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Germline but not Somatic *De Novo* Mutations Are Common in Human Congenital Diaphragmatic Hernia

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Abstract

Background—Congenital diaphragmatic hernia (CDH) is a developmental defect of the diaphragm that causes high newborn morbidity and mortality. CDH is considered to be a multifactorial disease, with strong evidence implicating genetic factors. Although recent studies suggest the biological role of deleterious germline *de novo* variants, the effect of gene variants specific to the diaphragm remains unclear, and few single genes have been definitively implicated in human disease.

Methods—We performed genome sequencing on 16 individuals with CDH and their unaffected parents, including 10 diaphragmatic samples.

Results—We did not detect damaging somatic mutations in diaphragms, but identified germline heterozygous *de novo* functional mutations of 14 genes in 9 patients. Although the majority of these genes are not known to be associated with CDH, one patient with CDH and cardiac anomalies harbored a frameshift mutation in *NR2F2* (aka *COUP-TFII*), generating a premature truncation of the protein. This patient also carried a missense variant predicted to be damaging in *XIRP2* (aka Myomaxin), a transcriptional target of *MEF2A*. Both *NR2F2* and *MEF2A* map to chromosome 15q26 where recurring *de novo* deletions and unbalanced translocations have been observed in CDH.

Conclusions—Somatic variants are not common in CDH. To our knowledge, this is the second case of a germline *de novo* frameshift mutation in *NR2F2* in CDH. Since *NR2F2* null mice exhibit

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CONFLICT OF INTEREST

No conflict of interest to report.

a diaphragmatic defect, and *XIRP2* is implicated in cardiac development, our data suggest the role of these two variants in the etiology of CDH, and possibly cardiac anomalies.

Keywords

congenital diaphragmatic hernia; *de novo*; *NR2F2*; *COUP-TFII*; *XIRP2*; 15q26

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a major birth defect, with a prevalence of about 1 in 3,500 live births (Parker and others, 2010; Shanmugam and others, 2017). CDH typically causes respiratory distress in newborns due to the associated pulmonary hypoplasia and pulmonary hypertension; and it has high mortality, between 30–40% (Tsao and Lally, 2012). CDH is a leading cause of healthcare expenditure in the United States (Metkus and others, 1995). Patients with CDH are known to have additional malformations. Heart anomalies such as ventricular septal defects and aortic arch obstruction are observed in about 10% of CDH patients (Graziano and Congenital Diaphragmatic Hernia Study, 2005; Shanmugam and others, 2017). The high frequency of heart anomalies might reflect a common mechanism. Although CDH might be caused by the interplay of genetic, environmental and nutritional susceptibilities (Holder and others, 2007), we recently provided additional evidence supporting the importance of genetic factors in CDH by identifying that patients with no immediate family history can sometimes be linked within multigenerational CDH pedigrees (Arrington and others, 2012). In addition, animal models, rare monogenetic disorders in humans, and chromosomal anomalies support a genetic contribution in CDH. More than 60 genes and loci have been associated with diaphragm development in animal models and humans (Brady and others, 2011; Slavotinek, 2014). Cytogenetic and array-based studies have identified rare recurrent deletions of 15q26, 8p23.1 and 1q41–q42 in CDH (Holder and others, 2007). However, causative genes and loci involved in human CDH remain unknown in most affected infants.

Recent studies in mice suggest the possibility that somatic gene variants might be involved in human CDH (Merrell and others, 2015). However, few studies have systematically evaluated the role of specific gene defects localized to the diaphragmatic tissue (muscle and/or membrane) in human CDH. Recent data however suggest that germline damaging *de novo* variants play a significant role in CDH (Longoni and others, 2017; Yu and others, 2015). We therefore performed whole genome sequencing (WGS) on 16 individuals with CDH and their unaffected parents, including 10 diaphragmatic samples, to test whether specific diaphragmatic defects might be associated with CDH and to identify germline functional *de novo* mutations in affected individuals.

MATERIALS AND METHODS

CDH Cohort Identification

We recruited 16 CDH trios (proband and unaffected parents) following University of Utah Institutional Review Board approval. Individuals and parents were enrolled in the neonatal intensive care unit at Primary Children’s Hospital after informed consent, and blood samples

were collected for DNA isolation. In 10 patients, diaphragmatic samples from the rim of the defect were collected during surgical repair. Medical records were reviewed for demographic and clinical data.

