Familial Ebstein’s anomaly: whole exome sequencing identifies novel phenotype associated with *FLNA.*

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**Running title:**

WES in familial Ebstein's anomaly

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**Abstract**

**Background** Familial Ebstein’s anomaly (EA) is a rare form of congenital heart disease (CHD). We report seven individuals among two generations of one family with Ebstein’s anomaly. This family was first reported in 1991 by Balaji *et al* in which family members were also reported to have a mild skeletal phenotype. The most likely mechanism of inheritance was concluded to be autosomal dominant. We sought to identify the genetic aetiology in this family using a next generation sequencing approach.

**Methods and Results** Whole exome sequencing (WES) was performed in two cousins in this family using the Agilent SureSelect Human all Exon 51 Mb version 5 capture kit. Data were processed through an analytical in-house pipeline.

WES identified a missense mutation in Filamin A (*FLNA*), an actin-binding protein located at Xq28, mutations in which are associated with the skeletal phenotypes Frontometaphyseal dysplasia, Otopalatodigital and Melnick-Needles syndrome, with X-linked periventricular nodular heterotopia and FG syndrome (Omim: # 305450). Review of the phenotypes of those with the mutation in this family shows increased severity of the cardiac phenotype and associated skeletal features in affected males, consistent with X-linked inheritance.

**Conclusions** Although congenital heart disease is reported in families with mutations in *FLNA*, this is the first report of individuals being affected by Ebstein’s anomaly due to a mutation in this gene and details the concurrent skeletal phenotype observed in this family.

**Keywords (up to 5)**

Familial Ebstein’s anomaly, whole-exome sequencing, *FLNA*

**INTRODUCTION**

Ebstein’s anomaly (EA) is a rare form of congenital heart disease (CHD) that occurs in approximately 1 per 200,000 births.1 In those with EA there is malformation of the tricuspid valve with adherence of the septal and posterior valve leaflets to the myocardium while the anterior leaflet is redundant, perforated or tethered. This is associated with apical displacement of the valve annulus and concomitant atrialisation and enlargement of the right ventricle.1 An associated interatrial communication can also be identified in the majority of cases with this anomaly.

Genetic factors are recognised in the aetiology of this condition as it is more common in those with a family history of (any) congenital heart disease 2 but families in which there are multiple cases of EA occurring in a Mendelian pattern of inheritance are rare.1 The only genes implicated to date are *NKX2-5*, identified in association with a variety of structural congenital heart defects with phenotypes including EA3 and the sarcomeric gene beta-myosin heavy chain (*MYH7*). Mutations in *MYH7* have been found in association with EA, initially in four individuals within the same family with this lesion and in 6% of a second cohort of unrelated individuals with this type of CHD.4

Over the last decade, next generation DNA sequencing (NGS) has become widely available. The techniques used allow capture of the whole exome and massively parallel DNA sequencing offers a valuable means to identify genes underlying Mendelian disorders.5 NGS has been widely applied to study familial and sporadic forms of CHD and helped to identify several genes underling these conditions6,7. Although these findings have helped understanding of CHD, they do not resolve the cause of most CHD cases7. While the majority of CHD is of unknown aetiology, next generation sequencing has led to the discovery of *NR2F2*, a pleiotropic transcription factor, mutations in which are associated with nonsyndromic atrioventricular septal defects.8

Here we report a familial case of Ebstein’s anomaly(Figure 1)9 manifesting across at least two generations, caused by a novel missense variant identified through whole-exome sequencing (WES) of an affected cousin pair. The clinical manifestation of this family was previously reported but the aetiology was not previously identified 9.

