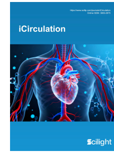
iCirculation
https://www.sciltp.com/journals/icirculation



Article

Genetic Variants with Cellular Dysfunction and Pathogenic Significance of *ACTC1* Gene Proximal Promoter in 634 Patients with Atrial Septal Defects and Controls

Zi-Fei Zheng [1,2,3,4,†], Huan-Xin Chen [1,2,3,4,†], Hai-Tao Hou [1,2,3,4,†], Qin Yang [1,2,3,4,†] and Guo-Wei He [1,2,3,4,*]

[1] Department of Cardiovascular Surgery & The Center for Basic Medical Research, TEDA International Cardiovascular Hospital, Tianjin University, Tianjin 300072, China
[2] Tianjin Key Laboratory of Molecular Regulation of Cardiovascular Diseases and Translational Medicine, Tianjin 300457, China
[3] The Institute of Cardiovascular Diseases, Tianjin University, Tianjin 300072, China
[4] TEDA International Cardiovascular Hospital, Chinese Academy of Medical Sciences, Tianjin 300457, China
* Correspondence: gwhezj@163.com or gwhe@tju.edu.cn; Tel.: +86-22-6520-9089
† These authors contributed equally to this work.

How To Cite: Zheng, Z.-F.; Chen, H.-X.; Hou, H.-T.; et al. Genetic Variants with Cellular Dysfunction and Pathogenic Significance of ACTC1 Gene Proximal Promoter in 634 Patients with Atrial Septal Defects and Controls. *iCirculation* **2026**, *1*(1), 7. https://doi.org/10.53941/icirculation.2026.100007

Received: 13 February 2026
Revised: 15 April 2026
Accepted: 13 May 2026
Published: 5 June 2026

Abstract: *Background*. Atrial septal defect (ASD), one of the most prevalent congenital cardiac malformations, has a well-established link to genetic factors. *ACTC1* gene plays important role in the development of the heart but the genetic variants within the proximal promoter region of the *ACTC1* gene in ASD pathogenesis are unclear. This case-control study aimed to investigate the variants of proximal promoter region of the *ACTC1* in ASD patients. *Methods*. We extracted genomic DNA from blood samples of 634 subjects (316 ASD patients and 318 matched healthy controls) and conducted sequencing of the *ACTC1* proximal promoter region. To assess the functional impact of these variants, we carried out *in vitro* dual-luciferase reporter assays. To further reveal the molecular mechanism, we employed electrophoretic mobility shift assays (EMSAs) and performed in silico predictions of the protein's binding affinity using the JASPAR database. *Results*. Our analysis identified 11 variants in the proximal promoter region. Six of these were exclusively found in ASD patients, one of which was a novel variant (g.5083G>A). The results indicated that all six variants led to a significant reduction in proximal promoter transcriptional activity ($p < 0.05$). An integrated analysis revealed that these variants disrupt or alter specific transcription factor binding sites, thereby interfering with the normal transcriptional regulation of the *ACTC1* gene. *Conclusions*. Our study identifies a novel mechanism in which *ACTC1* proximal promoter variants impair gene transcription by disrupting transcription factor binding. These findings provide new experimental evidence and a theoretical basis for the genetic etiology of ASD.

Keywords: atrial septal defect; *ACTC1*; genetic; congenital heart disease

1. Introduction

As the most prevalent birth defect (~1% of live births), congenital heart disease (CHD) poses a major threat to infant health, representing the foremost cause of non-infectious mortality in infancy [1,2]. CHD encompasses a broad spectrum of cardiac structural anomalies, which may also coexist with extra-cardiac malformations [3]. Over recent decades, advances in human genetics and genomic technologies have significantly deepened the understanding of CHD pathogenesis. Key genes underlying CHD encode proteins that orchestrate embryonic heart

Copyright: © 2026 by the authors. This is an open access article under the terms and conditions of the Creative Commons Attribution (CC BY) license (https://creativecommons.org/licenses/by/4.0/).
Publisher's Note: Scilight stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.

development, including transcription factors, signaling molecules, and structural components [4]. Extensive genetic studies have provided a foundation for improving genetic diagnosis, early prevention, and personalized therapeutic strategies in affected individuals.

Atrial septal defect (ASD) including ostium primum defect and secondum defect, is due to a defect of atrial septum during embryonic development [5].

The cardiac α-actin (*ACTC1*) gene, located at chromosome 15q14, encodes a key sarcomeric protein essential for normal cardiac development. It was previously found that the pathogenic variants of the *ACTC1* gene were the basis of CHD including ASD, cardiomyopathy, and left ventricular noncompaction [6–9]. In addition, variants of *ACTC1* gene affect two essential genes involved in apoptosis, Caspase-3 and *Bcl-2*, to induce myocardial apoptosis [10], and the variants negatively regulate the serum response (SRF)-signaling, thereby establishing a direct link to the pathogenesis of CHD [11,12]. Our previous studies found that the variants of gene proximal promoter regions are related to cardiac development in patients with CHD, such as *ISL1* and *CITED2* [13–19].

In this study, we characterized the variants of *ACTC1* proximal promoter (In subsequent sections, the term "promoter" is used to refer to the proximal promoter) region in ASD patients. We further assessed the functional consequences of these variants on transcriptional activity using *in vitro* assays and investigated their potential contribution to the pathological mechanisms underlying ASD.

2. Materials and Methods

2.1. Participants and Blood Samples

The study cohort comprised 634 participants: 316 patients with isolated, sporadic secundum ASD (159 males, 157 females) and 318 matched healthy controls.

The study protocol was conducted in compliance with the Declaration of Helsinki and received approval from the relevant Institutional Ethics Committee (TEDA International Cardiovascular Hospital). Written informed consent was obtained from the parents or legal guardians of all participants. All patients underwent surgical correction at TEDA International Cardiovascular Hospital, Tianjin, China. Control subjects underwent comprehensive health evaluations, including echocardiography, confirming the absence of cardiac defects and other major systemic diseases. A flowchart outlining the study design is presented in Figure 1.

A total of 634 participants were included in this study. We performed sequence analysis, cell function experiments, electrophoretic mobility analysis, and bioinformatics analysis sequentially to validate our hypothesis. ASD, atrial septal defect.

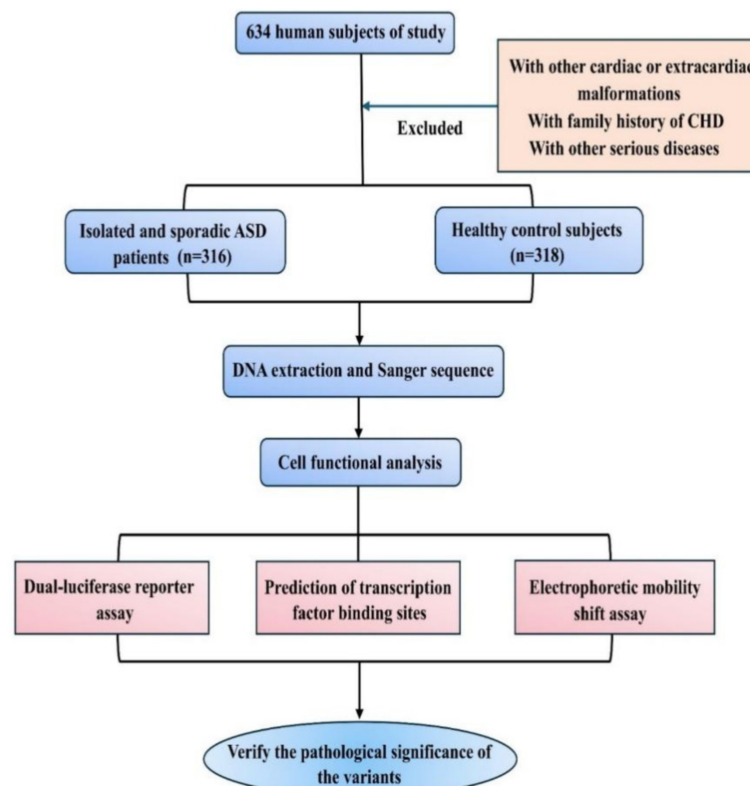


Figure 1. Study process diagram.

