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***FBN1* Contributing to Familial Congenital Diaphragmatic Hernia**

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Abstract

Congenital diaphragmatic hernia (CDH) is a relatively common, life-threatening birth defect. We present a family with recurrent CDH—paraesophageal and central—for whom exome sequencing (ES) revealed a frameshift mutation (c.4969_4970insA, p. Ile1657Asnfs*30) in the fibrillin 1 gene (*FBN1*) that causes Marfan syndrome. A diagnosis of Marfan syndrome had not been considered previously in this family. However, a review of the literature demonstrated that *FBN1* mutations have an unusual pattern of CDH in which paraesophageal hernias are particularly common. Subsequent clinical evaluations revealed evidence for ectopia lentis in affected family members supporting a clinical diagnosis of Marfan syndrome. Since only two other cases of familial CDH have been described in association with *FBN1* mutations, we investigated an oligogenic hypothesis by examining ES data for deleterious sequence changes in other CDH-related genes. This search revealed putatively deleterious sequence changes in four other genes that have been shown to cause diaphragm defects in humans and/or mice—*FREMI*, *DES*, *PAX3* and *MET*. It is unclear whether these changes, alone or in aggregate, are contributing to the development of CDH in this family. However, their individual contribution is likely to be small compared to that of the frameshift mutation in *FBN1*. We conclude that ES can be used to identify both major and minor genetic factors that may contribute to CDH. These results also suggest that ES should be considered in the diagnostic evaluation of individuals and families with CDH, particularly when other diagnostic modalities have failed to reveal a molecular etiology.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher's web-site.

Keywords

congenital diaphragmatic hernia; exome sequencing; oligogenic inheritance; Marfan syndrome; *FBN1*; *FREMI*; *PAX3*; *DES*; *MET*

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a relatively common birth defect with a high rate of mortality and long term morbidity [Brownlee et al., 2009; van den Hout et al., 2009]. CDH can present as an isolated defect, in association with other anomalies (CDH +), or as a part of a defined genetic syndrome that can be diagnosed based on specific clinical and/or molecular criteria [Poer, 2007; Scott, 2007]. Isolated CDH is associated with a low empiric recurrence risk (< 2%) suggesting that most cases may have a multifactorial inheritance pattern in which genetic, environmental and stochastic factors contribute to the development of the diaphragmatic defect [Edwards, 1960; Wolff, 1980; Norio et al., 1984]. Identifying the genetic factors that contribute to sporadic cases of CDH has proven to be challenging since the contribution of each factor to CDH may be relatively small and, in some cases, these minor genetic factors may be transmitted through multiple generations of unaffected individuals [Arrington et al., 2012]. Although sometimes difficult to identify, these genetic factors may have a significant influence on the penetrance of other CDH-related mutations in individuals, families and clans [Lupski et al., 2011]. This has been clearly demonstrated in mouse models in which the penetrance of CDH associated with specific mutations varies significantly in different genetic backgrounds [Wat et al., 2012; Beck et al., 2013a; Beck et al., 2013b].

Here we report on a family with recurrent CDH in which exome sequencing (ES) revealed a frameshift mutation (c.4969_4970insA, p.Ile1657Asnfs*30; NM_000138) in the fibrillin 1 gene (*FBN1*) which led to a molecular diagnosis of Marfan syndrome in both affected individuals. ES also revealed putatively deleterious sequence changes in four other CDH-related genes. Although it is unclear whether these changes contributed to the development of CDH in this family, our results demonstrate the ability of ES to identify both major and minor genetic factors that may contribute to the development of CDH.

CLINICAL REPORT

A Caucasian family consisting of parents and two children were referred for genetic evaluation due to recurrent CDH (Fig. 1). The parents were not related and the mother had no significant medical problems. The father was 27 years old and came to medical attention at age 10 with a history of gross motor and speech delay (walked at 18 months and began speaking shortly thereafter), multiple pulmonary infections, chronic iron deficiency anemia and chronic constipation. At the time, his height and weight were at the 3rd centile and he was noted to have a pectus carinatum, laxity of his finger joints, wind-swept hands with ulnar deviation of digits 2–5 bilaterally and lumbar scoliosis. A chest X-ray was obtained which showed a moderate-sized diaphragmatic hernia on the left side. At the time of surgical correction, the hernia was described as paraesophageal with nearly the entire stomach and colon residing in the chest. The left crus of the diaphragm was also noted to be very small.

