BMJ Open | Association of GDF1 rs4808863 with fetal congenital heart defects: a case–control study

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ABSTRACT

Background: Congenital heart defects (CHDs) are the most common fetal defects and the most important cause of child mortality and morbidity.

Objective: To investigate the association between growth/differentiation factor 1 (GDF1) polymorphisms and fetal CHDs, by evaluating the association of GDF1 rs4808863 with fetal CHDs.

Design: A case–control study.

Setting: Beijing, China.

Participants: We selected 124 fetuses with a CHD and a normal karyotype and normal array-based comparative genomic hybridisation analysis and compared them with 124 normal fetuses matched for gestational age and sex. Fetuses with a CHD, from 20 to 32 weeks of gestation were included. Fetuses with any chromosomal abnormalities, and fetuses from multiple pregnancies and those carried by pregnant women with chronic diseases, were excluded from this research. DNA extraction and genotyping were carried out for all cases to investigate the genotype distributions of GDF1 rs4808863.

Results: A significant difference was noted for the CT phenotype of GDF1 rs4808863 between the controls and the fetuses with CHDs using homozygote and heterozygote comparisons. The minor allele (T allele) of GDF1 rs4808863 was associated with an increased risk of CHD (p<0.05). A statistically significant difference between controls and fetuses with CHDs was noted in a comparison with the mutation genotype CT+TT and wild-type genotype CC (p<0.05) using dominant modal analysis. After stratification analysis, the CT phenotype, the minor allele (T allele) and the mutation genotype CT+TT of the rs4808863 polymorphism were associated with atrioventricular septal defect (AVSD), left ventricular outflow tract obstruction (LVOTO) and left–right laterality defects (p<0.05).

Conclusions: Our results suggest that the GDF1 rs4808863 polymorphism contributes to an increased risk of fetal CHDs, especially the subtypes of AVSD, LVOTO and left–right laterality defects.

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Strengths and limitations of this study

- This study is the first to investigate the relationship between growth/differentiation factor 1 (GDF1) polymorphisms and fetal congenital heart defects (CHDs).
- The study group excluded individuals with chromosomal aberrations.
- Stratification analyses were performed to detect possible differences in fetal CHD subtypes.
- The main limitation is the small sample size of study participants. More individuals should be included in a future study.
- An additional limitation is that GDF1 is a member of the transforming growth factor-β superfamily. More studies on the signalling pathway should be performed.

subsequently affect, cardiac function. CHDs are the most common fetal defects and the major cause of child mortality and morbidity.[1]

The aetiology of CHDs is not well understood. Earlier studies reported that genetic factors and environmental factors might play a major role in the occurrence and development of CHDs. Chromosomal aberrations are associated with the development of fetal CHD, but other multifactorial causes and genetic variants may play a part.[2] [3] Additionally, numerous signalling pathways mediate the development of CHDs, including the Notch signalling pathway, the transforming growth factor (TGF)-β signalling pathway and the Nodal signalling pathway.[4] [5] Previous studies have identified variable genes, members of CHD-associated signalling pathways and single-nucleotide polymorphisms (SNPs) that are associated with an increased risk for CHDs.[6] [7]

Growth/differentiation factor 1 (GDF1), a member of the TGF-β superfamily, was originally isolated from a day 8.5 mouse embryo cDNA library.[8] GDF1 can affect organ morphogenesis and establish and maintain asymmetric cues. GDF1 signal mutations can

INTRODUCTION

Fetal congenital heart defects (CHDs) are defined as conditions with fetal cardiovascular dysplasia which affect, or may

regulate left–right patterning via the Nodal signalling pathway, resulting in CHDs, such as right atrial isomerism.[9] [10] Sun et al[11] have shown that the GDF1 rs4808870 polymorphism is associated with an increased risk of pulmonary atresia in the Chinese Han population.

