ORIGINAL ARTICLE

Polymorphisms of the *RET* Gene in Hirschsprung Disease, Anorectal Malformation and Intestinal Pseudo-obstruction in Taiwan

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Background/Purpose: Mutations in the receptor tyrosine kinase *RET* gene are associated with Hirschsprung disease (HD), which is also known as congenital intestinal aganglionosis. We found an association with specific alleles in five single nucleotide polymorphism (SNP) sites of the *RET* gene in our HD patients.
Methods: We compared the association of specific *RET* SNP alleles in patients with severe GI disorders such as anorectal malformation (ARM) or pediatric intestinal pseudo-obstruction (IPO) to that in HD patients. Sixty-four HD, 23 ARM and 35 IPO patients were included. Genomic DNA extracted from blood samples was analyzed by polymerase chain reaction and DNA sequencing analysis.
Results: The allele distributions of all five *RET* SNPs in the HD patients deviated from those in the normal population ($p < 0.05$), whereas those of the ARM patients did not. The allele distributions of these *RET* SNPs in the IPO patients were all significantly different from those in the HD patients. Allele distributions of exon 2 and 13 in the IPO patients were also significantly different from those of the normal population. The frequencies of all the HD-predominant alleles were lower in the HD patients than the normal population, and were even lower in the IPO patients.
Conclusion: This study strengthens the association of specific *RET* SNP alleles with typical HD in Taiwan.
[*J Formos Med Assoc* 2010;109(1):32–38]

Key Words: anorectal malformations, Hirschsprung disease, intestinal pseudo-obstruction, *RET*

Hirschsprung disease (HD) is the most common cause of neonatal intestinal obstruction, and is characterized by the absence of intramural ganglion cells in the nerve plexuses of the distal gut. HD is present in neonates or early childhood, with symptoms ranging from chronic constipation to acute ileus. It affects one in 5000 live newborns, with a male predominance (3:1 to 5:1).[1] The disease has a complex genetic etiology with susceptibility genes including members of the *RET*,[2–5] or endothelin (*EDNRB*)-regulated signaling pathways,[6,7] and *SOX-10*-mediated transcriptional regulation.[8]

Coding sequence mutations in the receptor tyrosine kinase gene *RET*, the major HD gene, can be identified in up to 50% of familial and

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Received: January 12, 2009
Revised: May 7, 2009
Accepted: June 9, 2009

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7–35% of sporadic cases in various populations with different penetrance.[4,5,9–14] The rate of *RET* mutation in HD patients in Taiwan in our previous study was low (3.6%) compared with that reported in other populations. Nevertheless, we have demonstrated the association between HD and specific alleles in five single nucleotide polymorphism (SNP) sites in the *RET* coding regions.[15]

Anorectal malformation (ARM) caused by the absence or ectopic location of the anus is often associated with rectal ectasia, a state of massive dilation of the rectum and distal sigmoid colon. The incidence is approximately 1:5000 live births, with a male predominance. ARM can be primary at birth, with characteristic features such as dilated recto-sigmoid colon with a thin bowel wall, without hypertrophy of smooth muscles, or rectal ectasia with balloon-like rectum,[16] colonic inertia,[17] megarectum,[18] and pseudo-Hirschsprung disease.[19] As a result of intestinal obstruction and sepsis, many newborn ARM patients have to be treated surgically. Some ARM patients develop secondary rectal ectasia postoperatively as a result of bowel reaction to distal obstruction, or inadequate evacuation.[20] Despite its clinical relevance, the etiology and pathogenesis of typical HD and ARM associated with rectal ectasia are different.

Intestinal pseudo-obstruction (IPO) is a functional gastrointestinal (GI) disorder with symptoms including cramps, stomach pain, nausea, vomiting, bloating, fewer bowel movements than usual, and loose stools. Pediatric IPO refers to a heterogeneous group of disorders that are characterized by intestinal obstruction in the absence of mechanical evidence of obstruction.[21] The etiology of IPO can involve abnormalities of the neurogenic or myogenic control mechanisms of gut motility. Although most IPO patients are referred to as sporadic, familial forms also have been described with either autosomal recessive or dominant transmission.[22–24] Surgery usually plays a limited role in the treatment of IPO.