In his twenties, the father also required surgical intervention for severe scoliosis. Clinically-based genetic testing on the father included a normal G-banded chromosome analysis and a normal copy-number variation (CNV) detection analysis performed on a clinical basis by Stichting Klinisch-Genetisch Centrum Nijmegen using a 250 K Affymetrix SNP array with an average genome-wide resolution of 200 kb, excluding the Y chromosome.

The younger child was a 2-year-old boy with no significant medical problems. The older child was a 4-year old boy who was born at term and weighed 3.32 Kg (~25th centile). He was admitted at two months of age for feeding difficulties characterized by inconsolability after feeds with back arching and nasal regurgitation. An echocardiogram was normal but a barium swallow revealed what appeared to be a large right-sided posterolateral diaphragmatic hernia containing the fundus of the stomach. However, the operative report described a central diaphragmatic hernia through which the stomach had herniated. His physical examination at four years of age was unremarkable with the exception of ulnar deviation of the digits similar to that seen in his father.

METHODS AND RESULTS

Research-based exome sequencing (ES) analysis was performed on DNA samples from the affected father, the unaffected mother and the affected son as described in the Methods section of the Supplemental Materials (in supporting information online). Both the father and the affected son were found to have a heterozygous frameshift mutation (c. 4969_4970insA, p.Ile1657Asnfs*30; NM_000138) in the fibrillin 1 gene (*FBNI*) which was not identified in the unaffected mother. The variant:total reads ratio was 22:55 for the father's sample and 38:65 for the son's sample. Sanger sequencing confirmed the presence of the frameshift mutation in the father and his affected son with CDH. Sanger sequencing also revealed that the young, unaffected son in this family carried the same mutation (Fig. 1). No other *FBNI* mutations were identified in family members. Heterozygous truncating mutations in this region of the *FBNI* gene are a known cause of Marfan syndrome with associated aortic dilatation [Nijbroek et al., 1995; Pepe et al., 2001].

A clinical diagnosis of Marfan syndrome had not been previously considered in this family. However, after identification of the *FBNI* mutation, the father was re-evaluated to investigate for clinical findings which could provide additional evidence for a diagnosis of Marfan syndrome. His height was 1.73 m (10–25th centile) and his arm span was 1.63 m. He had a negative Marfan systemic score of three based on the Modified Ghent Nosology (Supplemental Table I) and had none of the facial features—dolichocephaly, downslanting palpebral fissures, enophthalmos, retrognathia or malar hypoplasia—typically observed in individuals with Marfan syndrome [Loeys et al., 2010]. Instead, he was noted to have a prominent metopic ridge, a widow's peak prominence and a bifid nasal tip with a recessed columella.

Similarly, his affected son with CDH also had a negative Marfan systemic score of 1 with reduced elbow flexion (Supplemental Table II). In contrast, his younger son, who did not have CDH, had more features suggestive of Marfan syndrome including positive thumb and

wrist signs, chest asymmetry, scoliosis, and reduced elbow flexion giving him a Marfan systemic score of 6 (Supplemental Table III).

Echocardiograms performed on the father and his younger, unaffected son showed no evidence for aortic dilatation or other anomalies. His affected son's echocardiogram revealed an aortic root diameter at the 95th centile ($Z = 1.96$) which is just below the cutoff at which a diagnosis of Marfan syndrome could be made ($Z \geq 2$). However, an ophthalmological evaluation on the father revealed a moderate degree of myopia ($-3D$), with iridodonesis and phacodonesis suggestive of ectopia lentis. His affected son with CDH was also found to have iridodonesis. Based on these ophthalmological findings—and the presence of a frameshift mutation in a region of *FBN1* in which heterozygous truncating mutations have been shown to cause aortic dilatation—both the father and the affected son with CDH were given a clinical diagnosis of Marfan syndrome. The medical evaluation of the younger son without CDH is ongoing.

