Original Article

Genetic association of *GJA8* with long-segment Hirschsprung's disease in southern Chinese children

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Background: Hirschsprung's disease (HSCR) is a complex congenital neurodevelopmental disorder affecting colons caused by both genetic and environmental factors. Although several genes have been identified as contributing factors in HSCR, the pathogenesis is still largely unclear, especially for the low prevalent long-segment HSCR (L-HSCR). Gap junction protein alpha 8 (*GJA8*) is involved in several physiological processes and has been implicated in several diseases. However, the relationship between *GJA8* single nucleotide polymorphism (SNP) rs17160783 and HSCR in the southern Chinese population remains unknown. The study aimed to explore the association of genetic variants in *GJA8* and HSCR susceptibility in southern Chinese.

Methods: SNP rs17160783 A>G in *GJA8* was genotyped by TaqMan SNP Genotyping Assay in all samples, which included 1,329 HSCR children (cases) and 1,473 healthy children (controls). Odds ratio (OR) and 95% confidence interval (CI) were used to evaluate the association of *GJA8* polymorphisms with HSCR susceptibility. The GTEx database and transcription factor binding site (TFBS) prediction were used to analyze the potential regulatory function of rs17160783.

Results: Genetic association analysis illustrated that rs17160783 could increase the risk of L-HSCR (P_{adj}=0.04, OR_{adj} =1.48, 95% CI: 1.02–2.14). We also found that *GJA8* expression was increased in HSCR and neurodevelopmentally impaired animal models. External epigenetic data revealed that *GJA8* rs17160783 may have the potential to regulate the expression of the *GJA8*, possibly by altering the binding of transcription factors for *GJA8*, and consequently impacting the PI3K-Akt signaling pathway during the enteric nervous system (ENS) development.

Conclusions: Our results suggested that rs17160783 might play a regulatory role in *GJA8* expression and increase the susceptibility of L-HSCR in children from southern China.

Keywords: Hirschsprung's disease (HSCR); gap junction protein alpha 8 (GJA8); single nucleotide polymorphism (SNP); genetics

Submitted Apr 22, 2024. Accepted for publication Aug 12, 2024. Published online Aug 28, 2024.
doi: 10.21037/tp-24-153
View this article at: https://dx.doi.org/10.21037/tp-24-153

Introduction

Hirschsprung's disease (HSCR) is a common congenital malformation of the digestive tract. Abnormal development of the enteric nervous system (ENS) is known to be involved in its pathogenesis. The formation of the normal ENS requires the completion of migration, proliferation and differentiation of enteric neural crest cells (ENCCs) in the intestine, culminating in mature neurons and glial cells (1). Overall, the global prevalence of HSCR is approximately 1/5,000, whereas the incidence of HSCR in Asia is 1.4/5,000 (2). The male-to-female ratio in HSCR is 4:1 (3). According to the extent of pathological changes in the intestine, HSCR can be divided into several clinical classifications including short-segment HSCR (S-HSCR), long-segment HSCR (L-HSCR), total colonic aganglionosis (TCA) and total intestine aganglionosis (TIA) (4).

L-HSCR is characterized by aganglionosis affecting a longer segment extending to the transverse colon. The prevalence of L-HSCR in patients with HSCR is approximately 10%, but compared with S-HSCR, patients with L-HSCR have different clinical and histological presentations (5,6). Due to its atypical clinical manifestations, L-HSCR is often misdiagnosed and more often diagnosed by surgical operation (6). Consequently, L-HSCR is often linked to severe complications, and the prognosis, even with surgical treatment, remains poor (7-9). Previous research has found different symptoms among various subtypes of HSCR, suggesting that they may have different pathogenic mechanisms (10). However, the mechanism underlying L-HSCR is still largely unknown.

Current studies suggest that the abnormal proliferation, migration, and differentiation of the ENCCs during the development of the ENS lead to the absence of neurons in the colon (11). Genetic variations in genes such as *RET* (2,12), *GDNF* (13), *PHOX2B* (14), *EDN3* (15,16), *EDNRB* (17,18), *SOX10* (19) have been proven to be associated with HSCR. However, this is far from enough to represent the genetic background of all HSCR subtypes, and more research is still needed to discover other genetic markers.

