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Molecular Characterization of two NR2F2 Exons identifies a Rare Intronic Variant Enriched in Indonesian Congenital Heart Disease Patients

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ABSTRACT:

Background: Congenital heart disease is a globally prevalent congenital disorder that has a complicated genetic etiology that includes both coding and non-coding variants. Although there is much evidence of pathogenic coding variants in key transcription factors, the role of regulatory variants in the Indonesian population is less known. The NR2F2 plays an essential role in cardiac development. To determine potentially pathogenic variation, we performed a case-control genetic study in Indonesian patients with CHD to find variants in the NR2F2 gene.

Methods: 27 ethnically matched controls and 35 CHD patients were examined, and genomic DNA was extracted from peripheral blood samples. Following targeted Sanger sequencing to identify potential pathogenic variants in exons 2 and 3 of the NR2F2 gene, extensive bioinformatic annotation was carried out using genomic visualization platforms.

Results: This study identified a rare intronic variation of the NR2F2 gene, rs753513812 (c.970+11G>C; g.8677G>C), in eight of 35 CHD patients, and it was absent in all control individuals and the majority of CHD subtypes. The variation was significantly enriched in CHD patients. This finding suggests that CHD susceptibility may play a role through non coding regulatory variations. Among the 35 patients examined, no pathogenic or likely pathogenic variants were detected in exons 2 and 3 of the NR2F2 gene.

Conclusion: These findings imply that pathogenic coding variants within these exons are unlikely to be a frequent genetic cause of CHD in Indonesian patients. Also, the study highlights the need for population-specific genetic investigations and their contribution to human cardiac development.

1. INTRODUCTION

Congenital heart disease (CHD) is the most prevalent class of congenital heart disease, which affecting 8–10 out of every 1,000 live births globally and contributing a major cause of infant morbidity and mortality (Hoffman and Kaplan, 2002)(Van Der

Linde et al., 2011). CHDs can be present at birth also can be isolated to complex cases. One kind of isolated CHD is a ventricular septal defect (VSDs), Ventricular septal defects (VSDs) are one kind of isolated CHD that, when combined with tetralogy of Fallot (TOF), can result in a complex form of CHD. About 80% of all CHD cases is Non-syndromic CHD cases, which are not linked to any other anomalies or abnormalities (Blue et al., 2012)(Nappi, 2024). Despite advances in medical until now CHD remains associated with long-term complications, reduced quality of life, and substantial healthcare burden. The etiology of CHD is highly heterogeneous, involving interactions between genetics, epigenetics, and environmental factors Despite medical advancement, CHD is still linked with long-term complications, healthcare costs and reduced quality of life. Genetic, epigenetic, and environmental variables interact to cause CHD, which has a very diverse etiology (Blue et al., 2017).

Genetics research conducted Over the last two decades, genetic studies have shown that a significant proportion of CHD cases have a heritable basis, particularly septal defects, left–right patterning problem, conotruncal anomalies, and left–right patterning disorders transcription factors and signalling molecules are playing key roles in heart morphogenesis, also both chromosomal abnormalities and single-gene variants are increase the risk of illness (Gelb and Chung, 2014)(Bruneau, 2008), numerous genetic and epigenetic factors control Cardio genesis through intricate spatiotemporal patterns. These elements include of transcription factors, structural proteins and non-coding RNAs (Mansoor et al., 2025)(Pierpont et al., 2018)(Wu et al., 2021). while initial research focused primarily on coding mutations that's disrupt protein structure, recent genomic studies have underscored the importance of non-coding and regulatory variants in cardiac development and congenital heart disease (Zaidi and Brueckner, 2017)(Gelb and Chung, 2014).

Recent evidence indicates that a diverse range of genes and genetic variations are constitute the primary driving forces behind the majority of congenital heart diseases (CHDs) (Morton et al., 2022)(Diab et al., 2021), Numerous studies conducted in Indonesia, characterized by a large and diverse population with very small amount of genetic data, have revealed a high prevalence of congestive heart failure CHD, which is frequently accompanied by late diagnosis with delayed access to specialized care , the human heart is developing according to a tightly regulated developmental pattern that necessitates exact temporal and spatial control over gene expression. Transcription factors are key regulators of this process, orchestrating cell fate decisions, chamber specification, and vascular development (Olson, 2006)(Ismail et al., 2015).

Transcription factors such as GATA4, members of the T-box family (TBX1 and TBX5), HAND family (HAND1 and HAND2), NKX2.5, NKX2.6, structural proteins like MYH6, and CITED2 are crucial and interconnected molecular factors that govern on cardiac development (Mansoor et al., 2025)(Khatami, Heidari, et al., 2018)(Khatami, Mazidi, et al., 2018)(Khatami et al., 2022)(Dianatpour et al., 2020)(Nees and Chung, 2020) (Odogwu, Hagen and Nelson, 2023) (Tabrizi t al. 2024).

