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Original Article

Discovery of *BMP10* as a new gene underpinning congenital heart defects

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Abstract: Objective: Aggregating evidence convincingly establishes the predominant genetic basis underlying congenital heart defects (CHD), though the heritable determinants contributing to CHD in the majority of cases remain elusive. In the current investigation, *BMP10* was selected as a prime candidate gene for human CHD mainly due to cardiovascular developmental abnormalities in *Bmp10*-knockout animals. The objective of this retrospective study was to identify a new *BMP10* mutation responsible for CHD and characterize the functional effect of the identified CHD-causing *BMP10* mutation. Methods: Sequencing assay of *BMP10* was fulfilled in a cohort of 276 probands with various CHD and a total of 288 non-CHD volunteers. The available family members from the proband harboring an identified *BMP10* mutation were also *BMP10*-genotyped. The effect of the identified CHD-causative *BMP10* mutation on the transactivation of *TBX20* and *NKX2.5* by BMP10 was quantitatively analyzed in maintained HeLa cells utilizing a dual-luciferase reporter assay system. Results: A novel heterozygous *BMP10* mutation, NM_014482.3:c.247G>T;p.(Glu83*), was identified in one proband with patent ductus arteriosus (PDA), which was confirmed to co-segregate with the PDA phenotype in the mutation carrier's family. The nonsense mutation was not observed in 288 non-CHD volunteers. Functional analysis unveiled that Glu83*-mutant BMP10 had no transactivation on its two representative target genes *TBX20* and *NKX2.5*, which were both reported to cause CHD. Conclusion: These findings provide strong evidence indicating that genetically compromised *BMP10* predisposes human beings to CHD, which sheds light on the new molecular mechanism that underlies CHD and allows for antenatal genetic counseling and individualized precise management of CHD.

Keywords: Congenital heart defect, medical genetics, transcriptional factor, BMP10, biological analysis

Introduction

Congenital heart defect (CHD), a wide spectrum of cardiovascular malformations resulting from anomalous development of the heart and cardiac valves as well as endo-thoracic large vessels, signifies the most prevalent kind of birth aberration in humans, occurring in roughly 1% of live neonates and in approximately 10% of miscarriages worldwide [1, 2]. Besides, when minor cardiovascular developmental deformities are encompassed, such as atrial septal aneurysm, patent foramen ovale, right aortic arch, and aortic bicuspid valve (the most common form of congenital cardiovascular abnormalities, occurring in 1% to 2% of people), the prevalence of CHD is as high as ~5% among live births [3-5]. As a vast collection of cardiovascular developmental anomalies, CHD is clinically assorted to >26 diverse isoforms, including patent ductus arteriosus (PDA), aortic/pulmonary atresia, aortic/pulmonary stenosis, aortic coarctation, aortopulmonary window, atrial/ventricular septal defect, tetralogy of Fallot (the

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commonest cyanotic CHD), atrioventricular septal defect, single ventricle, endocardial cushion defect, transposition of the major arteries, double outlet right ventricle, aortic arch interruption, abnormal coronary artery connection, cor triatriatum, and left heart hypoplasia/left ventricular noncompaction/spongy myocardium [2, 6-11]. Though some minor types of CHD do resolve spontaneously [2], severe types of CHD may give rise to degraded health-correlated quality of life [12-15], impaired exercise capacity [16-18], pulmonary arterial hypertension [19-21], acute brain injury and delayed neurodevelopment [22-25], thromboembolic/ischemic cerebral stroke [26-28], acute renal injury and chronic kidney disease [29-31], liver fibrosis and dysfunction [32], infective endocarditis [33-37], chronic/congestive heart failure [38-40], miscellaneous supraventricular and life-threatening ventricular dysrhythmias [41-45], and even premature cardiac demise [46-50]. Over the past decades, enormous advancements have been won in surgical and trans-catheter interventional therapies for CHD as well as in perioperative intensive care of CHD patients, which allow ~95% of live newborns inflicted with CHD (including those with complex CHD) to survive to adulthood, and as a consequence of longer life expectancy, now adults have already outnumbered children among the individuals living with CHD [51-56]. However, in comparison with the general population, adult survivors with CHD are associated with higher incidences of late comorbidity and mortality, including cerebrovascular thromboembolism, pulmonary hypertension, chronic renal disease, infective endocarditis, congestive heart failure, cardiac arrhythmias, cancer, and premature cardiac death [55-59]. Therefore, CHD has caused substantial mortality and morbidity as well as imposing vast economic encumbrance on individuals and society, underscoring the urgent necessity to ascertain the etiologies accountable for CHD [2].

