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NOTE

Mutations of Ret Proto-oncogene in 3 Korean Families with MEN 2A: Clinical Use of New Restriction Sites for Genetic Diagnosis

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Abstract. Abnormalities in the ret proto-oncogene are found in several disorders such as multiple endocrine neoplasia (MEN) 2, sporadic medullary thyroid cancer, congenital megacolon and papillary thyroid cancer. In MEN 2A or 2B, early genetic diagnosis before the development of clinical tumors is crucial for the cure of the disease. We studied mutations of ret proto-oncogene in 3 Korean families with MEN 2A and searched for new restriction sites that could be used for genetic diagnosis. By direct sequencing of exon 10 and 11 harboring 'hot' spots, heterozygous point mutation was detected at positions translating cysteine codon in all 3 families. In 2 families, mutations at codon 634 in exon 11 were found (from TGC to CGC or TAC), yielding a new *CfoI* or *RsaI* restriction site. In one family, a mutation was located at codon 618 in exon 10 (from TGC to CGC), generating a new *CfoI* restriction site. These new restriction sites were used in detecting 2 undiagnosed family members without clinical symptoms or signs. In one of them, thyroidectomy was performed to disclose a small medullary thyroid cancer. These results indicate that Korean MEN 2A patients have germ-line mutations in the ret proto-oncogene at the cysteine residues like patients of other races, and the strategy employing direct sequencing to find mutations at 'hot spot' and search for ensuing new restriction sites could be a useful approach for the molecular diagnosis of genetic diseases accompanied by mutations in restricted areas of disease genes such as MEN 2.

Key words: Ret, MEN, Restriction sites, Genetic diagnosis

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THE RET protein, a product of the ret proto-oncogene, is a receptor-type tyrosine kinase of neural crest origin expressed in endocrine tissue [1, 2], and is also related to the development of Wolfian ducts and enteric nerve plexus [3, 4]. Abnormalities of the ret protein are related to the pathogenesis of several diseases such as multiple endocrine neoplasia (MEN) 2A, MEN 2B, familial and sporadic medullary thyroid cancer, congenital aganglionic megacolon, papillary thyroid cancer and renal dysgenesis [3, 5–8].

MEN 2 is a genetic disorder with an autosomal dominant inheritance pattern and is characterized clinically by multiple endocrine tumors in the thyroid, adrenal medulla and parathyroid gland. Because of almost 100% penetrance of MEN 2 and its poor prognosis resulting from delayed diagnosis after the development of phenotypes, early diagnosis before the emergence of clinical tumors is crucial for the cure of disease [9, 10].

Traditionally, frequent pentagastrin stimulation

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tests have been advocated in family members of known MEN 2 families, which caused much trouble and poor compliance in them [11], but genetic diagnosis of MEN 2 with marker genes became available after linkage studies revealed that the disease gene of MEN 2 is located near the centromere of chromosome 10 [12, 13]. Furthermore, elucidation of ret as the disease gene of MEN 2 allowed an almost 100% accurate genetic diagnosis of the disease long before the development of disease phenotypes [5, 14, 15]. In MEN 2A or MEN 2B, mutations of the ret proto-oncogene are located in the restricted 'hot' areas offering technical advantages for molecular genetic diagnosis of the disease [15, 16]. Particularly in MEN 2A, mutations almost always occur in the cysteine residues in the extracellular domain of the ret protein, suggesting the possibility that dimerization of the ret protein resulting from mutations in cysteine residues renders constitutive signal transduction into the cells without ret-ret ligand interaction [15, 17, 18]. The ligand for ret has been unknown, but recent investigations revealed that the ret protein is the receptor for glial-cell-line-derived neurotrophic factor (GDNF) or molecules closely associated with the receptor for GDNF [19, 20]. These reports suggest that ret molecules have pathogenetic or therapeutic implications not only in diseases of the endocrine system or the peripheral nervous system but those of the central nervous system as well.

We searched for mutations of the ret proto-oncogene in 3 Korean families with MEN 2A and found generation of an abnormal restriction site in each of the 3 families that allowed a convenient genetic diagnosis and successful removal of a very small medullar thyroid cancer in one family member without clinical symptoms or signs of MEN 2A. Sequencing of the 'hot' areas with primers specific for the involved exons, search for abnormal restriction sites, and subsequent restriction endonuclease digestion of the polymerase chain reaction (PCR) products appear to be the most suitable strategies for the molecular diagnosis of genetic diseases involving mutations in a localized 'hot' area such as MEN 2A.