Disruption of transcription factor networks, whether through coding mutations or regulatory perturbations, can result in the development of structural heart defects. Among these transcription factors, NR2F2 (nuclear receptor subfamily 2, group F, member 2), also known as COUP-TFII, codes for three isoforms of COUP transcription factor 2 (COUP-TFII or COT2 [Uniport: P24468]). It has emerged as a critical regulator of cardiac development(Mansoor et al., 2025)(Ganapathi et al., 2023) , NR2F2 encodes a nuclear receptor transcription factor that is essential for regulating the development of epithelial to mesenchymal states, embryonic development, angiogenesis, and organogenesis (Pereira et al., 1999), It is also associated with the activation of vein and lymphatic vessel characteristics while suppressing artery-specific genes. In the developing heart, NR2F2 plays a pivotal role in atrioventricular differentiation, chamber identity, and vascular patterning (Polvani et al., 2020) (Wu et al., 2013a).

Experimental investigation utilizing NR2F2 plays a dosage sensitive role in heart morphogenesis experiment on animal models have shown that deletion or dysregulation of NR2F2 lead to severe cardiovascular abnormalities (Lin et al., 2012). Congenital cardiac abnormalities in humans are directly associated with pathogenic mutations affecting NR2F2 (Al Turki et al., 2014) reported that rare deleterious variants in NR2F2 cause a spectrum of CHD phenotypes, including atrial septal defects and atrioventricular canal defects, Furthermore, a study conducted in an Iranian CHD cohort identified five heterozygous single-nucleotide variants in exon 2 and exon 3 of the NR2F2 gene, in silico investigation predicted these novel variants are harmful implications on protein function which included four missense mutations and one synonymous variant, these mutation are not found in any public databases(Mansoor et al., 2025).

Although NR2F2 coding mutations have been associated to CHD, there is growing evidence indicates that noncoding variants may potentially play a major role in disease risk, Enhancers, silencers, and transcription factor binding sites are examples of regulatory elements found in the non-coding sections of the genome that controls gene expression (McLaren et al., 2016) mutation within these regions can disrupt transcriptional regulation without changing the protein sequence, these variants can cause to subtle and cause a significant effect during development, according to recent research have demonstrated that regulatory change in conserved non-coding regions can change the cardiac gene expression and cause congenital heart abnormalities (Gelb and Chung, 2014). According to genome-wide analysis have further revealed that de novo and uncommon inherited non-coding variants are enriched in patients with CHD, especially in regions controlling genes implicated in heart development (Zaidi et al., 2013) (Wang et al., 2019).

The study highlight the importance of investigate intronic and regulatory variants when evaluating the genetic architecture of CHD, Additionally advances in high-throughput sequencing methods and bioinformatic annotation tools has been made possible by evaluation of both coding and non-coding variants, The identification of rare variants with possible pathogenic significance is made easier by databases like dbSNP and gnomAD, which offer crucial data on variant frequency and population distribution (Sherry et al., 2001)(Karczewski et al., 2020).

Despite these developments, most genetic studies of CHD genetics research have been focused on populations of European ancestry, with small amount of information available from communities Southeast Asian populations, including Indonesia. It is crucial to include underrepresented communities in genetic research because population-specific genetic differences may affect illness susceptibility and variant interpretation (Sirugo, Williams and Tishkoff, 2019).

The lack of comprehensive genetic studies is the absence of thorough genetic research in Indonesian individuals with CHD, Given the critical role of NR2F2 in cardiac development, its intolerance to functional variation, and the growing recognition of regulatory variants in CHD, additionally the study Aim to expand the current knowledge of NR2F2 variation in CHD, that pathogenic coding variants in exon 2 and exon 3 of NR2F2 are not a common cause of congenital heart disease in Indonesian pediatric patients and emphasize the role of rare non-coding variants in underrepresented populations by integrating genomic and bioinformatic data.

1.1 Ethical Considerations

The established ethical guidelines for research involving human subjects were followed at every stage of the investigation. Ethical approval for this study was granted by the Health Research Ethics Committee of RSUP Dr. M. Djamil Hospital, Padang, West Sumatra, Indonesia (Approval No: DP.04.03/D.XVI.10.1. /380/2027).

The Committee waived the requirement for WHO 2011 Standards, Scientific Values, Equitable Assessment and Benefits, Risk, Persuasion/Exploitation, Confidentiality and Privacy, and informed consent, referring to the 2016 CIOMS Guidelines because the study used anonymized clinical data collected from hospital records, with no direct contact with patients.

This research was conducted among children diagnosed with congenital heart disease (CHD) at RSUP Dr. M. Djamil General Hospital, Padang, Indonesia, between October 2025 to October 2026. Clinical information, including age, sex, CHD type, and ethnicity, was gathered for all patients and used for research purposes only.

