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Association between *miR-492* rs2289030 G>C and susceptibility to Hirschsprung disease in southern Chinese children

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Abstract

Objective: Hirschsprung disease (HSCR) originates from disruption of normal neural crest cell migration, differentiation, and proliferation during the fifth to eighth weeks of gestation. This results in the absence of intestinal ganglion cells in the distal intestinal tract. However, genetic variations affecting embryonic development of intestinal ganglion cells are unclear. Therefore, this study aimed to investigated the potential value of *miR-492* rs2289030 G>C as a marker of susceptibility to HSCR

Methods: In this case–control study in southern Chinese children, we collected samples from 1473 controls and 1470 patients with HSCR. TaqMan genotyping of *miR-492* rs2289030 G>C was performed by real-time fluorescent quantitative polymerase chain reaction.

Results: Multivariate logistic regression analysis showed that there was no significant association between the presence of the *miR-492* rs2289030 G>C polymorphism and susceptibility to HSCR by evaluating the values of pooled odds ratios and 95% confidence intervals. Similarly, among different HSCR subtypes, rs2289030 G>C was also not associated with HSCR in hierarchical analysis.

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Conclusions: Our results suggest that the *miR-492* rs2289030 G>C polymorphism is not associated with susceptibility to HSCR in southern Chinese children. These results need to be further confirmed by investigating a more diverse ethnic population of patients with HSCR.

Keywords
miR-492, rs2289030, polymorphism, Hirschsprung disease, susceptibility, intestinal ganglion cells

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Introduction

Hirschsprung disease (HSCR) is a common congenital gastrointestinal disease that is affected by genetics. The absence of intestinal ganglion cells in the distal intestinal tract is a major risk factor and a pivotal pathological feature of HSCR. Gastrointestinal motor function is mainly regulated by the enteric nervous system (ENS), and the absence or abnormal function of the ENS can lead to gastrointestinal dysfunction. Because of congenital loss of ganglion cells in the intestine of the distal colon, anal canal, or rectum, this intestinal segment cannot produce normal intestinal peristalsis.[1–4] The clinical symptoms of these patients mainly include delayed meconium discharge, abdominal distension, bloody stool, fever, and biliary vomiting. At present, the main treatment for HSCR is to remove the intestinal tract or ostomy.[5,6] However, impaired bowel function with poor prognosis has a negative effect on patients' quality of life. For these reasons, identification of the main molecules involved in HSCR is urgent for improving the clinical outcome. A growing number of genes and genetic pathways that play a critical functional role in neural crest cell development have been confirmed to be related to the pathogenesis of HSCR.[2,7–9]. However, mutations in genes account for only approximately half of the known mutations found in patients with HSCR.[10,11]

Methylation and histone modification have critical roles in embryonic development and neurogenesis.[2,9] Particularly, DNA methyltransferase 3B and methyl CpG-binding protein 2 expression are downregulated in neural stem cells obtained from patients with HSCR, which results in lower global DNA methylation.[12,13] Moreover, the protein complex homeobox B5 can regulate *RET* by changing chromatin conformation.[14] Additionally, GDNF family receptor alpha-4 has been widely proposed as an HSCR susceptibility gene.[15] Although the interaction between genetic modification factors, including methylation and histone modification, may reflect the pathogenesis of HSCR, the role of epitranscriptomics in this pathogenesis remains to be further explored.

Epitranscriptomics refers to RNA modifications/functional alterations in the transcriptome that do not change the ribonucleotide sequence.[8,16] MicroRNAs (miRNAs) are a class of single-stranded, small molecule RNAs that are derived from noncoding regions of chromosomes. These miRNAs can act as either an oncogene or a tumor suppressor to regulate a variety of biological processes, such as development, inflammation, and tumorigenesis.[17,18] The miRNA *miR-492* is involved in regulating progression of

hepatic cancer by targeting phosphatase and tensin homolog.[19] Furthermore, *miR-492* may be regulated by the hepatoblastoma marker gene keratin 19 and play an important role in progression of malignant embryonic liver tumors.[20] Wu *et al.*[21] reported that *miR-492* was involved in regulating psoriasis by suppressing the basigin protein. Genetic variations of miRNA genes or polymorphisms have important significance and potential value for identifying high-risk individuals, diagnosing patients early, and using interventions in development and susceptibility of disease.[22–24] However, the polymorphisms of *miR-492* have not been investigated in case–control studies related to HSCR. A polymorphism of *miR-492* may affect migration of embryonic intestinal neural crest cells. Therefore, we aimed to investigate the association of the *miR-492* rs2289030 G>C single nucleotide polymorphism (SNP) and the risk of HSCR in southern Chinese children in a case–control study.

