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Identification and functional analysis of genetic variants in TBX5 gene promoter in patients with acute myocardial infarction

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Abstract

Background: Coronary artery disease (CAD), including acute myocardial infarction (AMI), is a common complex disease. Although a great number of genetic loci and variants for CAD have been identified, genetic causes and underlying mechanisms remain largely unclear. Epidemiological studies have revealed that CAD incidence is strikingly higher in patients with congenital heart disease than that in normal population. T-box transcription factors play critical roles in embryonic development. In particular, TBX5 as a dosage-sensitive regulator is required for cardiac development and function. Thus, dysregulated TBX5 gene expression may be involved in CAD development.

Methods: TBX5 gene promoter was genetically and functionally analysed in large groups of AMI patients ($n = 432$) and ethnic-matched healthy controls ($n = 448$).

Results: Six novel heterozygous DNA sequence variants (DSVs) in the TBX5 gene promoter (g.4100A > G, g.4194G > A, g.4260 T > C, g.4367C > A, g.4581A > G and g.5004G > T) were found in AMI patients, but in none of controls. These DSVs significantly changed the activity of TBX5 gene promoter in cultured cells ($P < 0.05$). Furthermore, three of the DSVs (g.4100A > G, g.4260 T > C and g.4581A > G) evidently modified the binding sites of unknown transcription factors.

Conclusions: The DSVs identified in AMI patients may alter TBX5 gene promoter activity and change TBX5 level, contributing to AMI development as a rare risk factor.

Keywords: Acute myocardial infarction, TBX5, Gene expression regulation, Promoter, Genetic variants

Introduction

Coronary artery disease (CAD), including acute myocardial infarction (AMI), is a common complex disease. Although genome-wide association studies have identified a great number of genetic loci and variants for CAD and AMI, genetic causes and underlying mechanisms for CAD and AMI remain largely unclear [1, 2]. Recent epidemiological studies have demonstrated that incidence

of CAD and AMI is strikingly higher in patients with congenital heart disease than that in normal population [3, 4]. Dysregulation of cardiac genes and embryonic developmental genes have been associated with congenital heart diseases [5–9]. Therefore, it is postulated that dysregulation of cardiac developmental genes may also contribute to the CAD development.

T-box (TBX) transcription factors are a group of factors with a highly conserved DNA-binding domain (T-box), and play critical roles in embryonic development. There are 18 T-box family members, including TBX transcriptional factor 5 [TBX5] [10]. TBX5 gene is expressed in the embryonic and adult heart. TBX5 is required in the early heart tube formation, cardiac chamber morphogenesis and cardiomyocyte differentiation. During the cardiac

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development, TBX5 activates multiple downstream target genes, including atrial natriuretic factor (ANF), connexin 40 (CX40) and serum response factor (SRF). TBX5 functions alone or synergistically with interacting partners, such as NK2 transcription factor related locus 5 (NKX2-5), GATA transcription factor 4 (GATA4) and TBX20 [11–15]. TBX5 is also expressed in the central conduction system, and required for the patterning and maturation of the cardiac conduction system [16, 17]. In addition, TBX5 gene is essential to limb identity and limb development [18–20]. Mutations in TBX5 gene cause Holt-Oram syndrome (HOS), which is characterized by congenital heart defects and upper limb abnormalities [21, 22]. Haploinsufficiency of TBX5 in ventricle myocytes in mice leads to diastolic dysfunction of heart, indicating that TBX5 is required for heart function in adults [23].

In human embryonic heart, TBX5 gene is expressed in myocardium, embryonic epicardium and coronary vasculature [24]. Animal experiments have shown that TBX5 regulates epicardial formation and coronary vasculogenesis in a dose-dependent manner [25]. Coronary artery abnormalities have been reported in HOS patients, further suggesting that TBX5 is involved in the coronary vessel formation [26–29]. Developmental biological studies have shown that TBX5 is a dose-sensitive regulator [30]. A gain-of-function TBX5 gene mutation is associated with atypical HOS and paroxysmal atrial fibrillation [31]. In addition, TBX5 is crucial to the cytokine gene expression in human T cells and fibroblast cells, indicating TBX5 involvement in inflammation [32, 33]. Therefore, altered levels of TBX5 may affect coronary vessel formation and inflammation, contributing to AMI development. In this study, we genetically and functionally analyzed the TBX5 gene promoter in large cohort of AMI patients and ethnic-matched healthy controls.

