Characterization of Transcriptional Repressor Gene MSX1 Variations for Possible Associations with Congenital Heart Diseases

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Abstract

Background

The human heart consists of several cell types with distinct lineage origins. Interactions between these cardiac progenitors are very important for heart formation. The muscle segment homeobox gene family plays a key role in the cell morphogenesis and growth, controlled cellular proliferation, differentiation, and apoptosis, but the relationships between the genetic abnormalities and CHD phenotypes still remain largely unknown. The aim of this work was to evaluate variations in MSX1 and MSX2 for their possible associations with CHD.

Methods

We sequenced the MSX1 and MSX2 genes for 300 Chinese Han CHD patients and 400 normal controls and identified the variations. The statistical analyses were conducted using Chi-Square Tests as implemented in SPSS (version 19.0). The Hardy-Weinberg equilibrium test of the population was carried out using the online software OEGE.

Results

Six variations rs4647952, rs2048152, rs4242182, rs61739543, rs111542301 and rs3087539 were identified in the MSX2 gene, but the genetic heterozygosity of those SNPs was very low. In contrast, the genetic heterozygosity of two variations rs3821949 near the 5’UTR and rs12532 within 3’UTR of the MSX1 gene was considerably high. Statistical analyses showed that rs3821949 and rs12532 were associated with the risk of CHD (specifically VSD).
Conclusions

The SNPs rs3821949 and rs12532 in the MSX1 gene were associated with CHD in Chinese Han populations.

Introduction

Congenital heart diseases (CHD) are a group of complex congenital anatomic malformations worldwide with high morbidity and mortality. The incidence of the illness is about 7.5% in newborns [1] and 1% of the patients required clinical intervention [2]. There are numerous types of this disease, including ventricular septal defect, pulmonary stenosis, tetralogy of Fallot, patent ductus arteriosus, mitral valve insufficiency, etc. [3], which are often complicated with arrhythmias and heart failure [4]. So far, many gene mutations and chromosomal variants have been identified in familiar and sporadic CHD cases [5–7]. However, the relationships between those genetic abnormalities and CHD phenotypes still remain largely unknown.

The mammalian heart is a complex and also one of the first formed organs during embryogenesis [3], and the formation is strictly regulated and controlled by gene regulatory networks, consisting of signaling pathways, transcription factors, epigenetic factors and miRNAs [8, 9]. Among the regulatory networks, the Nodal/TGF-β signaling pathway has a key role in early stages of human embryonic stem (HES) cell differentiation, directing the cells to develop into different embryonic lineages. Any malfunctions in the pathway may lead to errors in the transformation of the embryonic lineages [10–12]. For example, defects in the transforming growth factors LEFTY in the Nodal/TGF-β signaling pathway may affect the signaling of NODAL and TGF-β[3, 13]. In a previous study, we found that single nucleotide polymorphisms (SNPs) of the Lefty genes are associated with the risk of CHD [3]. The Nodal gene in the Nodal/TGF-β signaling pathway can initiate a series of signal transduction events in the later stages of embryonic development [13, 14]. However, no variations in the Nodal gene so far have been associated with the risk of CHD [3]. Therefore, it is not clear whether it is the Nodal/TGF-β signaling pathway or only LEFTY that is associated with the risk of CHD. Additionally, SMAD3, an intracellular regulating factor in the Nodal/TGF-β signaling pathway to modulate the transcription of many genes [15, 16], together with LEFTY plays central roles in the signaling pathway [15, 17]. Our previous work has demonstrated that the variant rs2289263 before 5’UTR of the SMAD3 gene is associated with increased risk of VSD in the Chinese Han population [18]. Additionally, as the process of HES cell differentiation during embryonic development is very important for the heart development, it may also be involved in the pathogenesis of CHDs.

During embryonic development, HES cells differentiate to various cell types of ectoderm, endoderm and mesoderm, and the cardiomyocytes are generated and differentiated in the mesoderm [19]. As the heart consists of several cell types with distinct lineage origins [20], such as myocardium cells, cardiac neural crest (NC) cells, aortico-pulmonary septum cells and membranous ventricular septum cells, etc. [21], interactions between these cardiac progenitors are very important for the cardiac development, and any mistakes may result in congenital heart malformations [1]. The muscle segment homeobox gene family is an important transcriptional regulator during embryonic development and has an important role in cell morphogenesis and growth [22]. Muscle segment homeobox 1 (MSX1) and Muscle segment homeobox 2 (MSX2) are members of the muscle segment homeobox gene family that encode transcription factors, playing important roles in the organogenesis and tissue–tissue interactions during vertebrate embryonic development [23], and mutations in MSX1 or MSX2 have
been associated with impaired development of cranial neural crest-derived structures, oral clefts, and nonsyndromic oligodontia [23–27]. Studies with animal models also identified MSX1 and MSX2 double mutants in a broad range of heart malformations, such as tetralogy of Fallot and persistent truncus arteriosus [20, 23].

