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# The Functional Polymorphism R129W in the *BVES* Gene Is Associated with Sporadic Tetralogy of Fallot in the Han Chinese Population

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***Background:*** Tetralogy of Fallot (TOF) accounts for ~10% of congenital heart disease cases. The blood vessel epicardial substance (*BVES*) gene has been reported to play a role in the function of adult hearts. However, whether allelic variants in *BVES* contribute to the risk of TOF and its possible mechanism remains unknown.
***Methods:*** The open reading frame of the *BVES* gene was sequenced using samples from 146 TOF patients and 100 unrelated healthy controls. qRT-PCR and western blot assays were used to confirm the expression of mutated *BVES* variants in the TOF samples. The online software Polyphen2 and SIFT were used to predict the deleterious effects of the observed allelic variants. The effects of these allelic variants on the transcriptional activities of genes were examined using dual-fluorescence reporter assays.
***Results:*** We genotyped four single nucleotide polymorphisms (SNPs) in the *BVES* gene from each of the 146 TOF patients. Among them, the minor allelic frequencies of c.385C>T (p.R129W) were 0.035% in TOF, but ~0.025% in 100 controls and the Chinese Millionome Database. This allelic variant was predicted to be a potentially harmful alteration by the Polyphen2 and SIFT softwares. qRT-PCR and western blot analyses indicated that the expression of *BVES* in the six right ventricular outflow tract samples with the c.385C>T allelic variant was significantly down-regulated. A dual-fluorescence reporter system showed that the c.385C>T allelic variant significantly decreased the transcriptional activity of the *BVES* gene and also decreased transcription from the *GATA4* and *NKX2.5* promoters.
***Conclusions:*** c.385C>T (p.R129W) is a functional SNP of the *BVES* gene that reduces the transcriptional activity of *BVES in vitro* and *in vivo* in TOF tissues. This subsequently affects the transcriptional activities of *GATA4* and *NKX2.5* related to TOF. These findings suggest that c.385C>T may be associated with the risk of TOF in the Han Chinese population.

**Keywords:** Tetralogy of Fallot, *BVES*, single nucleotide polymorphism, second heart field genes

## Introduction

Congenital heart disease (CHD) is the most common congenital abnormality, with an incidence rate of 7–10 in 1000 live births (Mendieta-Alcantara *et al.*, 2013; Leirgul *et al.*, 2014). Tetralogy of Fallot (TOF) is the most common cyanotic CHD phenotype, and occurs in 1 in 2500 live births (Hoffman and Kaplan, 2002). The overall prevalence of CHD is ~10.0 per 1000 live births (Liu *et al.*, 2015; Qu *et al.*, 2016), with TOF accounting for ~10% of the total (Di Felice and Zummo, 2009; Morgenthau and Frishman, 2018).

TOF and its variants have been observed as part of heritable syndromes, such as Alagille syndrome and DiGeorge syndrome. Allelic variants in Jagged1 (*JAG1*) (Notch signal receptor) and Hes-related family BHLH transcription factor with YRPW motif 2 (*HEY2*) (Notch signal target gene) have been linked to Alagille syndrome (Eldadah *et al.*, 2001; Fischer *et al.*, 2004; Hofmann *et al.*, 2012); and microdeletions

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of 22q11 (Devriendt *et al.*, 1998; Ito *et al.*, 2002; Conti *et al.*, 2003; Xu *et al.*, 2011) affecting the T-Box 1 (*TBX1*) gene have been associated with DiGeorge syndrome (Devriendt *et al.*, 1998; Ito *et al.*, 2002; Conti *et al.*, 2003; Xu *et al.*, 2011). TOF has a high mortality rate (Bertranou *et al.*, 1978; Starr, 2010) resulting in only ∼30% of TOF cases being familial; the remaining 70% are sporadic and of unknown etiology (Kola *et al.*, 2011).

Although TOF is a CHD with complex structural defects, it is caused by a single structural dysplasia (Sarris *et al.*, 2006; Sommer *et al.*, 2008). In the late stage of looping, cells from the anterior cardiac region of the secondary heart field (SHF) are added to the outflow tract area, which promotes outflow tract prolongation and the correct fusion of the outflow tract myocardial wall and ventricular septum; TOF will occur if incorrect fusion takes place (Waldo *et al.*, 2005; Bajolle *et al.*, 2006; Schleich *et al.*, 2013). In the animal models, knockout or haploinsufficiency of key genes in the SHF regulatory network, such as *Gata4* (Laforest and Nemer, 2011), *Nkx2.5* (Hami *et al.*, 2011; Guner-Ataman *et al.*, 2013), *Tbx1* (Xu *et al.*, 2004), T-box 20 (*Tbx20*) (Takeuchi *et al.*, 2005), and heart and neural crest derivatives expressed 2 (*Hand2*) (Morikawa and Cserjesi, 2008) may lead to abnormal outflow tract development. Therefore, abnormal expression of genes in the SHF regulatory network likely plays an important role in the occurrence of TOF.

