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ORIGINAL ARTICLE

Association of genetic polymorphisms in the *RET-protooncogene* and *NRG1* with Hirschsprung disease in Thai patients

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Hirschsprung disease (HSCR) is a congenital developmental defect of the enteric nervous system known to be associated with the *RET-protooncogene* and other candidates. Recently, a genome-wide association study has added *NRG1*, a regulator of the development of the enteric ganglia precursors, as a new candidate gene. The aim of this study is to validate the association of the *RET-protooncogene* and the *NRG1* in HSCR in Thai patients. The study used TaqMan single-nucleotide polymorphism (SNP) genotyping and PCR–restriction fragment length polymorphism for genotyping of 10 SNPs within the *RET-protooncogene* and four SNPs within the *NRG1*, in 68 Thai sporadic HSCR cases and 120 ethnic-matched controls. On univariate disease association analysis, 9 of 10 *RET-protooncogene* SNPs and all four *NRG1* SNPs showed an association with HSCR. The rs2435357 (*RET-protooncogene*) and rs2439305 (*NRG1*) showed the strongest associations with the disease at *P*-values of 8.17E-09 (odds ratio (OR)=6.43, 95% confidence intervals (CI)=3.33–12.40) and 6.94E-03 (OR=3.28, 95% CI=1.28–8.38), respectively. The *RET-protooncogene* rs2435357 (TT genotype) in combination with the *NRG1* rs2439305 (GG genotype) was strongly associated with an increased risk of HSCR with a *P*-value of 1.99E-04 (OR=20.34, 95% CI; 2.54–162.78) when compared with a single SNP of the *RET-protooncogene* or *NRG1*. Genetic variation of the *RET-protooncogene* and *NRG1* is involved in the risk of HSCR development in the Thai population. Moreover, the study also detected a combined effect of SNPs by SNP–SNP interaction, which may help in predicting HSCR risk.
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INTRODUCTION

Hirschsprung disease (HSCR) or congenital megacolon (OMIM 142643), one of the complex genetic disorders, is characterized by functional colonic obstruction secondary to an absence of ganglion cells in the distal part of the gastrointestinal tract.[1] The incidence of this disease is generally 1 per 5000 of live births, with males about 3.5–7.8 times more likely to be affected than females.[2,3] There are also differences among races, with a higher incidence in Asians at 2.8 per 10 000 of live births.[4] The incidence of HSCR in Thailand has never been studied, but it is believed to be more prevalent in the southern part of the country.[5]

The etiology of HSCR is multifactorial, involving multiple genetic and environmental factors.[6] To date, at least 11 genes have been identified as susceptibility loci in HSCR: *RET-protooncogene*, *GDNF*, *NTN*, *EDNRB*, *EDN3*, *SOX10*, *ECE-1*, *ZFHX1B*, *PHOX2B*, *KIAA1279*[4,6] and the most recently found, *NRG1*, identified by a genome-wide association study (GWAS).[7,8]

The *RET-protooncogene* encodes a tyrosine kinase receptor and belongs to a signaling pathway, which has essential roles in the development of the enteric nervous system.[9–11] Recent studies have suggested the *RET-protooncogene* as a major locus involved in HSCR pathogenesis.[11–13] Single-nucleotide polymorphisms (SNPs) of the *RET-protooncogene* have been demonstrated to have an association with HSCR in various studies in Asians,[12–16] the majority of these studies were conducted in East Asian populations.

NRG1 was identified as a novel HSCR susceptibility gene in a recent GWAS report, having roles in neuronal development and migration, synaptogenesis and neuronal transmission by binding to ErbB receptors.[7] In this study, we selected two genes on the basis that the *RET-protooncogene* is already known to be associated with the disease and

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NRG1 as a novel gene that it would be of interest to have validated in our population. In addition, both genes have functions in neuronal development, where they possibly interact with each other.[4,7] However, an association in terms of genetic epidemiology between *NRG1* and *RET-protooncogene* has not been previously reported. In this study, we aimed to investigate any potential associations between SNPs on the *RET-protooncogene* and *NRG1* genes, and HSCR. A secondary objective was to study the SNP–SNP interactions between the two genes, with an aim to develop a useful marker for disease prediction.

MATERIALS AND METHODS

Study subjects

The study was approved by the Research Ethics Committee of the Faculty of Medicine, Prince of Songkla University. A total of 68 patients, ages 0–15 years, with histologically proven HSCR, who underwent surgery in our institute during the years 2003–2009, were recruited into the study following informed consent. The disease severity of each patient was categorized by the extent of aganglionosis. Long-segment HSCR was defined as aganglionosis that extended proximal to the recto-sigmoid region including total colonic aganglionosis, whereas the remaining cases were grouped as short-segment HSCR. The controls were 120 ethnic-matched healthy volunteers, aged >15 years, with no history of chronic constipation. A control group that was not age-matched was used on purpose, because we needed to be certain that the controls did not have the disease. None of the subjects were related to any other subjects in the study.

Selection of candidate genes and SNPs

The two candidate genes, *RET-protooncogene* and *NRG1*, were chosen based on the data from the GWAS referred to earlier,[7] and our own previous study.[5] The haplotype tagging SNPs were selected from the International HapMap project database, based on criteria of r^2 more than 0.8 and minor allele frequency more than 0.05 on individual genotyping.