**SUBJECTS AND METHODS**

The family was ascertained through the Wessex Clinical Genetics Service, having presented for genetic counselling regarding the likely recurrence risk of Ebstein’s anomaly following the death of the male proband. Twenty-five years after their original presentation, we obtained consent for detailed genetic investigations in surviving members of the family. The proband, male III (1) was severely affected; he was born at 33 weeks gestation and died at 10 days of age. Unfortunately, no genetic material was available from him. Whole exome sequencing was conducted on DNA samples from individuals III (3) and III (5). Six affected surviving members of the family were examined by a clinical geneticist and their echocardiograms reviewed by two cardiologists. Detailed phenotyping was completed following the identification of a likely causal mutation*.* Targeted characterisation of phenotypic features previously associated with the likely causal gene was undertaken*.*

**DNA extraction**

Genomic DNA was extracted from peripheral venous blood samples collected in EDTA. DNA concentration was estimated using the Qubit ® 2.0 Fluorometer and A 260:280 ratio calculated using a nanodrop spectrophotometer. The average DNA yield obtained was 150µg/ml and approximately 20ug of DNA was used for next generation sequencing for each patient.

**Whole exome sequencing data generation and data analysis**

Whole exome sequencing was performed using the Agilent SureSelect Human all Exon 51 Mb version 5 capture kit. As previously described,10,11 default parameters were applied: fastq raw data generated from Illumina paired-end sequencing were aligned against the human reference genome (hg19) using Novoalign (novoalign/2.08.02). SAMtools12 (samtools/0.1.19) was used to call variation and ANNOVAR (annovar/2013Feb21) 13 was applied for variant annotation against a database of RefSeq transcripts. A bespoke script was used to assign individual variants as “novel” if they were not previously reported in the dbSNP137 databases, 14 1000 Genomes Project 15, the Exome Variant Server (EVS) of European Americans of the NHLI-ESP project with 6500 exomes [http://evs.gs.washington.edu/EVS/], in 46 unrelated human subjects sequenced by Complete Genomics 16or in the Southampton database of reference exomes (n=329).

Resultant variants files for each individual were subjected to further in-house quality control tests to detect DNA sample contamination and ensure sex concordance by assessing autosomal and X chromosome heterozygosity. Variant sharing between all pairs of individuals was assessed to confirm sample relationships. Sample provenance was confirmed by independent genotyping of a validated SNP panel, developed specifically for exome data.17

**Tiered selection**

*Tier 1.* *MYH7* and *NKX2-5* are the two candidate genes known to be involved in Ebstein’s anomaly. These two genes were previously tested by Sanger sequencing within the family with negative findings. Nevertheless, these genes were selected as top priority to confirm negative results from this alternative sequencing resource.

*Tier 2*. The Human Gene Mutation Database Professional 2013.4 (HGMD®, BIOBASE Biological Databases)18 was interrogated using the inclusive term “heart disease” to retrieve an extensive list of causal genes known to be involved in heart conditions. The query returned 338 genes from which 219 were selected as associated with clinical phenotypes similar to congenital hearts defects using the following terms: e.g congenital heart defects, heart valve defect, atrial septal defects and postaxial hexodactyly, cardiomyopathy and hypertrophic (see Supplementary Table 1 for complete list).

*Tier 3.* Using WES on the affected cousin pair, their exomes were compared to identify all genetic variants shared between them. All shared variants were removed that were observed (in various states of zygosity) within the local Southampton control cohort of exomes (n=329). Of the remaining variants, synonymous variants with low likelihood to impact protein function, splicing variants with a MaxEnt score < 3, and variants with low conservation across species (PhyloP < 0.99) were removed. The known phenotypic effects of the remaining variants were examined for relevance to cardiac anomalies

**Ethical approval**

This study meets the standards expected by the governance structure of the NHS and a clear and informed consent was taken from the family regarding the potential benefits and limitations of such work.

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**RESULTS**

**Quality control analysis**

Exome data were high quality defined by 80% of mappable bases of the Gencode defined exome represented by coverage of at least 20 reads. The average depth of coverage was 60.52 and 54.81 for patients III.3 and III.5 respectively, Supplementary Table 2.  The two samples from the affected cousins selected for exome analysis exhibited expected variant sharing for third degree relatives and no excess of sharing with any other sample on same dispatch DNA plate. Autosomal and X-chromosome heterozygosity were consistent with gender and did not indicate any sample contamination. VerifyBamID19 did not indicate any presence of contamination, and the application of a SNP tracking panel17 confirmed sample provenance.

**Tiered analysis**

In the *Tier 1*, no coding variation was found in *MYH7* & *NKX2-5* consistent with previous Sanger sequencing results.

