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Connection of *GLI1* variants to congenital heart disease susceptibility

A case-control study

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Abstract

The purpose of this study was to investigate the relationship between glioma-associated oncogene homolog 1 (*GLI1*) rs2228226 and rs10783826 polymorphisms and congenital heart disease (CHD) risk in a Chinese Han population.

Genotyping for our interested polymorphisms was performed using polymerase chain reaction-restriction fragment length polymorphism in 106 CHD patients and 112 healthy controls. Hardy–Weinberg equilibrium status in the control group was also checked via χ^2 test. Differences in genotype and allele frequencies between the case and control groups were analyzed adopting Chi-Squared test as well, and the relative risk of CHD resulting from *GLI1* genetic variants was checked via calculating odds ratio (OR) and 95% confidence interval (95%CI).

CC genotype of rs2228226 showed significantly higher frequency in CHD patients than in controls ($P = .011$), indicating that it increased the disease risk (OR = 3.257, 95%CI = 1.280–8.287). Similarly, C allele of the polymorphism elevated CHD incidence by 1.609 folds, compared with G allele (OR = 1.609, 95%CI = 1.089–2.376). However, rs10783826 was not correlated with the occurrence of CHD.

GLI1 rs2228226 polymorphism may be a risk factor for CHD in Chinese Han population, but not rs10783826.

Abbreviations: 95%CI = 95% confidence interval, CHD = congenital heart disease, GLI1 = glioma-associated oncogene homolog 1, GATA4 = GATA binding protein 4, HWE = Hardy–Weinberg equilibrium, NKX2–5 = NK2 homeobox 5, OR = odds ratio, PCR-RFLP = polymerase chain reaction-restriction fragment length polymorphism, SHH = sonic hedgehog, SNP = single nucleotide polymorphism, SOX9 = SRY-box transcription factor 9.

Keywords: congenital heart disease, *GLI*, polymorphisms

1. Introduction

Congenital heart disease (CHD) is common in infants, with high lethality, and characterized by abnormal cardiovascular structure and function, and metabolism, including several sub-types.[1,2] It is a leading cause of infant heart failure and deaths in the world.[3] The incidence of CHD is 0.4% to 1% in live births but with a mortality of 24.1%,[4,5] which severely threatens the patients' health and life and brings about huge economic burden to and life stresses on family. The etiology of CHD is complex and not understood completely until now. Previous reports show that the development of CHD results from the combination of genetic and environmental factors.[6,7] Known environmental factors for this disease include infections, drugs, living habit, and other diseases.[8] In recent decades, multiple genes are discovered to participate in the pathogenesis of CHD, such as NK2 homeobox 5 (*NKX2–5*), GATA binding protein 4 (*GATA4*), and transcription factor AP-2 beta.[9,10] But current findings are not enough to reveal the pathology of CHD.

Glioma-associated oncogene homolog 1 (*GLI1*) is a transcription factor initially isolated from glioblastoma, encoded by *GLI1* gene.[11] *GLI1* participates in normal differentiation, development, and drug tolerance of several organs in humans, mainly through sonic hedgehog (SHH) signal pathway.[12,13] Aberrant expression and activation of GLI1 can affect DNA damage responses and the integrity of repairing signals.[14] *GLI1* is also found to be associated with the proliferation, migration, and invasion of tumor cells in various cancers.[15,16] In the meanwhile, activating SHH pathway can up-regulate the expressions of *NKX2–5*, catalase-like 4, and myocyte enhancer factor 2C, 3 myocardial markers assuming pivotal functions in SHH pathway, so *GLI1* would exert important roles in cardiac development. Therefore, the association of *GLI1* with the pathogenesis of CHD should be paid attention.

However, publications about the effect of *GLI1* on the onset of CHD are few, and its single nucleotide polymorphism (SNP) becomes a promising means in exploring disease mechanism. In

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the present study, 2 SNPs (rs2228226, rs10783826) in *GLI1* were selected to discuss their association with CHD occurrence risk so as to obtain more information on CHD etiology.

2. Materials and methods

2.1. The case and control groups

This study adopted a case-control design. One hundred six CHD patients and 112 healthy persons were enrolled into case and control groups, respectively. Controls were frequency-matched with cases in both age and gender. CHD cases were recruited from the Department of Cardiac Surgery in Cangzhou City Central Hospital, including 54 boys and 52 girls. Their age ranged from 6 months to 12 years with an average age of 5.36 ± 4.28 years. These cases showed different sub-types, such as atrial septal defect, ventricular septal defect, arteriostenosis, tetralogy of fallot, and like. All cases were in line with diagnostic criteria for CHD with typical clinical symptoms, and confirmed through heart color ultrasound, cardiac catheterization, or surgery. Meanwhile, we ruled out cases with known chromosomal aberrations or congenital lesions in other organs.