In sporadic TOF patients, allelic variants account for ∼10% of cases (Greenway *et al.*, 2009), and genes in the SHF regulatory network are most frequently involved (Buckingham *et al.*, 2005; Topf *et al.*, 2014). For example, the first allele etiologically linked with sporadic TOF cases was the R25C variant of the *NKX2.5* gene. The rate of allelic variants of *NKX2.5* in TOF patients is ∼4% with the majority occurring among African American populations (Goldmuntz *et al.*, 2001; Akcaboy *et al.*, 2008; Gioli-Pereira *et al.*, 2010; De Luca *et al.*, 2011; Cao *et al.*, 2015). Variants in the *GATA4* gene, including N285S, have been identified, which affects binding to transcription factor binding sites for downstream genes (Zhang *et al.*, 2008, 2009; Peng *et al.*, 2010; Yang *et al.*, 2013; Yoshida *et al.*, 2016). Other genes such as Forkhead Box C1/2 (*FOXC1/2*), *HAND1/2*, T-box 5 (*TBX5*), and *TBX1* in the SHF regulatory network have also been reported to have variants associated with TOF (Wang *et al.*, 2011; Baban *et al.*, 2014; Topf *et al.*, 2014; Lu *et al.*, 2016; Huang *et al.*, 2017).

BVES, a highly evolutionarily conserved membrane protein, is highly expressed in adult hearts and skeletal muscles in vertebrates (Reese and Bader, 1999; Smith *et al.*, 2008). The expression of *BVES* changes in patients with heart failure (Gingold-Belfer *et al.*, 2011) and congenital ventricular septal defects (Zhang *et al.*, 2006). In adult mice, *Bves* is highly expressed in the conduction system, and *Bves* knockout mice have abnormal pacing under stress (Froese *et al.*, 2012). *BVES* variants are also identified in familial muscular dystrophy with arrhythmia (Schindler *et al.*, 2016) and in sporadic TOF patients (Wu *et al.*, 2013). Nevertheless, a clear association with TOF and an understanding of the possible mechanisms remain unclear.

In this study, we sequenced the *BVES* open reading frame using 89 tissue samples and 57 blood samples from patients with TOF, and identified four SNPs. Further analyses on the function of the SNPs were performed.

## Materials and Methods

### Samples

This study was approved by Ethics Committee of Hunan Normal University, Guangdong General Hospital, the Institutional Ethics Committee of Guangdong Academy of Medical Sciences, and the Institutional Ethics Committee of Sichuan Provincial People's Hospital. Written informed consent was obtained from each subject or their guardian.

Tissue and blood samples were obtained from patients and controls. TOF tissue samples were obtained from the hypertrophic muscle tissue of the right ventricular outflow tract (RVOT) during open-heart surgery for TOF. Normal samples were obtained from the RVOT tissue of persons who died accidentally and signed the consent for organ donation. Samples from patients with other congenital heart-related diseases were excluded from this study. After obtaining the tissue, it was quickly cut into small, 4–5 mm pieces with surgical scissors, placed into an Eppendorf tube, snap frozen in liquid nitrogen, and stored at −80°C before analysis.

### Sequencing and analysis of the purified DNA

Human genomic DNA was isolated from EDTA-anticoagulated blood or RVOT tissue using the proteinase K method as previously described (Tian and Wang, 2006). Each *BVES* exon was amplified with 10 ng of DNA template with the fragment sizes ranging from 400 to 700 bp; the PCR primers are shown in Table 1. The amplified DNA fragments were purified by gel extraction (TianGen) and subjected to direct sequencing on an ABI 3130XL instrument using BigDye terminators (v3.1). *BVES* produces three transcripts (A, B, and C) all of which encode proteins of 360 amino acids in length. We sequenced the coding sequence (from exon 2 to exon 8) of the *BVES* transcript B (NM_147147) (Wu *et al.*,

Table 1. Sequences of Primer Pairs for DNA Amplification and Mutational Analysis

| *Exon* | *Forward primer (5′-3′)* | *Reverse primer (5′-3′)* | *Length (bp)* | *Annealing temperature (°C)* |
|---|---|---|---|---|
| E02 | ATATATGTGCCTATGAGGGAATAGT | GCCTGACAAAGAATACTCTGAAGC | 615 | 57 |
| E03 | CTTCAGAAAAGGGAGGACTACTGTA | CAGAAAGCCTAAACTTCAGAGAAAT | 369 | 57 |
| E04 | AACTTCTATCACCATTTCACTTGCT | AACTCTGTGACTGCTTTCTCCATT | 479 | 57 |
| E05 | CATACCTCTGGCTCAATACTGGA | CCCAATAATTCATAGCAGTGTTTCT | 427 | 57 |
| E06 | ACGTGTAGAAGCTTGTGTGCA | TCTCAAGATTACAAGTATCCCCA | 464 | 57 |
| E07 | ATTTCTAGGAAAAGTACATGCTGCT | AAAGTATATGGCAAAACCAGTGAAC | 479 | 57 |
| E08 | TTCCTACAGTTTTATTTTCCAGTTG | CCATCCTTGACCCTTCCTCAC | 382 | 57 |

2013). The sequencing results were analyzed by Sequencher 5.4.5 against references (downloaded from the NCBI database of Human GRCh38/hg38).

