DNA sequencing of TGFβ2 in sporadic patients with tetralogy of Fallot

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Abstract. Transforming growth factor β2 (TGFβ2) plays an essential role in cardiac morphogenesis. However, the prevalence of TGFβ2 mutations in congenital heart disease (CHD) and the correlation between the TGFβ2 genotype and the CHD phenotype have not been studied extensively. The aim of this study was to examine DNA sequence changes in the TGFβ2 gene in sporadic patients with tetralogy of Fallot (TOF), and to observe whether TGFβ2 is the susceptibility gene for TOF. A cohort of 100 pediatric patients with TOF was recruited to the study; 200 healthy children were used as controls. PCR and genotyping were conducted for the detection of DNA changes in TGFβ2. The exons and the 5' untranslated region (5'UTR) sequences of the TGFβ2 gene were amplified. No mutations were identified in the coding region in any of the TOF patients. However, three single nucleotide changes, including 9126 A>AC, 9353 A>AG and 9040_9043 del CTTC, in the 5'UTR were found. There were no significant differences in allelic frequencies and genotype frequencies of position 9126 and 9353 between the TOF group and the control group. On the contrary, a significant difference was identified in the allelic frequencies (χ^2=17.469, P<0.001) of position 9040_9043 in the 5'UTR between the TOF group and the control group. Our results suggest that TGFβ2 may be a potential candidate gene of TOF. SNPs at position 9040_9043 del CTTC in the 5'UTR of TGFβ2 may be associated with susceptibility to TOF. The CTTC allele may be the susceptibility allele for TOF. However, the exact effect of these sequence changes requires further study using functional experiments.

Introduction

Congenital heart disease (CHD) is one of the most common types of congenital malformations in children, which are capable of severely influencing the mortality and quality of life of affected children (1). CHD affects 1-2% of newborn children and represents the largest class of birth defects (1). Conotruncal defect (CTD) is a type of complex congenital heart disease, which can lead to hypoxemia and irreversible acidosis during the neonatal period, thus leading to early mortality. Despite the prevalence and clinical significance of CTD, the causes are largely unknown. The pathogenesis of CTD has previously been identified. The interaction of multiple genes and the environment is the accepted cause of CHD. Tetralogy of Fallot (TOF) is a common type of CTD. The heart is the first organ to form in vertebrates. The formation of a four-chambered heart from a straight tube is a very complex process. Cardiogenic lineages originating from the anterior lateral mesoderm following gastrulation, develop into the cardiac primordia that fuse together to form the primitive heart tube along the ventral midline of the embryo. Subsequent looping, chamber maturation and alignment with the vasculature give rise to the multichambered heart (2). Errors in this process lead to CHD. Transforming growth factor β2 (TGFβ2) gene knockout animals have a CTD phenotype (3). Targeted disruption of TGFβ2 severely impairs the development of the right ventricle, the outflow tract and the aortic arch arteries, indicating that TGFβ2 is indispensable in the formation of the cranial elements of the developing heart (4). Thus, TGFβ2 is considered to be a candidate gene for conotruncal development. This study reports the findings of a cohort study, which aimed to detect TGFβ2 sequence changes in 100 Chinese TOF children. The results enabled the evaluation of the prevalence of TGFβ2 sequence changes in Chinese TOF children and the correlation between the genotypes and phenotypes.

Materials and methods

Cohort study and clinical features. A total of 100 children with TOF, who had been admitted to the Cardiac Center in the Children's Hospital of Fudan University, Shanghai between April 2007 and December 2009, were recruited for this study (Table I). TOF was confirmed by clinical symptoms, echocardiography, cardiac surgery or catheterization. Patients with any other abnormalities or known syndromes, including Holt-Oram, Marfan, Noonan, Alagille, DiGeorge and Char

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Table I. Characteristics of the 300 children in the study.

Cohort	No. of children with tetralogy of Fallot	No. of healthy children
Male	70	140
Female	30	60
Total	100	200

syndrome, were excluded from the study. Family history of CHD was not present in any of the cases. DNA samples from the 200 children who did not reveal any signs of genetic diseases or birth defects were used as the controls. Controls were recruited while undergoing health examinations; they all underwent cardiac evaluation by echocardiogram. All patients and control subjects were from the Chinese Han population. Informed consent was obtained from their parents. Study protocols were approved by the Institutional Research Ethics Committee of the Children's Hospital of Fudan University.

DNA sequencing in TGFβ2 exons and the 5' untranslated region (5'UTR). Genomic DNA was isolated from peripheral blood leukocytes using standard salt fractionation. Primers (Table II) were designed to amplify the exons (including their flanking intron) and the 5' UTR. The TGFβ2 gene sequences (GenBank accession no. 027721.1) were used to amplify the isolated DNA of the patients and controls using the polymerase chain reaction (PCR). Following purification by the AxyPrep DNA Gel Extraction kit (Axygen, Union City, CA, USA), the PCR products were sequenced on a 3730XL sequencer (ABI, Hitachi High-Technologies, Tokyo, Japan). For the sequence variant samples, the PCR products were sequenced once more from the opposite direction to confirm the nucleotide change.

Table II. Primers used to amplify the exons (including their flanking intron) and the 5'UTR sequences of the TGFβ2 gene.

