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SHORT COMMUNICATION

Analysis of *HOXB1* gene in a cohort of patients with sporadic ventricular septal defect

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Abstract

Ventricular septal defect (VSD) including outlet VSD of double outlet right ventricle (DORV) and perimembranous VSD are among the most common congenital heart diseases found at birth. *HOXB1* encodes a homeodomain transcription factor essential for normal cardiac outflow tract development. The aim of the present study was to investigate the possible genetic effect of sequence variations in *HOXB1* on VSD. The coding regions and splice junctions of the *HOXB1* gene were sequenced in 57 unrelated VSD patients. As a result, a homozygous c.74_82dup (p.Pro28delinsHisSerAlaPro) variant was identified in one individual with DORV. We also identified five previously reported polymorphisms (rs35114525, rs12946855, rs14534040, rs12939811, and rs7207109) in 18 patients (12 DORV and 6 perimembranous VSD). Our study did not show any pathogenic alterations in the coding region of *HOXB1* among patients with VSD. To our knowledge this is the first study investigating the role of *HOXB1* in nonsyndromic VSD, which provide more insight on the etiology of this disease.

Keywords Genetics · Ventricular septal defect · *HOXB1* · Variant · Congenital heart disease

Introduction

Congenital heart diseases (CHD) are the most common human birth defects, affecting nearly 8–10/1000 live births [1]. Ventricular septal defect (VSD) including outlet VSD of

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double outlet right ventricle (DORV) and perimembranous VSD (pmVSD) constitutes one of the major categories of CHD, with a prevalence of 30–40%, and can lead to significant mortality and morbidity both in children and adults if not repaired [1–3]. The cause of the CHD is often difficult to determine with certainty, nonetheless, studying factors that control heart development can help to better understand the etiology of these defects.

Heart development is a complex and ordered process that is spatially and temporally regulated [4]. Recent studies have shown that elongation of the embryonic heart is driven by addition of cardiac progenitor cells from adjacent pharyngeal mesoderm to the arterial and the venous poles [5]. This cell population named the second heart field (SHF), is surrounded by neural crest cells. The SHF is characterized by the expression of different genes including the transcription factors *Islet1, Nkx2-5* and *T-box1* (see [5, 6]). Genetic lineage tracing and retrospective clonal analysis experiments have shown that anterior SHF cells contributes to the formation of outflow tract (OFT) and right ventricular myocardium, whereas cardiac progenitors at the posterior region of the SHF contributes to a large part of the atrial myocardium [7]. Since its discovery, there has been accumulating evidence that direct or indirect perturbation of the SHF is associated with heart defects including DORV and VSD [8–10].

HOX genes form a large family of homeodomain transcription factors that play an important role in regulating positional identity along the anterior–posterior axis [11]. Mammalian *HOX* genes are organized into four different clusters and are expressed in defined and often overlapping domains along the body axis in a manner corresponding to their position along the chromosome (temporal and spatial colinearity) [12]. *HOX* gene expression in the mammalian heart was initially characterized in the chick, where expression of *HOXA4, HOXD3* and *HOXD4* was detected at early stages of cardiogenesis [13]. A recent study indicated that targeted deletion of the *HOXA1* gene in mouse embryos results in cardiovascular malformations including great artery patterning defects, VSD, and Tetralogy of Fallot (ToF) [14]. Interestingly, Soshnikova N et al. recently showed that when both *HoxA* and *HoxB* clusters were deleted together the embryos failed to undergo cardiac looping [15].

Our recent works indicated that *HOXB1* gene is potentially related to mammalian cardiovascular development. In the mouse, the *Hoxb1* gene is expressed in the posterior region of the SHF that contribute to both poles of the heart tube, including the atrio-ventricular canal and atrial myocardium at the venous pole, and the inferior wall of the mid-gestation OFT, which gives rise sub-pulmonary myocardium at the arterial pole [16]. Consistent with this observation, we have demonstrated that loss of function of *Hoxb1* in mouse embryos results in misalignment of the great arteries (7%), interrupted aortic arch (4%) and VSD (21%) [17, 18]. Together these finding suggest that *HOXB1* gene plays a previously unknown role in cardiovascular development and particularly to the formation of the inter-ventricular septum. The purpose of this study was to examine whether a mutation in *HOXB1* gene may be associated with nonsyndromic VSD in humans.

