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FOXC1 haploinsufficiency due to 6p25 deletion in a patient with rapidly progressing aortic valve disease

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6p25 deletion is a rare but well-known entity. The main clinical features include an abnormal facial appearance, developmental delay, and ocular anomalies. Cardiac anomalies are frequently seen but remain poorly delineated. We describe a 4-year-old girl with 6p25.3 deletion, which includes the *FOXC1* gene, typical dysmorphic features associated with developmental delay and oculo-motor anomalies. Aortic valve dysplasia was diagnosed early in life. The cardiac lesion progressed very rapidly between the age of 3 and 4 years requiring aortic valve replacement. Genomic analysis of blood and excised valve tissue showed down-regulation of *FOXC1* but also *FOXC2* expression in the diseased aortic valve. This allows us to speculate on the potential role of *FOXC1* in aortic valve anomalies.

KEYWORDS

6p25 deletion, aortic valve disease, *FOXC1*, genetics

1 | INTRODUCTION

Patients with 6p25 deletion syndrome present a variable phenotype of malformations and developmental delay. The spectrum of anomalies includes anterior segment dysgenesis ranging from isolated posterior embryotoxon to Rieger anomaly, brain anomalies, heart defects, hearing loss, and facial gestalt that includes hypertelorism, down-slanting palpebral fissures, and midface hypoplasia (de Vos, Stegmann, Webers, & Stumpel, 2016; Gould et al., 2004). Heart defects are seen in up to 60% of the patients. Atrial and ventricular septal defects, outflow tract abnormalities, and patent ductus arteriosus are most frequently reported; valvular abnormalities are less frequently described (de Vos et al., 2016).

Human *FOXC1* is located on chromosome 6 (6p25) and *FOXC1* haploinsufficiency is considered responsible for most of the clinical features of 6p25 deletion syndrome, leading to disturbed neural crest development (de Vos et al., 2016). Moreover, isolated mutations in *FOXC1* are associated with anterior segment dysgenesis and extra

ocular anomalies such as hearing loss, hypodontia and heart defects (Du et al., 2014; Gripp, Hopkins, Jenny, Thacker, & Salvin, 2013).

We describe a young patient with 6p25 deletion who presented a rapidly evolving aortic valve disease. Genomic analysis of blood and excised aortic valve was conducted.

2 | CLINICAL REPORT

A 4-year-old girl was referred to the pediatric cardiology department for treatment of a rapidly evolving aortic stenosis. She was the fourth child of healthy unrelated parents. Increased nuchal translucency was noted during pregnancy but fetal karyotype was normal (46,XX). At the age of 4 months, she required surgery for a vallecular cyst. Cardiac ultrasound examination performed at that stage showed a dysplastic but non-stenotic and non-regurgitant tri-leaflet aortic valve. At the age of 17 months, global developmental delay became apparent. Somatic growth was preserved with weight, height, and head circumference at

the 50th centile. Genetic expertise described an abnormal facial appearance with hypertelorism and downslanting palpebral fissures (Figure 1). Ophthalmic exam was normal, in particular free of anterior segment dysgenesis.

Chromosome microarray analysis (see section 3) showed an interstitial 6p25.3 deletion, extending from position 599.174 to 1.970.348 using UCSC Genome Browser (<http://genome.ucsc.edu/>; February 2009 Assembly, hg19). The deletion encompassed nine genes including *FOXC1* and *EXOC2*, and was approximately 1.4 Mb. No other clinically significant copy number variations were detected. This deletion was confirmed using FISH analysis. Parental analysis was normal.

