DNA hypermethylation of the *NOX5* gene in fetal ventricular septal defect

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Abstract. Ventricular septal defect (VSD) is the most comon form of congenital heart disease (CHD). DNA hypermethylation analysis may provide an insight into the molecular features and pathogenesis of this heart disease. Although aberrant DNA hypermethylation is implicated in the pathophysiology of this heart disease, only a limited number of genes are known to be epigenetically altered in VSD. We previously identified regulation of the *NOX5* gene by hypermethylation in VSD fetuses by promoter methylation microarrays. This study was designed to detect the expression of *NOX5* mRNA in VSD and normal fetuses. We also verified the results of promoter methylation microarrays by methylation-specific PCR. DNA extraction and nested methylation-specific PCR were performed on myocardial tissue samples from 21 VSD and 15 normal fetuses. The primers specific for methylated vs. unmethylated DNA were designed and amplified by nested PCR. The products were visualized on agarose gel. Hypermethylation of the *NOX5* promoter was more frequent in VSD fetuses (66.67%) than in normal fetuses (20%). There was a significant concordance between *NOX5* methylation and a decrease in its mRNA expression. Taken together, our results demonstrate that hypermethylation of the *NOX5* gene may be involved in the pathogenesis of VSD.

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Introduction

Congenital heart diseases (CHDs) are common congenital malformations. They are caused by abnormalities in the embryonic heart and vasculature. The reported incidence of CHDs varies between 12 and 14 per 1,000 live births (1). In China, approximately 100,000 neonates are born with CHDs every year. Only one-fifth of these neonates obtain treatment in the approximately 300 units that perform cardiac surgery (2). Ventricular septal defect (VSD) is the most common form of CHD. Currently, even though many genes related to cardiac development have been identified and correction by surgery has yielded favorable results as the main treatment option, the etiology and pathological mechanisms of the disease are unknown.

The NADPH oxidase (NOX) family consists of the homologs of $gp91^{phox}$ (glycosylated subunit of phagocyte NADPH oxidase flavocytochrome); this family includes the *NOX1*, *NOX2*, *NOX3*, *NOX4* and *NOX5* genes. ROS are derived by the NOX family as secondary messenger molecules that participate in cell proliferation, transformation, differentiation and apoptosis (3-5). The embryonic development of the ventricular septum involves a balance between various cell proliferation, differentiation and apoptosis processes. *NOX5* is a highly expressed embryonic gene, which may be involved in this process.

Epigenetic mechanisms, such as miRNA expression and histone modification, are crucially responsible for dysregulated gene expression in CHD (6). By contrast, the role of DNA methylation remains unknown. DNA methylation is catalyzed by DNA methyltransferase and involves the addition of a methyl group to the carbon-5 position of the cytosine ring converting it to methyl cytosine (7), which is another well-characterized epigenetic mark. DNA methylation plays an important role during embryogenesis, normal mammalian development, cellular differentiation and chromosome integrity. *NOX5*, a promoter hypermethylation gene, was found in our laboratory by promoter methylation microarrays (8). The aim of this study was to verify the results of promoter methylation microarrays and determine whether there is concordance between *NOX5* methylation and a decline in its mRNA expression in VSD and normal fetuses.