### Quantitative real-time PCR

Total RNA was isolated from RVOT tissue homogenized in TRIzol (Life) and extracted with chloroform isoamyl alcohol; 0.5–1 μg RNA was used for cDNA synthesis using TransScript One-Step gDNA Removal and cDNA Synthesis SuperMix (Trans). Quantitative real-time PCR (qRT-PCR) for BVES transcripts (Primer: Forward, 5'-3'ccactctctaccgatgtgcc; Reverse, 5′-3′ ccggtacatgccactgagtt) was performed using standard PCR conditions using an Applied Biosystems Quanstudio 5 machine with SYBR Green PCR Master Mix (Takara). Expression levels were standardized to *GAPDH* (Primer: Forward, 5'-3cgaccactttgtcaagctca; Reverse, 5′-3′ cccctcttcaagggtctac) expression and all the data were analyzed using the $2^{-\Delta\Delta CT}$ Livak method.

### Western blot

Total protein samples were prepared in radioimmunoprecipitation assay (RIPA) buffer, and protein concentration was determined by BCA assay (Beyotime). Protein separations were carried out by electrophoresis through 12% SDS polyacrylamide gels. The proteins were then transferred to nitrocellulose membranes, blocked with 8% skim milk and incubated with anti-BVES antibody (1:1500 dilution; Absin); and anti-β-ACTIN antibody (1:3000 dilution, Proteintech). The signal densities of the BVES protein bands were quantified and normalized to β-ACTIN using Image J.

### Plasmid construction

The wild-type (WT) human *BVES* coding regions was amplified using 1–5 μg of cDNA as a template (primers in Supplementary Table S1). A high-fidelity thermostable DNA polymerase (Takara) reagent was used for PCR under the following conditions: 3 min at 94°C followed by 30 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 60 s, then 72°C for 5 min. *Sal*I and *Xho*I were used to digest the PCR products and the pCMV-Myc vector. Next, the PCR products were ligated using solution I (Takara) into the linearized pCMV-Myc vector. This WT construct, pCMV-*BVES*-WT, was then used in the construction of the *BVES* variants, pCMV-*BVES*-L56L and pCMV-*BVES*-R129W where the resulting allelic variant plasmids that were constructed using the Mut Express II Fast Mutagenesis Kit V2 (Vazyme) according to the instructions of primers in Supplementary Table S1. This kit was also used to construct pGL3-*BVES*-L56L, pGL3-*BVES*-R129W plasmids.

The promoters of *NKX2.5*, *GATA4*, and *MEF2C* were amplified using the primers in Supplementary Table S1. PCR products and plasmids (pGL3-Bias) were digested with the same enzymes (*NKX2.5*, *KPN*I+*XHO*I; *MEF2C*, *KPN*I; *GATA4*, *Sac*I) and the target fragments were ligated into vectors using ClonExpress Ultra One Step Cloning Kit (Vazyme).

### Luciferase reporter assays

Human HEK293T cell lines were cultured in DMEM with 10% FBS. HEK293T cells were seeded in 24-well plates ($1\times10^5$ cells/well) 24 h before transfection. The cells were then transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturers' suggestions. We used 600 ng each of the expression plasmid ($pCMV_{series}$), the fluorescent reporter plasmid ($pGL3_{series}$), and the Renilla luciferase plasmid (pRL-TK). After 24 h, luciferase activity was measured using Dual-Luciferase®Reporter Assay System (Promega).

### Statistical analyses

To assess whether the means of the two groups were statistically different from each other, we applied the Student's *t*-test. A *p*-value of <0.05 was considered statistically significant.

## Results

### Identification of genetic variants of BVES in TOF patients

To examine the allelic variants in the *BVES* gene, the entire *BVES* coding sequences for all of the patients were analyzed and compared with the GenBank human *BVES* coding sequence. We identified four novel single nucleotide polymorphisms (SNPs): rs143510978 (c.408T>G, p.R136H) in one sample, rs751152136 (c.195T>C, p.H65H) in one sample, rs2275289 (c.385C>T, p.R129W) in 10 samples with 4 occurring in blood and 6 in RVOT tissues, and rs183390165 (c.1068C>T, p.V356V) in one sample (Table 2). We found that two of these novel SNPs, rs143510978 (p.R136H) and

Table 2. Single Nucleotide Polymorphism of Blood Vessel Epicardial Substance in Tetralogy of Fallot Samples

| SNP | Tissue | | Nucleotide | Genotype | Exon | Protein | MAF in TOF | MAF in NCBI |
|---|---|---|---|---|---|---|---|---|
| | Blood (n = 57) | Tissue (n = 89) | | | | | | |
| rs143510978 | 1 | 0 | c.408T>G | +/– | Exon4 | p.R136H | G = 1/146 = 0.0034 | G = 0.0022/11 (1000 Genomes) |
| rs751152136 | 0 | 1 | c.195T>C | +/– | Exon2 | p.H65H | A = 1/146 = 0.0034 | C = 0.000008/1 (ExAC) |
| rs2275289 | 4 | 6 | c.385C>T | +/– | Exon4 | p.R129W | T = 10/146 = 0.0351 | T = 0.0050 (1000 Genomes) |
| rs183390165 | 0 | 1 | c.1068C>T | +/– | Exon8 | p.V356V | A = 1/146 = 0.0034 | A = 0.0004/2 (1000 Genomes) |

1000 Genomes, 1000 Genomes Project; ExAC, Exome Aggregation Consortium; +/–, heterozygote.
TOF, Tetralogy of Fallot; MAF, minor allelic frequencies; SNP, single nucleotide polymorphism.

