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FGFRL1 deficiency contributes to congenital diaphragmatic hernia development

Yoel Gofin[1,2,*], Laura Palmer Mackay[1,2,*]; Karen Machol[1,2], Sundeep Keswani[2,3], Lorraine Potocki[1,2], Valeria Giorgia Naretto[4], Alfredo Brusco[4,5], Andres Hernandez-Garcia[1,**], Daryl A. Scott[1,2,6,**†]

1) Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX 77030, USA

2) Texas Children's Hospital, Houston, TX 77030

3) Department of Surgery, Baylor College of Medicine, Houston, TX 77030, USA

4) Medical Genetics Unit, "Città della Salute e della Scienza" University Hospital, Torino, 10126, Italy

5) Department of Medical Sciences, University of Turin, Torino, 10126, Italy

6) Department of Molecular Physiology and Biophysics, Baylor College of Medicine, Houston, TX 77030, USA

*, ** Authors contributed equally

† Correspondence

Daryl A. Scott

R813, One Baylor Plaza

BCM225

Houston, TX 770303, USA

dscott@bcm.edu

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ABSTRACT

Fibroblast growth factor receptor-like 1 (*FGFRL1*) encodes a transmembrane protein that is related to fibroblast growth factor receptors but lacks an intercellular tyrosine kinase domain. *In vitro* studies suggest that it inhibits cell proliferation and promotes cell differentiation and cell adhesion. Mice that lack FGFRL1 die shortly after birth from respiratory distress and have abnormally thin diaphragms whose muscular hypoplasia allows the liver to protrude into the thoracic cavity. Haploinsufficiency of *FGFRL1* has been hypothesized to contribute to the development of congenital diaphragmatic hernia (CDH) associated with Wolf-Hirschhorn syndrome. However, data from both humans and mice suggest that disruption of one copy of *FGFRL1* alone is insufficient to cause diaphragm defects. Here we report a female fetus affected by CDH whose 4p16.3 deletion allows us to refine the Wolf-Hirschhorn syndrome CDH critical region to a ~1.9 Mb region that contains *FGFRL1*. We also report a male infant with isolated left-sided diaphragm agenesis who carried compound heterozygous missense variants in *FGFRL1*. These cases provides additional evidence that FGFRL1 deficiency may contribute to the development of CDH in humans.

INTRODUCTION

The fibroblast growth factors (FGFs) regulate a variety of cellular functions including cell proliferation, differentiation, migration, and apoptosis and play a critical role in embryonic development [Belov and Mohammadi 2013; Trueb 2011]. FGFs act by binding to and activating members of the fibroblast growth factor receptors (FGFR) subfamily of tyrosine kinases that are encoded by four genes (*FGFR1-4*) in mammals [Johnson and Williams 1993; Mohammadi et al., 2005]. FGFR1-4 are transmembrane proteins whose structure includes three immunoglobulin-like domains, a single transmembrane helix domain, and an intracellular domain with tyrosine kinase activity. Fibroblast growth factor receptor-like 1 (*FGFRL1*: MIM# 605830) encodes a transmembrane protein that is related to FGFRs but lacks an intercellular tyrosine kinase domain [Wiedemann and Trueb 2000]. *In vitro* studies suggest that FGFRL1 inhibits cell proliferation [Trueb et al., 2003], and promotes cell differentiation [Baertschi et al., 2007] and cell adhesion [Rieckmann et al., 2008].

In mice, *Fgfrl1* is expressed at a relatively high level in the developing diaphragm [Trueb and Taeschler 2006] and plays a critical role in diaphragm development. Mice that are homozygous for a deletion of the first two exons of *Fgfrl1* (*Fgfrl1*$^{-/-}$) die shortly after birth due to respiratory distress caused by generalized hypotrophy of the diaphragm muscle [Baertschi et al., 2007]. The diaphragmatic muscles of *Fgfrl1*$^{-/-}$ mice are ~60% as thick as those seen in wild-type controls. In some regions, the muscular diaphragm is replaced by connective tissue and in some cases the liver protrudes in the thoracic cavity [Gerber et al., 2009]. Decreased expression of FGFRL1 during the late stages of gestation has also been hypothesized to contribute to the development of congenital diaphragmatic hernia (CDH) in mice exposed to nitrofen in utero [Dingemann et al., 2011].