Materials and methods

Study population

57 individuals with DORV with outlet VSD (n = 38) and with pmVSD (n = 19) presenting with a sporadic and non-syndromic OFT defect were recruited in our study from the Necker-Enfants Malades Hospital. The DNA of each individual was conserved at the biological resources center of Necker-Enfants Malades Hospital. Personal health data and DNA from each patient are part of the CARREG study (http://carreg.fr/en/), which was declared to the French national committee for informatics and liberties (France; CNIL; No. 1734573V0). The CARREG study is a prospective monocenter study promoted by the Centre de Référence des Malformations Cardiaques Congénitales Complexes (M3C) located at the Pediatric cardiology department of the Necker-Enfants Malades Hospital, Paris, France. Clinical records were reviewed by pediatric-cardiologist before recruitment and cardiovascular diagnosis was obtained by echocardiography mainly. Computed tomography (CT) would be performed when needed. Patients with 22q11.2 deletion or other recognized syndromes were excluded. Patients were 7.6 ± 2.8 years old; 30 boys and 27 girls. Fully informed consent was obtained from all participants and the parent or guardians provided written informed consent.

Genomic DNA extraction and polymerase chain reaction (PCR) amplification

Genomic DNA was extracted from the peripheral leukocytes using the Wizard Genomic DNA Purification Kit (Promega). *HOXB1* maps on chromosome 17q21.3 and extends on 2.47 kilobases (kb). The gene has 2 exons and encodes two transcripts of 2.02 and 1.41 kb. The exons and flanking introns of *HOXB1* were generated with polymerase chain reaction (PCR). The design of primers was based on the genomic sequence of human *HOXB1* (Genbank access no. NM_002144), as shown in Table 1. The PCR products were sequenced using appropriate PCR primers and the BigDye Terminator Cycle Sequencing kit (Applied Biosystems) and run on an automatic sequencer ABI 3130XL (Applied Biosystems) to perform mutational analysis.

Table 1 PCR primer sequences for *HOXB1*

Exon	Forward primer	Reverse primer	PCR product (bp)
1.1	TTGTAGGGCAAGAGGGTGTC	AGGGATGAAAATAGCCTCCG	397
1.2	CTGCAGCCCCAGCTACG	TGCACAGATCAGGAAGTGGG	540
2	TGGCAAATGGAGGTCCAGAG	GTTGGGGAGAGCCTGGGATA	502

Sequence analysis

Sequences from patients were aligned to the reference sequence of *HOXB1* using the Seqscape software (Applied Biosystems). Pathogenicity of variants was evaluated by UMD-Predictor (http://umd-predictor.eu) [19]. SNPs frequencies in general population are extracted from the Genome Aggregation Database (GnomAD), Cambridge, MA (http://gnomad.broadinstitute.org/) [03/2017 accessed] [20]. The schematic representation of variations on the HOXB1 protein (UniPortKB-P14653) was done with the IBS software [21].

Results and discussion

Using the DNAs from 57 patients with outlet VSD of DORV (n = 38) and pmVSD (n = 19), we identified six different *HOXB1* variations. Four coding SNPs are synonymous: p.Leu52Leu, p.Ser79Ser, p.Ser82Ser and p.Ala150Ala (Table 2; Fig. 1b). One non-synonymous variant (rs12939811) leads to a missense substitution of a Glutamine into an Histidine residue (c.309A>T, p.Gln103His). This variant was observed at the homozygous state in two patients, one with outlet VSD of DORV and one with pmVSD (Table 3). It is considered as a natural variant as it is predicted as polymorphism by UMD-Predictor [19] and has a high reported allele frequency of 0.1606 in the Genome Aggregation Consortium (GnomAD) (Table 3). Indeed, expected allele frequency would be up to 0.003 according to VSD prevalence of 0.3% in the general population [22].

