Three rare variants of SOX7 impairing its interaction with GATA4 may be a predisposing factor to complete AVSD

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Research

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Abstract

Background

Atrioventricular septal defects (AVSD) are a complicated subtype of congenital heart defects for which the genetic basis is poorly understood. Many evidences have demonstrated that transcription factor SOX7 which can interact with GATA4 plays a pivotal role in the cardiovascular development. The critical role of GATA4 in the morphogenesis of atrioventricular septum implies SOX7 a potential involvement of AVSD. However, whether SOX7 variants are involved in the pathogenesis of AVSD needs to be explored.

Methods

100 sporadic non-syndromic AVSD Chinese Han patients were recruited and the variants of SOX7 in 100 patients were screened by target sequencing. Functional assays were performed to testify the potential pathogenicity of nonsynonymous variants of SOX7 found in these AVSD patients.

Results

Through target sequencing, we identified three rare variants c.40C>G, c.542G>A, and c.743C>T of SOX7 in 100 sporadic non-syndromic AVSD Chinese Han patients. All mutant sites were highly conserved in mammals. Compared to the wildtype, these variants of SOX7 increased mRNA expression and decreased protein. In the developing murine hearts, SOX7 along with GATA4 expressed highly in the region of atrioventricular cushions. Moreover, SOX7 overexpression promoted the expression of GATA4 in human umbilical vein endothelial cells. Chromatin immunoprecipitation assay uncovered that SOX7 could directly bind to the region of GATA4 promoter. Luciferase assays demonstrated that SOX7 activated GATA4 promoter and the variants impaired the transcriptional activity of SOX7. Furthermore, the variants of SOX7 altered the regulation to the activity of GATA4 on its target genes.

Conclusions

Our studies provide evidence that deleterious variants in SOX7 are potential contributors to human AVSD and provide novel insights into the etiology of AVSD.

Background

Congenital heart defects (CHDs), including a variety of structural heart malformations are the most common birth defects with an incidence of 8-10 per 1000 live births [1]. CHDs account for nearly 30% of all major congenital anomalies representing a major public health problem [2]. The etiologies for CHDs are believed to involve both genetics and environmental factors, which perturb the normal development of cardiovascular system. Cardiac septal defects including atrial septal defect (ASD), ventricular septal defect (VSD) and atrioventricular septal defect (AVSD; MIM# 606215) are major contributors to the spectrum of CHDs [3]. With an incidence of 4-5.3 per 10000 live births, AVSD accounts approximately for 7% of all CHDs, covering a spectrum of cardiac anomalies characterized by defective heart septation

accompanied with abnormal atrioventricular valves [4].The spectrum of AVSD is composed of partial AVSD, intermediate AVSD and complete AVSD (also known as complete atrioventricular canal defect, CAVC), among which CAVC accounting for 56%~75% is the most common, also the most severe type [5]. Most of patients with CAVC would die before 2 years old if the surgical interventions hadn't been performed [6]. Even the long-term survival after biventricular AVSD correction is relatively good, besides the high risk of reoperation, the standardized mortality ratio is still up to 17% [7].

The development of the heart from a primitive heart tube into a complete four-chamber structure is an extremely complex physiological process that requires precise regulation of substantial transcription factors, adhesion molecules, ion channels, signaling pathways, and structural proteins [8-10]. Early studies using mouse models have revealed that three embryonic structures: the endocardial cushions, mesenchymal cap and dorsal mesenchymal protrusion involve in atrioventricular septation [11]. Defects in any of the mentioned structures may affect septation leading to the occurrence of AVSD. In addition, insufficient looping and abnormal fetal blood flow also contribute to AVSD [12, 13]. As a result, the molecular mechanisms of AVSD may be elusive. Although AVSD is often associated with several genetic syndromes, such as Down syndrome and heterotaxia syndrome [14, 15], approximately 40% of patients have no extracardiac anomalies (non-syndromic AVSD) [16]. Previous studies have identified more than one hundred AVSD-related genes, most of which are associated with human syndromes [17]. In non-syndromic AVSD, only a few of genes including GATA4, TBX5 and NR2F2 have been uncovered [18-20], which are responsible for a very small number of patients. In thousands of cases, the etiologies for the morphogenesis of AVSD need to be identified.

The zinc finger transcription factor GATA4, has been identified as an essential role in cardiac septation. Human genetic studies have implicated deleterious GATA4 mutations in patients with AVSD [18]. GATA4-mutant mice also recapitulated the AVSD phenotypes observed in patients [21, 22]. Human SOX7 gene along with GATA4 is located on chromosome 8p23.1 and covers two exons and one intron. 8p23.1 deletions or duplications that including SOX7 and GATA4 in human display a high risk of cardiac abnormalities, indicating that underlying synergy of SOX7 and GATA4 functions in the cardiac morphogenesis [23-25]. As a transcription factor, SOX7 belongs to the SOX F subfamily and has three highly conserved domains: the N-terminal high mobility group (HMG) DNA binding domain, transactivation domain and C-terminal β-catenin binding domain [26]. SOX7 protein is highly expressed in cardiovascular tissues [27]. Many evidences have revealed that critical roles for SOX7 in cardiovascular development, especially in endothelial progenitor cells specification [28, 29], myocardial differentiation [30], arteriovenous morphogenesis [31, 32] and endothelium-hematopoietic transformation [33, 34]. Mouse models showed the complete or pan-endothelial knockout of SOX7 was embryonic lethal at E10.5 resulting from the cardiovascular defects [35]. In vitro embryonic stem cell differentiation has demonstrated that SOX7 affected the parietal endoderm differentiation through regulating GATA4 and GATA6 expression [36], while genome-wide transcriptomics analysis identified SOX7 as specifically regulated by GATA4 in cardiomyogenesis in the xenopus model [30]. These data suggest that SOX7 plays vital roles in the morphogenesis of cardiovascular system. However, the direct links between SOX7 and

CHDs including AVSD is unknown, and the interaction between SOX7 and GATA4 involved in this process still need to be further explored.

Our network analysis showed a close relationship between SOX7 and reported AVSD-related genes, suggesting that SOX7 may be involved in the etiology of AVSD. The importance in cardiac morphogenesis and the close interaction with AVSD genes of SOX7 make it reasonable to investigate whether genetic variants of SOX7 contributed to AVSD. In this study, we adopted target sequencing to screen for the coding variants of SOX7 in a cohort of 100 sporadic non-syndromic CAVC patients. Three rare heterozygous variants of SOX7 were identified in the patients, but none in the matched health individuals. In vitro, expression of SOX7 variants were significantly affected compared with the wildtype, while subcellular distribution of SOX7 variants were unaffected. Meanwhile, we observed the co-localization of SOX7 and GATA4 in developing atrioventricular canal. Chromatin immunoprecipitation and luciferase assay were used to in depth explore the interaction between SOX7 and GATA4. Furthermore, the results from luciferase assays showed that the variants of SOX7 may promote AVSD incidence through GATA4. Our study firstly associates single nucleotide variants of SOX7 with CAVC, providing novel significance into the genetic pathogenesis of AVSD.