At the age of 4 years, weight and height remained within normal range, respectively, 18 kg (75th centile) and 103 cm (75th centile) and head circumference at the 50th centile. Cardiac follow-up revealed significant worsening of the aortic valve stenosis. Six months later, the peak left ventricle to aorta gradient reached 120 mmHg and mean gradient 70 mmHg, and was associated with mild valve regurgitation. Aortic valve replacement was indicated. Aortic annulus was normal for weight and height (13 mm, z-score -0.8). The ascending aorta was mildly dilated (20 mm, z-score +2.6) (Figure 2). Significant left ventricle hypertrophy was noted with preserved systolic function. Mitral and

tricuspid valves were considered normal. Pulmonary valve was noted to be mildly regurgitant but non-stenotic (Figure 2). The main pulmonary artery was not dilated. Cardiac anatomy was otherwise normal; in particular no coarctation was associated. Pre-operative CT-scan showed a hypoplastic left internal carotid artery. Aortic arch was otherwise normal. Surgery consisted of aortic valve replacement with a 19 mm aortic homograft, under cardiopulmonary bypass. Macroscopic examination revealed a very thickened, dysplastic but tri-leaflet aortic valve. After parental consent, the excised aortic valve tissue was sent for RNA-extraction. Post-operative course was complicated by respiratory infection and failure requiring ECMO but the child eventually left the hospital after 2 months, in good condition. One year after surgery the patient was in good clinical condition with on cardiac ultrasound a non-regurgitant, non-stenotic aortic valve. Pulmonary valve regurgitation remained stable.

3 | METHODS

3.1 | Chromosome microarray

Chromosome microarray was carried out using an 180,000 oligonucleotide array (Agilent Human Genome CGH Microarray kit G4449A[®],



FIGURE 1 Clinical findings at the age of 17 months (a,b) and 3 years (c,d), showing hypertelorism, strabismus, and downslanting palpebral

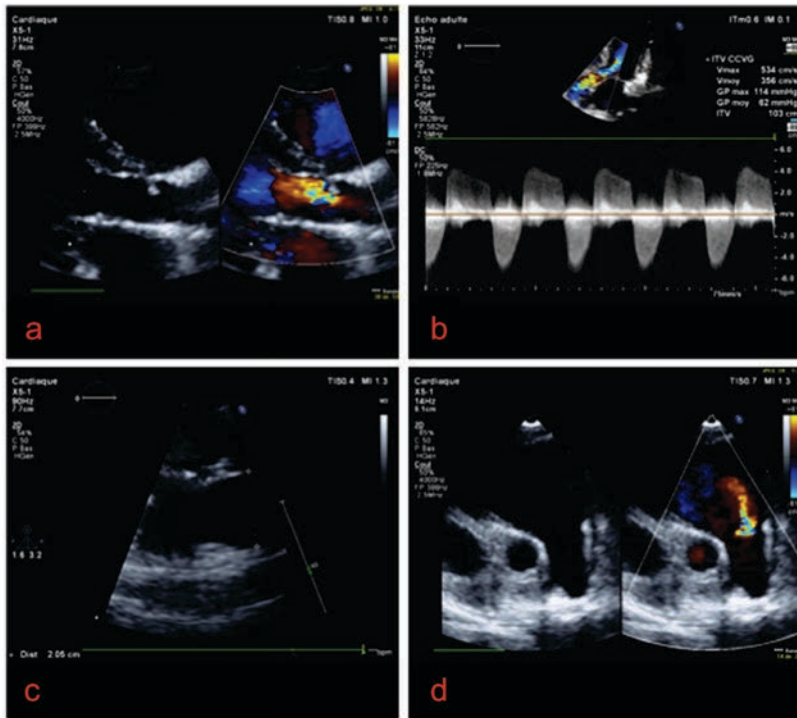


FIGURE 2 Pre-operative echocardiography data: (a) significant aortic insufficiency on color Doppler analysis, (b) Doppler analysis of severe aortic valve stenosis and insufficiency, (c) mild ascending aorta dilatation (2-dimensionnal echocardiography), (d) mild pulmonary valve insufficiency on color Doppler analysis. [Color figure can be viewed at wileyonlinelibrary.com]

Agilent Technologies, Santa Clara, CA). DNA was extracted from peripheral whole blood using a saline precipitation method. The data were processed using Agilent Feature Extraction (version 10.7.3.1) and DNA Analytics softwares (version 4.0.85) (both from Agilent Technologies), with the statistical algorithm ADM-2, sensitivity threshold 5 and at least 3 consecutive aberrant probes.