In vertebrate embryos, the heart is the first functioning organ to develop, and cardiac development experiences an exceedingly sophisticated biological process that involves a finely controlled sophisticated network, principally comprising cardiac structural proteins, transcription factors, signal-transducing molecules, and epigenetic modifiers [1, 60-64]. It has been validated that both non-heritable risk factors and inherited defectives may perturb this heart-forming process, leading to an extensive assortment of CHD [1, 3, 60-66]. It is estimated that acquired/environmental pathogenic factors contribute to ~10% of CHD, though their molecular mechanisms underlying CHD are largely obscure [1]. Well-established non-genetic factors that predispose someone to CHD include maternal disorders (obesity, essential hypertension, hyperlipidemia, diabetes mellitus, hyperhomocysteinemia, phenylketonuria, acute febrile illness, viral infections, epilepsy, pre-eclampsia, autoimmune imbalance, connective tissue disease, thyroid disease, and mental health disease), maternal medications (anti-depressant, anti-hypertensive, anti-convulsant, and anti-infective drugs), maternal ingestion of toxic substance (marijuana, alcohol, and tobacco), maternal malnutrition (folate deficiency), and maternal exposure of air pollutants, toxic chemicals, and heavy metals during the first trimester of pregnancy [1, 64, 67-70]. However, an ever-growing body of evidence substantiates that inherited components exert a predominant impact on the incidence of CHD [1, 3, 60-62]. In addition to chromosomal aneuploidies (Turner syndrome, trisomy 18, Down syndrome, DiGeorge syndrome, and trisomy 13) and copy number variations (losses and gains), deleterious mutations in more than 100 genes, encompassing *TBX20* and *NKX2.5*, have been causally implicated in CHD [1, 3, 60-62, 71-101]. Nevertheless, in the majority of cases, the heritable determinants for CHD remain indefinite [1], which underscores the conspicuous genetic heterogeneity of CHD and renders it warranted to discern new genes underpinning CHD.

Recently, research on the *Bmp10*-knockout animals unveiled the critical role of *Bmp10* in regulating cardiovascular development [102-104]. In mice, *Bmp10* knockout led to embryonic lethality mainly due to cardiac hypoplasia (hypoplastic ventricular walls) [102]. Morphological and histological analyses of *Bmp10*-deficient murine embryos and hearts revealed that myocardial growth was retarded with expanded pericardiac sacs, thinned myocardium (ventricular hypertrabeculation because of dramatic reduction in myocardial proliferation), and severe edema [102]. Additionally, acellular endocardial cushions developed in both the atrioventricular canal and outflow tract, result-

ing in failure to form normal endocardial cushions and ventricular trabeculae [102]. Besides, in the developing myocardium of *Bmp10*-deficient mice, the expression levels of multiple essential cardiogenic transcription factors, including NKX2.5 and MEF2C, were strikingly diminished [102]. Furthermore, a BMP10-conditioned medium could rescue *Bmp10*-null hearts in culture [102]. By utilizing conventional knockout as well as specific antibodies against BMP9 or BMP10, Chen and colleagues [103] demonstrated that BMP10 and BMP9 were concurrently expressed in the developing cardiovascular system with partially overlapping physiological roles, showing functional redundancy. Yet, analysis of *Bmp10*$^{9/9}$ mice, in which *Bmp10* was displaced by *Bmp9* (*Bmp9* knocked in at the *Bmp10* locus), showed that *Bmp10* possessed an exclusive function in cardiovascular morphogenesis, which couldn't be substituted or fully compensated by ectopic expression of *Bmp9* [103]. Specifically, *Bmp10*$^{9/9}$ hearts presented with hypoplasia with markedly thinner ventricular wall and apparent pericardial edema, and most *Bmp10*$^{9/9}$ hearts also manifested pronounced ventricular septal defects, in addition to significantly decreased myocardial proliferation and growth, enlarged heart volume, and changed cardiac shape [103]. Levet and partners [104] examined the impacts of *Bmp10* and *Bmp9* on the closure of the ductus arteriosus in mice and observed that *Bmp9* knockout caused an imperfect closure of the ductus arteriosus. Furthermore, at postnatal day 1 and day 3, administration of an anti-BMP10 neutralizing antibody aggravated the remodeling anomaly and resulted in a reopening of the ductus arteriosus at postnatal day 4 in these pups [104]. Collectively, these results from experimental animals establish a pivotal role of *BMP10* in proper cardiac organogenesis, especially in the myocardial growth, ventricular chamber maturation, and closure of ductus arteriosus, and prompt the hypothesis that a *BMP10* mutation contributes to CHD in humans.

Materials and methods

Human study subjects

The present retrospective human research was implemented in conformity with the ethical principles outlined in the Declaration of Helsinki. The protocols applied to this human research were approved by the local institutional review board of Tongji Hospital, Shanghai (approved protocol code: LL(H)-09-07). Written informed consent was provided by the research participants or their parents/legal guardians at the time of initial recruitment, prior to the commencement of the present human research. In compliance with approved guidelines and regulations, the personal identities of the consenting research participants were encrypted and secured. For the current human research, a cohort of 276 probands affected with various CHD was enlisted from the Chinese population of Han ethnicity, in addition 288 unrelated Han-race non-CHD volunteers were employed as control individuals. The available relatives of the CHD-affected probands were also enrolled. Each research participant was examined at least by a cardiologist and a pediatrician. Detailed personal, medical, and familial histories were retrieved, and thorough physical examinations, including echocardiography and electrocardiography, were completed for all study subjects. The affected individuals' congenital cardiovascular malformations were classified in terms of the nomenclature of the International Classification of Diseases, Eleventh Revision (ICD-11) [105]. The inclusion criteria for the patient group were having a diagnosis of CHD documented by the echocardiogram or confirmed by surgical proceedings and providing a signed informed consent form. The criteria for exclusion were patients with such syndromic CHD as Alagille syndrome, Down syndrome, Edward syndrome, Turner syndrome, DiGeorge syndrome, Patau syndrome, and Noonan syndrome. Patients with defined causes explainable for CHD were also excluded. The non-CHD individuals with a familial history of CHD were excluded from the control group. Demographic and clinical data along with 2 mL of whole blood were collected from each eligible study subject.

