Songklanakarin J. Sci. Technol.
42 (3), 596-601, May - Jun. 2020

http://www.sjst.psu.ac.th

Original Article

Genetic polymorphisms of the SOX10 gene in Thai patients with sporadic Hirschsprung disease

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Received: 14 February 2018; Revised: 15 January 2019; Accepted: 26 February 2019

Abstract

Hirschsprung disease (HSCR) is complex genetic disorder of the enteric nervous system (ENS) characterized by an absence of ganglion cells in various parts of the intestine. The disease has a strong genetic association with *RET-protooncogene (RET)* and various genes involved in neural crest development. *SOX10* is a transcriptional regulator whose expression is essential in the development of the ENS. The association between single-nucleotide polymorphisms (SNPs) in *SOX10*, rs139883 and rs139884, and sporadic HSCR has not been reported. We evaluated the genetic associations between two SNPs in *SOX10* and Thai sporadic HSCR, using a case-control design. The study included 120 HSCR and 242 sex-matched controls whose DNA was genotyped using the RFLP (rs139883) and Taqman SNP genotyping (rs139884) methods. The study found minor allele frequencies (MAF) at 0.25 and 0.24 for rs139883 (allele C) and rs139884 (allele A), respectively. Both SNPs complied with the Hardy-Weinberg equilibrium and were further examined through disease association. The association analysis found that the risk-genotype of rs139883 (CC) was associated with HSCR (odds ratio [OR] 2.09; 95% confidence interval [CI] 1.07-4.08; P=0.03). When a subgroup analysis was performed, CC genotype of the rs139883 was significantly associated with HSCR only in females (OR 5.6; 95% CI 1.1-19.5; P=0.01). Moreover, the long segment disease group significantly harbored the risk genotype at a higher frequency (28.6%) compared to the short HSCR (12.0%, P=0.02). In conclusion, the study suggests a genetic association between rs139883 in *SOX10* and Thai-female HSCR.

Keywords: Hirschsprung disease (HSCR), ganglion cells, single nucleotide polymorphisms (SNPs), SOX10, RET-protooncogene

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1. Introduction

Hirschsprung disease (HSCR, MIM142623) is a congenital disorder of the enteric nervous system (ENS) characterized by an absence of intramural ganglion cells in the distal colon that results in functional colonic obstruction (Amiel *et al.,* 2008). The incidence of HSCR is generally estimated at 1 in 5,000 live births with male predominance at a sex ratio of 4:1 (Arshad, Powell, & Tighe, 2012). The incidence varies among ethnic groups with a reported higher incidence in Asians at 2.8 per 10,000 live births (Torfs & Christianson, 1998). The majority of HSCR occurs as sporadic cases although a certain proportion of patients have associated anomalies (Amiel *et al.,* 2008).

The embryopathogenesis of HSCR is currently explained by the concept of neurocristopathy or failure of neural crest cells to migrate and differentiate into mature enteric ganglions (Austin, 2012). Molecular pathology in a number of genes has been implicated in HSCR development including the genes that encode for factors that are crucial for neural crest development (i.e., *RET protooncogene (RET), GDNF, EDNRB, EDN3, NRTN,* and *PHOX2B*) (Amiel *et al.,* 2008; Sangkhathat *et al.,* 2006). Variants/mutations on the RET sequence have been extensively reported in all types of HSCR (Emison *et al.,* 2010). In addition, rare coding mutations were reported in other genes such as *NRG1, ECE1, TCF4,* and *SOX10* (Emison *et al.,* 2010). Recent studies have suggested an interplay between the major players, *RET* and *NRG1* (Gui *et al.,* 2013; Phusantisampan *et al.,* 2012).