Methods

Study population

During the period from April, 2014 to August, 2016, the AMI patients ($n = 432$, male 284, female 148, median age

62.00 years) were recruited from Cardiac Care Unit, Affiliated Hospital of Jining Medical University, Jining, Shandong Province, China. AMI diagnosis criteria included typical clinical manifestations, changed ECG and biochemical markers (troponin or creatine kinase-MB), or coronary angioplasty. The ethnic-matched healthy controls ($n = 448$, male 235, female 213, median age 48.00 years) were recruited from the same hospital. The healthy controls with familial history of AMI, CAD and congenital heart diseases were excluded. This study protocol was approved by the Human Ethic Committee of Affiliated Hospital of Jining Medical University. This work was conducted according to the principles of the Declaration of Helsinki. Written informed consents were obtained from all participants.

DNA sequencing analysis of TBX5 gene promoter

Genomic DNAs were extracted from peripheral leukocytes isolated from venous blood. DNA fragments for TBX5 gene promoter were generated by PCR and directly sequenced as previously reported [6]. PCR primers were shown in Table 1. DNA sequence variants (DSVs) were identified by comparing to wild type TBX5 gene promoter.

TBX5 gene promoter activity with dual-luciferase reporter assay

Wild type and variant TBX5 gene promoters (1294 bp) were generated, and inserted into pGL3-basic for reporter gene expression constructs. The constructs were transiently transfected into cultured human embryonic kidney cells (HEK-293, CRL-1573, ATCC) or rat cardiomyocyte line cells (H9c2, CRL-1446, ATCC) for 48 h. Luciferases activities of transfected cells were measured using dual-luciferase reporter assay system. Relative activity of variant TBX5 gene promoters were calculated as previously reported [6].

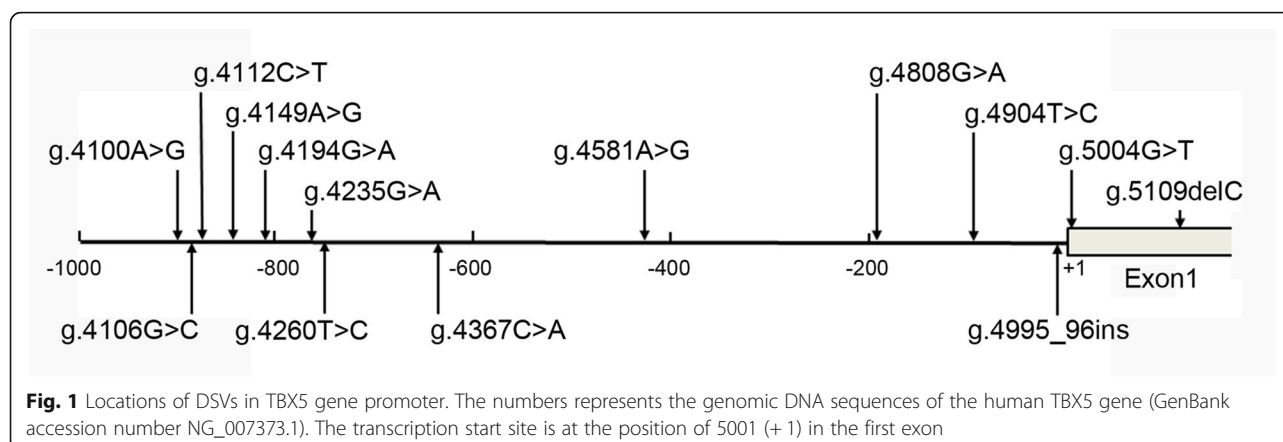
Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from cultured HEK-293 or H9c2 cells. As previously described, EMSA was performed with biotinylated double-stranded oligonucleotides (30 bp)