Materials And Methods

Study subjects and DNA extraction

We recruited 100 sporadic non-syndromic complete AVSD patients and 100 unrelated healthy controls without any heart disease from Shanghai Children's Medical Center in this study. All patients with complete AVSD in this study were diagnosed by echocardiography and cardiac surgery. Extracardiac abnormalities were excluded by reviewing clinical evaluations, medical records, and dysmorphology analysis. All subjects are Chinese Han ethnicity. The control group and patient group were well matched in terms of the mean age and sex ratio. The study in accordance with the Declaration of Helsinki was approved by Medical Ethics Committee of Shanghai Xinhua Hospital and Shanghai Children's Medical Center. Written informed consents were fully obtained from parents or legal guardians before their children were enrolled into this study. Total 2ml peripheral blood samples were collected from each subject for DNA extraction. The genomic DNA was purified by using the QIAamp DNA Blood Mini Kit (QIAGEN, Germany) following the standard protocol. The quality and concentration of the purified DNA was tested with a NanoDrop spectrophotometer (Thermo Scientific, USA). Then, the qualified DNA samples were stored at -80℃ until sequenced.

Target sequencing and mutations analysis

The Illumina HiSeq 2000 platform (Illumina, USA) was used to sequence the mutations of SOX7 (GenBank accession number NC_000008.11, NM_031439.4) in both patients and controls groups. The nonsynonymous mutations in the coding regions of SOX7 were called by Genome Analysis Toolkit (https://software.broadinstitute.org/gatk) and SAMtools (http://samtools.Source-forge.net), then validated by using Sanger sequencing. The primer pairs (see in Additional file 1) used for polymerase

chain reaction (PCR) were designed with Premier 5 software. The coding regions of SOX7 were amplified using KOD-Plus-Neo PCR kit (TOYOBO, Japan), following the manufacture's protocol. The PCR products were sent to a commercial provider, Beijing Genomics Institute, China, for Sanger sequencing. The sequencing peak maps were opened in Chromas and aligned with the reference sequence for SOX7 using Basic Local Alignment Search Tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The verified variants were checked in Single Nucleotide Polymorphism database (http://ncbi.nlm.nih.gov/SNP), Exome Aggregation Consortium database (ExAC, http://exac.broadinstitute.org) and 1000 Genomes Project (1000G, http://www.1000g enomes.org) to identify the novelty or rarity of the variants. Rare variants defined as minor allele frequency <0.005. To predict the impact of the amino acid substitutions in SOX7, three pathogenicity prediction tools including SIFT (http://provean.jcvi.org/index.php), Mutation Taster (http://www.mutationtaster.org/), and Polyphen-2 (http://genet-ics.bwh.havard.edu/pph2/) were used in our study.

Protein cross-species alignment

Multiple SOX7 protein sequences from *Homo sapiens* (NP_113627.1), *Pan troglodytes* (XP_009438512.2), *Bos taurus* (XP_024851815.1), *Ovis aries* (XP_027820413.1), *Oryctolagus cuniculus* (XP_008247184.2) and *Mus musculus* (NP_035576.1) were downloaded from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) and aligned using Clustal X software.

Plasmid construction and site-directed mutagenesis

SOX7 coding sequences including two exons were amplified from the human cDNA by PCR following the standard protocols. The human cDNA was obtained by the reverse transcriptional process from human umbilical vein endothelial cells (HUVECs). The primer pairs with two restriction endonuclease sites were provided in Additional file 1. Then, the PCR products and pcDNA3.1(+) vector were cut by NheI and BamHI restriction enzymes (New England Biolabs, USA), and spliced with each other using T4 DNA Ligase (New England Biolabs, USA). Single nucleotide variants c.40C>G (p.L14V), c.542G >A(p.G181E) and c.743C>T (p.P248L) were introduced into the pcDNA3.1(+)-SOX7 expression vectors by Quickchange Site-Directed Mutagenesis Kit (Stratagene, USA). The pcDNA3.1(+)-GATA4 expression vector was obtained from Origene, USA. For recombining GATA4 luciferase reporter plasmid, an approximate 3Kb upstream of the transcription start site of GATA4 was subcloned into MluI and XhoI sites of the pGL3-basic vector (Promega, USA). The BNP luciferase reporter plasmid was created by inserting the 181 bp 5'-flanking region of the BNP coding sequence to the pGL3-basic vector (Pro-mega, USA). All the expression vectors and the luciferase reporter were verified by Sanger sequencing.

Cell culture and transfection

HEK 293T and HUVEC cellswere cultured with the DMEM (HyClone, USA) mixed with 10% fetal bovine serum (MP Biomedicals, USA), penicillin (100 unit/ml), and streptomycin(100 μg/ml) (Gibco, USA), and incubated with 5% CO_2 at 37°C. The transient transfections were conducted with Fugene HD (Promega, USA) following the manufacturer's instructions.

Real-time quantitative PCR

HEK 293T or HUVEC cells were harvested 36-48 hours after the transient transfections. Total RNA was extracted using TRIZOL regent (Invitrogen, USA) in accordance with the standard protocol. Then, the purified total RNA was reverse transcribed into cDNA with Prime Script RT Master Mix (Takara, Japan). Real-time quantitative PCR was performed on an Applied Biosystems7500 system (Applied Biosystems, USA) using SYBR Premix Ex Taq (Takara, Japan). Primer sequences of SOX7, GATA4, and GAPDH for real-time quantitative PCR are listed in Additional file 1.

Western blot

HEK 293T or HUVEC cells were harvested 48 hours after the transient transfections and lysed in RIPA buffer (Beyotime, China) with 1% PMSF (Beyotime, China). For protein degradation inhibition experiments, 10 mM MG132 solution dissolved in DMSO was added into the cell culture mixture for 8-10 h before harvest. The protein lysates were purified by high speed centrifugation and quantified with the BCA Protein Assay Kit (Beyotime, China). Then, the protein solutions mixed with 5X protein loading buffer (Beyotime, China) were added to a 10% SDS-PAGE gel for the electrophoresis and then transferred onto the nitrocellulose membrane (Millipore, USA). The protein-loaded nitrocellulose membrane was blocked with 5% nonfat milk for 2 hours at room temperature and incubated with rabbit anti-SOX7 antibody (1:5000, Proteintech, USA), rabbit anti-GATA4 antibody (1:5000, Abcam, UK), or mouse anti-GAPDH antibody（1：10000, Sigma-Aldrich, USA）overnight at 4℃. Next, the washed membrane was immersed in HRP-conjugated secondary antibodies (1:10000, Proteintech, USA) for 2 hours at room temperature. The enhanced chemiluminescence (ECL) reagents (Millipore, USA) and Imagelab program (BioRad, USA) were used for bands detection and analysis.

Cell immunofluorescence staining

HEK 293T or HUVEC cells were plated into a 12-well plate paved with cover slips for 24 hours before transfections. 36 hours after transfections, cells were harvested and fixed with 4% paraformaldehyde for 15 min. The well-fixed cells were permeabilized by 0.3% TritonX-100 for 10 min and were then blocked with 5% bovine serum albumin (BSA) solution for 60 minutes at routine temperature. Then, the cells were incubated with goat anti-SOX7 antibody (1:200, R&D systems, USA) at 4℃ for overnight, followed by incubation with Alexa Fluor 488 secondary anti-goat IgG (1:200, Abcam, UK) for 1 hour. 4,6-diamidino-2-phenylindole (DAPI，Servicebio, China) was used to stain the nuclei. The slides were observed by the Olympus BX43 microscope (Olympus, Japan).

Embryo collection and transcriptome array

Human embryos of Carnegie stage 10-16 were collected at Shanghai Xinhua hospital, approved by the medical ethics committee. Embryonic hearts were observed and separated under a stereo microscope. Total RNA of the separated embryonic hearts was extracted by RNAprep Pure Tissue Kit (TIANGEN,

China) following the manufacturer's protocol. The transcriptome array was performed using Affymetrix Human Transcriptome Array 2.0 (USA) at Bohao Biotechnology Co, Ltd, China.

