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Missense mutations in the CITED2 gene may contribute to congenital heart disease

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Abstract

Background Congenital heart disease (CHD) is a lifelong abnormality present from birth. Multiple studies have shown that mutations in genes involved in heart development could cause congenital heart disease. The *CITED2* gene works as a transcription factor in the hypoxic pathway for the development of the heart. Therefore, five CHD types, ventricular septal defect, atrial septal defect, atrioventricular septal defect, tetralogy of fallot, and patent ductus arteriosus, were evaluated by conducting a targeted single nucleotide polymorphism (SNP) analysis of the CITED2 gene variant rs375393125 (T > C). This study aimed to identify the association of *CITED2* gene mutations in CHD patients.

Methods Three hundred fifty samples, 250 from patients and 100 from controls, were collected for this genetic analysis. Allele-specific PCR and gel electrophoresis were used to identify the target missense mutations. The genotypic results of the CHDs were further validated through Sanger sequencing.

Results The frequency of the homozygous mutant (CC) in CHD patients was 48.4%, and of the heterozygous mutant (TC) genotype was 11.4%; these percentages are higher than controls (1%). The control samples had only one heterozygous TC and no homozygous CC genotype. The chi-square value was obtained at 103.9 with a probability of 0.05, more significant than the significance value of 21.03. The odds ratio was 43.7, which is > 1. The calculated value of ANOVA was 11.6, which was more significant than the F critical value of 3.7. As a result of sequencing, the mutant sample of each selected CHD type was found heterozygous or homozygous, and the results were like those obtained through conventional PCR.

Conclusion The samples of CHD patients showed mutations. Therefore, the CITED2 gene SNP might be associated with CHD.

Keywords Congenital heart disease (CHD), CITED2 gene, Single nucleotide polymorphism (SNP), Allele-specific PCR, Genotype, Sequencing

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Introduction

Structural abnormalities in the heart during different developmental stages can lead to congenital heart disease (CHD) [16, 19, 24]. The prevalence of CHDs was reported at 1% in previous research studies [25, 32]. The four chambers of the heart are formed through the transformation of a single tube through growth, remodelling, and morphogenesis during the development of the fetus [7, 12]. It has been demonstrated that any change in these processing steps could cause CHDs [10].

Congenital heart diseases are fundamentally divided into cyanotic and acyanotic CHDs. This research

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focused on patients with both elevated and low pulmonary blood flow. The pulmonary blood flow defects that increase in size include Ventricular Septal Defect (VSD), Atrial Septal Defect (ASD), Atrioventricular Septal Defect (AVSD), and Patent Ductus Arteriosus (PDA). In contrast, the pulmonary blood flow defect that decreases in size is Tetralogy of Fallot (TOF) [1]. Multiple genes are reportedly associated with CHD. Many of these are involved in the developmental pathways of the heart [11]. The mutation may result in congenital heart abnormalities and alter the transcriptional processes of these genes [4]. However, multiple factors involved in CHDs were found to be related ($p < 0.05$), which might be due to having children with congenital heart defects (CHDs), with some other causes being consanguinity, maternal diabetes mellitus (DM), maternal hypertension (HTN), maternal infection, maternal smoking, maternal caffeine intake, maternal history of medication use and low birth weight [9]. According to Şanlı et al. [26], Twenty-four to twenty-eight parents were observed in cases of consanguinity. The first-degree consanguinity was a constant factor for 5% of the patients, and it was featured in 18 patients (15%). The contribution of the genetic factors is estimated to be as high as 50%, and a distant connection to one or more relatives was found in 10 people (8%). Consanguinity was recognized as an independent risk factor for CHD in the multivariate analysis, with an adjusted odds ratio of 2. 59 (95% CI 1. 73 – 3. 87) [13]. Multiple research studies reveal that there is a similar prevalence of CHDs in both genders [15, 23]. In the period of study, the researchers identified 1,127 CHD patients with a male-to-female ratio of 1:1.1. There are few differences in gender composition in congenital heart disease. Khoshhal et al. [17] state that males are predominant for VSD, TOF, CoA, TGA, and Truncus arteriosus. However, PDA, ASD, and AVSD are more likely to appear in females.

