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Association between Haplotype Tagging Single Nucleotide Polymorphisms of the ret proto-oncogene *(RET)* and Hirschsprung Disease in Thais

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ABSTRACT

Hirschsprung disease (HSCR) is one of several considerably complex diseases. Several HSCR susceptibility loci have been reported through genome-wide association studies. However, haplotype tagging single nucleotide polymorphisms (htSNPs) of the ret proto-oncogene *(RET)* has been identified in the past as being associated with an increased risk for HSCR. This study genotyped 10 single nucleotide polymorphisms (SNPs) of the *RET* in 68 patients with HSCR and 120 controls using TaqMan SNP genotyping assays and restriction fragment length polymorphism. However, haplotype analysis of the *RET* was likewise associated with the disease which has been suggested to be more powerful than individual SNP analyses. The haplotype analysis was carried out using Haploview software. The results showed that htSNPs, located in the intron 1 of the *RET*, strongly associated with HSCR (odds ratio 3.64, 95% confidence interval 2.24–5.92, $P < 0.0001$). These findings suggested that *RET* plays a role in the pathogenesis of HSCR in the Thai population.

Keywords: Hirschsprung disease, ret proto-oncogene (*RET*), single nucleotide polymorphisms, Haploview software, haplotype tagging single nucleotide polymorphisms

INTRODUCTION

Hirschsprung disease (HSCR) is a health problem, characterized by the absence of enteric neurons along a variable length of the intestine, which must be corrected by pediatric surgery (Amiel and Lyonnet, 2001). HSCR occurs in 1 out of every 5,000–10,000 live births and the rate is about four times higher in males than females (Badner *et al.*, 1990). The frequency of the disease is higher in Asia at 2.8 per 10,000 live births than in other parts of the world (Amiel *et al.*, 2008). The male-female ratio in patients with Short-segment Hirschsprung disease and Long-segment Hirschsprung disease is 4:1 and 1:1, respectively (Amiel and Lyonnet, 2001). The etiology of HSCR is multifactorial, involving multiple genes and environmental factors (Tam and Garcia-Barcelo,

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2009). The prevalence of HSCR is thought to be high in Southern Thailand Thailand (Sangkhathat *et al.*, 2006; Phusantisampan *et al.*, 2012).

Recent studies have identified susceptibility loci for HSCR in 11 genes: ret proto-oncogene *(RET)*, *GDNF*, *NTN*, *EDN3*, *EDNRB*, *ECE-1*, *SOX10*, *PHOX2B*, *ZFHX1B*, *KIAA1279*, *NRG1* (Carrasquillo *et al.*, 2002; Garcia-Barcelo *et al.*, 2009; Tam and Garcia-Barcelo, 2009). Recently, a set of genome-wide association studies (GWAS) have identified genes affecting risk for HSCR in large-scale, case-control samples that were replicated in several populations. Single nucleotide polymorphisms (SNPs), with the *RET* and *NRG1*, are loci reported by both GWAS and the candidate gene approach (Garcia *et al.*, 2009; Phusantisampan *et al.*, 2012).

However, there have been very few studies of haplotypes in this gene, and both SNP discovery and genotyping areas have usually focused on exons, although several non-synonymous polymorphisms are well-known SNPs in the *RET* (Iwashita *et al.*, 2001; Garcia-Barcelo *et al.*, 2003; Kim *et al.*, 2006). The level of linkage disequilibrium (LD) across the whole region of the gene in the Thai population is unclear. This study first defined the LD block that encompasses the rs1800858, rs1800861, rs1800862 SNPs and then computationally-generated a list of potentially functional SNPs within the block. These data were then used to select haplotype tagging single nucleotide polymorphisms (htSNPs) to examine the effect of the 10 study SNPs and their haplotypes with HSCR risk in a case-control study within the Southern Thai population.

MATERIALS AND METHODS

Subjects

In total, 68 patients with sporadic HSCR and 120 ethnically-matched healthy volunteers as control subjects, were enrolled in this study. Patients were recruited at the Pediatric Surgery Unit, Department of Surgery, Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand. The criteria for diagnosis of HSCR were based on a histological examination that showed a part without ganglion cells. Control subjects, enrolled at the Blood Bank Unit in Songklanagarind Hospital, Songkhla, Thailand and Pattani Hospital, Pattani, Thailand were collected from ethnically-matched healthy volunteers who were less than 15 years of age and had no history of chronic constipation.