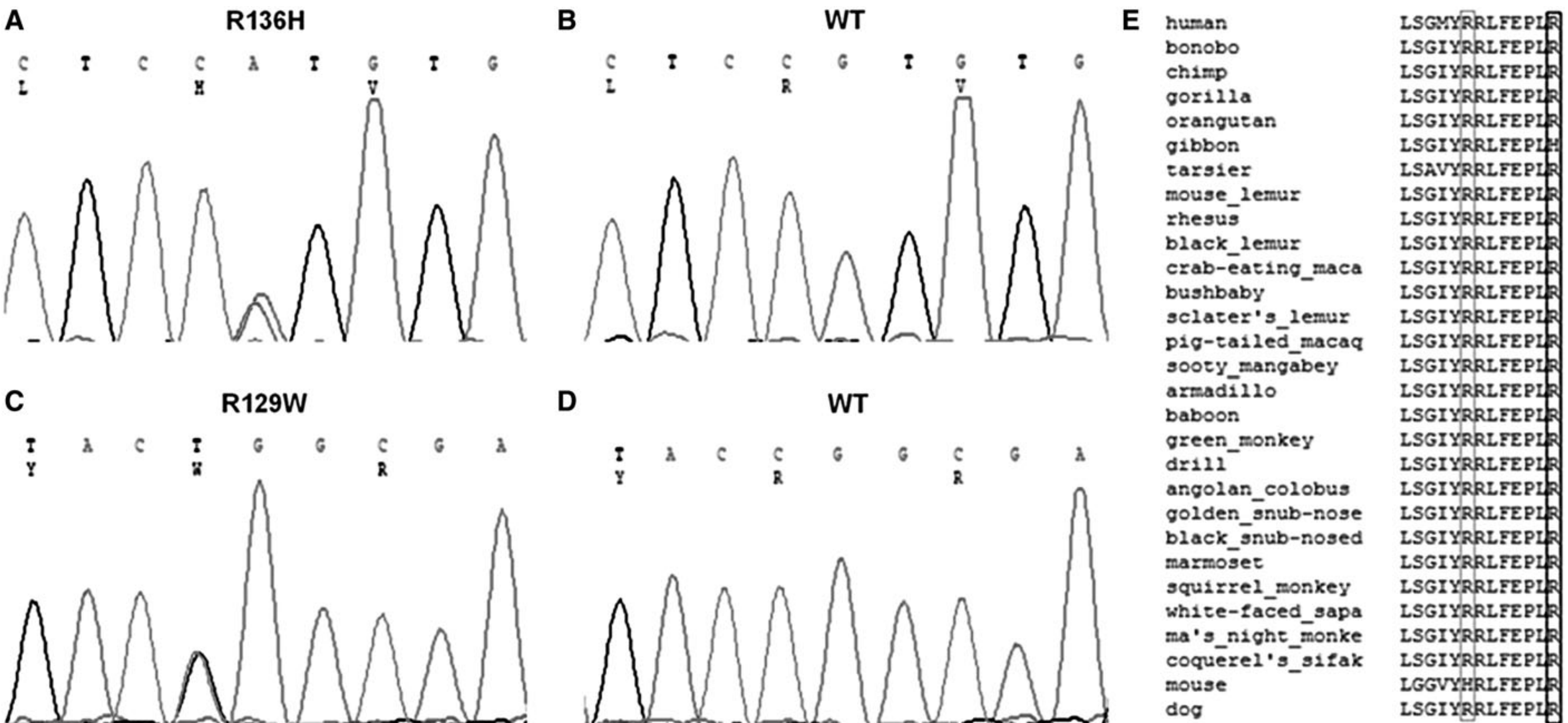
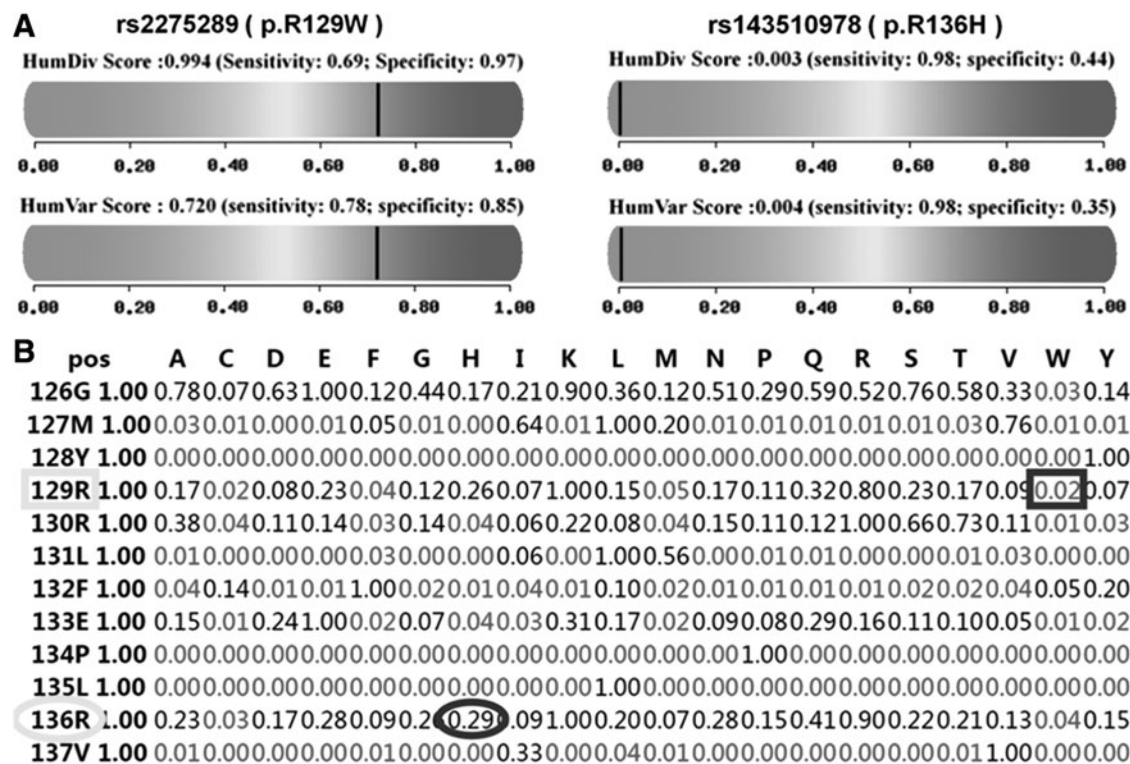