RET encodes a tyrosine kinase receptor of the signaling pathway that plays a necessary role in the development of the enteric nervous system (Kusafuka, Wang, & Puri, 1997). Common and rare variants in *RET* are implicated in the HSCR phenotypes in various populations (Kusafuka *et al.,* 1997; Nunez-Torres *et al.,* 2011; Sangkhathat *et al.,* 2006; So *et al.,* 2011). In Thai patients, our previous study showed a strong genetic association between a single nucleotide polymorphism (SNP) within the enhancer sequence on intron 1 of *RET*, rs2435357, and the disease (Phusantisampan *et al.,* 2012). Recent studies have suggested that *SOX10,* a transcriptional controller whose expression is detected during neural crest lineage development, naturally interacts with a transcriptional regulator which then stimulates the transcription process of *RET* (Emison *et al.,* 2010; Leon *et al.,* 2009). Mutations of *SOX10* have been reported in syndromic HSCR although in much lower incidence compared to RET. The majority of reported *SOX10* mutations in HSCR were deletion and truncation mutations which involved a highly conserved DNA-binding high mobility group domain and a transactivation domain at the carboxyterminal. Concerning common variants, a SNP in *SOX10* rs139883 was reported to be associated with schizophrenia (Maeno *et al.,* 2007; Yuan *et al.,* 2012). However, a study in Han Chinese did not identify any association between genetic polymorphisms in the coding regions of *SOX10* and HSCR (Pan, Lou, Luo, Yu, & Li, 2011). In this study, we performed a genetic association study of *SOX10* in HSCR that focused on SNPs on the exon 4, rs139884, which encodes for C-terminus and a SNP on the 3'UTR, rs139883 using a sex-matched case-control design. In addition, we looked for any interaction between *RET* rs2435357 and the disease association of *SOX10*.

2. Materials and Methods

2.1 Study subjects

DNA samples from 120 HSCR patients and 242 sex-matched controls were used in this case-control study. The cases were pediatric patients aged 0–15 years with pathologically confirmed HSCR who underwent surgery in our institute during the years 2003–2014. HSCR patients with a family history of the disease or chronic constipation in the first degree relative were not included. Case identification used the operation registry of the pediatric surgery unit. HSCR cases were categorized according to the extent of aganglionosis: (1) short segment in whom the aganglionosis was confined to the rectosigmoid colon and (2) long segment whose extent of aganglionosis was longer than the rectosigmoid colon but less than total colonic aganglionosis. Controls were sex-matched volunteers who resided in lower southern Thailand and had no clinical history of chronic constipation and no childhood history of abdominal operation. Written informed consent was obtained from all contributors for use of their clinical data and molecular genetic studies. The study was approved by the Human Research Ethnics Committee of the Institution.

2.2 SNP selection

Three prime UTR SNPs in SOX10, rs139884, and rs139883 were selected based on our previous study (Sangkhathat *et al.,* 2006) and associated data reported by others (Maeno *et al.,* 2007; Yuan *et al.,* 2012). A polymorphism of rs139884 on exon4 was demonstrated in Thai HSCR cases in our previous mutation screening study. The variant rs139883, located on the 3'UTR, was reported for its association with schizophrenia in Asians and was predicted to have influence on SOX10 mRNA stability (Maeno *et al.,* 2007). According to the 1000 Genomes Browser, the minor alleles of rs139884 and rs139883 in Asians are allele A (minor allele frequency, MAF, 0.25) and allele C (MAF 0.26), respectively (browsers.1000genomes.org).

The SNP rs2435357 on *RET* was reported to have a strong association with HSCR (Emison *et al.,* 2010). Our recent publication confirmed the disease association of this variant in the Thai population (Phusantisampan *et al.,* 2012). Since our previous study suggested a physical interaction between *SOX10* and *RET* in the signaling pathway that promotes neuronal differentiation (Emison *et al.,* 2010; Leon *et al.,* 2009), we were interested to test for the statistical interaction between *RET* and *SOX10* in HSCR association.

2.3 SNP genotyping and quality control

Genotyping of rs139884 and rs2435357 were performed by TaqMan SNP genotyping, using assay-by-design TaqMan minor groove binding probes labeled with FAM and VIC dyes (Applied Biosystem, Foster City, CA, USA). Each reaction mix contained 50 ng of DNA, 2 μ**L** of 2X TaqMan Genotyping Master Mix, 0.5 μL of 40X primers/probe mixture, and Milli-Q water to a total volume of 20 μL. The PCR reactions were performed in a 96-well plate format on an ABI Prism 7500 Fast Realtime PCR (Applied

Biosystem, Inc.). For positive controls, samples with known genotypes by DNA sequencing were used while negative controls used reactions with no DNA template. The primers and probes used in this study are shown in Table 1.