Co-immunofluorescence assay

Paraffin sections of human embryos were deparaffinized in xylene. Sections were permeabilized in 0.3% Triton X-100 in PBS at room temperature for 10 minutes and then blocked by 5% BSA at room temperature for 60 minutes followed by incubation with goat anti-SOX7 antibody (1:200, R&D systems, USA) and rabbit anti-GATA4 antibody (1:200, Abcam, UK) overnight at 4℃. Next, sections were stained with Alexa Fluor 488 anti-goat IgG and Alexa Fluor 594 anti-rabbit IgG for 1 hour. Images were captured by the Olympus BX43 microscope (Olympus, Japan).

Dual luciferase reporter assay

HUVEC cells were seeded into a 24-well plate for 24 hours before transfection and co-transfected with recombinant plasmids including wildtype or mutated SOX7 vector, the GATA4 vector, the luciferase reporters and the internal control reporter (pRL-TK, Promega, USA). The Dual Luciferase Reporter Assay Kit (Promega, USA) and the Dual-Glo luciferase assay system (Promega, USA) were used to measure the luciferase activities at 36 hours after the co-transfection.

Chromatin immunoprecipitation

HUVEC cells were cultured in a 15cm dish and transfected with pcDNA3.1(+)-SOX7 vector 36 hours before chromatin immunoprecipitation assay (ChIP). The ChIP assay was performed with EZ-ChIP ^{TM} (Millipore, USA) following the manufacturer's protocol. Cells were fixed and cross-linked with paraformaldehyde. Chromatin were then segmented by Ultrasound. For immuno-precipitation, the primary rabbit anti-SOX7 antibody (1:50, Proteintech, USA) was added to the chromatin solutions overnight at 4℃, followed by collection of immune complex precipitates, de-crosslinking and DNA purification. PCR was used to amplify the putative promoter region of GATA4 from enriched DNA targets. The binding sites of SOX7 to GATA4 promoter were putative according to the reported SOX7 binding motifs [37]. The primer pairs were designed using Primer5 software and shown in Additional file 1.

Network analysis

To detect potential relationships between SOX7 and identified AVSD-related genes, total 112 AVSD-related genes were listed [17] and inputted to STRING database (http://string-db.org), a tool can easily assess and integrate protein-protein interactions [38]. The STRING network of SOX7 and 112 AVSD-related genes was downloaded and visualized using Cytoscape software [39].

Statistical analysis

The results are shown as the mean ± SD of at least 3 independent replicates. Statistical comparisons were measured using student's t test, and P value < 0.05 was considered significant.

Results

Three rare nonsynonymous variants of SOX7 were identified in CAVC patients

By network analysis, we identified a close relationship between SOX7 and reported AVSD-related genes, of special note are GATA4, GATA6, BMP4, NR2F2 and HHEX (Fig 1). The findings inspired us to investigate whether mutations of SOX7 contribute to the pathogenesis of AVSD. We screened 100 unrelated non-syndromic CAVC patients and 100 unrelated healthy individuals by target sequencing. Three heterozygous nonsynonymous variants of SOX7 were identified in 3 CAVC patients but none in our healthy controls. And none other known AVSD pathogenic genes were identified in patients with mutated SOX7 gene. The variants of SOX7 were c.40C>G (p.L14V), c.542G>A (p.G181E) and c.743C>T (p.P248L), respectively, all of them were validated by Sanger sequencing (Fig 2). Among the three variants, c.542G>A and c.40C>G were found in ExAC database, but both of their minor allele frequency were lower than 0.005. Notably, Variant c.743C>T was neither found in ExAC nor 1000G. To predict the pathogenicity, we performed bioinformatics analysis on the mutations using Polyphen2, SIFT and Mutation Taster. All the three rare variants were likely to be damaging as predicted to be pathogenic in at least 1 program (Table 1).

Alignment of multiple SOX7 protein sequences

The human SOX7 gene located at chromosome 8p23.1, spans 6807 base pairs which covers two exons and one intron (Fig 3a), encoding the SOX7 protein lengths of 388 amino acids. The human SOX7 protein is composed of two conserved domains: the high mobility group (HMG) DNA binding domain and the transactivation domain (Fig 3b). Although the variants G181E and L14V were not located in these conserved domains while P248L in the transactivation domain, multiple protein sequences alignment across species revealed that all mutant sites in this study were highly conserved in mammals (Fig 3c), indicating that these variants might result in SOX7 protein structure and function alterations.

Reduced protein expression of SOX7 variants

We further detected the expression of the wildtype and mutant SOX7 by real-time quantitative PCR and Western blot. The wildtype and mutant SOX7 vectors were respectively transfected into HEK293T cells or HUVEC cells. Real-time quantitative PCR results demonstrated that mRNA expressions of these three variants were greater than those of the wildtype (Fig 4a, b). However, the protein expressions of the variants were significantly lower than those of the wildtype (Fig 4c, d, e, f), which were completely the opposite to the mRNA levels. The distinctly different results between the mRNA expression and the protein turnover indicated that the mutants might change the stability of SOX7 protein through promoting protein degradation. The ubiquitin-proteasome system is the major pathway for degradation of intracellular proteins [40]. Therefore, we emphatically investigated the effect of ubiquitin-proteasome degradation. With the addition of the special protease inhibitor, MG-132, the reduced turnover of P248L and G181E mutants were notably elevated, while L14V mutant took no significant changes (Fig 4g, h, i, j). These data indicated that certain SOX7 mutations altered protein levels by abnormal protein degradation.

Nuclear localization of SOX7 variants

To detect whether the variants affect the trafficking of the SOX7 protein, we transiently transfected HEK293T cells or HUVEC cells with wildtype or mutant vectors followed by immunofluorescence staining. The results revealed that the subcellular distribution of SOX7 mutant proteins was similar with those of wildtype proteins, as all of them showed diffuse nuclear localization (Fig 5a, b).

Co-expression of SOX7 and GATA4 in the developing human heart

To explore the likely disease-causing mechanisms of SOX7 variants in AVSD, we focused on the interaction between SOX7 and GATA4 since many studies had shown that GATA4 played vital role in atrioventricular septation [18, 21, 41, 42]. Affymetrix human transcriptome array demonstrated that both of SOX7 and GATA4 were highly expressed in human embryonic hearts of Carnegie stage 11-15 (Fig 6a), which is the critical period of the cardiac septum formation [43]. Immunofluorescence studies revealed that SOX7 and GATA4 were co-localized in the developing atrioventricular cushions (Fig 6b, Additional file 2), which is the most important embryonic structure for the atrioventricular septum [44]. These results indicated that SOX7 and GATA4 might have joint function in the development of the atrioventricular septum.

Identification of GATA4 as a direct downstream of SOX7

We firstly carried out real-time PCR and Western blot analysis to clarify the interaction between SOX7 and GATA4. Overexpression of SOX7 in HUVEC cells elevated expression of endogenous GATA4 (Fig 7a, c). We further reviewed the reported SOX7 binding motifs [37], and identified five putative SOX7 binding sites in about 3kb upstream of the GATA4 gene transcriptional start site (Fig 7d). ChIP assay demonstrated that SOX7 could bind to GATA4 promoter region (Fig 7e). Luciferase reporter assay revealed that SOX7 was evidently able to activate GATA4 promoter (Fig 7g). Taken together, the results suggested that SOX7 might act directly through GATA4.