2. MATERIALS AND METHODS

2.1 Study Material

The tools and materials used in this study were microtubes, cryotubes, sterile swabs, gloves, voculab tubes, micropipettes, magnetic stirrers, measuring cups, filters, glass funnels, 50 ml centrifuge tubes, petri dishes, gloves, blood and 2X Taq DNA Polymerase Master Mix, absolute ethanol, distilled water, vortex machine, centrifugates and PCR machine. The materials used were Blood samples (5 mL) Will be collected from each individual into K2-EDTA tubes, 96% alcohol, agarose gel, 1% TBE buffer, Ethidium Bromide (EtBr), PCR GoTaq green super mix, nuclease free water, loading dye, DNA ladder, Forward and Reverse primers, DNA Isolation Kit consisting of BL buffer, CL buffer, BW buffer, TW buffer, AE buffer, as well as GeneJET Genomi DNA Purifiation Kit.

2.2 Sample Collection

We Collected a blood from the group of 35 patients with non-hereditary congenital heart disease (CHD) at the M. Djamil Heart Canter in Padang. Furthermore, the baseline characteristics of the patients were recorded during hospital admission, these group of patients were diagnosed with CHD by both a paediatric surgeon and a cardiologist and were undergoing open-heart surgery **(Table 1**), All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (Andalas University, Indonesia) and with the Helsinki Declaration of 1975, as revised in 2000 and Informed consent was obtained from all patients for being included in the study (Mansoor et al., 2025).

We also selected 27 healthy children without any prior record of CHD in their pedigree or syndromic abnormalities These children were of similar age, gender, and region to our patient group. After taking Blood sample from patients and control we Perfume DNA Extraction by using GeneJET Genomi DNA Purifiation Kit protocol to extract whole genome DNA from Blood, followed by Electrophoresis to check DNA Extraction quality and Band (quality and quantity) after confirmation, Followed by PCR Amplification to screen for point mutations in coding regions of two Exon of NR2F2 gene by Using two Pair of PCR Primer that we design by Gene Runner and Oligo Analyzer.

Table 1 Baseline Clinical Characteristics of patients

Clinical Characteristics	Number of Patients(%)
Male/Female	19/16 (54.28 / 45.71)
Age (years)	Average age/; 30 days - 17 years
Ventricular Septal Defect (VSD)	12 (34)
Atrial Septal Defect (ASD)	3 (9)
Patent Ductus Arteriosus (PDA)	8 (23)
Tetralogy of Fallot (TOF)	3 (9)
Atrial Ventricular Septal Defect (AVSD)	1 (3)
Transposition of the Great Arteries (TGA)	1 (3)
VSD + PDA	6 (17)
TGA + VSD	1 (3)

2.3 Genomic analysis

Blood samples (5mL) were collected from a group of CHD patients with non-hereditary congenital heart disease (CHD) at M. Djamil Heart Centre in west Sumatra Padang City, Indonesia from each individual into K2-EDTA tubes and soon Separate it into two tube each into K2-EDTA tubes, one (3ml) blood tube for blood profile and another (2ml) blood tube for DNA isolation, The tubes were stored at -20°C. The GeneJET Genomi DNA Purifiation Kit protocol, were employed to extract whole genome DNA, in order to screen for point mutations in coding regions of the NR2F2 gene, two exons of this gene were amplified through Polymerase Chain Reaction (PCR) using two pairs of primers designed via Gene Runner and Oligo Analyzer.

The first PCR reaction was performed for primer (F: 5'-TGAGGCTGGTCATTAACTGTGG-3' (R:5'GAAGACAGGGCTCCTTCCTAC-3'). was designed to amplify a (594) bp product in exon 2 and Intron 2 chr15: 96334031 to 96334624, While the second PCR reaction was performed for primer: (F:5'-ATGCCTGTGGTCTCTCTGATG-3') and (R:5' CTCTGTTTCACTCCCCCTTC -3'), was designed to amplify a (308) bp product in exon 3 chr15:96337348 to 96337655 (Mansoor et al., 2025).

The process of amplifying DNA fragments involved subjecting them to specific PCR conditions. In the initial stage, the DNA fragments were denatured at 95°C for 5 minutes. Following that, 5 cycles of annealing at 64°C for 50 seconds and extension at 72°C for 5 minutes were carried out. Subsequently, 30 cycles of denaturation, annealing was carried out, Following PCR amplification, the concentration and purity of DNA amplicons were evaluated using a Nanodrop spectrophotometer (Thermo Fisher Scientific, USA) to ensure adequate quality for downstream Sanger sequencing. The amplified DNAs were then observed

by running them on a 1.5% agarose gel containing ethidium bromide and subjecting them to UV-transillumination. The samples were analysed using Sanger Sequencing Genetic Analyzer, additionally sequencing results were studied to identify homozygous or heterozygous single nucleotide variations (Mansoor et al., 2025).

