Mutations and polymorphisms of Hirschsprung disease candidate genes in Thai patients

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Abstract Mutation and polymorphism data for Hirschsprung disease (HSCR) varies among ethnic groups. Single nucleotide polymorphisms (SNP) of RET proto-oncogene (RET) were recently shown to be associated with the disease, and with disease severity, in different populations. In this study, comprehensive analysis of RET, GDNF, EDNRB, ET-3, and SOX-10 genes among sporadic HSCR in Thailand was conducted by standard PCR-SSCP, RFLP, and sequencing methods. Of 41 patients, 30 cases had rectosigmoid disease (RSD) and 11 cases were assigned to the long-segment disease (LSD) group. Four missense mutations of RET, S100M, R231H, T278N, and G533S, were identified in three patients. One novel missense mutation, V111Q, was detected in EDNRB. For ET-3, two novel missense mutations, D166E and C173R, occurred concomitantly in a patient. The incidence of missense mutation was significantly higher in our female HSCR patient than in the male counterpart. Statistical

analysis of the SNPs revealed a significant difference between allele distribution of RET L769L in patients in the LSD and RSD groups. The predominant genotype construct of RET A45A/L769L in our HSCR was GG/GG, which is obviously different from results from all previous studies. The GG/GG genotype construct was associated with RSD and with males. The study also detected a variant allele of RET S836S which has never been reported in Asian cohorts.

Keywords Hirschsprung disease · RET · GDNF · EDNRB · ET-3 · SOX-10

Introduction

Hirschsprung disease (HSCR) is a congenital anomaly of the enteric nervous system which causes functional colonic obstruction. Pathologically, the disease is characterized by the absence of enteric ganglion cells in submucosal and myenteric nervous plexuses (Martucciello et al. 2000). The severity of HSCR is classified, on the basis of the extent of aganglionosis, into rectosigmoid disease (RSD) or long segment disease (LSD), according to whether the aganglionic portion is confined within the rectosigmoid colon or extends more proximally.

HSCR is a good and classic model for understanding the pathogenesis of multifactorial disease. During the past decade, at least eight genes associated with the disease have been identified (reviewed by Kusafuka and Puri 1997, 1998; Amiel and Lyonnet 2001; Puri and Shinkai 2004). Most candidate genes belong to two major signaling pathways which have essential roles in migration and maturation of an enteric neuroblast,

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RET transmembrane receptor system (RET, GDNF), and endothelin receptor system (EDNRB, ET-3). Not concentrating solely on missense mutations, recent studies have also explored a potential association between allelotypes of synonymous polymorphisms within RET and the occurrence of the disease (Borrero et al. 1999, 2000; Sancandi et al. 2003; Garcia-Barcelo et al. 2003; Wu et al. 2005). Apart from these two pathways, heterozygous mutations of SOX-10, a member of Sry type HMG family of transcription factors, have been detected in patients with syndromic HSCR (Pingault et al. 1998).

Although the main disease susceptibility genes stated above and candidate single nucleotide polymorphisms (SNPs) of RET has been extensively investigated in HSCR patients from different ethnic groups, genotype data for South-East Asians remains unavailable. In this study, we performed a comprehensive genetic study for RET, GDNF, EDNRB, ET-3, and SOX-10 genes in sporadic HSCR patients in southern Thailand.

Materials and methods

Patients

Snap-frozen colon specimens from 41 consecutive cases of HSCR who were operated on at Songklanagarind Hospital, Thailand, from May 2003 to September 2005 were included in this study. Cases with family history of HSCR were excluded. Definitive diagnosis of aganglionosis was confirmed by histological examination. Patient demographic data were collected from hospital records using a standardized data-extraction form. Retrieval of clinical information and the genetic study were performed with permission from the Research Ethic Committee of the Faculty of Medicine, Prince of Songkla University. We have had no opportunity to investigate the genotype in non-disease controls.