Sequencing analysis of human BMP10

Extraction of genomic DNA from the study participants' blood leucocytes was fulfilled by employing the GeneJET™ Genomic DNA Purification Kit (Thermo Scientific, USA) as per the manual. The unique oligonucleotide primers applied to amplify the whole coding regions as well as the splicing boundaries of *BMP10* (NC_000002.12) were described elsewhere [106]. Amplification of *BMP10* from each

research subject's genomic DNA through polymerase chain reaction (PCR) was conducted under the SimpliAmp™ Thermal Cycler apparatus (Applied Biosystems, USA) using the AccuPrime™ *Taq* DNA Polymerase Kit (Invitrogen, USA) along with the *BMP10*-specific primers, according to the manufacturer's instructions. The amplicons were resolved via 1.4% agarose gel electrophoresis and purified with the GeneJET™ Gel Extraction Kit (Thermo Scientific, USA) following the protocol. The amplified products purified are subject to sequencing analysis as described previously [106]. Sanger sequencing analysis of *BMP10* was performed in all the study participants, including 276 CHD-affected index patients and 288 non-CHD volunteers as well as the available pedigree members of the proband who carried an identified *BMP10* mutation. Additionally, for an identified *BMP10* mutation, several online population genetics databases, including the Genome Aggregation Database (gnomAD; http://gnomad-sg.org/gene/ENSG00000163217?dataset=gnomad_r2_1/; logged in on 15 September 2023), and the Single Nucleotide Polymorphism database (SNP; https://www.ncbi.nlm.nih.gov/snp/?term=BMP10/; logged in on 15 September 2023), were retrieved to authenticate its novelty.

Construction of gene expression vectors

The wild-type human BMP10-pcDNA3.1 vector (WT) was constructed as described previously [106]. The Glu83*-mutant BMP10-pcDNA3.1 vector (Glu83*) was generated through PCR-based site-targeted mutagenesis utilizing the GENEART® Site-Directed Mutagenesis System (Invitrogen, USA) along with a complimentary pair of primers (forward: 5'-AAGGTGGACCCACCATAGTACATGTTGGAAC-3'; reverse: 5'-GTTCCAACATGTACTATGGTGGGTCCACCTT-3') and was verified by direct sequencing assay. The TBX20-luc and NKX2.5-luc vectors, where the promoters of the human *TBX20* and *NKX2.5* genes transactivate the expression of firefly luciferase reporter, respectively, were created as described elsewhere [94]. All the constructed eukaryotic expression vectors were confirmed by direct sequencing assay.

Cellular transfection with gene expression vectors and reporter gene assay

The HeLa cells were cultured as described previously [106]. Cells were grown in a 24-well plastic plate (Corning, USA) at a density of 1 × 10^5 cells per well, incubated for 36 h to reach ~80% confluency, and then transfected with various expression vectors through the Lipofectamine® LTX & PLUS™ Reagent (Invitrogen, USA). As an internal control vector that expresses renilla luciferase, pGL4.75 (Promega, USA) was applied for normalizing/standardizing transfection efficiency. The empty pcDNA3.1 vector was used as an external negative control. As described in detail previously [106], 12 ng of pGL4.75 (Promega), 1.2 μg of TBX20-luc or NKX2.5-luc, and 0.4 μg of each gene expression vector (empty pcDNA3.1 vector, wild-type human BMP10-pcDNA3.1 vector, or Glu83*-mutant human BMP10-pcDNA3.1 vector, singly or in combination) were used. Transfected cells with various expression vectors were harvested 36 h post cellular transfection and then lysed in the cell lysis buffer. The dual-luciferase activities of cellular lysates were quantitatively gauged on the GloMax® Luminometer using the Dual-Glo® Luciferase Assay Systems (Promega, USA) according to the instructions. The activities of the *TBX20* and *NKX2.5* promoters were expressed by the ratios of firefly luciferase activities to renilla luciferase activities. Cellular transfection with each expression vector was executed in three independent replicates.

Statistical analysis

For continuous/quantitative variables, resultant parameters are expressed as mean ± SD. Categorical/qualitative variables are presented by frequency number (n) and percentage (%). Quantitative parameters were compared with independent Student's *t*-test between the two groups. When quantitative parameters were compared among ≥ 3 groups, a one-way analysis of variance (ANOVA) with a Tukey-Kramer HSD post hoc test was applied. Qualitative parameters were compared utilizing Pearson's chi-square test or Fisher's exact test between the two groups. A two-tailed *P*-value of less than 0.05 indicated a statistically significant difference. All statistical analyses were accomplished by exploiting the SPSS software, version 22.0 (SPSS, USA).