2.4 Analysis of the Variant by Using Bioinformatic Tools

The identified variants were annotated using multiple bioinformatics tools and public databases (Table 3). Population allele frequencies and ancestry-specific distribution of rs753513812 were assessed using the Genome Aggregation Database (gnomAD). Variant identity and reference information were confirmed using the dbSNP. Functional consequence prediction was performed using the Ensembl Variant Effect Predictor (VEP) with the GRCh38 reference genome to determine variant classification and predicted impact.

Genomic localization and regulatory context were examined using the LUMC Mutalyzer 3, Mutation Taster 2025, UCSC Genome Browser, and NCBI Variation Viewer. In silico transcription factor binding site analysis was conducted using the FABIAN variant tool to evaluate potential regulatory effects. The statistical association between the cases and controls was evaluated using Fisher's exact test.

2.5 Statistical Analysis

Statistical analyses were performed using standard methods for the categorical genetic data. Variant frequencies between patients with congenital heart disease and control individuals were compared using Fisher's exact test due to the small sample size and low expected cell counts. Odds ratios were calculated to estimate the strength of the association between rs753513812 and disease status. All statistical tests were two-tailed, and a p-value of < 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

A total of 35 pediatric patients diagnosed with congenital heart disease (CHD) were enrolled in this study. The clinical spectrum of CHD included twelve patients of ventricular septal defect (VSD), patent ductus arteriosus (PDA) in eight patients, atrial septal defect (ASD) in three patient, atrioventricular septal defect (AVSD) in one patient, tetralogy of Fallot in three patients, transposition of the great arteries (TGA) in one patient, TGA combine with VSD in one Patients and combined VSD with PDA in six patients **(Table 1**).

All patients were clinically diagnosed by pediatric cardiologists and cardiac surgeons prior to enrolment, and genomic DNA of adequate quality and quantity was successfully extracted from all blood samples, following by targeted genetic analysis performed by Sanger sequencing of exon 2 and exon 3 of the NR2F2 (COUP-TFII) gene, using previously validated primers.

Polymerase chain reaction (PCR) amplification yielded clear and specific amplicons of the expected sizes (594 bp for exon 2 and 308 bp for exon 3), confirming the technical reliability of the assay.

Subsequent sequence alignment and variant analysis demonstrated that all examined sequences were identical to the reference *NR2F2* sequence. No pathogenic, likely pathogenic, missense, nonsense, synonymous, or frameshift variants were identified in either exon 2 or exon 3 in any of the 35 CHD patients. The absence of pathogenic variants in exon 2 and exon 3 of *NR2F2* in this Indonesian cohort suggests that coding mutations within these regions are not a common etiological factor for CHD in this population, although rare pathogenic *NR2F2* variants have been reported as potential etiological causes of congenital heart disease in other populations, the present study demonstrates that pathogenic coding variants in exon 2 and exon 3 of *NR2F2* are not a common cause of CHD in Indonesian children.

3.1 Study Cohort and Variant Detection

Congenital heart disease (CHD) is the most prevalent congenital defect in the world and has a strong genetic basis (Zhang et al., 2019). in this study were included 35 Indonesian patients with CHD, including those with patent ductus arteriosus (PDA), atrial septal defects (ASD), and ventricular septal defects (VSD), The comparative investigation also included 27 ethnically comparable Indonesian control subjects who did not have CHD, A rare single nucleotide variant, *rs753513812*, corresponding to a G>C substitution at chromosome 15 location 96334614 (GRCh38), was detected by targeted Sanger sequencing of the

NR2F2 gene (**Figure 1,** Error! Reference source not found.) Eight out of 35 CHD patients (22.9%) had the variant, while n one of the control group carried it, suggesting a significant enrichment in the affected group.