Materials and methods

Patients

Our study was prospectively conducted and planned as a case–control study. We included southern Chinese children with HSCR and controls who were recruited from the Guangzhou Women and Children's Medical Center from February 2010 to November 2015.[25–27] The controls were children who were matched geographically and ethnically with children with HSCR and had no history of HSCR or neurological disorders. According to the relevant regulations, this study was approved by the institutional review board of Guangzhou Women and Children's Medical Center (Ethical approval No. 201943800). All patients were diagnosed with HSCR via a histopathological examination and clinical imaging diagnosis. Clinical information was collected through medical records, and all subjects or their guardians signed informed consent forms.

Genotyping of the miR-492 *rs2289030 G>C SNP*

On the basis of our previous criteria,[28,29] in the present case-control study, we selected the potential rs2289030 G>C polymorphic locus of *miR-492*. Using a TIANamp Blood DNA Kit (DP348; TianGen Biotech Co., Ltd., Beijing, China) and TIANquick FFPE DNA Kit (DP330; TianGen Biotech Co., Ltd.), DNA was extracted from peripheral blood leukocytes of all participants and paraffin-embedded samples of the patients' intestines. DNA samples were diluted to 5 ng/mL in 96-well plates and stored at −20°C for later use. DNA samples were then added to TaqMan Genotyping PCR PreMix (probe) (FP211; TianGen Biotech Co., Ltd.) in 384-well plates. Real-time fluorescent quantitative polymerase chain reaction was used to detect genotyping of the *miR-492* rs2289030 G>C SNP.[30–32] At least 10% of samples were randomly selected for repeated genotyping and the same genotypic results were obtained.

Statistical analysis

Differences in demographic characteristics and distribution of genotype frequency between patients with HSCR and controls were assessed using the bilateral χ^2 test. The Hardy–Weinberg equilibrium was used to assess the genotype frequencies of the goodness-of-fit χ^2 test in the controls. Measurement data are presented as the mean ± standard deviation. Multivariate logistic regression was used to adjust for the effects of age and sex. The association between the *miR-492* SNP and susceptibility of HSCR was estimated by multivariate

logistic regression analysis using odds ratios (ORs) and 95% confidence intervals (CIs). Furthermore, stratified analyses were performed to calculate different subtypes of HSCR on the basis of the length of the aganglionic tract. All statistical analyses and tests were performed using SAS software (version 9.4; SAS Institute, Cary, NC, USA). Statistical significance was set at $P < 0.05$.

Results

Baseline demographics

We studied 1470 southern Chinese children with HSCR (mean age: 8.37 ± 20.50 months; 83.67% boys) and 1473 controls (mean age: 18.61 ± 19.75 months; 34.35% girls).

Association of the miR-492 *SNP with susceptibility of HSCR*

Using real-time fluorescent quantitative polymerase chain reaction, we successfully genotyped the *miR-492* rs2289030 G>C SNP in 1428 patients and 1463 controls. The distribution of genotype frequency of *miR-492* rs2289030 G>C was consistent with the Hardy–Weinberg equilibrium (rs2289030 G>C, $P=0.059$) in the controls. We found that the genotype results in patients with HSCR were not significantly different compared with those of the controls. Therefore, none of the rs2289030 genotypes were associated with susceptibility to HSCR (**Table 1**).

Stratification analysis of the risk between miR-492 *rs2289030 G>C and different HSCR subtypes*

Anatomically, HSCR was divided into three categories of short-segment HSCR, long-segment HSCR, and total colonic aganglionosis on the basis of differences in distal intestinal vascular malformations.[4,5,33] After adjusting for age and sex, we found that there were no significant associations between *miR-492* rs2289030 G>C and the three HSCR subtypes (Table 2). This finding suggests that the *miR-492* rs2289030 G>C SNP is not associated with susceptibility to HSCR.

Discussion

The complex subclinical manifestations of HSCR and the unclear pathogenesis of this disease limit the development of personalized diagnosis and treatment.[4,8] In our case–control study of southern Chinese children, we investigated the association between an *miR-492* SNP and susceptibility to HSCRs. To the best of our knowledge, rs2289030 G>C has not been examined in any previous studies on HSCR. Our study showed that *miR-492* rs2289030 G>C was not significantly associated with a risk of HSCR in southern Chinese children.

