

Clinical study of prenatal ultrasonography combined with T-box transcription factor 1 as a biomarker for the diagnosis of congenital heart disease

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Abstract. Congenital heart disease (CHD) seriously threatens fetal health. Therefore, prenatal examination to detect deformity is extremely important. The present study aimed to investigate the clinical application value of prenatal ultrasonography combined with molecular biology methods in the diagnosis of fetal CHD. A total of 1,000 pregnant women who had received fetal ultrasonography to examine fetal CHD were enrolled. Ultrasounds were performed for fetal heart examination and diagnosis, mainly on fetal heart position, size, structure and function, and heart valve morphology and function. These indexes were tested again 2 weeks after birth. Blood samples were collected from pregnant women with fetal CHD. Polymerase chain reaction (PCR) and western blotting were performed to detect the association between heart development and T-box transcription factor 1 (TBX1) expression. The results revealed that 10 fetuses had CHD (1%), of which ultrasound detected 9 cases. The specificity and sensitivity of ultrasounds were 100 and 90%, respectively. Of the 9 cases were identified by prenatal ultrasound screening, including 2 cases had endocardial cushion defect, 1 case had pulmonary stenosis combined with right ventricular dysplasia, 1 case had tetralogy of Fallot combined with a cleft lip and palate, 2 cases had ventricular septal defect, 1 case had a single ventricle defect, 1 case had Ebstein and 1 case had a triatrial heart. One case of ventricular septal defect was missed prior to delivery. PCR and western blotting demonstrated that TBX1 expression may be associated with CHD. Therefore, ultrasonography combined with laboratory examinations represent efficient, economic and safe methods for fetal CHD detection.

These methods may be significant to improve the rate of CHD diagnosis, and require further investigation.

Introduction

Fetal congenital heart disease (CHD) is a serious heart defect with high incidence (1). Its incidence is about 1-40/1,000 worldwide (2). In China, there ~300,000 fetuses with CHD (3), bringing a heavy burden to families and society (4). Therefore, prenatal detection of fetal malformation and elucidating the molecular mechanism of fetal CHD has important clinical significance and practical value (5). More importantly, prenatal examination also has great significance to reduce fetal CHD birth rate and promote prenatal and postnatal care (6).

Following science and technology developments, increasing methods for early fetal CHD diagnosis have been applied with high accuracy. Among them, color doppler ultrasound technique is one of the most important screening methods for detecting fetal CHD (1,7-9). With the improvement of ultrasonic detecting instrument resolution, color ultrasonic detection serves an important role in reducing fetal CHD incidence and mortality (8,10).

Laboratory testing methods, especially polymerase chain reaction, western blotting and gene sequencing, also have critical roles in fetal cardiac function screening. At present, laboratory testing methods have become an important supplement of ultrasonography for fetal CHD (11-13). Recent research suggested that the T-box transcription factor (TBX) family of proteins, especially TBX1, has a close association with fetal CHD occurrence and development (14,15). TBX1 is a transcription factor expressed in several tissues but its early expression in mesodermal tissue is particularly important for normal development of second heart field-derived heart segments, especially the outflow tract. Animal experiments have revealed that TBX1 overexpression is positively associated with CHD, indicating that TBX1 may be associated with fetal CHD occurrence (16-18). The aim of the present study was to investigate the clinical application value of prenatal ultrasonography combined with molecular biology methods in the diagnosis of fetal CHD.

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Materials and methods

Experimental subjects. According to the inclusion and exclusion criteria (19,20), 1,000 cases of pregnant women who had received fetal ultrasonography between June 2012 and June 2015 in Jining No. 1 People's Hospital (Jining, China) were enrolled. The inclusion criteria consisted of pregnant women at 18-25 gestational weeks and aged 18-30 years old, with no CHD family history or other serious disease history. Exclusion criteria were the subjects unmatched to the inclusion criteria. All subjects and families were informed of the purpose of the study and signed informed consent was obtained. The present study was approved by the ethics committee of Jining No. 1 People's Hospital. Ultrasounds were performed at 18-25 gestational weeks. The mean age of pregnant women was 24.9 ± 4.2 years.