Mutations analysis

Genetic analysis was conducted at Osaka University, Japan. Mutation screening was performed by the polymerase chain reaction–single-stranded conformation polymorphism (PCR-SSCP) technique, which has been described elsewhere (Sangkhatat et al. 2005). Whole coding sequences of RET (20 exons), GDNF (2 exons), EDNRB (7 exons), and ET-3 (5 exons and promoter region) were amplified. Screening of SOX-10 mutations covered exon 3 to exon 5. With some

exceptions, primer sequences used in this study followed previously published data (Kusafuka et al. 1997; Inoue et al. 1998; Sakai et al. 2000). Primer sequences used in our study can be provided on request.

Genotype study of RET SNP A45A and S904S in exons 2 and 15 was performed by restriction fragment length polymorphism (RFLP) analysis, using restriction enzyme Eco52I and RsaI (Toyobo, Japan).

Reference sequences and SNP information were obtained from the Ensemble Human Genome Browser; (http://www.ensembl.org/Homo_sapiens) and the National Center for Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>).

Statistical analysis

Testing of the Hardy–Weinberg equilibrium (HWE) of allele distribution in each SNP with minor allele frequency larger than 0.01 was performed by chi-square-based methods, using the program Popgene (University of Alberta, Canada). The pairwise linkage disequilibrium (LD) test was performed by use of the same program. Results from the LD test were displayed as normalized linkage disequilibrium coefficients (D'), together with the P -value. Univariate analysis using chi-square test or Fisher's exact test, as appropriate, was performed for each SNP to determine the significance of differences between allele frequencies in the two length groups. Statistical analysis was performed using statistics software SPSS version 14.0 (SPSS, Chicago, IL, USA). Values are reported as raw numbers and percentages, where applicable. Categorical variables were compared using chi-square or Fisher's exact tests, as appropriate. The crude odds ratio with a 95% confidence interval (CI) was also derived. Statistical significance was assumed for $P < 0.05$, for all comparisons.

Results

Demographic data

Of the 41 cases investigated the male to female ratio was 34:7, or 4.9:1. There was no difference between sex ratios for RSD (5.0:1) and LSD (4.5:1). With regard to the detailed extent of aganglionosis, in 30 patients (73.2%) the lesion was confined to the rectosigmoid region, in nine cases (22%) (including two cases with trisomy-21) the aganglionic colon extended to the transverse or descending colon, and two cases (4.9%) had total colonic aganglionosis. With regard to racial grouping, 25 patients (61.0%) were Chinese–Thai and

16 were Malay–Thai. None of the subjects were biologically related. The incidence of missense mutation in female patients was higher than in male counterparts at a statistically significant level (P -value: 0.027; odds ratio: 12.0, 95% CI: 0.953, 168.328).

Sequence variants of RET and GDNF

RET mutations were detected in five cases (Table 1). This figure does not include known SNPs with a frequency of minor allele at 0.02 or more (variants found in more than one allele in our HSCR population). Among seven points of sequence alteration, four were missense mutations. Three, S100M, T278N, and G533S, have not been reported previously. Interestingly, one infant with total colonic aganglionosis harbored two points of missense mutation on RET. The incidence of missense mutation of RET among Thai sporadic HSCR in our screening was, therefore, 3/41 or 7.3%. The study detected no aberrant band in fragment analysis of the GDNF amplicons.

Sequence variants of EDNRB, ET-3, and SOX-10

The study identified novel mutations of EDNRB in 3 of 41 cases analyzed, including one missense mutation at codon 111 and two silent mutations at codons 148 and 411, as summarized in Table 2. Excluding silent mutations which should be regarded as polymorphisms, the incidence of EDNRB mutations potentially related to the disease development was 1/41 or 2.44%.

Screening of ET-3 detected four novel mutations in five cases, including two novel missense mutations (D166E and C173R) which occurred simultaneously in one case, one silent mutation in three cases, and one heterozygous G to A change in the promoter region (Table 2). The case that had a silent mutation at codon 411 of EDNRB also harbored a mutation at codon 142 of ET-3. The A142A polymorphism is reported here for the first time.

On the study of SOX-10, a deletion mutation involving 11 bases was found at position minus 58, upstream to exon 5. This case with the large deletion was the same as that harboring a heterozygous change at codon 148 of the EDNRB gene.

