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Genetic Variant Screening and Association Study of *NKX2-5* in Congenital Heart Disease Patients From North India

Shadab Ahamad[1]*; Ramya Krishna[2]; Jayashree V[2]; Amrutha Shivaraju[2]; Kavya GK[2]; Ajay Kumar[1]; Subramanian Chellappan[3]; Prachi Kukshal[1]

[1]Sri Sathya Sai Sanjeevani Research Foundation, Palwal, Haryana, India-121102.

[2]Sri Sathya Sai University for Human Excellence, Muddenahalli, Karnataka, India-585313.

[3]Sri Sathya Sai Sanjeevani International Centre for Child Heart Care & Research, Palwal, Haryana, India-121102.

***Corresponding Author(s):**

Shadab Ahamad & Prachi Kukshal

Sri Sathya Sai Sanjeevani Research Foundation, Palwal, Haryana, India-121102.

Tel: +91-8077055361 & 9313254416

Email: shadab.ahamad@srisathyasaisanjeevani.org & prachi7k@gmail.com

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Abstract

Background: Globally 1% of the live births are affected by some form of congenital heart anomaly. Genetics and environment both play a role in its causation but very little of these aspects are explored from the Indian subcontinent. One of the first and key transcription factors required for the formation of the heart during development is *NKX2-5.* Several mutations in this gene have been identified for Congenital Heart Diseases (CHDs). In this study, we screened for known and novel variants to understand their role in CHDs.

Methods: Two exons and flanking 3' and 5' UTR regions of *NKX2-5* were sequenced in n= 71 CHD cases, followed by a case-control test of association and haplotype study.

Results: Only 3 known variants namely rs2277923 (c.63A> G), rs3729753 (c.606G>C), and rs703752 (c.61G>T) were identified in a total of n= 69 cases. Case-control test of association revealed no significant allelic or haplotypic association. A genotypic association was observed for rs703752 in a recessive model ($\chi 2 = 4.4702$; p=0.03; Risk score=0.33), along with a trend of association for rs3729753 ($\chi 2 = 3.73$; p=0.053; Risk score=1.68) and rs703752 (p=0.082).

Conclusion: Although we did not identify any new mutations in the coding regions of the NKX2-5 gene, our findings are important observations and incite for establishing the association between *NKX2-5* variants and cardiac defects in the context of the north Indian population. There is a need to explore the role of other transcription factors, and cardiac developmental pathways and establish their interaction and their role in disease biology in the Indian Subcontinent.

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Introduction

Congenital diseases are defects present at birth, and are assumed to be caused by both genetic and epigenetic factors [1], and are significant public health concerns. Globally 3-4% of the populations of live births are affected by some form of congenital anomaly [2]. Among these diseases, Congenital Heart Disease (CHD) is the most common form with a prevalence of approximately 9/1000 live births worldwide [3]. In India alone every year approximately 2,40,000 children are born with CHD [4]. In low and middle-income countries, the disease burden is often high due to limited access to prenatal care, screening, and treatment, leading to the cause of infant morbidity and mortality [5]. Clinically, CHDs are categorized as cyanotic and acyanotic [6], and the most common sub-phenotypes are Ventricular Septal Defect (VSD), Atrial Septal Defects (ASD), Tetralogy Of Fallot (TOF), Patent Ductus Arteriosus (PDA), and transposition of great arteries (TGA) [7].

Genetics plays a vital role in understanding the recurrence of congenital anomalies in families [8]. Chromosomal aneuploidies (9-18%), Copy Number Variants (CNVs) (10-15%; 3-25% in syndromic and 3-10% in non-syndromic CHD), and single gene disorders (12%) are the main genetic causes of CHD [9]. The transcription factors expressed predominantly in the heart mediate the expression of genes encoding cardiac structural proteins or regulatory proteins, which are essential for the normal development of the heart. Non-syndromic CHDs are usually characterized by multiple mutations that affect intricate inter-connected processes and can regulate several downstream developmental pathways leading to congenital defects [10].