On the other hand, 112 healthy children who had a medical examination in the hospital during the same period were selected, consisting of 52 boys and 60 girls aged from 1 to 15 years old. The research objects were all Chinese Han people and they had no blood relationship with each other. Afterward, professionally trained epidemiological investigators recorded relevant information of all subjects, and collected blood samples. This research was authorized by the Ethics Committee of Cangzhou City Central Hospital. Eligible participants' family knew about the research process and signed written informed consents. Sampling was conducted according to the national ethics criteria of human genome research.

2.2. DNA extraction

2 mL fasting peripheral blood from every subject was collected into blood collection tube with ethylenediaminetetraacetic acid-2Na anticoagulation. Peripheral blood leucocyte genome DNA was extracted for all samples using Blood Genome DNA Extraction Kit (TIANGEN, Beijing), according to the manufacturer's instructions, and then stored at −20°C in a refrigerator for standby application.

2.3. GLI1 polymorphism genotyping

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) was applied for the genotyping of *GLI1* rs2228226 and rs10783826 polymorphisms. First, we designed PCR primers using Primer Premier 5.0 software, based on the sequences of *GLI1* published in Genebank database on NCBI website, and synthesized in Shanghai Sangon biotech Co., Ltd. Detailed information of primer sequences is listed in Table 1. PCR reaction was conducted in a solution of 25 μL, including 1.0 μL genomic DNA, 1.5 μL $MgC1_2$, 1.0 μL dNTPs, 0.5 μL forward primer, 0.5 μL reverse primer, 2.0 μL 10 × Buffer, 0.5 μL Taq enzyme, and 18.5 μL sterile ddH_2O. Amplification conditions were 94°C pre-denaturation for 5 minutes, followed by 35 cycles of 94°C degeneration for 45 seconds, 58°C annealing for 30 seconds, 72°C extension for 45 seconds, and finally 72°C extension for 10 minutes. PCR products were checked using 1.0% agarose gel electrophoresis (AGE).

Table 1

Primer sequences of *GLI1* rs2228226, rs10783826 polymorphisms.

SNP	Location		Primer sequence
rs2228226	Exon12	Forward	5'-CAGGTATGTAACCCCCTGGA-3'
		Reverse	5'-TCCCCCAATTTTTCTGGAAG-3'
rs10783826	5'UTR	Forward	5'-GAGGCGGAGAGGGTATATAATCTT-3'
		Reverse	5'-GAGAGATGTTGCGTCTGGAG-3'

5'UTR = 5'untranslated region.

Eligible PCR products were digested by restriction enzyme. Enzyme reaction system was a mixture of 20 μL, including 10.0 μL PCR products, 1.0 μL 10 × Buffer solution, 2.0 μL restriction enzyme (*Ear*I for rs2228226 and *Eco*RI for rs10783826), and 7.0 μL double distilled water. Afterwards, the mixture was digested overnight in water bath at 37°C. Enzyme-digested products were separated employing 1.5% agarose gel electrophoresis, and final outcome in imaging system was observed.

2.4. Statistical analysis

PASW Statistics 18.0 software was used for data analysis, and measurement data were expressed by $\bar{x} \pm s$ or %. Genotype and allele frequencies of *GLI1* polymorphisms were gained through direct counting, and comparisons on the frequency of genotypes and alleles between the case and control groups were conducted applying Chi-Squared test. Before this, genotype distributions of *GLI1* polymorphisms in the control group were tested employing χ^2 test to determine whether they were consistent with Hardy–Weinberg equilibrium (HWE). Odds ratio (OR) with 95% confidence interval (95%CI) was calculated to express the effect of *GLI1* polymorphisms on CHD risk. $P < .05$ was considered as the statistically significant threshold.

3. Results

3.1. HWE test

Genotype frequencies of *GLI1* polymorphisms in 2 groups are displayed in Table 2. Accordingly, genotype distributions of *GLI1* rs2228226 and rs10783826 in the controls conformed to HWE ($P = .122$, .463). Therefore, they came from a Mendelian population with general representativeness.

3.2. Relationship analysis between GLI1 polymorphisms and CHD risk

Referring to rs2228226, its GG, CG, and CC genotype frequencies were 33.02%, 49.06%, and 17.92% in CHD patients group, and 42.86%, 50.00%, and 7.14% in the controls. The frequency of CC genotype in the case group was obviously higher than that in control group ($P = .011$), which indicated its promoting effect on CHD risk (OR = 3.257, 95% CI = 1.280–8.287). C allele of rs2228226 also had significantly higher frequency in CHD patients than in control group (42.45% and 32.14%, $P = .017$), and was also a risk factor for the disease (OR = 1.609, 95%CI = 1.089–2.376). For rs10783826, neither genotypes or alleles showed remarkable association with CHD

Table 2

Frequency comparisons of genotypes and alleles in *GLI1* polymorphisms between CHD patients and the controls.