Four genes in the Glu/Asp-rich carboxy-terminal domain gene family are part of the Cbp/p300-interacting transactivator, transcriptional activator family [18]. Only the *CITED* [18] gene encodes the hypoxia-inducible transcriptional coactivator nuclear protein. The regulation of *HIF1a-responsive* genes depends on the CITED2 protein through its binding with the p300/CBP protein [21, 28]. Hypoxia, cytokines, and oxidative stress stimulate the transcription of the *CITED2* gene. The "ACGTG" sequence of the *CITED2* promoter region is stably related to the SPI, AP-2, and HIF-1 [31]. The CITED2 protein is the only protein in this family that does not have a serine-glycine-rich junction (SRJ) [20]. The other proteins all had three conserved regions. This protein has CAD and NAD terminals [3]. Furthermore, this protein regulates the transcription of TFAP2, SMAD2, estrogen receptor, and hypoxia-inducible factor 1 [2].

HIF1a is known to be more highly expressed when the *CITED2* gene is mutated, even though the *CITED2* gene is thought to function as a transcription factor for the regular expression of HIF1a. In particular, overexpression of HIF 1a causes ASD, VSD, and TOF [30]. Therefore, the missense mutation rs375393125 (T > C) of the *CITED2* gene was chosen to evaluate CHD patients.

Methodology

Research plan

The current research work is a case-control study. The cases and controls were selected from the same population of the same age and gender. Conclusively, the results were investigated by comparing cases and controls.

Ethical approval and sample collection

This study was approved by the institutional review board of The Karachi Institute of Biotechnology and Genetic Engineering (KIBGE), University of Karachi, with Reference no: KIBGE/ICE/0271/16/02/2021, and National Institute of Cardiovascular Diseases (NICVD) Karachi "ERC-16/2019" with the confirmation of clinical trial number "ASBR/No/05275/Sci/2020". Before sample collection, informed consent was taken from all the study subjects (cases and controls). The detailed history of participants were taken through the study questionnaire to obtain retrospective information. Then, the whole blood from each subject was collected (2-5 ml) in an Ethylenediamine tetra-acetic acid (EDTA) tube and stored at -20 °C for further genomic analysis.

Sample size

The total samples for the current study were 350. There were 250 patients; among them, 50 samples were collected for each type of CHD (VSD, ASD, AVSDs, TOFs, and PDAs), while 100 samples were collected for the control with the same diagnosis procedure used for patients to validate the actual sampling.

Inclusion and exclusion criteria

The criteria for sample collection included patients who were diagnosed with congenital heart defects (CHDs) confirmed through echocardiography tests and who were less than 15 years of age. The controls samples were selected based on normal echocardiography results and had no history of cardiac disorders with same age range of patients (6 months to 15 years). Patients without diagnosed CHDs, syndromic CHD patients, those with a familial history of CHDs, and individuals with additional disabilities or complex congenital heart disease were excluded.

DNA extraction, amplification and sequencing

DNA extraction was performed manually from 1 ml of blood through the salting-out method. The quality and quantity of the DNA were checked using NanoDrop and gel electrophoresis techniques. The targeted SNP rs375393125 (T > C) was searched through PubMed. The allele-specific PCR was used to amplify targeted SNPs. The allele-specific primers (Table 1) were designed by using Primer 1 software through the template reference sequence > NC_000006.12. In each PCR, two DNA templates, 2 µl of nuclease-free water, 0.5 µl of each of the primers (0.5 µl forward wild type primer, 0.5 µl forward mutant primer, 0.5 µl reverse common primer, and 10 µl of the master mixture (Thermoscientific) were used. The total volume of a single reaction was 15 µl. In the optimized PCR condition, first denaturation temperature was 94 °C for 5 min, the second denaturation temperature was 94 °C for 30 s, the specific annealing temperature was 59 °C for 30 s, and the extension temperature was 72 °C for 45 s, overall PCR run was included 40 cycles. The last extension was for 5 min at 72 °C and held at 4 °C.

The amplified PCR products were subjected to agarose gel electrophoresis. The size of the targeted gene was 254 base pairs (bp). Therefore, a 2.5% agarose gel was prepared, and 6 µl of visualana dye was added to visualize targeted DNA bands. Then, 15 µl of amplified PCR product was loaded into the agarose gel and run for 45 min. The DNA bands were visualized through a gel documentation system (MoleQule-ON) under UV light to capture the image for genotyping (Fig. 2). After genotypic analysis, random samples were selected from each CHD type with mutant genotypes (CC or TC) and wild-type control (TT). The total volume of 0.6 µl purified DNA samples (Target region of CITED2 gene) were used for Sanger sequencing. Then, the results were visualized through Clustal W (Omega 7 software) to match the targeted DNA sequences of samples with the reference sequence. ABI sequencing software was used to validate the peak of nucleotides through the preprogram (Fig. 4). Omega 7 was used first to confirm the mutation, and then it was observed through ABI sequencing software to check the quality of nucleotide peak.

