Original article

Expression of Cx43-related microRNAs in patients with tetralogy of Fallot

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Background: **Abnormal expression of connexin 43 (Cx43) has been reported to play an important role in the development of conotrunccal anomalies. However, less is known about the underlying reason for its abnormal expression. MicroRNAs (miRNAs), as an important part of gene expression regulation, have been implicated in some cardiac diseases. This study aimed to investigate the expression of Cx43 and its related miRNAs in patients with tetralogy of Fallot (TOF), and illustrate the potential role of abnormal miRNAs regulation to Cx43 expression in the pathology of TOF.**

Methods: **Real-time polymerase chain reaction was used to detect the expression of Cx43 and 10 Cx43-related miRNAs in the myocardium from 30 TOF patients and 10 normal controls. Immunohistochemistry was used to detect Cx43 protein expression. Putative miRNA binding sites in the 3'UTR of Cx43 were examined in 200 TOF patients and 200 healthy individuals, using Sanger sequencing, to exclude sequence variations resulting in binding difficulties of miRNAs.**

Results: **Cx43 mRNA and protein expression in the myocardium tissue was significantly increased in TOF patients. The expression of MiR-1 and 206 was significantly decreased in the TOF patients as compared with the controls ($P<0.05$). No obvious difference was observed in the expression of the other 7 miRNAs between the TOF patients and controls ($P>0.05$). No meaningful sequence variation was detected in the putative miR1/206 binding sites in the 3'UTR of Cx43.**

Conclusions: **This study indicated that miR-1 and 206 is down-regulated in TOF patients, which may cause an up-regulation of Cx43 protein's synthesis. It provided a clue that miR-1 and 206 might be involved in the pathogenesis of TOF, additional experiments are needed to determine if in fact, miR-1 and 206 contribute substantially to TOF.**

World J Pediatr 2014;10(2):138-144

Key words: **congenital heart disease;**
Cx43;
miRNA;
tetralogy of Fallot

Introduction

Congenital heart disease (CHD) is the most prevalent form of birth defect in the structure of the heart or intrathoracic great vessels, affecting approximately 1%-2% of newborns, and is the leading cause of infant death resulting from birth abnormality, with more than 29% deaths of infants.[1] Tetralogy of Fallot (TOF), which accounts for 10% of CHD, is the most common cyanotic congenital heart defect and characterized by obstruction of the right ventricular outflow tract (RVOT), ventricular septal defect, aortic dextroposition (AD) and right ventricular hypertrophy (RVH).[2] Without surgery, TOF patients have poor prognosis, and even after intracardiac repair, the incidence of late complications is still high.[3] Despite its prevalence and clinical significance, the etiology of TOF in the majority of cases is unclear.[4]

TOF is considered by some authors to be a neural crest cell-related conotruncal heart defect that occurs during embryonic development of the heart.[5] Proper growth of conotruncal structure requires a unique combination of morphogenetic mechanisms associated with intercellular junctions.[6] As the most prevalent connexin (Cx) in mammal heart, connexin 43 (Cx43) plays an important role in cardiac development. Six integral membrane Cx proteins compose membrane-associated channels, which provide direct intercellular communication during cardiac development.[7] In Cx43 over-expressed transgenic mouse, over-expression

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doi: 10.1007/s12519-013-0434-0

of Cx43 resulting in an elevation of gap junction communication affects the migration of neural crest cells, which takes a role in modulating the growth and development of non-neural crest derived tissues. Thus, Cx43 over-expression leads to abnormal cell proliferation changes and obstruction of the RVOT.[8] These studies confirmed that over-expression of the *Cx43* gene participates in the pathogenesis of TOF. However, the expression and regulation of Cx43 in the process of TOF is not completely understood.