Gap junction protein alpha 8 (*GJA8*), which encodes one of the transmembrane junction proteins (also known as connexins), is recognized for its pivotal role in the ocular lens, particularly in maintaining lens transparency and development (20,21). It is noteworthy that mutations in the *GJA8* gene have been associated with a spectrum of ocular pathologies, including congenital cataracts (22-25). Recent research indicates that the effects of *GJA8* mutations are not limited to the eyes and may be associated with certain pathological conditions in the nervous system. Connexin 50 encoded by *GJA8* is an adhesion molecule (26), is believed to play a role in communication between nerve cells (27), potentially affecting how neural signals are transmitted and how cellular activities are coordinated within the nervous system. *GJA8* is also thought to be involved in regulating oxidative stress and cell death, which could contribute to processes that protect the nervous system (23). Moreover, studies reveal that connexins play crucial roles in the growth, migration, and apoptosis of nerve cells (28,29). Studies have shown that overexpression of *GJA8* inhibits cell migration and increases expression of the adhesion molecule N-cadherin (30-32). Overexpression of *GJA8* increases expression of the astrocyte marker GFAP and decreases expression of the neuronal marker Tuj1, suggesting that *GJA8* impairs neuronal differentiation (33).

Therefore, it is hypothesized that the *GJA8* may play a role in the development of the ENS. Given that the single nucleotide polymorphism (SNP) rs17160783 is located at a regulatory genomic region of *GJA8*, we conducted a study to explore the genetic association between *GJA8* and HSCR in children from southern China. We present this article in accordance with the MDAR reporting checklist (available at https://tp.amegroups.com/article/view/10.21037/tp-24-153/rc).

Highlight box

Key findings

- Gap junction protein alpha 8 (*GJA8*) rs17160783 polymorphism is associated with increased long-segment Hirschsprung's disease (L-HSCR) risk in the southern Chinese population.
- Single nucleotide polymorphism rs17160783 may have the potential to regulate the expression of the *GJA8*, possibly by altering the binding of transcription factors for *GJA8*, and consequently impacting the PI3K-Akt signaling pathway during the enteric nervous system development.

What is known and what is new?

- Hirschsprung's disease (HSCR) is a congenital disorder of the colon in children and causes significant morbidity and even death without proper treatment.
- We found that *GJA8* rs17160783 might increase the susceptibility of L-HSCR in the southern Chinese population.

What is the implication, and what should change now?

- Surgery is the main therapy for HSCR; however, the outcomes are often unsatisfactory. Our findings provide a new candidate gene for the exploration for the underlying pathology, which could be a potential target for the purpose of disease prevention and therapy.

Table 1 Clinical characteristics of the subjects

Characteristics	HSCR (n=1,329)	Controls (n=1,473)	P
Gender (female/male)	217/1,112	458/1,015	<0.001†
Age (months), mean ± SD	8.37±20.50	18.61±19.75	<0.001‡
Ethics	Chinese Han	Chinese Han	
Clinical characteristics			
S-HSCR	1,034	N/A	
L-HSCR	295	N/A	

†, χ^2-test; ‡, Mann Whitney *U* test. SD, standard deviation; S-HSCR, short-segment Hirschsprung's disease; L-HSCR, long-segment Hirschsprung's disease; N/A, not available.

Methods

Study subjects

All participants' data and samples in this study were obtained from Guangzhou Women and Children's Medical Center. This study was approved by the ethics committee of Guangzhou Women and Children's Medical Center (No. 2018052406). Informed consent was obtained from the participants' legal guardians. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). In the case group, patients were diagnosed with HSCR by histological examination of colon biopsies after surgical resection and were classified into S-HSCR and L-HSCR according to the length of the aganglionosis colon by pathologists. There were 1,473 individuals without gastrointestinal diseases or other systemic diseases enrolled in the control group from the physical examination center. Demographic information, including age and gender, was obtained from the medical record system for both HSCR cases and controls (*Table 1*).

Gene function enrichment analysis

A microarray dataset of containing four pairs of *Hmx1*-KO and wild-type mice were downloaded from the Gene Expression Omnibus (GEO) database (GSE47002). The *Hmx1*-KO mouse model is a valuable tool in neuroscience research, with applications including the study of neural development, particularly in sensory organs and the central nervous system, as *Hmx1* is a homeobox gene that plays a crucial role in the development of the nervous system (34).