Table 1 Allele-specific primers were used for the *CITED2* gene

Gene	Exon	Forward Primer	Reverse Primer (C)	Size
CITED	2	W: GGG GGTAGG GGTGAT GGT M: GGG GTAGGG GTGATGGC	CGCCTT CAACGC CCTAAT	254 bp

M Mutant, *W* Wild type, *C* common

Results

Genotyping of the *CITED2* SNP (rs375393125) in CHD patients

All samples and controls were genotyped from the PCR gel-electrophoresis image results. The genotypic frequencies of the controls and CHD patients were CHD patients and controls. The homozygous wild-type TT genotype frequency was more significant in controls than in CHD patients. In contrast, the frequency of the homozygous mutant CC genotype was greater in CHD patients than in controls, although the TC genotype was rare in all CHD samples (Figs. 1 and 2).

Frequency of rs375393125 mutation (T > C)

The number of T alleles was more significant in controls than in patients (Fig. 3A & B). All the controls had T alleles; consequently, higher C allele frequency was found in CHD patients, indicating that the mutant allele might affect the heart's development.

Statistical analysis

Statistical tests, chi-square tests, odds ratios, and ANOVA were performed to analyze *CITED2* gene mutations in CHD patients.

Chi-square test

The chi-square test showed a greater tabulated value than was calculated (103 > 21.03), with a p value < 0.05 and 12 degrees of freedom (Table 2). Missense mutations contribute to CHD incidence.

Odds ratio

The odds ratio, found to have a value greater than the critical value of 1 (43.7), was used to evaluate the strength of association between research subjects (CHD patients and controls) and the *CITED2* genotypes TT, TC and CC (Table 3). It is suggested that 43.7 times more *CITED2* gene mutations were found in CHD patients than in controls. This strong association suggested that it might be associated with CHDs.

Variation analysis

A one-way ANOVA found significant differences among the different CHD types and genotype groups. The data for the selected SNPs were arranged according to different genotypes. The three groups, TT, TC, and CC, exhibited significant variation. The CC genotype showed more variation than the TC genotype, while the F value 11 was more significant than the F critical 3.7

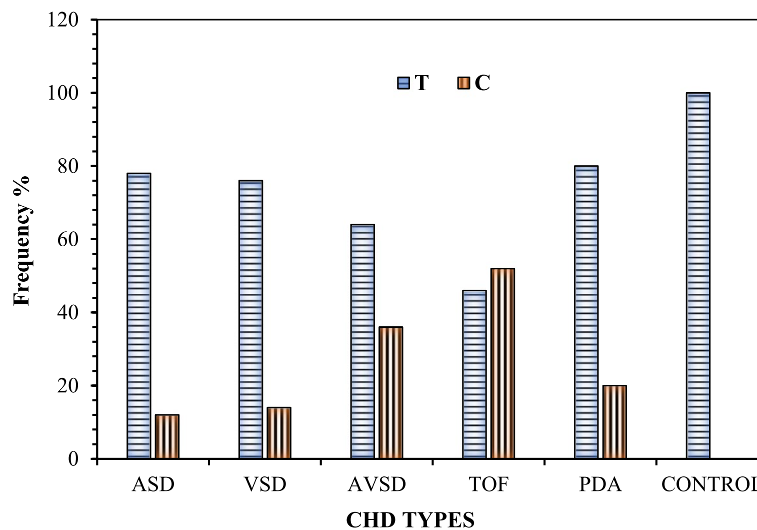

Fig. 1 Different colors differentiate the genotypes. The numbers on the bar graph represent the exact genotypic frequencies of CHD types and controls

with a degree of freedom of 2 and a *p*-value of 0.00091 (Table 4). Furthermore, CHD did not significantly vary from the control. The control group was found to have only the TT genotype, while CHD with the TC and CC genotypes may be correlated with CHD. Therefore, different CHD types are associated with genotypic variations.

Sanger sequencing

Sanger sequencing revealed that all CHD samples had *CITED2* mutations. VSD, ASD, AVSD, TOF, and PDA were found with the TC and CC genotypes, while the control had both TT genotypes (Table 4). All the results were validated against completely aligned PCR results.