Table 1 PCR primers for the human TBX5 gene promoter

PCR primers	Sequences	Location	Position	Products
Sequencing				
TBX5-F1	5'-GGGTTTGGGAGAAGGATTC-3'	3981	-1020 bp	688 bp
TBX5-R1	5'-GAGGCACGAGGCACTCTATT-3'	4668	-333 bp	
TBX5-F2	5'-AGAAATTGTGCCCATGATC-3'	4593	-408 bp	677 bp
TBX5-R2	5'-TCTCCGCTTCGCCTATCAG-3'	5269	+ 269 bp	
Functioning				
TBX5-F	5'-(KpnI)-CGCTCGAGTTTCCCCTTTT-3'	3877	-1124 bp	1294 bp
TBX5-R	5'-(HindIII)-CGGAGGAATGAGGGTGATGAAC -3'	5170	+ 170 bp	

PCR primers are designed based on the genomic DNA sequence of the TBX5 gene (NG_007373.1). The transcription start site is at the position of 5001 (+ 1)



to examine whether DSVs affected binding sites for transcription factors [34].

Statistical analysis

Transfection data were expressed as means \pm standard errors of the means, and analyzed using two-way analysis of variance. DSV frequencies in AMI patients and controls were compared with SPSS v23.0 (SPSS, Chicago, IL, USA). $P < 0.05$ was considered as statistically significant difference.

Results

Identified DSVs in TBX5 gene promoter

Fourteen DSVs, including three single-nucleotide polymorphisms (SNPs), were identified in this study

population (Fig. 1 and Table 2). Six novel heterozygous DSVs (g.4100A > G, g.4194G > A, g.4260 T > C, g.4367C > A, g.4581A > G and g.5004G > T) were only identified in AMI patients, the chromatograms of which were shown in Fig. 2. Four novel heterozygous DSVs (g.4149A > G, g.4904 T > C, g.4995_96insTAATAA and g.5109delC) were only found in healthy controls. Three SNPs [g.4106G > C (rs79795050), g.4112C > T (rs7957609) and g.4808G > A (rs57820630)] and one heterozygous DSV (g.4235G > A) were found in both AMI patients and controls with similar frequencies ($P > 0.05$).

DSVs-affected activity of TBX5 gene promoter

Reporter gene expression constructs, including empty pGL3-basic (negative control), pGL3-WT (wild type

Table 2 The DSVs within the TBX5 gene promoters in AMI patients and controls

DSVs	Genotypes	Location ¹	Controls (n = 448)	AMI (n = 432)	P value
g.4100A > G	AG	-901 bp	0	1	–
g.4106G > C (rs79795050)	GG	-895 bp	360	362	0.601
	GC		66	65	
	CC		2	5	
g.4112C > T (rs7957609)	CT	-889 bp	418	413	0.125
			27	19	
			3	0	
g.4149A > G	AG	-852 bp	1	0	–
g.4194G > A	GA	-807 bp	0	1	–
g.4235G > A	GA	-766 bp	3	5	0.499
g.4260T > C	TC	-741 bp	0	1	–
g.4367C > A	CA	-634 bp	0	1	–
g.4581A > G	AG	-420 bp	0	1	–
g.4808G > A (rs57820630)	GA	-193 bp	4	5	0.748
g.4904T > C	TC	-97 bp	2	0	–
g.4995_96insTAATAA	–/TAATAA	-6 bp	1	0	–
g.5004G > T	GT	+ 4 bp	0	1	–
g.5109delC	T/–	+ 109 bp	1	0	–

¹, DSVs are located upstream (–) to the transcription start site of TBX5 gene at 5001 of NG_007373.1