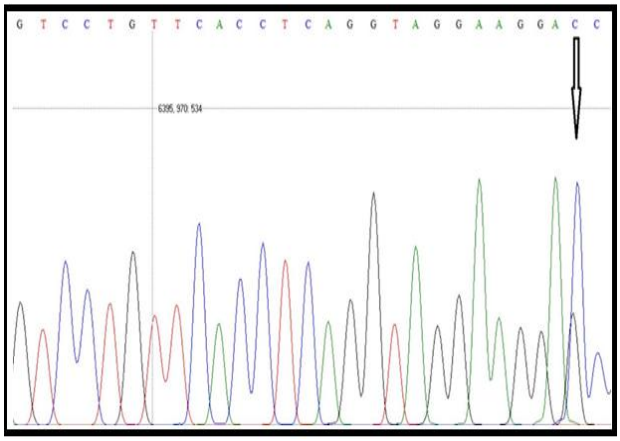

Figure 1 rs753513812 - c.970+11G>C

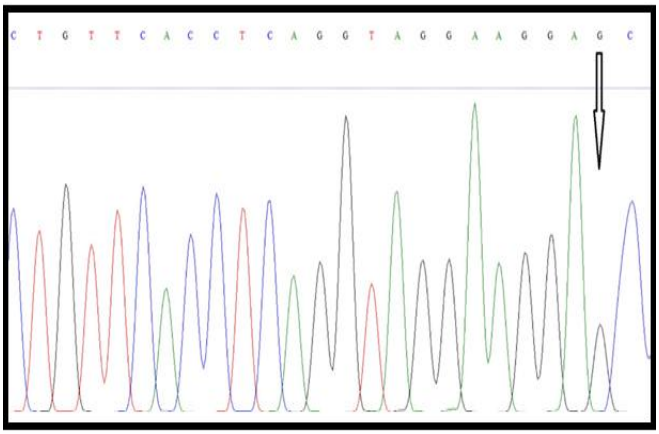

Figure 2 control - rs753513812

3.2 Genotype–Phenotype Correlation

No homozygous carriers of *rs753513812* were found, and all eight carriers were heterozygous. Three cases of VSD, two cases of ASD, and three cases of combined PDA and VSD were among the cardiac phenotypes linked to variant-positive patients. The occurrence of the variant across several CHD subtypes suggests that *rs753513812* may be a general contributor to cardiac developmental defects rather than being specific to a single problem type.

3.3 Population Frequency and Database Annotation

Analysis of Population database analysis revealed that *rs753513812* is very rare variant. With only two alleles reported and no homozygous individuals found, the variant has a worldwide allele frequency of roughly 1.27×10^{-6} in the gnomAD v4.1.0 database, which is consistent with variants under strong purifying selection (Karczewski et al., 2020).

According to Ancestry-specific data indicate that the variation is absent in East Asian populations, which is consistent with its absence in Indonesian controls, and has only been found at very low rates in African/African American and European (non-Finnish) populations **(Figure 3).**

Population-specific genomic architecture is a major key factor that could explain the discrepancy between our results and earlier reports. The prevalence of Rare pathogenic mutation frequency is frequently varying between ethnic groups because of founder effects, demographic history and genetic background. A key factor that may explain the discrepancy between our findings and previous reports is population-specific genetic architecture. Rare pathogenic variants often differ substantially in frequency between ethnic groups due to demographic history, founder effects, and genetic background. such population-dependent heterogeneity In CHD is widely recognized, and ancestry-specific research are necessary for affective interpretation of candidate gene associations (Fahed *et al.*, 2013)(Blue *et al.*, 2017).

According to dbSNP reports that *rs753513812* is a validated intronic single nucleotide variant with several reported alternative alleles (G>A/C/T) and no clinical significance claim in ClinVar (Sherry et al., 2001). This variant has not yet been documented in the literature, as there are No PubMed-indexed publications are currently linked with this variant.

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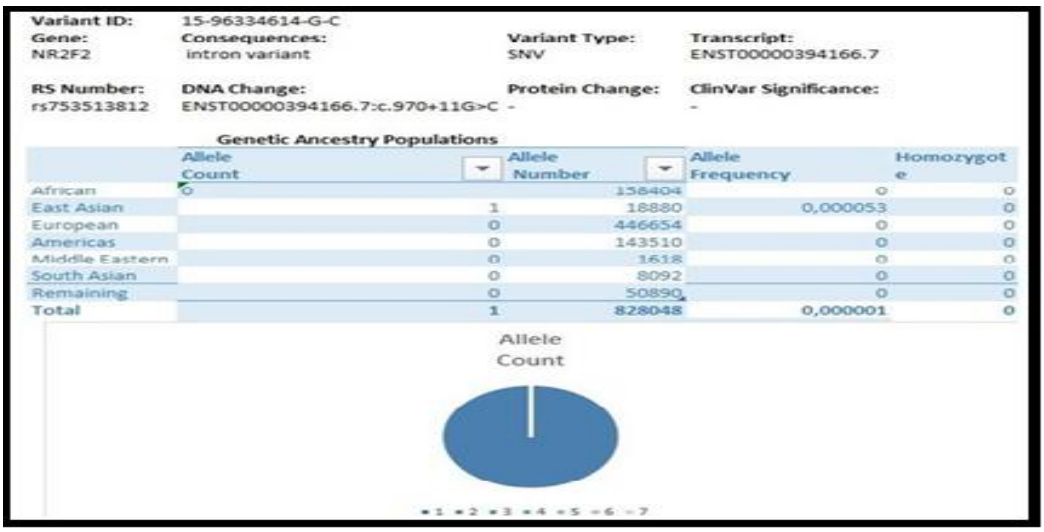

Figure 3 Genetic ancestry population gnomAD

3.4 Ensembl VEP Functional Annotation

Using Ensembl Variant Effect Predictor (VEP v115.1) for functional consequence annotation, *rs753513812* was classified as an intron variant with a predicted MODIFIER impact in the *NR2F2* gene (HGNC:7976) (McLaren *et al.*, 2016). According to this classification that the mutation may affect gene activity through non-coding regulatory rather than directly affecting the protein coding sequence mechanisms. The VEP results aligned with annotations derived from NCBI Variation Viewer and UCSC Genome Browser analysis.