The Gene Expression Omnibus 2R (GEO2R) was used to identify differentially expressed genes between the two groups using default parameters. The genes highly co-expressed with *GJA8* were identified (r>0.9) and gene functional enrichment analysis was performed on these genes.

Transcription factor binding sites prediction

HumanTFDB (http://bioinfo.life.hust.edu.cn/HumanTFDB/) was used to check whether polymorphism would destroy the putative transcription factor motif. We included the highly linkage disequilibrium (LD)-linked SNPs (r^2>0.8) to investigate the regulatory elements from the GTEx database (http://www.gtexportal.org/).

TaqMan genotyping

Genomic DNA was extracted from participants' peripheral blood using a Blood DNA Kit (TIANGEN, Cat No. DP348-03). PCR was performed in a 5-μL reaction, and thermal cycling conditions were set according to the manufacturer's protocol. Our primary investigation involved searching the dbSNP database and utilizing SNPinfo software to identify SNPs within the *GJA8* gene that may have functional implications. Here, the minor allele frequency (MAF) of the selected polymorphism among Chinese Han individuals exceeds 5%. Finally, we selected SNP rs17160783 and it was genotyped by the TaqMan SNP Genotyping Assay (Applied Biosystem, Cat No. 4351379) on all samples, which included 1,329 HSCR children (cases) and 1,473 healthy children (controls). We analyzed repeated genotyping by randomly selecting 10% of samples from

cases and controls. 100% consistency was achieved.

Cell culture

We used a method for reprogramming peripheral blood mononuclear cells (PBMCs) into human induced pluripotent stem cells (hiPSCs). PBMCs were obtained from healthy donors and transduced with a non-integrating Sendai virus vector carrying the reprogramming factors OCT4, SOX2, KLF4 and c-MYC, as previously described (35). The infected PBMCs were then cultured in StemPro-34 medium supplemented with the necessary growth factors to support the reprogramming process. Colonies with embryonic stem cell-like morphology were identified and selected for further expansion and characterization.

Neural crest differentiation of hiPSCs

When hiPSCs reached ~80% confluence, they were prepared to be dissociated into single cells using Accutase® and then seeded onto Matrigel-coated plates. To initiate induction (day 1), cells were incubated with day 1 medium for 24 hours and then cultured in N2 medium for 6 days (7 days in total) as previously described (36).

RNA extraction and real-time quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted using TRIzol reagent (Invitrogen™, Cat. No. 15596018) according to the standard protocol. Following the manufacturer's instructions, 1,000 ng of RNA was transcribed into complementary DNA using the HiScript II One Step qRT-PCR Probe Kit (Vazyme, Cat No. Q222-01). Target primers were amplified using ChamQTM Universal SYBR® qPCR Master Mix (Vazyme, Cat. No. q712).

Statistical analysis

In this study, the Chi-squared test was used to carry out the Hardy-Weinberg equilibrium (HWE) in the control group to understand whether there was heterogeneity in the sample. To verify the association between SNP and HSCR, MAF was compared between the case group and the control group under additive, dominant and recessive models, respectively. All this statistic calculation was explained by logistic regression with PLINK 1.9 software. The odds ratio (OR) and 95% confidence intervals (CIs) indicated the risk of allele for the disease. When the P value was less than 0.05, the results were considered statistically significant.

