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ORIGINAL ARTICLE

Association of maternal hypertension and diabetes with variants of the *NKX2-5*, *LEFTY1* and *LEFTY2* genes in children with congenital heart defects: a case-control study from Pakistani Population

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Abstract

Background Globally, congenital heart defect (CHD) is the most common congenital malformation, responsible for higher morbidity and mortality in the pediatric population. It is a complex multifactorial disease influenced by gene-environment and gene-gene interactions. The current study was the first attempt to study these polymorphisms in common clinical phenotypes of CHD in Pakistan and the association between maternal hypertension and diabetes with single nucleotide polymorphisms (SNPs) in children.

Methods A total of 376 subjects were recruited in this current case-control study. Six variants from three genes were analyzed by cost-effective multiplex PCR and genotyped by minisequencing. Statistical analysis was done by GraphPad prism and Haploview. The association of SNPs and CHD was determined using logistic regression.

Results The risk allele frequency was higher in cases as compared to healthy subjects, but the results were not significant for rs703752. However, stratification analysis suggested that rs703752 was significantly associated with the tetralogy of Fallot. The rs2295418 was significantly associated with maternal hypertension (OR = 16.41, p = 0.003), while a weak association was present between maternal diabetes and rs360057 (p = 0.08).

Conclusion In conclusion, variants in transcriptional and signaling genes were associated with Pakistani pediatric CHD patients that showed varied susceptibility between different clinical phenotypes of CHD. In addition, this study was the first report regarding the significant association between maternal hypertension and the *LEFTY2* gene variant.

Keywords Congenital heart defect · Maternal hypertension · Minisequencing · Single nucleotide polymorphism · Tetralogy of Fallot

Introduction

Congenital heart disease (CHD) is a functional and structural defect in the heart that arises during embryonic development. The clinical outcome and anatomic details of CHD are varied [1, 2]. Globally, more than 1 million fetuses with CHD resulted in a high disease burden [3]. Over the past few decades, the complex cyanotic CHD resulted in a higher mortality and morbidity rate in low-and middle-income countries (LMICs) [4]. The overall prevalence in Asian regions is higher due to milder lesions [5]. The clinical spectrum of cardiac malformations is broad that can range from solitary lesions such as ventricular septal defect (VSD) or atrial septal defect (ASD) to complex forms, including tetralogy of Fallot (TOF). Single nucleotide polymorphisms (SNPs) in more than 400 genes are identified to cause CHD in patients [6].

Congenital heart malformation is a multifactorial disease that involves a complex interaction between genetic and environmental factors [7]. Environmental factors are implicated in 2 to 10% of CHD, including maternal chronic disease conditions such as maternal diabetes, obesity, infection, or nutritional deficiencies [8, 9]. In 90% of cases,

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molecular mechanisms underlying disease pathogenesis are still not completely understood [10]. However, linkage disequilibrium (LD) analysis help in a better understanding of complex genetic diseases [11]. Genes involved in cardiac development fall into three functional categories: structural proteins, transcriptional factors, and signaling molecules [12]. The *NKX2-5* gene is the earliest known marker that expresses both in the fetal and adult heart located at chromosome 5, the 5q34-q35 region. Approximately 105 polymorphisms have been reported in this homeobox gene, including missense SNPs, deletion mutations, synonymous variants, and insertion polymorphisms. The two most studied markers are rs2277923 SNP results in the substitution of T to C (glutamine to glutamine at position 21 in the protein), and rs3729753 (C to G) is a synonymous variant (leucine to leucine at position 202 in the protein sequence) [13]. Similarly, rs703752 is 3 prime UTR variant in which A replaces the reference C base, [14] while rs28936670 (G>A) is a missense variant that replaces the arginine (R) by cytosine (C) at position 25 [15]. Another selected gene in this study was the left-right determination factor (*LEFTY*) which is the negative regulator of the Nodal pathway and thus plays an essential role during left-right patterning and cardiac morphogenesis. The *LEFTY1* gene is primarily expressed on the prospective floor plate, and the *LEFTY2* gene is expressed predominantly in the left lateral plate mesoderm [16]. Thus, a missense variant rs360057 (T>G) (mutates aspartate to alanine at position 322) from *LEFTY1* and rs2295418 (G>A) that replaces the proline by leucine from *LEFTY2* were selected. To the best of our knowledge, this was the first comparative study from the Pakistani population, and there is no SNPs information available for this disease in this population. The purpose of these SNPs analysis in the Pakistani subjects was 1) the Pakistani population is a unique ethnic group, these genetic markers showed inconsistent results with CHD in different ethnic groups but have not been analyzed in Pakistani CHD patients. 2) Comparative evaluation between different phenotypes of CHD can provide us better insights into disease pathophysiology and genetic susceptibility to CHD. 3) Association of maternal chronic disease conditions with SNPs of the *NKX2-5*, *LEFTY1*, and *LEFTY2* in children with congenital heart malformations can further enhance our understanding of disease pathogenesis that may provide us with novel direction for future CHD prevention.