2.2. DNA Extraction and Sequence Analysis

Extraction of genomic DNA from peripheral blood leukocytes was performed with a commercial kit (centrifuge-column based). According to the manufacturer's protocol, 200 μL of whole blood was used, yielding DNA in a final elution volume of 50 μL, with storage at −20 °C. The promoter region of the *ACTC1* gene (a 953-bp fragment spanning from −1504 to −551 bp; GenBank accession no. NG_007553.1) was targeted for amplification using the 2×UltraTaq PCR StarMix (Dye) (GenStar, Beijing, China). A pair of primers was designed, and the polymerase chain reaction (PCR) conditions were optimized. Purified PCR amplicons were Sanger-sequenced. Resulting chromatograms from ASD patients and controls were analyzed by alignment against the reference sequence to identify variants (primer sequences in Table 1).

Table 1. The primer list used in this study.

Primers	Sequences 5′-3′	Location
PCR primers		
ACTC1-F1	5′-TGGGGTAAACCTGGTCCCTT-3′	4414−4423
ACTC1-R1	5-'ACCAAATCAAAAAGTGTGGGCTT-3′	5367−5389
Sequence primers		
ACTC1-F1	5′-TGGGGTAAACCTGGTCCCTT-3′	4414−4423
ACTC1-R1	5-'ACCAAATCAAAAAGTGTGGGCTT-3′	5367−5389
The double-stranded biotinylated oligonucleotides for the EMSA		
g.4701G>A-Forward	5′-GCAGCCCTGGAAGAT**(G/A)**AGAAGCCGCTGTTGC-3′	
g.4701G>A-Reverse	5′-GCAACAGCGGCTTCT**(C/T)**ATCTTCCAGGGCTGC-3′	
g.4832T>C-Forward	5′-TGCGGAGGACCGAA**(T/C)**CCACAGACCATCCA-3′	
g.4832T>C-Reverse	5′-TGGATGGTCTGTGG**(A/G)**TTCGGTCCTCCGCA-3′	
g.5063A>G-Forward	5′-TCCTAGCGGGTGCGA**(A/G)**GGGGACCAAATAAGG-3′	
g.5063A>G-Reverse	5′-CCTTATTTGGTCCCC**(T/C)**TCGCACCCGCTAGGA-3′	
g.5083G>A-Forward	5′-ACCAAATAAGGCAAG**(G/A)**TGGCAGACCGGGCC-3′	
g.5083G>A-Reverse	5′-GGCCCGGTCTGCCA**(C/T)**CTTGCCTTATTTGGT-3′	
g.5099C>T-Forward	5′-GCAGACCGGGCCC**(C/T)**CCACCCCTGCCCCC-3′	
g.5099C>T-Reverse	5′-GGGGGCAGGGGTGG**(G/A)**GGGCCCGGTCTGC-3′	
g.5124A>G-Forward	5′-TGCTCCA**(A/G)**CTGACCCTGTCCATCAGCGTT-3′	
g.5124A>G-Reverse	5′-AACGCTGATGGACAGGGTCAG**(T/C)**TGGAGCA-3′	
Nucleotide sequence for JASPAR prediction of TFBS		
g.4701G>A-Forward	5′-GCAGCCCTGGAAGAT**(G/A)**AGAAGCCGCTGTTGC-3′	
g.4832T>C-Forward	5′-TGCGGAGGACCGAA**(T/C)**CCACAGACCATCCA-3′	
g.5063A>G-Forward	5′-TCCTAGCGGGTGCGA**(A/G)**GGGGACCAAATAAGG-3′	
g.5083G>A-Forward	5′-ACCAAATAAGGCAAG**(G/A)**TGGCAGACCGGGCC-3′	
g.5099C>T-Forward	5′-GCAGACCGGGCCC**(C/T)**CCACCCCTGCCCCC-3′	
g.5124A>G-Forward	5′-TGCTCCA**(A/G)**CTGACCCTGTCCATCAGCGTT-3′	

PCR primers are designed based on the genomic DNA sequence of the *ACTC1* gene (NG_007553.1). The transcription start site is at the position of 5919 (+1). EMSA, electrophoretic mobility shift assay; TFBS, transcription factor binding sites; F, forward; R, reverse. The variation sites are presented in bold. Standard italics are used for the gene name *(ACTC1*) following standard nomenclature conventions.

2.3. Plasmid Construction, Cell Culture, and Cell Transfection

To assess the functional impact of the identified variants on promoter activity, we generated a firefly luciferase reporter construct (pGL3-WT) by cloning the wild-type *ACTC1* promoter region into the KpnI/SacI sites of the pGL3-Basic vector. Six variant constructs (pGL3-V4701, pGL3-V4832, pGL3-V5063, pGL3-V5083, pGL3-V5099, and pGL3-V5124) were generated using site-directed mutagenesis. For plasmid propagation, constructs were transformed into *E. coli* DH5α and grown overnight in LB medium (37 °C, 200 rpm). Plasmids were then purified using the Star Prep Rapid Plasmid Miniprep Kit and stored at −80 °C.

HL-1 atrial cardiomyocytes were routinely cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. For transfection, cells were seeded in 6-well plates; upon reaching 60–70% confluence, they were co-transfected with individual reporter plasmids, the pRL-SV40 *Renilla* luciferase plasmid (internal control), and the empty pGL3-Basic vector (negative control). Firefly luciferase activity was measured at 48 h post-transfection using a dual-luciferase assay and normalized to the *Renilla* signal. The overall workflow is schematized in Figure 2.

To confirm the impact of the variants on promoter activity, wild-type and variant-type *ACTC1* gene promoter fragments were generated to construct gene expression vectors and then transfected into HL-1. The dual-luciferase reporter assay was conducted to measure the wild-type and variant-type *ACTC1* gene promoter transcriptional activity.