*Tier 2*, the analysis was extended to the 219 genes prioritised from HGMD. A total of 429 variants were called in *either* affected cousin across the 219 genes. Given the apparent dominant mode of inheritance evidenced by the pedigree segregation and further supported by literature findings20,21,401 mutations seen in heterozygous form in a local reference database of 329 individual without heart defects were excluded. Of the remaining 28 variants, a further 27 variants were excluded from downstream analysis as they were not observed in *both* affected cousins and occurred in one individual only. A single variant on the X chromosome in the Filamin A (*FLNA)* gene remained (NM\_001110556, c.G4660A, p.G1554R). This variant was novel by our assessment and consistently predicted to be deleterious by several *in silico* software annotation tools (SIFT, Polyphen2, Mutation Taster and Gerp= 5.67).

Finally, for the *Tier 3* analysis we identified 16,543 variants shared between the cousins. These were filtered to yield a final list of 9 variants (supplementary Table 3). Comprehensive literature review excluded 8 variants within genes functionally irrelevant to phenotype (supplementary Table 3). The novel nonsynonymous *FLNA* variant (c.G4660A, p.G1554R) represented the only outstanding candidate variant. The variant was confirmed using Sanger sequencing. We confirmed the segregation of the genotypic variant assumed to be causal to be consistent with the inheritance patter of the family on the six living affected blood relatives diagnosed with Ebstein’s anomaly. The variant was confirmed as carried in heterozygous state in all affected females and in hemizygous state in all affected males. Patient IV(1) was not tested for the variant.

**Mutationwithin *FLNA*** The p.G1554R mutation segregating in our family with EA sits within the 14th repeated rod-domain, Figure 2. The mutation replaces a non-polar amino acid with a polar amino acid and is likely to impact tertiary structure as indicated by PolyPhen2 and GERP scores.

**Clinical Phenotype**

A summary of the cardiac, musculoskeletal and facial phenotypes of the family members

available for clinical examination is shown in table 1.

**Cardiac Phenotypes**

**Patient I(1):** Medical notes were not available but there was a history, given by surviving relatives of this individual having had a mitral valve replacement in middle age.

**Patient I (2):** Limited information was available but was reported by her daughter, II(2), to have no symptoms of cardiac disease at the age of 93 years. She also had a son and a daughter with a different partner and neither child, or in turn, any of their nine children have presented with cardiac disease.

**Patient II (1):** The echocardiogram demonstrated mild apical displacement of the tricuspid valve with a large antero-superior leaflet. There was no mitral valve anomaly and no mitral stenosis or regurgitation; the aortic valve was normal. The left atrium was mildly dilated, consistent with the age of the patient.

**Patient II (2):** In this description the following findings also relate to those found at surgery (repair of Ebstein’s anomaly and placement of a 32mm tricuspid annuloplasty ring). There was a large anterior leaflet of the tricuspid valve which was partially tethered. There was a partially tethered posterior leaflet and a fully Ebsteinised septal leaflet of the tricuspid valve.

**Patient III (1):** The infant was born at 33 weeks gestation with hydrops fetalis. Echocardiography showed severe Ebstein’s anomaly with absent flow into the pulmonary arteries. Despite optimum medical treatment, including with ventilation, dopamine and prostaglandin infusions, he died at ten days of age.

**Patient III (2):** The findings were very similar to III(4) above with mild apical displacement of the septal leaflet of the tricuspid valve (1.3cm) and mild associated tricuspid regurgitation.

**Patient III (3):** The following findings relate to those found at surgery (repair of Ebstein's anomaly and replacement of the mitral valve) and on preoperative 3D echocardiography. The tricuspid valve was typical of Ebstein's anomaly with a large mobile anterior-superior leaflet fused to the posterior leaflet which was partially tethered. There were some secondary cords to the back of both antero-superior and posterior leaflets, with complete tethering of the final portion of the posterior leaflet and Ebsteinisation of the whole of the septal leaflet. There was a funnel-like opening into the right ventricle with associated tricuspid stenosis and regurgitation. The mitral valve was stenotic with a fixed orifice (1cm2) and heavy calcification of the posterior leaflet and a parachute arrangement of the mitral chordae which were fibrosed and fused.