**FIG. 1.** Identification of the genetic variants of *BVES* in TOF patients. **(A, B)** Sequencing map of rs143510978 (p.R136H). **(C, D)** Sequencing map of rs2275289 (p.R129W). WT: Sequence maps of normal controls. **(E)** Evolutionary conservation analysis of rs143510978 (*black box*), rs2275289 (*light gray box*). *BVES*, blood vessel epicardial substance; TOF, Tetralogy of Fallot; WT, wild type.




| pos | | A | C | D | E | F | G | H | I | K | L | M | N | P | Q | R | S | T | V | W | Y |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 126G | 1.00 | 0.78 | 0.07 | 0.63 | 1.00 | 0.12 | 0.44 | 0.17 | 0.21 | 0.90 | 0.36 | 0.12 | 0.51 | 0.29 | 0.59 | 0.52 | 0.76 | 0.58 | 0.33 | 0.03 | 0.14 |
| 127M | 1.00 | 0.03 | 0.01 | 0.00 | 0.01 | 0.05 | 0.01 | 0.00 | 0.64 | 0.01 | 1.00 | 0.20 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.03 | 0.76 | 0.01 | 0.01 |
| 128Y | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 |
| 129R | 1.00 | 0.17 | 0.02 | 0.08 | 0.23 | 0.04 | 0.12 | 0.26 | 0.07 | 1.00 | 0.15 | 0.05 | 0.17 | 0.11 | 0.32 | 0.80 | 0.23 | 0.17 | 0.09 | 0.02 | 0.07 |
| 130R | 1.00 | 0.38 | 0.04 | 0.11 | 0.14 | 0.03 | 0.14 | 0.04 | 0.06 | 0.22 | 0.08 | 0.04 | 0.15 | 0.11 | 0.12 | 1.00 | 0.66 | 0.73 | 0.11 | 0.01 | 0.03 |
| 131L | 1.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.03 | 0.00 | 0.00 | 0.06 | 0.00 | 1.00 | 0.56 | 0.00 | 0.01 | 0.01 | 0.00 | 0.00 | 0.01 | 0.03 | 0.00 | 0.00 |
| 132F | 1.00 | 0.04 | 0.14 | 0.01 | 0.01 | 1.00 | 0.02 | 0.01 | 0.04 | 0.01 | 0.10 | 0.02 | 0.01 | 0.01 | 0.01 | 0.01 | 0.02 | 0.02 | 0.04 | 0.05 | 0.20 |
| 133E | 1.00 | 0.15 | 0.01 | 0.24 | 1.00 | 0.02 | 0.07 | 0.04 | 0.03 | 0.31 | 0.17 | 0.02 | 0.09 | 0.08 | 0.29 | 0.16 | 0.11 | 0.10 | 0.05 | 0.01 | 0.02 |
| 134P | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 135L | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 1.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 |
| 136R | 1.00 | 0.23 | 0.03 | 0.17 | 0.28 | 0.09 | 0.2[illegible] | 0.29 | 0.09 | 1.00 | 0.20 | 0.07 | 0.28 | 0.15 | 0.41 | 0.90 | 0.22 | 0.21 | 0.13 | 0.04 | 0.15 |
| 137V | 1.00 | 0.01 | 0.00 | 0.00 | 0.00 | 0.01 | 0.00 | 0.00 | 0.33 | 0.00 | 0.04 | 0.01 | 0.00 | 0.00 | 0.00 | 0.00 | 0.00 | 0.01 | 1.00 | 0.00 | 0.00 |