Among the 10 SNPs in the *RET-protooncogene*, we included one SNP (rs2435357) that indicated a marker SNP from the published GWAS,[7] one tagSNP rs2506011 in intron 1 based on the HapMap database and linkage disequilibrium (LD) information in the Chinese population, which was assumed to be closely related genetically to the Thai population. The SNP rs2506011 was in the same LD block with rs2435357.[7] To complete the fine mapping within the region where rs2506011 belonged to, five captured SNPs of rs2506011 (rs2505540, rs2506021, rs2506020, rs2506010 and rs3123655) were picked. In addition, three SNPs from our previous study[5] (rs1800858, rs1800861, rs1800862) were included for re-validation.

Regarding the *NRG1* SNPs, rs16879552 and rs7835688 were selected from the GWAS data.[7] We also included the tagSNP rs2439305, which was in the same LD block as rs16879552 and rs7835688, according to the HapMap database. The SNPs were located within the suspected HSCR-susceptible region in intron 1. As this study was considered an early association study on *NRG1* and HSCR, we did not focus on a complete fine mapping for the intron 1 of *NRG1*, and captured SNPs were not included. Additionally, we decided to explore the rs2919381, a non-coding SNP in intron 5, which was predicted to be an intronic enhancer by the FASTSNP program,[17] which is a program that prioritizes SNPs based on their functional effects. The rs2919381 was among the non-coding SNPs that had high-risk scores and were located some distance away from the first group.

SNP genotyping

Genomic DNA extraction. Genomic DNA was extracted from frozen colonic tissues taken from the 68 HSCR cases, and from peripheral blood leukocytes of the 120 control samples, using the QIAamp DNA Mini Kit (QIAGEN, Hilden, Germany).

TaqMan SNP genotyping assays. Thirteen SNPs from the two genes were genotyped using custom TaqMan SNP Genotyping Assays on the 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The TaqMan probes were VIC- and FAM-dye-labeled. The PCR used 10 ng of genomic DNA in a total reaction volume of 5 µl that consisted of TaqMan universal master mix (Applied Biosystems) 2.5 µl and a 40× primer-probe mixture 0.125 µl. Mock (no template) controls were included in each run. Each reaction was amplified at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min.

PCR–restriction fragment length polymorphism

To genotype the c135 G/A polymorphisms of the SNP rs1800858, PCR amplification of exon 2 of the *RET-protooncogene* was performed as presented in our previous study.[5] Briefly, the 390 bp amplified fragment was cut using the *Bst*ZI enzyme (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. The digested products were analyzed on 3% agarose gel, in which individuals with GG (major allele homozygosity) showed two bands of 107 and 283 bp, those with GA (heterozygosity) showed three bands of 107, 283 and 390 bp, and those with AA (minor allele homozygosity) showed a single undigested band of 390 bp (not shown).

Genotyping quality control

We compared genotype call rates and concordances among each individual study and the overall study. We used the following criteria as a measure of acceptable genotyping: (1) >10% sample duplicates included; (2) concordance rate for the duplicates ⩾98%; (3) overall call rate by study >95%, and (4) call rates >90% for each individual 384-well plate. The data for any SNP failing these criteria in any assays were excluded from the final analyses. The Hardy–Weinberg Equilibrium was checked for all SNPs ($P>0.05$).

Available oligonucleotide sequences and the genotyping method used with each SNP are shown in Table 1 according to their genomic context. The GenBank accession numbers of the *RET-protooncogene* and *NRG1* reference sequences used in this study were NT_033985.6 and NT_007995.14, respectively.

Statistical analysis

For statistical analysis of the SNP association with the disease, both overall analysis and subgroup analysis (Thai-Chinese and Thai-Muslim), we used Fisher's exact test with a two-by-two contingency table under allelic, dominant and recessive inheritance models. *P*-values were two-tailed and a value of less than 0.05 was considered statistically significant. Correction for multiple parameters used a conservative Bonferroni correction for the 14 SNPs studied ($P<0.05/14$ SNPs=0.0036). Odds ratios (OR) and 95% confidence intervals (CI) were calculated on the risk genotypes.

The pairwise LD tests and haplotype analyses were performed with the Haploview software (www.broad.mit.edu/personal/jcbarret/haploview).

The SNP–SNP interaction analysis was conducted on the two genes to construct a combined allelotype that was associated with the disease. To examine whether marker combination may possess better predictability than individual marker alone, based on the parameters such as the most significance level, OR and frequency of the SNP allele were selected for disease marker. We selected rs2435357 (*RET-protooncogene*) and rs2439305 (*NRG1*) for the SNP–SNP interaction analysis, as the two genes that showed the most significant disease association. For gene–gene interactions, the multiplicative interaction effect of the SNPs was estimated using a multiple logistic regression model. For each individual, key variables were defined as a binary variable indicating case–control status, with SNP variable ranging from 0 to 2, indicating the number of risk alleles in an individual subject, according to the method described by Lewis.[18]

RESULTS

Characteristics of the study population

Of the 68 Thai patients with sporadic HSCR included in the study, there were 50 patients with short-segment HSCR (73.53%), 14 patients with long-segment HSCR (20.59%) and four patients with total colonic aganglionosis (5.88%). There were two patients with Down's syndrome (2.94%). The male to female ratio was 59:9 or 6.6:1.