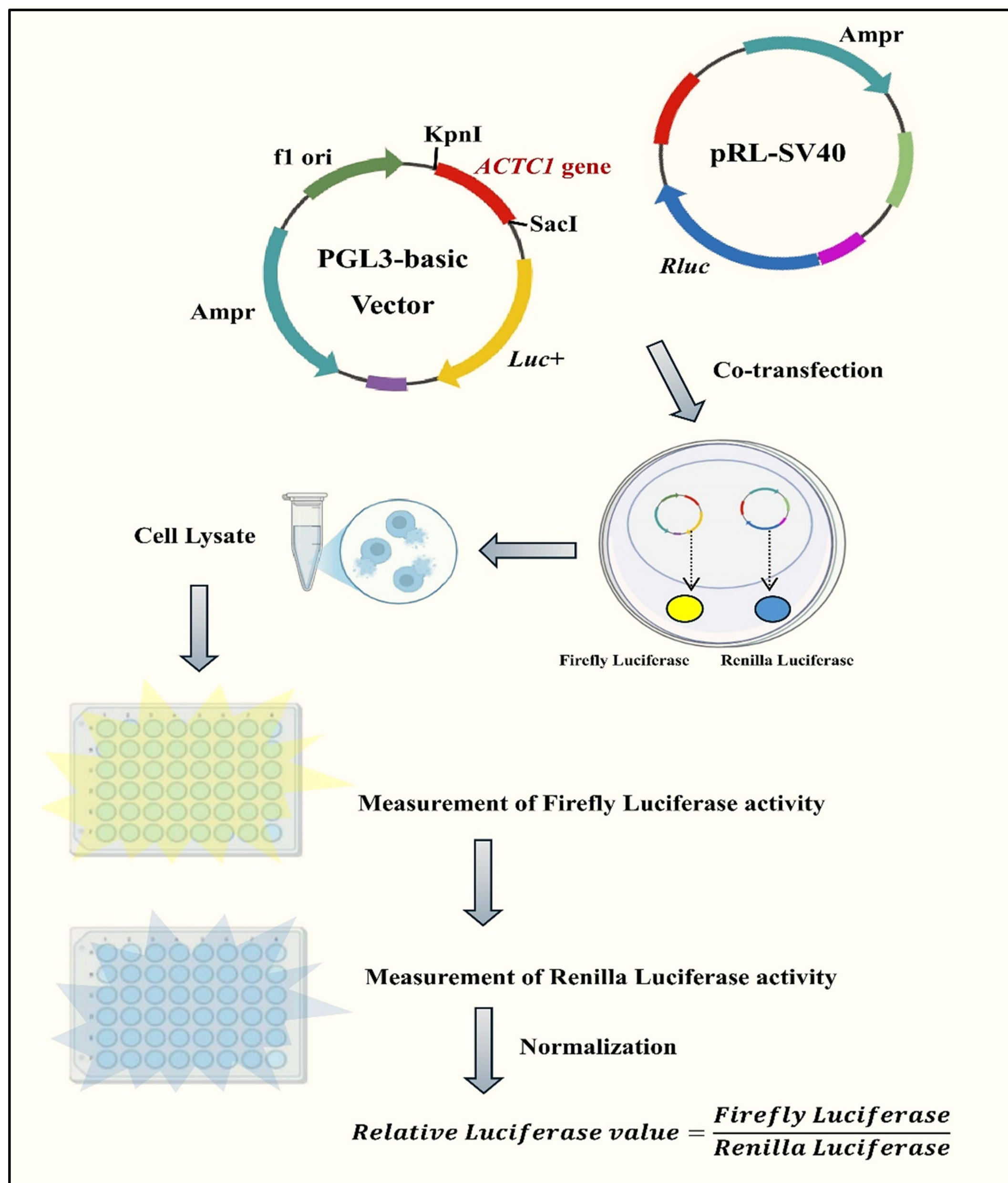


Figure 2. Dual luciferase reporter gene experiment workflow.

2.4. Dual Luciferase Reporter Gene Assay

Following the transfection period, cells were lysed directly using a passive lysis buffer. Luciferase activity in the cell lysates was quantified using the Dual-Luciferase® Reporter Assay Kit (Beyotime, Shanghai, China), with an equal volume of lysis buffer used as a blank control for background subtraction. Firefly luciferase activity was normalized to the corresponding Renilla signal to account for transfection variability. Relative promoter activity was calculated by defining the wild-type construct as 100%, with variant activities expressed as a percentage thereof. To ensure standardized conditions and strict comparability, all samples within a given experiment were harvested and assessed simultaneously. The assays were performed in three independent experiments, with each construct tested in triplicate per experiment. Data are representative of three independent experiments.

2.5. Electrophoretic Mobility Shift Analysis (EMSA)

For EMSAs, nuclear proteins were extracted from HEK-293 cells using a commercial kit (Beyotime) and stored at −80 °C. Complementary oligonucleotides were annealed to generate biotin-labeled probes for the wild-type and variant *ACTC1* promoter regions (Table 1). Subsequently, protein-DNA binding reactions were carried out with a commercial EMSA kit (Beyotime). Protein-DNA complexes were run on a 4% native polyacrylamide gel (90 V, 1 h) and blotted onto a nylon membrane (380 mA, 1 h). Protein-DNA binding affinity for the *ACTC1* promoter was assessed by EMSA using biotin-labeled probes, which were detected by chemiluminescence.

2.6. Transcription Factor Binding Site Prediction

To identify putative gains or losses of transcription factor binding sites (TFBS), we screened the identified *ACTC1* promoter variants against the JASPAR database. Predictions were considered high-confidence if they exceeded a relative profile score threshold of 85%. All sequences are provided in Table 1.

2.7. Statistical Analysis

For data reporting, continuous measures are reported as mean ± SD, while categorical measures are given as percentages. Inter-group comparisons were evaluated using one-way ANOVA. A probability value of less than 0.05 was defined as statistically significant. The SPSS statistical package, version 25.0, was used for all analyses.

3. Results

3.1. Variants Found in the ASD Patients

Comparative analysis of the *ACTC1* promoter sequence between 316 ASD patients and 318 healthy controls identified 11 variants. Six of the 11 variants were unique to ASD patients [g.4701G>A(rs11635130), g.4832T>C(rs1244826535), g.5063A>G(rs1487588877), g.5083G>A, g.5099C>T(rs2140434206), and g.5124A>G(rs1891814086)], whereas the other five were shared with the control group. Through a search of the NCBI SNP database, the g.5083G>A variant was identified as a novel variant, with no previously reported occurrences. In addition, in the databases of ALFA and gnomAD-Genomes, the allele frequency of 6 variants only existing in the ASD patients is lower than 0.001 in the East Asian population. This indicated that they have potential pathological significance for the formation of ASD. Consequently, we focused on functionally characterizing the six variants unique to the ASD cohort. The five variants also found in healthy controls were excluded from further analysis based on this finding. Details of all variants are provided in Table 2. The schematic in Figure 3A illustrates their positions within the *ACTC1* promoter, and Figure 3B presents the corresponding sequencing chromatograms.

Table 2. Variants in *ACTC1* gene promoter region of ASD patients and healthy controls.

	Position *	**Genotypes**	**ASD**	**Controls**	**Allele Frequency**			
Frequency in Control = 0 (Further Validation)					**ALFA [a]**		**gnomAD – Genomes [b]**	
					Total	**East Asian**	**Global**	**East Asian**
g.4701G>A (rs11635130)	−1218	G>A	2	0	T = 0.076	T = 0.0000	T = 0.062	T = 0.0000
g.4832T>C (rs1244826535)	−1087	T>C	1	0	G = 0.00000	G = 0.00	G = 0.000014	G = 0.0006
g.5063A>G (rs1487588877)	−856	A>G	1	0	G = 0.00000	G = 0.00	G = 0.000007	G = 0.0000
g.5083G>A	−836	G>A	1	0	None		None	
g.5099C>T (rs2140434206)	−820	C>T	1	0	None		None	
g.5124A>G (rs1891814086)	−795	A>G	1	0	C = 0.00000	C = 0.00	None	
Frequency in Control ≠ 0 (No Further Validation)								
g.4600G>A (rs79823729)	−1319	G>A	4	4	T = 0.012	T = 0.00	None	None
g.4636C>T (rs746216)	−1283	C>T	16	13	A = 0.069	A = 0.009	A = 0.074	A = 0.012
g.4922C>G (rs374099539)	−997	C>G	5	8	C = 0.00014	C = 0.02	C = 0.000350	C = 0.015
g.5016C>A (rs370499568)	−903	C>A	1	1	T = 0.00021	T = 0.01	T = 0.00021	T = 0.0045
g.5187T>C (rs113178069)	−732	T>C	21	12	G = 0.095	G = 0.00	G = 0.11	G = 0.015

* The position of variants is relative to the transcription start site at 5919 (+1) of *ACTC1* gene (NG_007553.1). [a] ALFA Allele Frequency, the ALFA project provides aggregate allele frequency from dbGaP. [b] The genome Aggregation Database (gnomAD), the data set provided on this website spans 123,136 exome sequences and 15,496 whole-genome sequences from unrelated individuals sequenced as part of various disease-specific and population genetic studies. (PRJNA398795). Standard italics are used only for the gene name (*ACTC1*) following standard nomenclature. ASD, Atrial septal defect.