Materials and methods

Recruitment of study population

This case-control study was approved by the ethical committee of Children's Hospital and The Institute of Child Health, Lahore (2021-282-CHICH) (2) Institutional Review Board Committee of Punjab University (84/DFEMS). This study followed the principles of the Declaration of Helsinki, and written informed consent was taken from the parents of diseased and healthy subjects before data and blood sample collection. A total of 376 subjects (151 controls, 225 cases) were recruited from hospitals for the study from 2021 to 2022. The CHD group was composed of non-syndromic patients. The CHD diagnosis was made through echocardiography and physical examination by pediatric cardiologists. All the included controls were healthy subjects that were age and gender-matched to the case group. Exclusion criteria were patients diagnosed with syndromic CHD, infectious diseases, and acquired heart diseases. A questionnaire was designed to record all the relevant clinical information from patients and parents.

Variants selection

The current study used an extensive literature survey through various databases, including the National Center for Biotechnology Information (NCBI) and the 1000 genome browser. The selection criteria for SNPs were based on their potential role in disease susceptibility reported in different populations and on identifying the polymorphisms that have not been investigated in Pakistani CHD patients. *LEFTY1* and *LEFTY2* genes' key role is to regulate human embryonic stem cells and mesodermal cell differentiation. The SNPs rs2295418 and rs360057 are present in the transforming growth factor beta (*TGFβ*) domain of the *LEFTY* protein hence the selected SNPs can affect the growth regulation mechanism of the heart. The homeobox *NKX2-5* gene plays a crucial role in the proliferation and differentiation of cardiac cells during embryonic development. The four SNPs in the *NKX2-5* gene were selected based on their significant haplotype associations or LD pattern in the various populations.

Genomic DNA extraction and quantification

The DNA was extracted from 200–500µl of anticoagulated peripheral blood using the salting-out method. The DNA was quantified manually through the agarose gel and by SepctraMax384 (Molecular Devices, CA, USA) and diluted to a final working concentration of 25ng/µl.

Multiplex amplification PCR and genotyping by using minisequencing

Multiplex polymerase chain reaction was performed to amplify the genomic regions containing the selected SNPs. Amplification primers and single base extension (SBE) probes were designed using primer3 (version 4) and Batch Primer 3 software (MA, USA), respectively. PCR reactions were performed in a total volume of 10µl containing the following working concentrations of reaction components: 25ng/µl DNA, 1.7X Taq buffer, 0.4mM DNTP's mix, 3mM $MgCl_2$, 2µl primer mix (Eurofins Genomics), and 2.0 Units of Taq polymerase (Thermo Scientific). The PCR program consisted of initial denaturation at 95°C for 5min, followed by 30 cycles of 95°C for 45s, 62°C for 45s, extension at 72°C for 45s, and a final extension at 72°C for 10min. The variants were genotyped by using ABI Prism SNaPshot™ Multiplex Kit (Applied Biosystems, Foster City, CA, USA). The multiplex PCR product was purified by using Exonuclease-I (2 units/µl) and FastAP (3 units/µl) (Thermo Scientific). Both enzymes and PCR product were incubated at 37°C for 1 hour followed by 80°C for 15min. The SNaPshot PCR was performed in a total volume of 5µl containing 2µl PCR product, 2µl SBE primer mix, and 1µl of nuclease-free water. The minisequencing PCR program consisted of 25 cycles at 96°C for 10s, 50°C for 5s, and 60°C for the 30s. The final purification was done by using 0.7 units of FastAP and incubating the samples at 37°C for 1 hour followed by 75°C for 15min. The final reaction mix containing 0.05µl of GeneScan-120 LIZ internal size standard (Applied Biosystems), 8.5µl Hi-Di Formamide, 1.45µl purified SNaPshot product was denatured at 95°C for 5min. Finally, the ABI-3130XL genetic analyzer was used for capillary electrophoresis and genotyping of different SNPs was analyzed by fragment length and fluorescent signals. GeneMapper ID-X (Applied Biosystem) software was used to analyze these selected variants.

