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Analysis of *FOXF1* and the *FOX* gene cluster in patients with VACTERL association

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Abstract

VACTERL association, a relatively common condition with an incidence of approximately 1 in 20,000 – 35,000 births, is a non-random association of birth defects that includes vertebral defects (V), anal atresia (A), cardiac defects (C), tracheo-esophageal fistula (TE), renal anomalies (R) and limb malformations (L). Although the etiology is unknown in the majority of patients, there is evidence that it is causally heterogeneous. Several studies have shown evidence for inheritance in VACTERL, implying a role for genetic loci. Recently, patients with component features of VACTERL and a lethal developmental pulmonary disorder, alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV), were found to harbor deletions or mutations affecting *FOXF1* and the *FOX* gene cluster on chromosome 16q24. We investigated this gene through direct sequencing and high-density SNP microarray in 12 patients with VACTERL association but without ACD/MPV. Our mutational analysis of *FOXF1* showed normal sequences and no genomic imbalances affecting the *FOX* gene cluster on chromosome 16q24 in the studied patients. Possible explanations for these results include the etiologic and clinical heterogeneity of VACTERL association, the possibility that mutations affecting this gene may occur only in more severely affected individuals, and insufficient study sample size.

Keywords

VACTERL; VACTERL association; *FOXF1*; *FOX* gene cluster; VATER; VATER association

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1. INTRODUCTION

1.1 VACTERL association

VACTERL is an acronym used to describe the non-random clustering of congenital anomalies: vertebral defects (V), anal atresia (A), cardiac defects (C) tracheo-esophageal fistula (TE), renal anomalies (R) and limb malformations (L). It is often termed an association in order to emphasize that these malformations appear together more often than would be expected by chance [2,13]. To diagnose the condition, most clinicians look for the presence of three constituent features of VACTERL association, without evidence of an alternate, overlapping diagnosis [17]. Diagnosis is difficult due to the number of disorders that have overlapping features with VACTERL, including Feingold syndrome, CHARGE syndrome, 22q11 deletion syndrome, Townes-Brocks syndrome, Pallister-Hall syndrome, Holt-Oram syndrome, Fanconi anemia, and Baller-Gerold syndrome [16].

VACTERL is likely a defect of blastogenesis, with an estimated incidence of 1 in 20,000 to 1 in 35,000 births [2,11,13]. Although relatively common, the causes of VACTERL have yet to be elucidated in the majority of patients. VACTERL is thought to largely occur sporadically, though there is evidence for inheritance in at least a subset of patients [17], suggesting that genetic factors may play a key role. A number of studies give evidence of heterogeneous etiologies [2,7,13,17].

Although few large studies looking for genetic or other causes of VACTERL have been performed to date, likely due to relatively few familial cases and the heterogeneity, scattered case reports describe possible genetic causes. Thus far, patients with VACTERL association have been found to have mutations in *HOXD13*[4], *ZIC3* [20], *PTEN* [12], and mitochondrial genes [3]; additionally, mutations and deletions involving *FOXF1* and the *FOX* gene cluster [15,19,21] have been identified in patients with VACTERL association features as well as a specific pulmonary phenotype (see below). The pathogenicity of each of these mutations as they relate to VACTERL association is not uniformly clear. However, animal models of the *FOXF1* gene and related signaling pathways have provided clues as to its pathogenicity, making *FOXF1* an intriguing candidate gene for VACTERL association.

1.2 *FOXF1* and VACTERL

Recently, Stankiewicz et al. (2009) identified overlapping microdeletions in 16q24.1q24.2 in seven patients with component features of VACTERL association, namely vertebral anomalies, gastrointestinal atresia, TE fistula, and cardiac malformations. In addition, these patients also had alveolar capillary dysplasia with misalignment of pulmonary veins (ACD/MPV), a rare and lethal developmental pulmonary disorder [19]. These deletions included the FOX transcription factor gene cluster at 16q24.1q24.2. All but one of these deletions contained *FOXF1*, a gene that plays a pivotal role in the development of the lung and foregut [10].Interestingly, the one patient with the deletion not involving *FOXF1* had a distinct phenotype with less overlap with VACTERL association in terms of the type of congenital malformations. Similar clinical and genetic findings have been reported by Yu et al (2009), who reported a patient with a 1.37 Mb deletion of chromosome 16q24.1-q24.2. In addition to ACD/MPV, this patient had genitourinary, cardiac, and intestinal anomalies [21].