Table 2 compares sex, age, and race between the cases and the control group. There were significant differences in sex and age, but

Table 1 Oligonucleotide sequences and methods for genotyping of the 14 study SNPs

SNP_ID	*Sequence name*	*Sequence*	*Genotype*	*Method*
rs2506010	rs2506010_F	5′-CTGGGCCTGGTGAAACCT-3′	C/T	TaqMan SNP genotyping
	rs2506010_R	5′-GGGTCGATGACCATCTTTGCAA-3′		
	rs2506010_VIC	5′-CCTAATGCTGGGATCCT-3′		
	rs2506010_FAM	5′-ACCTAATGCTAGGATCCT-3′		
rs2506011	rs2506011_F	5′-AGAGAACCTCCATCTAAAAGTTACATTCATTTAAA-3′	T/C	TaqMan SNP genotyping
	rs2506011_R	5′-GACTCAGTTTCCTCATCCAAGCAA-3′		
	rs2506011_VIC	5′-CTGGCATTTCATTGCTCA-3′		
	rs2506011_FAM	5′-TGGCATTTCACTGCTCA-3′		
rs2506020	rs2506020_F	5′-ACCCCAGGAGGCTTCAAGTA-3′	T/C	TaqMan SNP genotyping
	rs2506020_R	5′-GCAGAAGTGTGTGCAGGTACA-3′		
	rs2506020_VIC	5′-CCCGCATCCTCTG-3′		
	rs2506020_FAM	5′-CCCGCGTCCTCTG-3′		
rs2435357	rs2435357_F	5′-AGCCCTGCAGCCAAGG-3′	T/C	TaqMan SNP genotyping
	rs2435357_R	5′-GGACTGGCCACCCAAGTG-3′		
	rs2435357_VIC	5′-TGTGGATGACCATGTAAG-3′		
	rs2435357_FAM	5′-TGTGGATGACCGTGTAAG-3′		
rs2506021	rs2506021_F	5′-TGGGTACAGAGGAGAAACTGAGAT-3′	T/C	TaqMan SNP genotyping
	rs2506021_R	5′-GTGTGTGTTGCAGTGGTAATGAG-3′		
	rs2506021_VIC	5′-TGGAGCAAGGAACATAT-3′		
	rs2506021_FAM	5′-TGGAGCAAGGGACATAT-3′		
rs2505540	rs2505540_F	5′-GCGGGTATATACCAGTATGAGCAAA-3′	A/G	TaqMan SNP genotyping
	rs2505540_R	5′-TCACACATTCTCACACTGCCAATAG-3′		
	rs2505540_VIC	5′-CATGTGCTTGTTAGAGCC-3′		
	rs2505540_FAM	5′-ATGTGCTTGCTAGAGCC-3′		
rs3123655	rs3123655_F	5′-GCCACAGGCAGCCAGT-3′	C/G	TaqMan SNP genotyping
	rs3123655_R	5′-GCGCACATGGCATGATTCTG-3′		
	rs3123655_VIC	5′-CTGATCCATTGCCTAACAT-3′		
	rs3123655_FAM	5′-TGATCCATTGGCTAACAT-3′		
rs1800858	rs1800858_F	5′-AGCCTTATTCTCACCATCCC-3′	A/G	PCR–RFLP
	rs1800858_R	5′-CAGTGTCAGCGGCTGTGATA-3′		
rs1800861	rs1800861_F	5′-CTGCTCTGTGCTGCATTTCAG-3′	G/T	TaqMan SNP genotyping
	rs1800861_R	5′-GGGTGGTTGACCTGCTTCAG-3′		
	rs1800861_VIC	5′-CAGGTCTCGCAGCTCA-3′		
	rs1800861_FAM	5′-AGGTCTCGAAGCTCA-3′		
rs1800862	rs1800862_F	5′-CTGGGCAGTGGAGGCA-3′	C/T	TaqMan SNP genotyping
	rs1800862_R	5′-GTGAGGGCCCGCTCATC-3′		
	rs1800862_VIC	5′-CAACTCCAGCTCCCTG-3′		
	rs1800862_FAM	5′-CAACTCCAGTTCCCTG-3′		
rs16879552	rs16879552_F	5′-AGAGTTGATGCTTAAACCTCACTTTATTGTATAG-3′	C/T	TaqMan SNP genotyping
	rs16879552_R	5′-AGGTTGGTGCACACTTTTGTTTT-3′		
	rs16879552_VIC	5′-CTGCAATTTATACGAGTCCCA-3′		
	rs16879552_FAM	5′-CTGCAATTTATACAAGTCCCA-3′		
rs7835688	rs7835688_F	5′-TCAATGTTC TGTTAGCATTCTCCAAGT-3′	G/C	TaqMan SNP genotyping
	rs7835688_R	5′-GGTTTGTTTTCATCTATCCTAACAGAACATG-3′		
	rs7835688_VIC	5′-AACAAGTTAAATTGGATTTGA-3′		
	rs7835688_FAM	5′-ACAAGTTAAATTCGATTTGA-3′		
rs2439305	rs2439305_F	5′-ACACATGGCCATAAAAATAGTCAGAGA-3′	G/A	TaqMan SNP genotyping
	rs2439305_R	5′-GGACAAAAAGCTAGCAATAAACTGTGT-3′		
	rs2439305_VIC	5′-ACTCAATCCGTAAACTG-3′		
	rs2439305_FAM	5′-CTCAATCCATAAACTG-3′		
rs2919381	rs2919381_F	5′-AGCTGAGTAGGCAAACAAGAAAGAA-3′	A/G	TaqMan SNP genotyping
	rs2919381_R	5′-GTCCATTTGACTTGGTCTGTAGCTT-3′		
	rs2919381_VIC	5′-CATACAGTTTTATCAATTTATC-3′		
	rs2919381_FAM	5′-ACAGTTTTATCAGTTTATC-3′		