Severe CHDs are more likely to be detected before birth than minor CHDs. Women carrying fetuses with the most severe CHD are more likely to terminate the pregnancy given the poor outcome.[12] Previous studies on the association between SNPs and CHDs were conducted in adult or child populations and little is known about GDF1 polymorphisms in fetuses with critical CHDs. Therefore, this study aimed to investigate the GDF1 gene SNP in Han Chinese fetuses with CHDs.

MATERIALS AND METHODS

Ethics statement
We performed a case–control study. This study was approved by the ethics committee of Beijing Obstetrics and Gynecology Hospital, Capital Medical University. Each patient provided written informed consent to participate in this study.

Study population
We selected 124 sporadic fetuses with CHDs as the case group and 124 unrelated normal fetuses, matched for gestational age and gender, as the control group. All the cases of CHD were diagnosed in Beijing Obstetrics and Gynecology Hospital, Capital Medical University between January 2012 and December 2014. Inclusion criteria required that the fetuses were 20–32 weeks and had a CHD. All fetuses received a routine anomaly ultrasound scan in the second trimester, and echocardiography (Voluson E8, GE Healthcare, Milwaukee, Wisconsin USA) was performed when fetuses were found to exhibit a heart abnormality. The diagnosis of CHD was made by two fetal echocardiologists. Karyotype analysis and array comparative genome hybridisation were performed for all fetuses. Cases with any chromosomal abnormalities, such as trisomy syndrome and pathogenic copy number variants, were excluded. Fetuses from multiple pregnancies and those carried by pregnant women with chronic diseases (such as gestational diabetes mellitus, chronic alcoholism and connective tissue diseases) were excluded from this research.

Single nucleotide polymorphism selection
GDF1 (OMIM: 602880) maps to chromosome 19p13.11. After a small sample study, we found that the rs4808863 SNP (NM_001492.5:c.353C>T) presented genetic variants. We searched for the GDF1 rs4808863 SNP in the Ensembl genome browser (http://www.ensembl.org/index.html) and information was obtained from the SNP database. The Ensembl genome browser showed that the allele change of rs4808863 was Ala→Val (GCC→GTC), so we selected exon 8 including SNP rs4808863 as our research object.

DNA extraction and genotyping
We obtained umbilical cord blood by fetal blood sampling at the time of labour induction in the CHD group and after delivery of the baby, in the control group. All patients provided 2 mL samples of umbilical cord blood that were collected in Vacutainer tubes containing EDTA by invasive testing. We used the blood genomic DNA extraction kit (Be creative Lab (Beijing) Co Ltd, Beijing, China), according to the manufacturer's instructions. SNP genotyping was conducted by direct sequencing. PCRs were performed using specific primers for the GDF1 SNPs. Primers were designed by Primer Premier and Oligo as presented in table 1. PCR was performed in a 25 µL total volume reaction mixture containing 1.0 µL genomic DNA, 0.3 µL Taq polymerase (5 U/µL) (Omega Genetics Co Ltd, Beijing, China), 0.4 µL dNTPs (10 mM), 0.5 µL of each primer, 0.62 µL 10× buffer (Mg2+Plus), 9.37 µL 2×GC buffer I and 12.31 µL double distilled water). The PCR reaction consisted of an initial denaturation step at 95°C for 5 min; 5 cycles at 94°C for 30 s, 68°C for 30 s and 72°C for 1 min; 12 cycles at 94°C for 30 s, 65°C for 30 s and 72°C for 1 min; 12 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min; 10 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 1 min and an additional extension at 72°C for 7 min in a thermal cycler. We used an ABI Prism 3730 xl sequencer (Applied Biosystems, Foster City, California, USA) to sequence the PCR products after purification.

Statistical analysis
All statistical computations were performed using the SPSS statistical software package (V.17.0 for Windows, SPSS Inc, Chicago, Illinois, USA). p Values <0.05 were considered statistically significant. Allele frequencies were assessed for Hardy–Weinberg equilibrium using the χ^2 test or Fisher's exact test. The differences in the genotype frequencies between the two groups were also assessed using these tests.

