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Identification of Two Novel Mutations of the *HOMEZ* Gene in Chinese Patients with Isolated Ventricular Septal Defect

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Objectives: Ventricular septal defect (VSD) is the most common congenital heart disease (CHD). Genome-wide linkage analysis revealed a potential CHD susceptibility locus in the homeodomain leucine zipper-encoding (*HOMEZ*) gene in a South Indian population. The present study aimed to identify potential pathogenic mutations for *HOMEZ* and to provide insights into the etiology of isolated VSD in the Chinese population. *Methods:* Case–control mutational analysis was performed in 400 patients with isolated VSD and 400 healthy controls. Protein-coding exton of *HOMEZ* and their flanking sequences were amplified by polymerase chain reaction and sequenced on an ABI3730 Automated Sequencer. CLC workbench software was used to compare the conservatism of the HOMEZ protein with other multiple species. The ExPASy-ProtScale online tool was used to predicate the alignment of the hydrophobic features. *Results:* Two novel heterozygous missense mutations (c.116 C > T; c. 630T > A) were identified in *HOMEZ* gene exon-2. The two mutations lead to alanine to valine substitution at position 39 and serine to arginine at position 210, which are highly conserved among many species. The hydropathicity of the valine and arginine residue at the position 39 and 210 were significantly different from the wild type. *Conclusions:* We have identified two novel heterozygous missense mutations in *HOMEZ* gene exon-2 in isolated VSD patients in the Chinese population and have found that these two mutations resulted in alteration of the hydropathicity of the HOMEZ protein. Therefore, the two missense mutations of the *HOMEZ* gene are directly linked with the etiology of isolated VSD in the Chinese population.

Ventricular septal defect (VSD) is the most common congenital heart disease (CHD) and is present in 33% of all affected infants (Correa-Villasenor *et al.,* 1993). Reports of prevalence of VSD in population-based studies have shown large variations: 0.9–6.0 per 1000 live-births (Ferencz *et al.,* 1985). Recent studies have suggested that up to 4% of asymptomatic neonates, at birth, had VSD (Sands *et al.,* 1999). The causes of VSD are largely unknown. However, epidemiologic studies revealed a significant environmental contribution to the pathogenesis of VSD (Burki and Babar, 2001; Burdn *et al.,* 2006). Familial aggregation and twin studies indicated the presence of genetic factors for susceptibility to this condition (Maestri *et al.,* 1988; Oyen *et al.,* 2010).

The homeodomain leucine zipper-encoding (*HOMEZ*) gene is a vertebrate homeobox gene. Bayarsaihan and coworkers first identified *HOMEZ* through database analysis by using the mouse sequence as query and mapped it to chromosome 14q11.2 by genomic sequence analysis (Nagase *et al.,* 2000; Bayarsaihan *et al.,* 2003). The gene contains two exons, the second of which contains most of the coding sequence (Bayarsaihan *et al.,* 2003). The deduced 549-amino acid protein, which is translated with the gene has a calculated molecular mass of about 61 KD (Bayarsaihan *et al.,* 2003). The *HOMEZ* gene encodes a protein with an unusual structural organization, which contains three atypical homeodomains, two leucine zipper-like motifs, proline- and serine-rich motifs, and an acidic domain. It also has a putative nuclear localization signal within homeodomain 2 (Bayarsaihan *et al.,* 2003).

Studies demonstrated that a first-cousin marriage might be a significant risk factor for specific types of CHD in a consanguineous population (Becker *et al.,* 2001; Tadmouri *et al.,* 2009). Inbreeding studies suggest an autosomal recessive component in the cause of some congenital heart defects (Rose *et al.,* 1985; Badaruddoza *et al.,* 1994). Recently, a genome-wide linkage analysis implicated linkage of CHD with the polymorphism (rs1055061; c.1505G > A, p.Arg502Gln) of *HOMEZ* in a family-based South Indian population study (McGregor *et al.,* 2010). Moreover, mutational analysis revealed three novel heterozygous sequence variations of *HOMEZ* in 54 Indian probands with CHD (McGregor *et al.,* 2010).

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Based on the above studies, we hypothesized that *HOMEZ* may play an important role in the development of VSD. The aims of our study were to identify potential pathogenic mutations for *HOMEZ* and to provide insights into the etiology of isolated VSD in the Chinese population.