Abbreviations: RFLP, restriction fragment length polymorphism; SNP, single-nucleotide polymorphism.

other parameters including ethnic group and severity of the disease were comparable between the two groups. There was no statistically significant difference in genotype distribution between the two ethnic groups studied. Also, the genotype distribution had no significant difference between sexes.

Table 2 Demographic characteristics of subjects

Characteristic	*Cases*	*Controls*	P-*value*
No of subjects	68	120	—
Age+s.d. (years)	1.2 ± 2.1[a]	34.5 ± 10.3[a]	0.0001
Sex (no.)			
Female	9	55	<0.0001
Male	59	65	—
Ethnic groups			
Thai-Chinese	38	79	0.21
Thai-Muslim	30	41	—

[a]Mean ± s.e.m.

Association between the SNPs in the *RET-protooncogene* and HSCR

A total of 10 SNPs within the *RET-protooncogene* were genotyped in 68 cases and 120 controls. Of these, nine SNPs were successfully genotyped by the TaqMan SNP genotyping assay technique, and the remaining one (rs1800858) was studied by PCR–restriction fragment length polymorphism. The results of allelic and genotyping associations in the *RET-protooncogene* are summarized in Table 3. Significant associations between seven haplotype tagging SNPs in intron 1 of the *RET-protooncogene* and HSCR were demonstrated in our study. As shown in Table 3, the most significant *P*-value was 8.17E-09 with an OR of 6.43 (95% CI 3.33–12.40). The allele, genotype and haplotype block identified by seven SNPs (rs2506010, rs2506011, rs2506020, rs2435357, rs2506021, rs2505540 and rs3123655), spanning an area of 23-kb core-risk haplotype surrounding intron 1 of the gene, are strongly associated with the disease as shown in Figure 1. Pairwise LD for all possible pairs of SNPs was calculated for cases and controls.

Table 3 Summary results of the association between *RET-protooncogene* and HSCR

			Case				*Control*						
			Genotype				*Genotype*						
SNP_ID	*Position*[a]	*Allele (1/2)*	*11*	*12*	*22*	*MAF*	*11*	*12*	*22*	*MAF*	*Risk allele*	P-*value*[b]	*OR (95% CI)*
rs2506010	42893564 (intron 1)	C/T	2	5	31	0.118	7	42	30	0.354	T	9.91E-06[c]	7.23 (2.83–18.47)[c]
			1	6	23	0.133	2	17	22	0.256	T	7.99E-02[d]	2.84 (1.00–8.07)[d]
			3	11	54	0.125	9	59	52	0.321	T	1.57E-06[e]	5.04 (2.53–10.05)[e]
rs2506011	42894942 (intron 1)	C/T	2	5	31	0.118	7	42	30	0.354	T	9.91E-06[c]	7.23 (2.83–18.47)[c]
			1	6	23	0.133	2	17	22	0.256	T	7.99E-02[d]	2.84 (1.00–8.07)[d]
			3	11	54	0.125	9	59	52	0.321	T	1.57E-06[e]	5.04 (2.53–10.05)[e]
rs2506020	42899079 (intron 1)	C/T	2	5	31	0.118	7	42	30	0.354	T	9.91E-06[c]	7.23 (2.83–18.47)[c]
			1	6	23	0.133	2	17	22	0.256	T	7.99E-02[d]	2.84 (1.00–8.07)[d]
			3	11	54	0.125	9	59	52	0.321	T	1.57E-06[e]	5.04 (2.53–10.05)[e]
rs2435357	42902062 (intron 1)	C/T	2	9	27	0.171	19	43	17	0.513	T	3.26E-07[c]	8.95 (3.70–21.64)[c]
			5	5	20	0.250	6	21	14	0.402	T	8.69E-03[d]	3.86 (1.42–10.45)[d]
			7	14	47	0.206	25	64	31	0.475	T	8.17E-09[e]	6.43 (3.33–12.40)[e]
rs2506021	42904154 (intron 1)	C/T	2	5	31	0.118	7	42	30	0.354	T	9.91E-06[c]	7.23 (2.83–18.47)[c]
			1	6	23	0.133	2	17	22	0.256	T	7.99E-02[d]	2.84 (1.00–8.07)[d]
			3	11	54	0.125	9	59	52	0.321	T	1.57E-06[e]	5.04 (2.53–10.05)[e]
rs2505540	42910246 (intron 1)	G/A	2	5	31	0.118	7	42	30	0.354	A	9.91E-06[c]	7.23 (2.83–18.47)[c]
			1	6	23	0.133	2	17	22	0.256	A	7.99E-02[d]	2.84 (1.00–8.07)[d]
			3	11	54	0.125	9	59	52	0.321	A	1.57E-06[e]	5.04 (2.53–10.05)[e]
rs3123655	42915200 (intron 1)	G/C	2	5	31	0.118	7	42	30	0.354	C	9.91E-06[c]	7.23 (2.83–18.47)[c]
			1	6	23	0.133	2	17	22	0.256	C	7.99E-02[d]	2.84 (1.00–8.07)[d]
			3	11	54	0.125	9	59	52	0.321	C	1.57E-06[e]	5.04 (2.53–10.05)[e]
rs1800858	42915974 (exon 2)	G/A	22	13	3	0.250	31	30	18	0.418	G	1.38E-02[c]	2.13 (0.97–4.68)[c]
			14	10	6	0.367	9	21	11	0.476	G	4.01E-02[d]	3.11 (1.11–8.72)[d]
			36	23	9	0.301	40	51	29	0.454	G	4.32E-03[e]	2.25 (1.22–4.14)[e]
rs1800861	42933849 (exon 13)	T/G	4	10	24	0.237	19	41	19	0.499	G	7.42E-05[c]	5.41 (2.34–12.50)[c]
			3	11	16	0.283	7	17	17	0.378	G	2.83E-01[d]	1.61 (0.62–4.17)[d]
			7	21	40	0.257	26	58	36	0.458	G	1.13E-04[e]	3.33 (1.79–6.20)[e]
rs1800862	42935100 (exon 14)	T/C	0	0	38	0	0	1	78	0.006	C	1.00E+00[c]	NA[c]
			0	1	28	0.017	0	2	39	0.024	C	1.00E+00[d]	1.44 (0.12–16.62)[d]
			0	1	66	0.007	0	3	117	0.013	C	1.00E+00[e]	1.69 (0.17–16.60)[e]