Ultrasonography. A GE Voluson E6 color doppler ultrasonic detector at 6-10 MHz was used for fetal cardiac ultrasonography (21). Routine inspection (blood test) was applied before ultrasonography to test fetal development and position. Special examination (electrocardiogram and echocardiography) was then performed to test fetal heart. During color doppler ultrasonography, the pregnant women took a supine posture to reduce the probe pressure. Their posture was modulated to obtain the best image and different view angle. Ultrasound examination was conducted by the chief physician. Fetal heart position, size, structure, function, and heart valve morphology and function were examined. Fetal heart development condition was analyzed, including the right ventricular outflow tract, left ventricular outflow tract, pulmonary stenosis, atresia, aortic abnormalities, endocardial defects, valve deformity, cardiac neoplasm, tricuspid mobile and ventricular septal defects. Finally, aortic arch defects, tetralogy of Fallot, aortic stenosis, heterotopic heart, pericardial effusion and cardiothoracic ratio were examined. All pregnant women received fetal CHD screening and review in strict accordance with the world and domestic standards (22).

Prenatal intervention method. For the cases diagnosed as fetal CHD, subjects and their families were informed as soon as possible and accurately to decide on the following examination. Termination was proposed for the cases that were determined to be incurable (23). For the fetal CHD cases that progressed to induction of labor, the families agreed to scientific examination.

Pregnant women blood sample collection and analysis. For the cases with fetal CHD, 3 ml venous blood was extracted from the pregnant women with heparin anticoagulation (24). Genomic DNA was extracted using a DNA extraction kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol.

PCR. PCR was applied to test whether fetal CHD was associated with TBX1 (25). TBX1 gene primer sequences were: Forward, 5'-AATTTGGCCTCACCTGTGTC-3' and reverse, 5'-ACTCAGCCCTGACTCAATAG-3'. β -actin (internal reference control) primer sequences were: Forward, 5'-CTCACC

Table I. Ultrasound examination and pathological result comparison.

CHD type	Pathological result	Ultrasound examination
Triatral heart	1	1
Ebstein	1	1
Single ventricle	1	1
Ventricular septal defect	3	2
Tetralogy of Fallot	1	1
Pulmonary stenosis	1	1
Endocardial cushion defect	2	2
Total	10	9

CTGTCTGAATTTGG-3' and reverse, 5'-AACCTTAACTAG GCGACTCC-3'. The primers were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

The PCR system contained 1 μ l DNA template, 2.0 μ l 10X PCR Buffer, 2.0 μ l dNTP Mixture (2 mM), 1 μ l primer 1 (10 μ M), 1 μ l primer 2 (10 μ M), 0.25 μ l Taq DNA Polymerase (Thermo Fisher Scientific, Inc.), 1.75 μ l $MgCl_2$ (25 mM) and 16 μ l H_2O . The PCR reaction conditions were: Initial denaturation at 94°C for 7 min, followed by 28 cycles of denaturation at 94°C for 30 sec, annealing at 50°C for 60 sec, extension at 72°C for 30 sec and elongation at 4°C for 5 min.

Agarose gel electrophoresis. PCR products (500 ng/ μ l) 10 μ l were mixed with sample buffer and separated by 0.8% agarose gel electrophoresis at 80 mV for 15 min. The electrophoresis gel was stained by ethidium bromide and analyzed using a gel imaging system (26).

Experimental results were analyzed using BandScan 680 software (Glyko; <http://bandscan.software.informer.com/>). All electrophoresis bands were tested independently three times. TBX1 relative expression was defined as the ratio between TBX1 and β -actin optical density.

PCR product sequencing and analysis. TBX1 gene PCR products were sequenced by Sangon Biotech Co., Ltd., following agarose gel electrophoresis detection (27). TBX1 sequencing result was analyzed and compared with TBX1 gene sequence in NCBI database (www.ncbi.nlm.nih.gov/). The association between TBX1 gene sequence and fetal CHD was analyzed.