ARM and IPO patients represent a population with severe GI disorders other than HD, which is frequently encountered in pediatric surgery departments. We thus analyzed the five *RET* SNP sites of our patients to further support the non-random association of the *RET* polymorphisms in HD patients in Taiwan.

Materials and Methods

Samples

Sixty-four HD patients were diagnosed at infancy and underwent a definite pull-through operation at Chung Shan Medical University Hospital and Affiliate Hospitals from 1976 to 2005. Fifty-two patients had short-segment aganglionosis and 12 had long-segment aganglionosis, including two with total colon aganglionosis. Patients with Down syndrome or ARM were excluded from this group. Twenty-three ARM patients and 35 functional GI disorder patients with IPO were under medical treatment at Chung Shan Medical University Hospital from 1999 to 2006. The ARM patients included 14 with dilated rectal pouch and six with Down syndrome (three combined with dilated rectal pouch). The IPO patients suffered from severe constipation, but rectal biopsies revealed the presence of ganglion cells. There were four familial cases. Control DNA samples were obtained from normal individuals in Central or Southern Taiwan, who did not have HD, ARM or IPO. The study was approved by the local Institutional Review Board.

Polymerase chain reaction (PCR) and DNA sequence analysis

Genomic DNA was extracted from blood samples by the Puregene Genomic DNA Purification Kit (Gentra Systems, Big Lake, MN, USA), according to manufacturer's instruction. PCR amplification of the five *RET* exons (2, 7, 11, 13 and 15) was performed as described previously.[15] Basically, 150 ng of genomic DNA was amplified in a 50-μL reaction that contained 20 pmol each primer and 2 U *Taq* DNA polymerase (Gene Pure Technology, Taichung, Taiwan). The PCR products were purified by a PCR-M® Clean Up System

(Viogene, Taipei, Taiwan) and subjected to automated sequencing. For some patients or controls, polymorphisms in exons 2, 13, and 15 of the *RET* gene were examined by restriction enzyme cleavage as described previously.[15] The PCR products of exon 2 or 15 were digested with *Eag*I or *Rsa*I, respectively, at 37°C for 2–4 hours. The PCR products of exon 13 were cleaved with *Taq*I at 65°C for 1.5 hours. DNA fragments were then analyzed by agarose gel electrophoresis.

Immunohistochemistry

Bowel tissue sections were obtained from a HD patient with typical rectosigmoid and an ARM patient with rectal ectasia. Immunohistochemical studies were performed on paraffin-embedded tissue sections. The slides were incubated at 50°C overnight, then soaked in xylene for 10 minutes for three times, and immersed in 100% ethanol for 5 minutes, 95% ethanol for 5 minutes, and 80% and 70% ethanol for 1 minute each. After rehydration for 5 minutes, the slides were boiled in citrate buffer (pH 6.1, Target Retrieval Solution; Dako, Carpinteria, CA, USA) for 25 minutes, rinsed with water and 1 × PBS, and treated with 3% hydrogen peroxide for 10 minutes. After that, the slides were incubated with anti-Neuronal Nuclei (NeuN) antibodies (1:100 dilution; Chemicon, Temecula, California, USA) at room temperature for 30 minutes and then biotinylated secondary antibody (Dako) for 30 minutes, and finally in a solution of streptavidin–horseradish peroxidase (LSAB2 System; Dako) for 30 minutes. Color was with the Substrate–Chromogen Solution (LSAB2 System; Dako) for 5 minutes, and counter-stained with Mayer hematoxylin (Merck, Darmstadt, Germany) for 1 minute. At last, the slides were dehydrated in 50%, 75% and 100% ethanol for 1 minute each, and sealed with mounting medium (Cytoseal™ 60 Thermo Scientific, Waltham, MA, USA).

Statistical analysis

The χ^2 test was used to determine the significance of the association of the allele frequencies between patients and normal control groups.