**FIG. 2.** Predicted protein function with the SNP rs143510978 (p.R136H) and rs2275289 (p.R129W). **(A)** Polyphen2. HumVar analysis of the rs2275289 (p.R129W) variant (*left*) gives scores close to 1, suggesting a harmful effect of the variation. Analysis of the rs143510978 (p.R136H) variant (*right*) produces a lower score, suggesting a neutral effect of the substitution. **(B)** SIFT. Analysis of rs2275289 (p.R129W) (*black box*) shows a significant probability of a deleterious effect, whereas for rs2275289 (p.R129W) (*black oval*), the probability of it being deleterious is not significant. *Light gray oval* and *light gray box* indicates the location of the variant. SNP, single nucleotide polymorphism.

rs2275289 (p.R129W), caused amino acid changes (Fig. 1A–D) in regions that are highly evolutionarily conserved among primates (Fig. 1E). No significant differences were found in the minor allelic frequencies (MAF) of three of these SNPs (rs143510978, rs751152136, and rs183390165) between the TOF patients and the normal controls. Interestingly, the rs2275289 SNP occurred more frequently in TOF patients (TOF MAF = 0.0351, Normal group in 1000 Genomes = 0.0050) (Table 2). To exclude the possibility that variants in other genes involved in cardiac development were causative, the TOF-related genes (i.e., *GATA4*, *NKX2.5*, and *TBX20*) were sequenced from these patients' tissue samples; however, no deleterious allelic variants were detected. In addition, no damaging sequence variants were detected in the *TBX1* gene, which has been identified as a candidate TOF gene based on its inclusion within the microdeletion of 22q11 associated with CHD (Yagi *et al.*, 2003; Arnold *et al.*, 2006; Zweier *et al.*, 2007; Theveniau-Ruissy *et al.*, 2008; Aguayo-Gomez *et al.*, 2015).

We evaluated the allelic variant frequency of the rs2275289 polymorphic loci in the Chinese Millionome Database (CMDB), which is a unique large-scale Chinese genomics database produced by BGI (The Beijing Genomics Institute) and hosted in the National GenBank (Kinnunen *et al.*, 2018). We found that the T allele frequency was 0.025% in the normal Chinese population, which is the same as we detected in the 100 normal controls. Owing to the fact that two SNPs (rs2275289 and rs143510978) caused changes in the protein sequence, we analyzed the protein function of the variants and the wild type using Polyphen2 (Adzhubei *et al.*, 2010) and SIFT (Ng, 2003). In Polyphen2, the HumVar score is used in the diagnoses of Mendelian diseases for distinguishing between allelic variants with likely drastic effects from other more mildly deleterious alleles. The HumDiv score, which ranges from zero to one, is used to evaluate rare alleles at loci potentially involved in complex phenotypes. A zero score is assigned when the variant is considered neutral, the higher the score (closer to 1) the more likely the variant is to be dangerous. Both of the scores suggest that rs2275289 was likely damaging (HumVar score, 0.720; HumDiv score, 0.994), whereas the rs143510978 variant was likely benign (HumVar score, 0.004; HumDiv score, 0.003) (Fig. 2A). The SIFT prediction results also suggest that the SNP rs2275289, which changed a basic R into a nonpolar W, would not be tolerated and would be deleterious (Fig. 2B), no matter which transcript of the *BVES* gene is used for translating into a protein sequence (deleterious, score <0.05) (Supplementary Table S2). The SNP rs143510978, which encodes a conservative basic R to basic H switch, was predicted to have minimal effect on the protein function (score = 0.29).

Clinical diagnosis of 10 TOF patients containing the c.385C>T (p.R129W) allelic variant showed no clinical manifestations of cardiomyopathies or other self-reported inherited diseases. It was not known whether other family members of the *BVES* allelic variant carriers had the same alteration due to a failure to obtain a signed consent form (see information on patients containing c.385C>T (p.R129W) in Supplementary Table S3).

### *Effects of variant c.385C>T (p.R129W) on the expression of* BVES *in* in vitro *and in* in vivo

We examined the effect of the c.385C>T variant on the expression of *BVES* itself in six RVOT tissue samples from patients with this variant. The qRT-PCR analysis showed that expression of *BVES* with the c.385C>T variant was significantly downregulated compared with normal RVOT tissue samples that did not contain this variant (Fig. 3A). This was also true for BVES protein expression in the tissues harboring the c.385C>T variant; we identified a 70% reduction as measured by quantitative western blot (Fig. 3B, C). See the information of normal samples in Supplementary Table S4. To examine if this variant affects the transcriptional expression of *BVES in vitro*, a double luciferase reporter system was developed (see Materials and Methods section). Results showed that the variant *BVES* containing the c.385C>T (p.R129W) allelic variant had significantly reduced expression compared with the wild-type *BVES* (Fig. 3D), indicating that this functional SNP reduces the activity/expression of *BVES* both in *in vivo* and in *in vitro*.