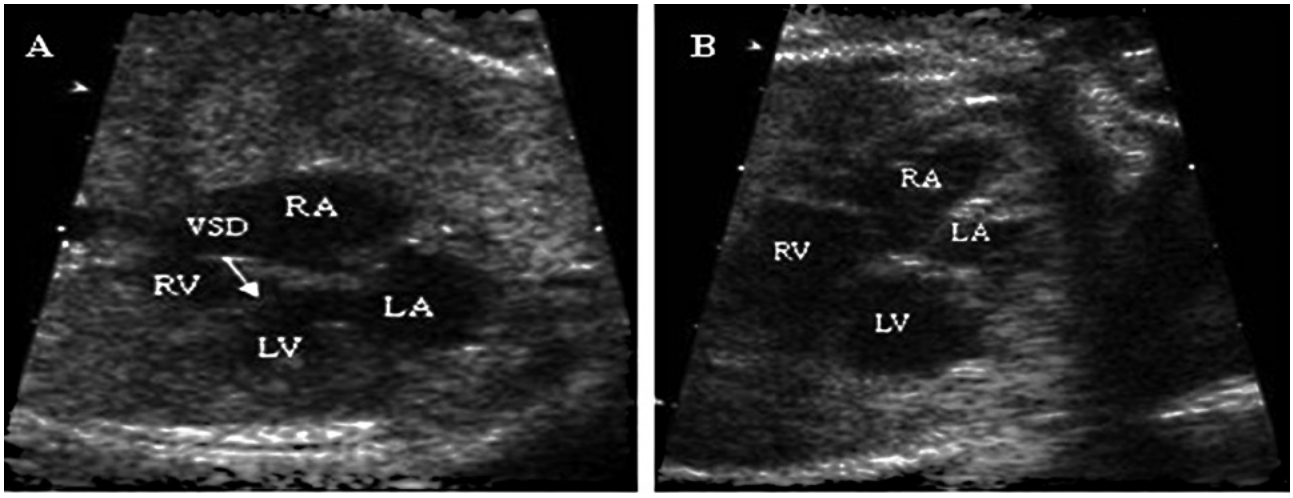

Figure 1. Two-dimensional echocardiographic image of a fetus at 26 weeks of gestation. (A) VSD and (B) normal images show disruption of the interventricular septum of VSD in the four-chamber view. RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle.

Table I. Primer sequences for the methylated (M) and unmethylated (U) *NOX5* gene.

Primers		Sequences	Temperature (˚C)
First cycle	M forward	5'-TATAGGGATCGCGTTTAAATTAC-3'	57
	M reverse	5'-ACTAAAAACTTCATAAACGTCGTC-3'	
	U forward	5'-TTTTATAGGGATTGTGTTTAAATTAT-3'	
	U reverse	5'-AAACTAAAAACTTCATAAACATCATCC-3'	
Second cycle	M forward	5'-TATAGGGATCGCGTTTAAATTAC-3'	55
	M reverse	5'-TATCAACGAAATACCGTCCTAC-3'	
	U forward	5'-TTTTATAGGGATTGTGTTTAAATTAT-3'	
	U reverse	5'-TATCAACAAAATACCATCCTACCTC-3'	

Materials and methods

Tissues and DNA. Fetal myocardial tissue samples were obtained from the Nanjing Maternal and Child Health Hospital. The representative four-chamber view of VSD is shown in Fig. 1. Thirty-six fetuses at 26 weeks of gestation were obtained during surgery for termination of pregnancy owing to trauma of the pregnant women. All samples were collected with the approval of the ethics committee of the appropriate institute, and written consent was provided by each pregnant woman and her family. The specimens were snap frozen in liquid nitrogen immediately and then stored at -80˚C until analysis. Genomic DNA was purified from tissue specimens using the conventional proteinase K digestion and phenol/chloroform extraction method. After purification, genomic DNA was treated with sodium bisulfite. The treatment converts unmethylated cytosines to uracils, while leaving the methylated cytosines unaffected (9).

Methylation-specific PCR. The *NOX5* gene sequence was obtained from GenBank (Gene ID 79400). Methyl Primer Express® Software was used to design the primer of methylation-specific PCR. The primer designed for the unmethylated sequences does not amplify the methylated sequence and vice versa. Bisulphite-modified DNA was amplified by nested PCR using the primer sets described in Table I. Primers were purchased from Invitrogen (Carlsbad, CA, USA). Myocardial tissue DNA samples, untreated or methylated *in vitro* by excess CpG (Sss.I) methyltransferase (NEB, USA), were used as positive controls for unmethylated and methylated DNA, respectively. Distilled water was used as a negative control. The first cycle of nested PCR was carried out in a final volume of 25 μl, containing 2.5 μl 10X buffer, 2.5 μl 10 mM dNTP mixture, 2.0 μl 50 mM $MgCl_2$, 1.0 μl of each of the primers, 0.25 μl (5 U/μl) *Taq* DNA polymerase and 2.0 μl DNA sample containing 10 ng DNA. The amplification conditions were as follows: an initial incubation at 94˚C for 2 min, followed by 36 cycles at 94˚C for 30 sec, 57˚C for 30 sec and 72˚C for 45 sec; and a final extension at 72˚C for 7 min. A 20-fold dilution of the product from the first cycle was the template of the second cycle, while the other components were similar. The amplification conditions for the second cycle were as follows: an initial incubation at 94˚C for 2 min, followed by 36 cycles at 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 45 sec; and a final extension at 72˚C for 7 min. PCR products were separated in a 1.5% agarose gel, stained with ethidium bromide and visualized under UV illumination. Each experiment was repeated at least three times.

