Homeobox C9 Is Not Potentially Related to Congenital Heart Disease in Chinese Patients

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Background: Congenital heart disease (CHD) is one of the most common human birth defects. The etiology and pathogenesis of CHD are complex and involve several genes as well as multiple changes in signaling pathways. The aim of this study was to identify potential pathological mutations in the Homeobox C9 (*Hoxc9*) gene in 350 Chinese children with CHD to further understand the etiology of CHD. **Method:** Sequence analysis of the *Hoxc9* gene in 350 nonsyndromic patients with CHD. **Result:** We did not identify any nonsynonymous variants in the coding regions of *Hoxc9* in the patients with CHD. We found one synonymous variant c.C564T (p. his188his) in one ventricular septal defect patient. We also identified four previously reported polymorphisms (rs56368105, rs12817092, rs34079606, and rs2241820) in CHD. **Conclusions:** We did not find any diagnostic alterations in the coding regions of *Hoxc9* among the patients with CHD. Nevertheless, to our knowledge, this is the first study of *Hoxc9* in nonsyndromic CHD and has expanded our overall knowledge of the etiology of this disease.

Introduction

CONGENITAL HEART DISEASE (CHD) is a common form of congenital malformation associated with significant morbidity and mortality. The worldwide incidence of CHD is between 8 and 10 per 1000 live births, 6.5 times higher than the rate of chromosomal formation abnormalities and 4 times higher than nerve tube flaw formation rate (Yagel *et al.*, 2009).

Hox genes are major regulators of development and play pivotal roles during adult tissue differentiation (Wang *et al.*, 2009). In mammals, there are 39 *Hox* genes organized into four genomic clusters, designated as groups A–D (Krumlauf, 1994). The *Hoxc* gene cluster was located in a sensitive area that has been identified as the human simple CHD susceptibility region (Gong *et al.*, 2005). The Homeobox C9 (*Hoxc9*) gene spans 3242 bp on human chromosome 12q13.13, comprises 2 exons, and encodes a 260 amino acid protein. The *Hoxc9* gene is required for the development of the neural tube in the mouse embryo, and disruptions in this gene lead to a deformed heart (Shashikant and Ruddle, 1996). Over the last few decades, *SMAD4* has been identified as a CHD-causing

gene (Qi *et al.*, 2007), and *Smad4*-MH1 could occupy one of the DNA binding sites on *Hoxc9*, inhibiting its transcription activity (Zhou *et al.*, 2008). From the results of these investigations, we predicted that *Hoxc9* may play a role in the development of CHD. The aim of our study was to identify potentially pathogenic mutations in *Hoxc9* among 350 Chinese children with CHD and to provide insights into the etiology of the condition.

Materials and Methods

Study population

A total of 350 nonsyndromic patients with CHD were recruited in this study. Informed consent was obtained from their parents or guardians. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committee of the National Research Institute for Family Planning. Clinical assessment was performed on each patient and included anthropometric measurement, physical examination for dysmorphism and malformation, and radiological evaluation. The patients also

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had peripheral blood taken, underwent chest X-ray examination, ultrasonic echocardiogram, and electrocardiogram.

DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes using standard methods. Two exons and nearby introns of *Hoxc9* gene were amplified by polymerase chain reaction using two pairs of *Hoxc9* gene-specific primers. PCR primer sequences were as follows: *Hoxc9* cDNA exon I, forward Primer 5'GGTGACCGGTGCCTTGG 3', reverse Primer 5'GCTCCCTGCACGGTTCG 3', amplified fragment length is 929 bp; exon II forward Primer 5'AACGGTTGGGAATTG GACTT 3', reverse Primer 5' CCTCCTCCTCGCTGGGTA GAAC 3', amplified fragment length is 787 bp. PCR products were sequenced using the BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA) and run on an automated sequencer, ABI 3730XL (Applied Biosystems) to perform mutational analysis.

Results

The clinical and molecular findings in patients with cardiac defects are shown in Table 1 and include ventricular septal defect (VSD), atrial septal defect, patent ductus arteriosus, pulmonary hypertension, tetralogy of Fallot, pulmonary atresia or stenosis, patent foramen ovale, tricuspid regurgitation, and certain other complex cardiac malformations.

The sequence analysis of *Hoxc9* in the 350 nonsyndromic patients with CHD did not identify any nonsynonymous variants in the coding regions. We found one synonymous variant c.C564T (p. his188his) in one VSD patient. In addition, we identified four previously reported polymorphisms in CHD : rs56368105, rs12817092, rs34079606, and rs2241820, two of these being in coding regions.