Candidate gene and selection of SNPs

Four SNPs representing the *RET* gene identified in recent GWAS (rs2435357) and in previous data (rs1800858, rs1800861 and rs1800862) were selected (Sangkhathat *et al.*, 2006; Garcia-Barcelo *et al.*, 2009). Six additional tag SNPs (rs2505540, rs2506021, rs2506020, rs2506010, rs2506011 and rs3123655) of the gene were also chosen. The SNPs and haplotype information from the International HapMap project database (http://hapmap.ncbi.nlm.nih.gov/) was used to generate haplotypes for a selection of htSNPs. The htSNPs of this gene with coefficients of determination (r^2) of more than 0.8 and a minor allele frequency of more than 0.05 from the International HapMap project database were selected for individual genotyping.

SNP genotyping

The 10 SNPs representing the *RET* gene were genotyped using TaqMan SNP genotyping assays and a polymerase chain reaction restriction fragment length polymorphism as described in Phusantisampan *et al.* (2012).

Statistical analyses

The distribution of genotypes was tested for deviations through the Hardy-Weinberg equilibrium (HWE) using Haploview software (www.broad.mit/edu/personal/jcbarret/haploview). The associations between the genotype and the risk of HSCR were estimated using odds ratios (ORs) and their 95% confidence interval (CI), which was performed using unconditional

logistic regression. Haplotype analysis was established based on the genotype data to draw a LD map using the Haploview software.

The arrangement procedure was used to estimate empirical and corrected empirical *P* values for the maximum statistic by incoherently permuting case-control labels 10,000 times under the global null hypothesis of no genetic association. Haplotype frequencies of samples less than 1% were excluded from the analysis and $P < 0.05$ was considered to indicate possible statistical significance.

RESULTS AND DISCUSSION

Relation of the *RET* SNPs and HSCR risk in Thais

All 10 markers in the controls harmonized to HWE ($P > 0.05$), data not shown. An unconditional logistic regression model was used to estimate the *RET* SNP markers effect as additive, dominant, or recessive. The frequencies of the T allele in rs2506010, rs2506011, rs2506020, rs2435357 and rs2506021, the A allele in rs2505540 and the C allele in rs3123655 were 87.5, 87.5, 87.5, 79.4, 87.5, 87.5 and 87.5%, respectively, which was higher than those of 67.9, 67.9, 67.9, 52.5, 67.9, 67.9 and 67.9% in the controls, respectively. As shown in Table 1, the most significant *P* value was 0.0003 with an odds ratio of 5.41 (95% CI; 2.09–14.04). To date, at least 11 genes (*RET*, *GDNF*, *NTN*, *EDN3*, *EDNRB*, *ECE1*, *SOX10*, *PHOX2B*, *ZFHX1B*, *KIAA1279* and *NRG1*) have been associated with HSCR (Amiel *et al.*, 2008; Garcia-Barcelo *et al.*, 2009). Among these reported genes, *RET* has been clearly presented to be a major genetic risk factor with sporadic HSCR (Emison *et al.*, 2005; Garcia-Barcelo *et al.*, 2009).