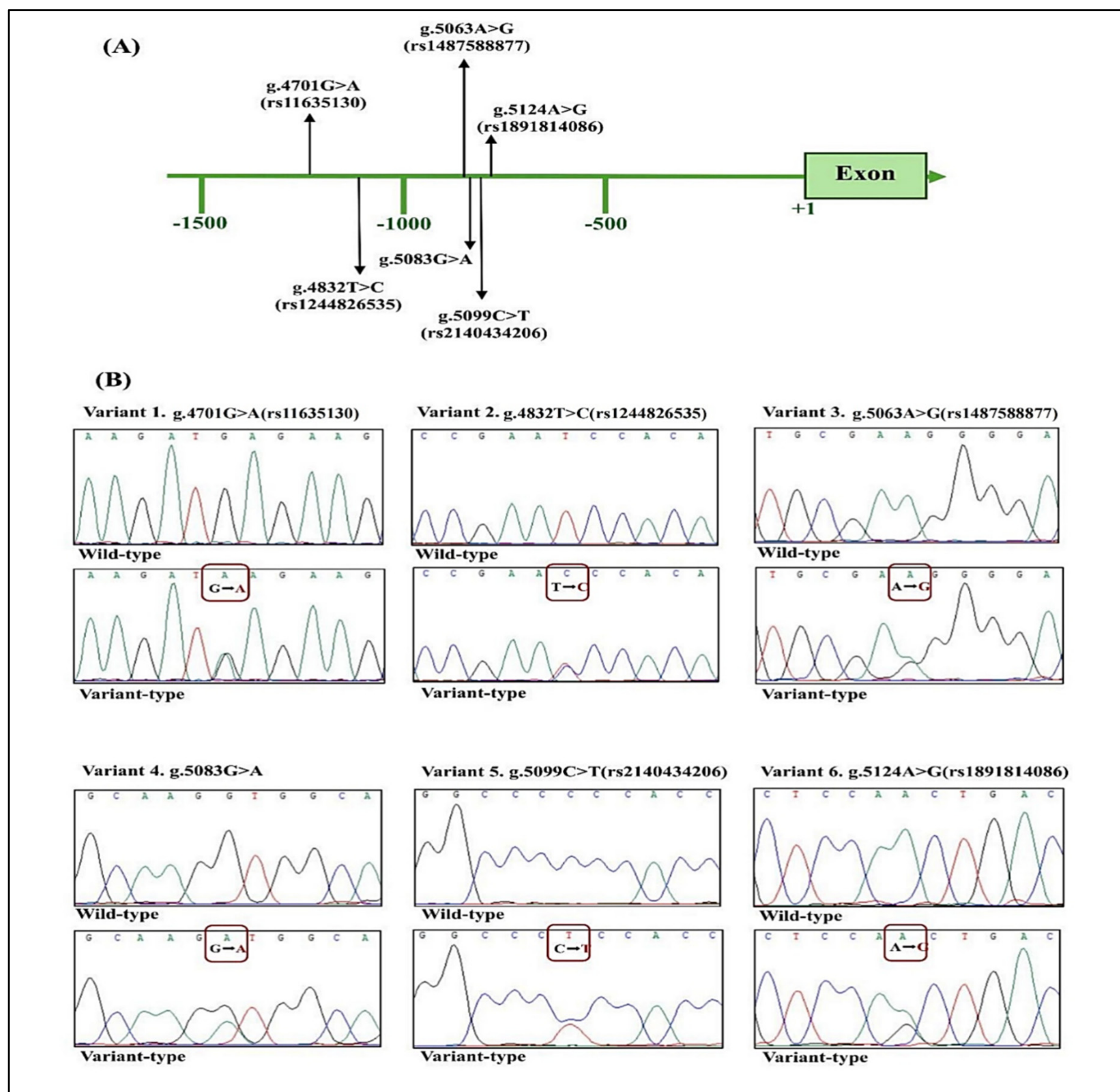


Figure 3. Locations and sequencing chromatograms of the variants of *ACTC1* gene promoter. (**A**) the genetic variants are named in accordance with the genomic DNA sequence of the human *ACTC1* gene (Genbank accession number NG_007553.1). The transcription start site is at the position of 5919 in the first exon. (**B**) sequencing chromatograms of the variants within the *ACTC1* gene promoter for all the heterozygous variants identified only in patients with ASD [g.4701G>A(rs11635130), g.4832T>C(rs1244826535), g.5063A>G(rs1487588877), g.5083G>A, g.5099C>T(rs2140434206), g.5124A>G(rs1891814086)]. Top panels show wild-type and bottom panels show heterozygous DNA sequences, marked with arrows.

3.2. Dual Luciferase Reporter Gene Assay Results

To determine the effects of the identified *ACTC1* promoter variants on transcriptional activity, dual-luciferase reporter assays were conducted. The wild-type promoter construct (pGL3-WT) and six variant constructs (pGL3-V4701, pGL3-V4832, pGL3 -V5063, pGL3 -V5083, pGL3-V5099, and pGL3-V5124) were individually co-transfected with the pRL-SV40 control plasmid (expressing *Renilla* luciferase) into HL-1 cardiomyocytes. After 24–48 h, luciferase activity was measured to quantify promoter-driven expression. For normalization, firefly luciferase activity was expressed as a ratio to the corresponding *Renilla* signal to account for transfection variability. The results showed that the transcription activity of all 6 variants found only in ASD patients decreased in different degrees. The transcription activities of promoters in V4701G>A(rs11635130), V4832T>C(rs1244826535), V5063A>G(rs1487588877), V5083G>A, V5099C>T(rs2140434206), and V5124A>G(rs1891814086) were reduced to 71.4%, 50.6%, 45.1%, 56.17%, 66.05%, and 69.82%, respectively (p<0.05). Transcriptional activity of the wild-type *ACTC1* promoter was normalized to 100%. The results are shown in Figure 4A. Representative parasternal short-axis color Doppler echocardiograms for five variants [g.4701G>A(rs11635130), g.4832T>C(rs1244826535), g.5063A>G(rs1487588877), g.5083G>A, and g.5099C>T(rs2140434206)] are shown in Figure 4B.