Functional impact of SOX7 variants on its transcriptional activity

Since SOX7 is a transcriptional factor, its function is fundamentally based on transcriptional activity. We had identified the activating effect of SOX7 on GATA4 promoter. To test the transcriptional activity of SOX7 variants, we therefore compared luciferase activity of GATA4 reporter activated by SOX7 wildtype proteins with that by the variants. We observed that the variants showed significantly lower transcriptional activity than the wildtype (Fig 7g). In consistent with the results of the luciferase assay, overexpression of these SOX7 variants in HUVEC displayed reduced expression of GATA4 mRNA (Fig 7b).

Despite we confirmed the transcriptional regulation between SOX7 and GATA4, as well as identified SOX7 as the upstream of GATA4, the interaction between these two transcription factors seems more complicated. We also observed that overexpression of GATA4 led to increased SOX7 expression in vitro (Fig 7a, c), suggesting potentially a positive feedback loop existing in SOX7 and GATA4. However, by using of the BNP luciferase reporter, a well-known GATA4-target downstream gene, to be co-transfected

with GATA4 and SOX7 into HUVEC cells, we revealed that SOX7 in low dose inhibited the activated activity of GATA4 to BNP promoter, and high-dose SOX7 took the opposite effect, suggesting SOX7 regulated the transcriptional activity of GATA4 in a dose-dependent manner (Fig 7h). Furthermore, the variants of SOX7 in the same dose with the wildtype showed notably different effects on the activation of BNP promoter by GATA4 (Fig 7i), indicating the variants impaired the physiological regulation of SOX7 on the transcriptional activity of GATA4.

As shown in Fig 8, we exhibited the illustration to summarize our findings. Three rare variants of SOX7 affecting its expression level and interaction with GATA4 contributed to complete AVSD.

Discussion

The completely four-chamber heart which forms from the primary heart tube is vital to the survival of mammals. During embryogenesis, a series of transcription factors are involved in the regulation of the cardiac morphogenesis [45]. Identification of cardiac transcription factor has provided novel insights into the pathogenesis of CHD. Substantial evidence indicates that transcription factor SOX7 plays multiple roles in cardiovascular development. Expression studies have implicated a conserved role for SOX7 in developing cardiovascular tissues across species. In stage 24-27 xenopus embryos, strong SOX7 expression was observed in the endocardium, procardiac tube, aortic arch and posterior cardinal veins [46]. In mouse embryos, SOX7 is expressed in the heart tube at E8.5 and in the endocardium as well as blood vessels at E12.5 [23, 47]. In human embryos, SOX7 is highly expressed in the heart throughout the period of cardiac development [48]. These expression data indicate a specific role of SOX7 in cardiovascular development. Reasonably, knockdown of SOX7 and SOX18 in zebrafish demonstrated notable vascular defects in arteriovenous differentiation, which resulted in pericardial edema and embryo death [32]. $Sox7^{-/-}$ mouse embryos showed widespread cardiovascular defects leading to embryonic lethal from E10.5 onward [35]. Moreover, disrupted SOX7 expression in embryonic stem cell differentiation models showed impaired cardiomyogenesis resulting from affected GATA4 and GATA6 [36]. Taken together, these in vivo and in vitro studies provide evidences that SOX7 interacting with GATA4 has pivotal functions in cardiovascular morphogenesis. Altered SOX7 may be responsible for a variety of congenital cardiovascular defects in human. Using network analysis, we further uncovered the tight contact between SOX7 and other AVSD-related genes, including GATA4, implying that SOX7 may be involved in the pathogenesis for AVSD.

In the present study, we explored the link between SOX7 variants and CAVC. Three heterozygous and missense mutations: c.40C>G (p.L14V), c.542G>A (p.G181E) and c.743C>T(p.P248L) of SOX7 were identified in 3 of 100 unrelated non-syndromic CAVC patients by using target sequencing, but none of them were found in the 100 healthy control individuals. Among these three variants, c.743C>T was reported for the first time, while the other two variants were previously reported in ExAC database, however, with the extremely low allelic frequency. Based on cross-species alignment of SOX7 protein sequences, all the three mutant sites were highly conserved, suggesting the mutants might alter the structure and function of SOX7 protein. Therefore, we performed bioinformatic analysis using Polyphen-

2, SIFT, and Mutation Taster to predict the potential effect of these variants on protein function. All three variants were predicted as possibly damaging in at least one program. These results inspired us to further investigate the pathogenicity of these variants.

We found that these SOX7 variants led to increased mRNA level but reduced protein expression compared with those of the wildtype group. The contradictory expression levels between RNA and protein was speculated that these single amino acid changes might affect the structure of SOX7 protein, resulting in decreased stability and increased degradation. It is well-known that there are mainly two distinct pathways for protein degradation in eukaryotic cells, namely the ubiquitin-proteasome and endosome-lysosome pathways [49]. We further examined our speculation by inhibiting the ubiquitin proteasome system using MG132, a widely used proteasome inhibitor [50]. The presence of MG132 remarkably enhanced the protein levels of p.G181E and p.P248L, whereas the protein level of the wildtype was not significantly affected by MG132 treatment. It strongly suggested that the lower protein expression level of p.G181E and p.P248L is attributed to ubiquitin mediated protein degradation. However, the protein level of p.L14V was not significantly increased with the treatment of MG132, suggesting that it might be degraded via another pathway. Moreover, we detected the subcellular distribution of the wildtype and mutant SOX7 protein. The immunofluorescence staining assay showed that both wildtype and mutant proteins were expressed in nucleus, indicating these variants did not affect the cellular location of SOX7.

In human, SOX7 is located on chromosome 8p23.1, where GATA4 exists. Patients with deletions or duplications of 8p23.1 region often display a spectrum of abnormalities including congenital heart defects, diaphragmatic hernia, and developmental delay [25]. Previous studies had implicated a complicated interaction between SOX7 and GATA4. In the parietal endoderm of E7.5 mouse embryos, SOX7 is co-expressed with GATA4 and competes with GATA4 to activate the Fgf3 promoter [51]. In the embryonic stem cell differentiation model, SOX7 affects the parietal endoderm by regulating GATA4 and GATA6 [36] . However, in the xenopus model, genome-wide transcriptomics analysis demonstrates that SOX7 is regulated by GATA4 directing cardiomyogenesis [30]. Doyle et al demonstrate a protein-protein interaction between SOX7 and GATA4 in cardiovascular progenitor cell differentiation [52]. In the present study, we showed that both SOX7 and GATA4 were highly expressed in the developing hearts of Carnegie stage 11-15 human embryos. Especially, SOX7 and GATA4 were co-expressed in the presumptive atrioventricular canal during the development of atrioventricular valves. These data are consistent with Doyle's research that SOX7 was expressed in a similar temporal pattern as GATA4 during embryoid body differentiation and co-expressed with GATA4 in a subset of cardiovascular progenitor cells [52]. Taken together, both previous studies and our findings demonstrate that SOX7 with GATA4 plays a crucial role in the atrioventricular septum development. Moreover, we found that overexpression SOX7 in HUVEC resulted in elevated expression of GATA4, vice versa, suggesting a potential feedback loop exists between SOX7 and GATA4. Our data of ChIP and reporter assays have firstly identified SOX7 as a direct upstream of GATA4. Therefore, the functional effects of the SOX7 variants may be implicated by analyzing its transcriptional activity on GATA4 promoter.