3.5 Genomic Context and Gene Constraint Analysis

The UCSC Genome Browser Visualization verified that *rs753513812* is located within an intronic region of NR2F2 and in a genomic context regulatory-rich with multiple regulatory annotation and close to the MIR1469 locus (James Kent *et al.*, 2002), *NR2F2* is expressed in a variety of tissues, including those related to cardiovascular development according to GTEx Expression data.

According to Gene-level constraint analysis from gnomAD shown that *NR2F2* is extremely intolerant to functional variation, with a probability of loss-of-function intolerance (pLI) score of 1.0 and a significantly lower observed-to-expected ratio for predicted loss-of-function variants (o/e = 0.06), indicating strong selective pressure acting on this gene (Karczewski *et al.*, 2020).

3.6 In Silico Regulatory Analysis

An in-silico transcription factor binding site (TFBS) study was conducted Using the FABIAN-variant tool to investigate the possible regulatory impact of *rs753513812***.** According to this analysis, the G>C substitution may result in the gain or loss of transcription factor binding sites within the intronic region of *NR2F2*, suggesting a potential impact on transcriptional regulation with possible effect on transcriptional regulation (da Cruz, Ivy and Jaggers, 2014)(Go S *et al.*, 2014).

Table 2 : Predicts DNA variant effects on transcription factor binding sites (TFBS), indicating potential TFBS gain (+1), loss (−1), or NA

TFBSs	**Database**	**c.970+11G>C (g.8677G>C)** **Prediction (Score)**
CHD1: Chromodomain helicase DNA binding protein 1	SwissRegulon	Gain (0.0046)
CLOCK: clock circadian regulator	jolma2013, HOCOMOCOv11, SwissRegulon, jaspar2022, cisbp_1	Gain (0.0080)

CTCF: CCCTC-binding factor	HOCOMOCOv11, jolma2013, SwissRegulon, jaspar2022	Loss (-0.0010)
EP300: E1A binding protein p300	SwissRegulon	NA (0.0000)
FOXK2: fork head box K2	jaspar2022	Loss (-0.0096)
PAX8: paired box 8	HOCOMOCOv11, SwissRegulon	Loss (-0.0043)
RBFOX2: RNA binding fox-1 homolog 2	hPDI	NA (0.0000)
TAF1: TATA-box binding protein associated factor 1	HOCOMOCOv11, SwissRegulon	Loss (-0.0258)
TBP: TATA-box binding protein	SwissRegulon, jaspar2022, HOCOMOCOv11	Gain (0.0037)
YBX1: Y-box binding protein 1	SwissRegulon	Gain (0.0283)
ZFX: zinc finger protein X-linked	HOCOMOCOv11, SwissRegulon, jaspar2022	Loss (-0.0388)

3.7 Case Control Association Analysis

Fisher's exact test was used to compare the case and control group. The *rs753513812* variation was found in 0 of 25 control individuals and 8 of 35 CHD patients (22.9%) indicating a statistically significant enrichment in the CHD group ($p = 0.0162$, two-tailed Fisher's exact test), because of the absent of the variant in controls, the odds ratio was infinite ($OR = \infty$), suggesting a strong correlation between rs753513812 and congenital heart disease in this population.

3.8 ACMG Interpretation and Classification

According to the American College of Medical Genetics and Genomics and the Association for Molecular Pathology (ACMG/AMP) guidelines, the NR2F2 variant *rs753513812* is classified as a Variant of Uncertain Significance (VUS). The variant fulfils PM2 (supporting) based on its extremely low allele frequency in global population databases and its absence in ethnically matched Indonesian control individuals. In addition, PS4 (supporting) is suggested by the statistically significant enrichment of the variant in congenital heart disease patients compared with controls (Fisher's exact test, $p = 0.0162$). Functional annotation using Ensembl Variant Effect Predictor classified *rs753513812* as a non-coding intronic variant with MODIFIER impact, and in silico regulatory analyses predicted potential effects on transcription factor binding, However, definitive pathogenic classification is currently not possible due to the absence of independent replication cohorts, familial segregation data, and experimental functional validation, it is therefore appropriate to categorize *rs753513812* as a Variant of Uncertain Significance.