3.2 | Real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR)

The pediatric cardiac team collected the aortic valve tissue. The protocol was evaluated and authorized by the local ethical board ("CPP Sud Méditerranée I" n°: 1657 MS 1). Control aortic valve was collected from a 7-year-old boy with normal karyotype. Immediately following surgical removal, a portion of the aortic valve leaflet was immersed in RNA_{later} solution (Sigma-Aldrich, Saint-Louis, MI) overnight and stored at -80°C . Total RNA was purified using Trizol (Life Technologies, Carlsbad, CA) and RNeasy mini Kit (Qiagen, Venlo, The Netherlands). RNA was reverse transcribed using the AffinityScript Multiple Temperature cDNA synthesis kit (Agilent Technologies, Santa Clara, CA). Quantitative PCR was performed using SYBR Green (Roche, Basel, Switzerland) based pPCR on a LightCycler 480 (Roche). Primers used in real-time PCR analysis of all genes examined were:

FOXC1: Forward: 5'AGCCATGAGCCTGTACGC and Reverse: 5'GTGACCGGAGGCAGAGAGTA;

FOXC2: Forward: 5'GAGATGTTCAACTCCCACCG and

EXOC2: Forward: 5'AGGCTTGACCATTGTGGAC and Reverse: 5'CATTITTTGGCTTGCCCACT;

DUSP22: Forward: 5'ATATTTTCCCCTTATCCCCACTG and Reverse: 5'TCACAGGAAGCAATCAGAG.

Graphs show the mean \pm standard deviation for three biological replicates. Samples were normalized to *GAPDH* as endogenous housekeeping gene. Level changes were calculated by the comparative cycle threshold ($\Delta\Delta\text{CT}$) method.

4 | RESULTS

RNA sequencing analysis performed in our laboratory on normal aortic valves revealed that among the 9 deleted genes included in the 1.4 Mb microdeletion, only *EXOC2* and *FOXC1* are expressed in the normal valve (unpublished data).

Based on these findings we first performed quantification for *EXOC2* and *FOXC1* mRNAs. Real-time quantitative PCR revealed significant reduction of these two transcripts (Figure 3). We further examined expression level of *FOXC2*. Surprisingly, *FOXC2* transcript levels were also reduced in the excised aortic valve when compared to control tissue. To assess the copy numbers of *FOXC2*, we performed quantitative PCR on genomic DNA extracted from the excised aortic valve tissue. As expected, we did not find any modification in *FOXC2*, while *FOXC1* and *EXOC2* were reduced as a

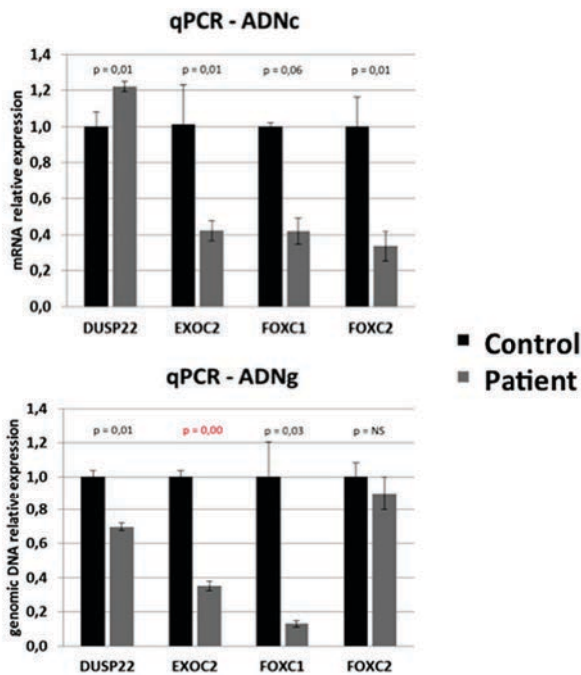


FIGURE 3 Results of quantitative PCR for mRNA (upper figure) and for genomic DNA (lower figure). In the upper figure, real-time quantitative PCR for *EXOC1*, *FOXC1*, and *FOXC2* mRNAs showed significant reduction in the excised aortic valve tissue (gray bars) when compared to normal tissues (black bars). In the lower figure, quantitative PCR on genomic DNA extracted from the excised aortic valve tissue (gray bars) did not find any modification in *FOXC2*, while *FOXC1* and *EXOC2* were reduced when compared to normal (black bars) as a result of the deletion. [Color figure can be viewed at wileyonlinelibrary.com]

5 | DISCUSSION

The spectrum of anomalies seen in 6p25 deletion syndrome varies. Axenfeld-Rieger syndrome is the most striking feature and has clearly been linked to the *FOXC1* gene. Cardiac anomalies are described in up to 60% of the patients reported.