Our analysis revealed also one intragenic duplication in the homozygous state for one patient with outlet VSD of DORV (Tables 2, 3). The same variation was detected in the heterozygous state in 8 patients with outlet VSD of DORV and 5 patients with pmVSD (Table 3). This in frame insertion of 9 nucleotides in the codon 27 (c.74_82dup) predicts the duplication of the $His_{25}Ser_{26}Ala_{27}$ sequence (p.Pro28delinsHisSerAlaPro) and was previously reported as allele *HOXB1* [23]. Although predicted as a "disease causing" by Mutation Taster [24] and not previously reported in ExAC database this variation is found in the GnomAD with a high allele frequency of 0.1740 in the European population. The electropherograms displaying the identified heterozygous and homozygous *HOXB1* duplications in contrast to its corresponding control sequence are showed in Fig. 1a. The duplication is located at the N-terminal region of HOXB1 protein.

CHD, which is seriously harmful to the health of young children, arises from abnormal heart development during embryogenesis. Embryological mechanisms of CHD have been extensively studied [2]. Our recent studies have indicated that *HOXB1* plays a formerly unrevealed role during heart development. In situ hybridization experiments showed

Table 2 In silico analyses of identified variants in the exon regions of *HOXB1*

Exon	Sequence variation	Amino acid variation	rs number	UMD-predictor	Mutation taster	Human splicing finder
1	c.74_82dup	p.Pro28delinsHisSerAlaPro	rs145570960	NA	Disease causing	Significant alteration of ESE/ESS motif ratio
1	c.154C>T	p.Leu52Leu	rs35114525	Polymorphism	Disease causing	Significant alteration of ESE/ESS motif ratio
1	c.237C>T	p.Ser79Ser	rs12946855	Polymorphism	Polymorphism	No significant impact on splicing signal
1	c.246G>A	p.Ser82Ser	rs145345040	Polymorphism	Disease causing	No significant impact on splicing signal
1	c.309A>T	p.Gln103His	rs12939811	Polymorphism	Polymorphism	Significant alteration of ESE/ESS motif ratio
1	c.450G>A	p.Ala150Ala	rs7207109	Polymorphism	Polymorphism	No significant impact on splicing signal

