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Association Analysis of the *RET* Proto-Oncogene with Hirschsprung Disease in the Han Chinese Population of Southeastern China

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Abstract Hirschsprung disease (HSCR) is a complex congenital disorder characterized by intestinal obstructions caused by the absence of the intestinal ganglion cells of the nerve plexuses in variable lengths of the digestive tract. This study investigated a possible role of the *RET* proto-oncogene in sporadic HSCR patients in the Han Chinese population. Our results indicated that rs1800858, rs1800860, rs1800863, and rs2075912, located in exons 2, 7, 15, and intron 19 of *RET*, are strongly associated with the disease ($P < 0.01$), with rs1800860 and rs1800863 playing a protective role in the pathogenesis of HSCR in the Chinese population. We also showed that the haplotype consisting of four SNPs is significantly associated with HSCR. We did not find a significant difference in the CA-repeat in intron 5 of *RET* between cases and controls. Our study provided further evidence that the *RET* gene is involved in the susceptibility to HSCR in the Han Chinese population.

Keywords Hirschsprung disease · *RET* proto-oncogene · Single nucleotide polymorphism · Haplotype · Linkage disequilibrium

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Introduction

Hirschsprung disease (HSCR, OMIM 142623) represents the main genetic cause of functional intestinal obstruction with an incidence of 1/5000 live births. Males are affected four times more often than females. This developmental disorder is a neurocristopathy and is characterized by the absence of the intestinal ganglion cells of the nerve plexuses in variable lengths of the digestive tract. The disease is usually present in infancy, although some patients present with persistent, severe constipation later in life. Symptoms in infants include difficult bowel movements, poor feeding, poor weight gain, and progressive abdominal distension (Amiel et al. 2008; Heanue and Pachnis 2007). HSCR can be either familial or sporadic and is further classified into two types, according to the extent of aganglionosis. In short-segment HSCR (S-HSCR, 80% of cases), the aganglionosis does not extend beyond the upper sigmoid. In long-segment HSCR (L-HSCR, 20% of cases), the aganglionosis extends more proximally and in rare cases presents as total colonic aganglionosis or total intestinal aganglionosis (Dasgupta and Langer 2004).

Hirschsprung disease is a heterogenic disorder; a number of genes have been shown to play a role in the disease etiology (Heanue and Pachnis 2007). Much genetic and functional evidence points to the *RET* gene (MIM 164761), located on chromosome 10q11.21, as a major disease-causing locus in HSCR (Edery et al. 1994). *RET* encodes a receptor tyrosine kinase, which is expressed in cell lineages derived from the neural crest; plays a crucial role in the regulation of cell proliferation, migration, differentiation, and survival during embryogenesis; and functions as a receptor for growth factors of the glial cell line-derived neurotrophic factor (GDNF) family (Burzynski et al. 2005).

Loss-of-function *RET* mutations have been shown to account for approximately 50% of familial and 7–35% of sporadic HSCR cases (Attie et al. 1994; Lyonnet et al. 1994). Furthermore, *RET*-specific single nucleotide polymorphisms (SNPs) or a combination of these SNPs, as low-susceptibility alleles or factors, have been implicated as having an important role in the pathogenesis of HSCR (Fitze et al. 2002; Griseri et al. 2005; Lantieri et al. 2006). In this study, we tested for allelic and haplotypic association of *RET* with HSCR and investigated five SNPs and one microsatellite in *RET* for association with HSCR in a cohort of Han Chinese patients from southeastern China.

Materials and Methods

Subjects

This study was approved by the Ethics Committee of Zhejiang University, and all subjects gave informed consent for the genetic analyses. Blood samples were obtained from 125 unrelated individuals (96 males, 29 females) diagnosed with sporadic HSCR. Their diagnoses were based on the histological examination of either biopsy or surgical resection material for the absence of enteric nerve plexuses (Martucciello et al. 2005). Of these cases, 26 patients were affected with L-HSCR

(including 4 with total colonic aganglionosis), and 99 with S-HSCR. Control DNA samples were obtained from a panel of 148 unaffected individuals of Han Chinese background.

SNP Selection and Genotyping

We selected five SNPs and one microsatellite located in the *RET* gene from the SNP database (http://www.ncbi.nlm.nih.gov/snp/) for study (Table 1). Genomic DNA was isolated from peripheral blood leukocytes by standard procedures. Genotyping was performed by the ligase detection reaction, which has been demonstrated to be a highly specific and sensitive assay for SNP detection. Fluorescent dye-labeled PCR products were used with an ABI Prism 377 DNA Sequencer for genotyping, and data were analyzed using GeneMapper software (Applied Biosystems, USA).