In humans, *FGFRL1* resides on chromosome 4p16.3. Deletions of 4p16.3 cause Wolf–Hirschhorn syndrome (WHS; MIM# 194190) which is characterized by distinctive

facial features, delayed growth and development, intellectual disability, seizures, and structural birth defects that can include CDH [Paradowska-Stolarz 2014]. *FGFRL1* is located in an ~2.3 Mb CDH critical region on 4p16.3 defined by a patient described by Casaccia et al. [Callaway et al., 2018; Casaccia et al., 2006]. Deletion of *FGFRL1* has been previously hypothesized to contribute to the development of CDH associated with WHS [Callaway et al., 2018; LopezJimenez et al., 2010]. However, the low loss-of-function intolerance of *FGFRL1* in the Genome Aggregation Database (gnomAD ver2.1.1, https://gnomad.broadinstitute.org/; pLI = 0.01; e/o ratio = 0.37) suggests that haploinsufficiency of *FGFRL1* alone is unlikely to be sufficient to cause CDH [Karczewski et al., 2020]. This is consistent with the observation that heterozygous *Fgfrl1*$^{+/-}$ mice are asymptomatic [Baertschi et al., 2007]

Here we refine the CDH critical region associated with WHS to an ~1.9 Mb region of 4p16.3 based on a deletion identified in a female fetus with CDH. We also report a male infant with isolated left-sided diaphragm agenesis who was compound heterozygous for missense variants in *FGFRL1*. These cases provides additional evidence that FGFRL1 deficiency may contribute to the development of CDH in humans.

MATERIALS AND METHODS

Editorial Policies and Ethical Considerations

Subjects 1 and 2 were enrolled in research studies in accordance with protocols approved by local institutional review boards. The procedures followed were in accordance with the ethical standards of Baylor College of Medicine's committee on human research and were in keeping with international standards.

Molecular Testing

The 4p16.3 deletion in Subject 1 was detected by array-comparative genomic hybridization (a-CGH) performed according to manufacturer's recommendations using a 60K Agilent array. Genetic testing for Subject 2 was performed on a clinical basis at Baylor Genetics using a cord blood sample and included a chromosome analysis with a band resolution of 550, array-based copy number variant (CNV) analysis (CMA-HR+SNP version 11.2) and Critical Trio Whole Exome Sequencing [Meng et al., 2017]. Tests for maternal blood contamination were negative.

RESULTS

Clinical presentations and molecular studies

Subject 1

Subject 1 (DECIPHER ID: 339928) was a 21 week gestation female fetus with left-sided CDH identified by prenatal ultrasound. The left lung was found to be hypoplastic and the heart was displaced to the right. The stomach was in the thorax, but the remaining thoracic and abdominal organs had a normal conformation and position. The brain had a normal size and conformation for the gestational age. Based on these findings, parents chose to terminate the pregnancy. Biometric values were consistent with a 21 week gestation with a weight of 480 grams (82^{nd} centile), a length of 28.5 cm (94^{th} centile), and the occipitofrontal circumference of 21.3 cm (99^{th} centile, Z-score = 2.59). The fetus was found to carry a *de novo* 4p16.3 deletion (minimum deletion chr4:71,552-1,800,425, maximum deletion chr4:1-1,875,255; hg19) by array-comparative genomic hybridization (Figure 1).

Subject 2

Subject 2 was a Hispanic male of Mexican descent who was conceived by healthy, non-consanguineous parents with the aid of oral medications for ovulation induction. His

parents had one spontaneous abortion at eight weeks gestation and have a five-year-old son who is in good health. There was no family history of functional or structural birth defects. The pregnancy was complicated by diet-controlled gestational diabetes.

At thirty-one weeks gestation, an ultrasound examination showed a left-sided CDH. A fetal MRI at thirty-eight weeks gestation confirmed a left-sided diaphragmatic hernia with herniation of the stomach, spleen, and a portion of the left lobe of the liver, with no evidence of a hernia sac. The total fetal lung volume was 16.8 mL, with a right lung volume of 15.6 mL and a left lung volume of 1.2 mL. The observed/expected total fetal lung volume was 15.4% based on the formula proposed by Rypens et al. and 19% based on mean values reported by the same group [Rypens et al., 2001]. The lung area to head circumference ratio was 1.0, the observed/ expected lung to head ratio was 30%, and 24% of the liver was herniated. A fetal echocardiogram performed four days prior to delivery showed mild hypoplasia of the left heart structures secondary to mass effect with normal aortic and mitral valve function, dextroposition with a leftward apex, normal biventricular systolic function, and no obvious intracardiac abnormalities.

The patient was born at 39 3/7 weeks gestation via an induced vaginal delivery. Apgar scores were 8 at one minute and 9 at five minutes. The patient had a birth weight of 3.06 kg (27^{th} centile), a birth length of 50.8 cm (69^{th} centile), and a head circumference of 35 cm (66^{th} centile). Due to his prenatal diagnosis of CDH, he was intubated and sedated immediately after delivery and transferred to the neonatal intensive care unit where he was put on an oscillator and started on inhaled nitric oxide and pressor support. On physical exam, he did not have any dysmorphic features or other congenital anomalies. A head ultrasound, performed on the first day of life, was normal.

