Analysis of the *RET* Gene in Subjects with Sporadic Hirschsprung's Disease

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Background: Hirschsprung's disease (HSCR), or aganglionic megacolon, is a hereditable disease of the enteric nervous system. It is an embryonic developmental disorder characterized by the absence of ganglion cells in the lower enteric plexus. Gut motility is compromised in HSCR, with consequent risk of intestinal obstruction.

Methods: We sequenced the *RET* gene and characterized the clinical manifestations in 15 unrelated Chinese patients (9 males, 6 females; age range, 2–21 years) with sporadic HSCR. Genomic DNA extraction, PCR and DNA sequence analysis were performed according to standard procedures.

Results: We identified heterozygous *RET* gene mutations in 2 patients. The mutations included a missense mutation in exon 2 (CGC → CAC) resulting in a substitution of arginine by histidine at codon 67 (patient 1), and a missense mutation in exon 3 (TAC → AAC) resulting in a substitution of tyrosine by asparagine at codon 146 (patient 2). The pathological findings disclosed short-segment HSCR in patient 1 and long-segment HSCR in patient 2, respectively.

Conclusion: We identified *RET* gene mutations in 2 of 15 patients with HSCR in Taiwan. The Y146N mutation we identified was novel. [*J Chin Med Assoc* 2008;71(8):406–410]

Key Words: Hirschsprung's disease, novel mutation, *RET* gene, Taiwan

Introduction

Hirschsprung's disease (HSCR), also called aganglionic megacolon, is a hereditable disease of the enteric nervous system.[1,2] It is an embryonic developmental disorder characterized by the absence of ganglion cells which are derived from the neural crest in the lower digestive tract. Due to the absence of enteric plexus, gut motility is seriously compromised in HSCR patients, resulting in intestinal obstruction. There is a racial and ethnic variation in the prevalence of HCRS, and it is more often found among Asians as compared with Caucasians (2.8 *vs.* 2 per 10,000 live births). About 20% of HSCR cases are familial ones, with considerable genetic diversity in HSCR arising either because of locus or allelic heterogeneity. Studies of the genetic bases of HSCR have identified several disease-causing genes, including *RET* (receptor tyrosine kinase, 10q11.2),[3–7] GDNF (glial cell derived neurotrophic factor, 5p12-3.1),[8,9] NTN (neurturin, 10q13.3),[10] EDN3 (endothelin 3, 20q13.2),[11] and EDNRB (G protein-coupled–endothelin receptor B, 13q22).[12] In the case of *RET* mutation in several families, the HSCR phenotype was found to cosegregate with MEN2A, particularly when the *RET* gene mutation is in codon 618 or 620.[3] Thus, the clinical manifestations of *RET* mutations are highly dependent on the location of the mutation.

Heterozygous germline mutations in the *RET* gene that act as dominant inhibitors account for around 50% of familial HSCR and 7–35% of sporadic HSCR.[5,7,13,14] These mutations include missense, nonsense, deletion, insertion and frameshift mutations and occur throughout the *RET* gene.[5,7,13–15] Most missense mutations result in *RET* inactivation, therefore implying that

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Table 1. Oligonucleotide primers used for PCR amplification, PCR product sizes and annealing temperatures

Exon	Forward primer	Reverse primer	Product size (bp)	Annealing temperature (°C)
1	GGGCGGCCAGACTGAGCGC	AACTTCGCCCTGGCCCTGCGG	207	67
2	AGCCTTATTCTCACCATCCC	ATAAGGGCGGCTTGAGGAAG	373	56
3	GGTTTACACCAGCCCTGGAG	TGTGTCAAGGGCTCGCAGAG	456	56
4	CTTCCCGAGGAAAGCGGCTG	CACGGACACTAAACCGACCG	393	56
5	CATCCTAAGGTCTCTGGTTTT	GAGCGAGCACCTCATTTCCT	331	56
6	CAGAGCAGCTTGGTGGTCA	AGTCTACTCTGTGCTGGTTGG	349	56
7	GAATCTCTACCCTCAGGCCATT	ACCCTCCCTCCCTGGAG	358	56
8	GCACTAGCTGGACGCTGG	GAGACCATCCCAGGCTGGC	280	67
9	TAGAGGGGCAGGATCTGC	GCAACTCTGGCTGAAGTGC	282	67
10	AGAGAATGGTCAGTAGGGACACT	GGACCTCAGATGTGCTGTT	531	56
11	ATGAGGCAGAGCATACGCAG	AACGGCACCTCATCACAGTC	535	56
12	CTTTTCCCCCCTCTTCTCC	GCATTGGGGGCTCTTCAGGGT	293	56
13	GCAGGCCTCTCTGTCTGAACTT	GGAGAACAGGGCTGTATGGA	296	56
14	TGTGTCCACCCCCTTACTCATTGG	CGTGGTGGGTCAGGGTGTGG	399	67
15	CCCCCGGCCCAGGTCTCAC	GCTCCACTAATCTTCGGTATCTTT	358	56
16	CCTCCTTCCTAGAGAGTTAG	CCCCACTACATGTATAAGGG	191	56
17	GGCTCTGTGAGGGCCAGGT	CCCTTCCCAAGTGAGGCT	232	56
18	GGCTGTCCTTCTGAGACC	ACTGCCCTGGGGTGAGGCT	233	56
19	TAGTTGTGGCACATGGCTTG	CAGAGCAGACTTTGGTTTTG	313	56
20	TGCACTTGAAGTTTTGGTTCTT	CTCAGAGCTCTTACCCGGTGT	439	56
21	CCGGGCCCACCACATCATC	AGCCCAAATTAGAGCCAGGTTACG	835	56