Results

Demographic and phenotypic characteristics of the recruited probands affected with CHD

In this human research, a cohort of 276 probands suffering from a wide variety of CHD (121 female probands and 155 male probands,

Table 1. Demographic and phenotypic characteristics of the 276 probands affected with congenital heart defects

Parameters	Number or mean	Percentage or range
Demographics		
Female probands	121	43.8
Male probands	155	56.2
Age (years)	4.1 ± 2.7	0.5-9.6
Positive family history of CHD	38	13.8
Distribution of distinct types of CHD		
ASD	66	23.9
VSD	62	22.5
PDA	55	19.9
TOF	29	10.5
DORV	7	2.5
AS	3	1.1
TGA	3	1.1
PS	2	0.7
PTA	1	0.4
VSD + PDA	17	6.2
DORV + VSD	11	4.0
TGA + VSD	7	2.5
ASD + PDA	6	2.2
VSD + ASD	4	1.4
TOF + ASD	2	0.7
PTA + VSD	1	0.4
Arrhythmias		
AVB	21	7.6
AF	9	3.3
Medical treatment		
Catheter-based therapy for CHD	130	47.1
Cardiac surgery for CHD	108	39.1
Follow-up examination	38	13.8

Data are shown as a mean ± standard deviation, frequency number, percentage, or range. CHD: congenital heart defects/disease; ASD: atrial septal defect; VSD: ventricular septal defect; PDA: patent ductus arteriosus; TOF: tetralogy of Fallot; DORV: double outlet of right ventricle; AS: aortic stenosis; TGA: transposition of the great arteries; PS: pulmonary stenosis; PTA: persistent truncus arteriosus; AVB: atrioventricular block; AF: atrial fibrillation.

with an average age of 4.1 ± 2.7 years) was clinically analyzed in contrast to 288 unrelated volunteers without CHD (127 female volunteers and 161 male volunteers, with a mean age of 4.2 ± 2.6 years). All the research participants were recruited from the Chinese Han-race population in Shanghai, China. The included CHD-affected probands possessed definite echocardiographic evidence, whilst the enlisted control volunteers' echocardiograms were normal, without proof showing cardiovascular structural malformations. Of the 276 probands inflicted with CHD, 38 probands had a familial history of CHD, whereas the 288 control individuals' parents explicitly denied a familial history of CHD. No study individuals had recognized secondary precipitating factors prone to CHD, encompassing maternal hyperhomocysteinemia, phenylketonuria, diabetes mellitus, hypothyroidism, essential hypertension, acute febrile illness, epilepsy, pre-eclampsia, nutritional deficiency, connective tissue disease, as well as exposure to therapeutic drugs, toxicants, and ionizing radiation during the first trimester of gestation, and most CHD-affected probands underwent catheter-based cardiac intervention or cardiac surgery. The demographic and phenotypic characteristics of the 276 probands affected with CHD are summed in **Table 1**.

Discovery of a CHD-causing mutation in BMP10

Through sequencing assay of the coding regions along with the splicing junction boundaries of the *BMP10* gene in 276 index patients affected with distinct kinds of CHD, a new *BMP10* mutation, namely NM_014482.3:c.247G>T;p.(Glu83*), was discovered to be in a heterozygous status in one seven-year male index patient with congenital PDA. PCR-sequencing analysis of *BMP10* in the available pedigree members of the mutation carrier revealed that the nonsense mutation was in co-segregation with the PDA phenotype, which was inherited in an autosomal dominant pattern in the whole family (arbitrarily designated as Family C01), with complete (100%) penetrance. Additionally, two PDA-inflicted family members (II-7 and III-4 from Family C01) of the mutation-carrying proband also suffered from a congenital ventricular septal defect (VSD). All the five affected relatives (III-2, III-4, II-3, II-7, and I-1 from Family C01) of the mutation-carrying proband underwent transcatheter cardiovascular interventional repair for CHD. The mutation was neither observed in 288 non-CHD control people nor released in such genetics databases as SNP