Relation of the *RET* haplotypes and risk of HSCR in Thai population

The association between haplotypes of the *RET* gene and sporadic HSCR was evaluated in Thai populations. It was found that block1 contained seven SNPs (rs2506010, rs2506011, rs2506020, rs2435357, rs2506021, rs2505540 and rs3123655) associated with HSCR which spanned a 23-kb genomic region. A total of 3 common haplotypes (TTTTTAC; CCCCCGG; TTTCTAC), were observed and for the analyses considered all haplotypes with a frequency ≥ 5% in the studied population (Table 2). This overrepresentation remained significant after a block-wide permutaion-based correction, thus supporting the results of the sing-marker association test. As shown in Figure 1, the best observed chi-square was 28.394 in Block1 (TTTTTAC). The haplotype TTTTTAC was highly associated with an increased risk of HSCR in the Southern Thai population of all subjects (OR = 3.64, 95% CI = 2.24–5.92; $P < 0.0001$), of Thai-Chinese subjects (OR = 5.44, 95% CI = 2.77–10.67; $P < 0.0001$) and of Thai-Muslim subjects (OR = 2.02, 95% CI = 0.97–4.20; $P = 0.0728$). As shown in Figures 2–4, this finding was similar to a study on Asian and Caucasian populations (Emison *et al.*, 2005; Liu *et al.*, 2008). If this haplotype is functional, 80% of Thai patients could share the same genetic aetiology. A very strong association was observed for seven SNPs in the intron 1 region which is an intronic enhancer of the *RET* gene. As seen in Figures 1 and 2, the dark cells in the left of the Figures indicate strong linkage disequilibrium. Another haplotype (CCCCCGG) was a protective haplotype with frequency in controls of 0.312 and a frequency in cases of 0.125, respectively, (OR = 0.32; 95% CI = 0.18–0.57; $P < 0.0001$) in all subject analysis, in Thai-Chinese subjects (OR = 0.26; 95% CI = 0.12–0.56; $P = 0.0004$) and in Thai-Muslim subjects (OR = 0.45; 95% CI = 0.18–1.09; $P = 0.0925$). The SNP rs2506011 in intron 1 of the *RET* was selected as the tagging SNP that strongly predicted the common haplotypes (frequency ≥ 5%). To assess how well the five tagging SNPs (rs2506010, rs2506020, rs2506021, rs2505540 and rs3123655) captured the measured SNPs, the pairwise r^2 values (correlations) were

computed of the tagging SNPs to each measured SNP. All r^2 values were above 0.8 in Thais. Thus, it was concluded that the selected tagging SNPs provide good prediction of all SNPs assayed in the studied population and that common variation was thoroughly characterized at this locus. All SNPs conformed to HWE among control subjects with each ethnic group (data not shown). The role of common SNPs, as well as variation in the non-coding region that may influence risk through expression levels, is considered to be an intronic enhancer. There are several ongoing efforts to systematically characterize genetic polymorphisms, such as the HapMap database, to provide the foundation for conducting comprehensive association studies of common

Table 1 Ten single nucleotide polymorphisms (SNP)s of the ret proto–oncogene *(RET)* gene investigated in case–control study.

SNP_ID	Genotype	Cases (N, %)	Controls (N, %)	Odds ratio (95% CI); P^a
rs2506010	CC	3 (4%)	9 (8%)	Reference
	CT	11 (16%)	59 (49%)	0.56 (0.13–2.40); 0.681
	TT	54 (79%)	52 (43%)	3.12 (0.80–12.15); 0.128
rs2506011	CC	3 (4%)	9 (8%)	Reference
	CT	11 (16%)	59 (49%)	0.56 (0.13–2.40); 0.681
	TT	54 (79%)	52 (43%)	3.12 (0.80–12.15); 0.128
rs2506020	CC	3 (4%)	9 (8%)	Reference
	CT	11 (16%)	59 (49%)	0.56 (0.13–2.40); 0.681
	TT	54 (79%)	52 (43%)	3.12 (0.80–12.15); 0.128
rs2435357	CC	7 (10%)	25 (21%)	Reference
	CT	14 (21%)	64 (53%)	0.78 (0.28–2.16); 0.790
	TT	47 (69%)	31 (26%)	5.41 (2.09–14.04); 0.0003
rs2506021	CC	3 (4%)	9 (8%)	Reference
	CT	11 (16%)	59 (49%)	0.56 (0.13–2.40); 0.681
	TT	54 (79%)	52 (43%)	3.12 (0.80–12.15); 0.128
rs2505540	GG	3 (4%)	9 (8%)	Reference
	GA	11 (16%)	59 (49%)	0.56 (0.13–2.40); 0.681
	AA	54 (79%)	52 (43%)	3.12 (0.80–12.15); 0.128
rs3123655	GG	3 (4%)	9 (8%)	Reference
	GC	11 (16%)	59 (49%)	0.56 (0.13–2.40); 0.681
	CC	54 (79%)	52 (43%)	3.12 (0.80–12.15); 0.128
rs1800858	AA	9 (13%)	29 (24%)	Reference
	GA	23 (34%)	51 (43%)	1.45 (0.59–3.56); 0.510
	GG	36 (53%)	40 (33%)	2.90 (1.21–6.94); 0.025
rs1800861	TT	7 (10%)	26 (22%)	Reference
	TG	21 (31%)	58 (48%)	1.34 (0.51–3.56); 0.637
	GG	40 (59%)	36 (30%)	4.13 (1.60–10.65); 0.003
rs1800862	TT	0 (0%)	0 (0%)	Reference
	TC	1 (1%)	3 (2%)	0.56 (0.13–2.40); 0.681
	CC	66 (99%)	117 (98%)	3.12 (0.80–12.15); 0.128