ESE exonic splicing silencer, *ESS* exonic splicing enhancer

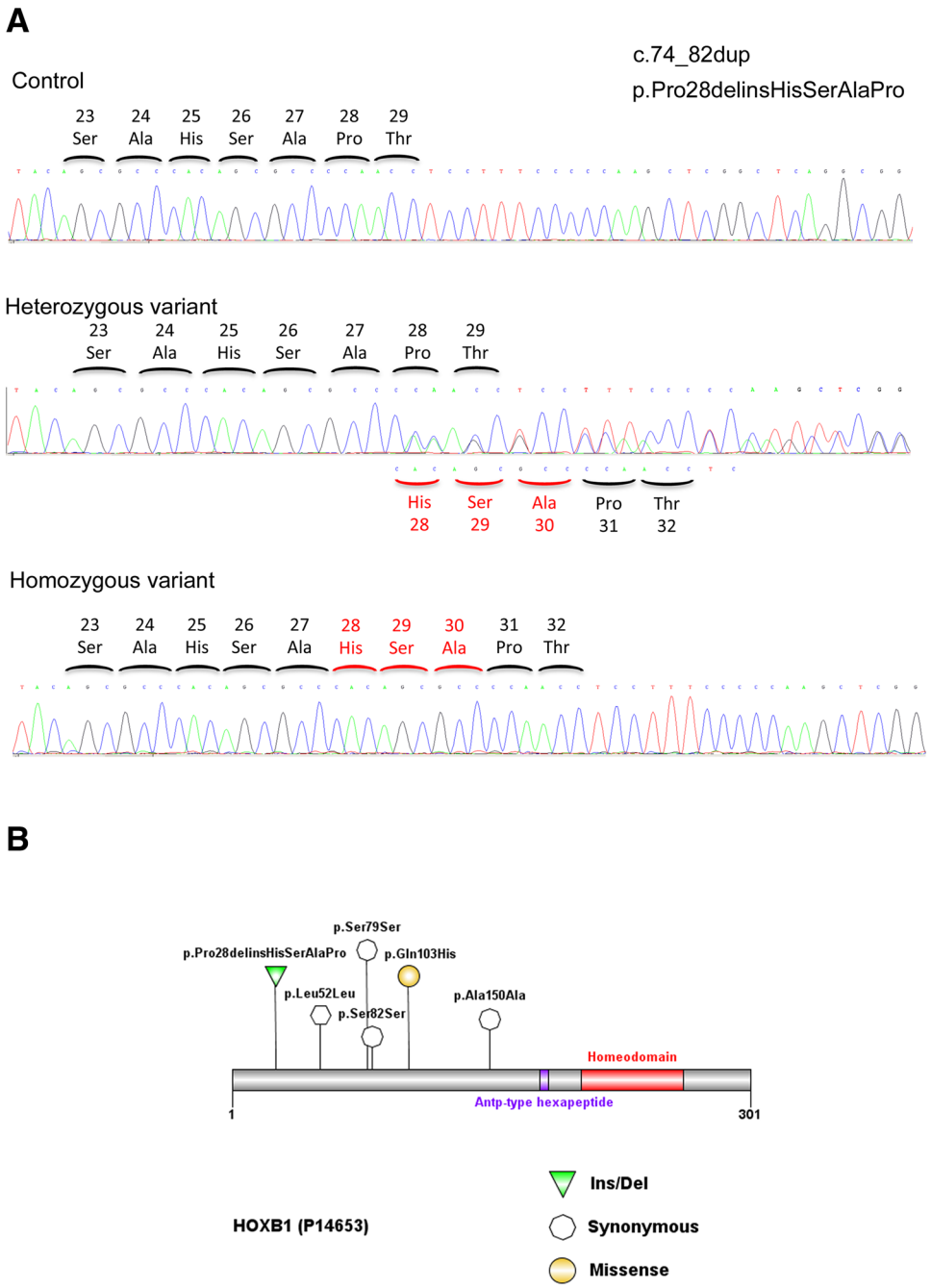

Fig. 1 **a** DNA sequencing electropherograms showing duplication variant identified in *HOXB1*. **b** Schematic representation of the structure of the human HOXB1 protein and the positions of variants in the HOXB1 identified in VSD patients

that *Hoxb1*, as a retinoic acid target gene, is expressed in a distinct subdomain of the SHF contributing to the inferior myocardial wall of the OFT [16]. Further analysis revealed that *Hoxb1* is necessary to correct balance between proliferation and differentiation of cardiac progenitors located within the SHF. In addition, in *Hoxb1*$^{-/-}$ embryos the length midgestation OFT is shorter compared to wild-type littermates [17]. Consistently, VSD are observed in *Hoxb1*-null mice. Because *HOXB1* is highly conserved during evolution it is probable that it could play a role in heart development in humans.

We therefore speculated that *HOXB1* is related to the development of the heart and that *HOXB1* mutations might cause CHD such as VSD. In this study, the exons and flanking regions of *HOXB1* were examined for potential pathogenic variants in patients with outlet VSD of DORV and pmVSD. However, we did not find any pathogenic variants in the coding regions. Therefore, we conclude that VSD cannot be a clinical isolated manifestation of *HOXB1* mutations. In the study by Webb et al. [25], homozygous missense (p.Arg207Cys) mutation in *HOXB1* was identified in two families with congenital facial paralysis

Table 3 Genotype of patients with Double outlet right ventricle (DORV) or Perimembranous ventricular septal defect (VSD) compared to gnomAD population