RESULTS

GDF1 SNP rs4808863 genotype distribution
Successful genotyping of rs4808863 in CHDs was not achieved in three cases. We identified two alleles (C and T) and three genotypes (CC, CT and TT) at rs4808863 (figure 1).

As presented in table 2, the distribution of genotype frequencies in both the CHDs (n=121) and the controls (n=124) were in Hardy–Weinberg equilibrium (p>0.05). In our controls, the genotype distributions and minor allele frequencies of GDF1 SNP rs4808863 were similar to the Han Chinese in Beijing genotype data in the HapMap database.

Association between genotype and allele frequencies of GDF1 SNP rs4808863 in fetuses with CHD and controls
The rs4808863 CC, CT and TT genotype distributions in CHD cases and controls are presented in table 3. The

Table 1 Primers for GDF1 exon 8

Primer name	Primer sequence	Sequence amplified regions (GRCh38)	Amplified fragment length (bp)	GC
GDF1–8F	CCCCAGCGTTCACCTTCCTC	chr19:18868321–18869484	1164	74%
GDF1–8R	AGACAGGCAAAGCCCAGAAGG			

Primers were designed by Primer Premier and Oligo.

frequencies of GDF1 rs4808863 genotypes were 58.6% (CC), 39.7% (CT) and 1.7% (TT) in CHD fetuses and 76.6% (CC), 21.0% (CT) and 2.4% (TT) in control subjects. The CT genotype frequency of rs4808863 in fetuses with CHDs differed significantly from that of the controls (p=0.002). The frequency of the minor T allele of rs4808863 was significantly increased in the CHD cases compared with healthy controls (p=0.012, OR=1.847, 95% CI 1.141 to 2.990). However, we found no significant differences in the TT genotype in CHD cases compared with healthy controls. According to dominant model analysis between controls and CHD cases, a statistically significant difference was found for the mutation genotypes CT+TT and wild-type genotype CC (p=0.003, OR=2.307, 95% CI 1.329 to 4.003).

Stratified subgroup analysis of GDF1 SNP rs4808863

Eight major groups of human heart malformations have been reported, including conotruncal defects (CTDs); atrioventricular septal defects (AVSD); anomalous pulmonary venous return (APVR); left ventricular outflow tract obstruction (LVOTO); right ventricular outflow tract obstruction (RVOTO); and septal, heterotaxy and complex (table 4).[13]

We analysed the genotypes and allele frequencies of the GDF1 rs4808863 SNP in different subgroups of CHDs (table 5). The results showed that the rs4808863 CT genotype was associated with an increased risk of AVSD and LVOTO compared with healthy controls

(p=0.001, OR=7.308, 95% CI 2.296 to 23.261; p=0.018, OR=4.176, 95% CI 1.386 to 12.586, respectively). In addition, the frequency of the T allele also significantly differed in patients with AVSD and LVOTO compared with healthy controls (p=0.007, OR=3.375, 95% CI 1.450 to 7.857; p=0.013, OR=3.068, 95% CI 1.332 to 7.069; respectively). Moreover, dominant model analyses of the subtypes of CHD, such as AVSD and LVOTO, exhibited significant difference (p=0.001, OR=6.552, 95% CI 2.072 to 20.717; p=0.013, OR=4.212, 95% CI 1.442 to 12.300; respectively).

However, no significant differences in rs4808863 genotypes, alleles and dominant models were noted in the analysis among the various subtypes, including CTDs, APVR, RVOTO, septal patients, heterotaxy, complex cases and controls.