In this study, we analyzed the transcribed regions and splicing sites of the MSX1 and MSX2 genes and compared the sequences between 300 Chinese Han CHD patients and 400 controls to validate the possible associations of MSX1 and MSX2 with CHDs. We found that variations rs3821949 near the 5’UTR and rs12532 within the 3’UTR of the MSX1 gene were closely associated with the risk of CHD (specifically, VSD).

Results

Patients

Clinical diagnosis of all recruited members was confirmed at the Fourth or the Second Affiliated Hospitals of Harbin Medical University. The CHD patients had no history or manifestations of any other systemic abnormalities. We also established that their mothers did not take medicines or attract infections during gestation, because these factors had been found to be associated with heart malformation in pregnancy [28, 29].

A total of 300 CHD patients (male 136, female 164, the min and max age were 0.2 and 61.0 respectively, and the average age was 15.47 years) and 400 unrelated controls (male 173, female 227, the min and max age were 0.3 and 60.0 respectively, and the average age was 13.68 years) were recruited for this study, and there was no statistical differences of the gender composition and age between the two groups (Table 1). The 300 CHD patients contained 128 with ventricular septal defects (VSD), 107 with atrial septal defects (ASD), 44 with patent ductus arteriosus (PDA), 6 with tetralogy of Fallot, 4 with pulmonary stenosis, and 11 with other types of congenital heart defects.

MSX1 and MSX2 gene analysis

We sequenced the MSX1 and MSX2 genes to test the hypothesis that germline common genetic variants in MSX1 or MSX2 may confer susceptibility to CHD. Upon analyzing the transcribed regions and splicing sites of MSX1 and MSX2, we identified rs4242182, rs61739543 and rs111542301 within the translated region, and rs2048152 within an intron, and rs4647952 and rs3087539 within 5’UTR and 3’UTR respectively of the MSX2 gene, but the genetic heterozygosity of all these SNPs loci was low. On the other hand, we identified rs3821949 near 5’UTR and rs12532 within 3’UTR of the MSX1 gene, and the genetic heterozygosity of the two SNPs was considerably high (Fig 1).

Table 1. Clinical characteristics and analysis of the study population.

| Parameter       | CHD     | Control | F  | t   | P    | 95%CI-Up | 95%CI-Low |
|-----------------|---------|---------|----|-----|------|----------|-----------|
| Sample (n)      | 300     | 400     | -  | -   | -    | -        | -         |
| Male/Female (n) | 136/164 | 173/227 | -  | -   | 0.583| -        | -         |
| Age (years)     | 15.47±17.47 | 13.68±10.22 | 147.419 | -1.579 | 0.115 | -4.00676 | 0.43695  |

Data are shown as mean±SD; between the two groups, there were no statistical differences of the age and gender composition.
SNP rs3821949 and rs12532 genotyping statistical analysis

To test possible associations between MSX1 and CHD, we conducted SNP analyses and found that both rs3821949 and rs12532 were associated with the risk of CHD (specifically VSD) in Chinese Han population (Tables 2 and 3). The Hardy-Weinberg equilibrium test for the CHD and controls were conducted and it was in line with the equilibrium.

Discussion

In this study, we analyzed the transcribed regions and splicing sites of the MSX1 and MSX2 genes in a large cohort of CHD patients and controls, and found that the variations rs3821949 and rs12532 in the MSX1 gene were associated with the risk of CHD in the Chinese Han population, demonstrating the involvement of the MSX1 gene in the CHD etiology.

The mammalian heart is a complex organ, starting to form in the mesoderm 18 or 19 days after fertilization, and many genes with strict temporal, spatial, and sequential expression are involved in the formation [3]. The genes MSX1 and MSX2 encode the transcription factors that play key roles in the survival and differentiation of secondary heart field precursors [20, 30].
MSX1 and MSX2 double mutants in mice show malposed, elongated or spiral rotated heart outflow tract [20, 31, 32], and MSX1 and MSX2 null mutants in embryos can increase apoptosis in the secondary heart field, hence leading to many heart malformations [33]. Mutations in MSX1 and MSX2 may perturb the differentiation of heart field precursors and myocardial cells in the heart outflow tract [34–36], while mice with mutation in only MSX1 or MSX2 did not show obvious cardiac defects [24, 25], suggesting that there may be some complementary roles of the MSX1 and MSX2 transcription factors in heart development. However, in this study, the genetic heterozygosity of the SNPs located within the MSX2 gene was very low in the Chinese Han population.