Results

GJA8 may be involved in ENS development

Studies have shown that *GJA8* is expressed in ependymal stem progenitor cells and several neurons, such as several groups of neurons in the cerebellum and related areas at the midbrain-hindbrain boundary, and in neurons in the ganglion cell layers (33,37). It was reported when *GJA8* is overexpressed, *GFAP* expression is increased, while *TUJ1* expression is decreased, suggesting that *GJA8* may facilitate the differentiation of neural precursor cells into glial cells and impair neuronal differentiation (33). Additionally, overexpression of *GJA8* stimulated autophagy (38). In neurodevelopmentally impaired animal models (39), we found that *GJA8* expression was increased (*Figure 1A*). We further identified the genes highly co-expressed with *GJA8* expression (r>0.9) and performed Gene Ontology (GO) enrichment analysis. We found that the gene set was significantly enriched in GO/KEGG terms, including PI3K-Akt signaling pathway, neurogenesis, generation of neurons, and neuronal differentiation (*Figure 1B*). The PI3K-Akt signaling pathway has been reported as a key signaling pathway for regulating cell survival, autophagy, neurogenesis, neuron proliferation, and differentiation (40,41). In HSCR, the PI3K/AKT pathway is thought to be involved in the migration, proliferation, and differentiation of ENCCs, which are essential for the formation of the ENS (42). Moreover, studies have shown that *RET* can affect the proliferation and survival, apoptosis, migration, and differentiation of ENCCs by activating the PI3K-Akt pathway (43,44). Hence, we hypothesized that *GJA8* may play a role in the ENS development by affecting the PI3K-Akt pathway. To test our hypothesis, we first collected both the ganglionic and aganglionic colons from the patients to check the expression of *GJA8* in HSCR colons. Compared to normal colons, *GJA8* expression was increased and the PI3K-Akt pathway was activated in aganglionic colons (*Figure 1C*). Using hiPSCs-derived ENCCs, we also found that the expression of *GJA8* was increased in HSCR patients-derived ENCCs (*Figure 1D*). Taken together, these findings suggested that the *GJA8* may be involved in the ENS development.