HSCR is due, in part, to the loss of RET function or a reduced amount of RET protein. Previous studies found the prevalence of *RET* gene mutations among patients with sporadic HSCR to be 14.3% in Japanese[16] and 19% in southern Chinese.[15] In contrast, the prevalence of *RET* gene mutations among patients with sporadic HSCR has been reported to be as low as 3.6% in Taiwan,[17] far below the global average. To address this discrepancy, we investigated the *RET* gene in 15 Chinese with HSCR in Taiwan.

Methods

Patients

Fifteen unrelated Han Chinese (9 males, 6 females; age range, 2–21 years) with sporadic HSCR were studied. Some of them have been described in a previously published report.[18] The clinical diagnosis of HSCR was based on previously described criteria.[2] An additional 50 unrelated normal Han Chinese subjects were recruited as controls to determine whether any sequence changes might be a common polymorphism. Demographic data and a detailed family history were obtained by an interview with each patient and their family members. This study was approved by the institutional review board of the hospital, and informed consent was obtained from each individual.

Analysis of the RET gene

Genomic DNA was isolated from EDTA-preserved whole blood using the GFX Genomic Blood DNA purification kit (Amersham Biosciences, Piscataway, NJ, USA). The 21 coding exons of the *RET* gene were amplified by polymerase chain reaction using primers and conditions as described previously[16,19,20] or modified empirically (Table 1). For all reactions, a 25-μL reaction mixture contained 200 ng of genomic DNA, 2.0 mM $MgCl_2$, 0.2 mM of each dNTP, 0.15 μM of each primer, 1× reaction buffer and 1 unit of FastStart Taq DNA polymerase (Roche, Indianapolis, IN, USA). PCR products were purified by spin column using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) and sequenced by automated DNA sequencing analysis with fluorescence-labeled dideoxyterminators (BigDye Terminator V3.1 Cycle Sequencing Kits, Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions (ABI 377-36 Autosequencer; Applied Biosystems).

Mutation confirmation

We used restriction analysis to confirm the presence of mutations in the *RET* gene. Restriction endonucleases were selected on the basis of whether a mutation created or destroyed a restriction endonuclease site. Digested PCR products were analyzed by electrophoresis on 12.5% polyacrylamide gels with fragments visualized

Table 2. Clinical summary and mutational analyses for all studied subjects

Proband	Sex	Age	Age at onset	Operative finding	Mutation
1	F	7 yr	4 yr	Short segment	Exon 2, R67H (CGC → CAC)
2	F	2 yr	9 mo	Long segment	Exon 3, Y146N (TAC → AAC)
3	M	2 yr	9 mo	Short segment	Exon 9, D489N (GAT → AAT)*
4	M	17 yr	16 yr	Long segment	
5	M	3 yr	2 yr	Long segment	
6	M	4 yr	4 mo	Short segment	
7	F	5 yr	1 mo	Long segment	
8	F	5 yr	1.5 mo	Short segment	
9	M	6 yr	1.5 mo	Short segment	
10	M	11 yr	1.5 mo	Short segment	
11	M	21 yr	3 yr	Short segment	
12	M	8 yr	3 yr	Short segment	
13	M	4 yr	2 yr	Short segment	
14	F	6 yr	1 mo	Short segment	
15	F	9 yr	1 yr	Long segment	

**Considered to be a polymorphism. Short segment HSCR involves the rectum and the sigmoid colon only, while long segment HSCR extends toward the proximal end of the colon.*

by a silver staining kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden). If the mutation neither created nor destroyed any restriction endonuclease site, we sequenced 50 unrelated normal subjects to study whether the DNA variation was a common polymorphism.

Results

The clinical features, pathologic findings and mutational analyses of all the individuals are shown in Table 2 and Figure 1.