Materials and Methods

Families

Three families with a clinical diagnosis of MEN 2A were included. This project was approved by the Ethical Committee of the Samsung Medical Center, and informed consent was obtained from the patients. The first family consisted of 3 MEN 2A patients and 7 apparently healthy members without clinical symptoms or signs of the disease (Fig. 1A). All 3 patients had medullary carcinoma of the thyroid and pheochromocytoma. Genomic DNA was available from 2 of the patients (II-1 and II-3) and 5 of the apparently healthy family members (II-2, III-1, -2, -3 and -4). It was not available from 1 patient and 2 apparently healthy members in this study. The second family consisted of 2 MEN 2A patients (II-1 and II-2) and their 2 undiagnosed offspring without clinical symptoms or signs of the disease (III-1 and III-2) (Fig. 1B). II-1 had only medullary thyroid carcinoma, while II-2 had both medullary thyroid carcinoma and pheochromocytoma. The third family comprised 2 MEN 2A patients and 4 apparently healthy family members without symptoms or signs of MEN 2A (Fig. 1C). Both patients had medullary carcinoma of the thyroid and pheochromocytoma. Genomic DNA was available only from the 2 patients.

Mutation search

Genomic deoxyribonucleic acid (DNA) was isolated from peripheral blood with Wizard prep kits (Promega, Madison, WI, USA). Exon 10, containing a 'hot' area of the cysteine residues in the extracellular domain, was amplified for 25 cycles with specific primers (forward, GGGATTAA AGCTGGCTATGGC; reverse, CTTGTTGGACCTC AGATGTGC) at 60 °C annealing temperature. Exon 11, also containing a 'hot' area, was amplified in the same way (forward, GCAGCCTGTACCCAGT GGTG; reverse, GAGACCTGGTTCTCCATGGAG). Bands migrating to expected sizes were purified with Jetsorb kits (Genomed, Research Triangle Park, NC, USA), and directly sequenced with an automated sequencing kit (Perkin Elmer, Foster City, CA, USA) and the respective ret primers.