MicroRNAs (miRNAs) are a class of 22 nucleotide non-coding RNAs that regulate gene expression at the post-transcriptional level. miRNAs are incorporated into the RNA-induced silencing complex (RISC), which inhibits gene expression by cleaving mRNAs or blocking their translation.[9,10] miRNAs are increasingly reported to play important roles in diverse biological and pathological processes. Several studies revealed that miRNAs are essential for cardiac development and function. Moreover, genetic studies have identified distinct roles or specific miRNAs during cardiogenesis, cardiac hypertrophy, and electrical conduction. Mice lacking miRNA-1-2 died before ED12.5 due to pericardial edema and ventricular dysplasia.[11,12] In myocardial cells of a coronary atherosclerosis mouse model, miR-1 affected Cx43 expression.[13] Some other miRNAs such as miR-206, miR-133, miR-130, miR-143 and miR-145 were reported to be important in cardiac development.[14]

This study aimed to investigate the expression of Cx43 and its related miRNAs in the RVOT myocardium of TOF patients, and to illustrate the potential role of abnormal miRNAs regulation to Cx43 expression in the pathology of TOF.

Methods

Patient population and materials

Myocardial samples from the right RVOT were collected from 30 patients (average age: 1.3±0.9 years; 21 males, 9 females) undergoing cardiac surgery in Children's Hospital of Fudan University between January 2009 and March 2011.Ten normal RVOT myocardial samples (average age: 1.8±0.9 years; 7 males, 3 females) from victims of traffic accidents were collected at the Forensic Medicine Department of Fudan University. All the myocardium tissue samples were quickly frozen in liquid nitrogen immediately after surgical resection or autopsy and stored in liquid nitrogen until analysis. Blood samples from 200 TOF patients and 200 controls were collected from Children's Hospital of Fudan University. The age and gender of the controls were matched as closely as possible to those of the TOF patients. This project was approved by the Ethics Committee of Children's Hospital of Fudan University. All patients signed the informed consent form voluntarily. They were subjected to anthropometric measurement and physical examination for dysmorphism and malformations. They also underwent a chest X-ray examination, electrocardiogram and an ultrasonic echocardiogram. All of the patients were diagnosed by echocardiography, and then confirmed by surgery. Physical examination revealed no facial dysmorphism or other malformations in all patients. Chest X-ray examination also demonstrated no thymic agenesis in the patients. Ultrasonic echocardiogram detected 3 patients with right aortic arch. Since these patients had no special facial features and malformations of other organs, we did not perform chromosome examination.

Preparation of RNA and real-time PCR analysis

Total RNA was extracted from myocardial tissues with TRIzol (Life Technologies, Inc., Grand Island, NY), according to the manufacturer's guidelines. For mRNA detection, cDNA was prepared in a reaction with a total volume of 10 μL, including 500 ng of total RNA, 2 μL of 5×Primerscript buffer, 0.5 μL of 1×Randommers (100 μm), 0.5 μL of 1× Primerscript RT Enzyme Mix, and 0.5 μL of 1×oligo DT primer (50 μm) (TaKaRa, Shiga, Japan). The reactants were incubated at 37°C for 15 minutes and then terminated by heating at 85°C for 5 seconds. Real-time PCR amplification reactions were performed using the SYBR Premix Ex Taq (perfect real time) kit (TaKaRa, Shiga, Japan), which was used to quantify the Cx43 mRNA transcript level, with the glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA transcript as the endogenous reference. Specific mRNA quantifications were performed in triplicate.

For miRNA detection, polyA tail was added to RNase-free DNase-digested total RNA using the *E.coli* polyA polymerase (NEB, Ipswich, USA). Two micrograms of the tailed total RNA were reversely transcribed with ImProm-II (Promega, Wisconsin, USA). Cx43-related miRNAs, including miR-1, miR19a, miR-130a, miR-130b, and miR-206, miR-30a, miR-30c, miR-30d, miR-30e, miR-144, were predicted using computational and bioinformatics-based approach online (Table 1). miRNAs predicted in ≥3 databases

Table 1. Online database used in predicting Cx43-related miRNAs

Database	Websites
miRanda	http://www.microrna.org/microrna/home.do
TargetScan	http://www.targetscan.org/
DIANA-microT	http://diana.cslab.ece.ntua.gr/microT/
PicTar	http://pictar.mdc-berlin.de/
RNA22	http://cbcsrv.watson.ibm.com/rna22.html

were recruited in our study. Real-time PCR amplification reactions were carried out as mentioned above.

