Characterization of human bone morphogenetic protein gene variants for possible roles in congenital heart disease

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Abstract. Congenital heart disease (CHD) is a complex illness with high rates of morbidity and mortality. In embryonic development, the heart is the first formed organ, which is strictly controlled by gene regulatory networks, including transcription factors, signaling pathways, epigenetic factors and microRNAs. Bone morphogenetic protein (BMP)-2 and -4 are essential in cardiogenesis as they can induce the expression of transcription factors, NKX2-5 and GATA binding protein 4, which are important in the development of the heart. The inhibition of BMP-2 and -4 inhibits the late expression of NKX2-5 and affects cardiac differentiation. The aim of the present study was to investigate whether BMP-2 and -4 variations may be associated with CHD in Chinese Han populations. The rs1049007, rs235768 and rs17563 single nucleotide polymorphisms (SNPs), which are genetic variations located within the translated region of the BMP-2 and -4, were evaluated in 230 patients with CHD from the Chinese Han population and 160 non-CHD control individuals. Statistical analyses were performed using the χ^2 test, implemented using SPSS software (version 13.0). The Hardy-Weinberg equilibrium test was performed on the population using online Online Encyclopedia for Genetic Epidemiology studies software, and multiple-sequence alignments of the BMP proteins were performed using Vector NTI software. No statistically significant associations were identified between these genetic variations and the risk of CHD (rs1049007, P-value=0.560; rs235768, P-value=0.972; rs17563, P-value=0.787). In addition, no correlation was found between the patients with CHD and the non-CHD control individuals. Therefore, the rs1049007, rs235768 and rs17563 genetic variations of BMP-2 were not associated with CHD in the Chinese Han population.

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Introduction

Congenital heart disease (CHD) is a common, complex illness with high rates of morbidity and mortality, the genetic etiology of which emains to be fully elucidated (1,2). Worldwide, the incidence of moderate and severe CHD is ~6/1,000 live births, however, if small muscular ventricular septal defects (VSDs) and other minor lesions are included, the incidence is ~75/1,000 live births (3). Appropriately, 1% of patients with CHD require intervention (4), and ~13% of patients with CHD show recognizable chromosomal variants (5,6). The majority of adult patients with show a variety of cardiac complications, including coronary heart disease, arrhythmias and heart failure (7). Although extensive genetic investigations and high-resolution technologies have revealed subtle defects in familiar and sporadic cases of CHD (2,8), the subtle genetic causes and molecular mechanisms of CHD remain to be fully elucidated.

In embryonic development, the heart is the first formed organ, which is strictly controlled by gene regulatory networks, including transcription factors, signaling pathways, epigenetic factors and microRNAs (2,9,10). During the last few decades, several cardiac-enriched transcription factors have been identified, and a variety of CHD-causing mutations have been identified in those factor genes, which provide molecular markers and models for the analysis of heart development and the molecular mechanisms underlying CHD (6,11). For example, NKX2-5 is a member of the NK-2 class homeodomain proteins, and is among the earliest markers of cardiac specification. *Drosophila* embryos fail to form a heart in the presence of a mutant NKX gene (12) and, if the functions of the NKX family members are inhibited, vertebrate embryos also fail to form a heart (13). The zinc finger binding proteins, GATA binding protein (GATA)-4, -5 and -6, are also members

of cardiac-enriched transcription factors, and are expressed early in heart development, being involved in the initial steps of cardiomyocyte differentiation (14). Mutations in GATA-4 have been identified to be associated with CHD (15), and a variety of other CHD-causing gene mutations have been identified, includingCITED2 (16), CFC1 (17) and TBX1 (18). These gene are critical in cardiac development; mutations in these genes lead to cardiovascular malformations and contribute to CHD (19).

In non-precardiac mesoderm, the expression of the heart-specific transcription factors, NKX2-5 and GATA-4, can be induced by BMP-2 and -4, and inhibition of BMP-2 and -4 signaling inhibits the late expression of NKX2-5 and affects the cardiac differentiation (20). In zebrafish, the BMPs also have a direct role in development of heart tube looping (21), and in Xenopus, BMP-2 and -4 are broadly expressed in the anterior endoderm and cardiac mesoderm, prior to cardiac differentiation in heart development (22-24). The inhibition of BMP signaling in mice lacking Sma6, leads to the development of hyperplasia in heart valves and defects in the aorticopulmonary septum (25).