A gene regulatory network that includes several signal transduction pathways and cardiac transcription factors like GATA binding protein 4 (*GATA4)*, NK2 homeobox 5 (*NKX2-5)* and T-Box factors (*TBX*) tightly control the events that take place during the development of the heart and interacts with *GATA4*, *TBX*, serum response factor (*SRF*) [11], Helix-loop-helix (*HEY2)* [12] and Myocyte Enhancer Factor *(MEF2)* [13] in early cardiac development. Rare alleles and mutations in such transcription factors would lead to malformation of the heart. The NKX2-5 gene is the most studied and the first gene reported to be associated with cardiogenesis [14]. It regulates cardiac progenitors, cardiac morphogenesis, cardiomyocyte differentiation, and conduction system development [15] and is involved in a spectrum of CHD subphenotypes [16]. It has also been shown to be involved in conditional tumor suppressor genes [17], thyroid hemiagenesis [18], and dilated cardiomyopathy [19]. *NKX2-5* is also required for adult myocardial repair [20]. A total of 11 miRNAs expressed in *NKX2-5* expressing cardiac Vs non-cardiac mesoderm were seen [21]. Expression of *NKX2-5* in mice [22], zebrafish [23], and chick [24] early cardiac progenitor cells suggests that it is essential for cardiomyogenesis. Animal Models suggest haploinsufficient experimental studies on mice with germline disruption of the *NKX2-5* have shown that mice before birth with abnormal looping hearts and *NKX2-5* knockout at the mid-embryonic stage cause premature death and defective cardiac morphogenesis [15]. Thus, *NKX2-5* has been a much-explored candidate gene for CHDs and is associated with both syndromic and non-syndromic CHDs [25].

Structure of NKX2.5

NKX2-5 is a DNA-binding transcriptional activator and a member of the evolutionarily conserved NK homeobox gene family, which plays a crucial role in organogenesis, and is expressed in a variety of tissues during development, including the heart, lung, and thyroid gland [26]. The *NKX2-5 (*5q35.1), which codes for a 324 amino acid protein, has two primary exons [27]. Similar to other members of the NK2 family of transcription factors, it contains a highly conserved Homeodomain (HD) (residues 138-197), that has a helix-loop-helix domain with three alpha helices, allowing it to interact with DNA.

The protein typically includes several other highly conserved structural domains in addition to the homeobox. Near the protein's N-terminal region (10-21 AA), a Tinman domain likely functions as a transcriptional repressor. A less conserved linker region connects an NK2-Specific Domain (NK2-SD, 212-234 AA) to HD [28]. A Single-Nucleotide Polymorphism (SNP) and mutations in this gene can alter the function of a gene, which would cause abnormal cardiac morphogenesis [10]. A total of 7 mutations [rs17052019 and rs2277923 (exon 1), rs3729938 rs3729753, and rs3729754 (exon 2), & rs703752 and rs11552707 (3'UTR)] have been reported to be involved in congenital heart malformation [29]. Earlier, mutations and variations in the *NKX2-5* have been linked to various CHDs, including ASD, VSD, TOF [30], and other subphenotypes [31]. The relationship between NKX2-5 gene variations and CHD susceptibility was assessed using a thorough meta-analysis program, and the results indicated that the rs703752 and rs2277923 polymorphisms of the NKX2-5 gene are associated with CHD [32]. In addition, altered expression of *NKX2-5* has been observed in various forms of heart disease, such as dilated cardiomyopathy and arrhythmogenic right ventricular dysplasia [33]. To date, more than 60 mutations have been found in the NKX2-5 gene [34]. A systematic study on genetic variants of NKX2-5 showed approximately 970 variants in the global population, of which 143 are identified to be pathogenic for humans. Among these 143, around 30 SNPs are found in the non-coding region which impacts transcription factor-DNA binding affinity, and ultimately can be suggested as key to establishing a pathogenic mechanism [35]. The homeodomain of this gene carries the maximum pathogenic variant i.e. 49 in 60 residues [36]. The variants rs703752 and rs2277923 have been previously associated with CHDs with p-values of 0.049 and 0.036 respectively in global populations [32]. As per the Genome-wide association studies catalog, two SNPs, rs6891790-T and rs6882776-A, are highly associated, with Minor Allele Frequency (MAF) of 0.346 and 0.418, and p-values of 3×10^{-26} [37] and 1×10^{-22} [38] respectively and both having role in atrial fibrillation. Seven novel mutations, including 4 missense variants were identified in *NKX2-5* among 26 individuals having atrioventricular conduction block with/without other CHDs [10]. However, c.95 A > T missense mutation in this gene is found to be pathogenic and may cause CHD when studied *in silico* in an Iranian population [28]. Though several associations from the intronic regions also have been seen there are difficulties in establishing their role in disease biology [35]. Very few of these have been functionally characterized [10]. These results strongly imply that *NKX2-5* is a key player in cardiac morphogenesis, cardiac function, and maturation. Therefore, the current study aimed to screen for the known genetic variants and identify novel mutations if any in *NKX2-5*.