We identified heterozygous missense mutation in the *RET* gene in 2 of 15 patients. The mutations found were a point mutation in exon 2 (CGC → CAC) resulting in a substitution of arginine by histidine at codon 67 (patient 1) and a point mutation in exon 3 (TAC → AAC) resulting in a substitution of tyrosine by asparagine at codon 146 (patient 2). The pathological finding disclosed short-segment HSCR in patient 1 and long-segment HSCR in patient 2. The R67H mutation neither created nor destroyed any restriction endonuclease site, so we sequenced 50 normal subjects and found that it is not a common polymorphism. The Y146N mutation destroys the restriction site for *AccI*. The D489N variation in family 3 was considered to be polymorphic as in D571N variation, which has been reported in 7% of individuals in a Korean population.[21]

There were no relationships between the specific mutations with clinical features of HSCR disease (sex, age at onset, length of anatomic involvement).

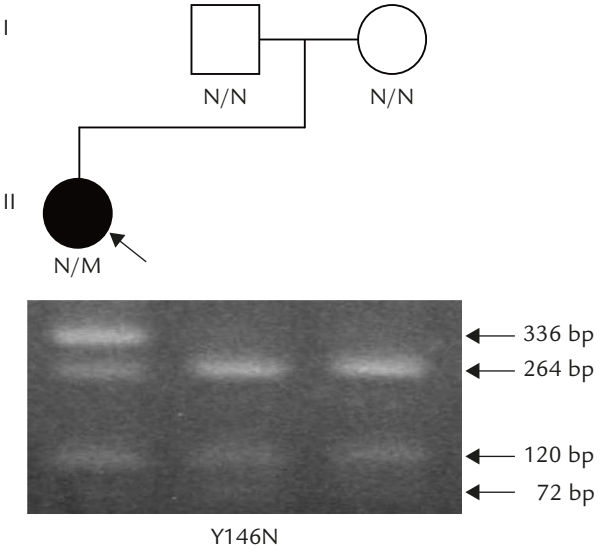

Figure 1. Pedigree of family 2 with *RET* gene mutation. Affected patients are indicated by black symbols. Normal individuals are indicated by white symbols. The arrow indicates the proband. The *RET* genotype of each individual is indicated below each symbol: N = normal allele; M = mutant allele. The size of the PCR product of family 2 (Y146N) is 456 bp. After *AccI* treatment, 3 restriction products of 264 bp, 120 bp and 72 bp in the normal type and 2 restriction products of 336 bp and 120 bp in the Y146N mutant were produced.

Discussion

The R67H mutation that we identified in patient 1 has been previously reported in a 5-year-old Japanese girl with central hypoventilation syndrome, chronic intestinal pseudo-obstruction and intractable hypoglycemia

in infancy,[22] and is not present in the normal population. Thus, R67H in the *RET* allele is likely to be disease-causing.

Patient 2 had a novel heterozygous mutation at codon 146 of exon 3 (TAC → AAC) that resulted in a substitution of tyrosine by asparagine. Evidence that the Y146N mutation was related causally to HSCR in this patient is based on the following: (1) the Y146 residue is highly conserved in human, chimpanzee, dog, rat, mouse, and chicken *RET* genes, suggesting that substitutions at these codons are not tolerated; (2) this base change was not found in 50 unrelated subjects, suggesting that it is not a common polymorphism; (3) the amino acid changes from hydrophobic amino acid to polar amino acid with an uncharged R group. Since both parents of patient 2 were normal in both phenotype and genotype, this mutation most likely arose *de novo* in this subject. The R67H and Y146N mutations resulted in short-segment and long-segment HSCR, respectively. Both mutations alter amino acids that are in the extracellular portion of the *RET* proto-oncogene. Amino acid substitution in the extracellular domain (class I mutation) has been predicted to impair RET maturation and prevent protein from reaching the cell surface,[23] and might alter protein folding and cause inappropriate intracellular trafficking.[24,25] However, there does not appear to be a significant relationship between the specific missense mutations or their location with specific clinical features of HSCR disease (sex, age of onset, length of anatomical involvement) in this or another study.[15]

In contrast with the low frequency of *RET* gene mutations previously observed in patients with HSCR disease in Taiwan,[17] we found a more typical prevalence (13.3%) of *RET* mutations in this study despite the small number of subjects analyzed. This discrepancy probably reflects an ascertainment bias. Compared with the *RET* gene mutations in southern Han Chinese with HSCR,[15] we found no similar mutations in the Han Chinese in Taiwan.

In conclusion, we have identified 2 missense mutations in the *RET* gene in 15 patients with HSCR disease in Taiwan. The Y146N mutation we identified was novel.

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