DISCUSSION

CHD is a major cause of neonatal morbidity and mortality.[1] To explore the potential mechanisms involved in fetal CHDs, we investigated the association of GDF1 rs4808863 and the risk of fetal CHDs in our study. We found that the minor T allele, the CT phenotype and dominant models of rs4808863 were significantly associated with susceptibility to CHDs, especially for two subtypes, AVSD and LVOTO. The results suggest that fetuses with the CT phenotype may be more easily affected by AVSD and LVOTO. Additionally, the inheritance patterns of GDF1 in AVSD and LVOTO may tend to exhibit

Figure 1 Partial sequence analysis of rs4808863 genotypes. (A) CC genotype. (B) TT genotype. (C) CT genotype.
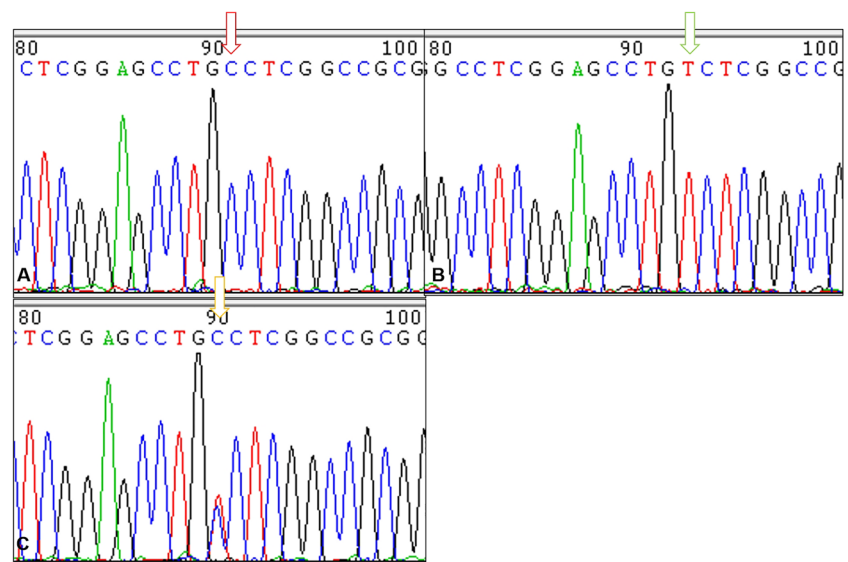
Table 2 Hardy–Weinberg equilibrium of GDF1 SNP rs4808863 allele frequencies

SNP	Group	Genotype			χ^2	p Value
		CC	CT	TT		
rs4808863	CHDs	71	48	2	3.74	0.053
	Controls	95	26	3	0.02	0.886

CC, CT and TT were genotypes of GDF1 SNP rs4808863. CHDs, congenital heart defects; SNPs, single-nucleotide polymorphisms.

Table 4 Details of the eight major subgroups of CHDs

Groups	Details
CTDs (n=56)	Tetralogy of Fallot
	Transposition of the great arteries
	Double outlet right ventricle
	Truncus arteriosus
	Interrupted aortic arch
AVSD (n=15)	Primum type atrial septal defect
	AVSD
APVR (n=4)	Total APVR
	Partial APVR
LVOTO (n=16)	Hypoplastic left heart syndrome
	Aortic stenosis
	Aortic coarctation
RVOTO (n=6)	Hypoplastic right heart syndrome
	Tricuspid atresia
	Ebstein
Septal (n=16)	Perimembranous ventricular septal defect
	Muscular ventricular septal defect
	Secundum atrial septal defect
Heterotaxy (n=4)	Laterality defects with simple cardiovascular malformation
	Laterality defects with complex cardiovascular malformation
Complex (n=4)	Single ventricle
	Complex heart anomaly

APVR, anomalous pulmonary venous return; AVSD, atrioventricular septal defects; CHDs, congenital heart defects; CTDs, conotruncal defects; LVOTO, left ventricular outflow tract obstruction; RVOTO, right ventricular outflow tract obstruction.

dominant models. In our study, no significant difference in the frequency of the TT genotype was noted between controls and those with CHDs, suggesting that the homozygous mutation might not be associated with CHDs. Bengtsson et al[14] reported that GDF1 null mice die during late embryogenesis. This phenomenon may explain the small sample size of the TT phenotype in the CHD cases.