The sequencing results were evaluated through Mega 7 software, revealing the proper mutation detected through PCR. Furthermore, ABI software was used to assess the program peaks and validate the results. Each sample had clear and prominent peaks in homozygous and heterozygous forms (Fig. 4). Finally, the sequencing and PCR results were cross-matched, and similar results were found.

Reported frequencies

Multiple studies have shown that rs375393125 is a sporadic mutation in a different population. Likewise, the Grand Opportunity Exome Sequencing Project (Go-ESP) found the C allele frequency is approximately 0.000077/1, whereas the Genome Aggregation Database Exome project (Gnom-AD) showed 0.000004/1, Gnom-AD 0.000032/1 (Fig. 5). The present study revealed a 30.8% C allele frequency in CHD patients, while only one was observed in the heterozygous form in normal individuals. The results suggest that CHD patients are susceptible to the C allele.

Analysis of risk factors

The gender of the patient, consanguinity and addiction history of the parents were taken as risk factors for CHD. The results showed that both groups had approximately male to female ratio, consanguinity, and bad addiction of smoking or chewing. However, comparing the two groups, the controls had greater proportions of consanguinity (Fig. 6). These findings showed that the general risk factors evaluated in the present study may not be associated with CHDs.

Discussion

The developmental pathways of genes *GATA4*, *NKx2.5*, *FOXH1*, *HAND2*, *20TBX20*, *MEF2c*, and *CITED2*) result in a healthy functional heart, whereas mutated genes result in a heart malfunctioning [11, 30]. In China and Germany, the serine-glycerin-rich region of the *CITED2* gene is reportedly the highly mutated domain [8]. The common defects are VSD, ASD, AVSD, TOF, and PDA [27]. The current research is based on five heart defects.

The activity of heart-associated genes (HIF-1α and TFAP2c) is influenced by the methylated and mutated transcription factor gene *CITED2* in CHD patients. According to one study, CHDs can be caused by