Exon	Sequence variation	gnomAD			Our total populations			DORV			pmVSD		
		HZ wt	HTZ	HZ mt	HZ wt	HTZ	HZ mt	HZ wt	HTZ	HZ mt	HZ wt	HTZ	HZ mt
1	c.74_82dup	82.550	14.500	2.950	75% (43)	23% (13)	2% (1)	74% (26)	23% (8)	3% (1)	74% (14)	26% (5)	0% (0)
1	c.154C>T	99.520	0.450	0.030	98% (56)	2% (1)	–(0)	100% (35)	0% (0)	0% (0)	95% (18)	5% (1)	0% (0)
1	c.237C>T	82.400	14.200	3.400	72% (41)	25% (14)	3% (2)	67% (22)	30% (10)	3% (1)	69% (13)	26% (5)	5% (1)
1	c.246G>A	99.397	0.600	0.003	93% (53)	7% (4)	– (0)	88% (29)	12% (4)	0% (0)	100% (19)	0% (0)	0% (0)
1	c.309A>T	83.900	13.200	2.900	77% (44)	20% (11)	3% (2)	76% (28)	21% (8)	3% (1)	79% (15)	16% (3)	5% (1)
1	c.450G>A	82.650	14.000	3.350	72% (41)	25% (14)	3% (2)	72% (26)	25% (9)	3% (1)	69% (13)	26% (5)	5% (1)

HZ wt homozygous wild-type, *HTZ* heterozygous carrier of the variation, *HZ mt* homozygous carrier of the variation

reported as Moebius syndrome (MIM 157900). While Moebius syndrome is a spectrum of congenital malformations, which essentially comprises a complete or partial facial nerve palsy, cardiac anomalies were rarely reported including dextrocardia, atrial septal defect, supracardiac variety of total anomalous pulmonary venous connection, single ventricle, transposition of the great arteries, and VSD [26–29]. The presence of VSD in patients with Moebus syndrome anomalies, suggests that VSD could be one characteristic of *HOXB1* mutations but not necessary an isolated sign of the mutation.

Interestingly, we found a *HOXB1* gene duplication previously reported [23, 30]. The homozygous variation was observed in one individual with outlet VSD of DORV. This insertion is located in the N-terminal region at the amino acid 28 of the protein and is predicted as causing disease in Mutation Taster [24]. While the role of this particular N-terminal region of *HOXB1* has not been fully established, the study by Di Rocco et al. [31], has revealed that deletion of the N-terminal region (amino acid 1-155) of HOXB1 caused a reduction in the activity of the HOXB1-PBX1 complex compared to the wild-type HOXB1. However, further investigation is necessary to fully characterize potential functional effect of the in-frame insertion identified in our study.

Our study has certain limitations. It analyzed a small cohort of patients with VSD including outlet VSD of DORV and pmVSD. Moreover, we cannot exclude that potential damaging variation in *HOXB1* may be present in the general population without causing cardiovascular anomalies, due to incomplete penetrance or modifying factors, increasing therefore its frequency in the databases. We cannot also exclude a potential effect of some of these variants through splicing alterations as predicted by HSF analyses (Table 3) [32]. Our recent studies demonstrated an overlap function between Hoxa1 and Hoxb1 during cardiovascular development in mice, which might be explained by the overlapping expression pattern and redundancy of these two factors during early development [17, 18]. While early *HOXA1* and *HOXB1* expression patterns have not been identified yet in human embryos, we cannot exclude that compensatory effect observed in the mouse was conserved during evolution. Expression and functional data gained in mouse models suggest that the role of *Hoxb1* is crucial for correct SHF formation, whereas *Hoxa1* function in the SHF is only revealed when one copy of *Hoxb1* is deleted [17, 18]. Interestingly, clinical studies showed that individuals carrying a homozygous truncating mutation in *HOXA1* have cardiovascular malformations including VSD, ToF and double aortic arch [33, 34]. These studies further suggest that *HOXA1* mutations might cause isolated VSD. Based on these findings we speculate that isolated VSD can be also caused by a combination of heterozygous variation in *HOXA1* and *HOXB1* genes. In conclusion, further studies are needed to explore

the role of *HOXB1* alone or in combination with that of *HOXA1* during heart development in humans.

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Author Contributions AP, NE, MG, JM, and JO performed the experiments. GC-B supervised the genetic and statistical analysis and drafted parts of the manuscript. FB and DB coordinated the CARREG protocol and validated the diagnosis of patients. SZ coordinate the genetic investigation and wrote the manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2008. The study subjects included seventy-seven patients from the Necker-Enfants Malades Hospital. This study was approved by the Committee for the Protection of Persons (Comité de Protection des Personnes (CPP) Paris, France No. 2009-164).

Informed consent Informed consent was obtained from all patients for being included in the study.

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