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Identification of non-synonymous Variations in *ROBO1* and *GATA5* genes in a Family with Bicuspid Aortic Valve Disease

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Running title *ROBO1* and *GATA5* variants in BAV

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Conflict of interest

The authors declare no conflict of interest.

Abstract

Bicuspid aortic valve (BAV) is the most common congenital heart defect with a high index of heritability. Patients with BAV have different clinical course and disease progression.

Herein, we report three siblings with BAV and clinical differences. Their clinical presentations include moderate to severe aortic regurgitation, aortic stenosis, and ascending aortic aneurysm. Genetic investigation was carried out using Whole Exome Sequencing for the three patients.

We identified two non-synonymous variants in *ROBO1* and *GATA5* genes. The *ROBO1*: p.(Ser327Pro) variant is shared by the three BAV affected siblings. The *GATA5*: p.(Gln3Arg) variant is shared only by the two brothers who presented BAV and ascending aortic aneurysm. Their sister who is affected by BAV without aneurysm does not harbor the *GATA5*: p.(Gln3Arg) variant. Both variants were absent in the patients' fourth brother who is clinically healthy with tricuspid aortic valve.

To our knowledge, this is the first association of *ROBO1* and *GATA5* variants in a familial BAV with a potential genotype/phenotype correlation. Our findings are suggestive of the implication of *ROBO1* gene in BAV and *GATA5*: p.(Gln3Arg) variant in ascending aortic aneurysm.

Our family-based study further confirms the intrafamilial incomplete penetrance of BAV as well as the complex pattern of inheritance of the disease.

Keywords: Ascending aortic aneurysm; Aortic regurgitation; Bicuspid Aortic Valve; *GATA5* gene, *ROBO1* gene.

Introduction

Bicuspid aortic valve (BAV) is a complex disease with significant clinical outcomes. It is the most common congenital heart defect occurring in 1% to 2% of the population. A bicuspid valve has only two leaflets (cusps) instead of three (1,2).

Although BAV is congenital, the disease may remain clinically silent until adulthood as heart and valve function can be preserved throughout life. Rarely, the disease can be severe at birth when the newborn develops aortic valve stenosis and congestive heart failure. Adult patients may present several symptoms, as the valve ages, linked to aortic stenosis and/or regurgitation, ascending aortic aneurysm, endocarditis, and aortic dissection (2,3).

Initially, BAV has been considered as a sporadic defect. However, several studies reported familial cases suggesting that BAV is highly heritable (3–6). Moreover, BAV has been observed in monozygotic twins (7,8). The prevalence of BAV is estimated to be 10-fold higher in first-degree relatives of a proband(5). Mostly, BAV has an autosomal dominant inheritance with incomplete penetrance and variable expressivity (2,3). Indeed, no monogenic model of inheritance can clearly explain BAV, suggesting a complex model of inheritance involving several genes in the same or in different pathways.

Genes associated with BAV include members of TGF β family, Notch signaling pathway, Wnt/ β -catenin pathway, and several transcriptional factors such as *GATA5* (9–11). *GATA5* gene is essential for aortic valve morphogenesis (12).

Thereafter, the Slit-Robo pathway was identified as a new contributor in cardiac cushion development. Robo receptors are required for normal expression levels of the Notch signaling pathway. Indeed, in *ROBO1* mutants mice, the expression of *Notch*, *Hey* and *Hes* genes is down-regulated (13,14). Subsequently, rare deleterious variants in *ROBO* genes were identified in patients with BAV and other heart defects (10,15,16).

Our understanding of how variants cause BAV phenotype and why the clinical course of patients is so different is incomplete. Moreover, the underlying embryological development process of BAV remains poorly known owing to the involvement of several

transcriptional factors, signaling, and cellular pathways during arteriogenesis. Furthermore, we cannot exclude the potential contribution of rare or frequent variants that may act as modifiers in the pathogenesis of BAV and could explain the high variable expressivity of the disease. Herein, we performed a clinical and genetic study of a family with BAV disease including three affected siblings, a healthy brother, and a mother, both with tricuspid valve.