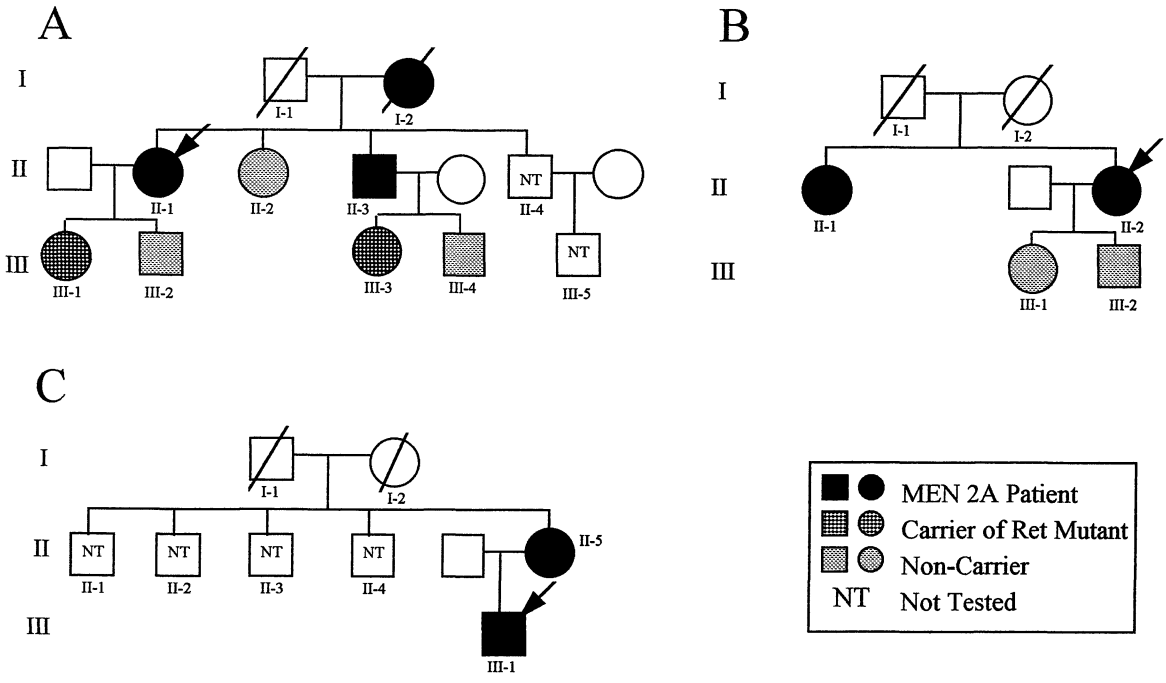

Fig. 1. Pedigrees of the 3 Korean families with MEN 2A. MEN 2A patients in these pedigrees denote family members who had medical records of MEN 2A before genetic diagnosis was performed. Carriers of the ret mutant were apparently healthy family members who did not have clinical symptoms or signs of MEN 2A and were later found to have the ret mutation by molecular genetic studies. One of them had very small medullary thyroid cancer.

Genetic diagnosis

Search for the possible appearance of new restriction sites or disappearance of preexisting restriction sites in the mutated sequences of the ret proto-oncogene was carried out with the DNASIS program (Hitachi Software Engineering). Once a new restriction site was suspected after analyses of the DNA sequences involved, its presence was confirmed by the restriction endonuclease digestion of the PCR products with the patients' genomic DNA and the same primers specific for the exon involved. Then genomic DNA from apparently healthy family members was amplified with the same primers for restriction endonuclease digestion. In brief, 8 μl of PCR products, 1 μl of restriction endonuclease and 1 μl of digestion buffer were mixed and incubated at 37 °C for 4 h. The digested material was analyzed on 1% agarose gels.

Pentagastrin stimulation test

Pentagastrin, 0.5 μg/Kg, (Ayerst Laboratories Inc., Philadelphia, PA, U. S. A.) was intravenously infused. Serum samples were collected at 0, 2, 5 and 10 min after infusion. The serum calcitonin level was measured with an immunoradiometric assay kit (CT-IRMA, Medgenex, Fleurus, Belgium). Samples and I^{125}-labeled anti-calcitonin antibody were incubated in tubes coated with anti-calcitonin antibody at room temperature for 24 h. The calcitonin concentration was measured by plotting the ratios of the bound count to the total count, against those of the standard samples.

Results

In the first family, genomic DNA from 2 patients (II-1 and II-3) was subjected to a mutation search (Fig. 1A). With automated sequencing of the cloned PCR products from the forward orientation, a heterozygous mutation of the ret proto-oncogene

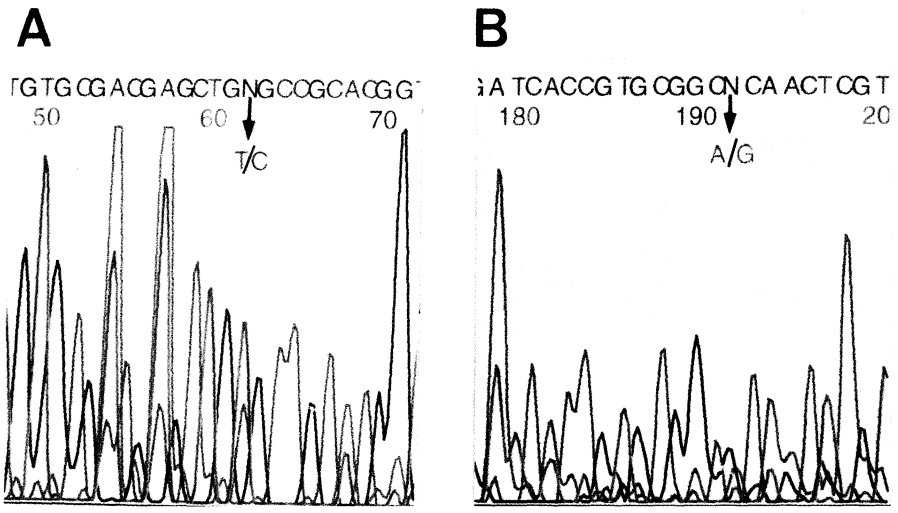

Fig. 2. Direct automated sequencing of the 'hot' area of the ret proto-oncogene with genomic DNA from the index case of family A in Fig. 1. Nucleotide sequencing in both the forward (A) and reverse (B) orientations revealed a heterozygous mutation from TGC to CGC in exon 11, generating a new *CfoI* restriction site.