Stankiewicz et al (2009) further identified four unrelated patients with ACD/MPV who had heterozygous mutations in *FOXF1*. In addition to ACD/MPV, all of these patients had associated malformations, namely cardiac, intestinal, and urinary tract malformations [19]. Specifically, one patient had a cardiac malformation, with a partial atrio-ventricular canal defect and a patent ductus arteriosus. Three patients had gastrointestinal malformations,

which included intestinal malrotation, annular pancreas, duodenal stenosis, congenital short bowel, omphalocele, and a Meckel's diverticulum. Anomalies affecting the urinary tract were present in three patients, and were described as including hydronephrosis, hydroureter, bladder dilatation, and obstructive renal dysplasia [19]. As the pattern of these malformations is similar to those seen in patients with VACTERL association, these human studies provide compelling evidence for the possible role of FOXF1 in the pathogenesis of VACTERL.

The pivotal role of FOXF1 in the development of the lung and foregut has support from mouse models, where haploinsufficiency of *Foxf1* causes a variable phenotype including lung immaturity and hypoplasia, fusion of the right lung lobes, narrowing of the esophagus and trachea, esophageal atresia, and TE fistula. Of note, similar malformations have been found in *Sonic hedgehog (Shh)* mouse mutants [8,10]. Further evidence for the connection between these two genes has been shown in mice, where *Foxf1* expression was induced by exogenous *Shh* in the lung and gut [9–10]. In addition, expression patterns of *Foxf1* mirror the basal levels of *Shh* expression [10]. The reproduction of similar VACTERL-type anomalies in *Shh (−/−)* and *Foxf1* (+/−) mouse mutants implies that these genes share a common pathway whose aberration may contribute to the VACTERL phenotype [8–10].

If the pathway linking *SHH* and *FOXF1* is thus involved in VACTERL association, the logical question is at what point in that pathway might genetic changes result in the type of anomalies observed in patients with VACTERL association? Several factors hint that it is not likely that this pathway would be altered at the level of *SHH* itself. Not only do loss-of-function mutations in *SHH* cause holoprosencephaly in humans, but multiple studies have shown negative mutation analysis of the *SHH* gene in patients with VACTERL association features without any signs of holoprosencephaly [1,6,14]. These studies provide a basis for further investigations aimed at downstream targets of the *SHH* pathway, such as *FOXF1*.

In summary, due to the involvement of SHH signaling in gut development, as well as the role of its downstream effectors, including FOXF1, the demonstration of FOXF1 as a SHH target, the presence of *FOXF1* mutations and deletions in patients with features of VACTERL association, and the VACTERL association-type phenotype present in mice heterozygous for *Foxf1* mutations, we carried out a mutational analysis of the candidate gene *FOXF1* and a copy number analysis of the 16q24.1q24.2 region containing the *FOX* gene cluster in a cohort of 12 patients with VACTERL association but without clear evidence of the pulmonary condition observed in previous patients with mutations affecting this gene, with the hypothesis that mutations in *FOXF1* could result in a variable, and milder phenotype than in the patients originally described.

2. METHODS

2.1 Patients

Patients were recruited through our National Human Genome Research Institute/National Institutes of Health (NIH) (Bethesda, MD, United States) IRB-approved protocol on VACTERL association, with informed consent obtained from all participants. In order to be included in the research described here, patients had to have at least three major component features of VACTERL association. Patients with evidence of another overlapping, explanatory diagnosis, either due to certain clinical features or because of results of genetic testing, were excluded. A total of 12 patients who met the above criteria were included in this study (8 additional patients with features of VACTERL were also studied, but further details are not described here as these patients did not meet the above criteria). Nine of the12 patients were interviewed and examined in person at the NIH. For the patients who were not seen in person at the NIH, blood samples were sent to our laboratory, and available medical

records were reviewed, with further medical histories provided by patients, family members and referring clinicians via phone and e-mail interviews.

2.2 Sequence analysis of the *FOXF1* gene

2.2.1 DNA isolation and PCR amplification—Patient genomic DNA was extracted from peripheral blood using a Qiagen kit per standard protocol. All amplicons were amplified employing a Roche LightCycler®480 (Roche, IN) in a 25 μl reaction volume using 10 ng DNA template, 2.5 μl PCR buffer, 0.5 μl 10 mM dNTPs, 5–7.5 μl GC-rich solution, and 0.3 μM of each primer. PCR amplification parameters were variable for each amplicon (see supplemental table 1): incubation at 95°C for 10 min followed by 45 cycles of: denaturation at 95°C for 10 sec, annealing (depending on primer, see supplemental table 1) at 62–70°C for 15 sec, and extension at 72°C.