Material & Methods

Ethical compliance

This study was performed according to the principles of the Declaration of Helsinki and to the ethical standards of the first author's institutional review board. The patients provided their written informed consent to participate in this study.

Patients and Whole Exome Sequencing

Peripheral blood samples were collected from the three affected patients (Figure1: II-2; II-3 and II-4).

Family pedigree is shown in Figure1-A.

Genomic DNA was extracted from the samples according to standard techniques. Whole-exome sequencing (WES) was performed for the three siblings.

WES was performed by the Genomics and Bioinformatics Platform (GBiM) of the INSERM U1251 Marseille Medical Genetics facility using the NimbleGen SeqCap EZ MedExome kit (total design size 47 Mb) according to the manufacturer's protocol (Roche Sequencing Solutions, Madison, USA). All DNA and libraries preparations (KAPA HyperPrep Kits (Roche)) were performed according to the manufacturers' instructions. The DNA libraries were subjected to paired-end sequencing using the Illumina NextSeq500 sequencing platform (Illumina, San Diego, CA, USA). Raw fastQ files were aligned to the hg19 reference human genome (University of California Santa Cruz, UCSC) using BWA software. Variant calling workflow was performed according to the GATK best practices. The output files were annotated using ANNOVAR software. Variant annotation process and exome analysis were performed with VarAFT software(17).

Variant prioritization

Variant prioritization was performed with the Variant Annotation and Filtering Tool (VarAFT), version 2.17-2 (<https://varaft.eu/>). To pinpoint putatively pathogenic and causal variants we first excluded variants with a minor allele frequency (MAF) >1% in the gnomAD database (<http://gnomad.broadinstitute.org/>). Then, we removed non-coding and

synonymous variants with no impact on splicing with HSF-Pro tool (18). Subsequently, the remaining variants were filtered based on their *in silico* pathogenicity prediction. To select potential pathogenic and clinically relevant variants, we focused on genes involved in aorta-related phenotypes (**Supp.File1**).

All identified variants with $MAF < 0.01$ shared by the three siblings are listed in the supplementary file as well as variants in genes related to aortic phenotypes (Supp.File2). Of note, WES data analysis and interpretation was performed of each patient separately and the identified variants were evaluated one by one in concert with the personal clinical features of each patient.

Sanger sequencing and familial segregation were performed for the likely causative prioritized variants.

Residue conservation was determined using the ConSurf server(<https://consurf.tau.ac.il/>). ConSurf is a bioinformatics tool for estimating the evolutionary conservation of amino and nucleic acid positions in a protein/DNA/RNA molecule based on the phylogenetic relations between homologous sequences.

Results

Clinical findings

The Proband (II-3) was admitted in our intensive care unit in December 2009 at the age of 30 years old for acute heart failure and suspicion of infective endocarditis. Work up revealed calcified typical BAV with Right-Left fusion and raphe (Sievers type I) with severe restrictive aortic regurgitation (AR), moderate aortic stenosis (mean gradient=25mmHg) and significant aortopathy of mixed phenotype involving both aortic root and tubular ascending aorta (sinus=53mm; junction=46mm and tubular aorta=53mm on CT scan). Left ventricle (LV) was dilated (LVED volume=280ml, LVES volume=59ml/m², LVES diameter=31mm/m²) and LV systolic function was severely depressed (LVES=32%). Diagnosis of endocarditis could not be formally confirmed in view of negative blood culture and lack of valvular vegetation but prolonged antibiotic treatment was prescribed in association with heart failure treatment. After hemodynamic stabilization, aortic valve replacement by mechanical prostheses associated with replacement of ascending aorta

through Bentall procedure was performed in March 2010. Pre-operative coronary angiogram was normal. The main post-operative event was the occurrence of conductive disorders requiring pacemaker implantation, upgraded one year later into cardiac resynchronization therapy defibrillator (CRT-D) for uncontrolled heart failure under optimal medical treatment. Six years later, *Staphylococcus aureus* device infection was treated by antibiotics and pacemaker removal followed by its re-implantation through epicardial approach. Aortic prosthesis was spared. At last follow-up in December 2021, the proband presented an improved LV systolic function (FE=50%) and normally functioning aortic valve. He has no progeny.