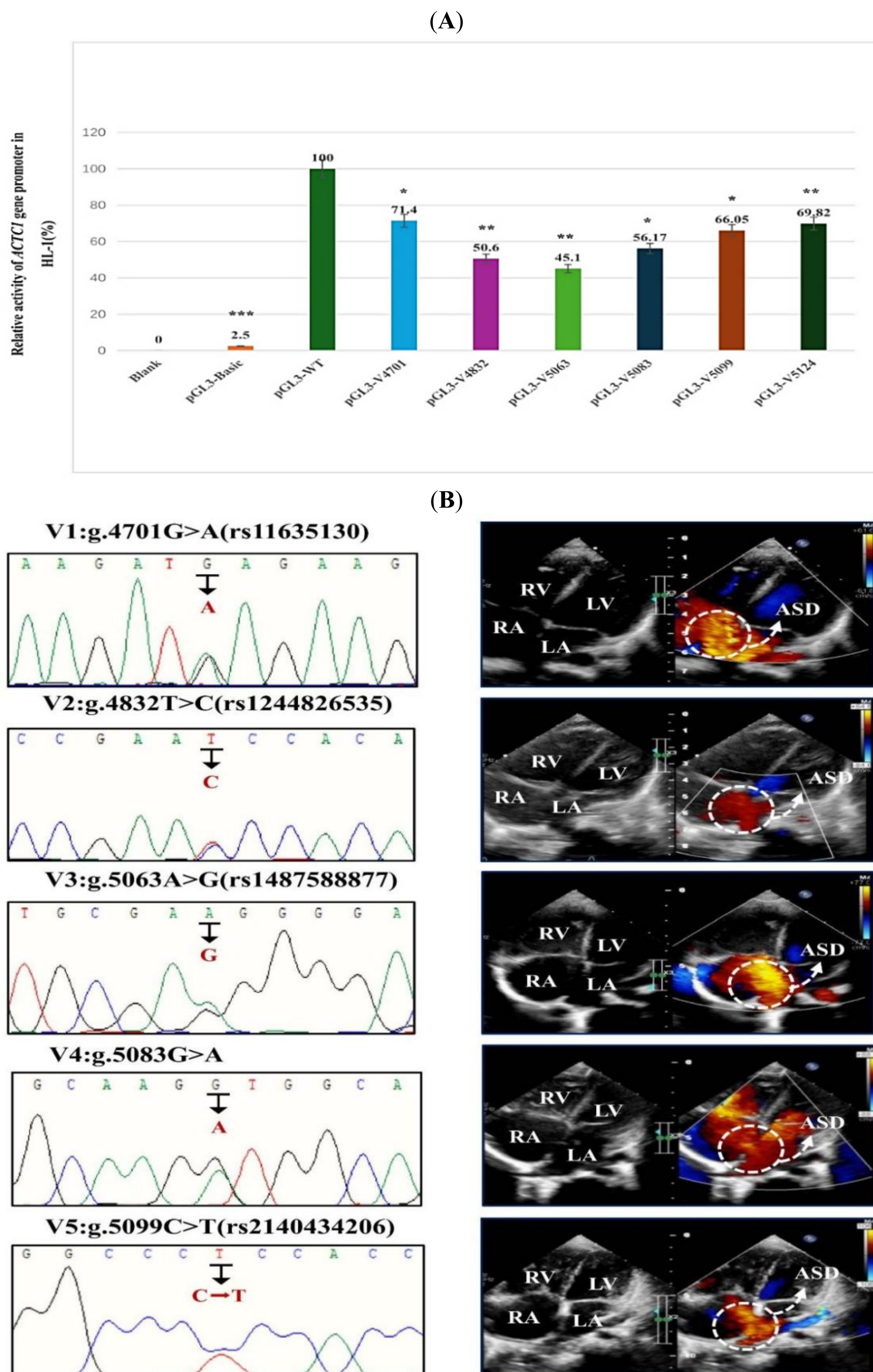


Figure 4. Dual luciferase reporter gene assay results. (**A**) Wild-type and variant-type *ACTC1* gene promoters were subcloned into pGL3-basic reporter vector and transfected into HL-1. Empty vector pGL3-basic was used as a negative control and dual-luciferase activities were assayed. The transcriptional activity of the wild-type *ACTC1* gene promoter was set as 100%. The relative activities of *ACTC1* gene promoters were calculated. The results of dual-luciferase activity showed that the transcriptional activity of the promoters with variants [g.4701G>A(rs11635130), g.4832T>C(rs1244826535), g.5063A>G(rs1487588877), g.5083G>A, g.5099C>T(rs2140434206), g.5124A>G(rs1891814086)] were reduced to 71.4%, 50.6%, 45.1%, 56.17%, 66.05%, and 69.82%, respectively. The quantitative data are represented as the means ± SD based on three independent experiments. * $p < 0.05$; ** $p < 0.01$. (**B**) Color Doppler echocardiography of parasternal short-axis view. The images show the cardiac chambers (left Echo panels) and the left-to-right blood flow shunt from the left atrium to the right atrium through the ASD (dotted line circle and the arrow), and show ASD (arrow) in patients with variants of g.4701G>A(rs11635130), g.4832T>C(rs1244826535), g.5063A>G(rs1487588877), g.5083G>A, and g.5099C>T(rs2140434206). LV, left ventricle; LA, left atrium; RA, right atrium; RV, right ventricle.

3.3. Results of EMSA

EMSAs with biotin-labeled probes were utilized to interrogate potential alterations in transcription factor binding caused by the six candidate variants. As shown in Figure 5, variants in band intensity reflect differences in transcription factor binding affinity. The results demonstrated that all six variants qualitatively altered the DNA-protein binding profile compared to the wild-type probe. Consistent with our in silico predictions indicating both the creation and disruption of binding sites, the variants resulted in a complex pattern involving both increased and decreased binding affinities for specific nuclear protein complexes.

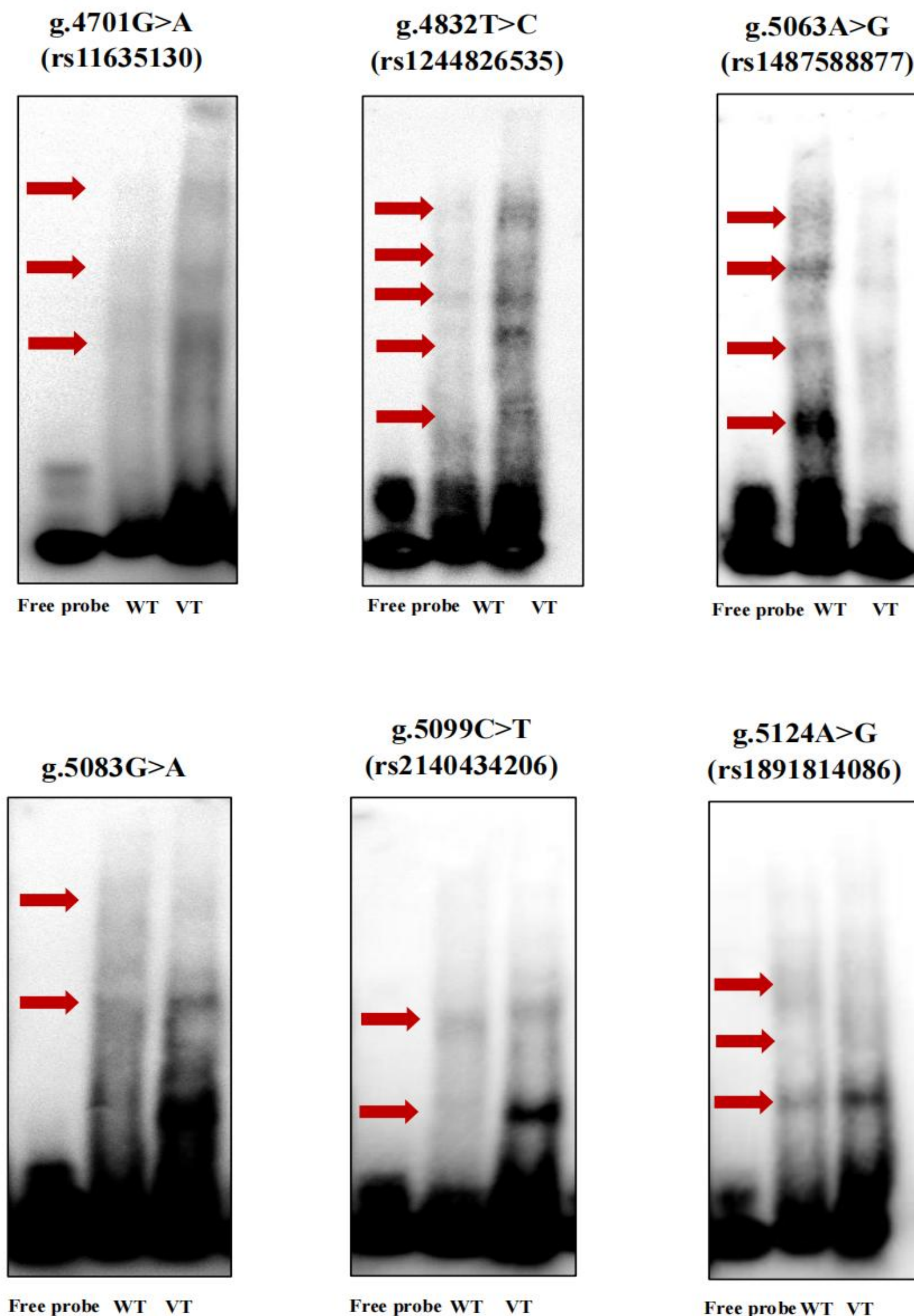


Figure 5. The results of electrophoretic mobility shift assay for variants in *ACTC1* gene promoter.

EMSA shows that variants may affect the binding ability of transcription factors. The free probe is marked with a black arrow at the bottom. The affected binding of an unknown transcription factor is marked with the upper double black. EMSA, electrophoretic mobility shift assay.

