

# Susceptibility of *PCSK2* Polymorphism to Hirschsprung Disease in Southern Chinese Children

Bingtong Wang<sup>1</sup>\*, Wenlin Fang\*, Dingjiang Qin\*, Qiuming He, Chaoting Lan

Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangdong Provincial Clinical Research Center for Child Health, Guangzhou, 510623, People's Republic of China

\*These authors contributed equally to this work

Correspondence: Chaoting Lan, Guangzhou Women and Children's Medical Center, Guangzhou Medical University, Guangdong Provincial Clinical Research Center for Child Health, Guangzhou, 510623, People's Republic of China, Email 415879043@qq.com

**Introduction:** Hirschsprung's disease (HSCR) is a developmental defect of the enteric nervous system (ENS), which is caused by abnormal development of enteric neural crest cells. Its occurrence is caused by genetic factors and environmental factors. It has been reported that single nucleotide polymorphisms (SNPs) of proprotein convertase subtilisin/kexin type 2 (*PCSK2*) gene are associated with HSCR. However, the correlation of HSCR in southern Chinese population is still unclear.

**Methods:** We assessed the association of rs16998727 with HSCR susceptibility in southern Chinese children using TaqMan SNP genotyping analysis of 2943 samples, including 1470 HSCR patients and 1473 controls. The association test between rs16998727 and phenotypes was performed using multivariable logistic regression analysis.

**Results:** We got an unexpected result, *PCSK2* SNP rs16998727 was not significantly different from HSCR and its HSCR subtypes: S-HSCR (OR = 1.08, 95% IC: 0.93~1.27,  $P_{adj}$  = 0.3208), L-HSCR (OR = 1.07, 95% IC: 0.84~1.36,  $P_{adj}$  = 0.5958) and TCA (OR = 0.94, 95% IC: 0.61~1.47,  $P_{adj}$  = 0.8001).

**Conclusion:** In summary, we report that rs16998727 (*PCSK2* and *OTOR*) is not associated with the risk of HSCR in southern Chinese population.

**Keywords:** Hirschsprung's disease, HSCR, single nucleotide polymorphism, SNP, proprotein convertase subtilisin/kexin type 2, *PCSK2*, genetic susceptibility

## Introduction

Hirschsprung's disease (HSCR) is a neonatal congenital disorder of the enteric nervous system (ENS) in which the myenteric and submucosal plexus of the distal intestinal wall are devoid of ganglion cells due to developmental impairment of the ENS during embryonic development.<sup>1</sup> The above pathological changes make intestinal peristalsis disorders and tonic contractions in children with HSCR, so that clinical manifestations such as delayed meconium, intractable constipation, abdominal distension and diarrhea happen.<sup>2,3</sup> The incidence of HSCR shows gender and racial differences, with a male-to-female ratio of 4/1, and an Asian population incidence of 1/5000.<sup>4</sup> There are three subtypes of HSCR, which can be divided into short-segment (S-HSCR), long-segment (L-HSCR), and total colonic aganglionosis (TCA) according to the length of ganglion lesions in the intestine.<sup>5</sup> In addition, HSCR can be divided into familial and sporadic, a few inherited in familial, most sporadic.

The occurrence of HSCR is a complex process. Its mechanism has not been fully elucidated, but studies have found that the occurrence of HSCR involves multiple signaling pathways and multiple genes. Many studies have shown that abnormal GDNF-GFR $\alpha$ 1-RET and EDN3-EDNRB signaling pathways are high-risk factors for HSCR, which play an important role in the migration, proliferation and differentiation of enteric neural crest cells during ENS development.<sup>6-8</sup>

Polymorphisms of *RET* can affect the binding of transcription factors (*NXF*, *ARNT2*, *SOX10*, *PHOX2B*, and *SIM2*) to *RET*, thereby affecting the expression of *RET* and leading to the occurrence and development of HSCR.<sup>9–11</sup> In addition, other mutant genes, like *NRG1* and *AUTS2* have been reported to be related to the occurrence of HSCR.<sup>12</sup> However, only a small fraction of HSCR cases can be explained by these identified variants, the heritability of a large proportion of cases remains to be determined.<sup>13</sup> Thus, we believe that the occurrence and development of HSCR may be the interaction of genetic factors and environmental factors. Next, it is necessary to identify unknown genes and other factors of HSCR.