Table 1. Mutations of the ret proto-oncogene in three Korean families with MEN 2A

Families	Exon number	Codon number	Nucleotide changes	Amino acid changes	New restriction sites
A	11	634	TGC→CGC	Cysteine→Arginine	*CfoI*
B	10	618	TGC→CGC	Cysteine→Arginine	*CfoI*
C	11	634	TGC→TAC	Cysteine→Tyrosine	*RsaI*

at codon 634 in exon 11 (TGC→CGC) was detected in the genomic DNA from both patients tested. The mutation was also observed when sequencing was done in the reverse orientation (Fig. 2). This mutation rendered replacement of the cysteine residue with arginine, and was found by DNASIS analysis to generate a new *CfoI* restriction site (GCGC) (Table 1). When the PCR products obtained with genomic DNA from the same 2 patients and the same primers specific for exon 11 were digested with *CfoI* (Boeringer Mannheim, Mannheim, Germany), cut bands as well as uncut bands were observed as expected, which indicated a heterozygous mutation (Fig. 3). The *CfoI* site was then used for the genetic diagnosis of 5 apparently healthy members in this family without clinical signs or symptoms of MEN 2A (II-2, III-1, -2, -3 and -4). Two of them had an abnormal *CfoI* site in exon 11 of ret (III-1 and III-3), indicating that they were heterozygous carriers of the ret

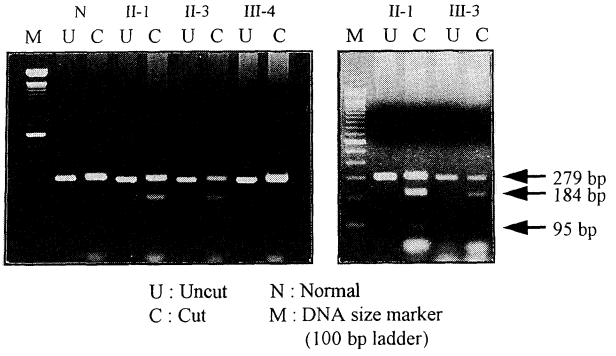

Fig. 3. The new *CfoI* site was used for the genetic diagnosis of family A members without clinical symptoms or signs of MEN 2A. The index case (II-1) and another patient (II-3) had the abnormal *CfoI* restriction site, as expected from the previous nucleotide sequence analyses. III-3 had the ret mutant containing the abnormal *CfoI* site, but III-4 did not. III-1 also had the ret mutation, but II-2 and III-2 did not (data not shown). N (Normal) is a normal control subject not related to this family.

Fig. 4. Histopathology of the thyroid gland from a carrier of the ret mutant (III-1) in family A (× 40). Two small medullary thyroid carcinomas, each 4 mm in diameter, were found.

mutant. The other 3 did not have such a mutant ret (Fig. 3).

Clinical work-up for III-1 and III-3 was carried out. III-1 (F/18) had calcitonin levels of 109, 2690, 2350 and 1320 pg/m*l* at 0, 2, 5 and 10 min after pentagastrin infusion, strongly suggesting the presence of subclinical medullary thyroid cancer. Ultrasonographic examination revealed 4 mm-sized thyroid nodules both in the left and right lobes, while physical examination failed to disclose palpable thyroid nodules. Computerized tomographic examination showed a right adrenal mass 3 cm in diameter. Twenty-four-h urine metanephrine was also increased to 3.0 mg/day (normal, 0–1.2 mg/day). Total thyroidectomy was performed after right adrenalectomy, which disclosed small medullary thyroid carcinomas in both the right and left lobes (diameter 4 mm each) with a clear resection margin, without lymph node involvement (Fig. 4). Post-operative calcitonin levels were 2, 3, 4 and 3 pg/m*l* at 0, 2, 5 and 10 min after pentagastrin infusion, indicating complete removal of the medullary thyroid carcinoma. III-3 (F/10) had serum calcitonin levels of 11, 242, 217 and 101 pg/m*l* at 0, 2, 5 and 10 min after pentagastrin infusion, suggesting the probable presence of early medullary thyroid carcinoma or C-cell hyperplasia [21]. She and her family refused further study.