Table II. Primer sequences for RT-PCR.

Gene		Primers	Temperature (˚C)
NOX5	Forward	5'-AAGACTCCATCACGGGGCTGCA-3'	65
	Reverse	5'-CCCTTCAGCACCTTGGCCAGAG-3'	
GAPDH	Forward	5'-CCATGTTCGTCATGGGTGTGAACCA-3'	60
	Reverse	5'-GCCAGTAGAGGCAGGGATGATGTTC-3'	

Table III. Hypermethylation status in the two groups.

Study group	No.	Hypermethylated cases	Hypermethylation yes (%)
VSD	21	14	66.67
Controls	15	3	20.00

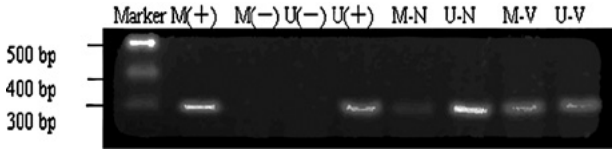

Figure 2. Representative results of the methylation-specific PCR analyses of myocardial DNA samples from normal and VSD fetuses. The PCR products in the lanes show the presence of methylated (M) and unmethylated (U) templates of the *NOX5* gene, respectively. The lanes marked N and V contain the control and VSD templates, respectively. Marker, 100-bp DNA ladder; M(+), methylated positive control; U(+), unmethylated positive control; M(-) and U(-), negative controls.

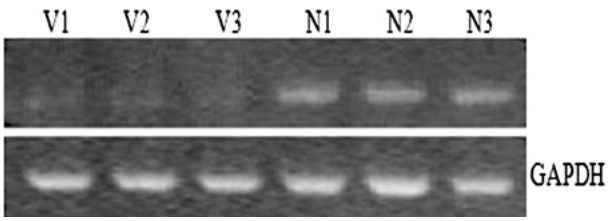

Figure 3. Expression of *NOX5* by RT-PCR. Representative results are expressed as the ratio between the intensity of bands corresponding to the gene studied vs. GAPDH. The lanes marked N and V contain the control and VSD templates, respectively.

RT-PCR. Total RNA from the myocardial tissue samples was extracted using the TRIzol method (Invitrogen). cDNA was synthesized from 1 μg of total RNA using an AMV Reverse Transcriptase kit (Promega A3500; Promega, Madison, WI, USA). An aliquot (10%) of the resulting cDNA was amplified for PCR with the primers listed in Table II. The number of cycles and reaction temperatures used in the PCR assay were optimized to provide a linear relationship between the amount of input template and the amount of PCR product.

Statistical analysis. Each experiment was performed at least three times. For the analysis, data were classified using Fisher's exact test and Student's t-test. A P-value <0.05 (2-sided) was regarded as statistically significant. All data were analyzed with SPSS 13.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results

Hypermethylation of the NOX5 promoter in VSD fetuses. The results of the methylation status analysis of the CpG islands in the *NOX5* promoter region in the myocardial tissue of normal and VSD fetuses are shown in Fig. 2. The *NOX5* promoter was hypermethylated in 66.67% of the VSD fetuses and in only 20% of the normal fetuses (Table III). Statistical analysis revealed that promoter hypermethylation of the *NOX5* gene was strongly correlated with the study groups (P=0.008).