Proprotein convertase subtilisin/kexin type 2 (*PCSK2*), which is mainly expressed in neurons and endocrine cells, belongs to the family of serine proteases that are responsible for the hydrolysis of precursor proteins to produce a variety of biologically active molecules such as hormones, neuropeptides, growth factors, cytokines and transcription factors.<sup>14–17</sup> The production of most biologically active molecules depends on proprotein convertase subtilisin/kexins (PCSKs), especially the proper processing of most neuropeptide and endocrine precursors by *PCSK2*.<sup>18</sup> It is worth noting that the regulation of gastrointestinal motility is controlled by a variety of biologically active peptides in central and intestinal neurons and peripheral endocrine cells.<sup>19</sup> Otoraplin (*OTOR*), recognized as a novel cochlear gene, has expression in the cochlear nerve. It has a predicted secretory signal peptide sequence and harbors a high degree of cross-species conservation. The rs16998727 SNP was validated in a study from the HSCR group (n = 181) and control group (n = 346) from the Hong Kong Chinese.<sup>20</sup> Considering that this result was not revalidated, we conducted a correlation study with a case group (n = 1470) and a control group (n = 1473) in a southern Chinese population to repeat the association of *PCSK2* polymorphisms with HSCR.

## Materials and Methods

### Study Subjects

The subjects of this study were from Guangzhou Women's and Children's Medical Center. This research project was approved by the hospital's Institutional Review Committee (Ethical Approval Number: 2018052406) and obtained written informed consent from each subject's guardian. The study included 1470 HSCR patients and 1473 controls, and specific clinical data can be found in previous studies, as shown in Table 1.<sup>21</sup> All cases were diagnosed as HSCR by postoperative biopsies after pull-through surgery showing lack of submucosal and intermuscular ganglia, and three subgroups were divided according to the length of enteric ganglionopathy, including S-HSCR (n = 1033), L-HSCR (n = 294), and TCA (n = 82). The control group had healthy populations without a history of nervous system diseases.

### SNP Selection and Genotyping

The selection criteria for rs16998727 (*PCSK2* and *OTOR*) were the same as described in our previous study.<sup>22</sup> Briefly, candidate SNPs to be validated are selected to satisfy minor allele frequency, Hardy-Weinberg equilibrium (HWE) and

**Table 1** Clinical Characteristics of the Study Subjects

Characteristic	Cases (n=1470)	Controls (1473)	P <sup>a</sup>
Gender			<0.001
Male (%)	1230(83.67%)	967(65.65%)	
Female (%)	240(16.33%)	506(34.35%)	
Age(month) <sup>b</sup> , mean±SD	8.37±20.50	18.62±19.75	<0.001
Clinical manifestation (%)			
S-HSCR	1034(70.34%)	N/A	-
L-HSCR	295(20.07%)	N/A	-
TCA	82(5.58%)	N/A	-
TIA	3(0.20%)	N/A	-
Unknown subtype	56(3.81%)	N/A	-

**Notes:** <sup>a</sup>Two-tailed  $\chi^2$  test of the distribution between HSCR cases and controls; <sup>b</sup>Age (month) of onset for HSCR cases (mean ± SD).

**Abbreviations:** SD, standard deviation; NA, no available; S-HSCR, short-segment HSCR; L-HSCR, long-segment HSCR; TCA, total colonic aganglionosis; TIA, total intestinal aganglionosis.

linkage disequilibrium criteria. TIANamp Blood Genomic DNA Kit (Tiagen Biotechnology Co., Ltd.) was used to extract genomic DNA from peripheral blood, then genotyping of *PCSK2* and *OTOR* polymorphisms was performed using the TaqMan real-time PCR blind method. Each sample was repeated three times. Moreover, we randomly selected 10% of individuals and obtained two DNA samples per person to evaluate the consistency of the genotyping.

## Statistical Analysis

We evaluated risk associations between *PCSK2* and *OTOR* polymorphisms with HSCR and HSCR subtypes by odds ratios (ORs) and 95% confidence intervals (CIs) of multivariable logistic regression analysis.  $P$  and  $P_{adj}$  denote the significance of the effect association without and with age and sex adjustment, respectively. Differences in age and gender between groups were compared using a two-tailed chi-square test. Hardy-Weinberg equilibrium test was used to assess genotyping quality, and  $P > 0.05$  was considered to indicate satisfactory goodness of fit. PLINK 1.9 software was used for statistical analysis and testing of additive, recessive and dominant genetic models.