2.2.2 DNA sequencing—Sequencing was performed bi-directionally at the core DNA Sequencing Facility, National Institute of Neurological Disorders and Stroke, NIH. DNA sequences were analyzed using Sequencher 4.10.1.

2.3 Copy number variation analysis of the *FOX* gene cluster

Genomic imbalances involving the 16q24.1q24.2 region were studied using the Illumina Omni-1-Quad SNP array. Three-hundred ng of DNA (4 μl of 75ng/μl DNA) was used for analysis using the Illumina “infinium assay” protocol [5]. Briefly, DNA was whole-genome amplified, fragmented, hybridized, fluorescently tagged, and scanned. The DNA samples were hybridized to the Illumina HumanOmni1-Quad BeadChips. These chips contain over 1 million SNP loci, enabling a high resolution CGH analysis.

The data was collected using BeadArray scanner, and visualized using the GenomeStudio (v2009.2, http://www.Illumina.com) genotyping module. The call rates for all the DNA samples were >99%. The results collected included the logR ratio and the B-allele frequency. The logR ratio represents a measure of the total fluorescent intensity signal for the given marker, and B-allele frequency values represent the relative ratio of the fluorescence for one allelic probe to the other. The regions with an increase in the logR ratio, and occurrence of two B allele frequency values (between 0 and 0.5, and 0.5 and 1) indicate a duplicated region, and correspondingly, a decrease in logR ratio and lack of heterozygosity (0.5) for B allele frequency, depict the regions of deletion.

3. RESULTS

3.1 Patients

For specific information on the patients studied, see Table 1. None of the 12 patients in our study had ACD/MPV, though 42% (5/12) patients had pulmonary findings (which does not, however, imply that these pulmonary findings are related to ACD/MPV). These pulmonary findings included abnormal bronchial branching,, broncheomalacia, persistent pulmonary hypertension, unilateral pulmonary agenesis, unilateral lobar agenesis, and pulmonic stenosis.

3.2 *FOXF1* mutation analysis

FOXF1 mutation analysis using PCR amplification and direct sequencing revealed no mutations or variants in the *FOXF1* gene coding sequence or the intron/exon boundaries in any of the 12 patients (or in 8 additional patients with features of VACTERL association who did not meet formal criteria for VACTERL association). Additionally, Illumina Omni1-

Quad high-density SNP array revealed no anomalies affecting the *FOXF1* region and the *FOX* gene cluster on chromosome 16, in particular in the region of 16q24.1q24.2.

4. DISCUSSION

Our analysis of *FOXF1* showed normal sequences and no genomic imbalances affecting the *FOX* gene cluster in the studied patients. Although the results of this study are negative, this does not necessarily exclude the possibility that *FOXF1* mutations are associated with VACTERL association.

Contributing to the negative results may be the small number of patients studied. Due to the likely clinical heterogeneity, it is difficult to pre-estimate the necessary sample size, but this study is almost certainly underpowered. There may still be utility in testing patients with VACTERL association for mutations in *FOXF1* on a research basis, as this gene has been shown in humans and animals to have a possible role in the pathogenesis of VACTERL association. Further, as the clinical findings in VACTERL association are highly variable, there could be a subgroup of patients in addition to those previously described [15,19] who should be tested on a clinical basis for *FOXF1* aberrations. Results of this and previous studies suggest that *FOXF1* may be a better candidate gene for more severely affected patients with a predominant respiratory component (such as ACD/MPV).

It is important to note that VACTERL association is likely very heterogeneous not only in etiology, but also in clinical presentation. VACTERL association may represent a spectrum from the less severely affected, such as those in our study, to the more severely affected, such as described by Stankiewicz et al (2009) and Yu et al (2010) [19,22]. This may contribute to the negative results of our study. The high perinatal mortality found in humans with *FOXF1* mutations is concordant with mouse studies, where mice homozygous for *Foxf1* mutations die before embryonic day 10 [10]. Interestingly, the only patient described by Stankiewicz et al. (2009) who had a microdeletion close to but not containing *FOXF1* was also the only patient without a severe respiratory phenotype [19].

Patients with mutations solely affecting *FOXF1* were reported as having additional features not typical of VACTERL association, including intestinal malrotation and congenital short bowel [19]. In contrast, patients with deletions including *FOXF1* as well as *FOXC2, FOXL1* and *MTHFSD*, have more typical features of VACTERL association (including vertebral anomalies, gastrointestinal atresias, urinary tract malformations, and cardiac anomalies). Further, when compared with the above-mentioned patient whose deletion did not include *FOXF1*, this latter patient has less typical features of VACTERL association [19].