Table 1 Six markers of the *RET* gene investigated in Han Chinese patients with Hirschsprung disease

dbSNP ID and position (125 cases; 148 controls)	Frequency						
	Allele		*P*	Genotype			H–W *P*
rs1800858, c135 G → A (exon 2)	G	A	4.06×10^{-11}	G/G	A/G	A/A	0.720
Cases	0.255	0.745		0.095	0.362	0.543	
Controls	0.548	0.452		0.292	0.507	0.201	
rs1800860, c1296 G → A (exon 7)	G	A	0.015	G/G	A/G	A/A	0.860
Cases	0.889	0.111		0.796	0.185	0.019	
Controls	0.810	0.190		0.653	0.313	0.034	
rs1800862, c2508 C → T (exon 14)	C	T	–	C/C	C/T	T/T	–
Cases	1.000	0.000		1.000	0.000	0.000	
Controls	1.000	0.000		1.000	0.000	0.000	
rs1800863, c2712 C → G (exon 15)	C	G	0.017	C/C	C/G	G/G	0.920
Cases	0.710	0.290		0.942	0.058	0.000	
Controls	0.920	0.080		0.847	0.146	0.007	
rs2075912, IVS19 + 47 C → T (intron 19)	C	T	2.95×10^{-11}	C/C	C/T	T/T	0.410
Cases	0.278	0.722		0.120	0.315	0.565	
Controls	0.575	0.425		0.347	0.456	0.197	
CA-repeats and position; global repeat association *P* value: 0.115							
Mint5, IVS5 + 989 $(CA)_n$ (intron 5)	$(CA)_{16}$	$(CA)_{17}$	$(CA)_{18}$	$(CA)_{19}$	$(CA)_{20}$	$(CA)_{21}$	$(CA)_{22}$	$(CA)_{23}$
Cases	0.005	0.000	0.010	0.035	0.177	0.227	0.333	0.212
Controls	0.003	0.007	0.010	0.045	0.280	0.234	0.234	0.185

Statistical Analysis

The deviation from Hardy–Weinberg equilibrium was examined in controls by a Chi-square test. The following statistical analyses were performed using SNPstats software (http://bioinfo.iconcologia.net/SNPStats). Using the logistic regression method, the case–control association of genotypes was tested, and the odds ratio (OR) and 95% confidence interval (CI) obtained. D' and r^2 were calculated to evaluate the magnitude of linkage disequilibrium. Haplotype frequencies were estimated using the EM algorithm coded into the haplo.stats package (http://mayoresearch.mayo.edu/mayo/research/biostat/schaid.cfm). The association analysis of haplotypes was similar to that of genotypes with logistic regression, and results are shown as OR and 95% CI. The most frequent haplotype was automatically selected as the reference category, and rare haplotypes were pooled together in a group. The log-additive inheritance model was assumed by default. The significance level of all these tests was 0.05.

Results

Single Nucleotide Polymorphism Analysis

Among the disease cases, the variant alleles of the SNPs rs1800858 and rs2075912 were significantly overrepresented ($P < 0.0001$), and rs1800860 and rs1800863 were underrepresented ($P < 0.05$) (Table 1). We did not find a significant difference in the CA-repeat in intron 5 between cases and controls. The variant allele of rs1800862 was not detected in this Han Chinese clinical population.

To investigate the association of *RET* SNPs with HSCR phenotypes, we compared the allelic distribution of the polymorphisms in cases with L-HSCR and with S-HSCR (Table 2). The variant A allele of rs1800860 was significantly underrepresented in the S-HSCR patients ($P = 0.006$). Other SNPs showed no significant difference between S-HSCR and L-HSCR patients.

Pairwise Linkage Disequilibrium

Pairwise linkage disequilibrium between four SNPs of the *RET* gene was calculated for the cases and controls in the Han Chinese population. We found strong linkage disequilibrium ($D' > 0.75$) between some pairs of markers in the *RET* gene, including rs1800858 and rs1800860 ($D' = 0.8575$), rs1800858 and rs1800863 ($D' = 0.8310$), rs1800858 and rs2075912 ($D' = 0.8498$), and rs1800863 and rs2075912 ($D' = 0.9166$) (data not shown).

Haplotype Analysis

The analysis of association of the haplotypes with HSCR was similar to that of genotypes by logistic regression (Table 3). We found that haplotype 2 (GGCC) showed significant association with the disease ($P < 0.0001$) and that this haplotype

Table 2 Allele frequency of SNPs in S- and L-HSCR cases

dbSNP ID, allele	Frequency			χ^2				
	S-HSCR (99 cases)	L-HSCR (26 cases)	Controls (148)	S-HSCR vs. L-HSCR (*P*)	S-HSCR vs. Controls (*P*)	OR (95% CI)	L-HSCR vs. controls (*P*)	OR (95% CI)
rs1800858, A	0.742	0.660	0.724	1.313 (0.252)	37.368 (0.000)	3.442 (2.296–5.161)	7.178 (0.007)	2.326 (1.240–4.366)
rs1800860, A	0.081	0.277	0.190	7.549 (0.006)	10.115 (0.001)	0.377 (0.203–0.700)	0.329 (0.566)	1.250 (0.583–2.680)
rs1800863, G	0.024	0.050	0.080	0.790 (0.374)	5.985 (0.014)	3.558 (1.210–10.474)	0.444 (0.504)	1.649 (0.374–7.276)
rs2075912, T	0.727	0.705	0.425	0.086 (0.769)	51.324 (0.000)	3.808 (2.618–5.540)	15.909 (0.000)	3.222 (1.798–5.776)