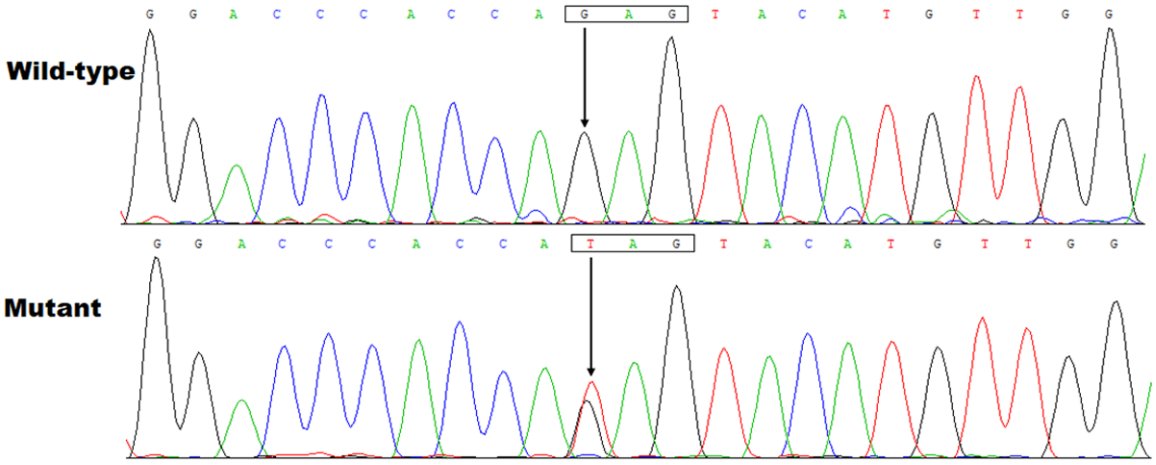

Figure 1. A novel *BMP10* mutation accountable for congenital cardiovascular deformities. The sequence chromatogram traces revealed the heterozygous *BMP10* mutation identified in the CHD-affected index patient (Mutant) as well as its homozygous control from an unaffected individual (Wild-type). A vertical arrow directs the nucleotide position where the mutation occurs. A rectangle delimits a codon of the *BMP10* gene.

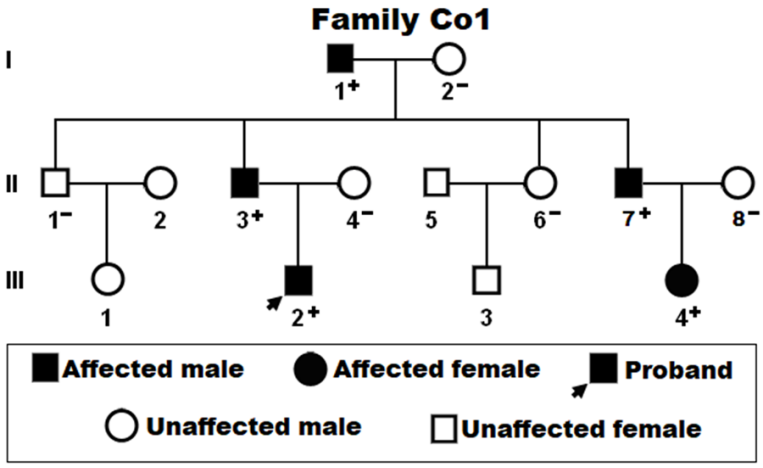

Figure 2. Pedigree inflicted with cardiovascular developmental malformations. An oblique arrow directs the proband. "+" signifies a carrier of the heterogeneous *BMP10* mutation; "-" denotes a non-carrier.

and gnomAD, highlighting the novelty of the identified BMP10 nutation linked to CHD. The sequencing chromatogram traces indicating the heterozygous c.247G>T mutation in *BMP10* and its control in a homozygous status are displayed in **Figure 1**. The pedigree of the proband harboring the identified BMP10 mutation is illustrated in **Figure 2**. In Family C01, there was a total of 14 family members available, encompassing seven male family members and the same number of female family members, with ages varying between 2 and 59 years. All the seven affected pedigree members from Family C01 had echocardiogram-documented PDA and experienced catheter-based interventional therapy for closure of PDA. No established environmental pathogenic factors susceptible to CHD were identified in all pedigree members. The clinical phenotypic profile and *BMP10* mutation status of the CHD-affected members from Family C01 are summarized in **Table 2**.

Functional impairment of Glu83-mutant BMP10 in transactivation of TBX20*

As depicted in **Figure 3**, in HeLa cells transfected with multiple expression vectors, including empty pcDNA3.1 vector as an external negative control (-), wild-type human BMP10-pcDNA3.1 vector (BMP10), and Glu83*-mutant human BMP10-pcDNA3.1 vector (Glu83*), singly or in combination, BMP10 and Glu83* transcriptionally activated the promoter of the *TBX20* gene by ~9-fold and ~1-fold, respectively (BMP10 vs Glu83*: t = 10.5275; P = 0.0005). When BMP10 and Glu83* were together transfected, the elicited transactivation effect was ~4-fold (BMP10 vs Glu83* + BMP10: t = 5.7909; P = 0.0044). Unanimous statistical results were yielded when multiple comparisons were conducted (F = 50.966, P =

Table 2. Clinical phenotypic profile and *BMP10* mutation status of the pedigree members from Family C01 suffering congenital cardiovascular structural deformities

Individual (Family C01)	Sex	Age (years)	Cardiovascular structural aberrations	BMP10 mutation (Glu83*)
I-1	Male	59	PDA	+/-
II-3	Male	33	PDA	+/-
II-7	Male	27	PDA, VSD	+/-
III-2	Male	7	PDA	+/-
III-4	Female	2	PDA, VSD	+/-

PDA: patent ductus arteriosus; VSD: ventricular septal defect; +/-: heterozygote for the *BMP10* mutation.