Primers used in real-time PCR amplification are summarized in Table 2. Depending on the transcript targeted, samples that revealed a low expression of GAPDH or 18sRNA [threshold cycle (CT) values greater than 30] were excluded from the analysis. The results were analyzed using the CT relative quantification method, and the data were averaged and then used for the $2^{-\Delta CT}$ calculation. $2^{-\Delta CT}$ corresponded to the ratio of each gene expression versus GAPDH/18sRNA and was expressed in a scatter plot.

Immunohistochemistry

The samples of myocardium tissue from the RVOT were fixed in 10% neutral-buffered formalin and then dehydrated and embedded in paraffin following routine methods. Sections of 4-5 μm thickness were cut using a Lecia RM2355 (Germany) and dried for 2 hours in a 56°C oven. The slides were deparaffinized and rehydrated using routine methods. The antigen was retrieved in sodium citrate buffer (0.03% citric acid, 0.2% sodium citrate) by heating in a microwave oven for 30 minutes and then rinsed 3 times for 2 minutes, each with distilled water. The myocardium tissue was blocked in 3% peroxide at room temperature by ablating endogenous peroxidase for 20 minutes and rinsing 3 times for 5 minutes, each with tris-buffered saline (TBST) and tween 20. The following steps were conducted in a moist chamber: (1) incubation with blocking buffer (3% bovine serum albumin, BSA) at room temperature for 20 minutes; (2) discarding the blocking buffer and adding monoclonal mouse anti-human antibody to Cx43 (sc-13558, dilution 1/400, Santa Cruz, USA), followed by incubating the sections for 2 hours at 37°C and rinsing in TBST 3 times for 5 minutes each; (3) dropping the goat anti-rabbit/mouse IgG (Genetech, Shanghai) and incubating the sections for 30 minutes at room temperature, followed by rinsing in TBST 3 times for 5 minutes each; (4) staining with 3,3-diaminobenzidin (DAB) for 1 minute, with termination of the staining with distilled water; and (5) counter-staining with Mayer's hematoxylin solution (Sigma-9627, USA) for 1 minute, dehydration, and mounting with per-mount mounting medium. In the negative control slide, 3% bovine serum albumin (BSA) was used and the primary antibodies were omitted.

Table 2. Primers for real-time PCR

Primers	Sequences
Cx43-RF	CAGCTTGTACCCAGGAGGAG
Cx43-RT	TGTCCCTGGCCTTGAATATC
hGAPDH-RF	CACCAGGTGGTCTCCTCTGAC
hGAPDH-RT	GGTGGTCCAGGGGTCTTACTC
hsa-miR-1	GGAGATGGAATGTAAAGAAGTATG
hsa-miR-19a	ACTGATGTGCAAATCTATGCAAA
hsa-miR-30a	GGAAGTGTAAACATCCTCGACT
hsa-miR-30c	TCAGCTGTAAACATCCTACACTC
hsa-miR-30d	GGAAGTGTAAACATCCCCGACT
hsa-miR-30e	GGAAGTGTAAACATCCTTGACT
hsa-miR-130a	GGCATCAGTGCAATGTTAAAAG
hsa-miR-130b	CAGUGCAAUGAUGAAAGGGCAU
hsa-miR-144	GTACTTACAGTATAGATGAT
hsa-miR-206	TGTGGTGGAATGTAAGGAAGTG
18S polyAF	AGTCGTAACAAGGTTTCCGTAGGTG
miR Hi-rev	CCAGTCTCAGGGTCCGAGGTATTC