3.4. Predictive Results of Affected Transcription Factor Binding Sites

The variants identified in this study were predicted to alter the transcription factor binding landscape within the *ACTC1* promoter. Given that in silico tools identify putative binding based on short, overlapping sequence motifs, a single variant can computationally alter scores for numerous overlapping position weight matrices, causing the local sequence to deviate from the consensus matrices. To reduce false positives and address tissue specificity, we rigorously filtered the initial JASPAR predictions to highlight only transcription factors with documented expression and functional relevance in the developing heart or atrial myocardium. The filtered, biologically plausible TFBS alterations (e.g., *TBX5*, *ZEB1*, *GATA4*, *RUNX2*) are summarized in Table 3.

Table 3. Predicted TFBS and Mutant-affected promoter Activity by JASPAR Database.

	Binding Sites for Transcription Factors		
Variants	**Create**	**Disrupt**	**Promoter Activity**
g.4701G>A(rs11635130)	GATA4, OTX2	ZNF394, NONO	↓
g.4832T>C(rs1244826535)	RUNX2, VDR, RUNX3, NR2C2, KLF3	MZF1, ZBTB20, RHOXF2, ZNF479, ZNF296	↓
g.5063A>G(rs1487588877)	TCFL5, VAX2, TBX1, NOTO, SNAI2, TBX15, LMX1B, GATA5, NKX6-2, KLF17, MAZ, PLAGL2, NR2C2, VEZF1, ZNF468, ZNF571	EOMES, TBR1, TBX18, RBPJ,	↓
g.5083G>A	YY1, YY2, NFIC, THAP1, ZNF597, ZNF534, ZNF891	ZEB1, TBX5, FIGLA, CTCFL, NR2C1, NR5A1, TBX18, TBX3, ZNF100	↓
g.5099C>T(rs2140434206)	ZNF263, NKX2-8, ZNF425, ZNF671, ZNF611, GLI4, ZNF366	MZF1, ZBTB7C, ZNF740, EGR4, KLF6, KLF11, SP9, GLI3, ZNF468, ZNF749, ZNF571	↓
g.5124A>G(rs1891814086)	ZNF354C, MYOG, TCF3, TFAP4, ASCL1, BHLHA15, ZBTB6, ZNF519, ZNF273, ZNF462	MYB, NFIC, NFIX, NR1D1, NR1D2	↓

TFBS, transcription factor binding sites.

4. Discussion

In this study, we for the first time found in the ASD patients in comparison to the normal controls that (1) there were 11 variants in *ACTC1* gene promoter. Among them, six variants only occurred in the ASD patients, and 1 variant (g.5083G>A) was a novel one that had not been reported before; (2) all six variants consistently impaired promoter function, thereby diminishing *ACTC1* transcriptional activity; and (3) all the variants affected the binding with TFBS and have pathological roles in the development of ASD.

The *ACTC1* gene is highly conserved from birds to mammals. It encodes a core sarcomeric protein essential for myocardial contraction, functioning through its direct interactions with troponin and tropomyosin [20,21]. *ACTC1* is the main actin subtype in the adult human heart. From early embryogenesis through to late fetal stages, it exerts a critical regulatory influence across multiple cardiac cell lineages [22]. In addition, knocking out *ACTC1* gene in chicken embryos can lead to delayed cardiac circulation and ASD. These findings implicate dysregulation of the *ACTC1* gene in the pathogenesis of ASD [8,23,24].

The occurrence of ASD is partly due to the variants of cardiac transcription factors. Genes such as *ACTC1*, *NKX2-5, LBX2* and *GATA4* are important to the development of the heart. They may affect the process of heart separation by affecting the migration of neural crest cells, thus leading to ASD [24–26].

Alterations in TFBS represent a key mechanism by which non-coding genetic variants can disrupt transcriptional regulation and contribute to the pathogenesis of CHD. Specifically, sequence variants within gene promoter regions can impair normal transcriptional activation, leading to aberrant gene expression with potential pathological consequences [27,28]. As summarized in Table 2 and illustrated in Figure 3, all 6 variants identified within the *ACTC1* promoter region were found to alter predicted TFBS profiles and significantly reduce transcription factor binding affinity. These functional findings strongly suggest that pathogenic promoter variants in *ACTC1* drive transcriptional dysregulation, thereby contributing to the molecular etiology of ASD.

While our in silico JASPAR analysis suggests potential candidates (such as TBX5 or ZEB1), we acknowledge that these predictions remain speculative and are intended as hypothesis-generating tools. The exact *in vivo* transcription factor repertoire altered by these variants requires further definitive validation using *in vivo* models or targeted chromatin immunoprecipitation assays. Nonetheless, based on our functional data and literature, we propose the following potential mechanisms. First, the reduced expression of the ACTC1 gene itself is a direct contributor to ASD [24]. Second, rather than acting as isolated events, our in silico data (Table 3) suggests the

variants disrupt the binding of master regulators like TBX5 to the ACTC1 promoter. TBX5 normally interacts with NKX2-5 and GATA4 to activate cardiac development, the disruption of TBX5 binding may destabilize this local multiprotein regulatory complex, hindering proper heart septation [29–31]. Third, the disruption of the ZEB1 binding site on the ACTC1 promoter impairs another critical pathway. ZEB1 orchestrates epithelial-mesenchymal transition (EMT), which is indispensable for normal cardiac septum formation. Furthermore, because the actin cytoskeleton closely regulates intracellular mechanotransduction, the profound loss of *ACTC1* structural protein may mechanically impair the essential interaction between *ZEB1* and Smad proteins, thereby inhibiting BMP/TGFβ signal transduction necessary for EMT [32–34]. Finally, this structural cytoskeletal deficit may trigger downstream compensatory signaling cascades leading to the upregulation of *VEZF1*. Increased *VEZF1* is known to repress CITED2 expression, a condition that our previous study has directly linked to the development of ASD [14,35]. Figure 6 presents a working model depicting the potential mechanism through which *ACTC1* promoter variants may contribute to the development of ASD, as inferred from our data.