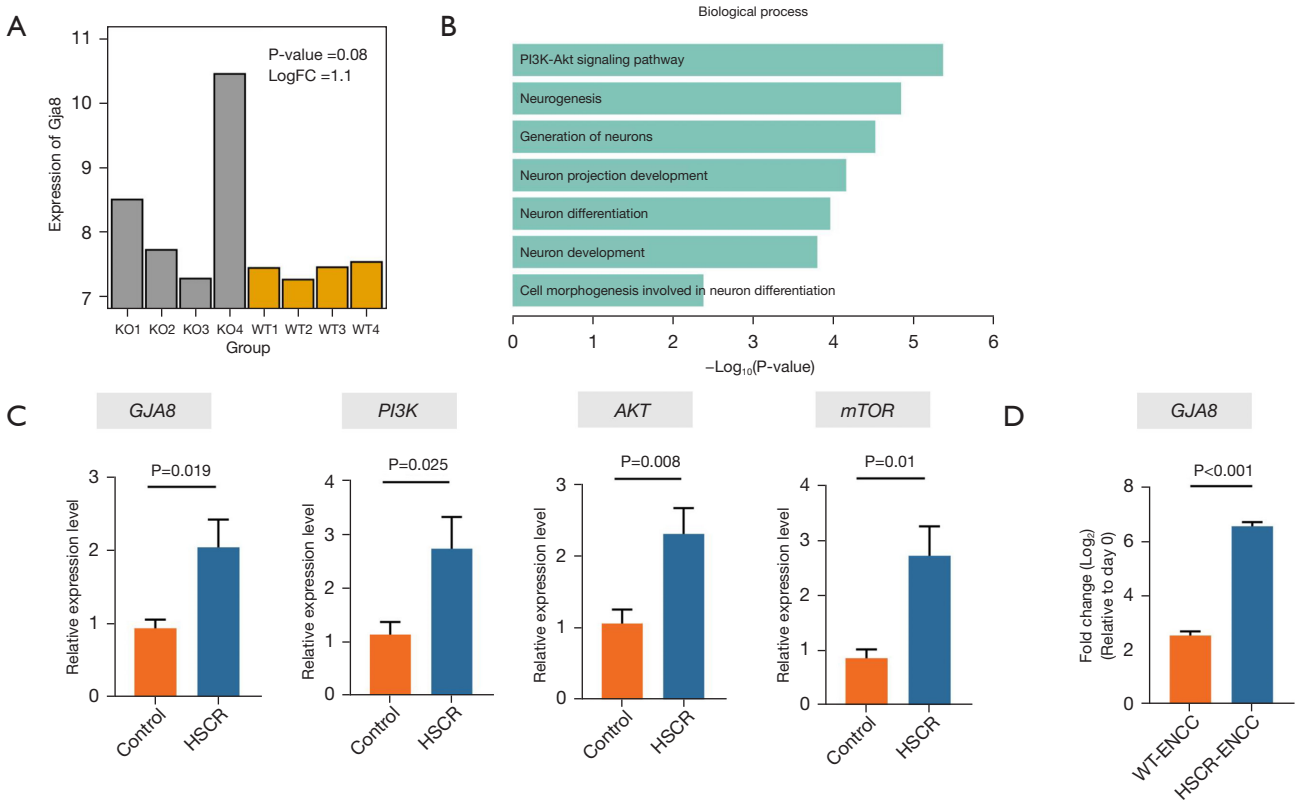

Figure 1 *GJA8* may be involved in ENS development. (A) *GJA8* expression was increased in neurodevelopmentally impaired animal models. (B) Functional enrichment of genes co-expressed with *GJA8* associated with neural development. (C) Expression of *GJA8* and the PI3K-Akt signaling pathway in HSCR patients and controls, respectively. n=10/group. (D) Expression of *GJA8* in ENCCs induced from control and HSCR patient-derived hiPSCs, respectively (n=3 biological replicates). FC, fold change; KO, *Hmx1*-KO mice; WT, wild-type mice; HSCR, Hirschsprung's disease; *GJA8*, gap junction protein alpha 8; ENS, enteric nervous system; ENCCs, enteric neural crest cells; hiPSCs, human induced pluripotent stem cells.

Association of GJA8 rs17160783 with L-HSCR susceptibility

We used 1,329 HSCR cases and 1,473 controls to assess the association between *GJA8* rs17160783 and HSCR. The clinical information of the participants was summarized in *Table 1*. The genotype frequency of rs17160783 and the association analysis between rs17160783 and HSCR were calculated, as shown in *Table 2* and Table S1. As shown, a total of 1,292 cases and 1,458 controls were successfully genotyped. HWE test showed no obvious heterogeneity for rs17160783 (P>0.05). The frequency of rs17160783-G was close to the frequency in East Asians from gnomAD (gnomad.broadinstitute.org). Subsequently, logistic regression tests were conducted using the additive, dominant, and recessive models respectively. We found that rs117160783-G was significantly associated with an increased L-HSCR risk in the dominant model (P_{adj}=0.04, OR_{adj} =1.48, 95% CI: 1.02–2.14, *Table 2*). Evidence of association between rs17160783 and S-HSCR is insufficient (P_{adj}=0.54, OR =1.08, 95% CI: 0.86–1.36, Table S1).

Regulatory potential of rs17160783

CCCTC binding factor (CTCF), a binding protein that influences the regulation of transcription, has been shown to play a key role in neural development (45), mainly by regulating chromatin structure and gene expression (46,47). Studies have shown that CTCF maintains the survival of neural progenitor cells, contributing to a delicate balance between neural progenitor cell proliferation and differentiation (48). Moreover, loss of the CTCF induces neuronal death (49). We found that rs17160783 had high

Table 2 Association between SNP rs17160783 and L-HSCR

SNP	A1/A2	MAF (gnomAD)	AFF	UNAFF	Model	OR (95% CI)	P	OR.adj	P.adj
rs17160783	G/A	0.07 (0.08)	1/51/234	7/189/1,262	DOM	1.43 (1.02–2.00)	0.04	1.48 (1.02–2.14)	0.04
					REC	0.73 (0.09–5.92)	0.77	0.60 (0.07–5.24)	0.64
					ADD	1.37 (0.99–1.88)	0.055	1.40 (0.99–1.98)	0.059

SNP, single nucleotide polymorphism; L-HSCR, long-segment Hirschsprung's disease; A1/A2, risk/non-risk allele; MAF, minor allele frequency in the control group; gnomAD, minor allele frequency in gnomAD East Asians; AFF, allele frequency in case group; UNAFF, allele frequency in the control group; DOM, REC, ADD, the association test following dominant, recessive and additive models; OR, odds ratio; CI, confidence interval; OR.adj, odds ratio after adjusting the genders of all the samples; P.adj, P values adjusted for gender and age by logistic regression.