Materials and Methods

Study design

The study was conducted at Sri Sathya Sai Sanjeevani Research Foundation, Palwal after Institution Ethics Committee (IEC) approval. A total of n= 86 non-syndromic CHD cases (n=21

ASD, n=20 VSD, n=20 TOF, and n=25 Misc.) from the north Indian cohort who underwent CATH or surgical corrective procedures at dedicated tertiary center Sri Sathya Sai Sanjeevani International Centre for Child Heart Care & Research, Palwal (Haryana) were included in the study. All the subjects underwent a detailed clinical investigation with a detailed medical history and confirmed by an Echocardiogram (ECHO). Informed consent was obtained from all the subjects.

Extraction of genomic DNA and PCR amplification

DNA extracted using the conventional phenol-chloroform method followed by requisite quality quantity check was used to screen for coding region with exon-intron boundaries (10 base pairs) of *NKX2-5.* Primers used to amplify the exons, and 5' and 3' untranslated (UTR) regions of *NKX2-5* were designed using Primer 3 tool (https://primer3.ut.ee / 4.1.0) (Supplementary Table 1). PCR was performed on a Veriti thermal cycle (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and amplified products were visualized on a 2% agarose gel. Samples were sequenced through a commercial facility. Out of 86 north Indian CHD samples screened, 15 were excluded either due to bad reads or incomplete information for all 4 sequence regions; hence n= 71 samples [n=15 ASD, n=18 VSD, n=18 TOF and n=20 Misc. including Tricuspid atresia-4, TGA-1, Atrioventricular canal defect-4, Total anomalous pulmonary venous connection-2, Aorto pulmonary window-3, Double outlet right ventricle-1, Coarctation of aorta-1, Pulmonary stenosis-1, Hemi-truncus-1, Sub-aortic membrane+PDA-1, Anomalous Left Coronary Artery From Pulmonary Artery (ALCAPA)-1] were finally included for analysis.

Variation detection and statistical analysis

The samples were anonymized and sequenced using the Sanger sequencing method at a commercial facility. Sequencing profiles were analyzed using ApE (A Plasmid Editor) software [39] and matched to the reference sequence. The 71 cases were compared to n=490 open source 1000 Genome South Asian data for a case-control allelic, genotypic, and sliding window haplotypic association using Plink 1.90 beta software (https://zzz.bwh.harvard.edu/plink/cite.shtml) [40]. P<0.05 was regarded as statistically significant.

Results

Using an ensemble database (https://www.ensembl.org/), more than 1300 variants have been reported, of these, we screened nearly 900-1000 screened (90 in 5'UTR; 231 in E1; 513 in E2; and 142 in 3'UTR) which included insertions, deletions, synonymous and missense mutations [36].

Mutational analysis: There were no novel mutations seen in the study cohort.

Variant screening: We detected 3 known benign variations, namely rs2277923 (c.63A>G) in exon 1, rs3729753 (c.606G>C) in exon 2, and rs703752 (c.61G>T) in 3'UTR of *NKX2-5* in the study population (Figure 1). rs2277923 is a highly polymorphic missense variant with a global and South Asian (SAS) MAF of 0.46. The other two SNPs, rs3729753 and rs703752 had a global/SAS MAF of 0.026/0.066 and 0.256/0.361 respectively.

Case-control association study: All three SNPs were in Hardy Weinberg Equilibrium (P≥0.01). No allelic association was observed for the three variants found in the study samples (Table 1). In the genotypic model, one SNP rs703752 showed association in a recessive inheritance model ($\chi2 = 4.47$; p=0.03; Odd ratio=0.3; Risk score=0.33), along with the trend of association ($\chi2 = 4.64$; p=0.082) in the genotypic association test. The variant, rs3729753, also showed a genotypic trend of association ($\chi2 = 3.73$; p=0.053; Odd ratio=1.84; Risk Score=1.68) in the dominant model (Table 2).

Sliding window haplotypic study: Haplotypic analysis also did not yield any clues (Table 3).

Table 1: Allelic association of reported SNPs.