LEFTY and SMAD3 play important roles in the Nodal/TGF-Lefty signaling pathway [3, 13, 15–18] and we have previously found that SNPs in Lefty and SMAD3 genes are associated with CHD.

### Table 2. The genotype and allele frequency of SNP rs3821949 and rs12532 in 300 Chinese Han CHD patients and 400 non-CHD controls.

| SNP          | Group | Genotype frequency (%) | Allele frequency (%) |
|--------------|-------|------------------------|----------------------|
| **rs3821949**|       |                        |                      |
|              | Genotype | CHD 300 | VSD 128 | ASD 107 | Controls 400 |
|              | G/G      | 189(63.0) | 81(63.3) | 63(58.9) | 292(73.0) |
|              | G/A      | 74(24.7)  | 31(24.2) | 29(27.1) | 72(18.0)  |
|              | A/A      | 37(12.3)  | 16(12.5) | 15(14.0) | 36(9.0)   |
|              | G        | 452(75.3) | 193(75.4) | 155(72.4) | 656(82.0) |
|              | A        | 148(24.7) | 63(24.6)  | 59(27.6)  | 144(18.0) |
|              | **rs12532** |       |       |       |                      |
|              | Genotype | CHD 300 | VSD 128 | ASD 107 | Controls 400 |
|              | G/G      | 121(40.3) | 55(43.0) | 31(29.0) | 126(31.5) |
|              | G/A      | 139(46.3) | 62(48.4) | 64(59.8) | 219(54.8) |
|              | A/A      | 40(13.3)  | 11(8.6)  | 12(11.2) | 55(13.8)  |
|              | G        | 381(63.5) | 172(67.2) | 126(58.9) | 471(58.9) |
|              | A        | 219(36.5) | 84(32.8)  | 88(41.1)  | 329(41.1) |

### Table 3. SNP rs3821949 and rs12532 within MSX1 gene associated with the risk of congenital heart diseases in Chinese populations.

| Genotyped SNP | Disease Type | Statistical Types | Value | Min counta | df | Asymp. Sig. (2-sided) | Value | Asymp. Std. errorb | Approx. Tc | Approx. Sig. |
|---------------|--------------|-------------------|-------|-------------|----|----------------------|-------|-------------------|----------|-------------|
| **rs3821949** | CHD-Control  | Genotype          | 7.974 | 31.29       | 2  | 0.019                | -0.098| 0.038             | -2.606   | 0.009d      |
|               |              | Allele            | 9.231 | 125.14      | 1  | 0.002                | -0.081| 0.027             | -3.046   | 0.002d      |
|               | VSD-Control  | Genotype          | 4.425 | 12.61       | 2  | 0.109                | -0.086| 0.045             | -1.976   | 0.049d      |
|               |              | Allele            | 5.376 | 50.18       | 1  | 0.020                | -0.071| 0.032             | -2.322   | 0.020d      |
|               | ASD-Control  | Genotype          | 8.029 | 10.76       | 2  | 0.018                | -0.118| 0.047             | -2.661   | 0.008d      |
|               |              | Allele            | 9.657 | 42.84       | 1  | 0.002                | -0.098| 0.034             | -3.119   | 0.002d      |
| **rs12532**   | CHD-Control  | Genotype          | 6.187 | 40.71       | 2  | 0.045                | 0.069 | 0.038             | 1.825    | 0.068d      |
|               |              | Allele            | 3.079 | 234.86      | 1  | 0.079                | 0.047 | 0.027             | 1.755    | 0.079d      |
|               | VSD-Control  | Genotype          | 6.509 | 16.00       | 2  | 0.039                | 0.110 | 0.042             | 2.536    | 0.012d      |
|               |              | Allele            | 5.627 | 100.12      | 1  | 0.018                | 0.073 | 0.030             | 2.376    | 0.018d      |
|               | ASD-Control  | Genotype          | 0.972 | 14.14       | 2  | 0.615                | 0.000 | 0.043             | 0.001    | 0.999d      |
|               |              | Allele            | 0.000 | 88.01       | 1  | 0.999                | 0.000 | 0.031             | 0.001    | 0.999d      |

a: The minimum expected count
b: Not assuming the null hypothesis
c: Using the asymptotic standard error assuming the null hypothesis
d: Based on normal approximation