An echocardiogram on the sixth day of life showed severe pulmonary hypertension, severe tricuspid regurgitation, a severely dilated right ventricle with qualitatively moderately

to severely depressed systolic function, and decreased left ventricle cavity size with hyperdynamic systolic function, likely secondary to a flattened septal configuration. There was also a small patent ductus arteriosus and patent foramen ovale/ small atrial septal defect, both with right to left shunting. Because of the severe pulmonary hypertension noted on this echocardiogram, sildenafil was initiated, along with prostaglandin E1 to enlarge the patent ductus arteriosus.

On day of life eleven, he was put on veno-arterial extracorporeal membrane oxygenation (VA-ECMO) due to respiratory failure and cardiac dysfunction. He underwent a CDH repair surgery on day of life twelve. The left-sided diaphragmatic defect was estimated as 95%, with no diaphragm along the left lateral aspect and the left anterolateral and posterolateral aspect of the chest. There was a rim of diaphragm medially that was about 5 mm in its largest dimension anteriorly, and a small crural remnant posteromedially. The abdominal contents were returned to the abdomen and diaphragmatic defect was closed with GORE-TEX Dual Mesh patch.

On day of life twenty-four, he had a successful trial off of VA-ECMO, but suffered an unexplained decompensation shortly thereafter. Despite maximal support, he died on day of life twenty-five. Postmortem CT and MRI evaluations did not identify additional birth defects.

A chromosome analysis revealed a 46,XY chromosomal complement. Array-based CNV analysis did not identify variants that were associated with known microdeletion or microduplication syndromes, deletions of the mitochondrial genome, or increased blocks of absence of heterozygosity (AOH). Trio exome sequencing performed on a clinical basis did not reveal any pathogenic variants, likely pathogenic variants, or variants of uncertain significance in known CDH genes. However, the same test revealed that the Subject 2 was compound heterozygous for two *FGFRL1* missense variants; a paternally inherited

c.886A>G, p.(I296V) variant located in a region that codes for an Ig-like C2-type 3 domain (amino acids 246-354; UniProt https://www.uniprot.org/uniprot/Q8N441), and a maternally inherited c.1328G>C, p.(G443A). All variants described in this paper are based on transcript NM_001004356.2. *In silico* analyses of these variants are summarized in Table 1.

DISCUSSION

Despite advances in diagnostic techniques, the molecular etiology of the majority of CDH cases remains undetermined [Longoni et al., 1993; Yu et al., 2019]. This is due, in part, to an incomplete understanding of the genes that contribute to the development of CDH. Often the first indication that a gene plays a role in diaphragm development comes from mouse models [Nakamura et al., 2020], with evidence of a role in human diaphragm development slowly accumulating over time [Ackerman et al., 2005; Brady et al., 2014; Longoni et al., 2015; Wat et al., 2011].

The first indication that FGFRL1 deficiency could cause CDH came from mouse models. *Fgfrl1*$^{-/-}$ mice die shortly after birth from respiratory distress and have abnormally thin diaphragms whose muscular hypoplasia allows the liver to protrude into the thoracic cavity [Baertschi et al., 2007; Gerber et al., 2009]. Decreased expression of FGFRL1 during the late stages of gestation has also been hypothesized to contribute to the development of CDH in mice exposed to nitrofen in utero [Dingemann et al., 2011].

In humans, *FGFRL1* is located in the previously defined ~2.3 Mb CDH critical region on chromosome 4p16.3 [Callaway et al., 2018; Casaccia et al., 2006], and haploinsufficiency of *FGFRL1* has been hypothesized to contribute to the development of CDH associated with WHS for over a decade [Callaway et al., 2018; LopezJimenez et al., 2010]. Among the ~61 RefSeq genes located in this region, *FGFRL1* was identified using a machine learning algorithm to be the second most similar to a group of training genes previously shown to

cause CDH [Callaway et al., 2018]. Among all RefSeq genes, *FGFRL1* was ranked at the 98.9th centile based on its similarity to genes in the CDH training set (CDH-specific pathogenicity score = 98.9%) [Callaway et al., 2018], suggesting that it represents an excellent positional candidate gene for CDH.