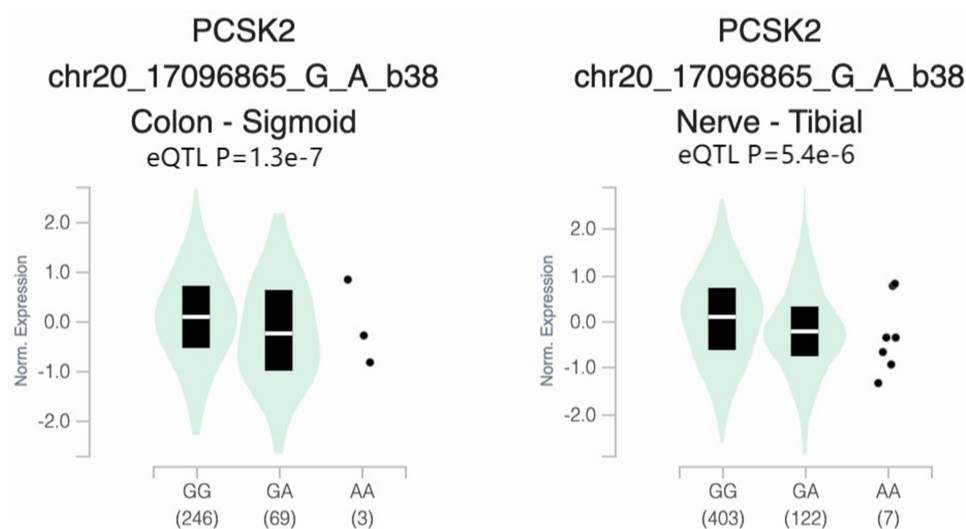
## Results

### eQTL Analysis

To explore the potential functions of SNPs, we used the Genotype Tissue Expression (GTEx) dataset to evaluate the association of the rs16998727 with *PCSK2* and *OTOR* gene expression. According to Figure 1, *PCSK2* rs16998727 was significantly different at the splicing quantitative trait locus (eQTLs) in colon tissue ( $P = 1.3e^{-7}$ ) and nerve ( $P = 5.4e^{-6}$ ), but no *OTOR* association was observed.

### Association of rs16998727 (*PCSK2* and *OTOR*) with HSCR Susceptibility

We calculated the association between the genotype frequency of rs16998727 and HSCR and are summarized in Table 2. The genotype of this SNP in the control group was in HWE ( $P > 0.05$ ). Additionally, logistic regression was performed on four different genetic models (allelic, genotypic, dominant and recessive) to predict the pattern of effects of rs16998727 *PCSK2* and *OTOR*. The results in Table 2 showed that the rs16998727 (*PCSK2* and *OTOR*) was not significantly associated with HSCR ( $P > 0.05$ ).



**Figure 1** Based on data from the GTEx Portal database (<https://www.gtexportal.org/home/>), boxplots show that the rs16998727 (chr20\_17096865\_G\_A\_b38) *PCSK2* genotype is associated with tibial nerve and sigmoid colon tissue expression.

**Table 2** Replication Results for rs16998727 A>G in a Southern Chinese Population of 1470 Cases and 1473 Controls

CHR	SNP	BP	A1/A2	Gene	Model	Patient	Control	OR (CI 0.95)	P	P_adj
20	rs16998727	17096865	A/G	OTOR, PCSK2	ALLELIC	474/2392	463/2457	1.09(0.94–1.25)	0.488	0.244
					GENO	50/374/1009	39/386/1038	–	0.441	0.584
					DOM	424/1009	424/1036	1.08(0.91–1.27)	0.747	0.381
					REC	50/1383	39/1421	1.32(0.86–2.04)	0.204	0.207

**Abbreviations:** CHR, chromosome; SNP, single-nucleotide polymorphism; BP, base pair where the SNP is located; A1/A2, minor allele/major allele; Gene, refgene, the gene where the SNP located; Model: ALLELIC, GENO, DOM, ADD, and REC corresponds to allelic association, genotypic association (2df), dominant, additive and recessive model, respectively; Freq, risk allele frequency of the SNP in cases or controls; the calculation of odds ratio (OR) is also based on the risk allele of each SNP; P, association test by logistic regression; P\_adj, P value adjusted by sex.

**Table 3** Stratification Analysis for the Association Between OTOR and PCSK2 rs16998727 A>G and Hirschsprung Disease Susceptibility (by Subtype)

CHR	SNP	BP	A1/A2	Gene	Aganglionic Status	Patient	Control	OR (CI 0.95)	P	P_adj
20	rs16998727	17096865	A/G	OTOR, PCSK2	SHSCR	327/1687	463/2457	1.08(0.93–1.27)	0.7239	0.3208
					LHSCR	95/483	463/2457	1.07(0.84–1.36)	0.7299	0.5958
					TCA	24/140	463/2457	0.94(0.61–1.47)	0.6782	0.8001

**Abbreviations:** CHR, chromosome; SNP, single-nucleotide polymorphism; BP, base pair where the SNP is located; A1/A2, minor allele/major allele; Gene, refgene, the gene where the SNP located; Freq, risk allele frequency of the SNP in cases or controls; the calculation of odds ratio (OR) is also based on the risk allele of each SNP; P, association test by logistic regression; P\_adj, P value adjusted by gender.