FOXC1 belongs to the forkhead family of transcription factors and is important for eye, genito-urinary, and cardiovascular development. However, the precise role of *FOXC1* in human cardiogenesis remains unclear. Human heterozygous mutations in *FOXC1* are associated with the Axenfeld-Rieger syndrome, and include some patients with additional congenital heart disease (Du et al., 2014; Gripp et al., 2013). Isolated, non-syndromic tetralogy of Fallot and coarctation have also been associated with mutations of *FOXC1* (Sanchez-Castro et al., 2016; Töpf et al., 2014).

The murine homologue *Foxc1* is expressed in periocular, prechondrogenic, and posterior fossa mesenchyme, meninges, endothelial cells, heart, and kidneys during embryonic development. It encodes a protein with virtually identical binding domains as the protein encoded by *Foxc2*, located on chromosome 8 (Ch16 in human). *Foxc1* and *Foxc2* have overlapping transcription domains in many embryonic tissues including paraxial, cephalic and nephrogenic

and mesenchyme of the aortic arches, valves, and outflow tract (Iida et al., 1997; Kume, 2009; Kume, Jiang, Topczewska, & Hogan, 2001; Winnier et al., 1999). Analysis of *Foxc1* and *Foxc2* mutant mice has demonstrated that these very similar transcription factors are both required for cardiovascular development. Homozygous *Foxc1* null mutants have skeletal, ocular and genitourinary abnormalities, and cardiovascular malformations such as interrupted aortic arch type-B, ventricular septal defect, and coarctation. Homozygous *Foxc2* null mutants have abnormal aortic arch patterning. Compound *Foxc1*;*Foxc2* homozygous null mutants have a lethal phenotype with multiple skeletal, ocular, and genitourinary abnormalities. Early remodeling of blood vessels is mostly normal but they do have cardiovascular defects, such as interruption or coarctation of the aortic arch. These compound mutant mice also lack the outflow tract and right ventricle (Seo & Kume, 2006). Compound *Foxc1*;*Foxc2* heterozygotes die mostly pre- and perinatally with a similar spectrum of cardiovascular, genitourinary, and eye abnormalities as those seen in each single homozygous null mutant (Kume et al., 2001; Seo & Kume, 2006; Seo et al., 2006; Winnier et al., 1999). Interestingly, compound *Foxc1*^{+/-};*Foxc2*^{-/-} mutants have a shortened outflow tract, a smaller right ventricle and a hypoplastic inflow tract as the other compound heterozygotes have not. The compound *Foxc1*^{+/-};*Foxc2*^{-/-} mutants have a reduced contribution of cardiac neural crest cells to the outflow tract, but also defects in the formation of endocardial cushions, which are required to outflow tract septation and arterial valve development. Those data led to the conclusion that these two closely related factors play a similar but not completely identical, dose-dependent, and synergistic role in the development of the cardiovascular system.

In our patient, it is of interest to note that valvular expression of *FOXC1* and *FOXC2* is significantly reduced when compared to the normal aortic valve tissue. Reduced *FOXC1* expression might be explained by the “haploinsufficiency” caused by the deleted segment. However, the reduction of *FOXC2* expression in the excised aortic valve tissue is not explained directly by the deletion and could result from an inter-regulation between these two factors. Consistently, in vitro experiments showed that transfection of *Foxc1* or *Foxc2* in 10T1/2 cells led to a slight up-regulation of *Foxc2* and *Foxc1*, respectively, suggesting a cross-regulation between these two factors (Seo et al., 2006). Our case report suggests that deletion of *FOXC1*, either directly or cooperatively with *FOXC2*, may interfere with arterial valve development, as seen in this patient.

CONFLICT OF INTEREST

None of the authors have conflicts of interest to declare.

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