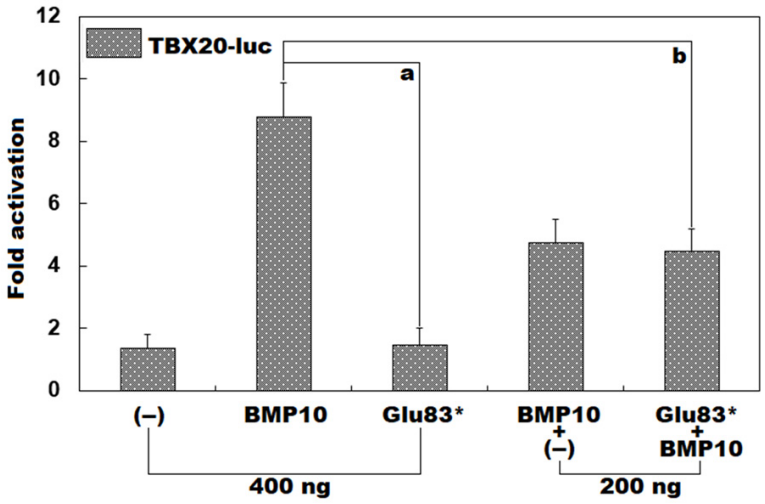

Figure 3. Functional failure of BMP10 caused by the Glu83* mutation. Dual-reporter gene analysis of the transactivation of the *TBX20* promoter-driven luciferase in maintained HeLa cells by wild-type human BMP10-pcDNA3.1 vector (BMP10) or Glu83*-mutant human BMP10-pcDNA3.1 vector (Glu83*), separately or together, revealed that Glu83* lost transactivation of *TBX20*. For each expression vector, reporter assay experiments were repeated three times in triplicate. Here, "a" and "b" signify $P < 0.001$ and $P < 0.005$, respectively, in comparison with BMP10 (0.4 μg).

1.289×10^{-6}). Specifically, for (-) vs BMP10, t = 7.4367; P < 0.0001; for (-) vs Glu83*, t = 0.0967; P = 0.9998; for (-) vs BMP10 + (-), t = 3.3800; P = 0.0016; for (-) vs Glu83* + BMP10, t = 3.1200; P = 0.0030; for BMP10 vs Glu83*, t = 7.3400; P < 0.0001; for BMP10 vs BMP10 + (-), t = 4.0567; P = 0.0004; for BMP10 vs Glu83* + BMP10, t = 4.3167; P = 0.0002; for Glu83* vs BMP10 + (-), t = 3.2833; P = 0.0020; for Glu83* vs Glu83* + BMP10, t = 3.0233; P = 0.0037; for BMP10 + (-) vs Glu83* + BMP10, t = 0.2600; P = 0.9917.

Failure of Glu83-mutant BMP10 to transcriptionally activate NKX2.5*

As presented in **Figure 4**, in HeLa cells transfected with multiple expression vectors, including empty pcDNA3.1 as a negative control (-), wild-type human BMP10-pcDNA3.1 (BMP10), and Glu83*-mutant human BMP10-pcDNA3.1 (Glu83*), singly or together, BMP10 and Glu83* transactivated the promoter of the *NKX2.5* gene by ~13-fold and ~1-fold, respectively (BMP10 vs Glu83*: t = 12.3295; P = 0.0002). When BMP10 and Glu83* were together expressed, the elicited transcription activity was ~7-fold (BMP10 vs Glu83* + BMP10: t = 6.0492; P = 0.0039). Congruous statistical results were acquired when multiple comparisons were implemented ($F = 88.45$, $P = 9.20 \times 10^{-8}$). Specifically, for (-) vs BMP10, t = 11.3200; P < 0.0001; for (-) vs Glu83*, t = 0.1400; P = 0.9996; for (-) vs BMP10 + (-), t = 5.9300; P = 0.0001; for (-) vs Glu83* + BMP10, t = 5.5267; P = 0.0001; for BMP10 vs Glu83*, t = 11.1800; P < 0.0001; for BMP10 vs BMP10 + (-), t = 5.3900; P = 0.0001; for BMP10 vs Glu83* + BMP10, t = 5.7933; P = 0.0001; for Glu83* vs BMP10 + (-), t = 5.7900; P = 0.0001; for Glu83* vs Glu83* + BMP10, t = 5.3867; P = 0.0001; for BMP10 + (-) vs Glu83* + BMP10, t = 0.4033; P = 0.9768.

Discussion

In the current human investigation, via Sanger sequencing assay of the *BMP10* gene in a larger cohort of 276 probands affected with distinct types of CHD, a new heterozygous *BMP10* mutation, termed NM_014482.3:c.247G>T;p.(Glu83*), was found in one seven-year male