Genotyping of rs139883 used the restriction fragment length polymorphism technique. Amplified PCR products were cut using the restriction enzyme HpaII (Thermo Scientific, Inc.). Each 25 μL of reaction contained 0.5 μL of restriction enzyme, 2.5 μL of 10X NE buffer, 2 μL of PCR products, and 20 μL of Milli-Q water. The digested products were electrophoresed on 2% agarose gel, stained with ethidium bromide, and visualized under UV transillumination.

The following criteria were used 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 96-well plate. The data for any SNP failing these criteria in any assay were excluded from the final analyses. The Hardy-Weinberg equilibrium (HWE) was used to check all SNPs (P>0.05).

2.4 Statistical analysis

The HWE of allele distribution in each SNP was determined by chi-square based methods. HSCR association of genotype variants was done in a recessive model using the chi-square test followed by univariate logistic regression to determine the OR and 95% CI.

SNP-SNP interaction analysis was conducted on the two genes (*SOX10* and *RET*) to construct a combined allelotype associated with the disease to examine which marker combination possessed better predictability than either individual marker alone based on parameters including the significance level and OR.

3. Results

3.1 Characteristics of the HSCR cases and controls

The 120 cases of 98 males and 22 females consisted of 92 patients with short segment HSCR (76.7%) and 28 patients with long segment HSCR (23.3%). Nine patients (7.5%) had Down syndrome. The average age at diagnosis was 15.0 months (median 3 months). Controls consisted of 195 healthy male and 47 healthy female volunteers whose ages ranged from 17 to 64 years. The frequency of long segment disease was found to be significantly higher in the female patients (40.9%) compared to the male patients (19.4%) (P=0.03).

3.2 Allele and genotype distribution

All genotyping data met quality control criteria. All SNPs conformed with HWE expectations: rs2435357 (P=0.10); rs139884 (P=0.20); and rs139883 (P=0.14). The minor allele frequencies of the 3 SNPs studied in the cases and controls are displayed in Table 2. Only the T allele of rs2435357 had a statistically significant association with HSCR.

When genotype distribution was analyzed in a recessive model, the TT genotype of rs2435357 and the CC genotype of rs139883 were significantly associated with HSCR at P<0.01 and P=0.03, respectively. Univariate logistic regression showed ORs of rs2435357(TT) and rs139883(CC) at 7.29 (95% CI 4.45−11.93) and 2.09 (95% CI 1.07−4.08), respectively. A subgroup analysis showed statistically significant associations in both sexes between the risk genotype of rs2435357(TT) and our HSCR cases, while the HSCR association of rs139883 (genotype CC) remained only in females (Table 3). When comparing genotype distribution between the aganglionosis length groups, the risk genotype CC of rs139883 was found at a significantly higher frequency in the long HSCR group (28.6%), compared to the short HSCR group (12.0%) (P=0.02). No significant correlation with aganglionosis length was found in the other two SNPs.

3.3 *RET-SOX10* interactions

The interactions between rs2435357 on *RET* and each *SOX10* SNP on HSCR association were tested by combining the SNPs into two pairs, giving nine combinations for each pair (Table 4 and 5). In the rs2435357/rs139883 pair, it was found that the T-containing genotypes of rs2435357 changed the OR of HSCR-rs139883CC association in the upward trend from 0.67 to 2.19 and 3.19 when the genotypes of rs2435357 were CC, CT, and TT, respectively. When combined with rs139884, the combination rs2435357-rs139884 TT-AG was associated with HSCR risk at an OR of 2.79 (95% CI 1.43−5.44).

Table 1. Primers and probes and genotyping methods used in this study.