Abbreviations: CI, confidence interval, HSCR, Hirschsprung disease; MAF, minor allele frequency; OR, odds ratio; SNP, single-nucleotide polymorphism.
Statistically significant associations after application of the Bonferroni correction are shown in bold, based on 14 independent effective tests ($P < 0.0036$).
[a]Positions of the SNPs were derived from NCBI build36.3_NT033985.6.
[b]The minimum *P*-values of Fisher's exact test were calculated by Fisher's exact test to compare the allelic and genotypic frequencies between cases and controls in three different models.
[c]Statistical analysis for the Thai-Chinese subjects.
[d]Statistical analysis for the Thai-Muslim subjects.
[e]Statistical analysis for all subjects.

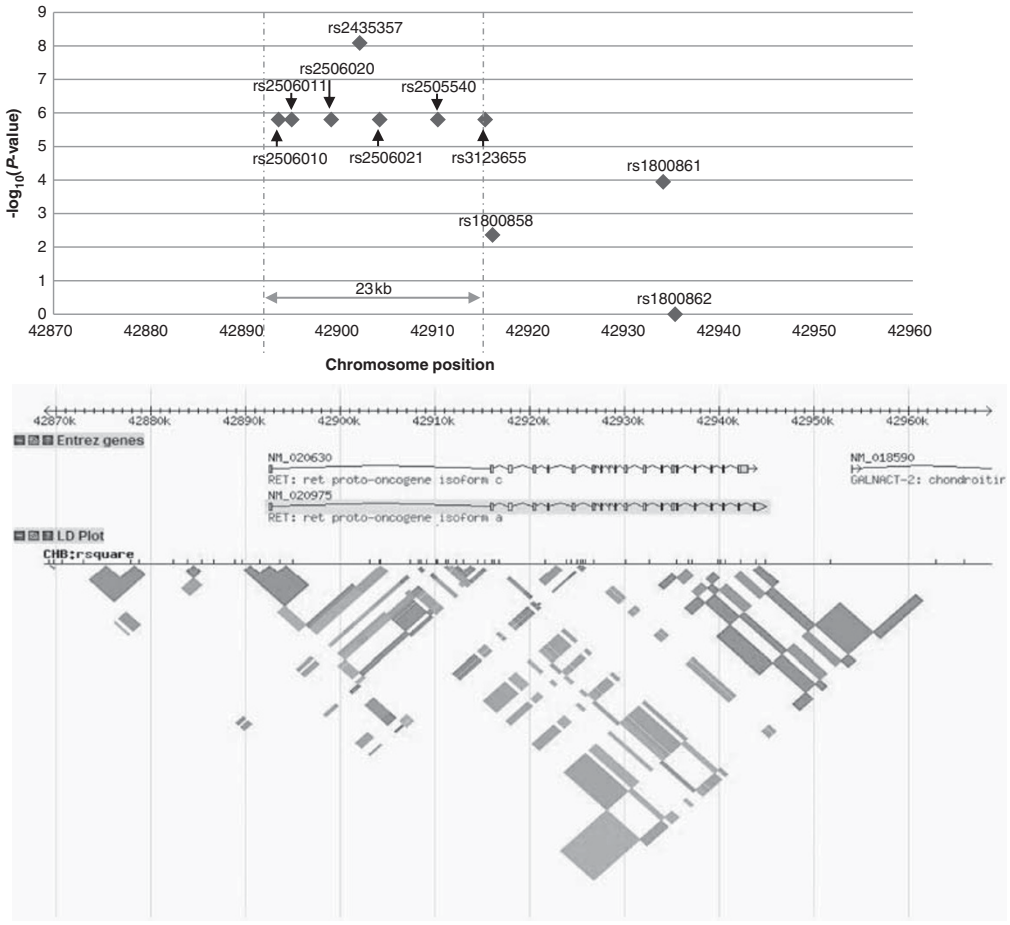

Figure 1 Case–control association *P*-value plots, LD map and genomic structure of the *RET-protooncogene* region on chromosome 10q11.2, spanning an area of 23-kb core-risk haplotype surrounding intron 1 of the gene. Pairwise LD (r^2) is based on the genotype data of Han Chinese in the HapMap database. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 4 Summary results for associations between the *NRG1* genotypes and HSCR