Table 3 Bioinformatics Tools

Tool / Database	Purpose in this study	URL / Citation
Mutalyzer 3	Validation of HGVS nomenclature and determination of cDNA and genomic variant positions	https://mutalyzer.nl
Mutation Taster 2025	Identification of variant location and preliminary functional context	https://www.mutationtaster.org
dbSNP (NCBI)	Verification of variant identity and reference annotation	https://www.ncbi.nlm.nih.gov/snp
ClinVar (NCBI)	Assessment of prior clinical reports and pathogenicity assertions for novelty evaluation	https://www.ncbi.nlm.nih.gov/clinvar
1000 Genomes Project	Evaluation of variant presence in global reference populations	https://www.internationalgenome.org

gnomAD	Assessment of global and ancestry-specific allele frequencies	https://gnomad.broadinstitute.org
Ensembl Variant Effect Predictor (VEP)	Functional annotation and impact classification of the variant	https://www.ensembl.org/info/docs/tools/vep
UCSC Genome Browser	Visualization of genomic context and regulatory features	https://genome.ucsc.edu
NCBI Variation Viewer	Confirmation of variant position and transcript-level annotation	https://www.ncbi.nlm.nih.gov/variation/view
FABIAN-variant	Prediction of transcription factor binding site (TFBS) gain or loss	https://fabian.bio

3.9 Discussion

We performed this study to gain insights into the prenatal diagnosis of congenital heart disease (CHD) and to develop gene-level interventions and treatments for CHDs, in this study, we analysis the two exon and one intron by using sangar sequencing to identified mutation in coding region of *NR2F2* gene the results revealed, no pathogenic or likely pathogenic variants were detected in exons 2 and 3 of *NR2F2* gene therefore we determined that patients with congenital cardiac disease in Indonesia are considerably more likely to have *rs753513812*, a rare intronic mutation in the NR2F2 gene.

The *NR2F2* gene is located at 15q26.2 and encodes a cardiac transcription factor protein consisting of 414 amino acids. *NR2F2* is a nuclear receptor (NR) transcription factor with a typical nuclear receptor structure that includes a ligand binding domain (LBD), a DNA binding domain (DBD), and two ligand-independent activation domains(Li *et al.*, 2022)(Ganapathi *et al.*, 2023)(Polvani *et al.*, 2020)

Human genetic studies have previously reported rare pathogenic *NR2F2* variants in patients with congenital heart defects, particularly atrioventricular septal defects and complex cardiac phenotypes , In addition, loss-of-function variants and copy number deletions involving *NR2F2* have been implicated in CHD, supporting haploinsufficiency as a disease mechanism .Therefore, the lack of pathogenic variants observed in this study does not negate the role of *NR2F2* in CHD pathogenesis but instead highlights population-specific and phenotype-dependent genetic effects (Al Turki *et al.*, 2014)(Bashamboo *et al.*, 2018)

A key factor that may explain the discrepancy between our findings and previous reports is population-specific genetic architecture. Rare pathogenic variants often differ substantially in frequency between ethnic groups due to demographic history, founder effects, and genetic background. In CHD, such population-dependent variability is well recognized, and ancestry-specific studies are essential for accurate interpretation of candidate gene associations. Notably, our previous study in an Iranian CHD cohort identified five heterozygous variants in exon 2 and exon 3 of *NR2F2*, including four missense variants predicted to be pathogenic by in-silico analysis (Mansoor *et al.*, 2025). The absence of similar variants in the Indonesian cohort suggests that NR2F2 coding variants may be rare or absent in certain populations.

Mostly Congenital heart disease have been linked with rare coding variants in NR2F2, especially in septal anomalies (Al Turki et al., 2014), the identification of *rs753513812* in patients with VSD, ASD, and PDA+VSD is therefore consistent with the established biological function of NR2F2 in heart development, although r*s753513812* is located within an intronic region and does not alter the protein coding sequence, non-coding variants are increasingly recognized as important contributors to congenital heart disease through disruption of gene regulation rather than protein structure, Since there are no earlier PubMed-indexed publications or clinical annotations are available, this is the first report of this variation in CHD patients in Indonesia. A *NR2F2* gene (nuclear receptor transcription factor) that is essential for heart morphogenesis, atrial ventricular differentiation, and vascular development(Pereira *et al.*, 1999)(Wu *et al.*, 2013b). Ensembl VEP discovered *rs753513812* as an intron variant with modifier influence and FABIAN analysis, which predicted changes in transcription factor binding site architecture, supporting a regulatory mechanism regulating *NR2F2* expression.

Methodological considerations further influence interpretation. Only exon 2 and exon 3 of *NR2F2* were analysed in this study, and pathogenic variants located in other coding exons, exon–intron boundaries, regulatory regions, or untranslated regions would not have been detected. Moreover, structural variants such as copy number variations affecting *NR2F2*, which have been implicated in CHD, cannot be identified by Sanger sequencing. Given that more than half of CHD cases remain genetically

unexplained, broader approaches such as full-gene sequencing, targeted CHD gene panels, or whole-exome sequencing may provide greater diagnostic yield.