BMP-2 and -4 have important functions at multiple stages of cardiogenesis, particularly in the initial induction of heart development. However, the exact nature of these roles, and the association between BMP-2 and -4, with CHD remains to be elucidated. To determine whether there is an association between BMP-2 and -4, and CHD, the gene sequences of BMP-2 and -4 were compared between 230 Chinese Han patients with CHD and 160 control individuals, focusing on the rs1049007, rs235768 and rs17563 SNP genetic variations. The results revealed no correlation between these candidate SNPs and the risk of CHD, and the BMP-2 rs1049007, rs235768 and BMP-4 rs17563 genetic variations may not be risk factors for CHD.

Materials and methods

Study population. A total of 230 patients with CHD and 160 control subjects with no reported cardiac phenotypes were recruited for the present study from the Second Affiliated Hospital of Harbin Medical University (Harbin, China), as shown in Table I. Written informed consent was obtained from each participant and the study was reviewed by the Ethics Committee of Harbin Medical University, consistent with the 1975 Declaration of Helsinki (26,27). The Ethics Committee approved the study. Detailed records on the medical history, physical examination and chest X-ray examination, electrocardiogram and ultrasonic echocardiogram were obtained.

BMP-2 rs1049007 and rs235768, and BMP-4 rs17563 SNP genotyping analysis and statistical methods. Whole genomic DNA was extracted from peripheral blood leukocytes using a QIAamp DNA Blood Mini Kit (cat. no. 51104; Qiagen, Hilden, Germany) (28). The genotypes for the rs1049007, rs235768 and rs17563 SNPs associated with the BMP-2 and -4 genes, respectively (Fig. 1), were determined using a two-stage method. First, rs1049007, rs235768 and rs17563 (Table II) were amplified using standard procedures (2,6,29), following which the PCR products were sequenced (Genewiz, Inc., South Plainfield, NJ, USA) to determine the genotype (Fig. 2). The statistical analyses were performed using χ^2 tests (descriptive statistic crosstalk) to calculate the odds ratios and P-values, implemented using SPSS software (version 13.0; SPSS, Inc., Chicago, IL, USA). In addition, Online Encyclopedia for Genetic Epidemiology studies (OEGE; http://www.oege.org/software) online software was used to perform the Hardy-Weinberg equilibrium test for the CHD and control populations.

Table I. Clinical characteristics of the study populations.

Parameter	CHD	Control
Sample (n)	230	160
Male/Female (n)	142/88	105/55
Age (years)	16.18±10.22	7.88±11.96

Data are presented as the mean ± standard deviation; CHD, congenital heart disease.

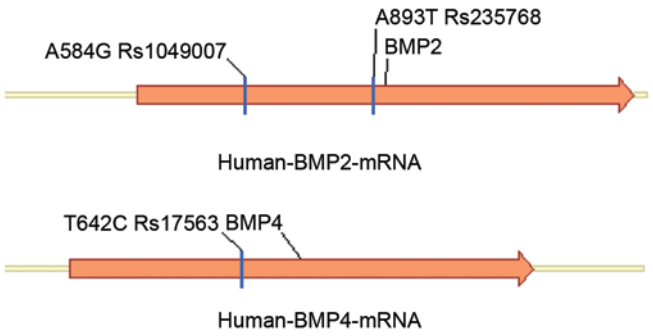

Figure 1. Schematic diagrams of rs1049007, rs235768 and rs17563 locations within the translated regions of the BMP-2 and -4 genes. BMP, bone morphogenetic protein.

Multiple sequence alignment. From the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/), the BMP-2 and -4 protein sequences of various species were obtained. Using Vector NTI software (Suite 9; Thermo Fisher Scientific, Inc., Waltham, MA, USA), multiple-sequence alignments of these proteins were performed.

Results

Patients. Clinical diagnosis of the recruited patients was confirmed at the Second Affiliated Hospital of Harbin Medical University. There were no histories of other systemic abnormalities in the patients with CHD, and their mothers had no history of taking medicines or contracting infections during pregnancy (30).

BMP genotyping and statistical analysis. To confirm the hypothesis that there are possible associations between BMPs, which inhibit the late expression of NKX2-5 and affects cardiac differentiation (20), and CHD, the present study performed SNP analyses. No significant differences

Table II. Polymerase chain reaction primers used for BMP genotyping sequence analysis.

Gene	Single nucleotide polymorphism	Primer	Size (bp)	Temperature (°C)
BMP2	rs1049007	Forward CGGGACCCGCTGTCTTCT Reverse TGGAAACGTCCGCTGGTG	455	60.5
	rs235768	Forward CCCACGGAGGAGTTTATC Reverse GCCACTTCCACCACGAAT	275	52.5
BMP4	rs17563	Forward CCCCACTTATCTGCTCCT Reverse AGTTTGGCTGCTTCTCCC	500	52.8

BMP, bone morphogenetic protein.