Further testing of a larger cohort of patients with VACTERL association for *FOXF1* mutations, as well as other mutations in genes in the 16q24.1q24.2 region, could help further delineate the spectrum. Because VACTERL association is thought to be causally heterogeneous, it is unlikely that there is one gene responsible for all cases, and it may be important to test multiple genetic factors to arrive at a satisfactory explanation of cause in a cohort of affected patients.

5. CONCLUSIONS

We did not find mutations in *FOXF1* or genomic anomalies affecting the *FOX* chromosome 16q24.1-q24.2 gene cluster in our small cohort of patients. Despite these negative results, this gene and genetic region remain interesting in the pathogenesis of VACTERL association in humans.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Abbreviations

ACD	alveolar capillary dysplasia
CGH	comparative genomic hybridization
CHARGE	coloboma, heart defect, atresia choanae, retarded growth and development, genital abnormality, ear abnormality
dNTP	Deoxynucleotide Triphosphate
IRB	Institutional Review Board
Mb	megabase
MPV	misalignment of pulmonary veins
NIH	National Institutes of Health
PCR	polymerase chain reaction
SNP	single nucleotide polymorphism
TE	tracheo-esophageal (as in TE fistula)
VACTERL	vertebral defects, anal atresia, cardiac defects, tracheoesophageal fistula with esophageal atresia, renal defects, limb anomalies
VATER	vertebral defects, anal atresia, tracheoesophageal fistula with esophageal atresia, renal defects, radial dysplasia

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Table 1

Summary of the 12 patients with VACTERL association.

#	Age at study	Sex	V	A	C	TE	R	L	Genetic Testing	Pulmonary	Other/Notes
1	8 months	M	Deformity of right 3rd rib; unclear if post operative or developmental	none	2 VSDs, PDA, PFO	Type C TEF	Slight fetal lobulation bilateral kidneys	none	Normal karyotype, normal *ZIC3*, *SHH* sequences	Respiratory insufficiency at birth	Hypospadias, hydrocele, cystic hygroma (self-resolving), anterior tongue tie
2	9 years	M	Sacral agenesis, fusion/block vertebrae at C5–C6, small, flattened C3 and C4, absent left 12th rib, abnormal segmentation at T1–T4 with hemivertebrae and spina bifida occulta, mild cervicothoracic scoliosis	Imperforate anus w/ rectourethral fistula	none	none	Left pelvic kidney, initially dysplastic and eventually ceased to function/ involuted	none	Normal microarray (Illumina Omni1-Quad), normal *ZIC3* sequence	Asthma	ADHD, history of left amblyopia, subtle clinodactyly
3	4 years	M	Tethered spinal cord (fatty filum terminale)	Anorectal stenosis, IAS (internal anal sphincter) achalasia	2 VSDs, persistent left superior vena cava, ASD, PFO, PDA, mild tricuspid regurgitation	Type C TEF	none	none	Normal microarray (Illumina Omni1-Quad), normal *ZIC3*, *TWSG1* sequences, normal chromosome breakage studies	Abnormal branching in two bronchi in right lung, mild bronchomalacia in left lung, mild persistent pulmonary hypertension	
4	11 years	F	Thoracic and lumbar scoliosis, hemivertebrae (3–4)	none	Congenital aortic stenosis, bicuspid aortic valve, dextroversion of cardiac mass due to TEF	TEF (w/esophageal atresia)	Grade II right kidney reflux (VUR)	none	Normal microarray (Illumina Omni1-Quad), normal *ZIC3*, *TWSG1* sequences	Agenesis of the right lung	
5	2 years	F	Tethered cord	Imperforate anus, cloaca	VSD, ASD, DORV	TEF	Multicystic kidneys, horseshoe kidney, hydronephrosis. Left kidney hypo-functioning and later removed	Left thumb aplasia, right thumb hypoplasia	Normal microarray (Illumina Omni1-Quad), normal *ZIC3*, *TWSG1* sequences	Pulmonic stenosis	