Discussion

More and more animal studies indicate that the *Hox* family plays an important role in embryogenesis and organ formation of vertebrates and works at different stages of tran-

scription and translation (Krumlauf, 1994; Veraksa *et al.*, 2000). In the past few years, research in *Drosophila* (Lovato *et al.*, 2002), zebra fish (Waxman and Yelon, 2009), and mouse (Martínez *et al.*, 2007) models have found that *Hox* genes were related to the development of the heart. A 3.4 cM candidate region of CHD that is located within 12q13.1~13.3 had shown significant correlation, by transmission disequilibrium test, in 62 nuclear family trios consisting of simple patients with CHD and their parents. There is an important gene cluster in this region—*Hoxc* gene cluster (Gong *et al.*, 2005). *Hoxc9* has been identified in the human blastocyst, where it was related to the homeobox transcription factor of embryonic origin (postzygotic gene activation) (Huntriss *et al.*, 2010). *Hoxc9* is required throughout embryo development for restricting expression of more anterior *Hox* genes, and has an essential role in organizing the motor system through global repressive activities. Genome-wide analysis of *Hoxc9* binding suggests that this mode of repression is mediated by direct interactions with *Hox* regulatory elements, independent of chromatin marks typically associated with repressed *Hox* genes (Jung *et al.*, 2010). In an ENU-induced recessive mouse mutation model with multi-organ abnormalities such as cardiac disorder, axial skeletal abnormalities, and limb malformations, it has been demonstrated that *Hoxc9*, *Hoxc10*, *Hoxd9*, *Hoxd10*, and *Hoxd12* were all significantly reduced (Szumska *et al.*, 2008). The defects may have been caused by the reduced production associated with these genes.

Zhou *et al.* (2008) used three techniques, GST pull-down assays, surface plasmon resonance analysis, and multidimensional nuclear magnetic resonance, to analyze the interaction between the recombinant homeodomain (HD) of *Hoxc9* and the MH1 domain of *Smad4*. He found that *Smad4*-MH1 can interact with the N-terminal part of the HD of *Hoxc9*, which, in turn, inhibits its transcriptional activity. *Smad4* is the main intracellular mediator of TGF- β signaling that has been shown to participate in many physiological and pathological processes, including heart development and heart disease. Recent studies have highlighted the importance of *Smad4* during embryonic cardiac developments (Lan *et al.*, 2007; Qi *et al.*, 2007; Song *et al.*, 2007, 2011) and show that knockout of the *Smad4* gene in mice leads to early embryonic lethality, showing attenuated vascular sprouting, ruptured blood vessels, poor trabeculation, hypoplasia of ventricles, and defects in ventricle morphogenesis. The embryos lacking *Smad4* in cardiomyocytes die between E10.5 and E15.5, and very few embryos were able to survive beyond E12.5. We speculate that there may be cross communication between *Hoxc9* and *Smad4* which affects the early development of the heart. So, we postulated that patients with CHD might exhibit mutations in the *Hoxc9* gene.

Marek *et al.* (2011) in a 21-year study in the Czech Republic reported on 1604 cases of prenatal diagnosis of CHD in the fetus. Termination of pregnancy occurred in 919 cases (57.3%), continuing pregnancy in 685 (42.7%) cases. Of the fetuses for which pregnancy continued, 59 (8.6%) ended in intrauterine fetal death and in 147 (8.6%), the fetus died shortly after birth. Of the 626 babies born alive (39% of those antenatally diagnosed with cardiac abnormalities), another 147 children died later. Thus, only 479 (29.9%) of the prenatally diagnosed 1604 diagnosed fetuses were alive at the end of their study. In addition to the CHD, 149 (31.2%) of these children also showed other structural malformations or chromosomal

TABLE 1. CARDIAC MALFORMATIONS
IN THE PATIENT COHORT

Transposition of the great arteries	1
Double outlet left ventricle	1
Double outlet right ventricle	2
Persistent left superior vena cava	2
Tricuspid insufficiency	6
Ventricular septal defect	133
Atrial septal defect	59
Tetralogy of fallot	19
Coronary artery fistulas	1
Pulmonal atresia or stenosis	6
Pulmonary hypertension	20
Aortic coarctation	1
Patent ductus arteriosus	25
Patent foramen ovale	14
Mitral vlve insufficiency	4
Stenosis of tight ventricular outflow tract	1
Tricuspid regurgitation	13
Secondary mitral regurgitation	3
Other	39

abnormalities. These data show that natural death and abortion account for a high proportion of fetal cardiovascular malformations. In the present study, we screened for potential pathogenic mutations in the *Hoxc9* gene in Chinese children with nonsyndromic CHD, but did not find any diagnostic mutations in the coding regions. These results could be explained in two ways. First, our blood samples were derived from living patients and it may be that, similar to *Smad4* mutations, any mutations in the coding sequence of *Hoxc9*, and the resultant early cardiac defects they cause, will be lethal. Mutations of the *Hoxc9* may also be accompanied by other important organ deformities such as motor system defects, which will greatly reduce the survival of the fetus. Second, it may simply be that there is very little relationship between the *Hoxc9* genes and the risk of CHD.

