

A Novel Mutation (D631del) of the RET Gene Was Associated with MEN2A in a Chinese Pedigree

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Abstract. Germline mutations in the RET proto-oncogene (RET gene) are well documented as the genetic causes of multiple endocrine neoplasia type 2A (MEN2A). We performed genetic analysis by direct RET gene mutation analysis in a Chinese MEN2A family and compared these results with biochemical screening tests and pathological examinations. Twenty-one exons and flanking introns of the RET gene were amplified using polymerase chain reaction (PCR). The PCR products were subjected to sequencing directly, or cloned into pGEM-T plasmids and sequenced. Restriction fragment length polymorphism (RFLP) was employed to confirm the mutation on the RET sequence. A novel heterozygous mutation of a 3-bp (GAC) deletion at codon 631 (D631del) of exon 11, resulting in the deletion of an aspartic acid at the locus, was identified in four MEN2A patients and one phenotypically normal family member. The average clinical onset-age of four MEN2A patients was 33.7 years, no cervical lymph node metastasis was found in MEN2A patients with medullary thyroid carcinoma in the family. The study indicated that the novel heterozygous deletion mutation at D631 of RET gene was co-segregated with MEN2A phenotype and promoted the development of MEN2A. This report is the first description of the D631del mutation in the family with MEN2A.

Key words: Multiple endocrine neoplasia type 2A, RET gene, Deletion mutation

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MULTIPLE endocrine neoplasia type 2A (MEN2A) is an autosomal dominant syndrome of multiple endocrine neoplasm, including medullary thyroid carcinoma (MTC), pheochromocytoma, and parathyroid adenoma or hyperplasia. Germline mutations in the RET gene are well documented as the genetic causes of MEN2A [1–2]. Patients with MEN2A have highly specific missense mutations in the extracellular cysteine-rich domain of the receptor, mainly at codon 609, 611, 618, 620, 630 and 634 in exons 10 and 11. All of these mutants result in the substitution of another amino acid in place of the cysteine. C634

mutants were the most common mutations associated with MEN2A, accounting for 100% of affected Chinese families ever reported [3]. Since MEN2A is a phenotypically and genetically heterogeneous disease, germline mutations also occur at non-cysteine codon in the intracellular domain in MEN2A. These non-cysteine mutations usually affected codon 533, 631, 666, 768, 790, and 791, and have been reported to be found in sporadic MTC or familial MTC and MEN2A [4–11]. Codon 631 is in the cysteine-rich domain of the RET gene. MEN2A linked to the RET mutations at codon 631 is extremely rare reported. To our knowledge, only 2 families were linked to D631 mutation so far [12], whereas other D631 mutations were associated with sporadic MTC [11]. Here we describe a novel mutation in a Chinese MEN2A family with a deletion of 3 bp in exon 11 result in a loss of non-cysteine codon at codon 631.

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Clinical Presentation

The proband (II₇) was a 38-year-old Chinese male who was hospitalized with diabetes and hypertension. Bilateral adrenal masses and bilateral thyroid nodular hyperplasia, but no cervical lymph node metastasis (LNM), were found by magnetic resonance imaging (MR), positron emission tomography (PET) and computerized tomography (CT). The proband had elevated serum calcitonin level (39.34 pmol/L, normal: <38.86 pmol/L) and 24-hour urinary vanillylmandelic acid (VMA) (89.3 mg/24 h, normal: 5–15 mg/24 h). Serum calcium and phosphorus were normal (2.3 mmol/L and 1.09 mmol/L respectively). Serum intact parathyroid hormone (iPTH) was within normal range. The ¹³¹I-metaiodobenzylguanidine (MIBG) scintigraphy showed increased uptake over the bilateral adrenal masses. No obvious abnormality of the parathyroid was detected by CT. The patient underwent bilateral adrenalectomy firstly, and bilateral pheochromocytomas were diagnosed by postoperative pathological examination. After bilateral adrenalectomy, the BP and VMA of the patient returned to normal, but serum calcitonin level remained high (54.76 pmol/L). Then the patient underwent a total thyroidectomy two months after the adrenalectomy, and multifocal MTC was confirmed by postoperative pathological examination.

The proband's elder sister (II₆), a 40-year-old female, presented paroxysmal hypertension. Laboratory data revealed increased 24-hour urinary VMA (86.9 mg/24 h) and normal calcitonin (20.87 pmol/L). Serum iPTH, calcium, phosphorus levels were within normal range. The CT and ultrasonography showed bilateral adrenal adenomas whereas thyroid and parathyroid were normal. Bilateral adrenal pheochromocytoma was confirmed by pathological examination after bilateral adrenalectomy.