Statistical analysis

The chi-square test was used to analyze the allelic and genotypic frequencies and the odds ratio (OR) was computed at a 95% confidence interval (CI). Hardy-Weinberg equilibrium (HWE) was evaluated by the chi-squared goodness of fit test. Logistic regression was used to analyze the association of polymorphism with variants. Haploview was used to construct the linkage disequilibrium map. SHEsis, SNPStats, and GraphPad prism was used for statistical and graphical analysis. Sample size estimation was based on disease prevalence. Power analysis was conducted using online calculators online power calculator OSSE (http://osse.bii.a-star.edu.sg/calculation2.php) and QUANTO program. The cut-off p-value for all statistically significant tests was < 0.05.

Results

Baseline characteristics study

The case and control subjects were age and gender-matched. The VSD (46.22%) and TOF (36.44%) were the most common types in our pediatric population. The maximum age limit in both studied groups was up to 15 years. The baseline parameters for each CHD type are given in Table 1.

Role of selected polymorphisms in CHD

The studied SNPs were in HWE ($p > 0.05$). Genotypic and allelic distribution suggested the independent role of polymorphisms in the *NKX2-5*, *LEFTY1, and LEFTY2* genes except for rs703752 ($p = 0.53$). The detailed results for all SNP's frequency distribution are given in Table 2. In addition, genetic contrast models also supported the results in Table 1, as each variant significantly increased the risk of disease (Table 3).

Table 1 Baseline parameters of population under study

Baseline characteristic	CHD (n = 225)						Controls (n = 151)
	VSD	TOF	TGA	ASD	PDA	CAO*	
Distribution (%)	46.22%	36.44%	7.56%	4.44%	4.44%	0.9%	100%
Gender:	68.27%	65.85%	59%	60%	60%	50%	65%
Male (%)	31.73%	34.15%	41%	40%	40%	50%	35%
Female (%)							
Age:	5.52 ± 2.78	5.62 ± 3.30	4.17 ± 3.64	5.33 ± 3.14	4.3 ± 2.48		5.71 ± 2.65
< 1Year	4.47 ± 3.46	4.23 ± 3.88	4.43 ± 4.09	5.45 ± 3.88	4.6 ± 4.39		4.07 ± 3.57
≥ 1 Year							
Hb level (g/dL)	12.03 ± 2.81	15.22 ± 4.70**	14.66 ± 1.33	12.33 ± 2.63	12.73 ± 3.91		Normal Range
RBC count (10^6/µl)	5.10 ± 1.89	7.32 ± 1.21**	5.50 ± 1.12	4.56 ± 0.40	5.18 ± 1.89		Normal Range
WBC count (10^3/µl)	8.09 ± 2.80	7.46 ± 2.59	7.11 ± 2.10	10.27 ± 3.08	7.88 ± 1.84		Normal Range
Platelet count (10^3/µl)	289.5 ± 155.27	214 ± 131.1	310.75 ± 104.09	306.24 ± 158.81	292 ± 165.67		Normal Range

*Mean ± SD was not calculated due to low frequency/single observation

** Significant findings ($p < 0.05$)

Comparative assessment of polymorphisms between different phenotypes

The comparison of polymorphisms association between VSD and TOF group suggested similar significant findings except for rs703752. The comparative evaluation of this SNP suggested non-significant results in ventricular septal defects (OR = 1.13, p = 0.69), while results in the TOF group were significant (OR = 2.71, p = 0.0008). The frequency distribution is given in Table 4.