			Case				*Control*						
			Genotype				*Genotype*						
SNP_ID	*Position*[a]	*Allele (1/2)*	*11*	*12*	*22*	*MAF*	*11*	*12*	*22*	*MAF*	*Risk allele*	P-*value*[b]	*OR (95% CI)*
rs16879552	32530758 (intron 1)	T/C	0	13	25	0.171	15	37	26	0.429	C	1.11E-04[c]	3.85 (1.70–8.72)[c]
			5	7	18	0.283	5	8	28	0.219	C	4.33E-01[d]	0.70 (0.26–1.86)[d]
			5	20	43	0.221	20	45	54	0.357	C	7.23E-03[e]	2.07 (1.12–3.81)[e]
rs7835688	32531041 (intron 1)	C/G	7	16	15	0.395	6	27	45	0.250	C	3.18E-02[c]	2.71 (0.84–8.72)[c]
			6	10	14	0.367	2	16	23	0.244	C	6.31E-02[d]	4.88 (0.91–26.14)[d]
			13	26	29	0.382	8	43	68	0.248	C	6.94E-03[e]	3.28 (1.28–8.38)[e]
rs2439305	32549006 (intron 1)	A/G	15	16	7	0.395	45	27	6	0.250	G	3.18E-02[c]	2.71 (0.84–8.72)[c]
			14	10	6	0.367	23	16	2	0.244	G	6.31E-02[d]	4.88 (0.91–26.14)[d]
			29	26	13	0.382	68	43	8	0.248	G	6.94E-03[e]	3.28 (1.28–8.38)[e]
rs2919381	32683466 (intron 5)	G/A	16	20	2	0.316	22	42	15	0.456	G	4.74E-02[c]	1.88 (0.84–4.24)[c]
			16	10	4	0.300	16	18	7	0.390	G	2.90E-01[d]	1.79 (0.69–4.63)[d]
			32	30	6	0.309	38	60	22	0.433	G	2.07E-02[e]	1.92 (1.04–3.54)[e]

Abbreviations: CI, confidence interval, HSCR, Hirschsprung disease; MAF, minor allele frequency; OR, odds ratio; SNP, single-nucleotide polymorphism.
Statistically significant associations after application of the Bonferroni correction are shown in bold, based on 14 independent effective tests ($P<0.0036$).
[a]Positions of the SNPs were derived from NCBI build36.3_NT007995.14.
[b]The minimum *P*-values of Fisher's exact test were calculated by Fisher's exact test to compare the allelic and genotypic frequencies between cases and controls in three different models.
[c]Statistical analysis for the Thai-Chinese subjects.
[d]Statistical analysis for the Thai-Muslim subjects.
[e]Statistical analysis for all subjects.

We found a strong LD ($D' > 0.94$) in all seven non-coding region SNPs. Haplotype analysis showed that these seven risk-associated alleles presented in the same risk haplotype (TTTTTAC) of all subjects; in both the Thai-Chinese and Thai-Muslim subgroups with a *P*-value=8.63E-08 (OR=3.64, 95% CI=2.24–5.92), a *P*-value=2.05E-07 (OR=5.44, 95% CI=2.77–10.67), and a *P*-value=7.28E-02 (OR=2.02, 95% CI=0.97–4.20), respectively (Supplementary Table S1).

Association between the SNPs in *NRG1* and HSCR

All four SNPs were successfully genotyped by the TaqMan SNP genotyping assay. The results of association analysis for SNPs of the *NRG1* and HSCR are summarized in Table 4. All SNPs studied had significant disease association; however, this association was shown to be marginal when the Bonferroni correction for multiple markers was performed (Figure 2). A pairwise LD test demonstrated that that three non-coding region SNPs in *NRG1* (rs16879552, rs7835688 and rs2439305) showed a strong LD ($D'=1$). Haplotype analysis showed significant associations between the risk alleles (CCG) and all HSCRs at a *P*-value of 6.72E-03 (OR=1.90, 95% CI=1.21–2.99). When ethnic subgroup analysis was performed, the *NRG1* haplotype showed disease association in the Thai-Chinese patients at a *P*-value of 2.25E-02 (OR=1.99, 95% CI=1.11–3.57), but not in Thai-Muslim with *P*-value=1.37E-01 (OR=1.79, 95% CI=0.87–3.72).