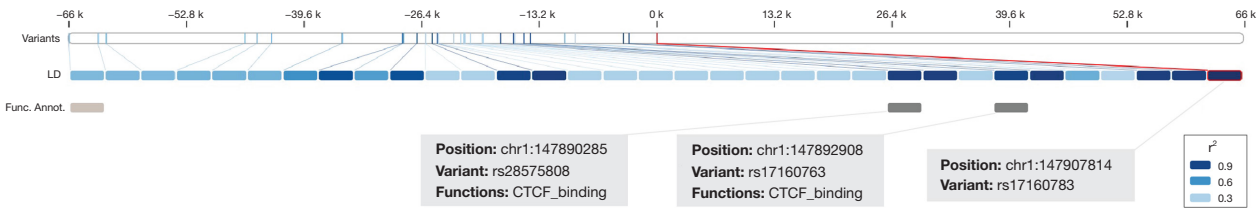

Figure 2 rs17160783 had a high linkage disequilibrium (r^2=0.91) with two SNPs (rs17160763 and rs28575808), both of which are located at CTCF binding sites. LD, linkage disequilibrium; SNP, single nucleotide polymorphism; CTCF, CCCTC binding factor.

Table 3 Prediction of transcription factor binding sites

SNP	Allele	TF	Start	Stop	Strand	Score	P value	Q value	Matched sequence
rs17160783	G (risk)	*SPIB*	12	27	–	12.1579	3.28E–05	0.00236	ACCCAGTGCTTCCTTT
	A (protective)	*SPIB*	12	27	–	11.2105	6.05E–05	0.00435	ATCCAGTGCTTCCTTT
rs17160763	A (risk)	*STAT5A*	23	30	–	8.34211	3.58E–05	0.00315	CACTTCTA
		PPARG	25	44	+	7.24444	6.35E–05	0.00406	GAAGTGCAGCATGACTCCAA
		RARA	26	41	–	9.82895	8.34E–05	0.00601	GAGTCATGCTGCACTT

'+' indicates the positive DNA strand, while the '–' indicates the negative DNA strand. SNP, single nucleotide polymorphism; TF, transcription factor; SPIB, Spi-B Transcription Factor; STAT5A, Signal Transducer And Activator Of Transcription; PPARG, Peroxisome Proliferator-Activated Receptor Gamma; RARA, Retinoic Acid Receptor Alpha.

LD (r^2=0.91) with two SNPs (rs17160763 and rs28575808), both of which are located at CTCF binding sites (*Figure 2*), indicating that they may regulate the transcription factor binding in this region. According to the prediction results from HumanTFDB (http://bioinfo.life.hust.edu.cn/HumanTFDB/), we found the rs17160783-G could change the binding scores of *SPIB*, while rs17160763-A could increase the binding of *STAT5A* (P=3.58e–5), *PPARG* (P=6.35e–5) and *RARA* (P=8.34e–5) (*Table 3*). Studies have shown that *SPIB* and *PPARG* mediate apoptosis through the PI3K-Akt signaling pathway (50,51). Moreover, *SPIB* (52,53), *STAT5A* (53), *PPARG* (54) and *RARA* (55) are related to cell differentiation respectively.

Discussion

As a condition characterized by a lack of intestinal ganglionic cells, children with HSCR are prone to several symptoms, including chronic constipation, abdominal distention, diarrhea and vomiting, which can be life-threatening in severe cases (56,57). The current treatment is surgical management, but serious complications such as enterocolitis may occur after surgery (58). However, the understanding of the pathogenesis of HSCR remains

considerably limited.

In this study, we mainly focused on the HSCR susceptibility of the *GJA8* gene. As previously described, *GJA8* encodes a gap junction protein that functions in intercellular communication and affects the exchange of ions and small molecules between different cells. Recent studies have shed light on the potential mechanisms by which *GJA8* mutations contribute to congenital cataracts, these mutations often result in amino acid substitutions that may affect the protein's function and are located in hotspot mutation regions, suggesting potential pathogenicity (27,59,60). Although current research focuses mainly on its role in ocular pathologies, *GJA8* may also have latent functions in the nervous system. Previous studies have revealed that *GJA8* is crucial for the differentiation of nerve cells (33). The proteins encoded by *GJA8* control the migration of neural crest cells *in vivo* by regulating the transcription of N-cadherin (28,32). Furthermore, studies have shown that *GJA8* regulates oxidative stress and cell death, potentially contributing to neuroprotective mechanisms (23). We also found that the expression of *GJA8* was increased in HSCR and neurodevelopmentally impaired animal models, and further GO analysis revealed that *GJA8* is involved in the PI3K/AKT signaling pathway. The PI3K/AKT pathway is activated by various extracellular stimuli and this activation is critical for regulating cell survival, proliferation, autophagy, neurogenesis, and synaptic plasticity in the nervous system (61,62). In neurodegenerative diseases such as Alzheimer's and Parkinson's, the PI3K/AKT signaling pathway is over-activated, leading to autophagy imbalance, pathological protein accumulation, and neuron loss (63,64). In HSCR, the PI3K/AKT pathway is thought to be involved in the migration, proliferation, and differentiation of ENCCs, which are essential for the formation of the ENS (42). Moreover, this signaling pathway has been reported as a key signaling pathway for neurogenesis and neuroprotection and is associated with *RET* and *RET*-regulating pathways in HSCR (40,43). In addition, we found that the PI3K-Akt pathway was activated in aganglionic colons by qRT-PCR analysis.