DNA Isolation and Whole Genome Sequencing

Genomic DNA was isolated from peripheral or cord blood samples using a Gentra Autopure LS (Qiagen Inc.) at the Center for Clinical & Translational Science at the University of Utah. DNA was also isolated from a diaphragm surgical specimen from patients when available (Supplementary Table 1). DNA samples were quantified using PicoGreen and were analyzed by agarose gel electrophoresis to confirm the integrity of the DNA. Whole genome sequencing was carried out as part of collaboration with the Utah Genome Project using the standard Illumina WGS pipeline.

Whole Genome Sequencing Data Analysis

Paired-end sequencing reads for each individual were mapped to the reference human genome (GRCh37, human_g1k_v37.fasta downloaded from ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/technical/reference/) using BWA-mem (Li and Durbin, 2010). Genome Analysis Toolkit (GATK) Best Practices pipeline (https://www.broadinstitute.org/gatk/) was then used for multiple realignment and base recalibration, and HaplotypeCaller in the GATK was used to call variants.

Confirmation of Family Relatedness and Gender on WGS samples

We performed family relatedness analysis of proband-parent trios by the pair-wise Identity by Descent (IBD) estimate analysis on WGS sequence variants. We used sequence variants on autosomal chromosomes that had minor allele frequency (MAF) over 0.05 and LD r^2 threshold of 0.2. Genders of samples were inferred based on variant heterozygosity rates on X chromosome for each subject. We used sequence variants with MAF over 0.05 and applied threshold of heterozygosity rate of 0.02 for calling males or females. Both analyses were carried out using the SVS software package (Golden Helix Inc.).

Sequence Variant Analysis

Functional annotation and filtering of the sequence variants were performed with the SVS and VarSeq software packages (Golden Helix Inc.). Variants were classified with respect to locations (exonic, intronic, splice site, 5′ UTR, 3′ UTR, upstream, downstream, or intergenic) and by exonic functions (missense, nonsense, frameshift, splice site, substitution, in-frame insertion/deletion, etc). Functional variants that were novel or rare (minor or alternative allele frequencies less than 0.01) in ExAC (Lek and others, 2016), NHLBI ESP6500 (http://evs.gs.washington.edu/EVS/) and 1kG Phase 3 (http://www.1000genomes.org/) databases were then analyzed in each trio under a *de novo* model in SVS and VarSeq software packages (Golden Helix Inc.). To predict deleterious effects of missense amino acid changes, various functional prediction programs were used including SIFT (Kumar and others, 2009), Polyphen2 (Adzhubei and others, 2010), MutationTaster (Schwarz and others, 2010), MutationAssessor (Reva and others, 2011), and FATHMM (Shihab and others, 2013). These predictions are consolidated in the dbNSFP database of

human nonsynonymous SNVs and their functional predictions (Liu and others, 2013) and this database is integrated into the SVS and VarSeq software packages (Golden Helix Inc.). Finally, we used the Integrative Genome Viewer (http://www.broadinstitute.org/igv/) and the GenomeBrowse (Golden Helix Inc.) to visually inspect the coverages and the quality of aligned sequence reads.

Validation of *de novo* Variants Identified by Whole Genome Sequencing

PCR primers were designed to amplify variants using Primer3Plus program (https://primer3plus.com/primer3web/primer3web_input.htm) and used to amplify DNA from the proband (see Supplementary Table 2 for primer sequences). The PCR product was purified with ExoSAP-IT (USB, Cleveland, OH), analyzed by agarose gel electrophoresis, and then submitted to the University of Utah DNA Sequencing Core Facility for Sanger DNA sequencing analysis. Once variants were detected in the proband, parents were also screened by Sanger DNA sequencing to confirm the absence of the *de novo* variants.

RESULTS

Confirmation of Family Relatedness and Genders

Family relatedness within all 16 trios was confirmed (Figure 1a). Genders of all participants were also confirmed except for one CDH male patient (101261) whose blood DNA was estimated as female due to high heterozygosity of X chromosome variants, although diaphragm DNA from the same patient was estimated as male. Blood DNA for this patient was isolated from cord blood but this patient's record indicates he had a dizygotic twin sister. The most likely explanation of gender mismatch was contamination or mishandling with his twin sister's cord blood and therefore this DNA was excluded from further analysis and we only used WGS data on diaphragm DNA.