**Patient III (4):** There was isolated mild apical displacement of the tricuspid valve (1.3cm) with mild associated tricuspid regurgitation.

**Patient III (5):** On examination of echocardiogram findings, the tricuspid valve was dysplastic with apical displacement of the septal leaflet (2cm), mild tricuspid regurgitation (TR) and mild tricuspid stenosis (TS) (peak gradient 7, mean 5mmHg) with E/A reversal. The mitral valve was also dysplastic with a large antero-lateral papillary muscle and three small postero-medial papillary muscles instead of one well-formed structure. Mild mitral regurgitation and mild mitral stenosis (peak gradient 14, mean 4mmHg) were seen with a dilated left atrium (LA length 8cm, volume 34ml and area 18.1cm2). There was a small amount of calcification on the anterior leaflet. An effectively bicuspid aortic valve was seen with two low commissures and a small right coronary cusp. There was mild Aortic regurgitation (AR) but no aortic stenosis; there was no pulmonary regurgitation (PR). A moderate secundum atrial septal defect with an associated left to right shunt was evident.

**Patient IV (1):** Neonatal **c**linical examination, including detailed cardiovascular examination was normal.At the time of assessment of other family members, patient IV(1)’s parents declined an echocardiogram, genetic testing or further clinical assessment.

**Non Cardiac phenotypes**

Although certain mutations in *FLNA* are associated with a neurological phenotype (periventricular nodular heterotopia) there was no history of seizures or unsteadiness in any members of the family. Cerebellar examination was normal in all. There was no history of learning difficulties in any family member examined. Systematic enquiry regarding urological diagnoses and deafness was also negative. One family member III(4) had a diagnosis of irritable bowel syndrome but no other abnormalities of bowel motility were reported. Regarding the skeletal phenotype, all family members examined gave a history of ‘stiff joints’ with both surviving males having fixed flexion of the knees and ankles. A history of joint stiffness was also reported by the family to have been present in family member I(1); anecdotally his gait was also reported to be the same as that observed by the family in both III(3) and III(5). All patients examined had proximally placed and externally rotated 5th toes; male III(5) also had short thumbs with hypoplastic distal phalanges. Both males and two of the females had limited supination of the elbows. A dental phenotype was observed in III(5) who had oligodontia with incomplete eruption of secondary dentition. Keloid scarring, noted in all members of the family who were examined was also reported to have been present in I(1) (see Table 1 for summary).

Although not examined as part of this study, individual IV(1) was described by the family as having no joint limitation, clearly demonstrated to them by her enthusiasm for exercise and general flexibility. The ease of movement in IV(1) was described as a direct contrast to the joint restriction seen in members of generation III during childhood. Patient I(1) was deceased at the time of the study; he died from bowel cancer at the age of 75 years.

**DISCUSSION**

Ebstein’s anomaly is a very rare condition and even less frequently observed segregating in families. We describe a family with evidence for this rare cardiac defect over at least two generations. This family was previously described but genetic aetiology remained elusive.

In this study we have applied contemporary sequencing technology and effective filtering of a cousin pair and identified a single variant within an X-linked gene known to cause cardiac developmental defects but not previously associated with Ebstein’s anomaly. We have confirmed the mutation using traditional Sanger techniques and demonstrated heterozygous and hemizygous carrier status for all female and male affected family members respectively.

The single base substitution (G>A) causes a missense mutation in which a non–polar Glycine amino acid residue is replaced with a positively charged Arginine. The variant was within a highly conserved (Polyphen=0.999) residue region of the protein that encodes for the 14th Ig-like domain. The tertiary structure of the Ig-like repeated domain consists in 7 antiparallel beta sheets and therefore it might be expected that the p.G1554R mutation causes a conformational change in the beta strands.

We have found no previous evidence of this variant in our local or public repository databases. However, mutations within the Ig-Rod domain are causal of a variety of congenital heart defects.