SNP	Category	A1	A2	F_A	F_U	ChiSq;p	OR
rs703752 3'UTR c.*61G > T	ASD	T	G	0.30	0.36	0.47; 0.49	0.76
	VSD	T	G	0.33	0.36	0.12; 0.74	0.89
	TOF	T	G	0.28	0.36	1.05; 0.31	0.68
	MISC	T	G	0.33	0.36	0.22; 0.64	0.85
	ALL	T	G	0.31	0.36	1.41; 0.23	0.79
rs3729753 Exon 2 c. 606 G > C	ASD	C	G	0.13	0.07	2.04; 0.15	2.16
	VSD	C	G	0.11	0.07	1.09; 0.29	1.76
	TOF	C	G	0.11	0.07	1.09; 0.29	1.76
	MISC	C	G	0.08	0.07	0.045; 0.83	1.14
	ALL	C	G	0.11	0.07	2.87; 0.09	1.66
rs2277923 Exon 1 63A > G	ASD	G	A	0.40	0.46	0.42; 0.52	0.78
	VSD	G	A	0.39	0.46	0.71; 0.39	0.75
	TOF	G	A	0.47	0.46	0.02; 0.89	1.05
	MISC	G	A	0.50	0.46	0.25; 0.62	1.17
	ALL	G	A	0.44	0.46	0.14; 0.71	0.94

A1: minor allele 1; A2: major allele 2; F_A: frequency of A1 in cases ; F_U: frequency of A1 in controls; ChiSq: Pearson's correlation; p: significance; OR: odd ratio; MISC: other miscellaneous CHD type.

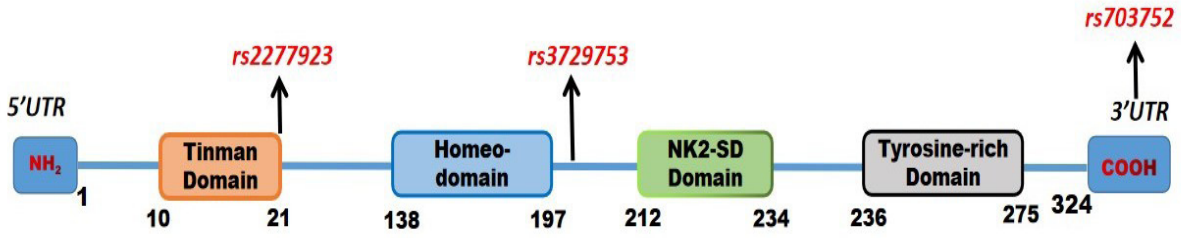

Figure 1: Diagrammatic representation of the structure of NKX2-5 and reported SNPs in our study.

*Fishers test p values (in cell counts less than five). Significant p value is in bold font

**genotype counts of variant homozygous\heterozygous\wildtype homozygous for each category are denoted

Table 2: Genotypic association of reported SNPs.

SNP	A1	A2	TEST	UNAFF	ASD		VSD		TOF		MISC		ALL	
					AFF	P	AFF	P	AFF	P	AFF	P	AFF	P
rs703752	T	G	GENO**	63\227\199	1\7\7	0.94	1\10\7	0.71	0\10\8	0.29	1\11\8	0.66	3\38\30	0.082
	T	G	TREND	353\625	9\21	0.49	12\24	0.73	10\26	0.30	13\27	0.64	44\98	0.23
	T	G	ALLELIC	353\625	9\21	0.56	12\24	0.86	10\26	0.38	13\27	0.74	44\98	0.26
	T	G	DOM	290\199	8\7	0.79	11\7	1*	10\8	0.81	12\8	1*	41\30	0.79
	T	G	REC	63\426	1\14	0.71	1\17	0.71	0\18	0.15	1\19	0.49	3\68	**0.0307**
rs3729753	C	G	GENO**	3\59\427	0\4\11	0.18	0\4\14	0.34	0\4\14	0.34	0\3\17	0.76	0\15\56	0.12
	C	G	TREND	65\913	4\26	0.16	4\32	0.30	4\32	0.30	3\37	0.83	15\127	0.091
	C	G	ALLELIC	65\913	4\26	0.14	4\32	0.30	4\32	0.30	3\37	0.75	15\127	0.11
	C	G	DOM	62\427	4\11	0.12	4\14	0.27	4\14	0.27	3\17	0.73	15\56	0.053
	C	G	REC	3\486	0\15	1*	0\18	1*	0\18	1*	0\20	1*	0\71	1*
rs2277923	G	A	GENO**	97\256\136	2\8\5	0.83	3\8\7	0.59	2\13\3	0.36	4\12\4	0.79	11\41\19	0.66
	G	A	TREND	450\528	12\18	0.50	14\22	0.39	17\19	0.88	20\20	0.61	63\79	0.70
	G	A	ALLELIC	450\528	12\18	0.58	14\22	0.49	17\19	1*	20\20	0.63	63\79	0.72
	G	A	DOM	353\136	10\5	0.77	11\7	0.29	15\3	0.42	16\4	0.61	52\19	1*
	G	A	REC	97\392	2\13	0.75	3\15	1*	2\16	0.55	4\16	1*	11\60	0.43

Sup Table 1: *NKX2-5* specific primers.