With advancement in research and technology, an increasing number of scholars believe that genetic factors play an important role in the development of HSCR. The genetic susceptibility of different individuals greatly varies. The major gene for HSCR encodes the receptor tyrosine kinase RET. The T variant of *RET* (*RET* rs2435357 C>T) can increase penetrance in patients with HSCR and RET coding mutations.[34] *RET* mutations affect the binding of RET to its ligands GDNF, neurturin, artemin, and persephin, leading to migration and differentiation disorders of neural crest stem cells.[10,35] Many studies have investigated the potential functional genetic variation sites and their genetic susceptibilities to HSCR.[1,4,5,8,36,37] Passarge and Lyonnet *et al*. suggested that a dominant gene for HSCR maps to 10q11.2, which encodes the *RET* receptor.[38–40] Additionally, semaphorin 3 and neuregulin

Table 1. Association between the *miR-492* rs2289030 G>C polymorphism and susceptibility of Hirschsprung disease.

Genotype	Patients (n = 1428)	Controls (n = 1463)	P^a	Crude OR (95% CI)	P	Adjusted OR (95% CI)[b]	P^b
rs2289030 G>C (HWE = 0.059)							
GG	784 (54.90)	828 (56.60)		1.00		1.00	
GC	545 (38.17)	527 (36.02)		1.09 (0.94–1.28)	0.263	1.09 (0.93–1.28)	0.286
GG	99 (6.93)	108 (7.38)		0.97 (0.73–1.29)	0.827	0.94 (0.70–1.28)	0.707
Additive			0.594	1.03 (0.92–1.16)	0.594	1.03 (0.91–1.16)	0.691
Dominant	644 (45.10)	635 (43.40)	0.359	1.07 (0.93–1.24)	0.359	1.07 (0.92–1.24)	0.411
Recessive	1329 (93.07)	1355 (92.62)	0.639	0.94 (0.70–1.24)	0.640	0.91 (0.68–1.22)	0.537

OR, odds ratio; CI, confidence interval; HWE, Hardy–Weinberg equilibrium.
[a]χ^2 test for the distribution of genotype between patients with Hirschsprung disease and controls.
[b]Adjusted for age and sex.

Table 2. Stratification analysis of the association between *miR-492* rs2289030 G>C and susceptibility of Hirschsprung disease by subtype.

Variable	rs2289030 (patients/controls)		Crude OR (95% CI)	P	Adjusted OR[a] (95% CI)	P^a
	GG	GC/CC				
SHCSR	554/828	453/635	1.07 (0.91–1.25)	0.436	1.07 (0.91–1.27)	0.427
LHCSR	157/828	127/635	1.06 (0.82–1.36)	0.683	1.03 (0.79–1.35)	0.813
TCA	45/828	33/635	0.96 (0.60–1.52)	0.849	0.94 (0.58–1.51)	0.790

OR, odds ratio; CI, confidence interval; SHCSR, short-segment Hirschsprung disease; LHCSR, long-segment Hirschsprung disease; TCA, total colonic aganglionosis.
[a]Adjusted for age and sex by omitting the corresponding stratification factor.

1 (*NRG1*) were also discovered in Europeans and Asians by genome-wide association study analysis.[41,42] These genes can affect migration, differentiation and viability of neuronal cells by binding to specific receptors. Subsequently, other researchers reported the existence of a cumulative genetic risk for HSCR susceptibility from *RET* (rs2506030 or rs2435357) and *NRG1* (rs2439302) polymorphisms in the Chinese population.[25,34,43] Furthermore, vesicle-associated membrane protein 5 rs1254900 and mutated in colorectal cancer rs11241200 contributed to the risk of HSCR by logistic regression and multifactor dimensionality reduction analyses in southern Chinese children and the Korean population.[27,44] These studies mentioned above indicate that SNPs may affect expression and function of target genes and may contribute to susceptibility of HSCR. Nonetheless, additional studies are required to improve the knowledge about the role of epigenetics in the pathogenesis of HSCR. Discovering SNPs and genetic mutations and determining their biological functions will help to understand the etiology of this disease.