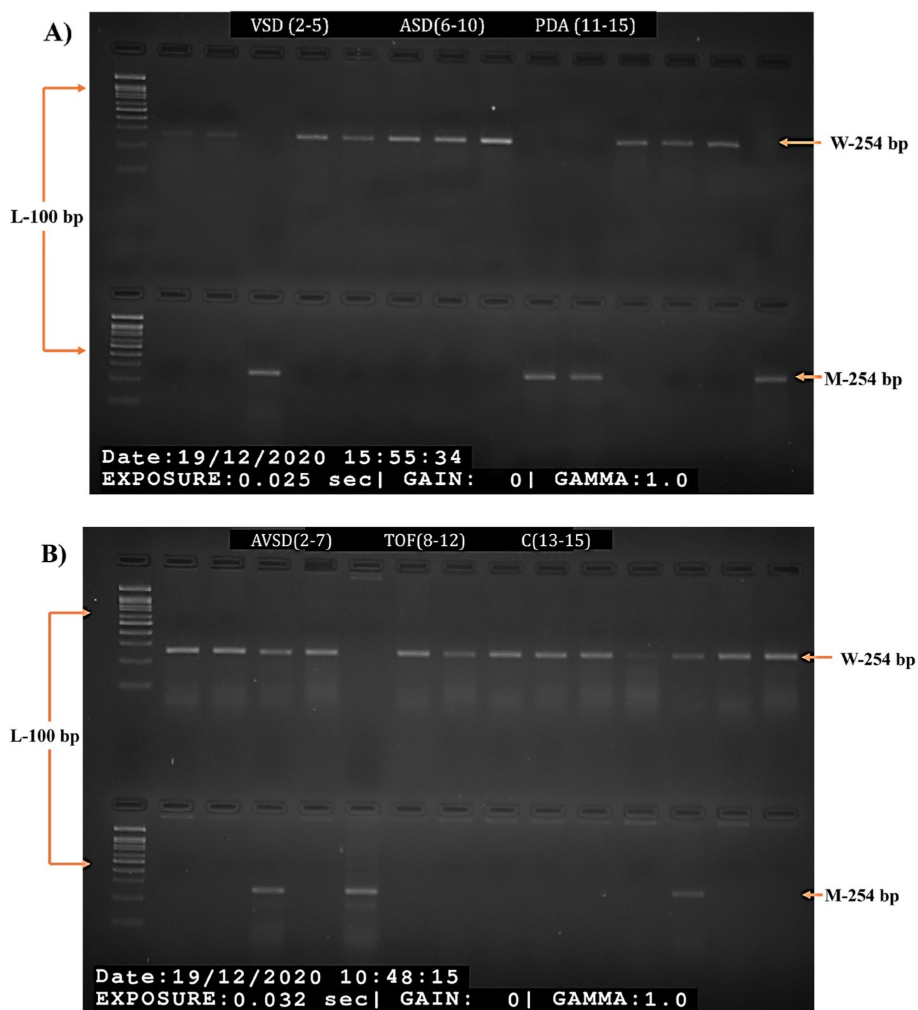

Fig. 2 The CHD and control sample genotyping. The first well represents the ladder (L), which is 100 bp in size and was used to confirm the target size of the template. **A** Represents the genotyping of 4 VSD, 5ASD, and 5 PDA samples. **B** Show the genotypes of 6 AVSD, 5 TOF, and 3 control samples. Initials for each CHD type and controls were used to label the gel images (VSD, ASD, AVSD, TOP, PDA, and C for controls). An amplified wild-type allele product is shown in the upper lane of the gel (W-254 bp), whereas a mutant allele with equal-sized bands measuring 254 bp is shown in the lower lane (M-254 bp)

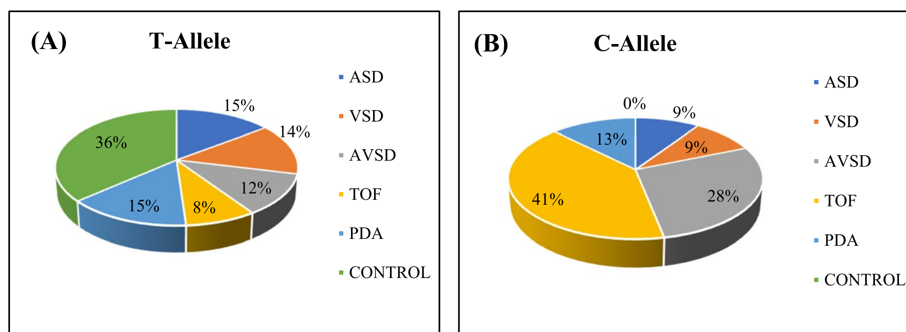

Fig. 3 **A** Pie chart showing the T allele frequency in all samples. **B** Frequencies of the C allele in all samples

Table 2 Chi-square analysis for association of CHDs

N	Samples groups		*P*	df	χ^2	S.V
350	CHDs patients 250	Controls 100	0.05	12	103.9	21.03

N Number of samples, X^2 chi-square table value, *df* degree of freedom, *S.V* significant value

Table 3 The odds ratio shows the strength of the association between the *CITED2* gene and CHDs()

Genotypes	CHDs	C	S. V	OD
C	76	1	> 1	**43.7**
T	186	100		

C Mutant, *T* Wild type or reference allele

Table 4 Sequencing results

CHD type	Sample ID	PCR results	Sequencing results
VSD	V-3	TC	TC
	V-7	CC	CC
ASD	A-3	TC	TC
	A-5	CC	CC
AVSD	AV-2	CC	CC
	AV-6	CC	CC
TOF	T-2	CC	CC
	T-8	CC	CC
PDA	P-3	TC	TC
	P-5	CC	CC
Control	C-1	TT	TT
	C-2	TC	TC

Digits 1, 2, 3, 5, 6, and 8 represent the sample ID numbers

mutations in the conserved segments of the *CITED2* gene (CR1, CR2, and CR3). Methylation in the promoter region of a gene likely influences transcription [31]. Research studies in different populations have shown an association between the *CITED2* gene and CHDs [8, 21]. A primary study revealed that mice with a loss-of-function mutation in the *CITED2* gene overexpress VEGF and HIF1a [3]. Thus, based on these studies, the mutation in exon 2 of the *CITED2* gene, SNP rs375393125, was selected for the genetic screening of 250 CHD patients for probable outcomes. The -results showed that the genotypic frequency of all samples was more significant for patients with a greater number of wild-type TT genotypes, while only CHD patients were found with mutant CC genotypes. Among all CHD groups, 40% of the TOF patients had a high percentage of mutant CC genotypes (Fig. 1). A study based on CHD in China revealed a decreased interaction between *CITED2* and p30°CH1 due to four mutated proteins. The significant interaction between p30°CH1 and HIF1A could upregulate VEGF promoter activity. This study revealed that mutations could downregulate TFAP2C in pitx2c transactivation and *CITED2* [5, 6, 29]. All CHD patients were found to have both T and C alleles. All CHD and control samples had a greater T allele frequency. On the other hand, the controls had no mutation C allele.