Table 3. Primers for miRNAs binding sites sequencing

Primers	Sequences
Cx43-miR-1-AF	GTGAATGAGCGGGTGGTAAT
Cx43-miR-1-AR	GGAGGGTCAGGCCTAGAAAG
Cx43-miR-1-BF	AACTGGTATTCTTGGGTTTTCCT
Cx43-miR-1-BR	ATCTCGGCAGGGGAAACA

DNA sequencing

Genomic DNA from 200 TOF patients and 200 healthy controls was extracted from peripheral blood leukocytes using the QIAamp DNA Blood Mini Kit (QIAGEN, Germantown, USA). miRNAs' binding sites in the 3'UTR of Cx43 were then amplified by polymerase chain reaction (PCR) with the following primers in all patients and controls (Table 3). Cycles were performed as follows: 94°C for 5 minutes, 35 cycles at 94°C for 30 seconds, 59°C for 30 seconds, 72°C for 30 seconds, and a final extension of 72°C for 5 minutes. The PCR products were sequenced using the PCR primers and BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and run on an automated sequencer, ABI 3730XL (Applied Biosystems).The sequence results were analyzed using Mutation Surveyor demo software. All variants found by sequencing were filtered from the National Center for Biotechnology Information human SNP database and the 1000 Genome Project database (http://browser.1000genomes.org/).

Statistical analysis

Comparisons between the groups were made using Student's *t* test. The Chi-square test was used to compare the differences in allele and genotype distribution in DNA sequencing. The significance was analyzed with SPSS 16.0 software and a P value <0.05 was considered statistically significant.

Results

Cx43 expression in the myocardium

Real-time PCR analysis revealed that the mRNA expression of Cx43 in the RVOT myocardium was

significantly increased in TOF patients compared with the controls (P=0.0006). Immunohistochemical staining showed that the *Cx43* gene was irregularly distributed in the cytoplasm and cell membranes of the RVOT myocardial cells in the TOF patients, whereas it was regularly distributed in the intercalated disc in the controls. Cx43 protein expression was increased in the TOF patients (Fig. 1).

Cx43-related miRNAs expression in the myocardium

Real-time PCR analysis of 10 putative Cx43-related miRNAs revealed that miR-1 and miR-206 expression was significantly decreased in the TOF patients (n=30) as compared with the controls (n=10) (P=0.0001, P=0.0112, respectively) (Fig. 2 A and B). No obvious difference was observed in the expression of miR-130a, miR-19a, miR-30a, miR-30c, miR-30d and miR-130b between the TOF patients and controls (Fig. 2 C-H). MiR-30e and miR-144 expressions were not detected in the RVOT myocardium.

Sequence variation of miRNAs binding sites

Since the expression of miR-1 and miR-206 was decreased in the TOF patients, we speculated that miR-1 and miR-206 might be associated with the over-expression of Cx43 in the TOF patients. The mechanism of miRNAs involves the incorporation of single-stranded miRNA into the RISC and its subsequent binding to the 3'UTR of the target mRNA through exact complementarity with the 5' end's 7-8 nt, and partial complementarity with the rest of the sequence.[15,16] Changes of miRNAs binding sites in the 3'UTR of the