Allele distribution of RET, EDNRB, ET-10, and SOX-10 SNPs

SNP genotyping revealed no missing data. The allele distribution of the seven SNPs on the three genes is displayed in Table 3. Of the seven SNPs analyzed, distribution of the allele in six SNPs (RET A45A, A432A, L769L, S836S, EDNRB L277L, and ET-3 A142A) conformed with the HWE whereas SOX-10 H309H deviated by a chi-square value of 7.60 (P -value 0.004). Because of limited collection of samples from healthy individuals, we compared allele frequency between cases with different disease severity (LSD against RSD).

In univariate analysis, no SNP was found to have significantly different allele distribution between the two ethnic groups in our population (data not shown).

Table 1 Sequence variants of RET found in 41 cases

RET exon	Nucleotide alteration	Codon position and amino acid change	Sex/length of aganglionosis group	Remarks
Exon 2	c135G > A c299G > T	A45A S100M	See Table 4 F/RSD	TCA
Exon 3	c438C > T ^a	Y146Y	M/LSD	
Exon 4	c692 G > A ^b c833 C > A ^b	R231H T278N	F/LSD	
Exon 7	c1296G > A	A432A	See Table 4	Down syndrome ^c
Exon 8	c1597G > A	G533S	M/LSD	
Exon 13	c2307T > G	L769L	See Table 4	Two cases ^a TCA, ileal involvement
Exon 14	c2508C > T	S836S	M, F/LSD	
Exon 15	c2712C > G	S904S	M/LSD	

Missense mutations are highlighted in bold

TCA—total colonic aganglionosis

^a One case with S836S polymorphism also had synchronous Y146Y silent mutation

^b These two missense mutations occurred in one infant with total colonic aganglionosis

^c Confirmed trisomy 21 by G-band karyotyping

Table 2 Sequence variants of EDNRB, ET-3, and SOX-10 found in 41 cases

	Gene and exon	Nucleotide alteration	Codon position and amino acid change	Sex/length of aganglionosis group	Remarks
Missense mutations are highlighted in bold ^a One case with ET-3 A142A polymorphism also had synchronous EDNRB K411K mutation ^b The two missense mutations occurred concomitantly in a male patient in the RSD group	EDNRB				
	Exon 1	c332T > A	V111Q	F/RSD	
	Exon 1	c442C > T	L148L	M/RSD	
	Exon 4	c831A > G	L277L	See Table 4	
	Exon 5	c966C > T	A322A	M/RSD	
	Exon 7	c1233A > G ^a	K411K	M/RSD	Malrotation
	ET-3				
	Promoter	c1(−125)G > A	Promoter	M/RSD	
	Exon 3	c426G > A ^a	A142A	2M/RSD, 1M/LSD	Three cases
	Exon 3	c498C > G^b c517T > G^b	D166E C173R	M/RSD	
	SOX 10				
	Exon 5	c927C > T	H309H	See Table 4	
	Exon 5	c698(−58), del 11 nt	Intron 4–5	M/RSD	

Table 3 Allele distribution of selected polymorphisms within RET, EDNRB, ET-3, and SOX-10, according to length of aganglionosis groups

	Gene, position, and SNP code	Nucleotide alteration	Allele frequency			<i>P</i> -value ^a	Crude odds ratio (95% CI)
			Total (N:82)	RSD (N:60)	LSD (N:22)		
<i>RSD</i> —rectosigmoid disease, <i>LSD</i> —long segment disease ^a Derived by use of the chi-square or Fisher's exact test as appropriate	RET						
	Exon 2 A45A	c135G > A	G: 64 (0.78)	G: 50 (0.83)	G: 14 (0.64)	0.056	0.350 (0.116, 1.054)
	rs1800858		A: 18 (0.22)	A: 10 (0.17)	A: 8 (0.36)		
	Exon 7 A432A	c1296A > G	A: 61 (0.87)	A: 51 (0.85)	A: 20 (0.91)	0.387	1.765 (0.350, 8.892)
	rs1800860		G: 11 (0.13)	G: 9 (0.15)	G: 2 (0.09)		
	Exon 13 L769L	c2307T > G	G: 63 (0.77)	G: 50 (0.83)	G: 13 (0.59)	0.021	3.462 (1.166, 10.273)
	rs1800861		T: 19 (0.23)	T: 10 (0.17)	T: 9 (0.41)		
	Exon 14 S836S	c2508 C > T	C: 80 (0.98)	C: 60 (1.00)	C: 20 (0.91)	0.070	NA
	rs1800862		T: 2 (0.02)	T: 0 (0.00)	T: 2 (0.09)		
	EDNRB						
	Exon 4 L277L	c831A > G	A: 51 (0.62)	A: 39 (0.65)	A: 12 (0.55)	0.387	1.548 (0.573, 4.177)
	rs5351		G: 31 (0.38)	G: 21 (0.35)	G: 10 (0.45)		
	ET-3						
	Exon 3 A142A	c426G > A	G: 79 (0.96)	G: 58 (0.97)	G: 21 (0.95)	0.613	0.727 (0.036, 44.73)
	(new)		A: 3 (0.04)	A: 2 (0.03)	A: 1 (0.05)		
	SOX 10						
	Exon 5 H309H	c927C > T	C: 64 (0.78)	C: 46 (0.77)	C: 18 (0.82)	0.767	0.730 (0.212, 2.517)
	rs139884		T: 18 (0.22)	T: 14 (0.23)	T: 4 (0.18)		