As a member of the TGF-β superfamily, GDF1 was mapped to chromosome 19p13.11.[8] GDF1 is homologous to Vg-1 in Xenopus and Dvr1 in zebrafish based on accumulating evidence.[15 16] Northern (RNA) analysis of GDF1 transcripts in murine embryonic mRNA detected two mRNA species 1.4 and 3.0 kb in length and only the 3.0 kb mRNA species persists throughout embryogenesis.[17]

In our study, GDF1 rs4808863 was mapped to exon 8 of GDF1. Recent studies have shown that GDF1 mutations are present in cardiovascular malformations, such as tetralogy of Fallot (TOF) and transposition of the great arteries (TGA). GDF1 also potentially decreases TGF-β signalling to cause some specific types of human CHDs.[18 19] To date, GDF1 mutations are reportedly involved in recessively inherited right atrial isomerism.[10] Our study showed that rs4808863 GDF1 polymorphism is associated with AVSD and LVOTO and these findings are not consistent with these studies. Furthermore, Sun et al[11] suggested that the GDF1 polymorphism might be associated with an increased risk of CTDs. However, De

Luca et al[20] investigated the prevalence of GDF1 mutations in patients with CTD and the results presented a contrasting view. No GDF1 mutations were noted in CTD, indicating that GDF1 is not a good candidate for CTDs.

In our study, we found that the GDF1 polymorphism is related to the non-CTD subtype, which is consistent with De Luca's study but differs from the research of Sun. CTDs, a group of abnormal great artery and outflow tract defects, account for about 15–20% of

Table 3 Genotype and allele frequencies of GDF1 SNP rs4808863 in controls and fetuses with CHD

Genotype/allele	Genotype/allele frequency		χ^2	p Value	OR (95% CI)
	CHDs	Controls			
CC	71	95	–	–	–
CT	48	26	**9.994**	**0.002**	**2.47 (1.400 to 4.358)**
TT	2	3	0.015	1.000*	0.892 (0.145 to 5.480)
C	190	216	–	–	–
T	52	32	**6.354**	**0.012**	**1.847 (1.141 to 2.990)**
CT+TT	50	29	**9.017**	**0.003**	**2.307 (1.329 to 4.003)**
CC+CT	119	121	0.180	1.000*	1.475 (0.242 to 8.98)

*p Value from adjusted χ^2 tests with continuity correction.
Significant differences between cases and controls are shown in bold.
CC, CT and TT are genotypes of GDF1 SNP rs4808863. C and T are alleles at rs4808863. CT+TT, dominant model; CC+CT, recessive model.
CHDs, congenital heart defects; SNPs, single-nucleotide polymorphisms.

Table 5 Genotype and allele frequencies of GDF1 SNP rs4808863 in controls and subtypes of CHDs

	CC	CT	TT	T vs C			CT vs CC			CT+TT vs CC		
				p Value	OR	95% CI	p Value	OR	95% CI	p Value	OR	95%CI
Controls	95	26	3	–	–	–	–	–	–	–	–	–
CTDs	36	19	1	0.147	1.558	0.853 to 2.845	0.066	1.928	0.953 to 3.903	0.085	1.820	0.916 to 3.617
AVSD	5	10	0	**0.007***	**3.375**	**1.450 to 7.857**	**0.001***	**7.308**	**2.296 to 23.261**	**0.001***	**6.552**	**2.072 to 20.717**
APVR	3	1	0	1.000*	0.9964	0.209 to 4.442	1.000*	1.218	0.122 to 12.201	1.000*	1.092	0.109 to 10.903
LVOTO	7	8	1	**0.013***	**3.068**	**1.332 to 7.069**	**0.018***	**4.176**	**1.386 to 12.586**	**0.013***	**4.212**	**1.442 to 12.300**
RVOTO	3	3	0	0.444*	2.250	0.578 to 8.752	0.260*	3.654	0.696 to 19.179	0.321*	3.276	0.627 to 17.116
Septal	11	5	0	0.880*	1.250	0.449 to 3.480	0.576*	1.661	0.530 to 5.207	0.704*	1.489	0.478 to 4.637
Heterotaxy	2	2	0	0.643*	2.250	0.435 to 11.632	0.217*	3.654	0.491 to 27.199	0.462*	3.276	0.442 to 24.293
Complex	4	0	0	–	–	–	–	–	–	–	–	–