S-HSCR short-segment Hirschsprung disease, *L-HSCR* long-segment Hirschsprung disease, *OR* odds ratio (95% confidence interval)

Table 3 Estimated haplotype frequency and association with HSCR

Haplotype no. and sequence	Frequency[a]			OR (95% CI)	*P*
	Cases	Controls	Total		
1 AGCT	0.663	0.391	0.505	1.00	–
2 GGCC	0.133	0.333	0.247	3.82 (2.29–6.38)	<0.0001
3 GACC	0.054	0.115	0.090	3.41 (1.57–7.44)	0.0022
4 AGCC	0.062	0.043	0.052	1.16 (0.49–2.75)	0.73
5 GAGC	0.014	0.052	0.036	5.24 (1.36–20.19)	0.017
6 GACT	0.028	0.014	0.020	0.93 (0.23–3.83)	0.92
7 GGGC	0.010	0.019	0.016	3.01 (0.46–19.95)	0.25
8 GGCT	0.016	0.015	0.015	2.14 (0.43–10.65)	0.35
Rare[b]	–	–	–	1.40 (0.28–6.96)	0.68

Global haplotype association $P < 0.0001$

[a] $N = 275$, adjusted by sex

[b] Rare haplotypes were pooled together in a group

was more frequently observed in cases than in controls (OR 3.82; 95% CI 2.29–6.38). Haplotypes 3 (GACC) and 5 (GAGC) also showed significant association with the disease ($P = 0.0022$ and 0.017, respectively). Other haplotypes showed no association with the disease.

Discussion

The associated haplotype was reconstructed with SNP marker alleles. Some polymorphic variants of the *RET* gene, including those found in the noncoding *RET* sequence, are believed to modify the effects of mutations in *RET* genes (Borrego et al. 2000; Fitze et al. 2002). Moreover, several SNPs in *RET* have been reported to show allelic association with HSCR. Some are overrepresented and others are underrepresented in HSCR patients (Borrego et al. 1999; Lantieri et al. 2006).

To refine the mapping of such alleles and to characterize their genetic behavior, we investigated a sample of 125 sporadic Han Chinese HSCR cases using SNP analysis across the entire *RET* gene. We found that four SNPs, located in exons 2, 7, 15, and intron 19, were strongly associated with the disease, revealing significant differences between patients and controls in frequencies of particular alleles. These results are similar to those obtained with a Caucasian population (Lantieri et al. 2006). We found that rs1800860 and rs1800863 played a protective role in the pathogenesis of HSCR in our Chinese population, which differed from the findings in the Caucasian population (Lantieri et al. 2006).

For rs1800858 and rs2075912, a large proportion of our cases proved to have homozygous genotypes, whereas these homozygous genotypes had a comparatively low frequency in controls. For rs2075912, the T/T genotype revealed a highly increased risk for the development of HSCR (OR = 7.790), and for rs1800858 the

OR for the A/A genotype was 2.056. We found that other SNPs (except for the A allele of rs1800860) showed no significant difference between S-HSCR and L-HSCR patients. It seems that the variants have little association with the severity of the disease.

In addition, we investigated the microsatellite CA-repeats within intron 5 of the *RET* gene in the Han Chinese population with HSCR. It is well known that microsatellite repeats are important informative markers for population genetic and genetic epidemiology studies because of the large numbers of alleles and high genetic variability within and between populations. Other studies that have compared the frequency of CA-repeats in HSCR patients and controls, to detect the possible association between this microsatellite marker and susceptibility for developing HSCR in Caucasian populations, suggest that this microsatellite might play a role in gene regulation and evolution (Burzynski et al. 2004; Lantieri et al. 2006). In our Chinese population, we did not find a significant difference between cases and controls. This may account, in part, for the ethnicity-dependent prevalence of the disease and therefore needs to be clarified further.

Predisposing haplotypes for HSCR have been extensively studied. Fitze et al. (2003) showed that the haplotype ACA, comprising alleles from SNP-5, SNP-1, and rs1800858, was overrepresented in their patient population (66.9% of 80 cases). Burzynski et al. (2004) found that a haplotype consisting of six SNPs (rs741763, SNP-5, SNP-1, rs2435362, rs2565206, and rs1800858) was transmitted from the parents in 55.6% of their patients with sporadic HSCR and was not transmitted in only 16.2%. Here, we showed that the haplotype consisting of four SNPs (rs1800858, rs1800860, rs1800863, and rs2075912) was significantly associated with HSCR in the Han Chinese population. These results suggest that the DNA variants of rs1800858, rs1800860, rs1800863, and rs2075912 may be good candidates for susceptibility to this disease, and the AGCT combination may represent the core of the susceptibility allele.

In conclusion, we observed a very strong association for four SNPs in the *RET* gene. The haplotype consisting of these markers showed similar results, indicating that a strong founder effect is present in our population. These results suggest that a common HSCR variant might play an important predisposing role in the pathogenesis of HSCR. Therefore, our data may provide new insights in the study of the precise molecular mechanism for the association of a *RET* haplotype with Hirschsprung disease susceptibility in different populations.

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