Table 4 Genotype distribution of RET polymorphisms A45A and L769L according to sex

Sex	Genotype of RET: A45A/L769L (%)			
	GG/GG	GA/GT	AA/GG	Other remaining genotypes
All (<i>n</i> =41)	23 (56.1%)	5 (12.2%)	1 (2.4%)	12 (29.3%)
Male (<i>n</i> =34)	21 (61.8%)	1 (2.9%)	0 (0%)	12 (35.3%)
Female (<i>n</i> =7)	2 (28.6%)	4 (47.1%)	1 (14.3%)	0 (0%)

The distribution in the male and female patient groups was significantly different, as analyzed by the chi-square test (*df* 3); chi-squared 22.4519, *P*-value 0.0001

One of four RET SNPs (L769L in exon 13) had a statistically significantly increased minor allele frequency in the LSD group (Table 3). Interestingly, the G allele of RET A45A was statistically associated with the male sex (P -value 0.048, odds ratio: 3.484, 95% CI 0.823, 13.909).

The pairwise LD test demonstrated that in our HSCR, RET A45A on exon 2 was in LD with RET L769L on exon 13 (D' 0.56, P -value <0.0001) and with S836S on exon 14 (D' 0.80, P -value 0.006). In the same analysis we also discovered allele association between EDNRB L277L and ET-3 A142A (D' 0.66, P -value 0.020).

Genotype distribution of RET SNPs in Thai HSCR

We combined RET SNPs A45A and L769L for genotype analysis on the basis of previously published data showing disease association. To our surprise, the most common genotype in our HSCR patients was homozygous for G in both SNPs, which differed from the other Asian studies. This predominant genotype construct “GG/GG” was found significantly more frequently in the RSD group (70% in RSD, compared to 18% in LSD; P -value 0.04, odds ratio: 0.095 and 95% CI: 0.017, 0.532). It should be noted that although GG/GG was the predominant genotype in both ethnic groups in our studied population, there were some differences between Chinese–Thai (GG/GG frequency 68%) and Malay–Thai (38%). The variation was not significant when analyzed statistically.

Because differences between missense mutation rate and allele frequency of RET A45A were apparent in the different sexes, we were interested to look at the distribution of genotype by sex. It was found that genotype distributions in female patients significantly differed from those in the whole patient population and in male patients (Table 4). Instead of GG/GG, which contributed 61.8% in male cases, GA/GT accounted for 57.1% of our female cases.

Discussion

We report a comprehensive genetic study of HSCR patients in southern Thailand. Geographically, the region is situated in the middle part of the Malayu peninsula, between the mainland of Thailand and Malaysia. Previous study has suggested that the population in this region was an admixture of two ethnic factions, the Chinese–Thai extract from the Thai mainland and the Malay–Thai extract, which is predominantly Muslim (Fucharoen et al. 1989; Laosombat et al. 1992).