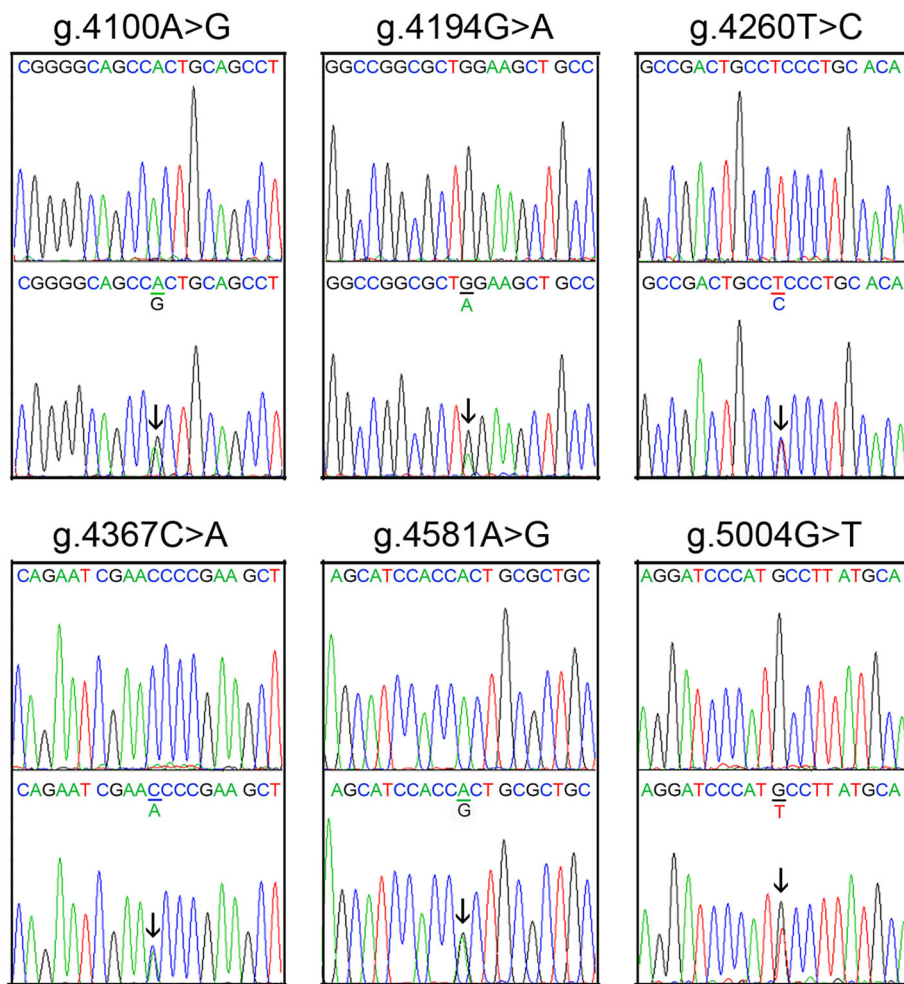


Fig. 2 Sequencing chromatograms of the novel DSVs in the TBX5 gene promoter in AMI patients. Sequencing orientations are forward. Top panels show wild type and bottom panels heterozygous DNA sequences. Heterozygous DSVs are marked with arrows

TBX5 gene promoter), pGL3-4100G, pGL3-4149G, pGL3-4194A, pGL3-4235A, pGL3-4260C, pGL3-4367A, pGL3-4581G, pGL3-4904C, pGL3-4915_16insTAATAA, pGL3-5004T and pGL3-5109delC, were transfected into cultured HEK-293 cells. The results showed that the DSVs in AMI patients either significantly increased (g.4367C > A and g.4581A > G) or decreased (g.4100A > G, g.4194G > A, g.4260 T > C and g.5004G > T) the activity of TBX5 gene promoter ($P < 0.05$). In contrast, the DSVs (g.4149A > G, g.4904 T > C, g.4995_96insTAA-TAA and g.5109delC) in controls or the DSV (g.4235G > A) in both AMI patients and controls did not significantly affect activity of TBX5 gene promoter ($P > 0.05$) (Fig. 3).

To examine tissue-specific effects of the DSVs, we measured the activities of variant TBX5 gene promoter in cultured H9c2 cells. As expected, the DSV in AMI patients either significantly increased (g.4367C > A and g.4581A > G) or decreased (g.4100A > G, g.4194G > A,

g.4260 T > C and g.5004G > T) the activity of TBX5 gene promoter ($P < 0.05$). Taken together, the DSVs in AMI patients significantly affect the activity of TBX5 gene promoter in a non-tissue specific manner.

DSVs-affected putative binding sites for transcription factors

To determine whether DSVs affect putative binding sites for transcription factors, TBX5 gene promoter was analyzed with JASPAR program (<http://jaspar.genereg.net/>). The DSV (g.4100A > G) may modify binding sites for basic leucine zipper transcription factor nuclear respiratory factor-1 (NRF-1), nuclear factor 1 X-type (NFIX) and nuclear factor kappa B subunit 2 (NFkB2). The DSV (g.4194G > A) may abolish binding sites for E2F transcription factor 4 (E2F4), a fork head factor, and basic helix-span-helix transcription factor AP-2 Alpha (TFAP2A). The DSV (g.4260 T > C) may create a binding site for zinc finger transcriptional repressor hypermethylated in cancer