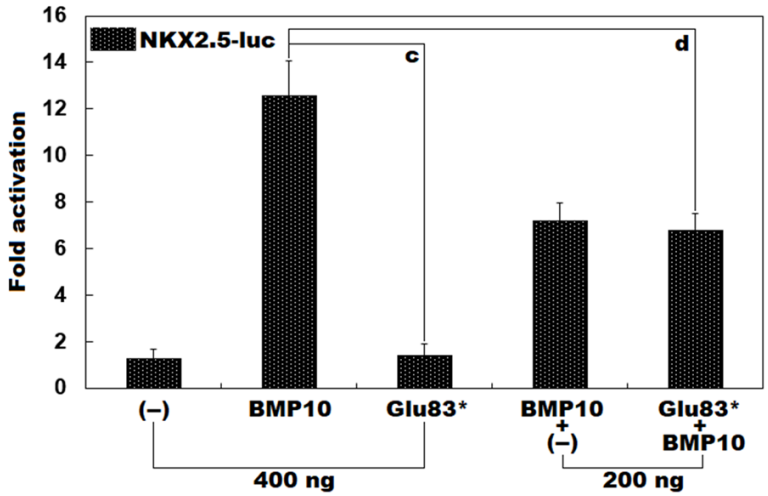

Figure 4. Diminished ability of Glu83*-mutant BMP10 to transcriptionally activate *NKX2.5*. In the HeLa cells grown in vitro, a dual-reporter gene gauge of the activation of the *NKX2.5* promoter-driven luciferase by wild-type human BMP10 expression vector (BMP10) or Glu83*-mutant human BMP10 expression vector (Glu83*), alone or in combination, unveiled that Glu83* possessed no ability to transactivate *NKX2.5*. For each expression plasmid utilized for reporter gene analysis, three cellular transfection experiments were fulfilled in triplicate. Here "c" denotes $P < 0.001$ and "d" indicates $P < 0.005$, in comparison with BMP10 (0.4 μg).

proband with congenital PDA. Sequencing assay of *BMP10* in the pedigree members available from the index patient harboring the *BMP10* mutation revealed that the mutation was co-segregated with congenital PDA in the mutation carrier's whole family. The nonsense *BMP10* mutation was neither observed in the 576 referential chromosomes from 288 non-CHD volunteers nor released from such genetics databases as HGMD, dbSNP, and gnomAD. Quantitative measurement of reporter gene activities in the HeLa cells grown in vitro unveiled that Glu83*-mutant BMP10 failed to transcriptionally activate its two representative target genes *TBX20* and *NKX2.5*, which were both commonly implicated with CHD [1, 3, 60-62]. These findings present convincing evidence supporting that genetically defective *BMP10* predisposes humans to CHD.

The human *BMP10* gene was located on chromosome 2p13.3, which encodes a growth factor peptide with 424 amino acid residues, an important player of the BMP family of ligands, pertaining to the TGFβ superfamily that profoundly mediates the genesis, growth, and maturation of cardiovascular system [106, 107]. The members of the BMP family regulate a diverse array of developmental events throughout embryogenesis in a broad range of species ranging from insects to mammals [106, 107]. Although all the BMP family members share a similar protein structure, each BMP member possesses a different profile of tissue expression and a unique physiological role [108, 109]. To date, no less than six members of the BMP family were substantiated to be expressed in the developing heart, encompassing BMP2, BMP5, BMP6, BMP4, BMP7, and BMP10, of which merely BMP10 was validated to be specifically and amply expressed in the developing heart [102, 110]. BMP10 is abundantly expressed in the hearts of humans as well as mice and chicks [107], and in the embryonic hearts, BMP10 is much more enriched in the myocardial trabeculae, though throughout the heart the expression of BMP10 can be detected [102]. It has been validated that BMP10 induces intracellular signaling through the receptor complex of ALK1 with morphogenetic protein receptor (type II) or activin receptor (type 2A) [104, 111]. Recent investigations have demonstrated that BMP10 activates two critical intracellular signaling pathways, namely the canonical pathway regulated by SMAD and the noncanonical pathway regulated by STAT3 [107], and elicits the expression of multiple downstream genes paramount to normal cardiovascular development via the SMAD-binding consensus sequences located in the promoters of downstream genes, such as *NKX2.5, MEF2C*, and *TBX20* [102, 112, 113], three key genes to proper cardiovascular morphogenesis and deleterious mutations in all the three genes have been identified to be accountable for CHD [114-121]. In the present human research, the found Glu83* mutation was predicted to create a truncated BMP10 protein without key structural domains, and biological analysis indicated that Glu83*-mutant BMP10 lost the ability to activate the expression of *NKX2.5* and *TBX20*. These results suggest that BMP10 haploinsufficiency

is one of the molecular mechanisms underpinning CHD in humans.

It may be attributed to cardiovascular developmental anomalies that genetically defective *BMP10* gives rise to CHD. In mice, homozygous knockout of *Bmp10* brought about embryonic demise because of severe defects in cardiovascular development, although the mice with heterozygous deletion of Bmp10 were viable with fertile function [102]. In the *Bmp10*-deficient embryos of mice, cardiac organogenesis was arrested with pronounced cardiac hypoplasia without ventricular trabeculae, mainly due to a strikingly diminished proliferation of Bmp10-null embryonic cardiomyocytes, and the anomalous morphogenesis of endocardial cushion was observed in the outflow tract and atrioventricular canal, which was terminated at the acellular stage [102]. Moreover, in mice, a double ablation of *Bmp10* and *Bmp9* led to vascular defects with high-output cardiac failure and pulmonary hemosiderosis, though the mice with a single ablation of *Bmp10* or *Bmp9* appeared to be normal with no obvious cardiovascular defects [122]. Additionally, analysis of *Bmp10*$^{9/9}$ mice, where *Bmp10* was replaced by *Bmp9* (*Bmp9* knocked in at the *Bmp10* locus), unveiled that *Bmp10* had an exclusive role in cardiovascular development, which couldn't be substituted or fully compensated by ectopic expression of *Bmp9* [103]. Specifically, *Bmp10*$^{9/9}$ hearts manifested hypoplasia with dramatically thinner ventricular walls and obvious pericardial edema, and most *Bmp10*$^{9/9}$ hearts also had congenital VSD, in addition to markedly reduced myocardial proliferation and growth as well as changed cardiac shape [103]. Notably, Levet and colleagues [104] explored the effects of *Bmp10* and *Bmp9* on the closure of the ductus arteriosus in mice and found that *Bmp9* knockout resulted in an imperfect closure of the ductus arteriosus. Furthermore, at postnatal day 1 and day 3, administration of an anti-BMP10 neutralizing antibody exacerbated the anomalous remodeling and caused a reopening of the ductus arteriosus at postnatal day 4 in these pups [104]. Taken together, these observational data from experimental animals establish an essential role of *BMP10* in cardiovascular morphogenesis, especially in myocardial growth, ventricular chamber maturation, and closure of ductus arteriosus.