Association of maternal hypertension and polymorphisms of *NKX2-5* and *LEFTY1* and *LEFTY2* in children with the congenital heart defect

Based on the above genetic association analysis, further association analysis was performed. And based on the prevalence, maternal hypertension, and diabetes were selected for association analysis with polymorphisms. The association of maternal hypertension was significant for only one SNP rs2295418 (OR = 16.41, p = 0.0032). However, the results for maternal diabetes were non-significant (p > 0.05) (Table 5). In addition, the selected SNP's association with maternal hypertension and diabetes is also non-significant for VSD and TOF groups (p > 0.05).

Table 2 Genotype and allele frequency distribution between case and control group

Genotypes/Alleles	CHD group (n = 225)	Control group (n = 151)	P value	OR(95%CI)
NKX2-5-rs2277923				
TT	0.187	0.457		
TC	0.738	0.483	< 0.0001	3.74 (2.33–5.99)
CC	0.076	0.060	0.01	3.10 (1.27–7.59)
T	0.56	0.7		
C	0.44	0.3	< 0.0001	1.85 (1.36–2.52)
NKX2-5-rs703752				
CC	0.693	0.755		
CA	0.302	0.212	0.018	1.55 (0.96–2.52)
AA	0.005	0.033	0.09	0.15 (0.02–1.27)
C	0.844	0.861		
A	0.156	0.139	0.53	1.14 (0.75–1.72)
NKX2-5-rs28936670				
GG	0.573	0.735		
GA	0.413	0.265	0.0019	2.00 (1.28–3.13)
AA	0.014	0.0		
G	0.780	0.868		
A	0.220	0.132	0.0024	1.84 (1.23–2.75)
NKX2-5- rs3729753				
CC	0.538	0.722		
CG	0.209	0.252	0.67	1.11 (0.68–1.84)
GG	0.253	0.026	< 0.0001	12.84 (4.51–36.55)
C	0.642	0.848		
G	0.358	0.152	< 0.0001	3.10 (2.14–4.48)
LEFTY2- rs2295418				
GG	0.827	0.914		
GA	0.120	0.079	0.15	1.67 (0.82–3.41)
AA	0.053	0.007	0.0092	8.90 (1.15–69.19)
G	0.887	0.954		
A	0.113	0.046	0.001	2.63 (1.43–4.84)
LEFTY1- rs360057				
TT	0.058	0.238		
TG	0.636	0.563	< 0.0001	4.66 (2.34–9.28)
GG	0.307	0.199	< 0.0001	6.37 (2.96–13.69)
T	0.376	0.520		
G	0.624	0.480	9.13×10^{-5}	1.80 (1.33–2.42)

Table 3 Genetic contrast models analysis between healthy and CHD subjects

Gene/SNP	Model	Genotype	Controls (%)	CHDs (%)	OR (95%CI)	P-value
NKX2-5-rs2277923	Dominant	T/T	45.7	18.7	1.00	< 0.0001
		T/C-C/C	54.3	81.3	3.67 (2.31–5.83)	
	Recessive	T/T-C/T	94	92.4	1.00	0.55
		G/G	6	7.6	1.29(0.56–2.97)	
NKX2-5-rs703752	Dominant	C/C	75.5	69.3	1.00	0.19
		C/A-A/A	24.5	30.7	1.36 (0.85–2.17)	
	Recessive	C/C-C/A	96.7	99.6	1.00	0.03
		A/A	3.3	0.4	0.13 (0.02–1.13)	
NKX2-5-rs28936670	Dominant	G/G	73.5	57.3	1.00	0.0012
		G/A-A/A	26.5	42.7	2.07 (1.32–3.23)	
	Recessive	G/G-G/A	100	98.7	1.00	
		A/A	0.0	1.3		
NKX2-5- rs3729753	Dominant	C/C	72.8	53.3	1.00	3×10^{-4}
		C/G-G/G	27.2	46.7	2.23 (1.43–3.47)	
	Recessive	C/C-C/G	97.3	74.2	1.00	< 0.0001
		G/G	2.7	25.8	12.47 (4.42–35.20)	
LEFTY2- rs2295418	Dominant	G/G	91.4	82.7	1.00	0.014
		G/A-A/A	8.6	17.3	2.23 (1.14–4.33)	
	Recessive	G/G-G/A	99.3	94.7	1.00	0.0069
		A/A	0.7	5.3	8.45 (1.09–65.59)	
LEFTY1- rs360057	Dominant	T/T	23.8	5.8	1.00	< 0.0001
		T/G-G/G	76.2	94.2	5.11 (2.60-10.01)	
	Recessive	T/T-T/G	80.1	69.3	1.00	0.018
		G/G	19.9	30.7	1.78 (1.09–2.91)	

