thyroid cancer. DNA testing identified the known mutation TGC to TAC at codon 634 and a second alteration GAC to GAA at codon 631. No further family information or other family members were available to pursue the clinical significance of the novel codon 631 variant or to determine whether the DNA changes were in a *cis*- or *trans*-configuration. The same two DNA changes were also detected in another unrelated patient (Case 6) with recurrent medullary thyroid cancer. No family history was obtained from this patient.

IVS 9 (-11) G→A in Case 7

This alteration was detected in a 68-year-old male with metastatic MTC without a family history of thyroid cancer.

The potential effect on splicing was determined using the Neural Network Splice Site Prediction Program (http://www.fruitfly.org/seq_tools/splice.html). Results indicated that the variant in the intron 9 (-11) G to A would interfere with normal RNA splicing by removing the donor site. No other family members were available for testing.

Exon 10 Codon 618 TGC→AGC (C618S) and Codon 623 GAA→AAA (E623K) in Case 8

These two alterations were first detected in an asymptomatic male who had a family history of MTC (see Figure 2). Further DNA studies on family members demonstrated that the two alterations were on different chromosomes. C618S, a known MEN2A/FMTC mutation, was present in the affected father and paternal uncle and also in an asymptomatic brother and a young asymptomatic paternal aunt. The codon 623 change was detected in the subject's brother, his mother, and the maternal grandfather, all of whom were clinically healthy.

Figure 2. Case 8. The alterations at codon 618 TGC→AGC (C618S) and codon 623 GAA→AAA (E623K) were first detected in a presymptomatic male with a family history of MTC (III:3). Testing of family members showed that C618S and E623K were on different chromosomes. Carriers of E623K alone did not have any clinical manifestations of MEN2 or FMTC (I:3, II:4, and III:1). Carriers of the C618S mutation either had thyroid cancer (II:1 and II:3) or were asymptomatic at relatively young ages (II:2, III:2, and III:3).

Exon 10 Codon 609 TGC→TAC (C609Y) and Codon 616delGAG in Case 9

Deletion of GAG at codon 616 (E616del) along with the C609Y mutation was observed in a 31-year-old man with elevated calcitonin levels. His father, who had a history of medullary thyroid carcinoma, also had the C609Y mutation but not the novel variant. Subsequently, the proband's mother and a brother, who were both phenotypically normal, were found to have the E616del variant only. His sister, who has had thyroidectomy due to medullary thyroid cancer, was also positive for both the C609Y and the E616del alterations. Her 10-year-old son was found to have inherited only the E616del variant and was asymptomatic at the time of the testing.

Exon 10 Codon 606 Tyr→Cys (Y606C) in Case 10

This alteration was detected in a woman who was diagnosed with medullary thyroid carcinoma. Her 9-year-old asymptomatic daughter was also found to have the same alteration. The family was considering a prophylactic thyroidectomy for the daughter. We do not have any more information on this family.

Exon 11 Codon 630 (TGC→CGC) Cys→Arg (C630R) in Case 11

This alteration was detected in three siblings of Middle Eastern ancestry. They all were reported to have thyroid goiter without any features of MEN2 and were all reported to have a family history of thyroid cancer. Because this family sent samples for analysis from outside the United States, retrieval of additional clinical information has not been possible. Thus, we are unable to determine whether the enlarged thyroid glands in these siblings were authentic goiter or thyroid carcinoma.

Exon 11 c.2101delAinsGACCTGTGCCGCC (R635-T636insELCR;T636P) in Case 12

This 13-bp insertion between codons 635 and 636 in conjunction with a 1-bp deletion was detected in a 28-year-old female patient with medullary thyroid carcinoma. Her two sons, who had elevated calcitonin levels, also tested positive for the same alteration. Both of the sons subsequently underwent prophylactic thyroidectomies. On histological examination, both were found to have microscopic foci of medullary carcinoma. Another family member who tested negative for this alteration is so far asymptomatic.

Discussion

Germline missense mutations at one of five cysteine codons in exon 10 (C609, C611, C618, and C620) and exon 11 (C634) in *RET* are known to be associated with

either MEN2A or FMTC.^{3-8,21,22} These residues are located in the extracellular, juxtamembrane cysteine-rich domain. Studies have shown that codon 634 mutations render the receptor tyrosine kinase (RTK) constitutively active by the tendency of mutant *RET* monomers to undergo dimerization, a situation that may mimic ligand binding. It is believed that whereas cysteine residues in wild-type *RET* form intramolecular disulfide bonds, mutation of a cysteine residue is predicted to leave an unpaired residue. Unpaired cysteines from two mutant *RET* monomers can then dimerize by formation of a disulfide bond.^{10,23} Simplistically, the same general mechanism may be proposed to explain the effects of mutations in the other cysteine codons. However, this generalized constitutive activation does not explain the mechanism for the non-cysteine codon mutations in *RET*.^{15,24,25,27}

Among the nine novel DNA changes we report, the alteration of K666E in exon 11 was detected in a total of 15 individuals in three unrelated kindred (cases 1, 2, and 3); eight had the clinical presentations of either MEN2A or FMTC, such as pheochromocytoma, MTC, C-cell hyperplasia, and positive pentagastrin stimulation test. An autosomal dominant-inherited pattern was observed in studying the family members in two/three generations of two of these families (see Figure 1). Although K666E was also seen in three healthy young relatives of case 1, two asymptomatic individuals in case 2 and one in case 3, pathogenicity is not ruled out since variable expression and reduced penetrance of this mutation could explain the observations. However, absence of this alteration in one family member with a positive pentagastrin stimulation test in case 2 may argue the possibility of other undetected mutation(s) segregating in the family.