Next, dual luciferase reporter assays revealed that all three variants of SOX7 indeed impaired the transcriptional activity on GATA4 promoter, which was consistent with the result of overexpression of the wildtype or mutant SOX7 induced mRNA levels of GATA4. Since SOX7 has been recently reported to co-regulate genes involved in heart development with GATA4 and inhibit the transcriptional activity of GATA4 via protein-protein interaction [52], we further employed dual luciferase reporter assays to measure the effects of SOX7 on GATA4's transcriptional activity using the well characterized BNP promoter which has been previously shown to be highly activated by GATA4 [53]. Interestingly, as the dose of SOX7 increased, the regulation effect of SOX7 on GATA4's transcriptional activity changed from inhibition to promotion, indicating that SOX7 regulates GATA4's transcriptional activity on BNP promoter in a dose-dependent manner. Accordingly, the variants of SOX7 affected the normal regulation of SOX7 to GATA4's transcriptional activity. However, it is difficult to clarify whether these alterations caused by the variants of SOX7 are due to protein instability or inactivity of GATA4 DNA-binding. Further studies will be needed to elucidate the specific mechanism.

Additionally, there were some limitations in our study. Firstly, the absence of parental samples limited clarifying the genetic background of these variants. Secondly, all functional analysis in this study were conducted in vitro. In vivo experiments using transgenic animal models to validate the pathogenicity of these variants need to be performed in future. Nevertheless, this study for the first time provides evidence that deleterious variants in SOX7 are potential contributors to human AVSD based on a large cohort, which may provide valuable information for prenatal diagnosis and consultation as well as therapy of AVSD.

Conclusion

In conclusion, our study suggests that the variants of SOX7 are associated with human AVSD and provides novel insight into the genetic etiology of AVSD, which may contribute to the prenatal diagnosis, consultation and treatment for AVSD.

Abbreviations

AVSD: atrioventricular septal defect

CAVC: complete atrioventricular canal

ASD: atrial septal defect

PH: pulmonary hypertension

Declarations

Ethics approval and consent to participate

Written informed consent was obtained from both patient and control group participants. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Xin Hua Hospital.

Consent for publication

Not applicable.

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests

The authors state that they have no conflicts of interest.

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Authors' contributions

SC and YL conceived and designed the study. BL, ZL, and NH performed the experiments; JY, LJ, YY, and KS analyzed the data; YX, QF, and JY collected the blood samples from all subjects. BL drafted the manuscript. SC and YL revised the manuscript. All authors contributed to writing this paper and approved the final manuscript.

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Table

Table 1 Clinical information and variant characteristics of SOX7 in patients with CAVC

Patient	Gender	Age	Diagnosis	Location in gene	Function	Nucleotide change	Amino acid change	Exac_MAF	SIFT score	Polyphen2	Mutation taster
1	Male	5 months	CAVC/ASD/PH	Exon 2	Missense	c.743C>T	p.P248L	/	0.256	0.367	Disease causing
2	Female	5 years	CAVC/PH	Exon 2	Missense	c.542G>A	p.G181E	0.000008979	1	0.475	Polymophism
3	Female	7 months	CAVC/ASD/PH	Exon 1	Missense	c.40C>G	p.L14V	0.00001267	0.039	0.731	Disease causing

VC: complete atrioventricular canal, ASD: atrial septal defect, PH: pulmonary hypertension

Supplemental Figure

Supplemental Figure 1. Immunofluorescence staining of SOX7 and GATA4 in Carnegie stage 13(13S) and Carnegie stage 16(16S) embryonic hearts demonstrated co-localization of them in the atrioventricular canal. The atrioventricular cushions were framed by blue rectangles and presented in higher magnification. V, ventricle; AVC, atrioventricular cushions.

Figures

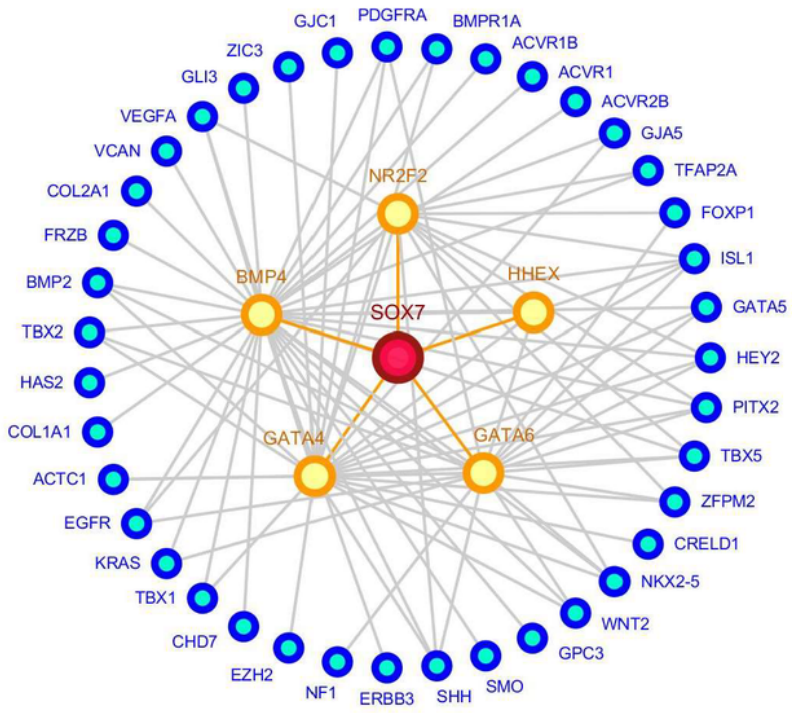

Figure 1

Gene regulatory networks between SOX7 and AVSD-related genes. Network analysis indicated the protein-protein interactions between SOX7 and other AVSD-related genes. The network was mapped in Cytoscape using annotations from the STRING 9.1 protein-protein interactions database. The red node represents SOX7, the yellow nodes represent the AVSD-related genes that directly interact with SOX7.