Expression of NOX5 gene mRNA in fetal myocardial tissue. The expression levels of *NOX5* in fetal myocardial tissue samples were measured by RT-PCR. Fig. 3 shows that the expression levels of *NOX5* were much higher in the controls than in the VSD fetuses. The result indicates that there is a significant association between low expression of *NOX5* with hypermethylation of the gene in VSD.

Discussion

DNA methylation is the main epigenetic modification in mammals and particularly in humans. The most striking feature of vertebrate DNA methylation patterns is the presence of CpG islands. Earlier studies estimated that ~60% of human genes are associated with CpG islands (10). Many studies of DNA methylation have been carried out in mammalian systems, in which genomic DNA methylation is found throughout the genome (11). Thus, DNA methylation provides information as to where and when the gene should be expressed, but does not alter the structure or function of a gene (12). DNA methylation patterns are required for normal embryonic development. The DNA methyltransferases *DNMT1*, *DNMT3A* and *DNMT3B* cooperatively regulate cytosine methylation in CpG dinucleotides in mammalian genomes (13); both Dnmt3a and Dnmt3b function as *de novo* methyltransferases that play important roles in normal development (14). Furthermore, the DNA methylation levels vary throughout the mammalian developmental process (15).

It has been recognized that environmental and genetic factors play important roles in VSD, as in other CHDs. However, recent studies have shown that CHD caused by single gene or single locus defects is more common than expected (16). The interaction between histone de-acetylation and DNA methylation existing between human end-stage cardiomyopathic and

heart failure has already been established (17). Most recent studies have focused on the crucial role of the NOX family in cardiac pathophysiology. In this respect, it has been shown that there is increased myocardial NOX family activity in the failing heart (18). Expression of *NOX2* has been demonstrated in human cardiomyocytes and was shown to be up-regulated during myocardial infarction (19).

NOX5 was the last discovered gene in the NOX family and it is highly divergent from other members of the family. Evolutionary tree analysis has revealed that *NOX5* may represent the gene which is closest to primordial NOX (20), and the gene is unique as it contains EF hand domains in the N-terminal region that bind calcium and permit activation of the enzyme by an increase in intracellular calcium (21). In prior studies, *NOX5* expression has been detected within blood vessels of the spleen and lung and also in coronary blood vessels (22,23).

In blood vessels from individuals without coronary artery disease, *NOX5* expression is very low, but it is substantially increased in blood vessels of individuals with the disease (24). In this study, we found that *NOX5* promoter hypermethylation occurred more often in VSD myocardial tissue by methylation-specific PCR. There was a significant concordance between *NOX5* methylation and a decline in its mRNA expression. Thus, the low expression of *NOX5* in individuals without coronary artery disease may account for the promoter hypomethylation of the gene. *NOX5* promoter hypermethylation in VSD myocardial tissue contributes to transcriptional silencing of the gene. The gene silencing leads to abnormal reduction in ROS production. ROS, as a signaling substance, stimulates the proliferation of mammalian cells. However, the formation of VSD is an extremely complex pathological process, which involves cell proliferation and differentiation during embryogenesis as well as apoptosis (25). *NOX5* silencing, which leads to reduction in ROS, may be involved in the pathological process of fetal VSD. On the other hand, in rodents, *NOX1* and *NOX2* are the primary isoforms expressed in the spleen (26), whereas in humans *NOX5* was initially characterized as a gene that is highly expressed in the testis, spleen and lymph nodes; in lymphocytes, this gene may participate in calcium signaling, proliferation, differentiation and apoptosis (21). The immunological profile in CHD children, including levels of IgG and IgA and complement components C3 and C4, was found to be significantly impaired in all children with CHD; T-helper cells were decreased and T-suppressor cells were increased in all groups with CHD as compared to controls (27). The B-cell percentage was increased in cyanotic children, but was not affected in acyanotic children (27). Thus, another possibility is that *NOX5* gene silencing in VSD fetuses may result in immune dysfunction and immunoregulatory disorders.

In summary, hypermethylation of the *NOX5* promoter was detected in 66.67% of the VSD fetuses using methylation-specific PCR. Our findings indicate that a high frequency of methylation of the *NOX5* gene promoter is an important mechanism for *NOX5* inactivation in VSD and normal fetuses.

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