SNP ID	Name	Sequence (5’ and 3’)	Alleles	Method
rs139883	rs139883_F	ACTGGGGGCTGTTTCTCAG	C/T	PCR-RFLP
	rs139883_R	AATGACCCTCTATCCCAGGAC		
rs139884	rs139884_F	CATAGCCGGCTGCTGAGTAG	A/G	TaqMan SNP genotyping
	rs139884_R	ACCTGCCGCCCAATGG		
	rs139884_VIC	VIC-CTGCTCACATGGCCTG-TAMRA		
	rs139884_FAM	FAM-TGCTCACGTGGCCTG-TAMRA		
rs2435357	rs2435357_F	AGCCCTGCAGCCAAGG	C/T	TaqMan SNP genotyping
	rs2435357_R	GGACTGGCCACCCAAGTG		
	rs2435357_VIC	VIC-TGTGGATGACCATGTAAG-TAMRA		
	rs2435357_FAM	FAM-TGTGGATGACCGTGTAAG-TAMRA		

Table 2. Results of association studies between each SNP and HSCR (Genotype distributions and allele frequencies).

SNP	Genotype	Case (N=120)	Percent (%)	MAF	Control (N=242)	Percent (%)	MAF	HWE P-value	P-value*
rs2435357	TT	75	62.50	0.25	67	27.69	0.45	0.10	<0.01
	CT	30	25.00		130	53.72			
	CC	15	12.50		45	18.60			
rs139883	TT	60	50	0.33	136	56.20	0.25	0.14	0.03
	CT	41	34.17		86	35.54			
	CC	19	15.83		20	8.26			
rs139884	GG	70	58.33	0.27	137	56.61	0.24	0.20	0.36
	AG	36	30.00		86	35.54			
	AA	14	11.67		19	7.85			

MAF=Minor Allele Frequency, HWE=Hardy-Weinberg equilibrium
*P-value for allele distribution

Table 3. Genotype distributions of rs2435357, rs139883, and rs139884 in Hirschsprung disease (HSCR) and controls according to sex.

Sex group/SNPs	HSCR	Controls	P-value
Male			
rs2435357			
TT	66 (62.86%)	39 (37.14%)	<0.01
CT/CC	32 (17.02%)	156 (82.98%)	
rs139883			
CC	14 (45.16%)	17 (54.84%)	0.14
CT/TT	84 (32.06%)	178 (67.94%)	
rs139884			
AA	12 (42.86%)	16 (57.14%)	0.26
AG/GG	86 (32.45%)	179 (67.55%)	
Female			
rs2435357			
TT	9 (60.00%)	6 (40.00%)	0.01
CT/CC	13 (24.07%)	41 (75.93%)	
rs139883			
CC	5 (62.50%)	3 (37.50%)	0.04
CT/TT	17 (27.87%)	44 (72.13%)	
rs139884			
AA	2 (40.00%)	3 (60.00%)	0.68
AG/GG	20 (31.25%)	44 (68.75%)	

4. Discussion

In this study, we evaluated the genetic associations between HSCR and the two genes that play important roles in the development of the enteric nervous system, *RET* and *SOX10*, using a case-control design. The study found significant HSCR associations in *RET* rs2435357 and *SOX10* rs139883 at ORs of 7.3 and 2.1, respectively. Although the association between *RET* rs2435357 and HSCR was previously validated in various populations, our study demonstrated, for the first time, that the CC variant in rs139883 within *SOX10* was a significant disease susceptibility variant of sporadic HSCR in the Thai population. In addition, disease associations between the variants in *SOX10* seemed to be modified when interacted with *RET* rs2435357 in the same pattern that we previously found in *NRG1* (Phusantisampan *et al.*, 2012), which suggested that *SOX10* might be a penetrance modifier of *RET*.

Genetic associations between polymorphisms in *SOX10* and human diseases were reported in early onset schizophrenia (Yuan *et al.*, 2012). In male Han Chinese, the CC genotype of rs139887, a SNP on intron 3 of *SOX10*, was found to have a significantly higher frequency in schizophrenic patients, and the genotype was also associated with earlier onset of the disease (Yuan *et al.*, 2012). The finding was reproducible in a Japanese study (Maeno *et al.*, 2007). Among male schizophrenic cases, minor allele carriers of rs139883 were diagnosed at a younger age (Yuan *et al.*, 2012). Since decreased expression of *SOX10* was demonstrated in brain tissue of schizophrenic patients (Iwamoto *et al.*, 2005; Wockner *et al.*, 2014), the evidence suggests that *SOX10* variants might influence neural tissue development through differential *SOX10* expression.