The deletion identified in Subject 1 allows us to refine the CDH critical region to a ~1.9 Mb that still includes *FGFRL1* (Figure 1). This interval includes ~48 RefSeq genes. Since *FGFRL1* has a low loss-of-function intolerance in gnomAD (pLI = 0.01; e/o ratio = 0.37), it is likely that haploinsufficiency of *FGFRL1* combined with other epigenetic, genetic, environmental, and/or stochastic factors is responsible for the CDH seen in a subset of individuals with WHS. The genetic factors involved may include the haploinsufficiency of other protein coding genes within the new critical region that have high CDH-specific pathogenicity scores including *FGFR3* (99.9%; MIM# 134934), *NSD2* (97.3%; MIM# 602952), and *ZNF141* (97%; MIM# 194648), *MAEA* (91%; MIM# 606801), *CPLX1* (89%; MIM# 605032) and/or *CTBP1* (85.6%; MIM# 602618) [Callaway et al., 2018]. Of these genes, only *CTBP1* has been clearly implicated in the development of the diaphragm with *Ctbp1*$^{-/-}$;*Ctbp2*$^{+/-}$ mouse embryos having abnormal muscle fiber formation in their diaphragms [Hildebrand and Soriano 2002].

The potential role of FGFRL1 deficiency as a contributor to CDH development is also supported by Subject 2, the first child with CDH that has been found to be compound heterozygous for variants in *FGFRL1* (c.[886A>G];[1328G>C], p.[(I296V)];[(G443A)]). As expected, based on the *Fgfrl1* mouse model, neither of Subject 2's parents, who carried only one affected *FGFRL1* allele, had CDH. Consistent with Subject 2's Hispanic (Mexican) ethnicity, these variants are seen most commonly in the Latino/Admixed American population of the gnomAD database. However, even in this population, they are rare (allele frequencies of 0.00032 and 0.00059, respectively) and have never been documented in the

homozygous state. *In silico* evaluations provide greater evidence for the deleterious nature of the c.886A>G variant (Table 1) that occurs in a region that codes for an Ig-like C2-type 3 domain of FGFRL1 and has a Combined Annotation Dependent Depletion (CADD; https://cadd.gs.washington.edu/) score of 23.9. In contrast, the c.1328G>C variant has a much lower CADD score of 8.5. If these variants contributed to the development of CDH in Subject 2, it is still possible that they did so in conjunction with other deleterious variants that were not identified on exome sequencing. Unidentified epigenetic, environmental and/or stochastic factors may have also played a role in Subject 2's CDH.

We conclude that the CDH critical region on 4p16.3 that is associated with WHS can be refined to an ~1.9 Mb telomeric region that contains *FGFRL1* and ~47 other RefSeq genes. We also conclude that *FGFRL1* deficiency may contribute to the development of CDH in humans, although definitive proof will require the identification of additional individuals with CDH that carry biallelic, deleterious variants in *FGFRL1*.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to declare.

DATA AVAILABILITY

The *FGFRL1* variants seen in Subjects 2 have been submitted to the ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/).

FIGURE LEGENDS

Figure 1. The deletion identified in Subject 1 defines the CDH critical region on 4p16.3 that is associated with Wolf-Hirschhorn syndrome. A schematic representation of molecularly defined, isolated 4p16 deletions associated with CDH [Callaway et al., 2018; Casaccia et al., 2006; Slavotinek et al., 2006; Tautz et al., 2010; Van Buggenhout et al., 2004]. In all cases, the maximal deletion is depicted. *FGFRL1* is located in the CDH critical region on 4p16.3 defined by the maximal deletion in Subject 1 (chr4:1-1,875,255; hg19).

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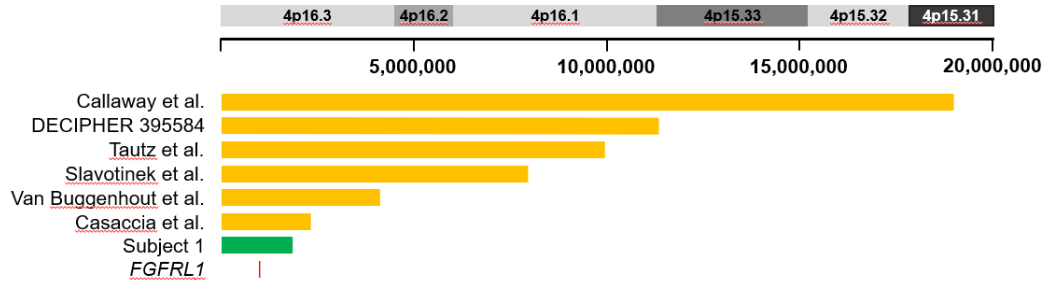

Table 1: Results of in silico analyses performed for the *FGFRL1* variants seen in Subject 2.

FGFRL1 Variant [NM_001004356.2]	SIFT	PolyPhen-2	Mutation1328Taster	CADD	Allele Frequency*
c.886A>G, p.(I296V)	Tolerated	Probably Damaging	Disease Causing	23.9	11/34456 (0.00032); no homozygotes
c.1328G>C, p.(G443A)	Tolerated	Benign	Polymorphism	8.5	20/33814 (0.00059); no homozygotes

* Latino/Admixed American population frequencies