Genotype/allele	CHD patients/% (n = 106)	The control/% (n = 112)	χ^2	*P*	OR (95%CI)	P_{HWE}
rs2228226						.122
GG	33.02 (35)	42.86 (48)	–	–	Ref.	
CG	49.06 (52)	50.00 (56)	0.677	.411	1.273 (0.716–2.266)	
CC	17.92 (19)	7.14 (8)	6.483	.011	3.257 (1.280–8.287)	
G	57.55 (122)	67.86 (157)	-	–	Ref.	
C	42.45 (90)	32.14 (72)	5.744	.017	1.609 (1.089–2.376)	
rs10783826						.463
TT	51.89 (55)	56.25 (63)	–	–	Ref.	
GT	34.90 (37)	35.71 (40)	0.039	.844	1.060 (0.596–1.883)	
GG	13.21 (14)	8.04 (9)	1.566	.211	1.782 (0.716–4.436)	
T	69.34 (147)	74.11 (166)	-	–	Ref.	
G	30.66 (65)	25.89 (58)	1.222	.269	1.266 (0.833–1.922)	

CHD = congenital heart disease, OR = odds ratio, 95%CI = 95% confidence interval, HWE = Hardy–Weinberg equilibrium.

occurrence, which suggested that it was not an independent risk factor for CHD.

3.3. Discussion

CHD is a congenital malformation caused by abnormal development of cardiovascular system in embryonic phase. In humans, there are mainly 4 molecules involved in cardiac development and CHD occurrence, namely signaling molecules, cell adhesion molecules, ion channel molecules, and transcription factors.[17] Among them, transcription factors are the most important regulators of cardiac development. They constitute exact regulatory network through interactions and participate in regulating the development of heart.[18]

In recent decades, multiple genes encoding transcription factors have been found to be involved in the onset of CHD. In the study of Pan et al, the loss-of-function mutation in T-box transcription factor gene *TBX1* significantly increased the susceptibility to CHD.[19] Pang et al found that sequence variants in *NKX2–5* gene promoter region could obviously enhance the transcription activities of *NKX2–5* promoter to change *NKX2–5* expression and the regulator network of cardiac development, when genetic mutations of *NKX2–5* in coding and promoter regions were also reportedly involved in the etiology of CHD.[20] Han et al explored the role of *GATA4* M310 V mutation in CHD development utilizing *GATA4* transgenic mice, and the results showed that this polymorphism was significantly correlated with the disease development.[21] According to the report of Sanchez-Castro et al, the upstream deletions of transcription factor gene SRY-box transcription factor 9 (*SOX9*) might contribute to the pathogenesis of CHD.[22]

Some CHD susceptible genes are reportedly located on chromosome 12q13 region, including homeobox C cluster genes, chromobox 5, diacylglycerol kinase alpha, and *GLI1*, of which *GLI1* is one of the most important candidates. This gene is located on chromosome 12q12.2–12.3, including 14 exons, and codes a transcription factor containing 5 zinc finger DNA-binding motifs. *GLI1* could bind to downstream target genes via specific zinc fingers to influence physical growth and development. Gianakopoulos et al revealed that in the cardiomyogenesis of P19 cells stably expressing *SHH*, *GLI1* interacted with *GLI2* to regulate the expressions of downstream genes associated with cardiomyogenesis, such as *NKX2–5* and *GATA4*, which suggested that *GLI1* may play an important role in cardiomyogenesis.[23] Weiterhin, according to the publication of Deng et al, *GLI1* could directly regulate *SOX9* transcription, and *SOX9* also facilitates nuclear *GLI1* expression through inhibiting the degradation of β-TrCP-mediated protein,[24] so *SOX9* was a candidate susceptible gene for CHD. However, the effect of *GLI1* on CHD occurrence is rarely studied until now.

With the development of molecular technique, SNP becomes a chief means to explore the pathogenesis of diseases.[25–27] In the present study, we selected 2 common SNPs, rs2228226 and rs10783826, in *GLI1* gene to investigate their influence on CHD susceptibility in a Chinese Han population. The frequencies of CC genotype and C allele of rs2228226 were significantly higher in CHD patients than in healthy controls, suggesting that rs2228226 might be a susceptible SNP for CHD. Rs2228226 is located on encoding region of *GLI1* and results in the change of amino acid sequence, which may lead to the spatial conformation of GLI1 protein to affect the combination of downstream target genes and then regulate heart development. Further reason still needs to be revealed. Differently, another SNP rs10783826 in 5'untranslated region of *GLI1* was not directly associated with the susceptibility to CHD either for genotypes or alleles.

In conclusion, *GLI1* rs2228226 polymorphism significantly elevates the risk of CHD occurrence in Chinese Han population, but not rs10783826. This study provides more evidences revealing the pathology and etiology of CHD. Unfortunately, the accuracy of final results was also limited by several defects, such as relatively small sample size, single study population, and the lack of considerations on gene–environment interactions. Beside, due to the small sample size, subgroup analysis based on disease type was not performed in this study. Therefore, in the future, we should conduct more studies with well-designed, large-enough sample sizes and different races to reveal the etiology of CHD for better and early diagnosis and timely treatment of CHD.

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