Western blotting. Western blotting was performed to detect TBX1 expression in blood samples (28,29). The protein was lysed by radioimmunoprecipitation assay lysis buffer (Thermo Fisher Scientific, Inc.) and incubated on ice for 30 min followed by centrifugation at 13,000 \times g for 30 min at 4°C. The protein concentration was determined by Pierce bicinchoninic acid Protein Assay kit (Thermo Fisher Scientific, Inc.). Then the extracted proteins (40-60 μ g) were separated by 8% SDS-PAGE at 60 V for 30 min and 120 V for 90 min. Following this, the protein was transferred to

Table II. Cases of ultrasound examination and pathological result comparison (n=9).

Case	Gestational week	Factor	Ultrasound diagnosis	Pathological result
1	21	None	Endocardial cushion defect	Endocardial cushion defect
2	22	Abortion	Ebstein	Ebstein
3	23	Family history	Ventricular septal defect	Ventricular septal defect
4	23	None	Single ventricle	Single ventricle
5	24	Influenza	Tetralogy of Fallot	Tetralogy of Fallot
6	24	None	Ventricular septal defect	Ventricular septal defect
7	25	None	Pulmonary stenosis	Pulmonary stenosis
8	25	None	Endocardial cushion defect	Endocardial cushion defect
9	26	Poison contact	Triatrial heart	None

All cases had an outcome of induced labor.



Figure 1. Fetal cardiac ultrasonography result. GE Voluson E6 color doppler ultrasonic detector was used for fetal cardiac ultrasonography. Ultrasound probe frequency was 6-10 MHz.

a polyvinylidene difluoride membrane at 300 mA for 3 h. Following blocking with 5% bovine serum albumin (Thermo Fisher Scientific, Inc.)/PBS with Tween-20 (PBST) for 2 h at 37°C, the membrane was incubated with anti-TBX1 (cat. no. ab18530; Abcam, Cambridge, MA, USA) or anti- β -actin (cat. no. ab8227; Abcam) primary antibodies (1:2,000) at room temperature for 2 h. Next, the membrane was further incubated with horseradish peroxidase-conjugated goat-anti-rabbit secondary antibody (cat. no. ab6721; Abcam; 1:2,000) at room temperature for 1 h, after washing with PBST three times. At last, the membrane was developed using Pierce ECL Western Blotting Substrate (Thermo Fisher Scientific, Inc.) and analyzed. The results were analyzed by BandScan 680 software. All electrophoresis bands were tested independently three times. TBX1 relative expression was defined as the ratio between TBX1 and β -actin optical density.

Statistical analysis. SPSS 18.0 (SPSS, Inc., Chicago, IL, USA) was used for data analysis. All data are presented as the mean \pm standard deviation. One-way analysis of variance with the Newman-Keuls multiple comparison post-hoc analysis was used for multiple comparisons. $P < 0.05$ was considered to indicate a statistically significant difference.

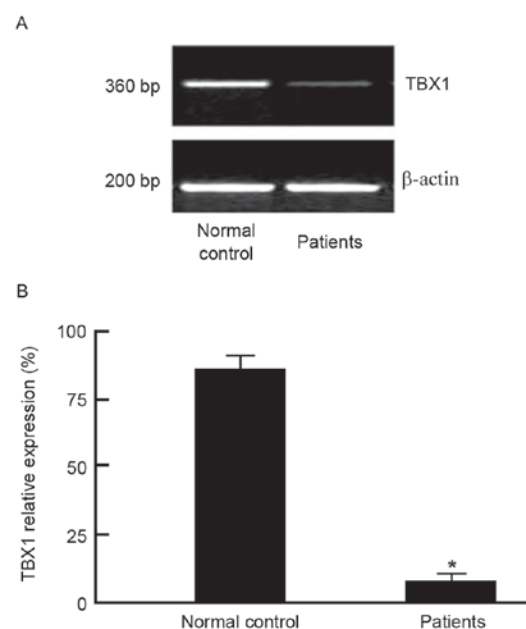


Figure 2. TBX1 gene polymerase chain reaction product agarose gel electrophoresis result. (A) Agarose gel electrophoresis and (B) quantification of TBX1 expression relative to β -actin. Data are expressed as the mean \pm standard deviation. * $P < 0.05$. TBX1, T-box transcription factor 1.