The phenotypic spectrum of disease associated with mutations in *FLNA* is broad and includes several conditions with a predominantly skeletal phenotype; FG syndrome, Frontometaphyseal dysplasia, Melnick-Needles syndrome,27 Otopalatodigital syndrome, types I and II27, and terminal osseous dysplasia.28 It also includes individuals with a predominantly neurological phenotype (periventricular nodular heterotopia)29 and those with cardiac manifestations of disease (X-linked cardiac valvular dysplasia).30 Its causality is further underscored by the missense and loss of function variants presented in ExAC.31 The family identified were examined for any features of the above conditions. Macrocephaly, seen in FG syndrome and was found in both of the males and one female, however, this condition also includes mental retardation, not present in this family. Survival to adulthood noted in this family is not typical in males affected by Melnick-Needles syndrome or Otopalatodigital syndrome type II. Skeletal features including joint contractures, prominent supraorbital ridges and proptosis observed in members of the family described are typically associated with Fibromuscular Dysplasia (FMD);27, however, typical of this condition is also conductive and sensorineural hearing loss, neither of which were present in this family. In addition, while cardiac anomalies are described in FMD, EA is not.

The data available for the 6 cases described above (based on a combination of the echocardiogram and MRI findings and the operative findings in the 2 patients who underwent surgery) clearly identify varying degrees of severity of Ebstein's anomaly in these cases. The phenotype varied between mild septal displacement of the septal leaflet of the tricuspid valve with mild associated tricuspid regurgitation, to severe Ebstein's anomaly with a combination of tricuspid stenosis and regurgitation. In 3 of the 4 females (II(1), III(4) and III(2)) the anomaly was subtle with isolated mild displacement of the tricuspid valve and only mild tricuspid regurgitation. Only in female patient II (2) was the anomaly more severe and required surgery. This more severe phenotype could be explained by a hypothesized skewed pattern of X-chromosome inactivation which in many other X-linked disorders has been considered as the cause of the phenotype in female carriers.

The males III (3) and III (5) had more severe cardiac presentation with both tricuspid and mitral valves involvement requiring surgery. It is unusual for patients with Ebstein’s anomaly to have mitral valve disease: this is congenital in nature, but has also been associated with calcification which was progressive in at least one of them. The patient III (3) developed severe mitral valve stenosis and this was an important factor in the timing of surgery. The other, III (5), had a dysplastic mitral valve with only mild calcification of the anterior leaflet and only mild stenosis and regurgitation. Only one patient has an atrial septal defect and one patient (operated) had a patent foramen ovale (PFO). However, it is not possible to exclude small PFO's in the other patients as they have not had a contrast echocardiogram.

The finding of mitral valve disease in addition to EA and abnormalities of the pulmonary and aortic valves in members of this family illustrates a phenotypic overlap with X-linked valvular dysplasia (23). The variability of the phenotype comparing affected males with each other (and similarly, comparing the affected females with each other) is consistent with that observed among individuals who harbour pathogenic variants in other genes that predispose to the manifestation of a cardiac phenotype, such as *GATA4*32.

In conclusion, the absence of a neurological phenotype, survival of some males to adulthood and the presence of EA in multiple members of this family mean that the *FLNA* mutation described is associated with the unique phenotype seen in this family and furthermore, it represents a novel cause for familial EA. However, due to the presence of additional features segregating with EA in this family, there is little evidence that mutations in *FLNA* will be a common cause of sporadic, non-syndromic EA.

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**CONFLICT OF INTEREST DISCLOSURES**