Region	Primer sequences (5'-3')	Annealing Temperature (°C)	Product size (bp)
Exon1 + 5'UTR (E1)	Forward: TATTAGGTGACACGAAACTGCTCAT Reverse: TTTCGTCCCCAAGAAACTCAGG	54	653
Exon 2 (E2)	Forward: GCGCTCCGTAGGTCAAGC Reverse: CGTGGGCCTCAATCCCTA	60	469
Exon2+ UTR (E2U)	Forward: ACCGGCGCTACAAGTGCAAGC Reverse: CGTTTGTTCTTGCGCACGGGA	58	585
3'UTR (U)	Forward: GATCCCACCTCAACAGCTCC Reverse: GCATTCCCTGAGCAAATTGATAACA	62	543

Table 3: Haplotype analysis of reported SNP.

DF: degree of freedom.

SNPS	HAPLOTYPE	F_U	ASD			VSD			TOF			MISC			ALL		
			F_A	CHISQ	DF	P	F_A	CHISQ	DF	P	F_A	CHISQ	DF	P	F_A	CHISQ	DF	P
rs703752|rs3729753	OMNIBUS	NA	NA	2.20	2	0.33	NA	1.11	2	0.57	NA	1.78	2	0.41	NA	0.23	2	0.89	NA	3.61	2	0.17
rs703752|rs3729753	CG	0.07	0.13	2.04	1	0.15	0.11	1.09	1	0.29	0.11	1.09	1	0.29	0.08	0.05	1	0.83	0.11	2.87	1	0.09
rs703752|rs3729753	AC	0.36	0.30	0.47	1	0.49	0.33	0.12	1	0.73	0.28	1.05	1	0.31	0.33	0.22	1	0.64	0.31	1.41	1	0.23
rs703752|rs3729753	CC	0.57	0.57	0.004	1	0.95	0.56	0.04	1	0.84	0.61	0.21	1	0.65	0.60	0.12	1	0.73	0.59	0.07	1	0.79
rs3729753|rs2277923	OMNIBUS	NA	NA	1.13	2	0.57	NA	2.54	2	0.28	NA	1.12	2	0.57	NA	0.25	2	0.88	NA	2.52	2	0.28
rs3729753|rs2277923	GC	0.07	0.10	0.58	1	0.45	0.11	1.09	1	0.29	0.11	1.09	1	0.29	0.08	0.05	1	0.83	0.09	1.97	1	0.16
rs3729753|rs2277923	CC	0.39	0.31	0.79	1	0.37	0.28	1.96	1	0.16	0.36	0.15	1	0.69	0.43	0.16	1	0.69	0.35	1.06	1	0.30
rs3729753|rs2277923	CT	0.54	0.59	0.24	1	0.63	0.61	0.71	1	0.39	0.53	0.02	1	0.89	0.50	0.25	1	0.62	0.55	0.08	1	0.77
rs703752|rs3729753|rs2277923	OMNIBUS	NA	NA	2.60	3	0.46	NA	4.07	3	0.25	NA	2.69	3	0.44	NA	0.28	3	0.96	NA	5.59	3	0.13
rs703752|rs3729753|rs2277923	CGC	0.07	0.10	0.58	1	0.45	0.11	1.09	1	0.29	0.11	1.09	1	0.29	0.08	0.05	1	0.83	0.09	1.92	1	0.16
rs703752|rs3729753|rs2277923	CCC	0.39	0.31	0.78	1	0.38	0.28	1.94	1	0.16	0.36	0.15	1	0.69	0.43	0.16	1	0.69	0.35	1.01	1	0.32
rs703752|rs3729753|rs2277923	ACT	0.36	0.31	0.30	1	0.58	0.33	0.11	1	0.74	0.28	1.03	1	0.31	0.33	0.21	1	0.65	0.31	1.24	1	0.26
rs703752|rs3729753|rs2277923	CCT	0.18	0.27	1.70	1	0.19	0.28	2.21	1	0.14	0.25	1.14	1	0.29	0.18	0.007	1	0.93	0.24	2.94	1	0.087

Discussion

Previous studies in the Indian population have reported 6 Novel mutations namely c.239A>G (p.E21E), c.896C>A (p.A240A), c.608A>G (p.E203G), c.646C>T (p.R216C), c.852G>A (p.N226D) and c.1212G>T in south Indian population have previously been reported [41]. Another study revealed a total of 7 mutations, 3 in the intronic region, 3 in the coding region, and 1 in 3' UTR [42]. A recent study by Dixit et al [43] in a nortern population of India has identified 13 genetic variants, out of which 3 were novel, namely c.182C > G, c.391G > A, and c.443C > A. A novel synonymous variation leading to G>C trans-conversion in the exon 2 region in the Kashmiri population was reported [44].