The proband's younger brother (II₈) was 35 years

old man who presented headache and palpitation. The 24-hour urinary excretion of both adrenaline and noradrenaline were high, 123.55 ug/24 h (normal: 0–90 ug/24 h) and 45.87 ug/24 h (normal: 0–20 ug/24 h), respectively. Bilateral adrenal masses were found by CT. The patient had bilateral multiple thyroid nodules detected by ultrasonography and CT. He underwent adrenalectomy and total thyroidectomy, and was diagnosed having pheochromocytoma and MTC by postoperative pathological examination.

Another younger brother (II₉) of the proband also presented headache and palpitation. His blood pressure was 170/110 mmHg, 24-hour urinary VMA was 56.3 mg/24 h, left adrenal mass and bilateral multiple thyroid nodules were found by CT. He underwent left adrenalectomy and total thyroidectomy and was diagnosed as left pheochromocytoma and bilateral MTC by postoperative pathological examination.

III₁₀, 10 years old, was the son of II₆, who did not present any symptoms related to MEN2A. The 24-hour urinary VMA was within normal range. The CT showed normal thyroid and adrenal gland images.

Clinical features of the 5 subjects carrying the RET gene mutations were summarized in Table 1.

Subjects and Methods

Subjects

Twenty-two members in a two-generation family were recruited for this study, the pedigree of the family was showed in Fig. 1. The diagnostic criteria used here for MEN2A was according to previously published criteria [13]. Four members (II₆, II₇, II₈, II₉) were diagnosed as MEN2A by postoperative pathological examination. On the basis of these episodes, an inherited disorder was speculated in this family. The molecular examinations for germline RET mutations

Table 1. Clinical features of the MEN2A patients in the study

Number	Gender	Age at onset	Initial demonstration	MTC	PCC	HPT	Mutation
II ₇	Male	31	PCC	++	++	—	DelD631
II ₆	Female	36	PCC	—	++	—	DelD631
II ₈	Male	35	PCC	++	++	—	DelD631
II ₉	Male	33	MTC	++	+-	—	DelD631
III ₁₀	Male	10 [#]	—	—	—	—	DelD631

MTC: Medullary thyroid cancer; PCC: Pheochromocytoma; HPT: hyperparathyroidism;

++: Bilateral; + -: Unilateral; #: age at tested

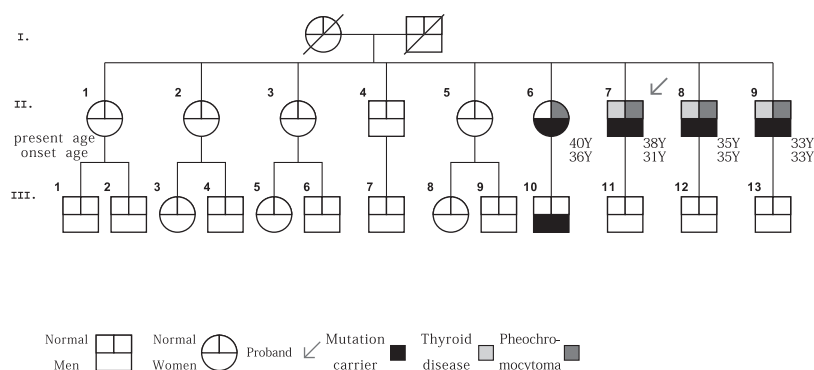


Fig. 1. The pedigree of the described family. II₇, II₈, and II₉ are the patients with both MTC and pheochromocytomas. II₆ is the patient only with pheochromocytomas. The III₁₀ is an phenotypically normal carrier of mutation.

in the family members were performed. All of the family members agreed to participate in this study. The study was approved by the Ethics Committee of the First Affiliated Hospital of Sun Yat-Sen University. Informed consents were obtained from all the subjects.

Methods

DNA Extractions and Mutation Screening

Genomic DNA was extracted from peripheral blood leukocytes using the classical phenol-chloroform methods [14]. Sets of oligonucleotide primers used for amplifying the 21 exons and flanking introns of the RET gene were designed using Primer3 software (SourceForge, Mountain View, CA <https://sourceforge.net/projects/primer3>). PCR reactions were performed using the genomic DNAs of the proband (II₇), proband's sister (II₆), and proband's two young brothers (II₈, II₉) and other 17 first degree relatives as templates in a 25 μ L reaction mixture containing 2.5 μ L DNA, 10 \times buffer 2.5 μ L, 2 mM dNTP 2.5 μ L, 25 mM MgCl₂ 3 μ L, 0.4 μ L of each specific primer, 1.5 U Taq DNA polymerase, and distilled water. The thermal cycling parameters were an initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 40 sec with a final extension at 72°C for 10 min (PCR Perkin-Elmer thermal cycler). The PCR products were subjected to sequencing directly, or 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 BigDye™ Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). Nucleotide sequences were determined by an automated Applied Biosystems sequencer model 3100 (Applied Biosystems, Foster City, CA, USA).