Moreover, it was found in an average of 30% of all the patients (Fig. 3A). These results showed that the high frequency of the mutant CC genotype or C allele in the respective samples may be associated with heart abnormalities. Furthermore, these results were confirmed through Sanger sequencing. The sequencing results showed that all the samples had the T > C mutation, and the PCR results were subsequently validated by cross-matching. All the samples were found to have similar PCR and Sanger sequencing results.

The chi-square test and odds ratio test were applied to determine the genetic association between the targeted *CITED2* gene and CHD type and the strength of the association. The observed chi-square value of 103 was significantly more significant than the tabulated value of 21.03 (Table 1). The odds ratio test found similar results with a more excellent calculated value of 43.7 than the threshold value of 1 (Table 2). Variance analysis revealed significant differences among the CHD groups and *CITED2* genotypes (Tables 3 and 5). Hence, *CITED2* gene mutation(s) are strongly associated with CHDs. The available data for rs13253637 in the NCBI database showed a rare and negligible mutant C allele in many different populations of ordinary individuals. Therefore, the outcomes of this research were compared with those from the Gnome-AD and GOE exome projects. The genome-AD project shows a 0.000004/1 distribution of the C allele. Similarly, the GOE sequencing project showed a 0.000077/1 frequency of the C allele. This research revealed a higher C allele frequency in CHD patients (Fig. 4).

An epigenetic study of congenital heart malformation revealed that environmental factors may change a gene's transcriptional activity, ultimately affecting the heart's