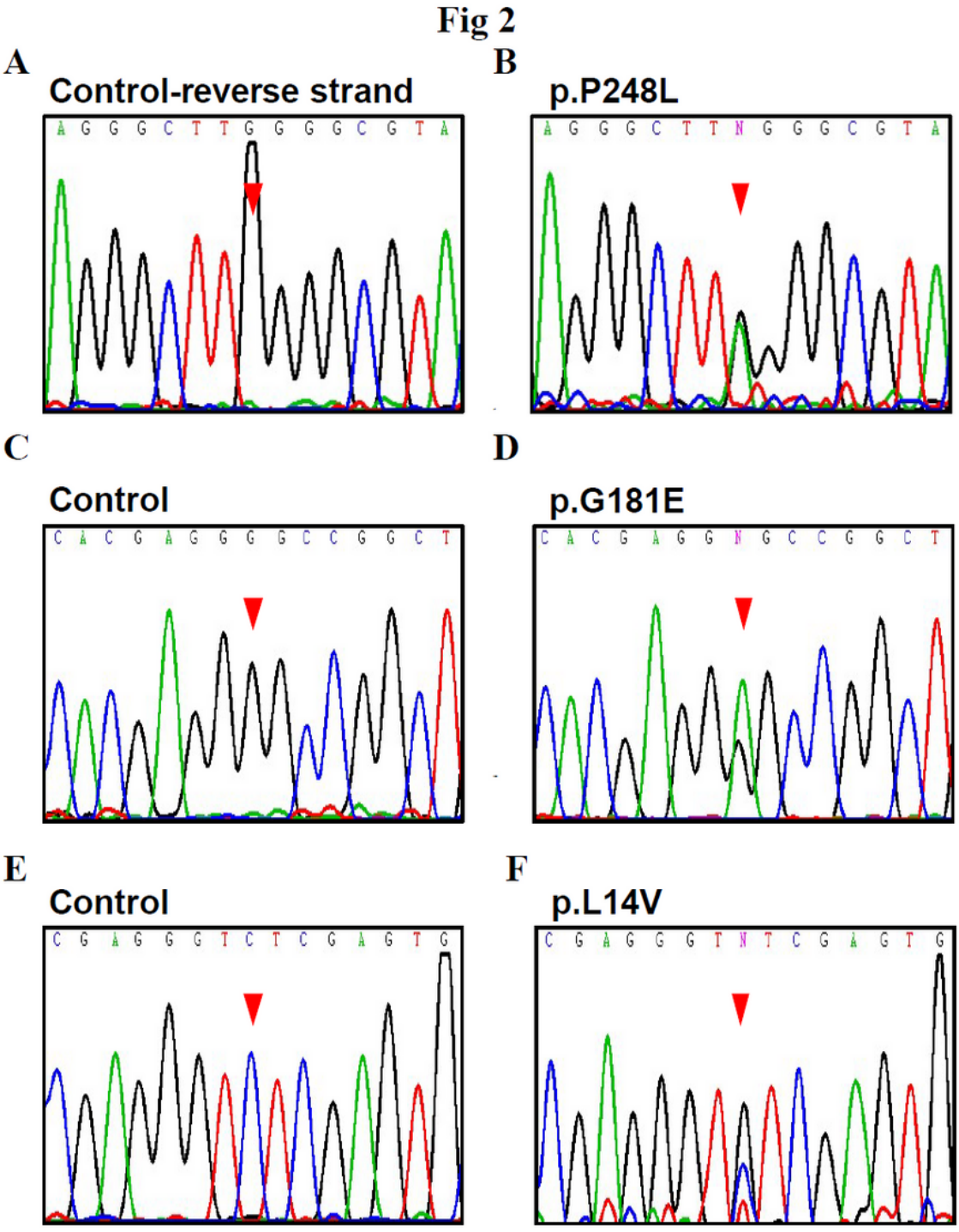

Figure 2

Sequence chromatograms of SOX7 missense variants in patients and controls. a, c, and e Chromatograms of normal controls. b, d, and f Chromatograms of the three heterozygous variants. Arrows show heterozygous nucleotide changes.

Fig 3

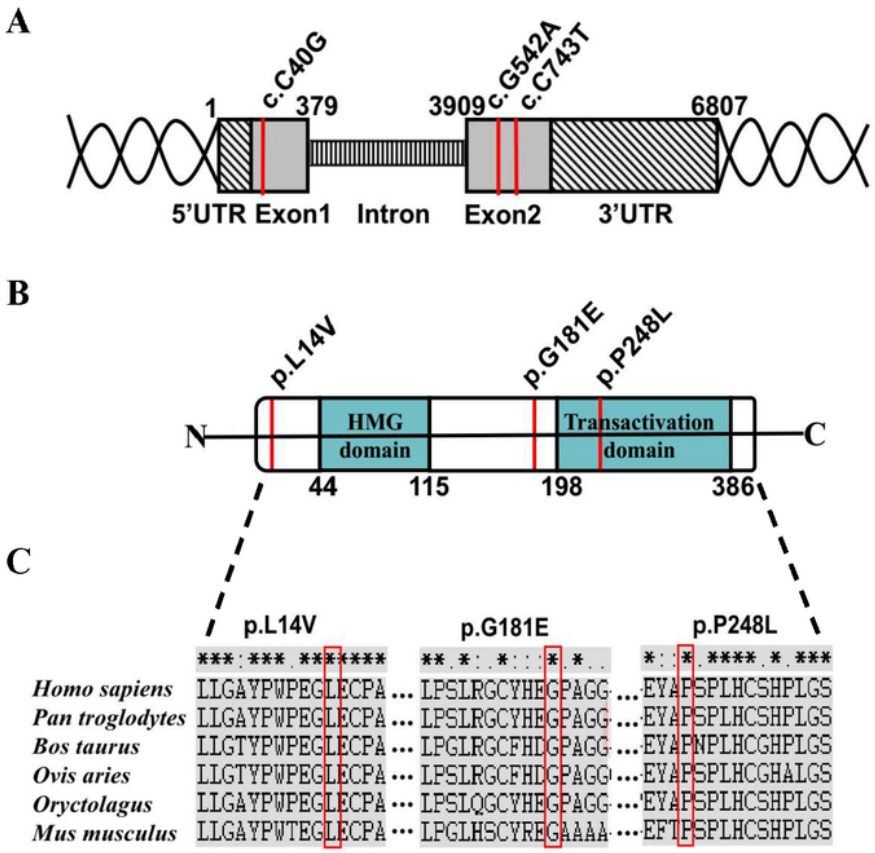

Figure 3

Location and conservation of SOX7 variants. a, b Schematics of SOX7 gene exons and protein with location of variants identified in this study. c Alignments of SOX7 protein among different species. All variants were highly conserved.