Results

RET gene polymorphisms in HD or ARM patients

We investigated 64 typical HD patients. The allele distributions of the five *RET* SNPs (c135G > A in exon 2, A45A; c1296G > A in exon 7, A432A; c2071G > A in exon 11, G691S; c2307T > G in exon 13, L769L; and c2712C > G in exon 15, S904S) in this HD population were very close to those in our previous study,[15] with significant deviation ($p < 0.05$) from those of the normal population (Table 1). The frequencies of allele A of c135G > A, allele G of c1296G > A, allele G of c2071G > A, allele G of c2307T > G and allele C of c2712C > G were significantly higher in our HD patients than in the controls.

To investigate whether the association of *RET* SNPs was specific to HD patients, we investigated *RET* polymorphisms in ARM patients. No significant differences were detected in the allele distributions of these five *RET* SNPs between the ARM patients and normal controls. Statistically significant deviations ($p < 0.02$) were detected in the allele frequencies of three *RET* SNPs (exons 7, 11, and 13) between HD and ARM patients (Table 1).

RET gene polymorphism in pediatric IPO patients

We determined the allele distributions of the *RET* SNPs in patients with IPO. There were significant differences in the allele frequencies of all five SNPs between patients with HD and IPO ($p < 0.001$; Table 2). In addition, significant differences were also detected in the frequencies of exons 2 and 13 SNPs between the IPO patients and normal controls ($p < 0.05$). The frequency of the G allele of c135 (exon 2) in HD patients was 0.31, while the frequency increased to 0.46 and 0.61 in normal and IPO patients, respectively. For the T variant of c2307 (exon 13), the frequency in HD patients was 0.30, while the frequency increased to 0.47 and 0.64 in normal and IPO patients, respectively. It was apparent that the G allele of the c135 SNP and the T allele of the c2307 SNP were more frequent in the normal and IPO

Table 1. Allele frequencies of *RET* polymorphisms in Hirschsprung disease patients, anorectal malformation patients, and normal population

Exon	Codon	Nucleotide change	Allele frequency*			Statistics					
			HD (128)	ARM (46)	N (100)	HD *vs*. N		ARM *vs*. N		ARM *vs*. HD	
						χ^2	*p*	χ^2	*p*	χ^2	*p*
2	A45A	c135G>A	G:0.31	0.43	0.46	5.19	0.023	0.081	0.776	2.24	0.135
			A:0.69	0.57	0.54						
7	A432A	c1296G>A	G:0.97	0.80	0.77	21.24	<0.001	0.217	0.641	13.23	<0.001
			A:0.03	0.20	0.23						
11	G691S	c2071G>A	G:0.98	0.89	0.91	4.98	0.026	0.127	0.772	5.61	0.018
			A:0.02	0.11	0.09						
13	L769L	c2307T>G	T:0.30	0.54	0.47	7.19	0.017	0.681	0.409	8.91	0.003
			G:0.70	0.46	0.53						
15	S904S	c2712C>G	C:0.99	0.96	0.92	7.71	0.005	0.660	0.418	2.54	0.111
			G:0.01	0.04	0.08						

**The numbers in the parentheses are the numbers of the alleles included in the analyses. HD = Hirschsprung disease; ARM = anorectal malformation; N = normal population.*

Table 2. Allele frequencies of *RET* polymorphisms in Hirschsprung disease patients, intestinal pseudo-obstruction patients and normal population

RET exon	Codon	Nucleotide change	Allele frequency*			Statistics			
			HD (128)	IPO (70)	N (100)	HD *vs*. IPO		IPO *vs*. N	
						χ^2	*p*	χ^2	*p*
2	A45A	c135G>A	G:0.31	0.61	0.46	16.92	<0.001	3.93	<0.050
			A:0.69	0.39	0.54				
7	A432A	c1296G>A	G:0.97	0.71	0.77	27.51	<0.001	0.68	0.411
			A:0.03	0.29	0.23				
11	G691S	c2071G>A	G:0.98	0.84	0.91	12.31	<0.001	1.78	0.181
			A:0.02	0.16	0.09				
13	L769L	c2307T>G	T:0.30	0.64	0.47	22.25	<0.001	4.96	<0.050
			G:0.70	0.36	0.53				
15	S904S	c2712C>G	C:0.99	0.87	0.92	13.76	<0.001	1.08	0.300
			G:0.01	0.13	0.08				

**The numbers in the parentheses are the numbers of the alleles included in the analyses. HD = Hirschsprung disease; IPO = intestinal pseudo-obstruction; N = normal population.*

populations than that in the HD patients. Although there are no statistically significant difference in the other sites (exon 7, 11, and 15) among the three groups, the frequencies of the HD-predominant alleles showed a decrease order of HD patients > normal population > IPO patients in exon 7 (0.97 > 0.77 > 0.71), exon 11 (0.98 > 0.91 > 0.84) and exon 15 (0.99 > 0.92 > 0.87), respectively.