Noticeably, rare *BMP10* variations have been involved in dilated cardiomyopathy in humans [106, 123]. Gu et al. [106] recruited a multigenerational pedigree suffering from autosomal-dominant dilated cardiomyopathy from the Chinese population of Han ethnicity and conducted a whole-exome sequencing assay of the DNAs available from the pedigree members. Consequently, a *de novo* rare *BMP10* mutation, NM_014482.3:c.166C>T;p.(Gln56*), was identified and confirmed by sequencing analysis to co-segregate with dilated cardiomyopathy in the family. Functional deciphering through dual-luciferase activity measurements showed that Gln56*-mutant BMP10 had no transcriptional activation on its two representative target genes *TBX20* and *NKX2.5* [106]. Nakano et al. [123] enrolled 36 patients suffering from familial dilated cardiomyopathy, 97 patients suffering from non-familial dilated cardiomyopathy, and 46 cases with hypertensive dilated cardiomyopathy, and performed the analyses of all exons of the human *BMP10* gene encompassing flanking 5'- and 3'-untranslated regions by using the single-strand conformation polymorphism, clone sequencing, and *Bam*HI enzyme digestion methods. As a result, in addition to two common single nucleotide polymorphisms, a novel rare *BMP10* variant, namely NM_014482.3:c.977C>T;p.(Thr326Ile), was discovered in a case suffering from hypertensive dilated cardiomyopathy and then detected in his/her father with hypertensive dilated cardiomyopathy. The significant association of this *BMP10* variation (c.977C>T) with hypertensive dilated cardiomyopathy was further confirmed by analyzing a larger population (1,382 elderly consecutive cases, including 616 hypertensive and 766 normotensive cases). The biological assay demonstrated that the Thr326Ile variant significantly decreased the ability of BMP10 to bind Titin-cap (Tcap) at Z discs of myocardial cells and facilitated the extracellular secretion of BMP10 (the intracellular amount of endogenous BMP10 was markedly decreased by the Thr326Ile variation) [123]. In the present research, a new *BMP10* mutation, namely NM_014482.3:c.247G>T;p.(Glu83*), was found in a pedigree affected with PDA, separately or in combination with VSD, hence expanding the phenotypic spectrum correlated to *BMP10* mutations.

There are many studies which have reported that BMP10, a cardiac-restricted BMP family member, plays a critical role in regulating the development of the heart. The most interesting feature of BMP10 is its transient presence in

the developing trabecular myocardium [102] and the BMP10-TBX20 signaling cascade is important for ventricular wall development and maturation [112]. NKX2.5 is one of the BMP10-activated cardiogenic transcription factors [124]. In this study, a novel *BMP10* mutation (c.247G>T;p.Glu83*) was identified in a family suffering from PDA/VSD, and quantitative analysis of dual-luciferase activities in maintained HeLa cells unveiled that Glu83*-mutant BMP10 had no transactivation on its two representative target genes TBX20 and NKX2.5. Hence, the current study firstly indicates that BMP10 loss-of-function mutation predisposes to CHD in humans, mainly by reducing the expression of TBX20 and NKX2.5.

There are some limitations to the present investigation. Firstly, a novel heterozygous *BMP10* mutation, NM_014482.3:c.247G>T;p.(Glu83*), was found in a family with PDA/VSD. There is a need to further investigate the mechanism that causes mutations in this gene. Secondly, a deleterious *BMP10* mutation was identified via candidate gene analysis. Hence, it cannot be ruled out that other genetic defects may also contribute to the pathogenesis of CHD. Whole genome or exome sequencing analysis may help address this issue. Thirdly, the subcellular distribution of the Glu83*-mutant BMP10 protein, as well as its ability to bind the promoters of target genes, remains to be clarified. Finally, the pathogenic effect of the *BMP10* mutation is still to be further investigated in a genetically modified animal model, such as a mouse model with the mutation knocked in.

Conclusion

The present human investigation firstly indicates *BMP10* as a novel gene causative for human CHD and adds new insight into the molecular pathogenesis of human CHD, which is conducive to prenatal genetic diagnosis, early clinical prophylaxis, and timely prognostic risk assessment of CHD in a subset of patients.

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Disclosure of conflict of interest

None.

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