Our investigation revealed that rs17160783 in the *GJA8* locus was associated with L-HSCR in southern Chinese. However, the association with S-HSCR was not significant. Previous studies have demonstrated that mutations in RET, GDNF, and SOX10 are associated with L-HSCR and syndromic HSCR, but they do not explain the susceptibility to S-HSCR, which is the predominant subtype of HSCR. The genetic mechanisms underlying the differential susceptibility to HSCR subtypes still need to be elucidated. rs17160783 is in high LD with two SNPs which have the potential to affect CTCF binding, suggesting that these genetic variants may regulate the expression of *GJA8* by changing the binding affinity of transcription factors. Next, we predicted the changes in binding transcription factors between the risk and non-risk alleles. The prediction results indicate that the HSCR-associated SNP may increase the binding of *SPIB*, *STAT5A*, *RARA*, and *PPARG*. *SPIB* and *PPARG* are important regulators of cell differentiation and can mediate apoptosis through the PI3K-Akt pathway (50,51,53), whereas, *RARA* plays a key role in neuronal differentiation (65). A balance between *STAT5A* and PI3K-Akt is essential for T-cell viability (66) and determines neurotrophic or neuroprotective effects in neurons (67,68). Together, SNPs in *GJA8* may regulate the expression of *GJA8* possibly by altering the binding of transcription factors for *GJA8*, and consequently impacting the PI3K-Akt signaling pathway during the ENS development (*Figure 3*).

In addition, the association of *GJA8* with L-HSCR suggests that genetic variations in this gene may influence disease risk, potentially offering a new avenue for early detection and personalized medicine approaches. However, the translation of this genetic insight into clinical practice is not without challenges. The biological function of *GJA8* in the context of HSCR and the specific role of its SNPs in disease pathogenesis requires further elucidation. Current limitations include the need for comprehensive functional studies to understand the mechanistic link between *GJA8* variants and disease phenotypes. Additionally, the clinical utility of *GJA8* SNPs as diagnostic or therapeutic targets must be validated through rigorous epidemiological and clinical studies, ensuring their predictive value and therapeutic relevance. To advance our understanding of *GJA8* in HSCR, future research should focus on interdisciplinary collaboration among experts in genetics, molecular biology, clinical medicine, and other fields.

Although our study is the first discovery of association between *GJA8* rs17160783 and L-HSCR, there are still some limitations in this study. Firstly, all participants were recruited from the same hospital, raising the possibility of selection bias. Multi-center studies in the future would help to reduce the incidence of this bias. Secondly, the sample size of L-HSCR in our study was small and there is an urgent need for multi-center studies with larger sample sizes to better understand this rare disease. Thirdly, the molecular mechanism relating *GJA8* to the PI3K-Akt