Materials and Methods

Study population

A total of 400 nonsyndromic VSD patients and 400 control subjects with no reported cardiac phenotype were recruited. All participates were matched by ethnicity, gender, and age. An informed consent form was obtained from their parents or guardians. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee (Internal Review Board) of TEDA-international Cardiovascular Hospital, Tianjin, China.

Clinical assessment of the patients included anthropometric measurement and physical examination for dysmorphism and malformation. The patients also underwent chest X-ray examination, electrocardiogram, and ultrasonic echocardiogram. All patients underwent open heart surgery for repair of VSD and were confirmed as isolated VSD without other major congenital malformations.

DNA extraction and mutational analysis

Genomic DNA was extracted from peripheral blood leukocytes by the QIAamp blood kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Then, the genomic DNA was tested on a 1% agarose gel and NanoDrop 2000 instrument. The DNA was stored at −20°C before use.

The protein-coding exon (exon-2) of the *HOMEZ* gene and the partial flanking sequences were amplified by polymerase chain reaction (PCR) with a pair of *HOMEZ* gene-specific primers (Exon-2 F: 5-AGTTGGGACGACAGGCACGAAC-3, Exon-2 R: 5-GCGGGTGAAACATAGTCAAGT-3; 2673 bp). The PCR primers were designed by using GeneTool Software. PCR cycling conditions were as follows: 94°C for 5 min once, 35 cycles of 94°C for 1 min, annealing temperature 60°C for 1 min, 72°C for 3 min, and 72°C for 10 min once.

The PCR products were sequenced on an ABI3730 Automated Sequencer (PE Biosystems, Foster City, CA). The data were compared with sequences from the NCBI GenBank (*HOMEZ*: NM_020834.2).

CLC workbench software was used to compare the conservatism of the HOMEZ protein with other multiple species. The alignment of the hydrophobic features between the wild type and mutant type was predicted by the ExPASy-ProtScale tool (www.expasy.org/cgi-bin/pro- tscale.pl). The online tool of Polyphen (http://genetics.bwh.harvard.edu/pph/) was used to predict possible impact of an amino acid substitution on the structure and function of a human protein.

Results

Two novel heterozygous missense mutations (c. 116C > T; c. 630T > A) were identified in *HOMEZ* gene exon-2 (Fig. 1) when mutational analysis was performed in 400 isolated VSD patients. By comparing with the GenBank data, the two mutations lead to an alanine to valine substitution at the position 39 (p.A39V) and serine to arginine at position 210 (p.S210R) in the HOMEZ protein. The position 39 and position 210 of the HOMEZ protein were known to be highly conserved among many species (Fig. 2), suggesting that this position might play an important role in maintaining the protein function.

To further explore the role of this amino acid, an online tool ExPASy-ProtScale was used. This approach was defined by a numerical value assigned to predict the hydrophobicity or hydrophilicity scales, the secondary structure conformational parameters, and other scales, which are based on different chemical and physical properties of the amino acids. The hydropathicity of the valine residue at the position 39 and the arginine residue at the position 210 were significantly different from the wild type and the substitution may, in turn, result in modification of the protein structure (Fig. 3, A). Furthermore, when predicted by Polyphen, the changes of these two amino acids were shown to probably damage the protein structure (Fig. 3, B).

A total of 400 unrelated healthy control subjects were also recruited for mutational screen, and the alteration was not detected in any of them.

We also identified some nonpathogenic variants, as follows: rs117273314, rs117434701, rs1055061, rs79723196, rs76331664, rs10131813, and rs10131813. All of those SNPs were performed association analysis between isolated VSD case patients and 400 healthy controls, but none of them showed statistical differences.

Discussion

Congenital heart malformations occur in more than 0.5% to 1% of live births and are the most common birth defects in newborns (D'Alton *et al.*, 1993). The atrial septal defect and VSD account for the greatest proportion of CHD (Hoffman *et al.*, 1995). In recent years, our knowledge of the etiology of certain CHD has increased significantly. Great strides have been made in the identification of genes responsible for both syndromic and nonsyndromic forms of CHD, and their role in