Even within the same racial group, incidences of mutation of HSCR-related genes differed according to the characteristics of the cases, the disease-causing mutation, and the method of screening. The mutation rate of RET among Asians is from 3.6 to 24% (Wu et al. 2005; Sakai et al. 2000; Tou et al. 2006; Garcia-Barcelo et al. 2004). Considering missense mutations in sporadic HSCR alone, figures reported are 7.1% for the Japanese (Sakai et al. 2000), 24% for Hong Kong Chinese (Garcia-Barcelo et al. 2004), and 0% for Taiwan Chinese (Wu et al. 2005). Incidence of RET missense mutation at 7.3% was in agreement with results from with other series. One distinguishing feature that should be noticed in our patients was that six of seven mutations of RET belonged to the LSD group whereas mutations found in other genes were confined to cases with rectosigmoid extent. In agreement with other reports, cases harboring heterozygous mutation of EDNRB were mainly short segment HSCR who had unaffected parents or were asymptomatic (Amiel and Lyonnet 2001; Puri and Shinkai 2004; Tam and Garcia-Barcelo 2004).

Reports from different geographic regions showed that some synonymous polymorphisms on RET had different allele distribution between HSCR cases and controls (Borrego et al. 2000; Sancandi et al. 2003; Garcia-Barcelo et al. 2003; Wu et al. 2005). Those studies also successfully identified a representative genotype or haplotype in their HSCR patients, and association between some SNPs and the severity of the disease. Statistical association between the SNPs A45A and L769L of RET and length of aganglionosis was observed for a cohort from Taiwan (Wu et al. 2005); this deviated from the previous series of Hong Kong Chinese, for whom analysis of RET A45A, A432A, and L769L revealed different allele frequency between length groups for RET A432A only (Garcia-Barcelo et al. 2003). Our current study confirmed association between allele distribution and the length of aganglionosis for the point RET L769L but rejected the point RET A45A, and A432A. Failure to demonstrate such association for RET A45A in our cases was probably a result of the inadequate sample size, because the P -value at 0.057 was close to the boundary of significance. Our data did, however, support a previous observation of an association between genotype of RET and severity of aganglionosis.

In the current series a variant allele of RET exon 14; S836S (c2508C > T) was detected that has been found in no other Asian series (Garcia-Barcelo et al. 2003; Wu et al. 2005; Sakai et al. 2000) and was detected in reduced frequency compared with healthy subjects in Italian HSCR (Griseri et al. 2000, 2002). In our HSCR,

the minor allele in this polymorphism was found exclusively in patients of Malay–Thai extraction who had LSD. This ethnic subgroup is descended from the Malay Archipelago population, which may explain the genetic variation from other East Asian populations. Another major discrepancy between our data and other reports was at the haplotype frequency of the RET A45A and L769L, of which G-G was probably the predominant haplotype in our HSCR, in contrast with A-G in Chinese, Italian, and Spanish patients (Borrego et al. 2000; Sancandi et al. 2003; Garcia-Barcelo et al. 2003; Wu et al. 2005). It has been shown in those population-based studies that the A allele of RET A45A has strong HSCR disease-association. Without a control panel, association of the G allele of this SNP with HSCR could not be tested in our cases. We did, however, deduce from our findings that GG/GG is the characteristic genotype construct for the RET A45A/L769L in HSCR patients in Southern Thailand and this genotype could be shown to have a significant association with short-segment phenotype and the male gender.

Sex is a known risk-modifying factor in HSCR phenotype development. A male individual has a greater risk or, in the other words, female patients may require stronger genetic alteration or more susceptible genetic background to develop HSCR phenotype. Our finding of higher mutation incidence in females was consistent with the observation in the Hong Kong cohort (Garcia-Barcelo et al. 2004). Our study revealed the existence of different genotype between male and female HSCR for the first time. A allele in RET A45A was found in significantly higher frequency in female subjects compared with the whole group or with male patients. In females we also found the GA/GT, rather than GG/GG, genotype to be the predominant genotype for RET A45A/L769L. The significance of this distinct genotype pattern should be subjected to further study.

In summary, we performed a comprehensive genetic screening and SNP genotyping of HSCR-related genes in Thai sporadic cases. The study found missense mutations of RET, EDNRB, and ET-3 at rates comparable with those of previously studied cohorts in the Asian population. The study also revealed GG/GG as a predominant genotype construct of the RET A45A/L769L which was associated with short-segment HSCR and the male gender.

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