In the second family, genomic DNA from 2 patients was tested for a possible mutation in the ret proto-oncogene (Fig. 1B, II-1 and II-2). A heterozygous mutation at codon 618 of exon 10 was detected in both patients, with automated sequencing of the PCR products with primers specific for exon 10, in both the forward and reverse orientations (data not shown). This mutation was also from TGC to CGC as in the first family, and yielded the replacement of the cysteine residue with arginine (data not shown). A new *CfoI* restriction site in exon 10 of the ret proto-oncogene was also generated from this mutation (Table 1), which was employed for the genetic diagnosis of the 2 apparently healthy offspring. Neither of the 2 offspring tested had an abnormal *CfoI* site in exon 10 of ret, indicating that they did not inherit the mutant ret proto-oncogene from their mother (data not shown).

In the third family, genomic DNA from 2 patients was studied. A heterozygous mutation of the ret proto-oncogene at codon 634 in exon 11 was detected in both patients (Fig. 1C, II-5 and III-1) with automated sequencing of the PCR products in both the forward and the reverse orientations. This mutation was from TGC to TAC, and yielded a substitution of tyrosine for the cysteine residue (data not shown). This mutation yielded a new *RsaI* restriction site (GTAC) in exon 11 of ret as tested with DNASIS analysis (Table 1), which was confirmed by the restriction enzyme digestion of the PCR products with *RsaI* (New England Biolabs, Beverly, MA, USA) in these 2 patients (data not shown), but genetic diagnosis was not performed because genomic DNA was not available from other apparently healthy family members.

Discussion

Our results confirm that germ-line point mutations in exon 10 or 11 of the ret proto-oncogene [5, 14, 22, 23] also occur in Korean patients with MEN 2A. The mutations were the same ones as those observed in other races [23, 24]. Our genetic diagnosis, based on the generation of new restriction sites due to the mutations, proved to be correct as 2 family members in the first family without symptoms or signs of MEN 2A but with the ret mutation had histology-proven medullary thyroid carcinoma and/or abnormal pentagastrin stimulation tests. Our observation that all of the 6 patients studied in families I and III with mutations at codon 634 had both medullar thyroid carcinoma

and pheochromocytoma is consistent with recent reports that mutations at this position are related to a high prevalence of pheochromocytoma and/or hyperparathyroidism [14, 25], but none of them had definite hyperparathyroidism.

The concentration of ret mutations in the localized 'hot' areas renders the search for mutations in MEN 2 families a relatively easy process compared to other diseases in which genetic abnormalities are biochemically diverse and scattered over a wide area in the disease genes. In particular, ret mutations in MEN 2A almost always involve the cysteine residues in the extracellular domain of the ret protein [15]. Changes from the TGC codon of the cysteine residue to another one is likely to generate a new cleavage site for restriction endonuclease digestion as we and others observed [22, 23], which will be extremely helpful for molecular genetic diagnosis of family members without symptoms or signs of the disease. Apart from the above advantages offered by the molecular biological characteristics of the ret mutation, the clinical characteristics of MEN 2 also favor genetic diagnosis. Almost all of the genetic carriers of the MEN 2A disease gene eventually develop phenotypes. Furthermore, medullary thyroid cancers, the most important determinant of the disease, could be prevented by prophylactic thyroidectomy without causing many adverse effects [22, 24]. Because of these clinical characteristics of MEN 2, its genetic diagnosis does not involve many practical problems or ethical controversies that could be frequently encountered in the diagnosis of other genetic diseases. Carriers of the ret mutation in families with MEN 2 can be followed up more closely with the pentagastrin stimulation test or other measures. Prophylactic thyroidectomy could also be indicated considering the high penetrance of medullar thyroid carcinoma and its impact on the longevity of the carrier [26, 27].

Our data suggest that identification of the responsible mutation in the 'hot' regions by direct sequencing approaches, search for an appearance or disappearance of cleavage sites, and subsequent PCR-restriction enzyme digestion of the gene segment harboring the mutated sequences are useful strategies for molecular diagnosis of genetic diseases involving mutations in restricted areas of the disease genes such as MEN 2.

Acknowledgments

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