The affected proband brother (II-2) had followed a similar cardiac history with a Bentall procedure performed in another center in 2006 at the age of 40 for severe regurgitation due to BAV associated with ascending aorta aneurysm of mixed phenotype and decreased LVEF. BAV phenotype could not be identified retrospectively nor were pre-operative aortic dimensions. Early post-operative echocardiographic data showed major LV dilation (LVED volume=380mL; LVES volume=112mL/m²; LVES diameter=23mm/m²) and decreased LVEF measured at 30%, which improved under medical treatment to stabilize around 40%. Subsequent medical history was marked by suspicion of negative blood culture prosthetic aortic valve endocarditis treated medically in 2014, followed by resuscitated cardiac arrest due to ventricular tachycardia treated by implantation of subcutaneous defibrillator. The latter was upgraded into cardiac resynchronization therapy defibrillator (CRT-D) in 2017 for increased lead impedance, decreased LVEF and QRS widening. Recurrent *Staphylococcus aureus* infective endocarditis was successfully medically treated in December 2019 but patient ultimately died from COVID infection in December 2020 with no progeny.

Recently, the proband's sister (II-4) underwent BAV screening at the age of 50 years old. She carries a typical BAV with Right-Left fusion and raphe (Sievers type I), with no aortopathy (sinus=36mm; junction=32mm; tubular aorta= 37mm). The BAV presents with slight calcifications, trivial restrictive regurgitation and mild aortic stenosis (mean gradient=11mmHg). LV dimensions (LVED volume=99 mL, LVES volume=22ml/m², LVES diameter=16mm/m²) and systolic function (LVEF=62%) are normal (Figure 1-B). She has

a 23-year-old son and two daughters of 25 and 20 years old respectively. The latter underwent BAV screening and has a tricuspid aortic valve with normal aortic diameters.

The proband's brother (II-1), a 51-year-old man, has a tricuspid aortic valve with normal aortic diameters. The proband's mother is alive with a tricuspid aortic valve and no aortopathy. The proband father died in 2010 at the age of 70 years from non-cardiac cause and had not been screened.

Genetic findings

WES data were analyzed with a focus on genes implicated in aorta-related defects which allowed us to prioritize two rare heterozygous missense variants: *ROBO1*: NM_002941: exon8: c.979T>C: p.(Ser327Pro) and *GATA5*: NM_080473: exon2: c.8A>G: p.(Gln3Arg), with a MAF, **in the European (Non-Finnish) population, 0.00032 and 0.0085**, respectively (gnomAD **(v2.1.1)**). Both variants were maternally inherited.

All affected patients (II-2; II-3 and II-4) carried the *ROBO1*: p.(Ser327Pro) variant. To our knowledge, no phenotypic data are associated to this variant (rs200951485). It is not reported in Clinvar database as well. The *ROBO1*: p.(Ser327Pro) variant is predicted deleterious by *in silico* prediction tools, namely LRT, Mutation Taster, Polyphen, SIFT and UMD-Predictor. Its CADD-phred score is **26.3**. The score cutoff of deleteriousness used >15 (<http://cadd.gs.washington.edu>).

Of note, the *ROBO1*: p.(Ser327Pro) variant is the only shared variant between the three siblings using the gene list indicated in the Supp.File1.

On the other hand, only the two severely affected brothers harbor the *GATA5*: c.8A>G: p.(Gln3Arg) variant. This variant (rs113068438) is predicted pathogenic by SIFT and CADD (score=21.7). The sister (II-4) do not harbor the *GATA5* variant (Figure1-C).