None

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**Table 1: Cardiac, musculoskeletal and facial phenotype for family members available for clinical examination**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
|  |  | **MALE** | | **FEMALE** | | | |
|  |  | III(5)\* | III(3)\* | II(2) | II(1) | III(4) | III(2) |
|  | Age at examination (years) | 31 | 27 | 57 | 59 | 34 | 35 |
| **Skeletal features** | Head Circumference | 60.0cm (91st-98th centile) | 60.5cm (91st-98th centile) | 58.5cm (98th-99th centile) | 54cm (9th-25th centile) | 55.5cm (50th centile) | 56.2cm (50th-75th centile) |
| Limb musculature | ↓↓ | ↓↓ | ↓ | - | - | - |
|
| Hypertelorism | ++ | ++ | - | + | - | - |
| Prominent supraorbital ridges | ++ | ++ | - | - | - | + |
| Proptosis | ++ | ++ | - | + | - | + |
|
| Elbow supination | ↓ | ↓ | ↓ | - | ↓ | - |
| Elbows | ++ | ++ | + | + | - | - |
| Fingers | - | + | + | + | - | + |
| Keloid scarring | ++ | ++ | + | + | + | - |
| Knees | ++ | ++ | + | - | - | - |
|  | **Intellectual delay** | - | - | - | - | - | - |
| **Cardiac features** | Aortic regurgitation | Mild | - | - | - | - | - |
| Aortic root aneurysm | - | - | - | - | - | - |
| Aortic valve insufficiency | Mild | - | - | - | - | - |
| Atrial septal defect (ASD) | + | **-** | **-** | - | - | - |
| Bicuspid Aortic Valve | + | **-** | - | - | - | - |
| Cardiomyopathy | - | - | - | - | - | - |
| Dysplastic pulmonary valve | - | - | - | - | - | - |
| Left ventricular noncompaction | - | - | - | - | - | - |
| Mitral regurgitation | Mild | Severe | - | - | - | - |
| Mitral stenosis | + | + | - | - | - | - |
| Mitral valve cleft | - | - | - | - | - | - |
| Mitral valve dysplasia | + | Severe | - | - | - | - |
| Mitral valve prolapse | - | - | - | - | - | - |
| Myxomatous valvular dystrophy | + | - | - | - | - | - |
| Patent ductus arteriosus | - | - | - | - | - | - |
| Patent foramen ovale (PFO) | - | **-** | **+** | - | - | - |
| Pulmonary regurgitation | + | + | - | - | - | Mild |
| Pulmonary stenosis | - | - | - | - | - | - |
| Surgical intervention | - | Monocuspid tricuspid valve repair in 2006 | Ebstein's repair in 2006 | - | - | - |
| Tricuspid regurgitation | Mild | Moderate | Residual post Ebstein's repair | Mild | Mild | Mild |
| Tricuspid stenosis | + | **+** | - | - | - | - |
| Tricuspid valve displacement | 2.0 cm | Surgical repair | Surgical repair | 1.4 cm | 1.3 cm | 1.3 cm |
| Ventricular septal defect (VSD) | - | - | - | - | - | - |

\*Exome sequenced patient; NA, not available;↓ Reduced; + Present; - Not present

**FIGURE LEGENDS**

Figure 1. Family pedigree showing segregation of Ebstein’s anomaly. Affected individuals are shown in dark symbols. Asterisk (\*) indicates patient selected for whole exome sequencing analysis. † Individual III (1) died at 10 days of age from cardiac failure. ‡ Individual I (1) is designated as likely affected following family description of surgical mitral valve replacement, keloid scarring, stiff joints and wide-based gait.

**Figure 2.** **Schematic representation of filamin A protein**. Filamin A acting-binding protein is encoded across 48 exons of the *FLNA* gene and acts as a regulator of the actin cytoskeleton20,21. The protein contains an N-terminal actin binding domain (ABD) and a region comprised of 24 repeated immunoglobulin-like (Ig-like) subdomains.22 The predicted tertiary structure of these repeated domains is 7 antiparallel beta sheets of 3 and 4 beta strands.23,24 The Ig-like domains are required for protein interactions with other Ig-like domains via their beta-sheets. 25,26 Mutations within *FLNA* have been associated with multiple conditions: cardiac valvular dysplasia, FG syndrome, frontometaphyseal dysplasia, periventricular heterotopia, Melnick-Needles syndrome, otopalatodigital syndrome, type I, otopalatodigital syndrome, type II and terminal osseous dysplasia. 24 The 107 different known disease causal mutations reported in HGMD are herein represented by diamonds (e.g. the five different mutations associated with Melnick-needles Syndrome fall within the 10th Ig-like domain whereas mutations associated with periventricular heterotopia are spread across multiple Ig-like domains and the actin binding domains. The G1554R mutation identified in the current family is shown. All known *FLNA* causal disease mutations, extracted from HGMD in January 2014, are in Supplementary Table 4.