Table III. Genotype and allele frequencies of the rs1049007, rs235768 and rs17563 SNPs in 230 Chinese Han patients with CHD and 160 non-CHD control individuals.

SNP	Group	n	Genotype frequency, n (%)			Allele frequency, n (%)	
rs1049007			G/G	A/G	A/A	G	A
	CHD	230	155 (67.4)	68 (29.6)	7 (3.0)	378 (82.2)	82 (17.8)
	Control	160	103 (64.4)	54 (33.8)	3 (1.9)	260 (81.3)	60 (18.8)
rs235768			T/T	T/A	A/A	T	A
	CHD	230	142 (61.7)	80 (34.8)	8 (3.5)	364 (79.1)	96 (20.9)
	Control	160	97 (60.6)	57 (35.6)	6 (3.8)	251 (78.4)	69 (21.6)
rs17563			T/T	T/C	C/C	T	C
	CHD	230	114 (49.6)	96 (41.7)	20 (8.7)	324 (70.4)	136 (29.6)
	Control	160	85 (53.1)	62 (38.8)	13 (8.1)	232 (72.5)	88 (27.5)

SNP, single nucleotide polymorphism; CHD, congestive heart disease.

Table IV. rs1049007, rs235768 and rs17563 SNPs within BMP-2 and -4 are not associated with risk of congenital heart disease.

			Pearson's χ^2				Pearson's R			
Genotyped SNP	Gene	Genotype/ allele	χ^2	Min count[a]	df	Asymp. P-value (2-sided)	Pearson's R-value	Asymp. SE[b]	Approx. T-value[c]	Approx. P-value[d]
rs1049007	BMP2	Genotype	1.160	4.10	2	0.560	0.017	0.050	0.337	0.737[d]
		Allele	0.108	58.26	1	0.742	0.012	0.036	0.329	0.743[d]
rs235768	BMP2	Genotype	0.568	5.74	2	0.972	0.012	0.051	0.239	0.811[d]
		Allele	0.054	67.69	1	0.816	0.008	0.036	0.233	0.816[d]
rs17563	BMP4	Genotype	0.479	13.54	2	0.787	-0.032	0.051	-0.623	0.534[d]
		Allele	0.393	91.90	1	0.531	-0.022	0.036	-0.626	0.531[d]

[a]Minimum expected count; [b]Not assuming the null hypothesis; [c]Using the asymptotic standard error assuming the null hypothesis; [d]Based on normal approximation. df, degrees of freedom; Asymp, asymptomatic; Approx, approximate; SNP, single nucleotide polymophism; BMP, none morphogenetic protein.

were found between the patients with CHD and the 160 CHD-free control individuals in relation to the risk of CHD (Tables III and IV). The present study also performed Hardy-Weinberg equilibrium tests for the patients with CHD and control individuals, and found that the χ^2 value was 3.06 for the patients with CHD and 2.86 for the CHD-free control individuals. The results were concordant with the Hardy-Weinberg equilibrium.