SNP–SNP interaction of the network that governs the development of the enteric nervous system-related genes

The rs2435357 (*RET-protooncogene*) and rs2439305 (*NRG1*) were identified as the most significant markers associated with HSCR in our cohort. Analysis of the combined genotypes of these two genes revealed a significant increase in HSCR risk with increasing numbers

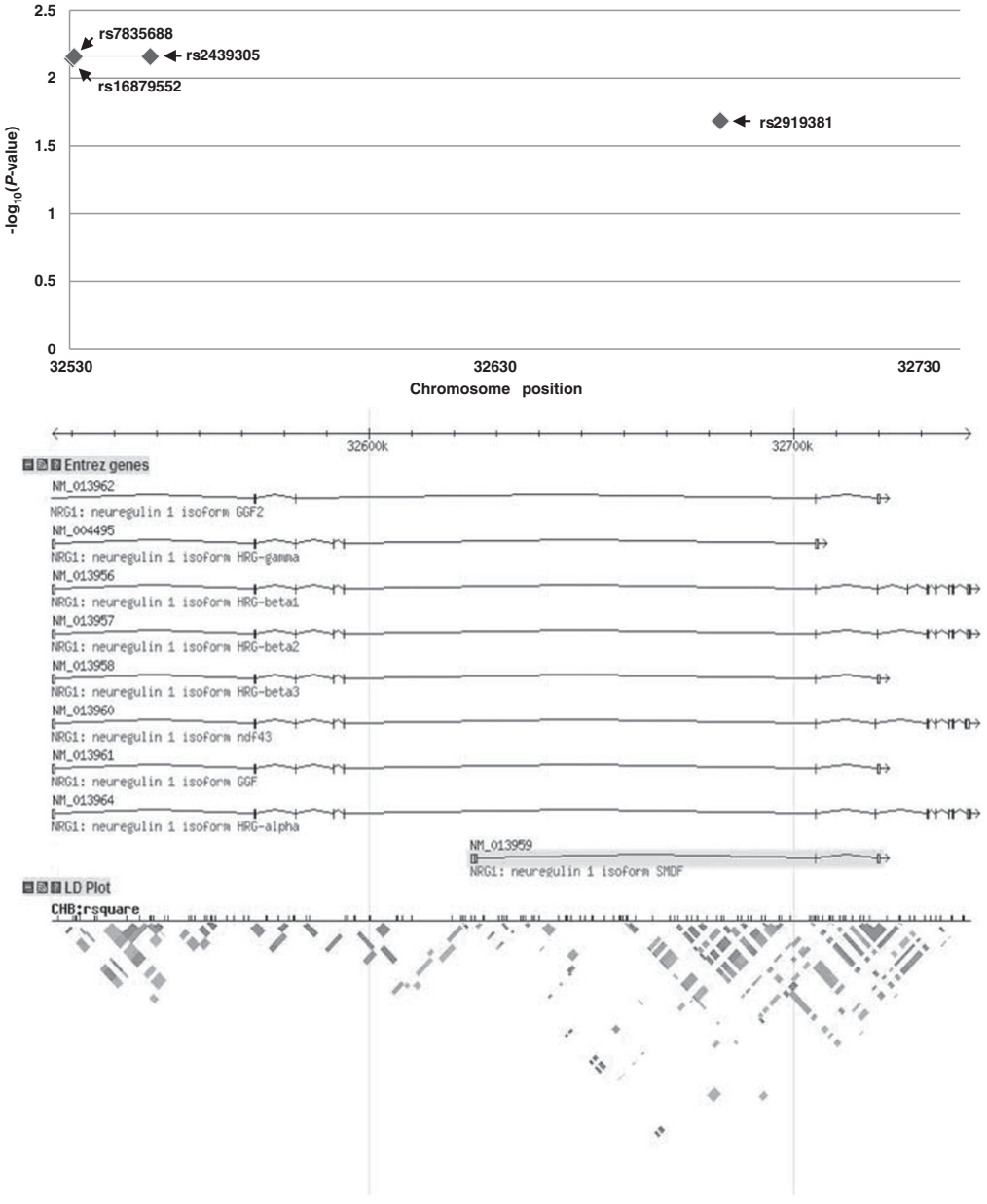

Figure 2 Case–control association *P*-value plots, LD map and genomic structure of the *NRG1* region on chromosome 8p12. Pairwise LD (r^2) was based on the genotype data of Han Chinese in HapMap database. A full color version of this figure is available at the *Journal of Human Genetics* journal online.

Table 5 OR and corresponding 95% CI for the joint effects of SNP–SNP interaction and HSCR risk

Gene–gene SNP–SNP interaction		*Combination*		*Frequency* N, *(%)*			
RET	*NRG1*	*Genotype*	*Genotype*	*Controls*	*Patients*	*OR ratio (95% CI)*	P-*value*[a]
rs2435357	rs2439305	CC	AA	14 (11.76)	6 (8.82)	Reference	
		—	AG	10 (8.40)	1 (1.47)	0.16 (0.02–1.30)	1.01E-01
		—	GG	1 (0.84)	0 (0)	NA	1.00E+00
		CT	AA	36 (30.25)	4 (5.88)	0.14 (0.05–0.43)	6.68E-05
		—	AG	21 (17.65)	7 (10.29)	0.54 (0.21–1.33)	2.06E-01
		—	GG	6 (5.04)	3 (4.41)	0.87 (0.21–3.59)	1.00E+00
		TT	AA	18 (15.13)	19 (27.94)	2.18 (1.05–4.51)	3.80E-02
		—	AG	12 (10.08)	18 (26.47)	3.21 (1.44–7.17)	4.15E-03
		—	GG	1 (0.84)	10 (14.71)	20.34 (2.54–162.78)	1.99E-04