## Stratification Analysis

Considering that HSCR is divided into different subtypes, SNP may play an important role in HSCR subtypes. Therefore, we want to analyze further whether there is an association between rs16998727 (*PCSK2* and *OTOR*) and HSCR subtypes (S-HSCR, L-HSCR and TCA). The results in Table 3 show that there is no significant difference between rs16998727 and S-HSCR (OR = 1.08, 95%IC = 0.93–1.27,  $P_{adj}$  = 0.3208), L-HSCR (OR = 1.07, 95%IC = 0.84–1.36,  $P_{adj}$  = 0.5958) and TCA (OR = 0.94, 95%IC = 0.61–1.47,  $P_{adj}$  = 0.8001).

## Discussion

HSCR is a neonatal ENS disease with congenital loss of enteric ganglion cells. Currently, the only treatment option for HSCR is to resect the ganglionic bowel and reconstruct the normally innervated bowel.<sup>23</sup> However, postoperative complications such as enterocolitis and intractable constipation often occur in children with HSCR.<sup>24</sup> It is generally known that HSCR is related to genetic factors, but only a few mutant genes of HSCR have been found so far, and it is still necessary to continue to identify the unknown mutant genes of HSCR.

The rs16998727 SNP was found to be associated with HSCR in a study from Hong Kong Chinese.<sup>20</sup> This SNP has not been replicated, so we replicated in a cohort of HSCR (n = 1470) in southern China. The closest genes to this SNP site are *PCSK2* and *OTOR*. The above studies in Hong Kong population in China only show that *PCSK2* rs16998727 is associated with HSCR. Additionally, the GTEx database searched for the association of potential functions of rs16998727 (*PCSK2* and *OTOR*) genes and found that the eQTLs of *PCSK2* rs16998727 were significantly different in colon tissue and nerves. Therefore, we next focused on the relationship between *PCSK2* rs16998727 and HSCR.

PCSK enzymes are a family of nine related serine proteases, of which *PCSK2* is abundant in endocrine and neuronal cells. There is a report of low birth weight and mild hypoglycemia in *pcsk2*-KO mice, in which part of this phenotype may be due to altered gastrointestinal physiology (GI) peptide response systems, primarily by altered bioactive peptide molecules produced from *PCSK2* precursors in the gastrointestinal tract, delaying gastrointestinal motility.<sup>19</sup> In addition, a study on the genome-wide association screening of sporadic amyotrophic lateral sclerosis (ALS) among American veterans showed that *PCSK2* rs6080539 was significantly different from the occurrence of ALS.<sup>25</sup> Another study found that *PCSK2* was strongly positive in most pheochromocytomas and paraganglioma in midgut neuroendocrine tumors (NETs).<sup>17</sup> Studies have shown that bioactive peptide molecules catalyzed by *PCSK2* are closely related to gastrointestinal motility and neural development. The regulation of

intestinal peristalsis in HSCR is controlled by a variety of bioactive peptides in central and intestinal neurons and peripheral endocrine cells.<sup>19</sup>

The results of this study were surprising, because we were not able to reproduce the same results as the original study, no correlation was observed between *PCSK2* rs16998727 HSCR and in the southern population (Table 2). Besides, a variant may only have a slight impact on a specific type of HSCR. Therefore, we speculate that this may partially explain the ineffective effects of *PCSK2* rs16998727. Each specific type of HSCR may have a specific genetic background. For further verification, we conducted a stratified analysis of all HSCR subtypes. The results showed that *PCSK2* rs16998727 and all specific subtypes were not significant (Table 3). The above results are contrary to our hypothesis, the reason why the results were not replicated consistently may be due to the limitations of this study. Firstly, only one SNP was selected in this study. The influence of other *PCSK2* SNPs has not been explored, and the functional variants responsible for the gene effects remain undiscovered. Secondly, HSCR is a disease that combines genetic factors, environmental factors and other factors, so it is possible that the effect of *PCSK2* rs16998727 on HSCR is masked by other factors. Lastly, our sample size is relatively large, but our study population is limited to Han people in southern China. Further studies should focus on the relationship between genotypes-phenotypes in multiple regions and ethnicities. In conclusion, this study demonstrated no correlation between rs16998727 and HSCR.

## Ethics Statement

This study was approved by the Institutional Review Board of Guangzhou Women and Children's Medical Center (Ethical Approval Number: 2018052406). All procedures performed in this study involving human participants were by the Declaration of Helsinki (as revised in 2013).

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors state that the study is published without any conflict of interest.

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