Results

Basic information. Fig. 1 presents a representative fetal ultrasonography result. In the present study, 10 fetuses were identified to have CHD (0.99%), of which ultrasound detected 9 cases. The specificity and sensitivity of ultrasound were 100 and 90%, respectively.

Comparison between ultrasound examination and pathological result. Of the 9 cases of CHD detected by prenatal ultrasound screening, 2 cases were endocardial cushion defect, 1 case was pulmonary stenosis combined right ventricular dysplasia, 1 case was tetralogy of Fallot combined cleft lip and palate, 2 cases were ventricular septal defect, 1 case was single ventricle defect, 1 case was Ebstein and 1 case had a triatrial heart. One case of ventricular septal defect was missed prior to delivery (Tables I and II).

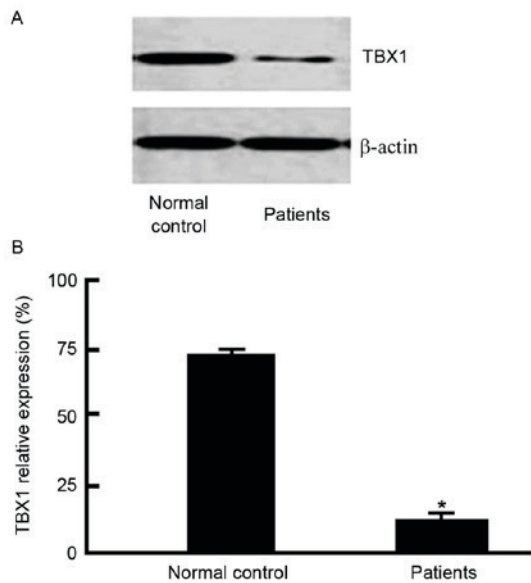


Figure 3. TBX1 protein expression levels, as determined by western blotting. (A) Representative western blot images and (B) quantification of TBX1 protein expression levels. β -actin served as an internal loading control. Data are expressed as the mean \pm standard deviation. * $P < 0.05$. TBX1, T-box transcription factor 1.

TBX1 gene PCR product agarose gel electrophoresis result. TBX1 was PCR amplified by using primer based on exon 1. PCR products were separated by agarose gel electrophoresis. The TBX1 gene PCR product was 360 bp in length (Fig. 2).

TBX1 western blotting result. Protein expression levels were analyzed by BandScan 680 software. Western blotting revealed that TBX1 protein expression levels were reduced in blood samples from pregnant women with fetal CHD (Fig. 3), suggesting that TBX1 reduction may be associated with fetal CHD occurrence and development.

TBX1 gene PCR amplification product sequencing comparison. The sequencing result of the TBX1 gene was compared with the TBX1 gene in NCBI database. The PCR product sequencing results of the present study coincided with the TBX1 gene sequence, and the sequence similarity achieved 100% (Fig. 4).

Discussion

With technology progression, prenatal examination is an important aspect to secure fetal safety and health. The present study investigated clinical application value of fetal cardiac ultrasound combined with molecular biology methods, such as PCR and western blot, in detecting fetal CHD.

Ultrasonic inspection is widely used for fetuses and has made a significant contribution for prenatal and postnatal care; however, the etiology of fetal CHD has various reasons. The present study verified ultrasonic diagnosis at the gene and protein level. The results demonstrated that consistent with previous studies (16–18), ultrasound scanning can effectively detect fetal CHD.

