

DNA sequencing of TGF β 2 in sporadic patients with tetralogy of Fallot

YAN GAO¹, XIAO-JING MA¹, GUO-YING HUANG¹, JING ZHANG¹,
HUI-JUN WANG², DUAN MA² and YAO WU¹

¹Cardiac Center, Children's Hospital of Fudan University, Shanghai 201102;

²Institute of Biomedical Sciences, Fudan University, Shanghai 200032, P.R. China

Received October 19, 2011; Accepted January 19, 2012

DOI: 10.3892/etm.2012.492

Abstract. Transforming growth factor β 2 (TGF β 2) plays an essential role in cardiac morphogenesis. However, the prevalence of TGF β 2 mutations in congenital heart disease (CHD) and the correlation between the TGF β 2 genotype and the CHD phenotype have not been studied extensively. The aim of this study was to examine DNA sequence changes in the TGF β 2 gene in sporadic patients with tetralogy of Fallot (TOF), and to observe whether TGF β 2 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 TGF β 2. The exons and the 5' untranslated region (5'UTR) sequences of the TGF β 2 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 ($\chi^2=17.469$, $P<0.001$) of position 9040_9043 in the 5'UTR between the TOF group and the control group. Our results suggest that TGF β 2 may be a potential candidate gene of TOF. SNPs at position 9040_9043 del CTTC in the 5'UTR of TGF β 2 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 β 2 (TGF β 2) gene knockout animals have a CTD phenotype (3). Targeted disruption of TGF β 2 severely impairs the development of the right ventricle, the outflow tract and the aortic arch arteries, indicating that TGF β 2 is indispensable in the formation of the cranial elements of the developing heart (4). Thus, TGF β 2 is considered to be a candidate gene for conotruncal development. This study reports the findings of a cohort study, which aimed to detect TGF β 2 sequence changes in 100 Chinese TOF children. The results enabled the evaluation of the prevalence of TGF β 2 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

Correspondence to: Dr Guo-Ying Huang, Cardiac Center, Children's Hospital of Fudan University, Shanghai 201102, P.R. China
E-mail: gaohuang2012@126.com

Key words: congenital heart disease, tetralogy of Fallot, transforming growth factor β 2, single-nucleotide polymorphism

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 TGF β 2 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 TGF β 2 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.

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 ($\chi^2=17.469$, $P<0.001$) 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 TGF β s, bone morphogenetic proteins, growth and differentiation factors, activins and Nodal, which are vital for the development and homeostasis of metazoans (5). TGF β superfamily members critically regulate a number of processes within the cardiovas-

Table II. Primers used to amplify the exons (including their flanking intron) and the 5'UTR sequences of the TGF β 2 gene.

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

F, forward primer; R, reverse primer; 5'UTR, 5' untranslated region; TGF β 2, transforming growth factor β 2.

cular system, including cardiac development and angiogenesis. The TGF β 2 gene has been mapped to chromosome 1 in the mouse and the human (6). Its functions are not overlapping with other TGF β subtypes (7).

A crucial function of TGF β ligands during murine heart development was first suggested by conventional knockout mice studies (7). While TGF β 1^{-/-} and TGF β 3^{-/-} mice demonstrated no evident signs of congenital heart defects, TGF β 2^{-/-} 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. TGF β 2 knockout mice display defective myocardialization associated with deregulation of neural crest cell apoptosis (10). It has been demonstrated that TGF β 2 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 TGF β 2 may reduce apoptosis in the endocardial cushions. However, recent studies have revealed that apoptosis in the endocardial cushions in TGF β 2 knockout mice is increased (10). In RXR α ^{-/-} mice, elevated levels of TGF β 2 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

^aPearson χ^2 test, ^bOR=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 TGF β 2 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 TGF β 2 for CHD patients. In this study, specific SNPs in the TGF β 2 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 ($\chi^2=17.469$, $P<0.001$) of position 9040_9043 between the TOF group and the control group. SNPs at position 9040_9043 del CTTC in 5' UTR of TGF β 2 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 TGF β 2 protein structure or function. However, the exact effects of these changes require further study using functional experiments.

Acknowledgements

This study was supported by the Natural Science Foundation of China (30930096) and Basic Research Projects (2010CB529505 and 2009CB941704). We would like to sincerely thank the patients and their families for the support of our study, the members of the Pediatric Heart Center and those of the Institutes of Biomedical Sciences for technical assistance.

References

1. Nemer M: Genetic insights into normal and abnormal heart development. *Cardiovasc Pathol* 17: 48-54, 2008.
2. Srivastava D and Olson EN: A genetic blueprint for cardiac development. *Nature* 407: 221-226, 2000.
3. Loffredo CA, Silbergeld EK, Ferencz C, *et al*: Association of transposition of the great arteries in infants with maternal exposures to herbicides and rodenticides. *Am J Epidemiol* 153: 529-536, 2001.
4. Dunker N and Kriegelstein K: Targeted mutations of transforming growth factor-beta genes reveal important roles in mouse development and adult homeostasis. *Eur J Biochem* 267: 6982-6988, 2000.
5. Feng XH and Derynck R: Specificity and versatility in TGF- β signaling through Smads. *Cell Dev Biol* 21: 659-693, 2005.
6. Barton DE, Foellmer BE, Du J, *et al*: Chromosomal mapping of genes for transforming growth factors beta-2 and beta-3 in man and mouse: dispersion of TGF-beta gene family. *Oncogene Res* 3: 323-331, 1988.
7. Sanford LP, Ormsby I, Gittenberger-de Groot AC, *et al*: TGF β 2 knockout mice have multiple developmental defects that are non-overlapping with other TGF β knockout phenotypes. *Development* 124: 2659-2670, 1997.
8. Proetzel G, Pawlowski SA, Wiles MV, *et al*: Transforming growth factor-beta 3 is required for secondary palate fusion. *Nat Genet* 11: 409-414, 1995.
9. Shull MM, Ormsby I, Kier AB, *et al*: Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. *Nature* 359: 693-699, 1992.
10. Bartram U, Molin DG, Wisse LJ, *et al*: Double-outlet right ventricle and overriding tricuspid valve reflect disturbances of looping, myocardialization, endocardial cushion differentiation, and apoptosis in TGF-beta(2)-knockout mice. *Circulation* 103: 2745-2752, 2001.
11. Azhar M, Schultz JEJ, Grupp I, *et al*: Transforming growth factor beta in cardiovascular development and function. *Cytokine Growth Factor Rev* 14: 391-407, 2003.
12. Kubalak SW, Hutson DR, Scott KK, *et al*: Elevated transforming growth factor beta2 enhances apoptosis and contributes to abnormal outflow tract and aortic sac development in retinoic X receptor alpha knockout embryos. *Development* 129: 733-746, 2002.
13. Hughes TA: Regulation of gene expression by alternative untranslated regions. *Trends Genet* 22: 119-122, 2006.
14. Gray NK and Wickens M: Control of translation initiation in animals. *Annu Rev Cell Dev Biol* 14: 399-458, 1998.
15. Mignone F, Gissi C, Liuni S, *et al*: Untranslated regions of mRNAs. *Genome Biol* 3: REVIEWS0004, 2002.