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the risk of CHD [3, 18]. Normal functions of the Nodal/TGF-β signaling pathway are very important for the early stages of HES cell differentiation into different embryonic lineages [10, 12]. During embryonic development the HES cells differentiate to various cell types including cardiomyocytes in the mesoderm [19]. The heart consists of several cell types with distinct lineage origins [20, 21], and interactions between these cells are very important for the cardiac development [1]. The muscle segment homeobox gene family controls cell morphogenesis, growth, proliferation, differentiation, and apoptosis during embryonic development [22]. So the differentiation of HES cells and cardiac progenitor cell interactions during embryonic development are important for the heart development and any defects or mistakes may cause CHDs.

MSX1 seems to be an active gene in the HES cells as implicated by its up-regulated expression when the HES cells are co-cultured with PA6 cells [37], and, conversely, the expression levels of MSX1 become lower when the HES cells are treated with dopamine [38]. In a genome-wide methylation-gene expression study between the epigenetic modifications of retinoic acid treated and undifferentiated HES cells, the author uncovered 166 differentially methylated CpG sites and 2,013 differentially expressed genes, in which 19 genes including MSX1 are highly correlated with each other [39].

The 3'UTR and 5'UTR sequences play important roles in regulating the expression of genes [40] [41]. Of great significance, variations within 3'UTR may be associated with human tumor-ogenesis and survival of the patient [42, 43]. The 5'UTR region of gene is a structural complex, which can bind with some miRNAs and may be involved in gene expression, protein translation and disease pathogenesis [44, 45]. In a recently study, we demonstrated that the variant rs2289263 before the 5'UTR of SMAD3 gene is associated with increased risk of VSD in the Chinese Han population [18]. Probably, the SNPs in 3'UTR or 5'UTR may affect the binding of the untranslated regions with regulatory factors such as miRNAs and eventually hamper the gene expression and function. Findings in this study with rs3821949 and rs12532 of the MSX1 gene have updated our understanding on 5'UTR and r 3'UTR and may lead to new insights into the pathogenesis of CHDs.

**Materials and Methods**

**The study population**

From the Fourth and the Second Affiliated Hospitals of Harbin Medical University, Harbin, China, we collected specimens of 300 CHD patients and 400 normal controls for this study (Table 1); specimens of 90 of the 300 CHD patients were overlapping with those used for another study [3, 18]. All the CHD patients and normal controls were given comprehensive physical examination, electrocardiogram and ultrasonic echocardiogram examinations. None of the patients showed any other cardiac or systematic abnormalities, and the normal controls did not show any defects in the heart or other body parts. For this work, we obtained a written informed consent from each participant or their parents on behalf of minors, and the Ethics Committee of the Harbin Medical University approved this work, consistent with the 1975 Declaration of Helsinki.

**DNA analysis**

Using standard protocols, we extracted the genomic DNA from the peripheral blood leukocytes of the participants. The human MSX1 and MSX2 genes each consist of two exons located on 6p16.2 and 5q35.2, respectively. To determine the SNP genotypes in the genes, we amplified
the four exons and splicing sites of the genes using polymerase chain reaction (PCR) method, and sequenced the products using standard protocols [46]. After that, the genotypes of the SNP were determined using PCR and gene sequencing methods [3].

Rs3821949 and rs12532 SNP genotyping analysis and Statistical methods

We determined genotypes of the rs3821949, rs12532 and rs4647952, rs2048152, rs4242182, rs61739543, rs111542301, rs3087539 in the MSX1 and MSX2 genes (Fig 2), and all the measurements were conducted by two independent researchers (Table 4). And then overall CHD meta-analysis was conducted according to the types of CHD and sample sizes.

The continuous variable (measurement data, such as age) statistical analyses were conducted using independent-samples T test and the discrete variable (enumeration data, such as gender composition and genotype frequency) statistical analyses were conducted using Chi-Square Tests to calculate odds ratios and P value as implemented in SPSS (version 19.0). P values less than 0.05 were considered statistically significant. The Hardy-Weinberg equilibrium test of the CHD and control population was conducted with the online software OEGE.
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Ethics approval: Ethics Committee of Harbin Medical University.

Author Contributions

Conceived and designed the experiments: FFL SLL. Performed the experiments: YH SS XL XDZ JZ QLS. Analyzed the data: FFL SLL YH SS XL. Contributed reagents/materials/analysis tools: YH SS XQL. Wrote the paper: FFL SLL. Specimen collection: YH SS XQL.

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