Immunohistochemistry of tissues from HD and ARM patients

A low incidence of ARM associated with typical HD has been reported.[26,27] To confirm our classification of patients as typical HD or ARM patients associated with rectal ectasia, we performed immunohistochemical studies. NeuN has been used as a neuron-specific marker for the

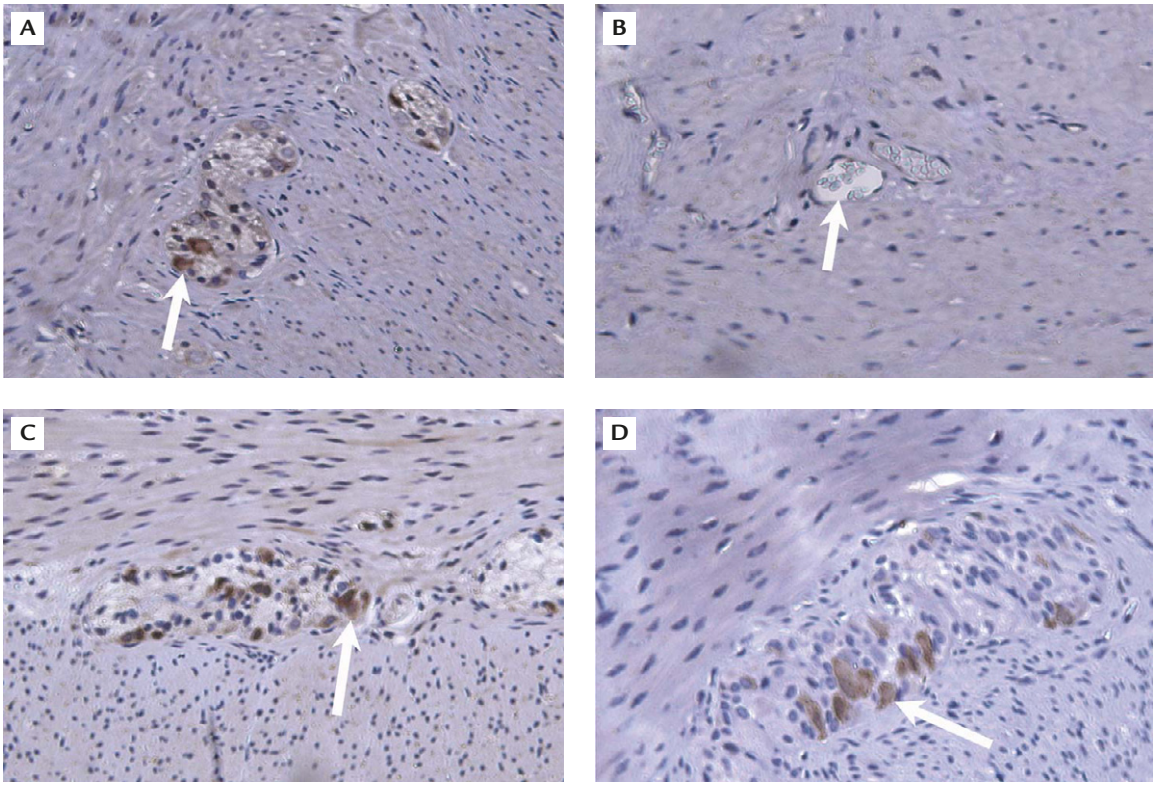

Figure. NeuN immunoreactivity in tissue sections from a typical Hirschsprung disease patient and an anorectal malformation patient associated with rectal ectasia. (A) Normal and (B) abnormal distal segments of a typical Hirschsprung disease patient. (C) Normal and (D) abnormal rectal ectasia segments, along with the distal pouch of an anorectal malformation patient with rectal ectasia. White arrows indicate positions of the myenteric plexuses in these sections (original magnification, 200×).

detection of ganglion cells in colonic plexuses.[28] Immunostaining results showed that mature large ganglion cells were present in the myenteric plexuses of the normal segments (Figure A) but was absent in those of abnormal distal segments in a typical HD patients (Figure B). Conversely, NeuN immunostaining results of an ARM patient associated with rectal ectasia showed mature large ganglion cells in the myenteric plexuses of normal segments (Figure C) and in those of the abnormal segments from rectal ectasia or the distal pouch (Figure D).