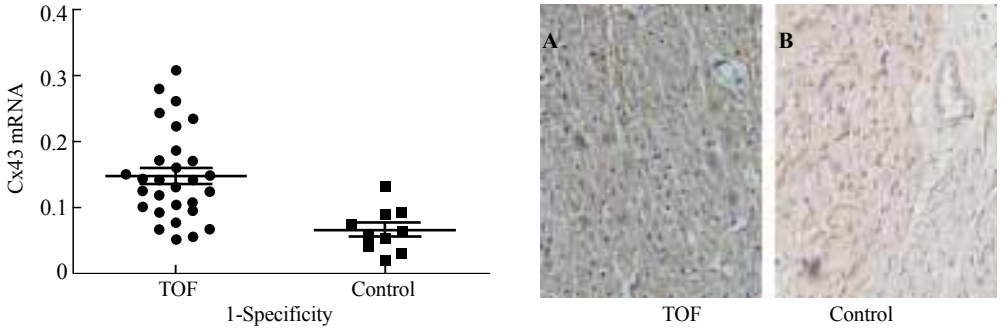

Fig. 1. Cx43 mRNA and protein expression. **A:** Real-time PCR analysis of Cx43 mRNA expression in the RVOT of the TOF patients (n=30) and controls (n=10). Cx43 gene expression levels were corrected by *GAPDH* gene expression levels. Cx43 expression was significantly increased in the TOF patients (P=0.006); **B:** Immunohistochemical staining of Cx43 in the RVOT in paraffin-embedded sections (original magnification × 200). Cx43 was distributed evenly in the intercalated discs of the controls, whereas the TOF patients had a heterogeneous Cx43 distribution in the cytoplasm and cell membrane. The staining intensity of Cx43 was also relatively weaker in the controls. PCR: polymerase chain reaction; Cx43: Connexin 43; RVOT: right ventricular outflow tract; TOF: tetralogy of Fallot.

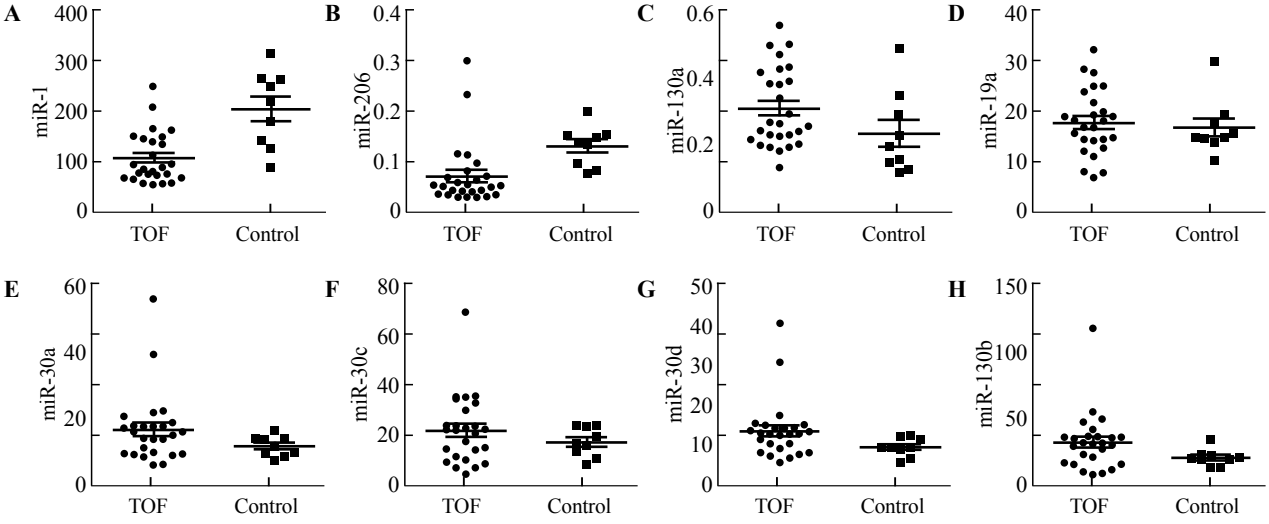

Fig. 2. Cx43-related miRNAs expression. **A&B:** miR-1 (P=0.0001) and miR-206 (P=0.0112) expressions were significantly decreased in the RVOT myocardium of TOF patients; **C-H:** No obvious difference was observed in the expression of miR-130a, miR-19a, miR-30a, miR-30c, miR-30d and miR-130b between the TOF patients and controls (P>0.05). Results were normalized to expression of 18sRNA, and unpaired Student's t test was used to evaluate the differences. Cx43: connexin 43; RVOT: right ventricular outflow tract; TOF: tetralogy of Fallot; miRNAs: microRNAs.