In this present study, we identified three previously reported benign variants in the NKX2-5 gene, namely rs3729753, a synonymous variant in Exon2 [45], rs2277923, a missense variant located in exon 1 [46] and a 3' UTR variant, rs703752 [41]. No mutation or rare variants were observed in the study. Of the three common variants only 3' UTR variant, rs703752 demonstrated marginal genotypic association in a recessive model (p=0.03). rs2277923 a well-associated exonic SNP [29,42] and significantly associated with the risk of CHD in offspring in Diabetic mothers [47] was non-significant in the present study confirming previous findings [48,46]. This SNP has also been associated with controlling blood pressure ($p= 7 \times 10^{-14}$) [49]. Another study on the same gene in the Indian population also did not find any mutation and the same SNP, rs2277923, was found significantly higher [50].

This may be due to the limited numbers of samples screened, genetic and phenotypic heterogeneity, and various other environmental factors not assayed here. While several studies primarily involved analyzing the exonic regions of the gene have variable findings the focus of recent research has shifted to exploring the promoter regions and analyzing intronic variants too. However, these limitations do not diminish the significance of our findings, which provide valuable insights into the genetic basis of congenital heart diseases. This work needs large-scale investigations and may suggest avenues for further research.

Conclusion

We understand little of the underlying molecular machinery of CHDs and efforts to understand its biology are growing by leaps and bounds. Although we did not identify any new mutations in the coding regions of the NKX2-5 gene, our findings are important observations and incite for establishing the association between *NKX2-5* variants and cardiac defects in the context of the north Indian population. These findings would further aim at exploring the variations in a larger population, targeting the specific variations, and the genetic variations with clinical outcomes which could trigger the development of gene therapies and targeted drugs. Further research in these areas could provide a valuable understanding of the complex mechanisms underlying congenital heart diseases and guide the development of more effective treatment strategies.

Abbreviations

AA: Amino Acid; ASD: Atrial Septal Defect; CHD: Congenital Heart Disease; CNV: Copy Number Variation; GATA4: GATA Binding Protein 4; HD: Homeodomain; HEY2: Helix Loop Helix; MAF: Minor Allele Frequency; MEF2: Myocyte Enhancer Factor; NK2-SD: NK-2 Specific Domain; NKX2-5:NK2 Homeobox 5; PCR: Polymerase Chain Reaction; PDA: Patent Ductus Arteriosus; SAS: South Asian Population; SNP: Single Nucleotide Polymorphism; SRF: Serum Response Factor; TBX: T-Box Factor; TGA: Transposition Of Great Arteries; TOF: Tetralogy Of Fallot; UTR: Untranslated Region; VSD: Ventricular Septal Defect.

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Authors' Contributions

Shadab Ahamad: Conceptualization, Experimental analysis, data curation, writing original draft, review, and editing. Ramya Krishna: Experimental work. Jayashree: Experimental work. Amrutha S: Experimental work. Kavya GK: Experimental work. Ajay Kumar: Sample collection and patient history taking. Subramanian Chellappan: Provided samples. Prachi Kukshal: Conceptualization, review, and editing of draft. All authors read and approved the final manuscript.

Declarations

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Conflict of Interest: The authors have no conflicts of interest to declare.

Ethics approval and consent to participate: Ethics approval from Institutional Ethics Committee (IEC), Sri Sathya Sai Sanjeevani Research Foundation (SSSSRF) registered with National Ethics Committee Registry for Biomedical and Health Research, Department of Health Research (File no.: EC/NEW/INST/2022/2673) was granted under the number PSR00007/1/IEC/2/2019. Written informed consent was obtained for all participants and parents/ guardians and assent was provided by children above 8 years of age as per IEC, SSSSRF, and ICMR guidelines. Involvement in the study was voluntary and there were no repercussions for non-participation. This study was performed in accordance with the declaration of Helsinki.

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