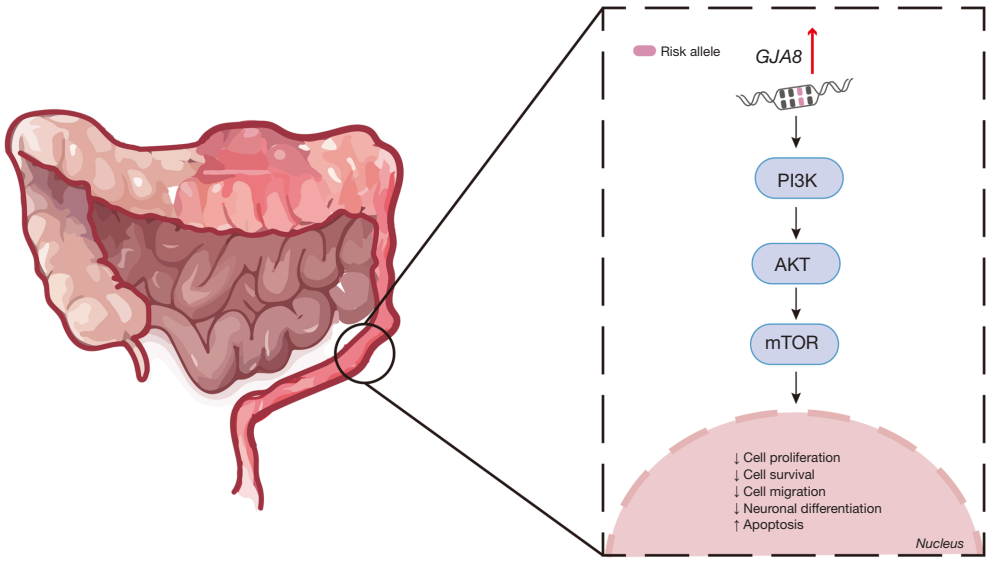

Figure 3 Hypothesis of rs17160783 risk on L-HSCR. rs17160783 may regulate the expression of *GJA8*, which further causes abnormal development of ENS through the PI3K-Akt signaling pathway. *GJA8*, gap junction protein alpha 8; L-HSCR, long-segment Hirschsprung's disease; ENS, enteric nervous system.

signaling pathway needs validation. We hope that future studies will pay more attention to this gene and confirm it with more experiments.

Conclusions

In conclusion, in our study, we found an association between L-HSCR and rs17160783 in southern Chinese. This SNP may have the potential to regulate the expression of the *GJA8*, possibly by altering the binding of transcription factors for *GJA8*, and consequently impacting the PI3K-Akt signaling pathway during the ENS development.

Acknowledgments

We thank the Clinical Biological Resource Bank of Guangzhou Women and Children's Medical Center for providing the clinical samples.

Funding: This work was supported by the grant of the Research Foundation of Guangzhou Women and Children's Medical Center for Clinical Doctor (No. 2023BS013, to Y.W.); the China Postdoctor Science Foundation (No. 2023M730791, to C.L.); the Guangdong Basic and Applied Basic Research Foundation (No. 2024A1515013190 to C.L., No. 202201010833 to W.Z.); the National Natural Science Foundation of China (No. 82301955 to C.L., No. 82370526 to W.Z.); the Science and Technology Project of Guangzhou (No. 2024A03J1238, to C.L.).

Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://tp.amegroups.com/article/view/10.21037/tp-24-153/rc

Data Sharing Statement: Available at https://tp.amegroups.com/article/view/10.21037/tp-24-153/dss

Peer Review File: Available at https://tp.amegroups.com/article/view/10.21037/tp-24-153/prf

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tp.amegroups.com/article/view/10.21037/tp-24-153/coif). Y.W. reports support from the grant of the Research Foundation of Guangzhou Women and Children's Medical Center for Clinical Doctor (No. 2023BS013). C.L. reports support from the Guangdong Basic and Applied Basic Research Foundation (No. 2024A1515013190), National Natural Science Foundation of China (No. 82301955), Science and Technology Project of Guangzhou (No. 2024A03J1238), and the China Postdoctor Science Foundation (No.

2023M730791). W.Z. reports support from the Guangdong Basic and Applied Basic Research Foundation (No. 202201010833) and the National Natural Science Foundation of China (No. 82370526). The other authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013). This study was approved by the ethics committee of Guangzhou Women and Children's Medical Center (No. 2018052406). Informed consent was obtained from the participants' legal guardians.

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Cite this article as: Ma Z, Zhong W, Song K, Chen J, Tian B, Chen Y, Li L, Lan C, Zhong W, He Q, Wu Y. Genetic association of *GJA8* with long-segment Hirschsprung's disease in southern Chinese children. Transl Pediatr 2024;13(8):1395-1405. doi: 10.21037/tp-24-153