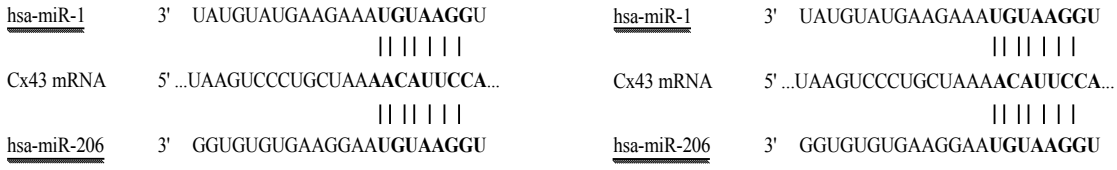

Fig. 3. The complementary sequences between miR-1/miR-206 and its putative sites within the 3'-UTR of Cx43 mRNAs, predicted with computational and bioinformatics-based approach using Target-Scan (http://www.targetscan.org/). Complementarity is shown in bold and connected by "|". Note that there are two putative miR-1/ miR-206 target sites in the 3'UTR of Cx43. Cx43: connexin 43.

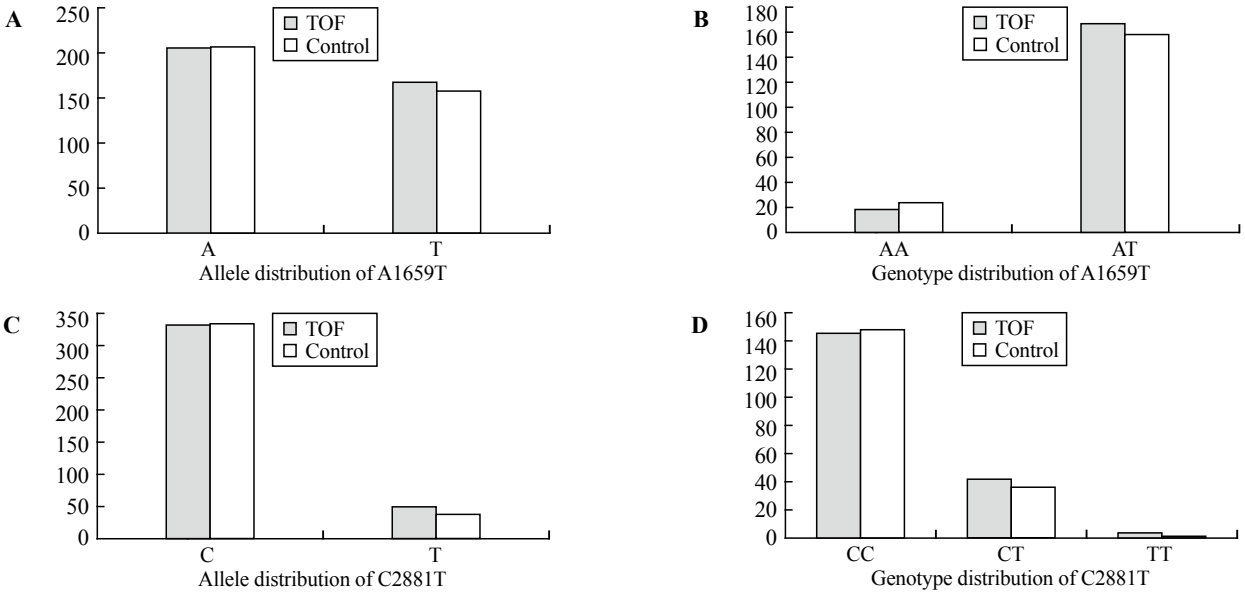

Fig. 4. *Cx43* gene 3'UTR sequencing. **A&B:** Novel SNP (A1659T) was found in the 3'UTR of Cx43, which was located in 24bp downstream of putative miR-1/miR-206 binding sites. The Chi-square test showed no differential distributions of the allele A/T (OR=0.942, 95% CI=0.703-1.259, *P*=0.684) and genotype of AA/TC/AT (OR=0.749, 95% CI=0.394-1.421, *P*=0.374); **C&D:** Another known SNP C2881T (rs78394273) was detected in 116bp downstream of other putative miR-1/miR-206 binding sites. No differential distributions of the allele C/T (OR=0.777, 95% CI=0.498-1.214, *P*=0.268) and genotype of CC/CT/TT (*P*=0.374) was found between the TOF patients and controls. Cx43: Connexin 43; TOF: tetralogy of Fallot; OR: odds ratio; CI: confidence interval.

target gene may affect the activity of miRNAs. We used a bioinformatics-based approach (Target-Scan) to find two identical potential binding sites of miR-1 and miR-206 in the 3' UTR of Cx43 (Fig. 3), and detected the sequence of the binding sites in 200 TOF patients and 200 controls to exclude mutations and sequence variations resulting in binding difficulties of miR-1 and miR-206.

Sequence analysis of miR-1 and miR-206 putative binding sites in the 3'UTR of Cx43 identified no mutations in the TOF patients. One novel SNP A1659T and another known SNP C2881T (rs78394273) were identified near the putative binding sites. The Chi-square test revealed no allele/genotype distribution differences of these two SNPs between the TOF patients and controls (site 1659, *P*=0.684; site 2881, *P*=0.268) (Fig. 4).

Discussion

As a congenital disorder due to combined effect of genetics and environmental alterations during gestation, TOF has been corrected by surgical repair for more than 30 years. Although this approach leads to low mortality and an excellent quality of life, late sudden cardiac death remains a persistent risk for patients with TOF, with an estimated incidence of 0.5% to 6%.