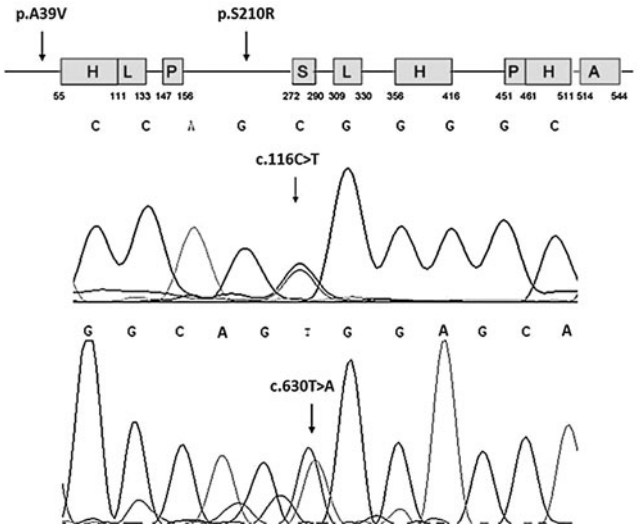

FIG. 1. Two missense mutations (p.A39V; p.S210R) in the protein of HOMEZ (indicated by an arrow; H, homeodomain; L, leucine zipper motif; P, proline-rich motif; S, serine-rich sequence; A, acidic domain.) (upper). Corresponding single transitions are observed at position 116 (C > T) and position 630 (T > A) of the *HOMEZ* gene as C/T and T/A double peak (indicated by an arrow) (lower). *HOMEZ*, homeodomain leucine zipper-encoding.

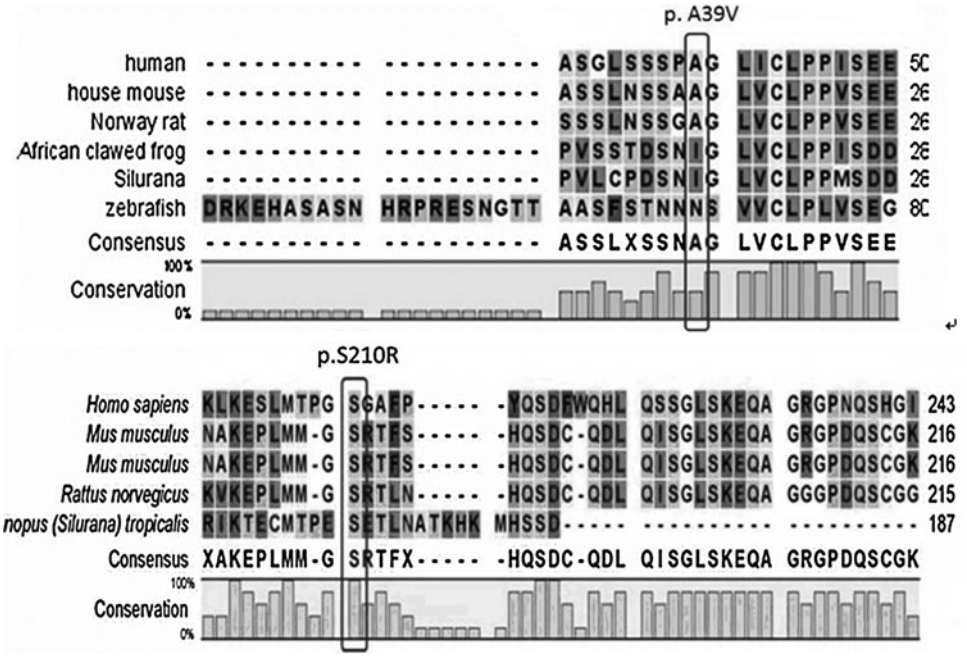

FIG. 2. Multiple-sequence alignment of partial amino acid sequence of the HOMEZ protein in different species is shown. The alignment data indicate that alanine at position 39 and serine at position 210 (indicated by pane) are conserved in different species in the HOMEZ protein.

cardiogenesis (Pierpont *et al.,* 2000; Bentham and Bhattacharya, 2008). Since the genome-wide association study (GWAS) technology was used for complex disease genetics, CHD has been at the forefront of a rapidly moving field (Perreault *et al.,* 2010; Wooten *et al.,* 2010). Studies with GWAS methods have identified many genetic variants and loci that are associated with CHD.