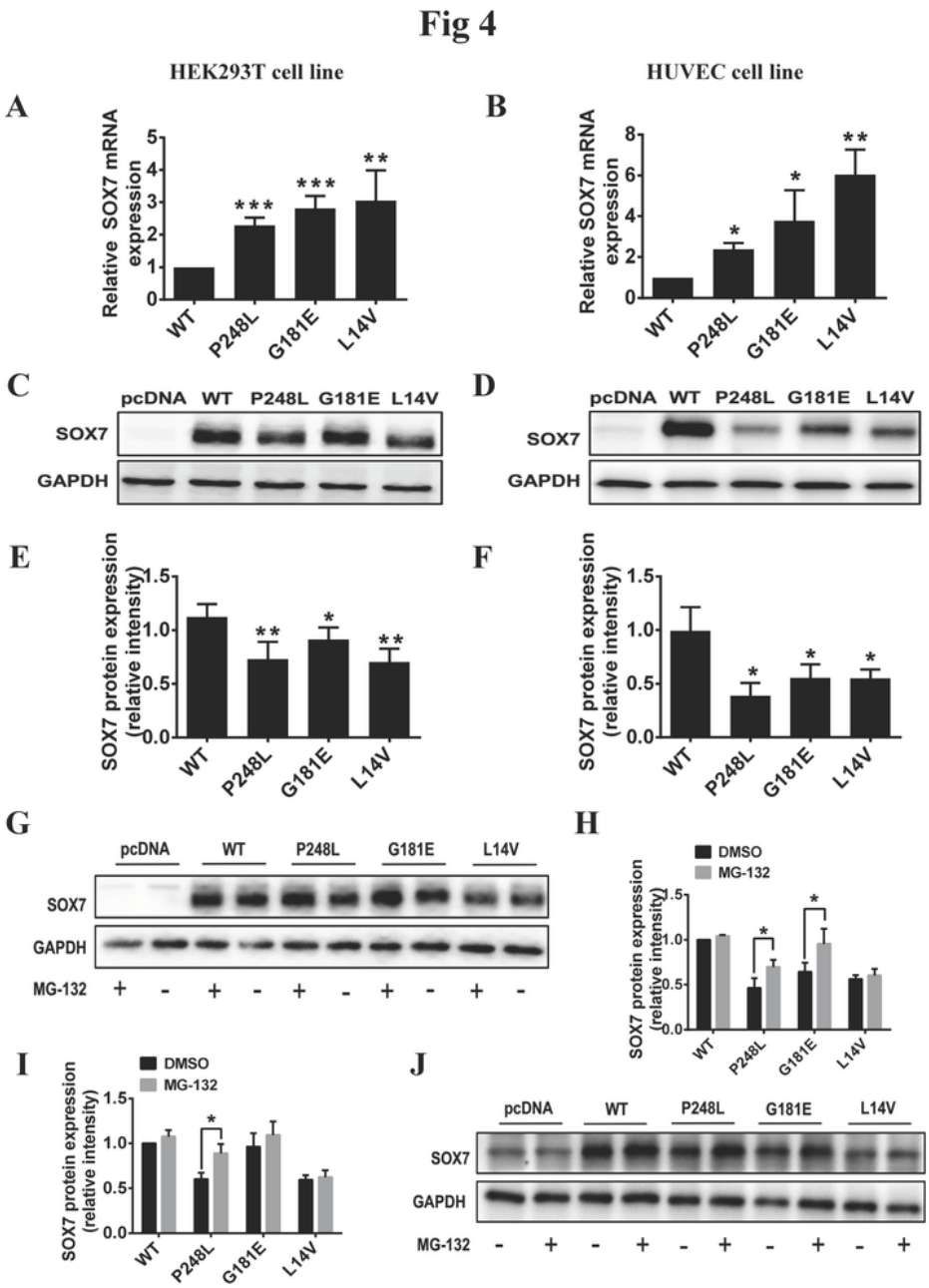

Figure 4

The mRNA and protein expression levels of wildtype and mutant SOX7. Control vector (pcDNA), SOX7 wildtype (WT) or mutant plasmids (P248L, G181E and L14V) were transfected into HEK293T cells and HUVEC cells and harvested. a, b Relative mRNA expression of the wildtype plasmid and variants of SOX7 in HEK293T cells and HUVEC cells, respectively (n=3). c, d Western blot analysis of the control vector, wildtype and variants of SOX7 in HEK293T cells and HUVEC cells, respectively. GAPDH was used as an

internal control. e, f Density quantitation of SOX7 variant protein expression as shown in c and d (n=3). g, j Western blot analysis of SOX7 mutant protein degradation through the ubiquitin-proteasome pathway in HEK293T cells and HUVEC cells, respectively. MG-132 was the proteinase inhibitor and DMSO was used as the control. i, h Density quantitation of SOX7 variant protein expression in the presence of MG132 as shown in g and j (n=3). *, P < 0.05; **, P < 0.01; ***, P < 0.001.

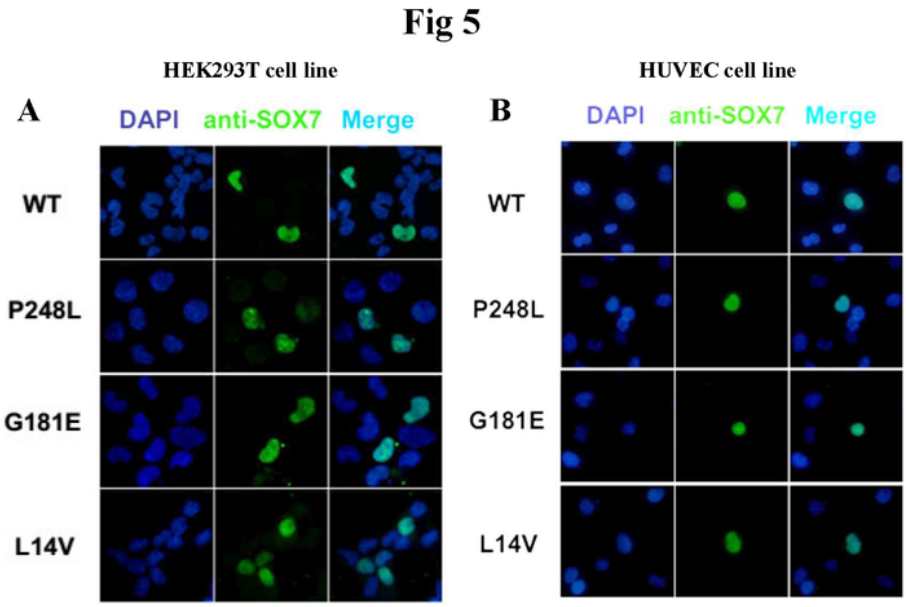

Figure 5

Subcellular distribution of wildtype and mutant SOX7 proteins in vitro. a Representative images of immunofluorescence staining for SOX7 wildtype and variants proteins in HEK293T cells. b Representative images of immunofluorescence staining for SOX7 wildtype and variants proteins in HUVEC cells. All the wildtype and mutant SOX7 (P248L, G181E and L14V) located in nucleus.

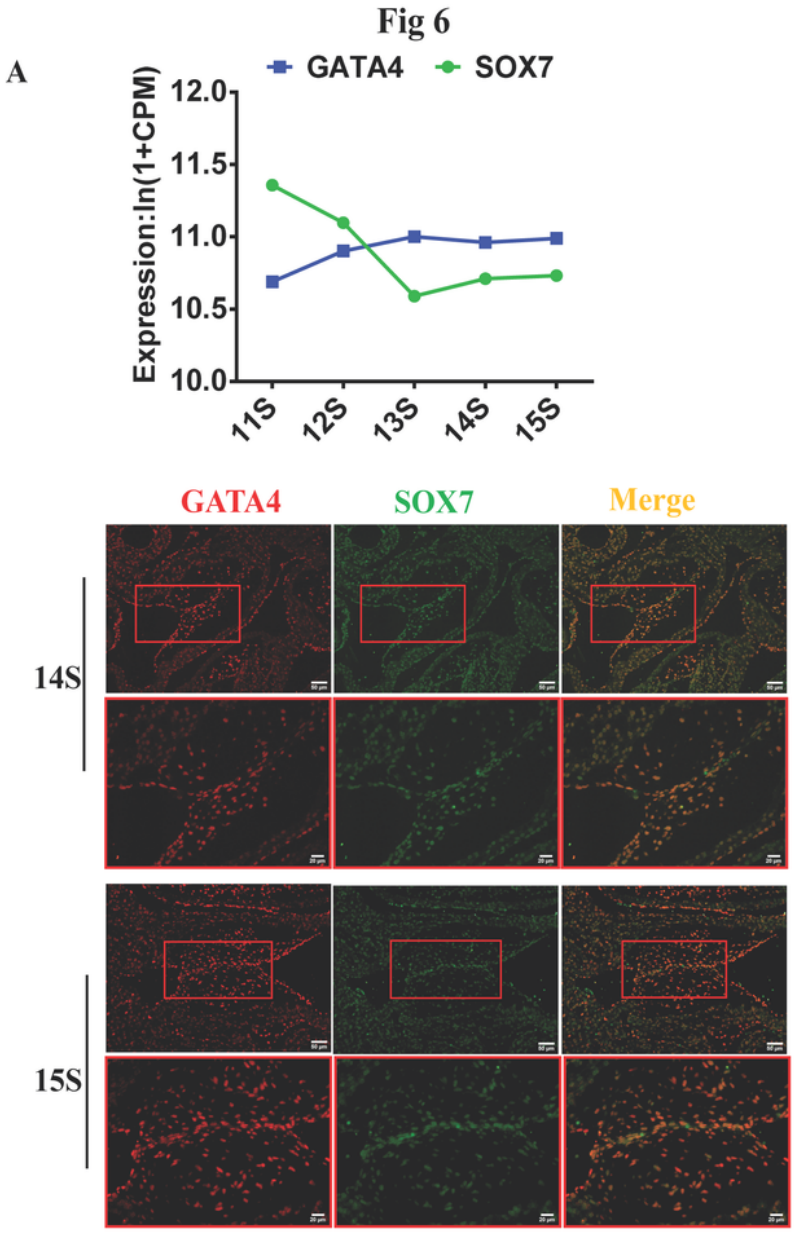

Figure 6

Expression of SOX7 and GATA4 in the embryonic human heart. Human embryos in Carnegie stage 10-16 (10S-16S) were collected and isolated. a The great expression level of SOX7 and GATA4 in Carnegie stage 11-15 embryos was detected by Affymetrix Human Transcriptome Array. CPM: counts per million reads. b Immunofluorescence staining of SOX7 and GATA4 in Carnegie stage 14(14S) and Carnegie stage 15(15S) embryonic hearts demonstrated co-localization of them in the atrioventricular canal. The atrioventricular cushions were framed by the red rectangles and presented in higher magnification.