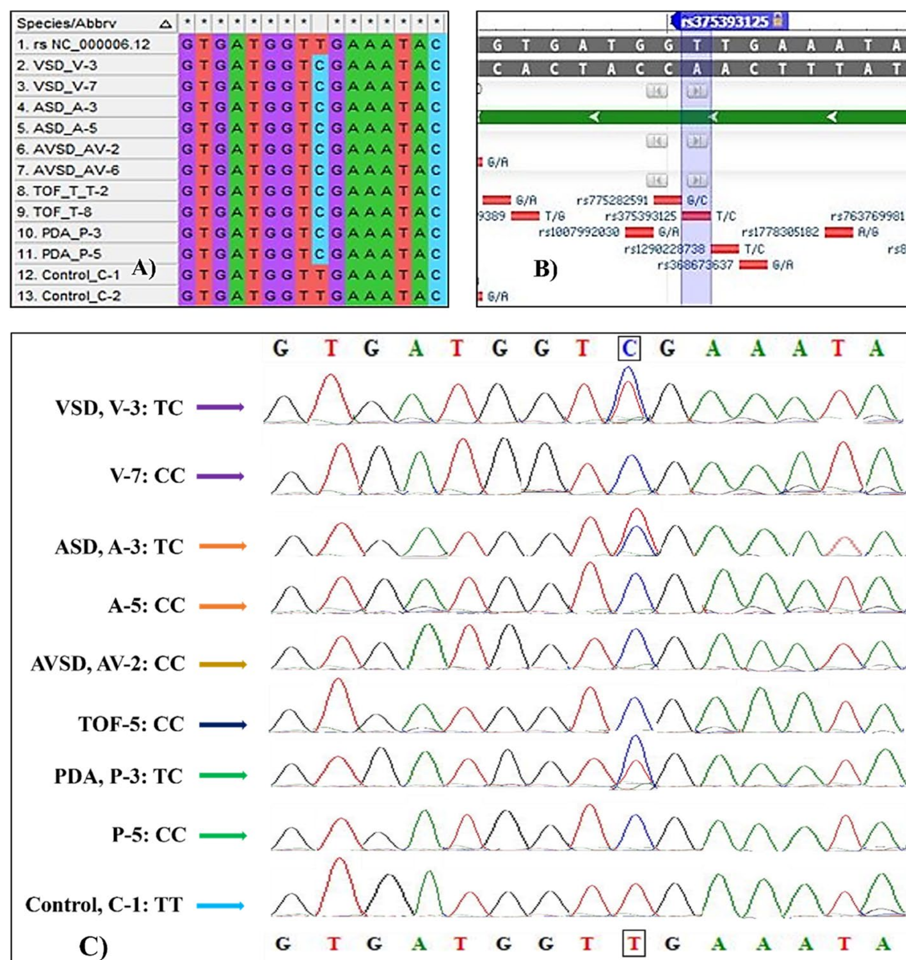

Fig. 4 Multiple sequence alignment. **A** All the samples were sequenced using Clustal-W through MEGA11 software. **B** The sequence was confirmed through the NCBI database. **C** The mutant target region was checked through the ABI, and the actual peak of the Sanger sequence was confirmed for each sample

developmental process [14, 22]. Additionally, the present study focused on identifying the associations of general factors, including addiction, sex, and consanguinity, with CHDs. The exact ratios of addiction (chewing and smoking), sex, and kinship were detected in both the cases and controls. However, these factors had an insignificant effect on CHDs (Fig. 5).

It has been reported that CHDs may be caused by *CITED2* gene mutation (s) in research involving varied populations. However, current research has also found similar findings. This SNP can be used as a marker for future theranostic approaches.

Conclusion

There are multiple gene(s) and mutation(s) that cause CHDs during heart development. Certainly, these defects impair the main functions of the heart. Many genetic and environmental factors are also responsible for such conditions. Among them, *CITED2* gene mutation(s) are also considered to cause CHDs. Research studies in different populations have reported that mutation(s) in the *CITED2* gene might be responsible for causing CHDs. Association analyses of the *CITED2* gene with CHD patients were performed in the present study due to differences in odds ratios and chi-square analyses, and a possible association between the gene mutations was observed. The CHD patients had a greater frequency of the rs375393125 (T > C) SNP than the healthy individuals (controls). Therefore, based on the results, it can be determined that the *CITED2* gene may be associated with structural heart defects. Large sample and protein analysis is required for a more in-depth study of the changes and mutations at the amino acid level.

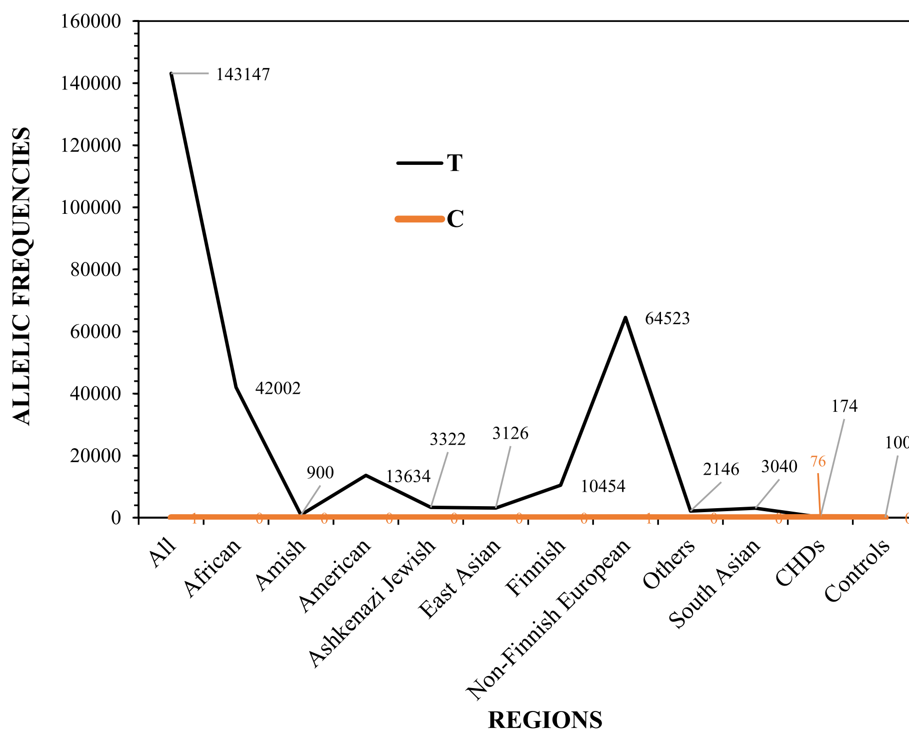

Fig. 5 Worldwide analysis of T and C allele frequencies relative to the present research