Query	1	ATGGCATTCAATCCATTTCGACCTGGACGCTCTGATCTTTTGTACCAATTTATGGGAGCA	60
Subject	1	ATGGCATTCAATCCATTTCGACCTGGACGCTCTGATCTTTTGTACCAATTTATGGGAGCA	60
Query	61	GGAGTTGGTCTCTGGAGCCGGTGTCCACCACCAAAATTTGTTTTTCAATGCTTCAA	120
Subject	61	GGAGTTGGTCTCTGGAGCCGGTGTCCACCACCAAAATTTGTTTTTCAATGCTTCAA	120
Query	121	GCTGGGTTCCACCAGGACCTGTTGGATCACCACCTGAAGATGATGGGGTAACGTATGAT	180
Subject	121	GCTGGGTTCCACCAGGACCTGTTGGATCACCACCTGAAGATGATGGGGTAACGTATGAT	180
Query	181	CCGAAAGTTGAGCTTGATGAACGAGAATATGGCAACAGTTTCACAATGTGGAACTGAA	240
Subject	181	CCGAAAGTTGAGCTTGATGAACGAGAATATGGCAACAGTTTCACAATGTGGAACTGAA	240
Query	241	ATGGTCATTACTAAAGTGAAGGTTTGTTTTATTAAGTTTTTTTCTAAATCTTCT	300
Subject	241	ATGGTCATTACTAAAGTGAAGGTTTGTTTTATTAAGTTTTTTTCTAAATCTTCT	300
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Subject	301	TGAAACTCTTTTGTCTGCTTTGATTTTGAACATATTTCTCAGTCTCAATCCGTT	360
Query	361	GTCATTAACTAGCTGAAAGTTCTCACTAAAAATCCATCTGTTTCTATATTTTG	420
Subject	361	GTCATTAACTAGCTGAAAGTTCTCACTAAAAATCCATCTGTTTCTATATTTTG	420
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Subject	481	TCCCATCACTTTACTTTGAGAGCTCATTGGGTTTATTGTAATCATCAAGTAATTA	540
Query	541	TAAATCTATAGAATGAAAAATAGAAAATAATTTTGACGGTGGAAATTTTGTATTA	600
Subject	541	TAAATCTATAGAATGAAAAATAGAAAATAATTTTGACGGTGGAAATTTTGTATTA	600
Query	601	CCTGAAAAAATGAGATATAAGTTGATAAAGTCGAGCAGTTGGGGTCAAGACTAGCAG	660
Subject	601	CCTGAAAAAATGAGATATAAGTTGATAAAGTCGAGCAGTTGGGGTCAAGACTAGCAG	660
Query	661	GGAATTTAAAAATACAAAAATACAAAAAATTTGAAATATATTAATAATTTATTATCA	720
Subject	661	GGAATTTAAAAATACAAAAATACAAAAAATTTGAAATATATTAATAATTTATTATCA	720

Figure 4. T-box transcription factor 1 gene polymerase chain reaction amplification product sequencing comparison.

Previous studies have demonstrated that the transcription factor TBX1 has a close association with CHD (20). The PCR and western blotting results of the present study demonstrated that TBX1 may be associated with CHD at the gene and protein level. Therefore, TBX1 downregulation may be associated with CHD occurrence. However, the underlying mechanism of TBX1 in CHD requires further investigation.

The present study had three primary results. Firstly, 10 fetuses were identified to CHD (0.99%), of which ultrasound detected 9 cases. The specificity and sensitivity of ultrasound were 100 and 90%, respectively. Secondly, of the 9 cases detected prenatal ultrasound screening, 2 cases had endocardial cushion defect, 1 case had pulmonary stenosis combined right ventricular dysplasia, 1 case had tetralogy of Fallot combined with a cleft lip and palate, 2 cases had a ventricular septal defect, 1 case had a single ventricle defect, 1 case had Ebstein and 1 case had a triatrial heart. One case of ventricular septal defect was missed prior to delivery. Lastly, PCR and western blotting demonstrated TBX1 expression may be associated with CHD. This implicates TBX1 as a biomarker of CHD.

However, the present study had several limitations. Firstly, the enrolled sample size was relatively small. Since the incidence of fetal CHD was low, a larger sample size is required in the future. Secondly, a single-blind method of analysis was used; multicenter double-blind clinical trials are required to investigate the association between TBX1 expression and CHD. Finally, the subjects were not classified by nationality, ethnicity and family history, which may have influenced the results.

In conclusion, ultrasonography combined with laboratory examination may represent efficient, economic and safe methods for fetal CHD detection. These methods may be significant to improve the rate of CHD diagnosis, and require further investigation.

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