Linkage disequilibrium and haplotype analysis

The linkage disequilibrium map showed moderate LD between rs703752 and rs2277923 (D'=80) (Fig. 1). The haplotype frequency distribution was significant for CC (OR = 1.78, p = 0.000359) and wild haplotype alleles CT (OR = 0.533, $p = 4.38 \times 10^{-5}$).

Discussion

Congenital heart defects are complex genetic malformations that involve interaction between different genes and the environment, particularly maternal factors [17, 18]. In the present study, six single nucleotide polymorphisms from three different genes were genotyped to find their independent association first time in Pakistani subjects. In addition, the association of maternal chronic disease conditions with variants in CHD children was further analyzed, which may provide us with novel and better prevention strategies. The Pakistani population represents a unique tool to find the contribution of genetic markers to complex disorders based on the restricted cultural, religious, and social settings.

The genotypic and allelic frequencies of selected markers suggested that compared to the healthy subjects, CHD had a higher minor allele frequency (MAF), except rs703752, for which results were not statistically significant. However, the comparative analysis between two clinically different phenotypes of CHD showed that rs703752 allelic and genotypic frequencies were statistically different in TOF ($p < 0.01$) as compared to ventricular septal defects ($p > 0.05$). Although, the remaining five SNP's results were similar in VSD and TOF. Similarly, a significant association of two variants (rs28936670 and rs2277923) in the *NKX2-5* gene was found in Egyptian CHD children [15]. Chen et al. performed a meta-analysis in the Chinese population to investigate the effect of the rs3729753 variant in CHD patients. The findings of their study were non-significant for rs3729753 [19]. In Greek patients, a significant association was observed for rs2277923 [20]. This study's findings were in accordance to those reported by Cao et al. in 2015. Similar to this study, they reported association of rs2277923 and rs703752 was significant and non-significant, respectively in the Chinese Bai population. The LD map analysis was also the same as strong linkage disequilibrium was shown between rs703752 and rs2277923 [21]. The current study also investigates the association of maternal hypertension and diabetes with polymorphisms that showed an independent significant role in CHD patients. This study suggested a significant association between maternal hypertension and CHD in children with variant rsrs2295418 (OR = 16.41, p = 0.003), while a weak difference was observed for variant rs360057 and maternal diabetes (OR = 21.60, p = 0.08). Zhao et al. suggested a significant association between maternal diabetes

Table 4 Comparative assessment of variants between different phenotypes of CHD.