[3] It is important to understand the genetic mechanisms underlying this pathologic condition. Point mutations[17-19] identified in the TOF patients are the beginning for us to comprehend this disorder genetically and enable us to hypothesize that this syndrome may be a result of altered proliferation, differentiation and migration of precardiac cells during heart development. However, heart development requires a unique combination of morphogenetic mechanisms, it is not enough to clarify the pathogenesis of multi-gene diseases by gene mutations. Non-coding region modifications, like miRNAs regulation, DNA methylation and histone modification, provide a way to clarify the effect of adverse environmental factors combining with genetics on the pathogenesis of multi-gene diseases, such as TOF.

Gap junctions, constituted by Cxs, play an essential role in heart morphogenesis, as they mediate the intercellular communication during cardiac development.[20] In the mammalian working myocardium, Cx43 is the major Cx protein expressed. Previous studies suggested that disturbances in protein arrangement and expression of Cx43 may influence both heart embryogenesis and myocardial maturation, resulting in hypertrophy and fibrosis of the right ventricle and the induction of severe arrhythmias in children with TOF. *In utero* hypertrophy and fibrosis of the right ventricular tissue were reported when the right ventricle is unloaded and oxygen is supplied by the placenta, RVOT pathology is associated with abnormal levels of Cx43 expression.[21] In the TOF patients of our study, we found increased expression of Cx43 mRNA and protein in the RVOT myocardium, which was accompanied by abnormal spatial distributions. We suggested that the over-expression of Cx43 mRNA might be associated with enhancer hypomethylation in transcriptional level (unpublished data). However, more obviously increased expression of Cx43 protein was also found in our study, indicating the coexistence of a post-transcriptional mechanism mediating Cx43 expression in the TOF patients.

miRNAs regulation is the most common post-transcriptional mechanism, and current knowledge supports that miRNAs regulate key genetic programs in cardiovascular biology, and are critical for cardiac development, endothelial function, lipid metabolism, ventricular hypertrophy, and post-infarction dysrhythmias.[22] miR-206 was found to regulate Cx43 expression during skeletal muscle development,[23] suggesting that Cx43 is the target gene of miR-206. Yang et al[13] verified that the 3'UTR of Cx43 contains nucleotides that are complementary to the 5' end of miR-1 and demonstrates that miR-1 can post-transcriptionally repress the expression of the gap-junction protein Cx43 in cardiac myocytes.