SNP_ID = SNP identification; N = Number of samples; CI = Confidence interval; P^a = Values were calculated using Fisher's exact test; N/A = Not available.

variation. This is the first time a significant haplotype analysis associated with HSCR has been shown in a sample of Thai lineage.

CONCLUSION

This study provided support for the hypothesis that genetic variants in the *RET* gene influence HSCR risk. The results supported the hypothesis that a haplotype of this gene conferred susceptibility to HSCR across different ethnicities. Moreover, certain *RET* genes might play an essential role in the development of HSCR in Thailand.

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Table 2 Haplotype association analysis using probability values (*P*) of single nucleotide polymorphism (SNP)s in the ret proto-oncogene *(RET)* with sporadic Hirschsprung disease.

Haplotype	Haplotype frequency	Haplotype frequency		*P* nominal	*P* permuted*
		Cases	Controls		
TTTTTAC	0.589	0.828	0.473	2.32E-07[a]	<1.00E-04[a]
	0.662	0.750	0.598	5.78E-02[b]	2.79E-01[b]
	0.616	0.794	0.516	9.00E-08[c]	<1.00E-04[c]
CCCCCGG	0.268	0.118	0.340	3.00E-04[a]	2.20E-03[a]
	0.204	0.133	0.256	7.31E-02[b]	3.33E-01[b]
	0.244	0.125	0.312	5.05E-05[c]	5.00E-04[c]
TTTCTAC	0.134	0.053	0.172	1.21E-02[a]	5.15E-02[a]
	0.134	0.117	0.146	6.08E-01[b]	9.83E-01[b]
	0.134	0.081	0.163	2.43E-02[c]	1.12E-01[c]

* = Permutation for 10,000 times.

[a] = Data analysis for the Thai-Chinese subjects.

[b] = Data analysis for the Thai-Muslim subjects.

[c] = Data analysis for the all subjects.

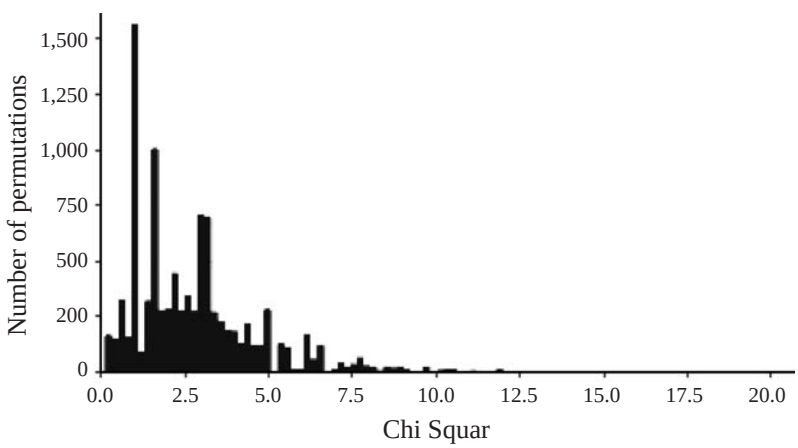

Figure 1 Permutation tests based on single markers in blocks. (Number of permutations =10,000).