Restriction Fragment Length Polymorphism (RFLP)

RFLP, digested with Hpy99I (New England Biolabs Inc.), was employed to confirm mutations found in the RET gene. A 12 μ L PCR product was digested in a final volume of 20 μ L with 0.5 unit Hpy99I under appropriate buffer conditions at 37°C for an hour. The digested products were resolved by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Digested D631 yielded fragments of sizes 243 and 95 bp whereas D631del yielded one fragment of 338 bp.

Biochemical Measurements

Serum iPTH and calcitonin were measured by IRMA (Diagnostic Systems Laboratories, Inc., normal: 11–55 pg/ml and <38.86 pmol/L, respectively). Urinary VMA, adrenaline and noradrenaline excretion for 24 h were measured using high performance liquid chromatography (HPLC).

Results

RET gene Sequence Analysis

A heterozygous germline deletion mutation was detected in the proband (II₇) at codon 631 of the RET

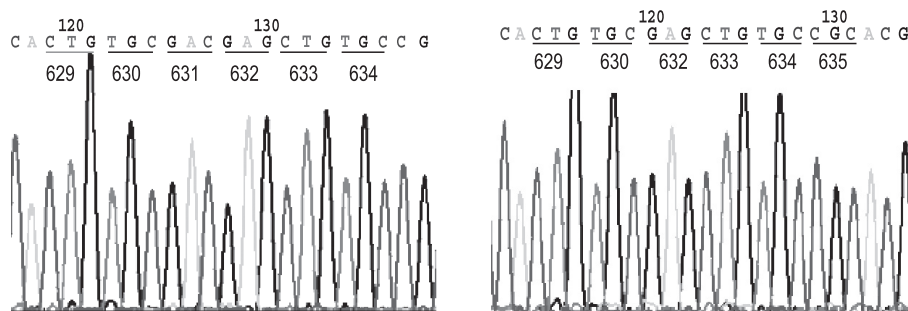


Fig. 2. Mutation analysis by cloning and sequencing of the amplified DNA from exon 11 of RET protooncogene showed the presence of the germline mutation D631del (GAC). *Left*, Wild-type homozygous sequence is displayed. *Right*, Mutant sequence is observed a deletion of 3 bp (GAC) codoning 631.

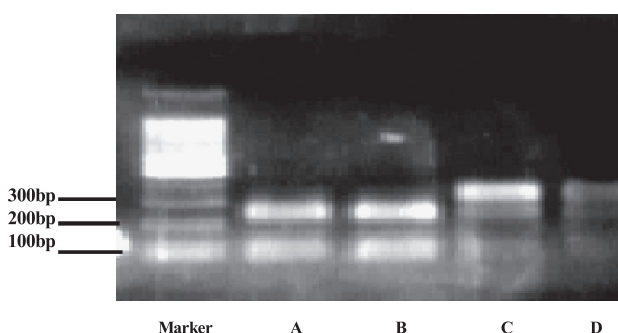


Fig. 3. PCR-RFLP analysis. Band A and B: normal control and wild-type homozygote (two fragments of 95 bp and 243 bp); Band C and D: D631del heterozygote (three fragments of 95 bp, 243 bp and 338 bp).

gene, resulting in the deletion of an aspartic acid at codon 631 (D631del) (Fig. 2). The proband's sister (II₆) and two younger brothers (II₈, II₉) developed MEN2A were also screened for RET mutations, and the same heterozygous deletion mutation at codon 631 was detected. The same D631del mutation was also detected in the proband's nephew (III₁₀) who was a phenotypically normal. The other 17 first degree relatives were negative for the mutation by direct DNA sequencing (data not shown). No other nucleotide alterations were found in the whole coding sequence of the RET gene in all the familial members.

RFLP Analysis

Digested wild-type homozygote yielded two fragments of 95 bp and 243 bp, and the D631del heterozygote yielded three fragments of 95 bp, 243 bp and 338 bp. Hpy99I-digested PCR product from patients

(II₆, II₇, II₈, II₉) and the mutant carrier (III₁₀) yielded three fragments of 95 bp, 243 bp and 338 bp (Fig. 3), and digested PCR product from family members without MEN2A and normal control yielded two fragments of 95 bp and 243 bp (Fig. 3).