Interestingly, the *GATA5*: p.(Gln3Arg) variant was previously reported in patients with atrioventricular septal defects and BAV associated to aortopathy (11,19,20). All the reported BAV patients carrying this variant were males (20).

The unaffected proband's brother (II-1) do not harbor *ROBO1* or *GATA5* variants (Figure 1-C).

Both variants were absent from our *in-house* exome database gathering 200 individuals and from our BAV adult and pediatric cohorts including 54 exomes.

The conservation analysis by ConSurf showed a high conservation score for both variants (Figure 2).

Discussion

The present study investigated a family with three BAV-affected siblings. We identified two heterozygous non-synonymous variants in *ROBO1* and *GATA5* genes.

Robo proteins are a family of transmembrane receptors with highly conserved intracellular domains and no autocatalytic or intrinsic enzymatic activity (21). In humans, variants in *ROBO4* gene have been identified in patients with isolated BAV as well as BAV with ascending aortic aneurysm (10,15,22).

In the present family, we identified a variant in *ROBO1* gene (p.Ser327Pro) in the three affected siblings. To date, loss of function variants in *ROBO1* gene have been linked only to tetralogy of Fallot and ventricular septal defects in humans (16). Robo1 receptor is widely distributed in the developing mouse heart and seems to be the most crucial Robo receptor during heart development (13,14,23). In mouse, deletion of *Robo1* results to several heart defects ranging from BAV to venous connections anomalies (13). Indeed, *robo1* knock-down inhibits endocardial and myocardial cell migration, resulting in unfused heart fields and *robo1* gain-of-function leads to an incomplete heart field fusion (13). Additionally, Robo1 can interact with Neuropilin1, a receptor required for outflow tract cushion development and membranous ventricular septum formation (23). Robo1 expression is also detected in pharyngeal regions populated by the neural crest cells, suggesting that Robo1 can contribute to normal neural crest cells migration. Several studies have shown that perturbation of cardiac neural crest cells causes abnormal pattern of outflow tract cushions and subsequently BAV (24,25). *Robo1;Robo2* double mutants mouse showed a wide range of heart defects and a high penetrance BAV phenotype (23).

We also identified a rare heterozygous non-synonymous variant in *GATA5* gene. The *GATA5*: c.8A>G: p.(Gln3Arg) variant was present in the affected brothers (II-2) and (II-3) only. Both patients presented a severe clinical presentation of BAV with ascending aortic aneurysm but not their sister (II-4) who does not present an aortopathy.

GATA5 protein is a transcription factor that contains two GATA-type zinc fingers. Diseases associated with *GATA5* include congenital heart defects, Multiple Types, 5 (OMIM 617912) and aortic valve disease (26,27).

Interestingly, the *GATA5*: c.8A>G: p.(Gln3Arg) variant has been reported only in male BAV cases (11,20). Padang et al investigated the role of different variants in *GATA5* gene in BAV patients with associated aortopathy and the identified variants were present only in male patients. The BAV cohort comprises 77% males which may reflect a reduced expressivity of *GATA5* mutations in females (20). These findings need further investigations.

The patient reported by Bonachea et al carrying the *GATA5*: p.(Gln3Arg) variant had a BAV with aortic coarctation (11). In the same study, only the *GATA5*:p.(Gln3Arg) demonstrated decreased transcriptional activity in vitro, ~~supporting its pathogenicity (11). Indeed, this variant is located in the first transcription activation domain of *GATA5* gene. The substitution affects a highly conserved residue among the *GATA4/5/6* subfamily and impact the transcriptional activation of *GATA5* target regions (20). Moreover, the substitution of the Gln3 residue with arginine in a construct containing *GATA4* activation domain-I resulted in an 80% reduction in transcriptional activity, supporting its functional importance and potential pathogenicity (28).~~

Taking into account the present family segregation and the aforementioned studies, we hypothesize that *GATA5*: p.(Gln3Arg) variant is most likely implicated in the associated aortic phenotypes in BAV, such as aortic coarctation and ascending aortic aneurysm.