These finding indicate the need of comprehensive population-specific genetic investigation and analysis to better understand the complicated genetic basis of congenital heart dieses and highlight the need of population specific genetic research, although rare pathogenic *NR2F2* variants have been reported as potential etiological causes of congenital heart disease in other populations, the present study demonstrates that pathogenic coding variants in exon 2 and exon 3 of *NR2F2* are not a common cause of CHD in Indonesian children, A link with the research is supported by statistically significant enrichment of *rs753513812* in CHD patients when compared to controls, However, because of the absence of functional validation and familial segregation data the variant is recognized as a Variant of Uncertain Significance (VUS) according to ACMG/AMP guidelines(Richards *et al.*, 2015).

The precise relevance of this variants in in heart development required to be clarified by Future research using transcriptomic analyses, functional assays, and larger population, Our research concludes that *rs753513812* as a rare intronic *NR2F2* variant that is enriched in Indonesian CHD patients, This finding suggest that routine screening of exon 2 and exon 3 of *NR2F2* alone is unlikely to have high diagnostic utility for Indonesian pediatric patients with predominantly isolated septal defects and emphasizes necessity of conducting ancestry-specific genetic studies, analysis other coding region such as a exon one and intron one and adopting broader genomic approaches, such as full-gene sequencing, targeted CHD gene panels, or whole-exome sequencing, to comprehensively elucidate the complex genetic basis of congenital heart disease also the significance of including underrepresented populations in genetic studies of CHD and highlights the possible role of non-coding regulatory variants in congenital heart disease.

4. CONCLUSION

Congenital heart disease (CHD) remains a major cause of childhood morbidity and mortality, particularly in developing countries such as Indonesia, where population-specific genetic data are still limited. In this study, we investigated the potential contribution of the *NR2F2* (COUP-TFII) gene to CHD etiology in an Indonesian pediatric cohort by targeted Sanger sequencing of exon 2 and exon 3. No pathogenic or likely pathogenic variants were identified in the coding regions among 35 CHD patients, therefore we recognized that patients with congenital cardiac disease in Indonesia are more likely to have enriched intronic variant, *rs753513812*, in the *NR2F2,* According to the results coding variants in exon 2 and exon 3 of *NR2F2* are not a common cause of CHD in Indonesian children, particularly in cases dominated by isolated septal defects such as ventricular septal defect and patent ductus arteriosus. Although *NR2F2* plays a well-established role in cardiac development by identifying the rare variant possibly linked with CHD in this population is supported by the fact that variants were absence in ethnically matched controls and affected individuals with CHD.

Rather than a defect-specific effect, the variant's existence across several CHD subtypes points to a wide contribution to heart developmental processes, although *rs753513812* does not alter the protein-coding sequence of *NR2F2*, growing evidence indicates that non-coding regulatory variants can play a crucial role in congenital heart disease by disrupting transcriptional regulation during embryonic development. Consistent with this concept, in silico analyses predicted alterations in transcription factor binding at the variant locus, and gene-level constraint metrics demonstrated that *NR2F2* is highly intolerant to functional variation, the biological plausibility of a regulatory mechanism affecting *NR2F2* expression is supported our finding. despite the absence of functional validation, segregation analysis, and independent replication, the variant is still recognized as a Variant of Uncertain Significance under ACMG/AMP standards, Despite the statistically significant enrichment seen in this population, as a result, care should be taken while interpreting clinical data.

Overall, this research results highlight important population-specific and phenotype-dependent differences in the genetic architecture of CHD, the absence of detectable pathogenic variants in this study does not exclude a role for *NR2F2* in CHD but suggests that its contribution may involve other regions of the gene. Importantly, this study adds to our under sanding of *NR2F2* variation in congenital heart disease and offers the first evidence of *rs753513812* in CHD patients from Indonesian, It emphasizes how crucial it is to investigating rare non-coding mutation and include underrepresented groups in genetic research, To point out the exact function of this variation in heart development and disease risk, Future studies using functional assays, transcriptome analyses, and larger multi-centre population will be crucial.

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AUTHOR CONTRIBUTIONS

Wahidullah Mansoor: Conceptualization, Data curation, Methodology, Writing – review & editing.

Dewi Imelda Roesma: Methodology, Project administration, Writing – review & editing.

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Hirowati Ali: Project administration, Writing – review & editing.

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CONFLICT OF INTEREST

All authors of this manuscript have viewed and approved the manuscript. There is no conflict of interest is existed between the authors.

ETHICS STATEMENT

Ethical approval was collected for this study from Research Ethics Committee of RSUP Dr. M. Djamil Hospital, Padang, West Sumatra, Indonesia (Approval No: DP.04.03/D.XVI.10.1. /380/2027).

DATA AVAILABILITY STATEMENT

The datasets used and/or analysed during the current study are available from the correspond author on reasonable request.

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