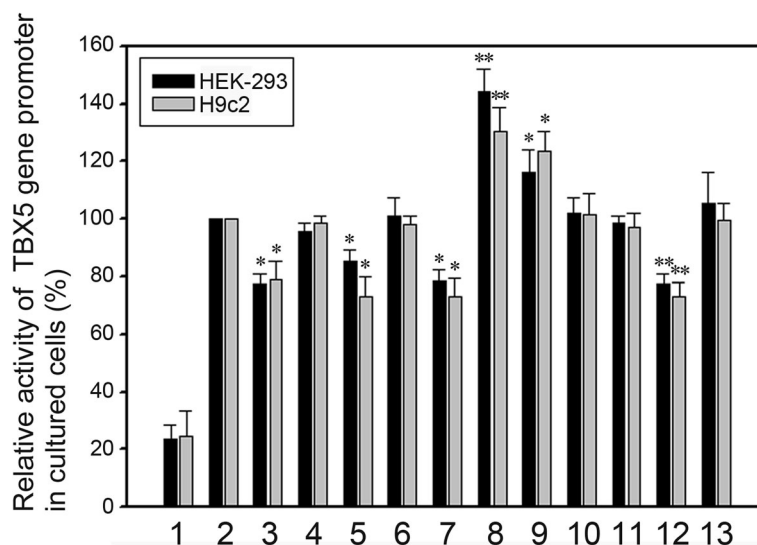


Fig. 3 Relative activity of wild type and variant TBX5 gene promoters. Empty vector pGL3-basic was used as a negative control. Transcriptional activity of wild type TBX5 gene promoter was designed as 100%. Relative activities of variant TBX5 gene promoters were calculated. Lanes 1, pGL3-basic; 2, pGL3-WT; 3, pGL3-4100G; 4, pGL3-4149G; 5, pGL3-4194A; 6, pGL3-4235A; 7, pGL3-4260C; 8, pGL3-4367A; 9, pGL3-4581G; 10, pGL3-4904C; 11, pGL3-4915_16insTAATAA; 12, pGL3-5004T; and 13, pGL3-5109delC. Dark bars indicate HEK-293 cells, and grey bars H9c2 cells. WT, wild type. *, $P < 0.05$; **, $P < 0.01$

2 (HIC2) and modify binding sites for TFAP2B and TFAP2C. The DSV (g.4367C > A) may create a binding site for nuclear factor of activated T-cells 5 (NFAT5) and modify binding site for TFAP2A. The DSV (g.4581A > G) may abolish binding sites for zinc finger protein 354C (ZNF354C) and zinc finger and BTB domain-containing protein 7C (ZBTB7C). The DSV g.5004G > T may create a binding site for TEA domain transcription factor 1 (TEAD1) and abolish a binding site for transcription factor EB (TFEB). Therefore, the DSVs in AMI patients may affect putative binding sites for transcription factors.

DSVs-affected binding site of transcription factors

To investigate whether the DSVs experimentally affect binding sites for transcription factors, EMSA was performed with wild type or variant oligonucleotides (Table 3). In both HEK293 and H9c2 cells, the DSVs (g.4100A > G and g.4260 T > C) enhanced the binding of unknown transcription

factors, which were likely transcriptional repressors as both DSVs decreased the TBX5 gene promoter activity. The DSV (g.4581A > G) almost abolished the binding of an unknown transcription factor, which acted as a transcription repressor since the DSV significantly increased the TBX5 gene promoter activity (Fig. 4). Other DSVs (g.4194G > A, g.4367C > A and g.5004G > T) did not evidently affect the binding of transcription factors, likely due to EMSA sensitivity.