Genotypes/Alleles	Control group	VSD group	TOF group	Control vs. VSD group		Control vs. TOF group	
				P value	OR (95%CI)	P value	OR (95%CI)
NKX2-5-rs2277923							
TT	0.457	0.18	0.17				
TC	0.483	0.74	0.76	< 0.0001	3.78 (2.07–6.90)	< 0.0001	4.19 (2.15–8.15)
CC	0.060	0.08	0.07	0.03	3.23 (1.10–9.50)	0.04	3.29 (1.01–10.71)
T	0.7	0.55	0.55				
C	0.3	0.45	0.45	0.0008	1.87 (1.29–2.70)	0.001	1.90 (1.28–2.82)
NKX2-5-rs703752							
CC	0.755	0.76	0.561				
CA	0.212	0.24	0.427	0.69	1.13 (0.62–2.05)	0.0008	2.71 (1.50–4.89)
AA	0.033	0.0	0.012			0.52	0.50 (0.06–4.36)
C	0.861	0.880	0.774				
A	0.139	0.120	0.226	0.53	0.84 (0.49–1.43)	0.017	1.80 (1.10–2.94)
NKX2-5-rs28936670							
GG	0.735	0.51	0.512				
GA	0.265	0.471	0.476	3×10^{-4}	2.57 (1.51–4.36)	0.0016	2.58 (1.46–4.54)
AA	0.0	0.019	0.012				
G	0.868	0.745	0.750				
A	0.132	0.255	0.250	0.0004	2.24 (1.41–3.53)	0.0014	2.18 (1.34–3.54)
NKX2-5- rs3729753							
CC	0.722	0.558	0.488				
CG	0.252	0.183	0.232	0.85	0.94 (0.50–1.78)	0.36	1.36 (0.70–2.63)
GG	0.026	0.260	0.280	< 0.0001	12.69 (4.23–38.01)	< 0.0001	15.67 (5.10-48.11)
C	0.848	0.649	0.604				
G	0.152	0.351	0.396	< 0.0001	3.009 (1.97–4.59)	< 0.0001	3.65 (2.35–5.69)
LEFTY2- rs2295418							
GG	0.914	0.837	0.817				
GA	0.079	0.106	0.122	0.39	1.45 (0.61–3.44)	0.23	1.72 (0.71–4.17)
AA	0.007	0.058	0.061	0.01	9.52 (1.13–80.41)	0.0099	10.30 (1.18–89.91)
G	0.954	0.889	0.878				
A	0.046	0.111	0.122	0.006	2.56 (1.28–5.10)	0.003	2.86 (1.40–5.82)
LEFTY1- rs360057							
TT	0.238	0.087	0.037				
TG	0.563	0.654	0.598	0.003	3.20 (1.44–7.10)	0.0005	6.92 (2.02–23.65)
GG	0.199	0.260	0.366	0.004	3.60 (1.47–8.12)	< 0.0001	12.0 (3.33–43.24)
T	0.520	0.413	0.335				
G	0.480	0.587	0.665	0.02	1.54 (1.07–2.19)	0.00014	2.15 (1.45–3.19)

and rs2277923 (OR = 17.72, p = 0.001) [22]. Another study reported that congenital heart defects in children were associated with maternal hypertension and diabetes [23]. However, no significant association was found between de novo variants in CHD children and maternal diabetes or obesity [24].

To the best of our knowledge, this was the first study that elucidates the association of maternal hypertension with variants in children. However, this study also had several limitations. The first sample size was not large enough due to social and psychological stigma among patients, parents, and their families. Thus current study has limitations for the stratification analysis of maternal risk factors, including the type of therapeutic strategies. The second three genes selected that are potentially involved in the pathogenesis of CHD were selected in this study. Third, information about maternal genotype and disease status should be included in future studies.

Conclusion

In conclusion, all variants showed an independent association with CHD except for one rs703752. However, detailed investigations between different clinical phenotypes suggested that this SNP was potentially involved in TOF patients as compared to VSD. Moreover, some variants showed a higher risk in diabetic mothers or maternal hypertension. This information can provide us with a new direction for CHD prevention so that high-risk mothers should

Table 5 Association of maternal hypertension and maternal diabetes with SNPs in children