Discussion

MEN2A is an autosomal dominant syndrome of multiple endocrine neoplasm, it is well known that MEN2A arises as a result of activating mutation of the RET gene [15]. In the present study, we reported a novel deletion mutation (D631del) of RET gene in a Chinese MEN2A family.

About 98% of MEN2A has germline missense point mutations in the cysteine codons of the RET receptor molecule. Of these cysteine mutations, mutations at the cysteine residue 634 in exon 11 are by far the most commonly found in MEN2A families [16]. Very few non-cysteine mutations have been found in patients with MEN2A. D631 was a non-cysteine residue in the cysteine-rich domain of the RET gene. Only two families with D631 mutation were reported to be associated with MEN2A [12]. In the present study, we reported a novel mutation (D631del) that led to a deletion of codon 631 which was different from the missense mutations at codon 631 reported by several previous studies [9, 11–12]. Because there were very few reports of D631 mutations, and no report of frame-shift mutations at codon 631 (duplications, insertions and indels), D631del might provide valuable information on clinical associations and mechanisms of development of MEN2A caused by RET mutations.

MEN2A arises as a result of activating mutation of the RET, but the mechanism of RET activation by these mutations is uncertain. RET protein is a tyrosine kinase-like cell surface receptor expressed primarily in neural crest and urogenital precursor cells [17–18]. Like other tyrosine kinase receptors, the structural domains of polypeptide products consist of an extracellular ligand-binding region with a cadherin-like site, as well as a large juxtamembrane cysteine-rich domain, a transmembrane region, and a conserved intracellular portion containing the tyrosine kinase domain (TK) [19]. The function of the RET protein is based on the extracellular binding of ligands and coreceptors, dimerization of receptor through cysteine-rich domain, and intracellular autophosphorylation of tyrosine kinase catalytic domain [20]. Normally, ligand binding results in conformational changes with receptor dimerization, followed by activation of TK domain and phosphorylation of intracellular substrates. Heterozygous point mutations convert RET into a dominant transforming gene with oncogenic activity. Asai *et al.* [21–23] demonstrated that cysteine mutations activated RET by inducing its ligand-independent dimerization. D631Y, an aspartic acid to tyrosine change in cysteine rich domain, was found to have high transforming activity by inducing its disulfide-linked dimerization and enhancing the activity of tyrosine kinase autophosphorylation [21]. It suggested that non-cysteine mutations in cysteine rich domain also activated RET by inducing its ligand-independent dimerization. Since D631del led to a deletion of codon 631, we supposed that D631del might have more greatly influenced on RET protein function than point mutation at codon 631 induced by a conformational change of the protein. Combined with the clinical character that LNM was absent in the patients with D631del, it suggested that the D631del might have less vigorous transforming activity in the thyroid and adrenal gland cells, resulting in delaying and weakening tumorigenesis. To answer the question whether the D631del mutation had a gain-of-function effect and

was able to convert RET into a dominantly transforming oncogene similarly to MEN2A-associated RET point mutations, further functional studies are necessary to explore the issue.

The clinical manifestations in MEN2A reflect differences in behavior and function among the mutant RET isoforms. The clinical significance of D631del needs assessment. However, MEN2A patients with D631del mutation are so extremely rare that no clinical information is available about penetration, onset and prognosis in D631del RET genotype. Our study explored that the average onset age of MEN2A was 33.7 years and no cervical lymph node metastasis was found in MEN2A patients with MTC in the family. II₆ was diagnosed as bilateral adrenal pheochromocytomas but having no symptoms and imaging presentation of MTC, which was also found in some D631Y MEN2A patients [12]. This might imply that pheochromocytoma developed prior to MTC commonly in the patient with D631 mutation. III₁₀ carried the D631del mutation, but did not present any symptoms related to MEN2A. It might be attributed to the young age of the subject. In general, prophylactic total thyroidectomy is recommended to MEN2A carriers [24]. But we could not persuade III₁₀ to receive prophylactic thyroidectomy, a close follow-up is necessary to carry out for him.

In conclusion, we identified a novel D631del mutation in a Chinese family with MEN2A. The D631del mutation locating in the cysteine-rich region of the RET protein, may account for the development of MEN2A. Functional studies are necessary to evaluate and understand the impact of D631del RET gene mutations on clinical traits.

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