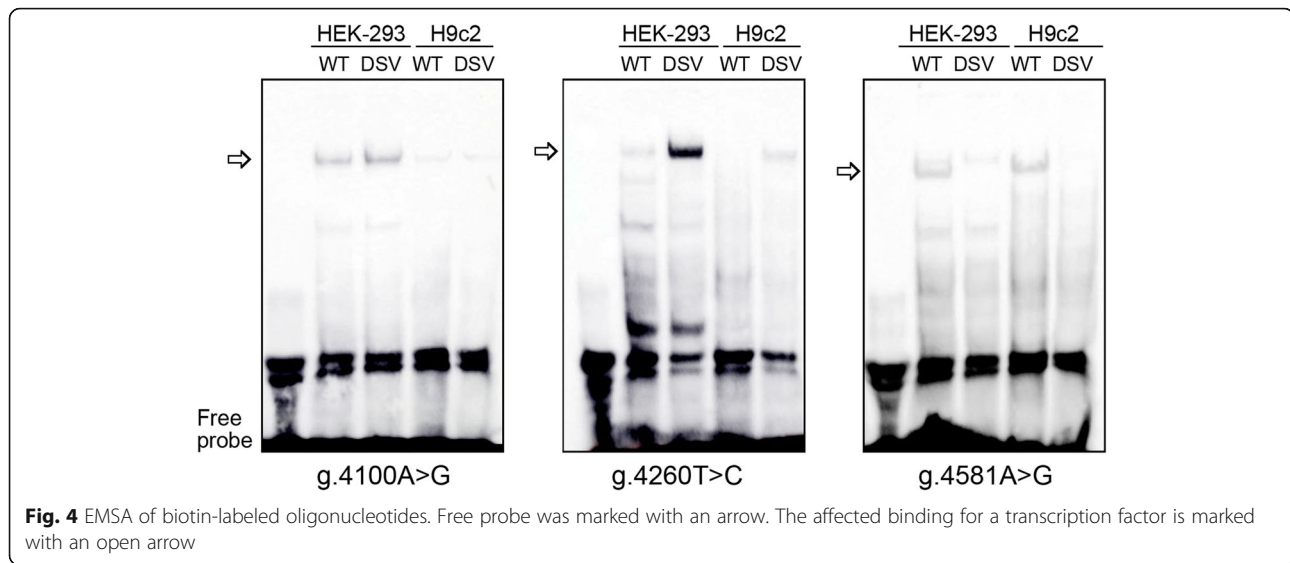
Discussion

Mutations in TBX5 gene have been implicated in HOS, a variety of congenital heart diseases, dilated cardiomyopathy and atrial fibrillation [35, 36]. Intron variants of TBX5 gene is associated with PR interval, QRS duration and QT interval, as well as atrial fibrillation [37–39]. Regulatory variation in a TBX5 gene enhancer causes isolated congenital heart disease [40]. Genetic variants and altered expression levels of TBX5 gene have been reported

Table 3 Biotinylated double-stranded oligonucleotides for EMSA

DSVs	Oligonucleotide sequences 1	Locations
g.4100A > G	5'-AAGCTCGGGCAGCC(A/G)CTGCAGCCTGGCTG-3'	4085–4114
g.4194G > A	5'-CTTGTGGCCGGCGCT(G/A)GAAGCTGCCCGCTC-3'	4179–4208
g.4260 T > C	5'-TGCCGGCCGACTGCC(T/C)CCCTGCACATTTTG-3'	4245–4274
g.4367C > A	5'-AAACCCAGAATCGAA(C/A)CCCGAAGCTGGGG-3'	4352–4381
g.4581A > G	5'-TTGCGAGCATCCACC(A/G)CTGCGCTGCTTAGA-3'	4566–4595
g.5004G > T	5'-TAATAAGGATCCCAT(G/T)CCTTATGCAAGAGA-3'	4989–5018

Bold letters in the oligonucleotide sequences indicate the DSVs



in patients with cancers [41, 42]. In previous studies, we have reported two heterozygous DSVs, g.4303C > G and g.4900C > T, in patients with ventricular septal defects [6]. In this study, six novel heterozygous DSVs (g.4100A > G, g.4194G > A, g.4260 T > C, g.4367C > A, g.4581A > G and g.5004G > T) were identified in six AMI patients, and significantly affected the transcriptional activity of TBX5 gene promoter. Further EMSA revealed that the DSVs modified binding sites for transcription factors. Collective frequency of the DSVs in AMI patients were 1.39% (6/432). The DSVs in TBX5 gene promoter identified in patients with ventricular septal defects were not found in this study, suggesting these DSVs were unique in AMI patients. Therefore, our findings suggested that TBX5 gene promoter DSVs may change TBX5 levels, contributing to AMI development at adult stage. As this is a single-center study, more AMI patients from multi-center will be needed to further confirm our findings.