Abbreviations: CI, confidence interval, HSCR, Hirschsprung disease; N, number of subjects; OR, odds ratio; SNP, single-nucleotide polymorphism.
[a]Multiple logistic regression comparison.

of putative high-risk alleles. Table 5 presents the SNP–SNP interaction results in all models. Under a recessive model, the risk of HSCR was highest in those subjects harboring homozygous risk alleles (TT) of rs2435357 and homozygous risk alleles (GG) of rs2439305 (OR=20.34, 95% CI=2.54–162.78), compared with other genotypes. There was no or minimal increased risk for HSCR at these two loci if only one risk allele was present: OR=0.74 (95% CI=0.18–2.95) for the presence of the *NRG1* risk allele but not the *RET-protooncogene* risk allele, and OR=3.54 (95% CI=1.88–6.66) for the presence of the *RET-protooncogene* risk allele but not the *NRG1* risk allele.

DISCUSSION

In this study, two candidate genes that are known to be involved with control of the development of the enteric nervous system, *RET-protooncogene* and *NRG1*, were simultaneously examined using a real-time PCR-based genotyping technique. Using the model of genetic susceptibility in multifactorial disease, we identified disease association of the genetic variants in *RET-protooncogene* and *NRG1* with sporadic HSCR in Thai patients.

The *RET-protooncogene*, located on chromosome 10, is composed of 21 exons and encodes a 52.42 kb size tyrosine kinase receptor involved in growth, migration and differentiation of enteric neurons.[19] According to GWAS data,[7] rs2435357 represents a marker for an LD locus in the *RET-protooncogene* that needs further investigation. Functionally, rs2435357 has been proven to lie in the enhancer-like sequence within intron 1 of the *RET-protooncogene*.[20] To conduct a fine-mapping study for the most significant genetic variants associated with HSCR in the Thai population, more haplotype tagging SNPs covering intron 1 of the *RET-protooncogene* were included in our analysis. We further genotyped for rs1800858, rs1800861 and rs1800862, which have been reported to have disease association in previous studies.[21,22] Using a high-throughput genotyping technique, our case–control study successfully validated an association between HSCR in the Thai population and the SNP within the LD block of the *RET-protooncogene* with a high OR.

NRG1, a 1.1-Mb gene located on chromosome 8p12, belongs to a family of structurally related glycoproteins that act as multifunctional factors that bind to ErbB receptors and are involved in neuronal cell differentiation.[23–26] A HapMap database constructed from a GWAS has suggested a statistically significant association between the SNPs under the LD block in *NRG1* and HSCR.[7] A recent validation study in the Chinese population also confirmed this disease association. Focusing on the SNPs within *NRG1*, our study found an association between *NRG1* polymorphisms and HSCR in Thais at a comparable OR with the Chinese study noted above.[7]

We further evaluated the interaction between the *RET-protooncogene* and *NRG1* by analyzing the combination of landmark SNPs from the two genes for any disease association. Our main finding was that genetic interactions significantly modulated the risk for HSCR in our Thai case–control subjects. Interestingly, the combination between polymorphisms of *RET-protooncogene* and *NRG1* was associated with a 20-fold increased risk of the disease in individuals who carried the risk alleles of these polymorphisms compared with individuals whose genotype contained neither risk allele. These results are in line with a previous study that demonstrated interactions between rs2435357 in *RET-protooncogene*, and the rs16879552 and rs7835688 in the *NRG1* in the Chinese population,[7] in the way that the two *NRG1* SNPs reported in their study were in high LD with the rs2439305 SNP reported in this study. *NRG1* is involved in neuronal development by promoting neuronal survival, whereas the *RET-protooncogene* may perform proliferative and/or main differentiation functions.[8,25] The mechanism of interaction between the two genes is still not well understood, because very few studies have considered such interactions, and therefore, it would be pure speculation to try to explain the interactions found in this study on the basis of known functions. However, epidemiological analyses of interacting variants will lead to a better understanding of the biological pathways underlying HSCR. Considering a relatively high specificity, a SNP or a combination of SNPs can be used as a disease marker together with clinical parameters for screening for HSCR in individual cases. Further prospective validation studies with a larger number of participants are required to confirm the predictive power of the markers.

Our study found that real-time PCR with Taqman probes is a rapid and simple method for SNP genotyping. To our knowledge, this is the first study that has used a fluorescence-based SNP detection assay for genotyping *RET-protooncogene* and *NRG1*, as previous studies have usually used PCR-direct sequencing, single-stranded conformation polymorphisms and restriction fragment length polymorphisms.[5,13,14,16,20,21] This fluorescence-based technique relies on different properties of two probes that are specific to the alleles of interest, each labeled with different fluorescent markers. It offers several advantages compared with older techniques; not only does it feature high specificity and accuracy, but also not requiring a post-amplification step reduces the time and cost of genotyping. In this study, only one of the SNP (rs1800858) failed to use this method.

In conclusion, we identified SNPs in *RET-protooncogene* and *NRG1*, which were disease-associated with Thai HSCR. We also demonstrated epidemiological interaction between the two genes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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