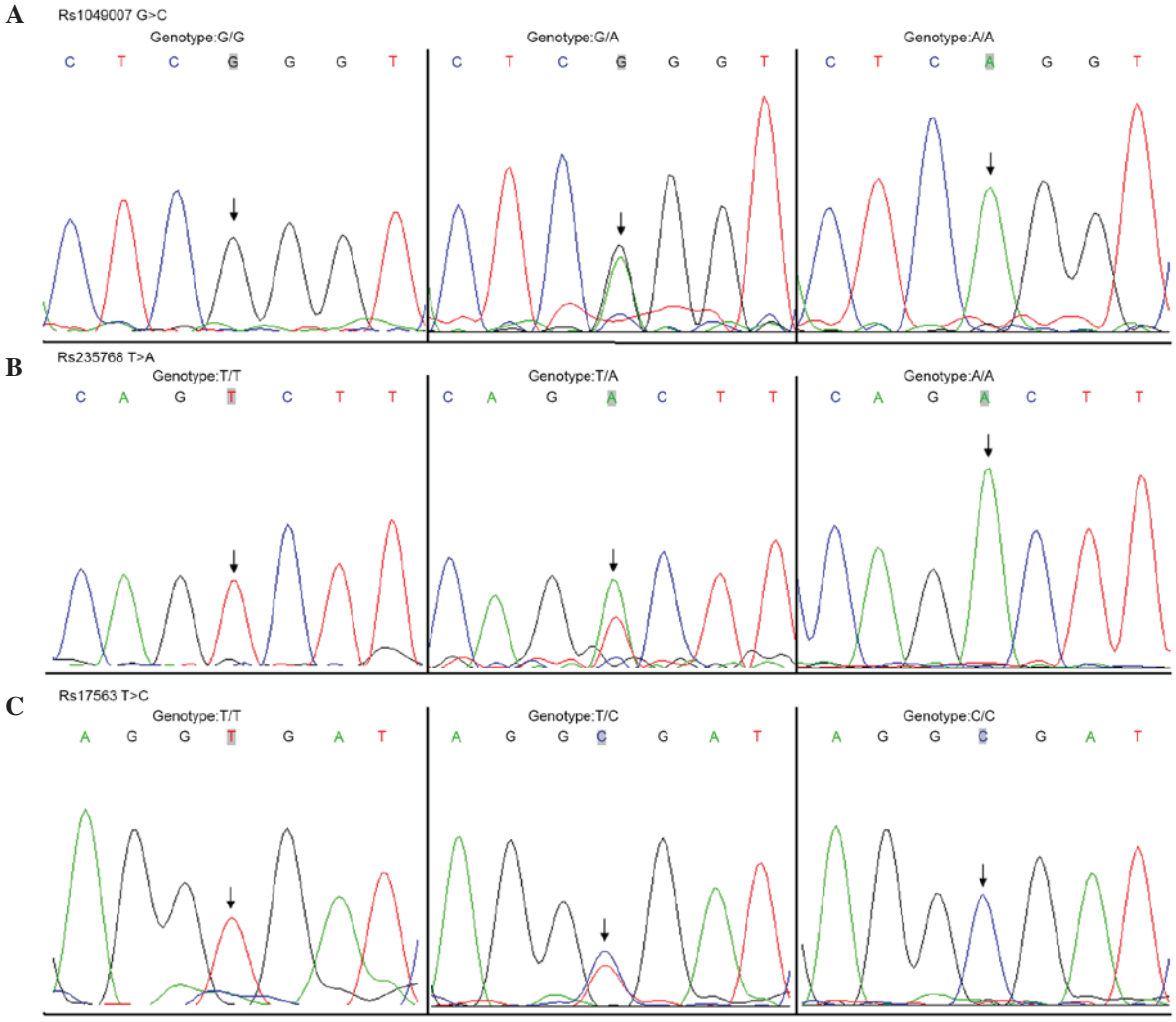

Figure 2. DNA sequence chromatograms of the rs1049007, rs235768 and rs17563 single nucleotide polymorphisms. (A) rs1049007; (B) rs235768; (C) rs17563. Arrows indicate the sites of variation.