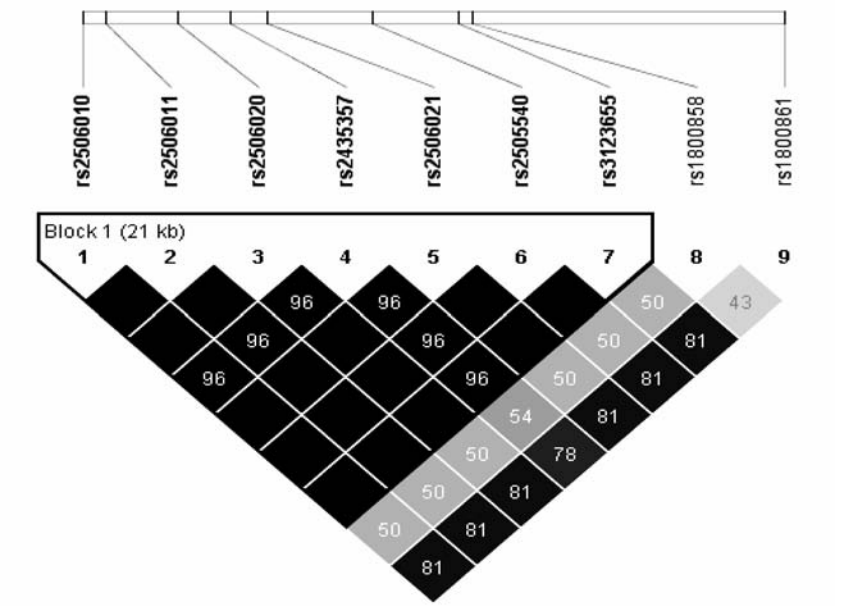

Figure 2 Haplotype blocks and linkage disequilibrium structure for single nucleotide polymorphism (SNP) nominally significant in the single-marker test evaluated as *D'* to define haplotype blocks in all subjects analysis. *D'* values are displayed in different shades from the light region to the dark region for $D' = 0$ to $D' = 1$, respectively. The dark shade indicates regions of high linkage disequilibrium (LD) and the light shade indicates regions of low LD.

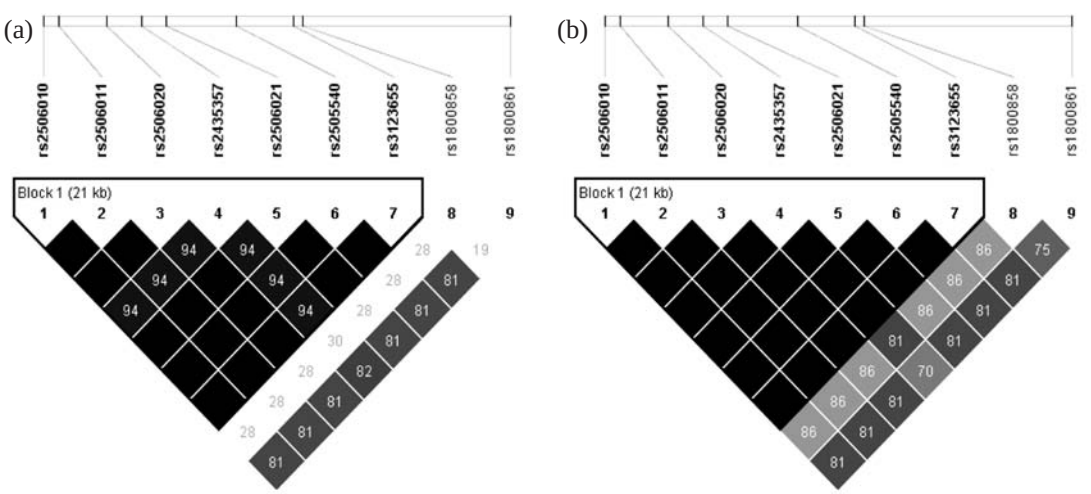

Figure 3 Linkage disequilibrium analysis of single nucleotide polymorphism (SNP) in the ret proto-oncogene *(RET)* region. The number at the intersection of each pair of SNPs represents the pairwise *D'* values between two SNPs in: (a) Thai-Chinese subjects analysis; and (b) Thai-Muslim subjects analysis.