In the present study we report a clinical and genetic investigation of a BAV family including three affected siblings. We identified a novel *ROBO1* variant and a previously reported variant in the *GATA5* gene. Our study further supports the implication of the slit-robo pathway in BAV ~~and the potential digenic inheritance pattern of the disease~~. Further studies are needed to assess the cumulative functional effect of rare non-synonymous *ROBO1* and *GATA5* variants in patients with BAV and its associated aortopathy.

Figure legends

Figure 1: A- Family pedigree: Darkened left upper quadrant: aneurysm; Darkened right lower quadrant: Bicuspid Aortic Valve; White symbols: healthy members with tricuspid aortic valve; Genotypes are indicated below symbols: (+) wild-type allele; (-) Alternative allele. B- TTE Diagnosis of BAV in patient II-4: B.1 Parasternal short-axis view of BAV in systole showing fusion with raphe between the left and right cusps. B.2 Supra-sternal view of the aortic arch showing the absence of aortic coarctation. B.3 Parasternal short-axis view of the aortic root and junction showing normal aortic diameters. C- Sanger electropherograms.

Figure 2: Conservation analysis result by ConSurf software. Mutated amino acids in our study are indicated by an arrow.

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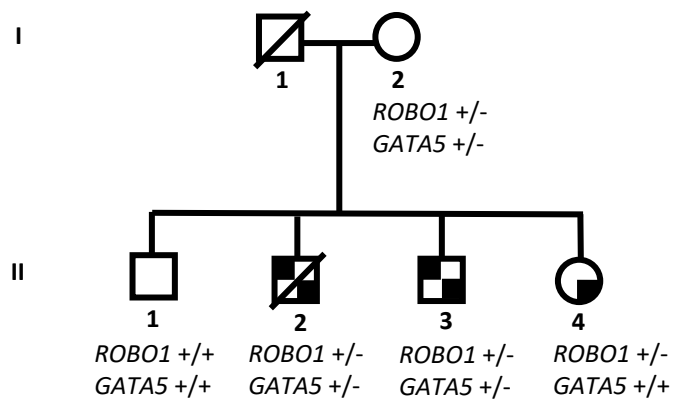
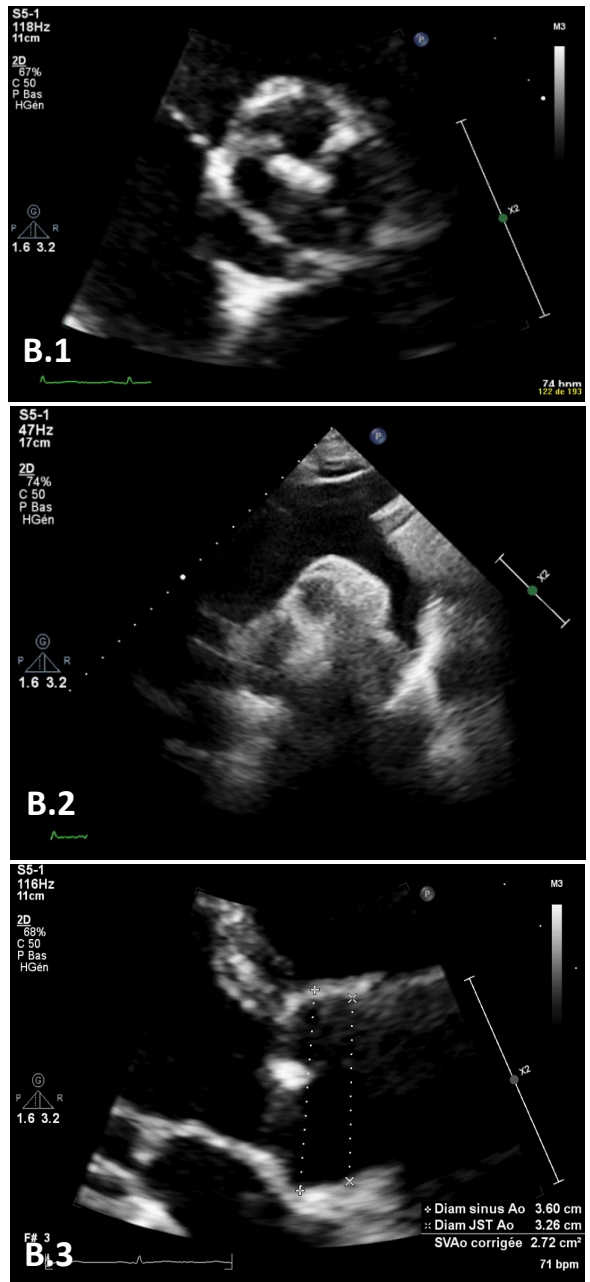
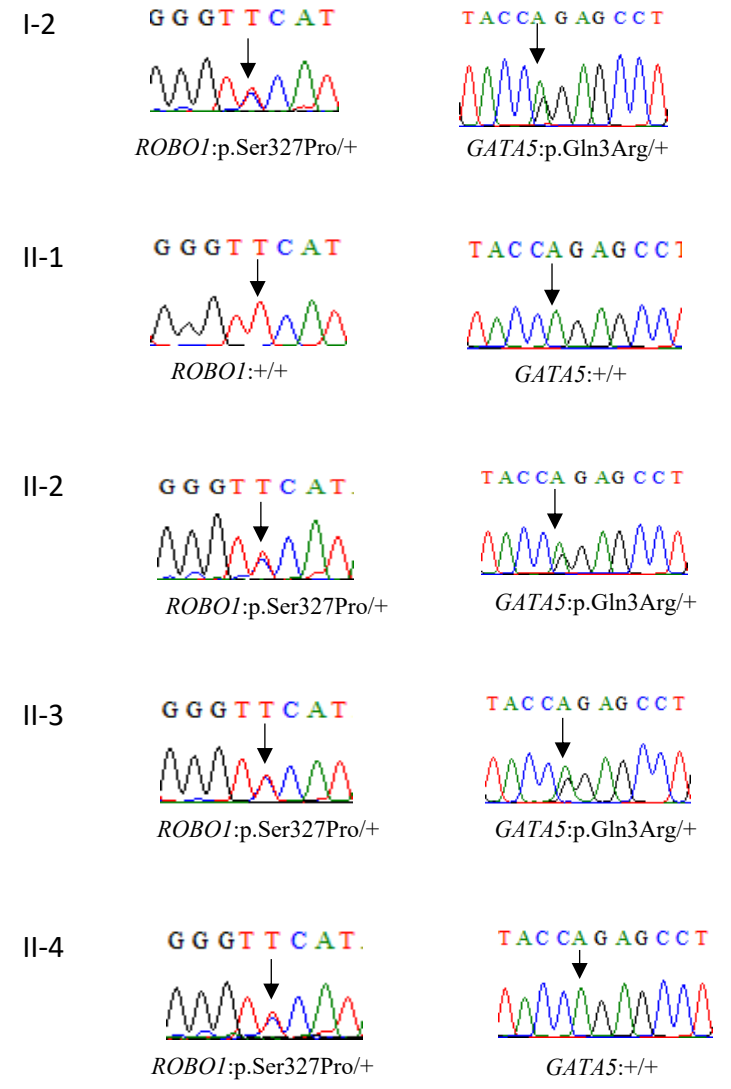
Author Contribution. Study concept and design: JFA, SZ.; Clinical Investigation of the patients and family members; HG, AT, FC, JFA; Analysis and interpretation of data: GCB, HJ. Molecular investigation: HJ.; Writing—Original Draft Preparation: HJ; HG; Critical—Review & Editing: AT, JFA, SZ. Supervision: SZ; Validation: SZ, JFA.

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A**B****C**

ROBO1: p.Ser327

S Y T C V A E N M V G K A

GATA5: p.Gln3

M Y Q S L A - L A A S P R

Conservation scale

1 2 3 4 5 6 7 8 9
Variable Average Conserved