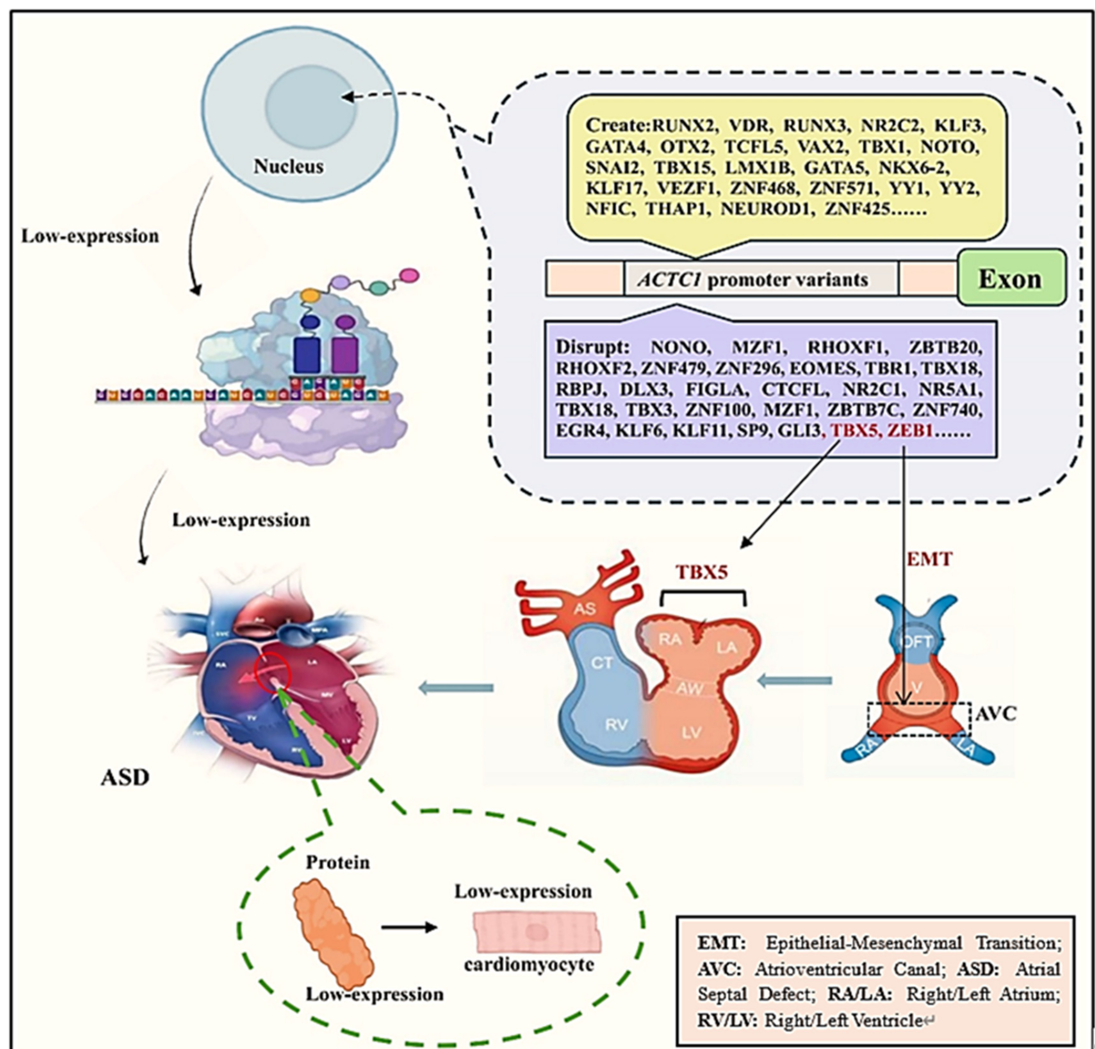


Figure 6. A schema describing the role of the promoter variant in the *ACTC1* gene.

In summary, our study demonstrates that all six identified variants in the *ACTC1 promoter* region lead to a significant reduction in transcriptional activity. Functional *in vitro* assays confirmed that the resultant alterations in *ACTC1* expression disrupt key cellular processes relevant to cardiac development. EMSA further revealed that all 6 variants reorganized the binding profiles of nuclear proteins, demonstrating both enhanced and diminished binding affinities. This reflects a complex alteration of the transcriptional machinery, ultimately contributing to the net reduction in promoter activity observed in our functional assays. Collectively, these findings suggest that *ACTC1* promoter variants may diminish the expression of downstream genes critical for cardiac morphogenesis, thereby potentially contributing to aberrant heart development and ASD pathogenesis.

This study, however, has several limitations. Rather than assay bias, this likely reflects our selection of variants unique to the ASD cohort, as ACTC1 loss-of-function is a known driver of cardiac defects. Future studies

should test the shared, benign variants as negative controls to confirm this finding. Second, while our study links *ACTC1 promoter* variants to ASD, it is imperative to note that CHD is a multifactorial disorder, whose etiology likely involves the convergence of numerous genetic and environmental determinants. Furthermore, the *in vivo* functional impact and pathogenic contribution of these specific variants remain to be validated using tissue or animal models. Another important avenue for future research is the elucidation of the regulatory interplay between the identified *ACTC1* promoter variants and their downstream target gene networks to fully understand their role in the genetic architecture of CHD. Future studies in these directions will help clarify the precise mechanisms through which non-coding variants contribute to cardiac malformations.

This model describes the role of variants in the promoter region of the *ACTC1* gene identified from this study. Variants in the promoter of the *ACTC1* gene identified in this study may alter the TFBS cluster, resulting in a decrease in gene expression. In addition, some of the disrupted TFBS play a key role in cardiac development. These factors contribute to the formation of ASD. EMT, epithelial-mesenchymal transition; AVC, atrioventricular canal; ASD, atrial septal defect.

5. Conclusions

We identified 11 variants in the *ACTC1* promoter. Notably, six were found exclusively in the ASD cohort [g.4701G>A(rs11635130), g.4832T>C(rs1244826535), g.5063A>G(rs1487588877), g.5083G>A, g.5099C>T (rs2140434206), g.5124A>G(rs1891814086)], one of which (g.5083G>A) is novel. Furthermore, functional assays demonstrated that all six variants abrogated promoter activity. These findings provide crucial genetic and molecular insights into the role of *ACTC1* in ASD.

Author Contributions

Z.-F.Z.: data curation, formal analysis, investigation, validation, writing—reviewing and editing; H.-T.H. and H.-X.C.: investigation; Q.Y.: investigation; G.-W.H.: conceptualization, methodology, visualization, supervision, funding acquisition, project administration, writing—reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Funding

This research was funded by the National Natural Science Foundation of China, grant numbers 82370350 and 82170353; Internal Fund from TEDA International Cardiovascular Hospital, grant number 2021-ZX-002; Tianjin Key Medical Discipline Construction Project (TJYXZDXK-3-036C) and Special Fund for High Quality Development Project.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Ethics Committee of TEDA International Cardiovascular Hospital (protocol code [2021]-0715-4 and date of approval: 15 July 2021).

Informed Consent Statement

Informed consent was obtained from all subjects involved in the study. Written informed consent has been obtained from the patients (or their parents/guardians) to publish this paper.

Data Availability Statement

Data used in the current study are available upon reasonable request from the corresponding author.

Acknowledgments

We thank the patients and their family members for cooperation. The assistance of surgeons and nursing staff at the Division of Pediatric Cardiac Surgery, Department of Cardiovascular Surgery is gratefully acknowledged.

Conflicts of Interest

The authors declare no conflict of interest. Given their editorial roles, Guo-Wei He (Editor-in-Chief) and Qin Yang (Editorial Board Member) had no involvement in the peer review of this paper and had no access to information regarding its peer-review process. Full responsibility for the editorial process of this paper was delegated to another editor of the journal.

Use of AI and AI-Assisted Technologies

No AI tools were utilized for this paper.