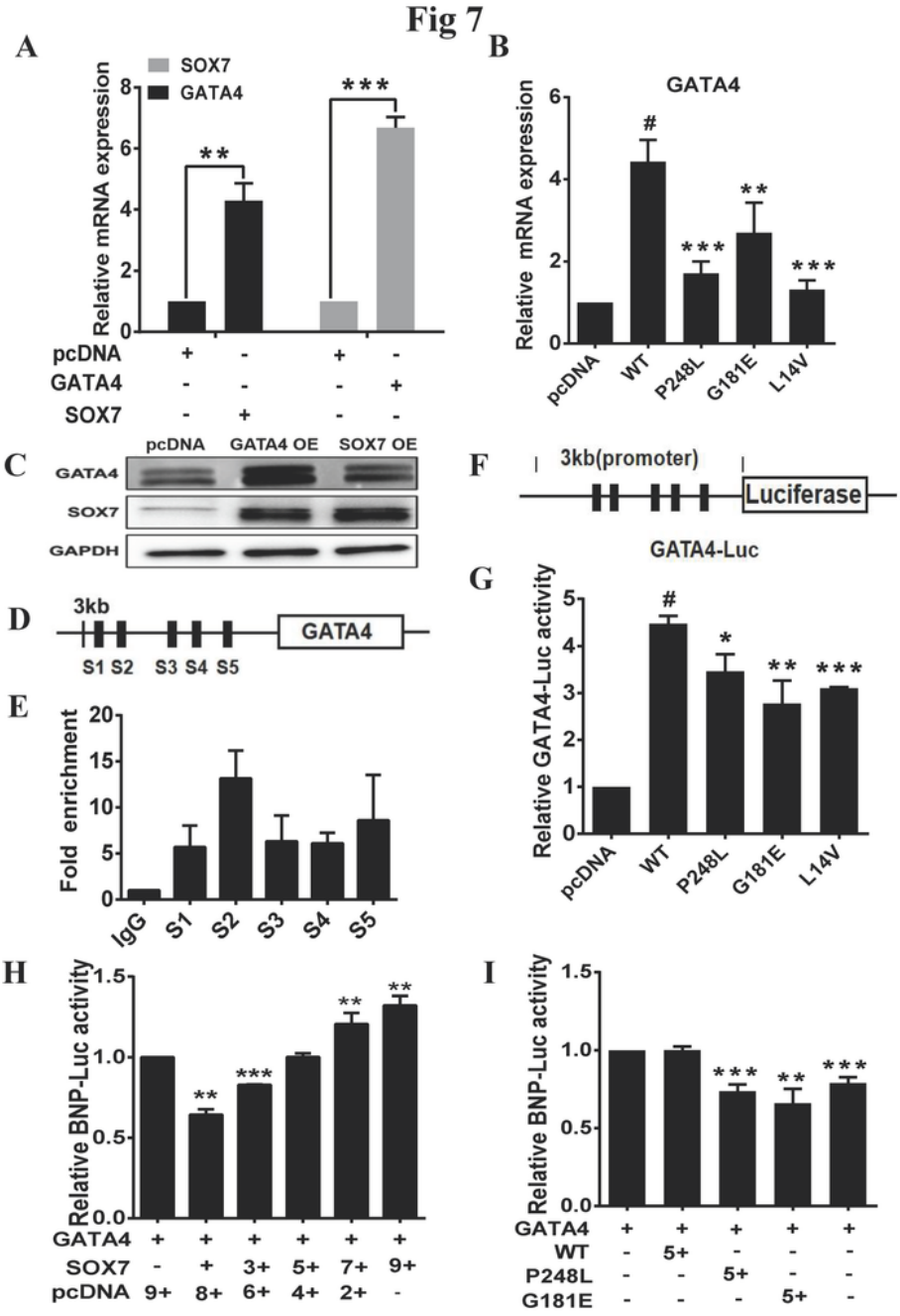

Figure 7

Interplay between SOX7 and GATA4 was altered by SOX7 variants. Plasmids were transfected into HUVEC cells followed by real-time quantitative PCR, Western blot, ChIP, and luciferase assays. a Relative mRNA expression of SOX7 or GATA4 detection after GATA4 or SOX7 overexpressed (n=3). b Comparison of GATA4 mRNA expression when overexpressed wildtype or mutant SOX7 (n=3). c Western blot analysis of SOX7 or GATA4 protein expression with overexpressed GATA4 or SOX7. d The diagram of five putative binding sites(S1-S5) of SOX7 in the promoter region of GATA4. e ChIP assay demonstrated SOX7 was able to bind to GATA4 promoter region directly (n=3). f The diagram of the structure of GATA4 luciferase reporter. g The transcriptional activity of GATA4 affected by wildtype or mutant SOX7. h The dose of SOX7 impacted the transcriptional activity of GATA4 to BNP and the variants of SOX7 affected its normal regulation on GATA4 transcription activity (i) (n=3). # , $P < 0.01$ versus pCDNA; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus WT.

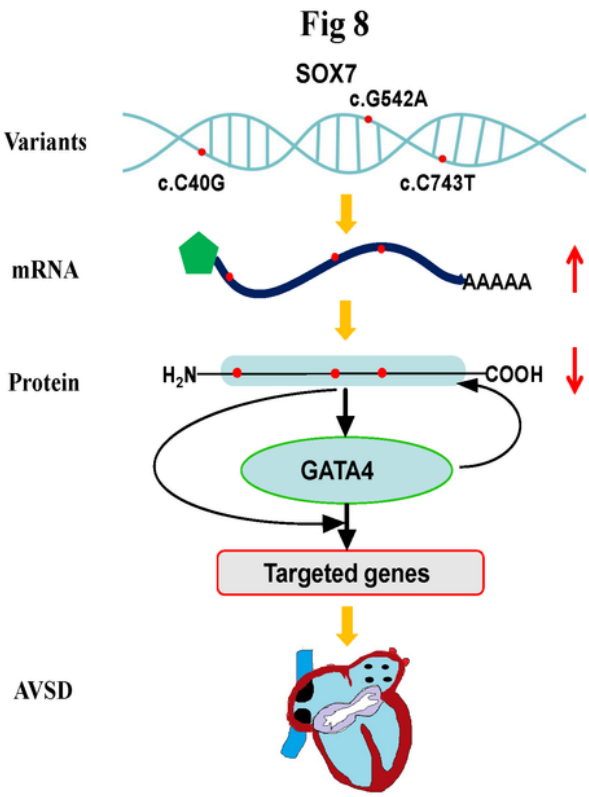

Figure 8

The schematic diagram of the potential mechanism of SOX7 variants involved in AVSD pathogenesis

Supplementary Files

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