In conclusion, our understanding of the functions of the *Hoxc9* gene is still very limited, and its exact role in heart development is still unknown. Further studies will be required to determine its full role, if any, in the development of the early embryonic heart.

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Author's Contributions

Lei Sun performed the experiment and drafted the article. Longfei Cheng participated in its design and coordination and helped draft the article. Binbin Wang participated in revision of the article. Jing Wang carried out the molecular genetic studies. Congmin Li, Bingren Gao, Xiaochen Wang, and Tianchu Huang participated in samples collection. Hui Li and Xu Ma conceived of the study, participated in its design and coordination, and helped draft the article.

Disclosure Statement

The authors declare that no competing financial interests exist.

References

- Gong LG, Qiu GR, Jiang H, *et al.* (2005) Analysis of single nucleotide polymorphisms and haplotypes in *HOXC* gene cluster within susceptible region 12q13 of simple congenital heart disease. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi* 22:497–501.
- Huntriss J, Hinkins M, Picton HM (2010) cDNA cloning and expression of the human *NOBOX* gene in oocytes and ovarian follicles. *Neuron* 67:781–796.
- Jung H, Lacombe J, Mazzoni EO, *et al.* (2010) Global control of motor neuron topography mediated by the repressive actions of a single *Hox* gene. *Neuron* 67:781–796.
- Krumlauf R (1994) *Hox* genes in vertebrate development. *Cell* 78:191–201.
- Lan Y, Liu B, Yao H, *et al.* (2007) Essential role of endothelial *Smad4* in vascular remodeling and integrity. *Mol Cell Biol* 27:7683–7692.

- Lovato TL, Nguyen TP, Molina MR, *et al.* (2002) The *Hox* gene abdominal-A specifies heart cell fate in the *Drosophila* dorsal vessel. *Development* 129:5019–5027.
- Marek J, Tomek V, Skovránek J, *et al.* (2011) Prenatal ultrasound screening of congenital heart disease in an unselected national population: a 21-year experience. *Heart* 97:124–130.
- Martínez L, Martínez-Calonge W, Matesanz R, *et al.* (2007) The etiology of congenital diaphragmatic hernia and esophageal atresia: the *Hox* genes. *Cir Pediatr* 20:223–228.
- Qi X, Yang G, Yang L, *et al.* (2007) Essential role of *Smad4* in maintaining cardiomyocyte proliferation during murine embryonic heart development. *Dev Biol* 311:136–146.
- Shashikant CS, Ruddle FH (1996) Combinations of closely situated cis-acting elements determine tissue-specific patterns and anterior extent of early *Hoxc8* expression. *Proc Natl Acad Sci U S A* 93:12364–12369.
- Song L, Yan W, Chen X, *et al.* (2007) Myocardial *Smad4* is essential for cardiogenesis in mouse embryos. *Circ Res* 101:277–285.
- Song L, Zhao M, Wu B, *et al.* (2011) Cell autonomous requirement of endocardial *Smad4* during atrioventricular cushion development in mouse embryos. *Dev Dyn* 240:211–220.
- Szumaska D, Pieles G, Essalmani R, *et al.* (2008) VACTERL/caudal regression/Currarino syndrome-like malformations in mice with mutation in the proprotein convertase *Pcsk5*. *Genes Dev* 22:1465–1477.
- Veraksa A, Campo MD, McGinnis W (2000) Developmental patterning genes and their conserved functions: from model organisms to humans. *Mol Genet Metab* 69:85–100.
- Wang KC, Helms JA, Chang HY (2009) Regeneration, repair and remembering identity: the three Rs of *Hox* gene expression. *Trends Cell Biol* 19:268–275.
- Waxman JS, Yelon D (2009) Increased *Hox* activity mimics the teratogenic effects of excess retinoic acid signaling. *Dev Dyn* 238:1207–1213.
- Yagel S, Silverman NH, Gembrum U (2009) *Fetal Cardiology*. Infirmary Healthcare, New York, pp 101–110, 305–328.
- Zhou B, Chen L, Wu X, *et al.* (2008) MH1 domain of *SMAD4* binds N-terminal residues of the homeodomain of *Hoxc9*. *Biochim Biophys Acta* 1784:747–752.

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