Since CDH is a relatively rare finding in Marfan syndrome, we searched for putatively deleterious sequence changes in other genes known to cause diaphragmatic defects in mice and/or humans that could potentially be contributing to the development of CDH in this family. Putatively deleterious sequence changes in four other CDH-related genes (*FREMI*, *DES*, *PAX3*, and *MET*) were identified in the father and his affected son and were confirmed by Sanger sequencing (Table I). The *FREMI*, *DES*, and *PAX3* changes were also seen in the younger son who did not have CDH (Fig. 1).

The first variant was a c.1394G>C, p.Gly465Ala change in the FRAS1 related extracellular matrix 1 gene (*FREMI*; OMIM #608944). Recessive mutations in *FREMI* have been shown to cause Bifid Nose with or without Anorectal and Renal anomalies (BNAR; OMIM #608980) and Manitoba OculoTrichoAnal (MOTA; OMIM #248450) syndromes and have recently been shown to also cause isolated CDH in humans and anterior sac hernias in mice [Beck et al., 2013a; Beck et al., 2013b].

The second variant was a c.638C>T, p.Ala213Val change in the desmin gene (*DES*; OMIM #125660) which encodes a constitutive subunit of the intermediate filaments in skeletal, cardiac and smooth muscles. Mutations in *DES* have been implicated in the development of a form of idiopathic dilated cardiomyopathy (OMIM #604765) but have not been shown to cause CDH in humans. All of the early stages of muscle differentiation and cell fusion occur normally in *Des*-null mice, but after birth a lack of desmin renders the muscle fibers of the diaphragm more susceptible to damage during contraction [Li et al., 1997]. The third variant was a c.944C>A, p.Thr315Lys change in the paired box 3 gene (*PAX3*; OMIM #606597). The father was found to be homozygous for this change and his affected son was heterozygous. Autosomal dominant mutations in *PAX3* cause craniofacial-deafness-hand syndrome (OMIM #122880) and Waardenburg syndrome types 1 (OMIM #193500) and type 3 (OMIM #148820) which are not associated with an increased risk for CDH. However, *PAX3*-deficient mice fail to form a muscular diaphragm [Li et al., 1999].

The fourth variant was a c.2975C>T, p.Thr992Ile change in the met proto-oncogene (*MET*) which is dysregulated in many types of human cancer but is not known to cause CDH in

humans. In *Met*-null mice, the diaphragm is not colonized by myogenic precursor cells and, as a consequence, the skeletal muscles of the diaphragm do not form [Bladt et al., 1995].

To determine if these sequence changes were recurrently seen in patients with CDH, we looked for identical changes in 66 unrelated individuals with CDH (29 Caucasians, 25 Hispanics, 5 African Americans, 3 Asians, 2 of mixed ancestry and 2 of other/unknown ancestry) for whom a molecular cause had not been identified. The *FREMI* c.1394G>C, p.Gly465Ala change was seen in two individuals, the *PAX3* c.944C>A, p.Thr315Lys change in three individuals and the *MET* c.2975C>T, p.Thr992Ile change in one individual. Their clinical features are described in Supplemental Table IV. The allele frequency of the *FREMI* c.1394G>C, p.Gly465Ala variant was higher in the CDH cohort (2/132 or 1.52%) than in European American control samples catalogued in the NHLBI Exome Variant Server (<http://evs.gs.washington.edu/EVS/>; version ESP6500SI-V2; 82/8444 or 0.97%) but this difference was not statistically significant ($P = 0.3721$, two tailed Fisher exact tests). The allele frequencies of the remaining variants were lower in the CDH cohort than in European American controls but, again, these differences were not statistically significant ($P > 0.05$; see Table I).