SOX10 has been shown to be one of the essential contributors in enteric nervous system development (Bondurand & Sham, 2013; Taylor, Montagne, Eisen, & Ganz, 2016). Mice with semidominant mutations of *SOX10* were demonstrated to have megacolon and aganglionosis (Lane & Liu, 1984). Missense mutations of *SOX10* in humans were found to be associated with intestinal aganglionosis, pigmentation defects, hearing loss, and demyelination of the nervous system compatible with a neurocristopathy syndrome called Waardenburg's syndrome (Inoue *et al.*, 2007; Pingault *et al.*, 2010). In addition, *SOX10* may have roles in the enteric nervous system-regulated gut inflammation (Rolig *et al.*, 2017). *SOX10* interacts with various transcription factors, especially *RET* during enteric nervous system development (Bondurand & Sham, 2013). In our study, the risk genotype of *RET* increased the OR of HSCR association in rs139883, which suggested that the two genes function together. The variant rs139883 is located on the regulatory element within 3'UTR of *SOX10*, which may regulate expression of the protein. Even though a relatively low case number was a significant limitation of our study, the subgroup analysis suggested that the association of *SOX10* and HSCR was stronger in females who tended to have long segment disease and hereditary influence. Although our study was limited to examining the HSCR group in sporadic cases, the association data suggested that an individual without a familial history of HSCR can be at a higher risk of having the disease when born with disease variants.

Table 4. Associations and combinations of *RET-protooncogene*, rs2435357 and *SOX10*, rs139883.

SNP-SNP interaction	Combination Genotype-Genotype		Case	Control	OR (95% CI)	P-value
rs2435357	CC	TT	8 (53.33%)	40 (59.70%)	Reference	
rs139883		CT	5 (33.33%)	21 (31.34%)	0.46 (0.17−1.24)	0.13
		CC	2 (13.33%)	6 (8.96%)	0.67 (0.13−3.35)	0.62
	CT	TT	11 (36.67%)	78 (60.00%)	Reference	
		CT	11 (36.67%)	44 (33.85%)	0.45 (0.23−0.91)	0.03
		CC	8 (26.67%)	8 (6.15%)	2.09 (0.76−5.71)	0.15
	TT	TT	41 (54.67%)	18 (40.00%)	Reference	
		CT	25 (33.33%)	21 (46.67%)	2.77 (1.48−5.20)	<0.01
		CC	9 (12.00%)	6 (13.33%)	3.19 (1.11−9.18)	0.03

CI=Confidence interval, OR=Odds ratio

Table 5. Associations and combinations of *RET-protooncogene*, rs2435357 and *SOX10*, rs139884.

SNP-SNP interaction	Combination Genotype-Genotype		Case	Control	OR (95% CI)	P-value
rs2435357	CC	GG	10 (66.67%)	38 (56.72%)	Reference	
rs139884		AG	3 (20.00%)	26 (38.80%)	0.21 (0.06−0.72)	0.01
		AA	2 (13.33%)	3 (4.48%)	1.35 (0.22−8.19)	0.74
	CT	GG	14 (46.67%)	77 (59.23%)	Reference	
		AG	11 (36.67%)	42 (32.31%)	0.48 (0.24−0.97)	0.04
		AA	5 (31.25%)	11 (8.46%)	0.91 (0.31−2.69)	0.87
	TT	GG	46 (61.33%)	22 (48.89%)	Reference	
		AG	22 (29.33%)	18 (40.00%)	2.79 (1.43−5.44)	<0.01
		AA	7 (9.33%)	5 (11.11%)	2.94 (0.91−9.45)	0.07

CI=Confidence interval, OR=Odds ratio

5. Conclusions

The study detected genetic association between rs139883 within SOX10 and HSCR. The association was stronger when an individual also harbored the TT allele of rs2435357 on the RET-protooncogene which suggested roles of both variants in disease development.

Acknowledgements

We thank Mr. Dave Patterson for assistance in proofreading and editing the English language of the manuscript.

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