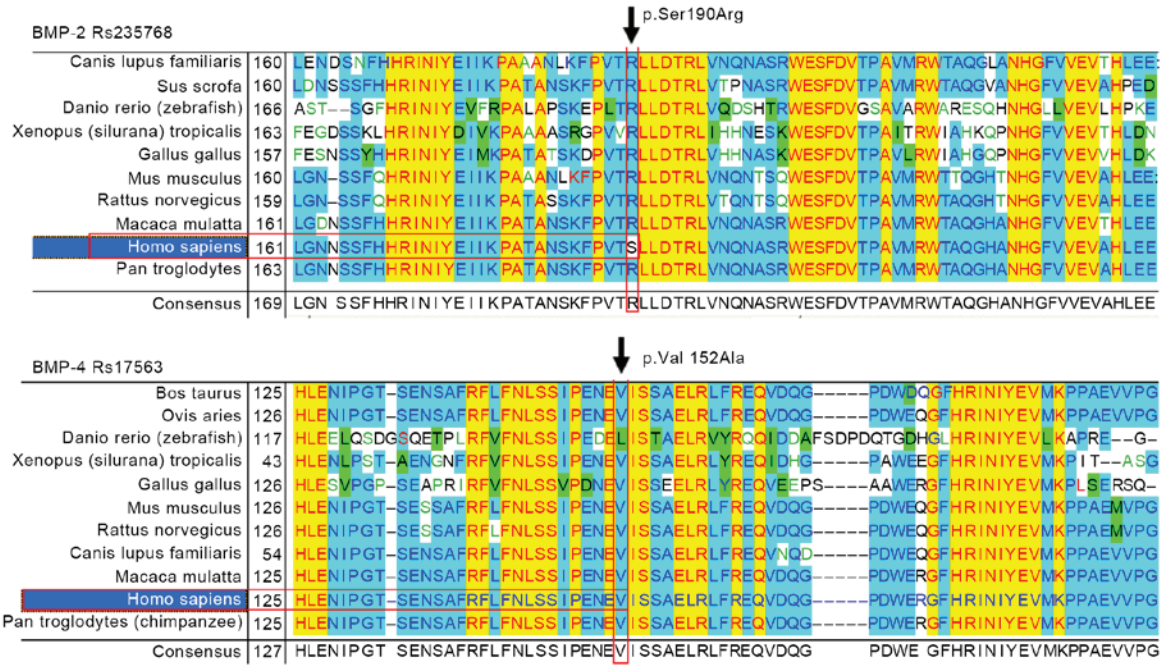

Figure 3. Multiple-sequence alignment of BMP-2 and -4 from birds, fish and mammals, including *Homo sapiens*, *Pan troglodytes* and *Macaca mulatta*. Conservation of 190Ser and 152Val residues in BMP-2 and -4, respectively, were high and in a highly conserved regions. BMP, bone morphogenetic protein.

Conservation of proteins in evolution. The comparison of the BMP-2 and -4 protein sequences between different species included birds, fishes, rodents and mammals, including *Homo sapiens*, *Pan troglodytes and Macaca mulatta*. The results of the multiple-sequence alignment analysis showed that the conservation of the rs235768 and rs17563 variations were high; the rs1049007 variation did not alter the protein sequence. The conservation of the 190Ser and 152Val residues in BMP-2 and -4, respectively, were high and were located in highly conserved regions of the proteins (Fig. 3).

Discussion

BMP4, a member of the transforming growth factor (TGF)-β family, is capable of causing human embryonic stem cells (hESCs) to differentiate, and this differentiation can occur without extensive generation of mesoderm and endoderm (31). When inhibiting the fibroblast growth factor (FGF)2 pathway and maximizing BMP4 signaling, BMP4 can direct the hESCs to differentiate towards syncytiotrophoblasts (32,33). The differentiation program induced by BMP4 involves rapid induction, and occurs prior to the expression of caudal type homeobox 2 and several other mesoderm marker genes (32,34). BMP2 is a homodimeric disulfide-bonded protein and is also a member of the TGF-β family (35-37). Despite substantial differences in amino acid sequences with cystine-knot growth factors, it has a similar monomer structure with the factor (38), and has a similar dimer structure with the TGF-β family (39,40).

Several previous studies have shown that BMP-2 and -4 are implicated in the formation of the heart from the overlying mesoderm (41). The expression of the early cardiac markers, NKX2-5 and GATA-4, can be induced by BMP-2 within anterior mesodermal cells, which are located close to the heart forming region (20,42). Following pretreatment with FGFs, BMP-4 can increase the activity of the posterior mesoderm cells (20,43). If the expression of BMP-2 and -4 are inhibited, cardiac differentiation can be inhibited (20,44). Therefore, BMP-2 and -4 are essential in cardiogenesis, and the inductive functions of BMP-2 and -4 may result from the interactions between BMP and other extracellular signaling molecules (45). BMP signaling is important in the formation of different early cell types from hESCs, including the mesoderm (46), endoderm (18) and trophoblasts (47). However, BMP signaling is not required for the expression of early markers of cardiac specification, including NKX2-5, and GATA-4, -5 and -6 (41,48). Therefore, the present study focussed on the rs1049007, rs235768 and rs17563 SNP genetic variations, the aim of which was to analyze the association between BMP-2 and -4, and CHD.

Previously, Qian *et al* found that the rs762642 polymorphism in BMP4 may increase susceptibility to sporadic CHD, and that the polymorphism contributes to the susceptibilities to atrial septal defects and VSDs (49). In addition, it was found that the rs17563 SNP in BMP4 was not associated with the risk of CHD or types of CHD (49). These previous results do not conflict with the results of the present study. The rs762642 SNP is located within the intron between the first and second exon of BMP4, which may affect the function of the promoter and enhancer regions of the gene. Although the rs17563 SNP within the fourth exon resulted in an amino acid change within the CDS region of the gene, the SNP may not be as important as the rs762642 SNP for the function of BMP4.

In conclusion, the present study compared the gene sequences of BMP-2 and -4 between 230 Chinese Han patients with CHD and 160 control individuals, focusing on the rs1049007, rs235768 and rs17563 SNP genetic variations. The meta-analysis assisted in clarifying the associations between these SNPs with CHD, the results of which revealed that there were no correlations between these candidate SNPs and the risk of CHD in the Chinese population. The risk of developing CHD in individuals with these variants of the BMP-2 and -4 genes may be low; the results of the present study demonstrated that these variations in the BMP-2 and -4 genes were not associated with CHD in the Chinese Han population.

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