### *Effects of the* BVES *c.385C>T (p.R129W) variant on transcriptional expression of the genes in SHF regulatory network related to TOF*

It is known that abnormalities of cardiac development controlled by the SHF regulatory network are a major cause of TOF (Ward *et al.*, 2005; Schleich *et al.*, 2013; Morgenthau and Frishman, 2018). To examine whether the allelic variant c.385C>T (p.R129W) in *BVES* affects the activities of genes in the SHF regulatory network, a luciferase reporter system was used to examine expression of *NKX2.5*, *GATA4*, and *MEF2C*. In this study, the allelic variant c.385C>T (p.R129W) and another *BVES* functional allelic variant c.166T>C (p.L56L), previously identified in TOF patients [47], were overexpressed using the eukaryotic transient overexpression vector (pCMV-Myc) (Zhou *et al.*, 2013) and analyzed. Transfection efficiency was standardized with reference to *Renilla* luciferase activity resulting from the parallel introduction of this plasmid into the test cells. Each group contains three plasmids, pRL-TK, pCMV-*BVES*-WT/pCMV-*BVES*-L56L/pCMV-*BVES*-R129W, and pGL3-*GATA4*/pGL3-*NKX2.5*/pGL3-*MEF2C*. Each group had three wells and was repeated in triplicate. The results show that the promoter activities of *NKX2.5* and *GATA4* were significantly decreased by both *BVES* allelic variants compared with WT (Fig. 4A, B). However, no effect was observed on the promoter activity of *MEF2C* by overexpressing *BVES* or *BVES* with p.R129W, except in one case. The overexpression of *BVES* with p.L56L increased its promoter activity for unknown reasons (Fig. 4C). These results indicate that both *BVES* polymorphisms may downregulate the expression of the key genes *NKX2.5* and *GATA4* in the SHF regulatory network related to TOF.

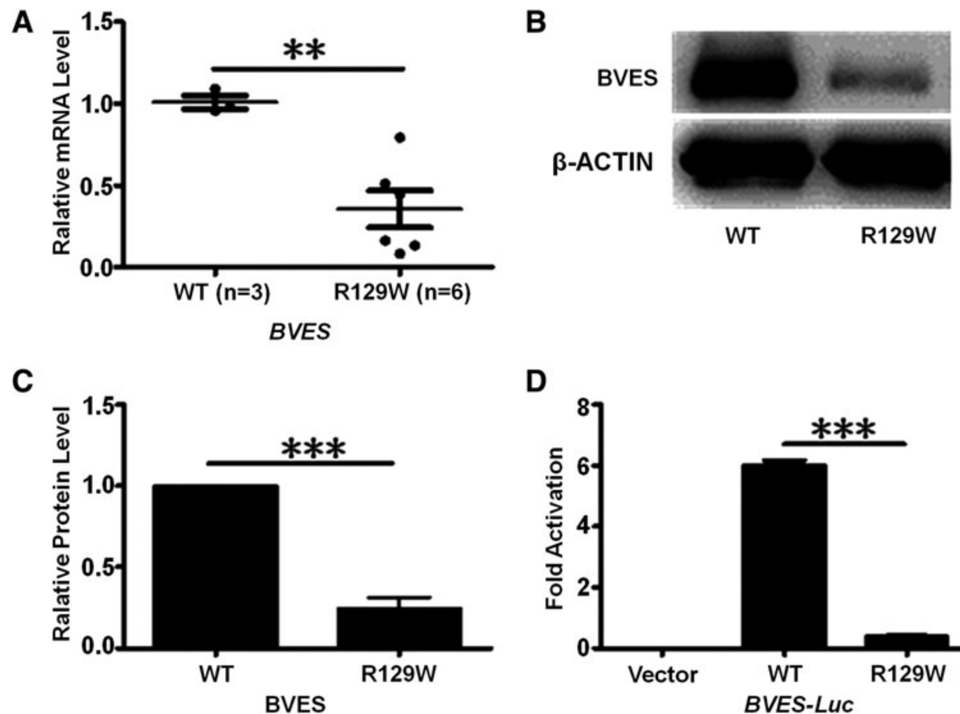



**FIG. 3.** Effects of the allelic variant c.385C>T (p.R129W) on expression of *BVES in vivo* and *in vitro*. **(A)** Expression of *BVES* mRNA in RVOT from TOF patients containing p.R129W relative to normal controls (WT). **(B)** Western blot detection of protein expression of BVES with p.R129W in TOF patients compared with the normal samples (WT). **(C)** Quantification of data from **(B)** by densitometric analysis. WT: Normal RVOT tissue samples; R129W: TOF patient RVOT tissue samples containing c.385C>T (p.R129W). **(D)** Results of dual luciferase reporter assays. Vector: Transfection with an empty vector (plasmid, pGL3-Bias); WT: Plasmid containing wild-type *BVES*; p.R129W: Plasmid containing *BVES* with the c.385C>T (p.R129W) variant. Each group contains two plasmids, pRL-TK and a reporting plasmid. Each group had three wells and was repeated in triplicate. $^{**}p<0.01$; $^{***}p<0.001$; Error bars show mean and SEM. RVOT, right ventricular outflow tract.