Discussion

RET coding region mutations are only found in a small fraction of typical HD patients but the association of variants or haplotypes has been shown in different populations.[29–32] We have reported previously the association of five different *RET* SNPs with typical HD in Taiwan.[15] The result was very similar to that in the Chinese population in Hong Kong[32] but different from other Asian populations in Korea[33] and Thailand.[34] We further analyzed these polymorphic sites in 23 ARM patients but did not detect any significant difference ($p > 0.05$) between the allele distributions of all five *RET* SNPs in patients and the normal population. However, the allele distributions of exon 2 and 15 SNPs in ARM patients did not differ significantly from those in HD patients. This was probably the result of the limited sample size of the ARM patients. Neuron-specific NeuN immunohistochemical analysis confirmed our clinical classification, and excluded the possibility of misclassification of the patients.

Pediatric IPO might arise from neuropathy or myopathy.[21] IPO patients included in the present study were characterized by signs and symptoms of intestinal obstruction without

mechanical evidence of obstruction. Consequently, typical HD patients were excluded from this group of patients. The *RET* SNPs in these IPO patients and their allele distributions were all significantly different from those in the HD patients ($p < 0.001$). Besides, for these IPO patients, allele distributions at the polymorphic sites of exons 2 and 13 were also significantly different ($p < 0.05$) from those of the normal control population. The frequencies of the HD-predominant A allele of c135 (at exon 2) and the G allele of c2307 (exon 13) was the highest in IPO patients and the lowest in HD patients. Even though the allele distributions in the other three SNP sites did not differ significantly between IPO patients and the normal population, the frequencies of the HD-predominant alleles in these three sites similar trends (HD patients > normal population > IPO patients). The composition of the 35 IPO patients was rather heterogeneous and included three patients born to Vietnamese mothers and one to a Korean father. Thus, the association of specific *RET* alleles with our IPO patients was not because the patients were from a subpopulation with common ancestors.

Decreased RET function caused by *RET* mutations is associated with HD. On the contrary, gain of function mutations in *RET* cause multiple endocrine neoplasia type 2 or medullary thyroid carcinoma.[35] Four SNPs in exons 2, 7, 13, and 15 that showed a strong association with HD in this study did not change the encoded amino acids. Association of reduced RET protein expression has been reported for a few variants or haplotypes of HD.[31,36] Whether the association of HD with *RET* SNPs results from closely associated functioning variants, or the SNP *per se* is responsible for the reduced RET protein function is still under investigation. It has not been reported that the *RET* gene is involved in IPO. One possible explanation of the putative association of specific *RET* alleles in our IPO patients is that variants associated with specific *RET* SNPs might affect the normal RET-related signaling in the enteric nervous system, even though neuron development might not be permanently defective as in HD. Balanced allele distribution for normal RET protein function might be required for normal intestinal motility.

In summary, neither ARM nor IPO patients showed an association of specific alleles in the *RET* coding regions as in typical HD. The results strengthen the association of specific *RET* SNP alleles with typical HD in Taiwan. Furthermore, specific *RET* alleles in exon 2 and 13 SNPs appear to be associated significantly with IPO, which provides a new aspect for considering the role of RET-related signaling in regulation of enteric movements.

Acknowledgments

This study was supported in part by the National Science Council (NSC 89-2314-B040-036, 89-2745-P040-002, and 90-2745-P040-002) and Chung Shan Medical University (CSMC 89-OM-A038). We thank the patients and their parents involved in this study. We appreciate the help received from Mr L.C. Yang for conducting immunohistochemical experiments and Ms Y.T. Shen for preparation of the manuscript.

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