RET mutations in *cis*-configuration have been previously reported in patients with MEN2A, FMTC, or MEN2B.²⁵⁻²⁷ The alteration of both codons 634 (C634W) and 635 (R635G) was found in one family with MEN2A.²⁷ A combination of both V804M and R844L was reported in a kindred with FMTC. Both V804M and R844L mutations are within the tyrosine kinase domain in exon 14. Family studies for this FMTC kindred indicated that the mutations cosegregated with disease and were not identified in 200 unrelated normal controls.²⁶ Furthermore, two germline mutations, V804M and Y806C, in *cis* were detected in a patient with MEN2. In this study, it was reported that the novel Y806C was inherited from the patient's father, but other carriers (the father and sibling) were not affected with MEN2. In contrast, V804M was a *de novo* mutation in this case, which has been previously reported in patients with familial medullary thyroid carcinoma. Combination of mutations of the *RET* proto-oncogene may cause oncogenic activities different from those of single mutations.²⁵ In our study, there were five independent cases that had two *RET* alterations (cases 4, 5, 6, 8, and 9). In case 8, family studies demonstrated a *trans*-configuration for C618S and E623K, and clinical information suggested that E623K is a benign variant. However, it is possible that E623K may interact with the C618S variant and affect the phenotype of the C618S mutation. Similarly in case 9, the C609Y mutation is present in the affected individuals, whereas the 616delGAG does not seem to segregate

with the disease. In cases 5 and 6, a known MEN2A/FMTC mutation, C634Y, is present in combination with D631E in two individuals with MTC and a family history of MTC. However, family members were not available to determine *cis*- or *trans*-configuration and segregation with disease. The possibility of the D631E variant being in the linkage disequilibrium with C634Y is very low, because of approximately 6700 cases tested, 66 had the C634Y mutation, of which only two showed associated the D631E variant. Alterations of D631V in *cis* with H665Q were detected in a male individual with pheochromocytoma (case 4) and also in his healthy mother and maternal grandmother. Interestingly, the maternal great-grandfather of case 4 had a history of pheochromocytoma, raising the possibility that one or both of the alterations could be incompletely penetrant in causing pheochromocytoma.

The remaining novel DNA changes were each detected in "single" families and were not associated with the presence of other "classical" MEN2A/FMTC mutations. In case 7, the DNA change of IVS9-11G→A was identified in a patient with sporadic metastatic MTC. On testing the alteration in the Splicing Site Prediction Program, the results demonstrated that this variant is likely to generate a new donor site and thus interfere with the normal RNA splicing. However, analysis of patient RNA and/or *in vitro* splicing and transcription studies are necessary to prove this possibility.²⁴

In case 10, a patient with MTC and her juvenile asymptomatic daughter carried the alteration Y606C. The daughter was lost to follow-up, which is unfortunate because further clinical evaluations would be important to determine significance of Y606C.

In case 11, C630R alteration segregated with the presence of thyroid goiter in three siblings of Middle Eastern ancestry. None of the siblings demonstrated any features of MEN2, but a family history of thyroid cancer was reported. Mutations at codon 630 that change cysteine to phenylalanine, serine, or tyrosine have been reported in patients with MEN2A or FMTC.^{19,28} However, the relationship between C630R variation and development of thyroid goiter should be further explored.

Case 12 is the only case with an indel mutation in the *RET* proto-oncogene. Interestingly enough, in 1997, Hoppner et al²⁹ reported a family with a 12-bp duplication between codons 634 (Cys) and 635 (Arg), which created an extra cysteine residue. Those patients were found to have a higher incidence of hyperparathyroidism along with medullary thyroid cancer. In the case that we report, there was a 13-bp insertion between codons 635 and 636 in conjunction with a 1-bp deletion resulting in substitution of a threonine to proline at codon 636 followed by an insertion of glutamic acid (Glu), leucine (Leu), cysteine (Cys), and arginine (Arg). The proband carrier of this mutation developed MTC at 28 years of age, and her two sons who were 9 and 11 years old at the time of their thyroidectomies had foci of C-cell hyperplasia. They were not reported to have hyperparathyroidism.

Overall, the germline gene variants that we identified in 12 unrelated cases are infrequent. The alterations T636insELCR;T636P and K666E are putative mutations, whereas E623K and 616 delGAG are likely to be benign

variants. The DNA changes IVS9-11G→A, D631V in *cis* with H665Q, D631E (with C634Y), Y606C, and C630R are of unknown significance. The clinical significance may be clearer if the same variants are identified in additional cases. Even alterations that apparently do not segregate with disease in family studies need to be further investigated to determine whether they modify the effects of "classical" MEN2A/FMTC mutations. Variable expression and reduced penetrance of *RET* mutations adds to the difficulty in establishing genotype-phenotype correlation. Long-term follow-up may be required, because onset of MEN2A or FMTC can be at a relatively late age. Age-appropriate screening measures should be considered for individuals with identified alterations of the *RET* proto-oncogene. Additional case reports, animal models, and/or functional studies are needed to be certain of the clinical association of these variants.

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