Gene/SNP	Genotype	Maternal Hypertension	OR (95%CI)	P value	Maternal Diabetes	OR (95%CI)	P value
NKX2-5-rs2277923	TT	No	1.00	0.25	No	1.00	0.45
	TC	No	3.41 (2.04–5.68)		No	4.13 (2.51–6.81)	
	CC	No	3.71 (1.28–10.75)		No	3.82 (1.18–8.21)	
	TT	Yes	2.47 (0.79–7.71)		Yes	6.70 (1.32–33.98)	
	TC	Yes	15.19 (5.49–42.04)		Yes	8.14 (2.54–26.04)	
	CC	Yes	3.09 (0.70-13.72)		Yes	7.66 (0.82–71.15)	
NKX2-5-rs28936670	GG	No	1.00	0.92	No	1.00	0.53
	GA	No	2.24 (1.38–3.63)		No	2.03 (1.27–3.25)	
	AA	No	---		No	---	
	GG	Yes	3.52 (1.69–7.32)		Yes	3.52 (1.13–10.94)	
	GA	Yes	5.73 (1.62–20.32)		Yes	4.07 (1.13–14.67)	
	AA	Yes	---		Yes	---	
NKX2-5- rs3729753	CC	No	1.00	0.52	No	1.00	0.17
	CG	No	1.04 (0.61–1.79)		No	1.24 (0.73–2.08)	
	GG	No	14.25 (4.27–47.59)		No	13.06 (3.93–43.43)	
	CC	Yes	2.56 (1.20–5.45)		Yes	4.30 (0.91–20.36)	
	CG	Yes	5.73 (1.24–26.56)		Yes	0.72 (0.16–3.28)	
	GG	Yes	16.68 (2.17-128.29)		Yes	15.29 (1.99-117.27)	
LEFTY2- rs2295418	GG	No	1.00	0.0032*	No	1.00	0.55
	GA	No	0.43 (0.15–1.18)		No	1.26 (0.58–2.77)	
	AA	No	3.91 (0.45–33.86)		No	6.18 (0.76–49.99)	
	GG	Yes	1.56 (0.77–3.16)		Yes	1.93 (0.73–5.11)	
	GA	Yes	16.41 (2.18-123.64)		Yes	6.95 (0.87–55.52)	
	AA	Yes	---		Yes	-----	
LEFTY1- rs360057	TT	No	1.00	0.7	No	1.00	0.08
	TG	No	4.42 (2.05–9.54)		No	5.65 (2.66–12.03)	
	GG	No	6.18 (2.67–14.31)		No	7.82 (3.42–17.89)	
	TT	Yes	2.40 (0.46–12.58)		Yes	----	
	TG	Yes	13.51 (4.89–37.32)		Yes	11.40 (3.59–36.17)	
	GG	Yes	41.60 (4.82-358.69)		Yes	21.60 (2.32-200.87)	

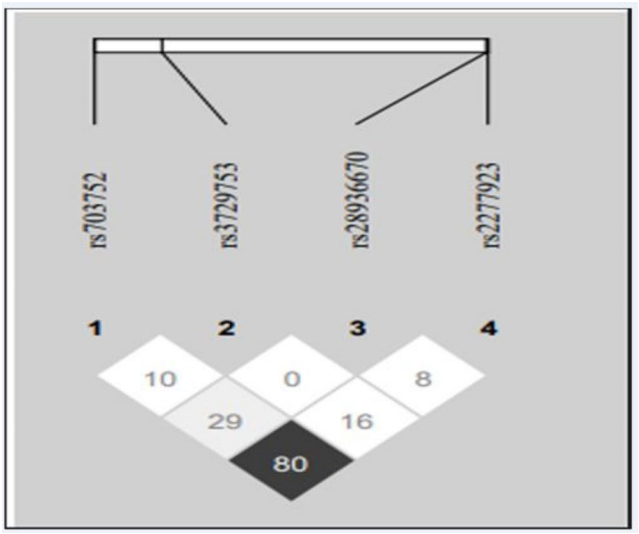

Fig. 1 Linkage disequilibrium map

be screened during pregnancy. Although to the best of our knowledge, this was the first study from a lower-income Pakistani population. However, there is a need to conduct more studies with a larger sample with other parameters analysis. Moreover, SNPs correlation analysis should include association analysis with maternal hypertension and diabetes that can provide more insights into disease pathogenesis and novel direction for future CHD prevention.

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Author Contribution SA conceptualized and designed the study, performed experiments, data collection, and analysis, and wrote, drafted, and proofread the manuscript. MFS supervised the study, designed the study, analyzed, drafted, and proofread the manuscript. All authors have read, approved, and agreed to be accountable for each aspect of the final draft of the manuscript.

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Data Availability The datasets for this manuscript are not publicly available because it is a part of the PhD research work of Ms. Sana Ashiq. Requests to access the datasets should be directed to Sana Ashiq, email sanaashiq72@gmail.com.

Declarations

Consent to participate Informed consent was obtained from the parents of study subjects.

Conflict of interest The authors declare that they have no conflicts of

interest.

Ethics approval This study was approved by the ethical committee of Children's Hospital and The Institute of Child Health, Lahore (2021-282-CHICH) (2) Institutional Review Board Committee of Punjab University (84/DFEMS).

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