Fragment		Primer	Amplicon (bp)
Exon 1	F	ACGTTTTTCTGTTGGGCATT	544
	R	CTCAGGGGATGGAAGTCAAA	
Exon 2	F	AGTTCTGTGCCAGGCATCTC	224
	R	CCCCACAGGAGACAAACACT	
Exon 3	F	TTAAACTGGCCGTTGGAAAC	473
	R	ACTCCTGCAGTCCCATTGAC	
Exon 4	F	TGTCAGAATGCCAACTCAGC	349
	R	TGCAGCAGGGACAGTGTAAG	
Exon 5	F	TGCTGTTCATGAATGGCTTC	293
	R	CCCCACCTCATATGACCAAG	
Exon 6	F	TAACTGTTGCCAGCTGATGC	475
	R	AAGCCTCTGGGAAGAATGGT	
Exon 7	F	TTTTTCTGGTTTGGGGTGAG	292
	R	TGCACATGFTAACCCAAGAA	
Exon 8	F	ACGAATTGCGTTCATTTTCC	247
	R	TTTGGTCTTGCCACTTTTCC	
5'UTR-1	F	GCAGACACGTGGTTCAGAGA	599
	R	TTGGTTACTCCACGTTGCTG	
5'UTR-2	F	GAAGCCTTCCCTTCTAGAGCA	600
	R	TTGGTTACTCCACGTTGCTG	
5'UTR-3	F	GGGAAGGTGGAACAGTGGTA	690
	R	GGTAAGGGAGGAAGGAGGTG	

F, forward primer; R, reverse primer; 5'UTR, 5' untranslated region; TGFβ2, transforming growth factor β2.

Results

In the present study, mutations in the coding regions were not identified in any of the TOF patients. However, three single nucleotide changes, including 9126 A>AC, 9353 A>AG and 9040_9043 del CTTC in the 5'UTR, were detected. These have been listed in the database of single-nucleotide polymorphism (SNP).

There were no significant differences in the allelic frequencies and the genotype frequencies of position 9126 and 9353 between the TOF group and the control group. However, there was a significant difference in the allelic frequencies (χ^2=17.469, P<0.001) of position 9040_9043 in the 5'UTR between the TOF group and the control group (Table III).

Discussion

The transforming growth factor β superfamily is a large family of genes containing over 30 members, including TGFβs, bone morphogenetic proteins, growth and differentiation factors, activins and Nodal, which are vital for the development and homeostasis of metazoans (5). TGFβ superfamily members critically regulate a number of processes within the cardiovascular system, including cardiac development and angiogenesis. The TGFβ2 gene has been mapped to chromosome 1 in the mouse and the human (6). Its functions are not overlapping with other TGFβ subtypes (7).

A crucial function of TGFβ ligands during murine heart development was first suggested by conventional knockout mice studies (7). While TGFβ1$^{-/-}$ and TGFβ3$^{-/-}$ mice demonstrated no evident signs of congenital heart defects, TGFβ2$^{-/-}$ embryos displayed multiple cardiac defects (8,9). These include the double-outlet right ventricle and TOF, which are known to derive from an error in the development of the second heart field that contributes to the formation of the outflow tract myocardium. TGFβ2 knockout mice display defective myocardialization associated with deregulation of neural crest cell apoptosis (10). It has been demonstrated that TGFβ2 knockout mice suffer from cardiovascular anomalies, including failure of normal completion of looping, septation of the outflow tract and ventricular remodeling (10,11). This suggests that lack of TGFβ2 may reduce apoptosis in the endocardial cushions. However, recent studies have revealed that apoptosis in the endocardial cushions in TGFβ2 knockout mice is increased (10). In RXRα$^{-/-}$ mice, elevated levels of TGFβ2 may contribute to abnormal outflow tract morphogenesis by enhancing apoptosis in the endocardial cushions

Table III. SNPs observed in the TOF patients and the controls.

Nucleotide change	Amino acid change	SNP	Genotype frequency		P-value[a]
			Patients (n=100)	Controls (n=200)	
9040_9043CTTC/-	5'UTR	rs9331507	80 (80.0%)	104 (52.0%)	0.0001[b]
9126A/C	5'UTR	rs7550232	78 (78.0%)	167 (83.5%)	0.858
9353A/G	5'UTR	rs10482718	88 (88.0%)	166 (83.0%)	1.008

[a]Pearson χ^2 test, [b]OR=3.692 (95% CI 1.971-6.917); SNP, single-nucleotide polymorphism; 5'UTR, 5' untranslated region; TOF, tetralogy of Fallot.

and promoting aortic sac malformations by interfering with the normal development of the aorticopulmonary septum (12). Therefore, too much or too little TGFβ2 may enhance apoptosis in endocardial cushions.

Regulation of gene expression is achieved at various levels, including transcription, post-transcriptional processing, mRNA stability, translation, post-translational modification and protein degradation. UTRs have significant regulatory roles (13). The 5'UTR of mRNAs contains motifs capable of regulating numerous aspects of mRNA function and can therefore influence gene function (14). Overall, translation rates are affected by characteristics of the 5'UTR, including length and start-site consensus sequences, the presence of secondary structures, upstream AUGs, upstream open reading frames (uORFs) and internal ribosome entry sites (14,15). In addition, the 5' UTR can contain sequences that function as binding sites for regulatory proteins.

To date, there have been no studies evaluating mutations in TGFβ2 for CHD patients. In this study, specific SNPs in the TGFβ2 gene were detected in certain Chinese Han TOF children and controls. Also, three single nucleotide changes, including 9126 A>AC, 9353 A>AG and 9040_9043 del CTTC in 5'UTR, were detected. There was a significant difference in the allelic frequencies (χ^2=17.469, P<0.001) of position 9040_9043 between the TOF group and the control group. SNPs at position 9040_9043 del CTTC in 5' UTR of TGFβ2 could be associated with TOF susceptibility. The CTTC allele may be the susceptibility allele for TOF. Due to the significant regulatory function of the 5'UTR, it was speculated that these sequence changes could theoretically affect the TGFβ2 protein structure or function. However, the exact effects of these changes require further study using functional experiments.

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