DISCUSSION

A review of the literature revealed that the diaphragmatic defects associated with mutations in *FBN1* have been documented in individuals with both classic Marfan syndrome and the early onset/rapidly progressive form of Marfan syndrome (Supplemental Table V). The early onset/rapidly progressive form of Marfan syndrome can present in the neonatal period with congenital contractures, dilated cardiomyopathy, congestive heart failure, pulmonary emphysema, and severe mitral or tricuspid valve insufficiency. *FBN1* mutations associated with the severe form of Marfan syndrome cluster in exons 24–32 but have also been described in other regions of the gene [Putnam et al., 1996; Faivre et al., 2009]. Neither of the affected individuals presented here had a severe form of Marfan syndrome. Indeed, a diagnosis of classic Marfan syndrome was not considered until the c.4969_4970insA, p.Ile1657Asnfs*30 frameshift mutation in *FBN1* was identified by exome sequencing.

The father in this family was diagnosed with a paraesophageal hernia at 10 years of age. It is unclear whether his hernia was present at birth or developed later in life. Although the exact incidence of congenital paraesophageal hernias has not been determined in large studies, they are considered a rare form of CDH in the general population [Kesieme et al., 2011]. In contrast, at least 42% (8/19) of the reported *FBN1*-related diaphragmatic defects identified in the prenatal/neonatal period were paraesophageal hernias. This suggests that *FBN1* mutations are associated with an unusual pattern of diaphragm defects and that identification of a congenital paraesophageal hernia should prompt a search for additional features that might suggest a diagnosis of Marfan syndrome [Jetley et al., 2009].

Since a diagnosis of Marfan syndrome has only been described in two other cases of familial CDH [Petersons et al., 2003; Jetley et al., 2009], we investigated an oligogenic hypothesis by examining ES data for deleterious sequence changes in other CDH-related genes that may have also contributed to the development of CDH in this family. This search revealed

putatively deleterious sequence changes in four other CDH-related genes—*FREMI*, *DES*, *PAX3*, and *MET*. Of these genes, only *FREMI* has been implicated in the development of CDH in humans but all four have been shown to play a role in diaphragm development in mice [Bladt et al., 1995; Li et al., 1997; Li et al., 1999; Beck et al., 2013a; Beck et al., 2013b]. It is unclear whether these changes, alone or in aggregate, are contributing to the development of CDH in this family. However, since the allele frequencies of these changes in the general population range from 0.97% to 3.8%, their individual contribution is likely to be small compared to that of the frameshift mutation in *FBN1*.

We conclude that ES can be used to identify both major and minor genetic factors that may contribute to the development of CDH. Our results also suggest that ES should be considered in the diagnostic evaluation of individuals and families with CDH, particularly when other diagnostic modalities have failed to reveal a molecular etiology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1.

CDH in a family with Marfan syndrome. (A) Pedigree showing the inheritance pattern of the *FBN1* frameshift mutation and putatively deleterious single amino acid changes in *FREM1*, *DES*, *PAX3*, and *MET*. Shading indicates that the individual has CDH. (B) Sanger sequencing results confirming the *FBN1* frameshift mutation (c.4969_4970insA) in the father and his affected son with CDH. The sequencing primer used to generate this sequence was oriented in a 3' to 5' direction. Hence the sequence appears to show the insertion of a "T" rather than an "A". (C) The effects of the c.4969_4970insA mutation on the *FBN1* protein (p.Ile1657Asnfs*30). Color figure can be viewed in the online issue, which is available at <http://onlinelibrary.wiley.com/journal/10.1002/> (ISSN) 1552-4833.

TABLE I

Additional Sequence Changes Identified In Family Members With CDH

Gene	Change	SIFT (Orthologues/Homologues)	Polyphen2	Mutation Taster	European American Frequency	Frequency in 66 unrelated individuals with CDH
<i>FREMI</i>	c.1394G>C, p.Gly465Ala	Tolerated/Damaging	Probably Damaging	Disease Causing	82/8444 (0.97%)	2/132 (1.5%)
<i>DES</i>	c.638C>T, p.Ala213Val	Damaging/Damaging	Possibly Damaging	Disease Causing	118/8600 (1.4%)	Not Found
<i>PAX3</i>	c.944C>A, p.Thr315Lys	Tolerated/Damaging	Possibly Damaging	Disease Causing	326/8600 (3.8%)	3/132 (2.3%)
<i>MET</i>	c.2975C>T, p.Thr992Ile	Damaging/Damaging	Probably Damaging	Disease Causing	102/8188 (1.2%)	1/132 (0.76%)