*p Value from adjusted χ^2 test with continuity correction.
Significant differences between cases and controls are shown in bold.
APVR, anomalous pulmonary venous return; AVSD, atrioventricular septal defects; CHDs, congenital heart defects; CTDs, conotruncal defects; LVOTO, left ventricular outflow tract obstruction; RVOTO, right ventricular outflow tract obstruction; SNPs, single-nucleotide polymorphisms.

CHDs. CTDs include TOF, TGA, double outlet right ventricle (DORV), interruption of the aortic arch, pulmonary atresia, persistent truncus arteriosus and atriopulmonary window.[13] In Sun's study, a correlations was found between GDF1 and the risk of pulmonary atresia. However, no cases of pulmonary atresia were included in our study. Therefore, the findings of our study are similar to those of both Sun and De Luca. Based on these data, GDF1 might not be associated with CTDs, especially in the clinical phenotypes of TOF, TGA and DORV. On the other hand, in comparison with other studies, our study focused on a different polymorphism site, a different population and more severe clinical phenotypes of CHDs. These differences might be the main reasons for the different result.

The mechanism by which GDF1 is involved in CHD remains unclear. However, several previous studies have investigated the potential mechanism. Baker et al[21] studied cardiac development in zebrafish and found that zebrafish heart development undergoes first, the process of cardiac jogging and second, asymmetry of cardiac looping. The Nodal signalling pathway may establish cardiac laterality by influencing the second asymmetry of cardiac looping. TGF-β signalling is critically important for the morphogenesis of atrioventricular cushions.[22] As a member of the TGF-β superfamily, GDF1 was first identified by Lee as the first molecule involved in organising left–right axis formation to regulate the Nodal pathway by heterodimerising with Nodal.[8 23 24] Shen[22] observed that the Nodal signalling pathway plays a critical role in normal embryogenesis and is especially important in the formation of the human heart. During the process of Nodal signal transduction, the heterodimerisation of GDF1 and Nodal stimulate activin receptor signalling. Then, type I and type II activin receptors activate the phosphorylation of Smad2 and Smad3. Next, Smad2 and Smad3 cooperate with Smad4 to translocate into the nucleus. The Smad complex binds DNA or DNA binding protein to prime the expression of tissue-specific genes.[9 25 26] In addition, Zhang et al[27] demonstrated that GDF1 plays a critical role in cardiac remodelling via the MEK-ERK1/2 and Smad signalling pathways. We hypothesise that GDF1 may play a role in CHD via these two signalling pathways, but more research is needed to verify this hypothesis.

By establishing mouse models with a targeted mutation in GDF1, researchers have shown that GDF1 is implicated in the establishment of left–right asymmetry. Mutant mice exhibited a range of congenital anomalies, such as CHD and visceral situs inversus.[28]

GDF1 is one of the known susceptibility genes for CHD, but the incidence of GDF1 SNPs had not been previously explored in the Chinese Han population. Our group designed this study to analyse the association between the GDF1 rs4808863 SNP and CHD. The study design enabled us to complete the experiment by using inexpensive instrumentation and reagents. In summary, our study demonstrated that GDF1 rs4808863 was

associated with an increased risk of fetal CHDs, especially AVSD and LVOTO. However, the main limitation of our study is the small sample size and a larger study should be conducted in the future. An additional limitation is that GDF1 is a member of the TGF-β superfamily and more studies on the signalling pathway should be performed. Although this study provides a basis for further investigation of the biological role of GDF1 in the development of fetal CHDs, further studies need to explore the molecular mechanisms of the interaction between GDF1 and fetal CHDs.

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