Familial aggregation studies have indicated that parental consanguinity is a risk factor for CHD (Maestri *et al.,* 1988; Oyen *et al.,* 2010). This implies that recessive inheritance contributes to the susceptibility of this condition. For example, unlike other Mendelian forms of CHD, the Ellis van-Creveld syndrome is inherited in an autosomal-recessive manner (Ellis and van Creveld, 1940). The hallmark features of the Ellis van-Creveld syndrome are congenital heart defects, which occur in at least 50% of affected individuals, as well as chondroectodermal dysplasia and bilateral postaxial polydactyly (McKusick *et al.,* 1964; da Silva *et al.,* 1980).

To explore the recessive model of CHD, McGregor and coworkers conducted a genome-wide linkage analysis utilizing high-density oligonucleotide microarrays and enrolling 83 Indian CHD probands (phenotypic distribution: conotruncal lesions, right-sided obstructive lesions, septal defects, single-ventricle lesions, valvular defects, and other complex defects) born to unaffected consanguineous parents. Then, mutational analysis in the coding regions of potential predisposing genes and genetic association study were performed in 54 Indian probands and a United States population (McGregor *et al.,* 2010). In the results, two-point linkage analysis resulted in two SNPs (rs1055061 and rs12433225) with log-of-odds scores above the standard threshold of 3.3. Of the two markers, rs1055061 lies within an exon of *HOMEZ*, causing a non-synonymous amino acid substitution (Arg > Glu). Three heterozygous variations were found in the 5′ UTR region (c.116G > A), in exon 1(Gly10Arg), and in the 3′UTR (c. + 193delT) of the *HOMEZ* gene when resequencing, and mutational analysis were performed in 54 Indian probands. In addition, no significant differences were detected in the genotype or allele distribution between 325 patients with CHD and 605 population-based controls in an American population of mixed European ancestry (McGregor *et al.,* 2010).

Our patients were sporadic and were from many provinces of China, with no specific parental consanguinity among the patients. In the results, our study for the first time has identified two heterozygous missense mutations (c.116 C > T, p.A39V; c. 630T > A, p.S210R) of the *HOMEZ* gene in the isolated VSD patients. The two mutations, respectively, lead to an alanine to valine substitution at the position 39 (p.A39V) and serine to arginine at position 210 (p.S210R) in the HOMEZ protein. Although the substitutions are not laid within the unusual structural organization, these positions are highly conserved among many species (human, rats, mice, etc.), which suggest that they might play an important role in maintaining the protein function. Additionally, the hydropathicity of the valine and arginine residue at the position 39 and 210 of the HOMEZ protein were significantly different from the wild type and the substitutions may, in turn, result in modification of the protein structure. Owing to the discussion above, we believe that these two novel heterozygous missense mutations of *HOMEZ* are directly linked with the etiology of isolated VSD in the Chinese population.

Limitation of study

There are limitations of this study that should be discussed. First, modern genetics demonstrated that the introns and noncoding exons might play an important role in the gene expression regulation (Mattick, 1994; Ren, 2010). In our study, only the coding exons and the partial flanking sequences of *HOMEZ* were amplified and sequenced. Therefore, our study does not rule out the role of introns and noncoding exons of *HOMEZ* in the development of isolated VSD that will be one of the future directions for genetic studies in CHD. In fact, there are other genetic factors involved in VSD in Chinese patients as we recently reported (Xuan *et al.,* 2012). Second, the findings from the present study that only involves the Chinese