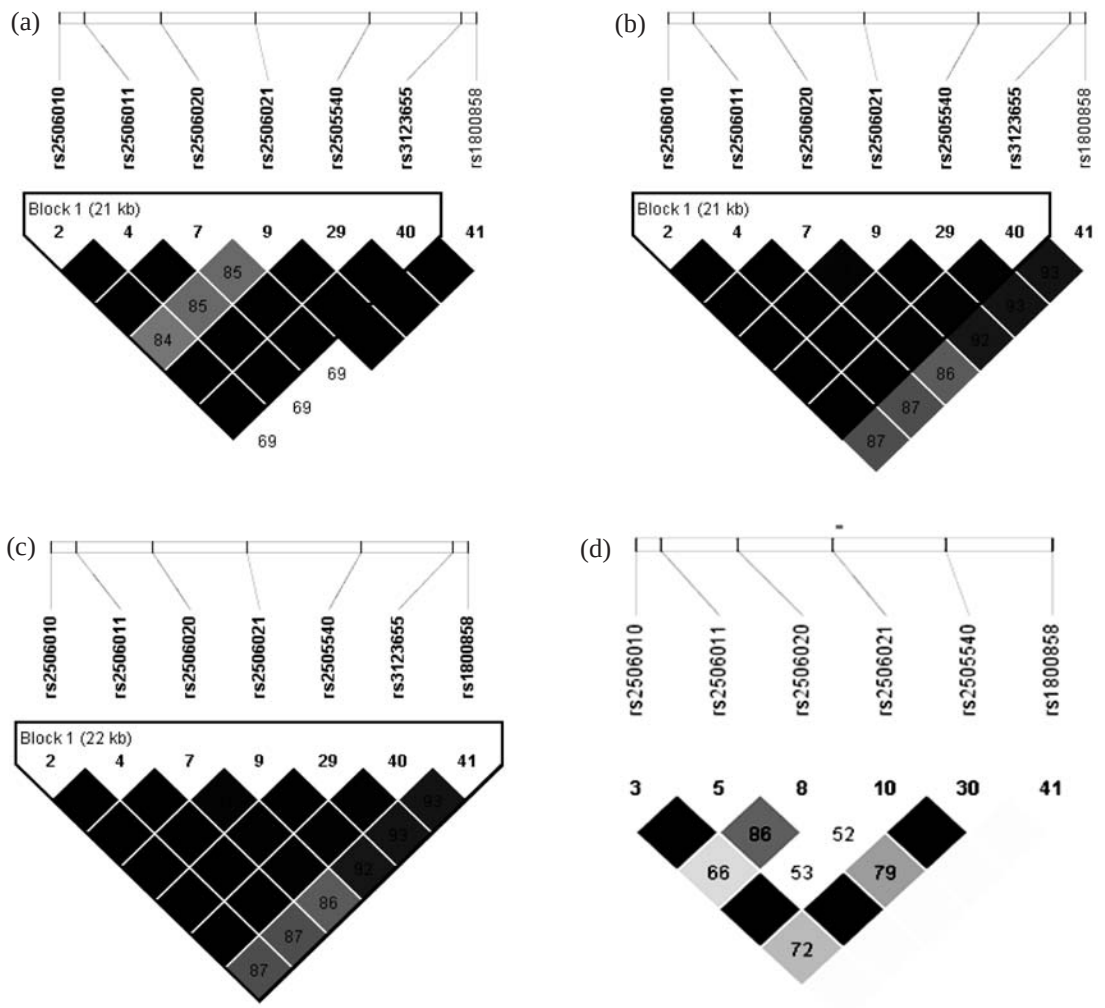

Figure 4 Linkage disequilibrium analysis of single nucleotide polymorphism (SNP) in the ret proto-oncogene *(RET)* in HapMap database, that utilizes *D'* to define haplotype blocks with other populations based on the genotype data of: (a) Utah, USA residents with Northern and Western European ancestry; (b) Han Chinese in Beijing, China; (c) Japanese in Tokyo, Japan; and (d) Yoruba in Ibadan, Nigeria.

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