References

1. Duarte, V.E.; Singh, M.N. Genetic syndromes associated with congenital heart disease. *Heart* **2024**, *110*, 1231–1237. https://doi.org/10.1136/heartjnl-2023-323126.
2. Garg, V. Insights into the genetic basis of congenital heart disease. *Cell Mol. Life Sci.* **2006**, *63*, 1141–1148. https://doi.org/10.1007/s00018-005-5532-2.
3. Morton, S.U.; Quiat, D.; Seidman, J.G.; et al. Genomic frontiers in congenital heart disease. *Nat. Rev. Cardiol.* **2022**, *19*, 26–42. https://doi.org/10.1038/s41569-021-00587-4.
4. Williams, K.; Carson, J.; Lo, C. Genetics of Congenital Heart Disease. *Biomolecules* **2019**, *9*, 879. https://doi.org/10.3390/biom9120879.
5. Geva, T.; Martins, J.D.; Wald, R.M. Atrial septal defects. *Lancet* **2014**, *383*, 1921–1932. https://doi.org/10.1016/s0140-6736(13)62145-5.
6. Chong, J.X.; Childers, M.C.; Marvin, C.T.; et al. Variants in ACTC1 underlie distal arthrogryposis accompanied by congenital heart defects. *HGG Adv.* **2023**, *4*, 100213. https://doi.org/10.1016/j.xhgg.2023.100213.
7. Ingles, J.; Goldstein, J.; Thaxton, C.; et al. Evaluating the Clinical Validity of Hypertrophic Cardiomyopathy Genes. *Circ. Genom. Precis. Med.* **2019**, *12*, e002460. https://doi.org/10.1161/circgen.119.002460.
8. Frank, D.; Yusuf Rangrez, A.; Friedrich, C.; et al. Cardiac α-Actin (ACTC1) Gene Mutation Causes Atrial-Septal Defects Associated With Late-Onset Dilated Cardiomyopathy. *Circ. Genom. Precis. Med.* **2019**, *12*, e002491. https://doi.org/10.1161/circgen.119.002491.
9. Finsterer, J.; Stöllberger, C. Non-compaction associated with an ACTC1 mutation. *Acta Cardiol.* **2015**, *70*, 88–89. https://doi.org/10.1080/ac.70.1.3064601.
10. Jiang, H.K.; Qiu, G.R.; Li-Ling, J.; et al. Reduced ACTC1 expression might play a role in the onset of congenital heart disease by inducing cardiomyocyte apoptosis. *Circ. J.* **2010**, *74*, 2410–2418. https://doi.org/10.1253/circj.cj-10-0234.
11. Rangrez, A.Y.; Kilian, L.; Stiebeling, K.; et al. Data on the role of cardiac α-actin (ACTC1) gene mutations on SRF-signaling. *Data Brief* **2020**, *28*, 105071. https://doi.org/10.1016/j.dib.2019.105071.
12. Rangrez, A.Y.; Kilian, L.; Stiebeling, K.; et al. A cardiac α-actin (*ACTC1*) p. Gly247Asp mutation inhibits SRF-signaling *in vitro* in neonatal rat cardiomyocytes. *Biochem. Biophys. Res. Commun.* **2019**, *518*, 500–505. https://doi.org/10.1016/j.bbrc.2019.08.081.
13. Yin, X.Y.; Chen, H.X.; Chen, Z.; et al. Identification and functional analysis of genetic variants of ISL1 gene promoter in human atrial septal defects. *J. Gene Med.* **2022**, *24*, e3450. https://doi.org/10.1002/jgm.3450.
14. Chen, Z.; Chen, H.X.; Hou, H.T.; et al. Genetic Variants of CITED2 Gene Promoter in Human Atrial Septal Defects: Case-Control Study and Cellular Functional Verification. *J. Cardiovasc. Dev. Dis.* **2022**, *9*, 321. https://doi.org/10.3390/jcdd9100321.
15. Zheng, S.Q.; Chen, H.X.; Liu, X.C.; et al. Identification of variants of ISL1 gene promoter and cellular functions in isolated ventricular septal defects. *Am. J. Physiol. Cell Physiol.* **2021**, *321*, C443–C452. https://doi.org/10.1152/ajpcell.00167.2021.
16. Yin, X.Y.; Chen, H.X.; Chen, Z.; et al. Genetic Variants of ISL1 Gene Promoter Identified from Congenital Tetralogy of Fallot Patients Alter Cellular Function Forming Disease Basis. *Biomolecules* **2023**, *13*, 358. https://doi.org/10.3390/biom13020358.
17. Chen, Z.; Chen, H.X.; Hou, H.T.; et al. Identification and Functional Verification of CITED2 Gene Promoter Region in Patients with Patent Ductus Arteriosus. *Int. J. Mol. Sci.* **2023**, *24*, 16204. https://doi.org/10.3390/ijms242216204.
18. Zheng, S.Q.; Chen, H.X.; Liu, X.C.; et al. Genetic analysis of the CITED2 gene promoter in isolated and sporadic congenital ventricular septal defects. *J. Cell Mol. Med.* **2021**, *25*, 2254–2261. https://doi.org/10.1111/jcmm.16218.
19. Chen, Z.; Chen, H.X.; Hou, H.T.; et al. Pathophysiological Role of Variants of the Promoter Region of CITED2 Gene in Sporadic Tetralogy of Fallot Patients with Cellular Function Verification. *Biomolecules* **2022**, *12*, 1644. https://doi.org/10.3390/biom12111644.

20. Wang, Y.; Du, X.; Zhou, Z.; et al. A gain-of-function ACTC1 3'UTR mutation that introduces a miR-139-5p target site may be associated with a dominant familial atrial septal defect. *Sci. Rep.* **2016**, *6*, 25404. https://doi.org/10.1038/srep25404.
21. Yang, Q.L.; Bian, Y.Y.; Wang, B.; et al. Novel phenotype-genotype correlations of hypertrophic cardiomyopathy caused by mutation in α-actin and myosin-binding protein genes in three unrelated Chinese families. *J. Cardiol.* **2019**, *73*, 438–444. https://doi.org/10.1016/j.jjcc.2018.09.005.
22. Latinkić, B.V.; Cooper, B.; Towers, N.; et al. Distinct enhancers regulate skeletal and cardiac muscle-specific expression programs of the cardiac alpha-actin gene in Xenopus embryos. *Dev. Biol.* **2002**, *245*, 57–70. https://doi.org/10.1006/dbio.2002.0639.
23. Greenway, S.C.; McLeod, R.; Hume, S.; et al. Exome sequencing identifies a novel variant in ACTC1 associated with familial atrial septal defect. *Can. J. Cardiol.* **2014**, *30*, 181–187. https://doi.org/10.1016/j.cjca.2013.12.003.
24. Matsson, H.; Eason, J.; Bookwalter, C.S.; et al. Alpha-cardiac actin mutations produce atrial septal defects. *Hum. Mol. Genet.* **2008**, *17*, 256–265. https://doi.org/10.1093/hmg/ddm302.
25. Wang, J.; Luo, J.; Chen, Q.; et al. Identification of LBX2 as a novel causal gene of atrial septal defect. *Int. J. Cardiol.* **2018**, *265*, 188–194. https://doi.org/10.1016/j.ijcard.2018.04.038.
26. Fan, D.; Pang, S.; Chen, J.; et al. Identification and functional study of GATA4 gene regulatory variants in atrial septal defects. *BMC Cardiovasc. Disord.* **2021**, *21*, 321. https://doi.org/10.1186/s12872-021-02136-w.
27. Vooght, K.M.; Wijk, R.; Solinge, W.W. Management of gene promoter mutations in molecular diagnostics. *Clin. Chem.* **2009**, *55*, 698–708. https://doi.org/10.1373/clinchem.2008.120931.
28. Chen, H.; VanBuren, V. A provisional gene regulatory atlas for mouse heart development. *PLoS ONE* **2014**, *9*, e83364. https://doi.org/10.1371/journal.pone.0083364.
29. Al-Khindi, T.; Sherman, M.B.; Kodama, T.; et al. The transcription factor Tbx5 regulates direction-selective retinal ganglion cell development and image stabilization. *Curr. Biol.* **2022**, *32*, 4286–4298.e5. https://doi.org/10.1016/j.cub.2022.07.064.
30. Garg, V.; Kathiriya, I.S.; Barnes, R.; et al. GATA4 mutations cause human congenital heart defects and reveal an interaction with TBX5. *Nature* **2003**, *424*, 443–447. https://doi.org/10.1038/nature01827.
31. Bruneau, B.G.; Nemer, G.; Schmitt, J.P.; et al. A murine model of Holt-Oram syndrome defines roles of the T-box transcription factor Tbx5 in cardiogenesis and disease. *Cell* **2001**, *106*, 709–721. https://doi.org/10.1016/s0092-8674(01)00493-7.
32. Postigo, A.A.; Depp, J.L.; Taylor, J.J.; et al. Regulation of Smad signaling through a differential recruitment of coactivators and corepressors by ZEB proteins. *Embo J.* **2003**, *22*, 2453–2462. https://doi.org/10.1093/emboj/cdg226.
33. Nebigil, C.G.; Désaubry, L. The role of GPCR signaling in cardiac Epithelial to Mesenchymal Transformation (EMT). *Trends Cardiovasc. Med.* **2019**, *29*, 200–204. https://doi.org/10.1016/j.tcm.2018.08.007.
34. Li, C.; Xia, J.; Yao, W.; et al. Mechanism of LncRNA XIST/ miR-101-3p/ZEB1 axis in EMT associated with silicosis. *Toxicol. Lett.* **2022**, *360*, 11–19. https://doi.org/10.1016/j.toxlet.2022.03.001.
35. AlAbdi, L.; He, M.; Yang, Q.; et al. The transcription factor Vezf1 represses the expression of the antiangiogenic factor Cited2 in endothelial cells. *J. Biol. Chem.* **2018**, *293*, 11109–11118. https://doi.org/10.1074/jbc.RA118.002911.