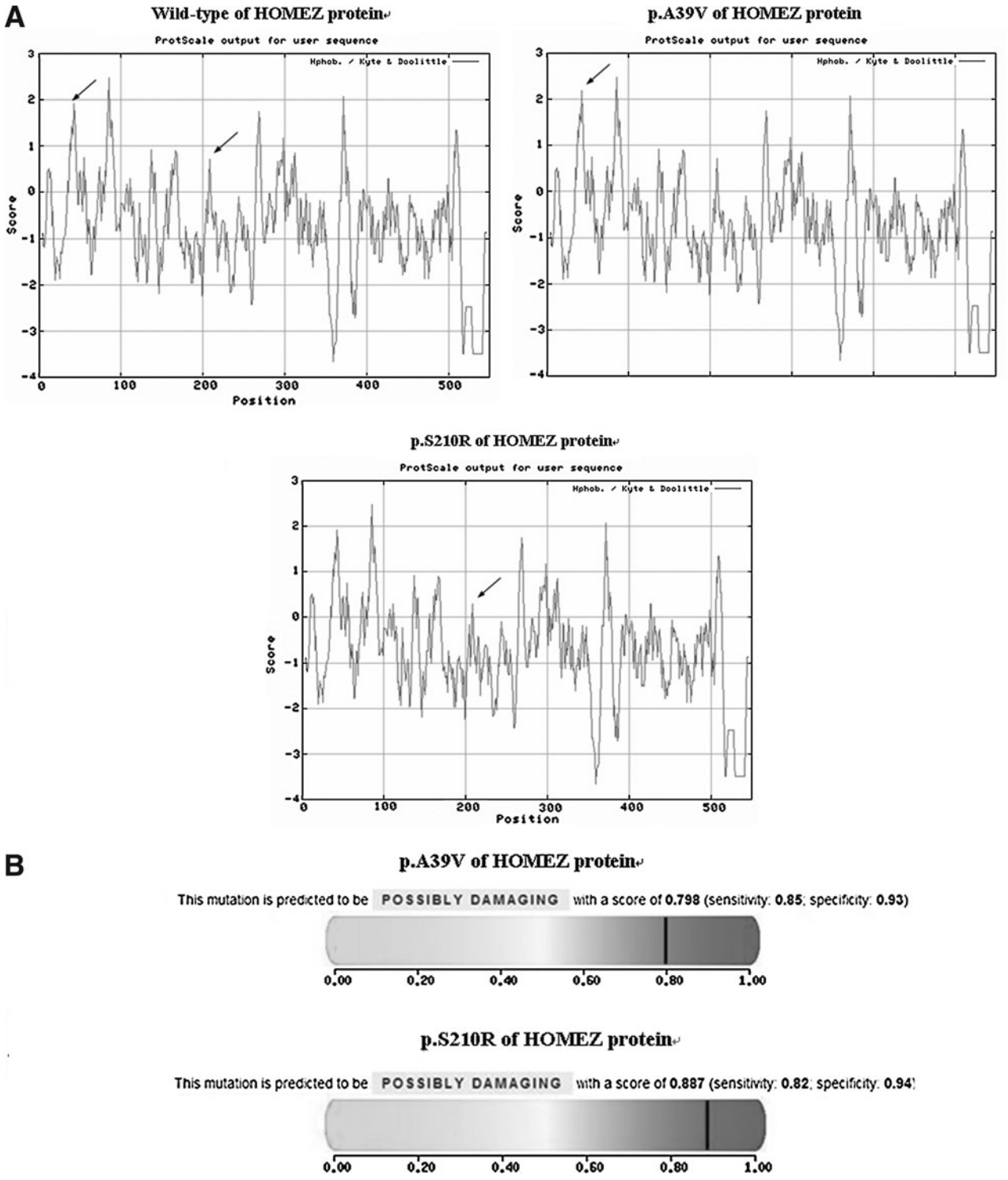

FIG. 3. **(A)** Hydropathy plot of the wild type, p.A39V, and p.S21P0R of the HOMEZ protein prepared in the Expasy ProtScale Web site according to the Kyte and Doolittle algorithm. Hydrophobic segments have values above zero in the Y-axis, amino acid position is plotted on the x-axis beginning with the N-terminus. The hydrophobicity scores of p.A39V of the HOMEZ protein are higher than the wild type, and p.S210R of the HOMEZ protein is lower than the wild type (the hydrophilicity of corresponding amino acids in HOMEZ protein is indicated by arrows). **(B)** The results of Polyphen indicate that the changes of these two amino acids were shown to probably damage the protein structure (possibly damaging score: p.A39V = 0.798, p.S210R = 0.887).

population should be carefully interpreted in other populations. The relationship between *HOMEZ* and isolated VSD in other populations warrants further study. In addition, the function of these two novel heterozygous missense mutations needs further experimental investigation to confirm.

In summary, the present study by using mutational analysis of *HOMEZ* in 400 Chinese patients with isolated VSD revealed two novel missense mutations (c.116 C > T, p.A39V; c. 630T > A, p.S210R) in exon-2. These two mutations have not been previously reported in CHD. It is possible that the etiology of isolated VSD might be directly linked with the two mutations. The effect of the mutations on the expression levels and the concrete functional role of these mutations in the development of the septum and the formation of isolated VSD should be addressed in further studies.

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Author Disclosure Statement

No competing financial interests exist.

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