Studies in both Drosophila[24,25] and mice[26] demonstrated the importance of miR-1 during cardiogenesis. In the absence of miR-1, mouse hearts became hyperplastic, indicating a role for miR-1 in negatively regulating proliferation in cardiogenesis.[11] However, a recent study[27] about non-coding RNA expression in the myocardium from infants with TOF found no evidence of abnormal miR-1 expression in 16 TOF patients by using microarray based miRNA profiles, whereas array based miRNA profiles might lose some important miRNA information, causing false negative. In 30 TOF patients we detected miRNA expression in myocardium samples, and found down-regulation of miR-1 in these patients, indicating a role it might play in the development of this disease.

The muscle specific miR-1 and miR-206 are closely related in terms of expression and function, but different in chromosomal location, targets gene, and transcriptional activation. Over-expression of miR-206 promotes myocardium differentiation.[28] However, the expression of miR-206 in heart tissue is varied among the studies published. Anderson et al[23] reported that miR-206 is expressed predominantly in adult skeletal muscle, skin, Ed11 whole embryo and lung, but not in heart muscle. However, microarray data detected the expression of miR-206 in the heart, a tissue that expresses high levels of Cx43.[29,30] In our study, real-time PCR analysis demonstrated low level expression of miR-206 in the RVOT myocardium of normal controls. And more obviously decreased expression was detected in the TOF patients, suggested that miR-206 might express little in some special regions of the heart and regulate target gene expression in this region. High level expression of miR-206 was also involved in the down-regulation of Cx during myogenesis *in vivo*.[28] Thus, it is reasonable to propose that the expression of miR-1 and miR-206 is down-regulated in TOF patients via some unknown mechanisms and the decreased miR-1 and miR-206 levels may cause an up-regulation of Cx43 protein's synthesis.

The mechanism of miRNAs depends on its intact binding to the 3'UTR of the target mRNA. AGTR1 polymorphics of miR-155 binding sites were reported to be associated with cardiac hypertrophy in Friedreich ataxia.[31] miR-135a might be involved in heart failure associated with 25 bp deletion of the *MYBPC3* gene.[32] In our study, we also detected the sequence of the putative miR-1 and miR-206 binding sites in the 3'UTR of Cx43. No mutations or differential distributed SNPs were found in this region, which suggested that the sequence variation of miR-1 and miR-206 binding sites in the 3'UTR of Cx43 might not have a role in its abnormal expression.

In conclusion, this study indicated that miR-1 and miR-206 are down-regulated in TOF patients, which may cause an up-regulation of Cx43 protein's synthesis. It provided a clue that miR-1 and miR-206 might be involved in the pathogenesis of TOF, and additional experiments are needed to determine that if miR-1 and 206 contribute substantially to TOF.

Acknowledgments

We thank all of the patients involved in our study.

Funding: This work was supported by the National Basic Research Program of China (2010CB529500, 2009CB941704), the Natural Science Foundation of China (30930096, 30901624), and the Shanghai Municipal Science and Technology Commission (11JC1401400).

Ethical approval: Approved by the Ethics Committee of

Children's Hospital of Fudan University.
Competing interest: No benefits in any form have been received or will be received from any commercial party related directly or indirectly to the subject of this article.
Contributors: Wu Y wrote the first draft of this paper. All authors contributed to the intellectual content and approved the final version. Huang GY is the guarantor.

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Accepted after revision September 28, 2012