The human TBX5 gene is localized to 12q24.1, contains 9 exons and spans more than 47 kb [21, 43]. There are GC box, T-box-like binding elements, and NKX2-5 binding site in the human TBX5 gene promoter. TBX5 gene is likely to be autoregulated at transcriptional level [44]. An enhancer located to ~90 kb downstream of TBX5 gene has been identified [36]. microRNAs (miR-10a and miR-10b) repress TBX5 gene expression and decrease TBX5 level by targeting the TBX5 3'-untranslated region [45]. In this study, the novel DSVs in the TBX5 gene promoter were biologically functional by affecting binding sites for transcription factors. Molecular mechanisms for these DSVs to influence TBX5 gene expression will be further explored and elucidated.

A number of downstream target genes and interacting partners for TBX5 have been reported. During the heart development, TBX5 regulates NKX2.5, atrial natriuretic

factor (ANF) and cardiac structural genes [11, 46–48]. TBX5 directs SRF gene expression by binding to its 3'-untranslated region [49]. TBX5 partners include GATA4, GATA5, MEF2C, NKX2-5, and other cardiac transcription factors [11, 12, 50–53]. These transcription factors form a complex cardiac regulatory network to coordinately control cardiac gene expression [54]. TBX5 forms a positive and negative feed-forward circuit with Sal-like protein 4 (SALL4), a zinc finger transcription factor, to ensure the morphogenesis of embryonic heart [55]. A member of Krüppel-like family of zinc finger proteins, Krueppel-like factor 13 (KLF13), is co-expressed with TBX5 in cardiomyocytes, which may act as a genetic modifier of TBX5 [56]. In a gene regulatory network to maintain cardiac rhythm, TBX5 directly activates paired like homeodomain 2 (PITX2) gene [57]. In addition, TBX5 binds to the proximal region of the NFAT3 (nuclear factor of activated T cells 3) gene in human T cells [32]. Therefore, changed TBX5 level may interrupt cardiac gene regulatory network, contributing to AMI development by affecting cardiac function and influencing coronary vasculature.

Conclusion

In this study, novel DSVs in TBX5 gene promoter were identified in AMI patients. The DSVs significantly altered TBX5 gene promoter activity in cultured cardiomyocytes. EMSA revealed that the DSVs affected binding sites for transcription factors. Therefore, TBX5 gene promoter DSVs may alter TBX5 gene promoter activity and change subsequent TBX5 level, contributing to AMI development as a rare risk factor.

Abbreviations

AMI: Acute myocardial infarction; ANF: Atrial natriuretic factor; CAD: Coronary artery disease; CX40: Connexin 40; DSVs: DNA sequence variants; E2F4: E2F transcription factor 4; GATA4: GATA transcription factor 4;

HIC2: Hypermethylated in cancer 2; HOS: Holt-Oram syndrome; KLF13: Krueppel-like factor 13; NFAT3: Nuclear factor of activated T cells 3; NFAT5: Nuclear factor of activated T cells 5; NFIX: Nuclear factor 1 X-type; NFKB2: Nuclear factor kappa B subunit 2; NKX2-5: NK2 transcription factor related locus 5; NRF-1: Nuclear respiratory factor-1; PITX2: Paired like homeodomain 2; SALL4: Sal-like protein 4; SRF: Serum response factor; TBX5: T-box transcription factor 5; TEAD1: TEA domain transcription factor 1; TFAP2A: Transcription factor AP-2 alpha; TFAP2B: Transcription factor AP-2 beta; TFAP2C: Transcription factor AP-2 gamma; TFE3: Transcription factor EB; ZBTB7C: BTB domain-containing protein 7C; ZNF354C: Zinc finger protein 354C

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Authors' contributions

YC and BY have conceived the project and planned the experiments, SW, JZ, XH and YZ conducted the experiments; JC, QS, SP and SZ analyzed and interpreted the data, SW, YC and BY wrote the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study available from the corresponding author on reasonable request.

Ethics approval and consent to participate

The study protocol was reviewed and approved by the Ethics Committee at the Affiliated Hospital of Jining Medical University, China, and conducted in conformance with the Helsinki Declaration. Informed written consent was obtained from all patients before all the study procedures.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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