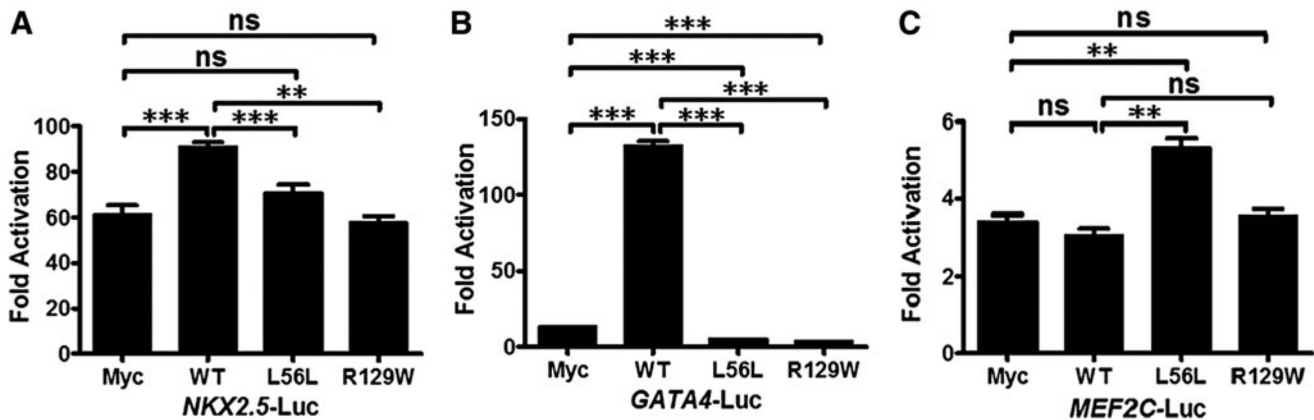



**FIG. 4.** Effects of *BVES* allelic variants on promoter activities of the key genes in the SHF regulatory network related to TOF. **(A)** Effects of both allelic variants p.L56L and p.R129W on the activity of the *NKX2.5* promoter. **(B)** Effects of both allelic variants p.L56L and p.R129W on the activity of the *GATA4* promoter. **(C)** Effects of both allelic variants p.L56L and p.R129W on the activity of the *MEF2C* promoter. Myc: pCMV-Myc, expression plasmid without a DNA fragment; WT: pCMV-*BVES*, wild type of *BVES*; L56L: pCMV-*BVES*-L56L, *BVES* with p.L56L; R129W: pCMV-*BVES*-R129W, *BVES* with p.R129W; ns, $p > 0.05$, no significance; $^{**}p < 0.01$; $^{***}p < 0.001$; Error bar shows SEM. SHF, secondary heart field.

## Discussion

In this study, the *BVES* open reading frame was sequenced in 146 samples (89 tissue samples and 57 blood samples) from patients with sporadic TOF. We identified a likely damaging SNP c.385C>T (p.R129W) with a frequency of 0.035% in TOF patients and compared its frequency with the general Chinese population by analyzing the CMDB.

SNPs have previously been linked to CHD. For example, the SNP rs3130933 in *POU5F1* (MAF: CASE-0.018, Controls-0.008) increases the risk of CHD through reduction of gene expression (Lin *et al.*, 2015). Our study classified the rs2275289 (p.R129W) SNP in *BVES* as detrimental by multiple software analyses, and we showed using both *in vitro* and *in vivo* analyses that this variant reduces the activity of the gene itself; as well as affecting activities of the downstream genes.

It is well known that the transcription factor *NKX2.5* is a key gene in the regulation of SHF formation; and that defects in this gene are associated with TOF (Goldmuntz *et al.*, 2001; Guner-Ataman *et al.*, 2013). The *Gata4*- and *Nkx2.5*-positive cells in the anterior mesoderm are recruited for the formation of the outflow tract (Guner-Ataman *et al.*, 2013). In chicken embryos, ablation of the anterior SHF cells is associated with a decrease in the expression of *Nkx2.5*, leading to TOF (Ward *et al.*, 2005). In zebrafish, *Nkx2.5* knockdown reduces the proliferation of SHF myocardial progenitor cells, leading to abnormal ventricular and outflow tract development (Guner-Ataman *et al.*, 2013). In mice, the *Gata4* heterozygous mutants exhibit double outflow tract, atrioventricular septal defect, mitral and tricuspid valve thickening, and myocardial thinning (Laforest and Nemer, 2011). Our results suggest that the rs2275289 (p.R129W) SNP in *BVES* reduces its own expression and likely decreases expression of *NKX2.5* and *GATA4*, suggesting a role for *BVES* in TOF etiology.

In conclusion, the allelic variant c.385C>T (p.R129W) in *BVES* is a functional polymorphism, which decreases the transcriptional activity of the gene itself as well as the transcriptional activities of key genes in the SHF transcriptional network related to TOF. However, the pathogenic mechanism of TOF caused by allelic variant c.385C>T (p.R129W) in *BVES* needs further studies.

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## Author Disclosure Statement

No competing financial interests exist.

## Supplementary Material

Supplementary Table S1
Supplementary Table S2
Supplementary Table S3
Supplementary Table S4

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