#	Age at study	Sex	V	A	C	TE	R	L	Genetic Testing	Pulmonary	Other/Notes
6	6 months	M	T8 butterfly vertebra, focal left convex scoliosis at T8, fatty filum terminale,	none	none	TEF (w/esophageal atresia)	Grade III–IV hydronephrosis, right malrotated and hypoplastic kidney	none	Normal microarray (Illumina Omni1-Quad), normal *ZIC3*, *TWSG1* sequences	none	Tracheomalacia, bifid uvula, high arched palate, umbilical hernia, extracardiac mass overlying anterior wall of RVOT (had hx mediastinal abscess s/p drainage with residual mass)
7	5 years	F	Congenital scoliosis, partial sacral agenesis, absent pair of ribs, dysplastic sacral vertebrae, partial hemisacrum	Imperforate anus, cloaca	VSD, PDA	none	Right kidney hypoplasia, hydronephrosis	none	Normal microarray (Illumina Omni1-Quad), normal *ZIC3*, *TWSG1* sequences	none	
8	18 years	F	Deformity of body of C2 with partial fusion deformity with C3, spina bifida occulta at C5, mild scoliosis of cervical, thoracic and lumbar spine, tethered cord	Low imperforate anus	VSD, ASD, PFO	none	Neurogenic bladder, vesicoureteral reflux	Minimal bowing deformities of the radius and ulna bilaterally	Karyotype - trisomy 21 but otherwise normal, normal chromosomal breakage studies, normal microarray (Illumina Omni1-Quad) other than trisomy 21, normal *ZIC3* sequence	none	While this patient had trisomy 21, her features of VACTERL are thought to be an independent clinical diagnosis and not a component of trisomy 21. Described in Solomon et al. Clinical Dysmorphology [18].
9	52 years	M	Vertebral fusion at C3–C4 and C5, congenital cervical (C6–C7) and lumbar (L3–L4) block vertebrae, cervical and lumbar hemivertebrae, lumbar scoliosis	Imperforate anus	none	none	Horseshoe kidney with a smaller left kidney component than the right, urinary tract fistula	none	Normal microarray (Illumina Omni1-Quad), normal *ZIC3* sequence	COPD/asthma	Hypospadias, hearing loss (hearing loss as child, otosclerosis of stapes, s/p stapedectomy), inguinal hernia, psoriasis

#	Age at study	Sex	V	A	C	TE	R	L	Genetic Testing	Pulmonary	Other/Notes
10	28 years	F	Congenital fusion C7-T1, T7–T8, T11–T12, bifid rib on left at T10, absence of 11th rib, lumbar pedicle dysplasia, scoliosis	none	Persistent left superior vena cava	Type C TEF	none	none	Normal *ZIC3* sequence	Recurrent pneumonia in childhood, persistent pulmonary hypertension at birth	Sensorineural hearing loss: L>R, bilateral hip dysplasia w/ short femoral necks and overgrowth of greater trochanter
11	4 years	M	Thoracic and lumbar scoliosis, fusion of 2nd and 3rd ribs on right, hemivertebrae at T1–T5, T9–10, S1 and S4	Imperforate anus	Bicuspid aortic valve, PFO, small PDA, ASD, small aortic arch, persistent left superior vena cava, dilated proximal ascending aorta	none	Left kidney crossed fused ectopia	none	Normal karyotype, normal microarray (Illumina Omni1-Quad)	none	Right inguinal hernia, bilateral clinodactyly
12	3 years	M	13 Ribs on the right, 1–2 extra lumbar vertebral bodies,deformed right lateral 6th rib, asymmetric vertebral bodies with left taller than right in lower lumbar area, thoracic rotoscoliosis, tethered cord, filum terminale lipoma	Imperforate anus	PDA, PFO	TEF w/esophageal atresia	Grade I VUR, penile chordee, hypospadias	none	Normal karyotype, normal microarray (Illumina Omni1-Quad)	Lack of RUL, chronic lung disease (but due to prematurity and prolonged ventilation with neonatal PNA), tracheomalacia	Twin gestation (monozygotic twin without any anomalies or issues), premature gestation (28 wks), eczema

Abbreviations: A: anal anomalies; ADHD: attention deficit hyperactivity disorder; ASD: atrial septal defect; C: cardiac malformations; COPD: chronic obstructive pulmonary disease; DOL: day of life; DORV: double outlet right ventricle; HTN: hypertension; IAS: internal anal sphincter; L: limb anomalies; PDA: patent ductus arteriosus; PFO: patent foramen ovale; R: renal anomalies; RUL: right upper lobe; RVOT: right ventricle outlet tract; TE/TEF: tracheo-esophageal fistula; UTI: urinary tract infection; V: vertebral anomalies; VSD: ventricular septal defect; VUR: vesicoureteral reflux.

Note: no patients had evidence for a duplication or deletion at 16q24.1-24.2 on high density SNP array, and all had normal FOXF1 mutation analysis;