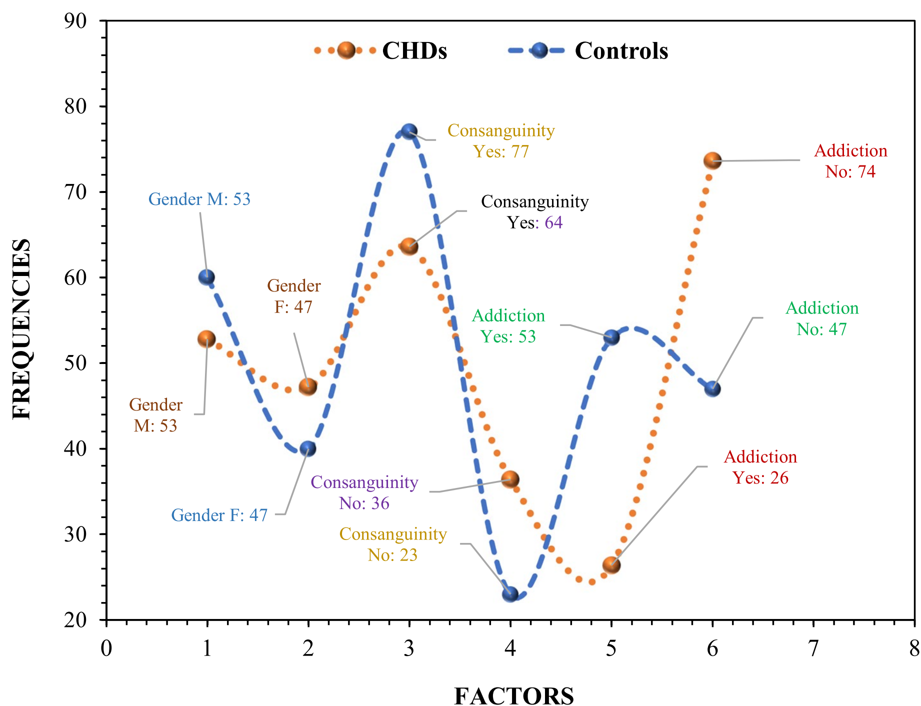

Fig. 6 The graph shows multiple risk factors associated with CHD incidence

Table 5 Variation analysis of groups

S. V	SS	Df	MS	F	*P*	F.C
B/G	6344.1	2	3172	11	0.0009	3.7
W/G	4108.3	15	273			
T	10452.4	17				

S.V source of variance, *SS* sum of the squares, *MS* mean of the squares, *F* f test, *B/G* between the groups, *W/G* within the groups

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12872-024-04035-2.

Supplementary Material 1.

Authors' contributions

HY: The project was conceptualized and designed, data was gathered and analysed, and the manuscript was prepared by the author. HA: The individual helped in the development of the research methodology and the interpretation of the collected data. Additionally, critically evaluated and revised the work to enhance its intellectual substance. Also Participated in data collection, reviewed the literature, and contributed to the manuscript's writing and editing.SIA: The individual helped out in collecting blood samples. NP: Expert Pediatric Consultant provided the facility for sampling. AA: Supervised the research project, idea and concept, provided expert input on the study design and analysis, and reviewed and approved the final manuscript. Have read and approved the final version of the manuscript, and contributed to the development and completion of this research.

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Self-funding.

Availability of data and materials

Subject to ethical and privacy rules being followed, qualified researchers may reasonably request access to the data used in this work. To help with the data-sharing process, interested parties can contact corresponding author: afsheen.arif@uok.edu.pk to seek access to the data. Our dedication is to advocate for openness and conscientious data exchange, all the while upholding the study participants' privacy and confidentiality. Adherence to appropriate data usage and privacy precautions is a need for data access.

Declarations

Ethics approval and consent to participate

The Committee on Health Research Ethics, Deanship of Scientific Research, University of Karachi, provided ethical Approval for this study under Ref No # KIBGE/ICE/027116/02/2021. The research adhered to ethical standards and all applicable laws and regulations to protect participants' rights and welfare during the entire study. All 350 study participants gave their informed consent, guaranteeing they understood the research goals and could withdraw from the study at any moment. Participants' safety and well-being were paramount, and steps were taken to swiftly address any adverse effects or discomfort.
I hereby declare that the work presented in this research is my own effort, except otherwise acknowledged, and that the research is my own composition. No part of this research has been previously published or presented elsewhere.

Consent for publication

Not Applicable.

Competing interests

The authors declare no competing interests.

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