A growing amount of research has shown that noncoding RNAs are potential markers of disease risk and prognosis in various diseases.[2,8,36,37,45–47] A decrease in *miR-141* can target downstream genes, increasing the expression of integrin-associated protein and cullin 3, which play important roles in inhibiting cell migration

and proliferation during the pathogenesis of HSCR.[48] The miRNA *miRNA-206* has also been shown to be downregulated and targets fibronectin 1 (FN1), serum deprivation response, and paired box 3 in Chinese patients with HSCR.[49] In contrast, Gunadi *et al*.[50] reported an increase in *miRNA-206* levels in Indonesian patients with HSCR. These authors showed that fibronectin 1 gene expression was significantly increased in Indonesian HSCR. Moreover, paired box 3 gene expression was not different between Indonesian patients with HSCR and the control group. Thus, the conflicting *miRNA-206* expression pattern in patients with HSCR and controls is mainly attributed to different detection levels and selection bias, with a genetically distinct group within Asia.

Nonetheless, whether *miR-492* is involved in the pathogenesis of HSCR has yet to be determined. *miR-492* affects proliferation, migration and invasion of hepatoblastoma by regulating cluster of differentiation 44 (CD44).[51] Furthermore, *miR-492* plays a role in promoting tumor cell growth by regulating suppressor of cytokine signaling 2, SRY-related high mobility group-box transcription factor 7, CD147, and p21-activated kinase 7 (PAK7).[52–55] Intriguingly, PAK7, which belongs to the p21-activated kinase family, is a highly conserved family of serine/threonine protein kinases that are regulated by Ras-related small G-proteins, such as Cdc42 and Rac1.[56] Ding *et al*. observed that PAK activation inhibited expression of endogenous nischarin and promoted growth of rat cortical neurites.[57] Moreover, PAK7 regulates the differentiation of oligodendrocyte precursor cells, which are myelin-forming cells of the central nervous system.[58] Collectively, these reports raised the question of whether *miR-492* can directly interact with phosphorylated PAK7 and regulate migration or differentiation of neural crest cells in the pathogenesis of HSCR.

Yu *et al*. reported[59] that patients carrying the *miR-492* rs2289030 C>G genotype had a significantly reduced risk of hepatocellular carcinoma. The miRNAs are short (20–24 nt) noncoding RNAs that are involved in posttranscriptional regulation of gene expression in multicellular organisms by affecting the stability and translation of mRNA. Because of the location of rs2289030 G>C in the transcription factor binding sites of *miR-492*, its functional regulatory role might be important. To determine whether this marker is a putative genetic factor that also confers susceptibility to HSCR, we analyzed the relationship between *miR-492* rs2289030 G>C and susceptibility of HSCR in southern Chinese children. However, we did not find an association between *miR-492* rs2289030 G>C and susceptibility of HSCR. Nevertheless, we cannot exclude the implication of other polymorphisms within *miR-492* in pathogenesis of HSCR. There is still a lack of research on *miR-492* and susceptibility mechanisms of HSCR, which has potential implications for HSCR therapy. Additionally, there are some differences in the etiology of this disease and genetic susceptibility among different ethnicities. Because all of the recruited subjects in this study were Han Chinese living in southern China, some of our results are not completely consistent with previous studies in other ethnic populations, such as Caucasians or Koreans.[59–61] This inconsistency between studies may be attributed to genetic variations and living environments between different ethnic groups.[62] Because of the possible differences in allele frequencies and SNP linkage imbalance patterns among different populations, only the rs2289030 C>G SNP from the *miR-492* gene was selected for association analysis in the current study. More gene polymorphisms should be further investigated in the

future. The incidence of neonatal HSCR is approximately 1/5000. In this study, we did not perform a sample size calculation, and the limited number of samples may have affected the significance of our results.

In summary, the *miR-492* rs2289030 G>C SNP is not associated with the risk of HSCR in southern Chinese children. The role of other SNPs in the pathogenesis of HSCR needs to be further examined. Future research should target studying genetic variants identified in HSCR to identify penetrance and the clinical effect. This may ultimately lead to better screening programs and prevention of HSCR.

Authors' contributions

Wei Zhong conceived and designed the study. Yi Zheng, Mi Wang, Yanqing Liu, Qiuming He, Xiaoli Xie, Lifeng Lu, and Wei Zhong collected samples and data. Yanqing Liu and Mi Wang provided technical support. Yi Zheng, Mi Wang, and Lifeng Lu collected and processed the data, and conducted experiments. Yi Zheng and Xiaoli Xie analyzed the data. Xiaoli Xie prepared all of the tables. Yi Zheng, Yanqing Liu, Mi Wang, and Wei Zhong wrote the manuscript. All authors approved the final manuscript.

Declaration of conflicting interest

The authors declare that there is no conflict of interest.

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