No Somatic Mutations in the Diaphragm Tissue Samples

We performed WGS on DNA isolated from the matching diaphragm tissues from 10 patients to examine possible contribution of somatic mutations in diaphragm to the etiology of CDH. However, we did not detect any damaging somatic mutations in the diaphragm. On the other hand, *de novo* functional mutations detected in blood samples were all present in the matching diaphragm tissue (Table 1 ; Supplementary Tables 1 and 2; Supplementary Data).

Germline *De Novo* Mutations

We identified and validated 14 *de novo* functional mutations in 9 patients (Tables 1 and Supplementary Data). These include two frameshift variants, one loss of start codon variant, and 11 missense variants predicted to be damaging by at least one functional prediction program among SIFT, Polyphen2, MutationTaster, MutationAssessor, and FATHMM (Table 1; Supplementary Table 2). All variants were either novel (10 variants) or rare with allele frequencies between 0.000008297 and 0.001235 (4 variants) in the ExAC exome database (Lek and others, 2016) (Table 1). Among these 9 patients, 5 had isolated CDH and 4 had additional malformation(s) (Table 1 and Supplementary Table 1). Among the 16 tested infants, most were inborn and six presented additional anomalies, four of them in the group with positive genetic findings (Supplementary Table 1). Interestingly, in this small cohort,

most infants with positive genetic findings displayed a large size 3 or 4 diaphragmatic defect (Putnam and others, 2016). All infants survived to discharge except two in the group with positive genetic findings (Supplementary Table 1).

A CDH Patient with *De Novo* Damaging Variants in *NR2F2* and *XIRP2*

An infant with left CDH with a difficult resuscitation also had severe coarctation of aorta and hypoplastic mitral valve. Due to the difficult clinical situation, parents declined extracorporeal membrane oxygenation (ECMO) and the infant died within the first 24 hours of life. This infant was found to harbor a heterozygous *de novo* frameshift mutation p.Leu264Ser*fs*Ter55 (NM_021005.3) in *NR2F2* (the nuclear receptor subfamily 2, group F, member 2, also known as chicken ovalbumin upstream promoter transcription factor two or *COUP-TFII*) causing a termination codon after 54 aberrant amino-acids generating a premature truncation of the protein (Figure 1b, c). The frameshift mutation p.Leu264Ser*fs*Ter55 (NM_021005.3) detected in this study is in an exon of four isoforms of *NR2F2* (Figure 1d). This exon contains the protein-protein interaction domains to form heterodimers with ZFPM2 (FOG2) which, in turn, modulates the transcriptional activity of GATA4, GATA5, and GATA6 (Holder and others, 2007; Pereira and others, 2000). This infant also carried a missense variant predicted to be damaging in *XIRP2*, also known as Myomaxin, a target of muscle-specific transcription factor *MEF2A*.

DISCUSSION

We have been unable to detect specific gene variants limited to the diaphragmatic tissue, but we have identified germline heterozygous *de novo* functional mutations in 14 genes in 9 CDH patients. Although the majority of these genes are not previously known to be associated with CDH, one CDH patient harbored a heterozygous *de novo* frameshift mutation in *NR2F2*. This mutation was not reported in the ExAC exome database of more than 60,000 unrelated individuals. *NR2F2* (*COUP-TFII*) null mice generated by conditional deletion in the mesentery exhibit a diaphragmatic defect similar to the human Bockdalek-type CDH (You and others, 2005). To our knowledge, this is the second case of a *de novo* frameshift mutation in *NR2F2* found in human CDH with cardiac anomalies (High and others, 2016). Interestingly, rare variants in *NR2F2* can cause congenital heart defects in humans (Al Turki and others, 2014), a phenotype often associated with CDH (Graziano and Congenital Diaphragmatic Hernia Study, 2005; Shanmugam and others, 2017). In the study by Al Turki and others (2014), none of the congenital heart defect patients with *NR2F2* missense or loss-of-function sequence variants manifested CDH. However, our and other recent (High and others, 2016) findings of *NR2F2 de* novo frameshift mutations in CDH patients with cardiac anomalies suggest a common developmental pathway. Furthermore, our data showing germline heterozygous *de novo* frameshift mutation in *NR2F2* and missense mutation in *XIRP2* are consistent with the possibility of digenic inheritance in this patient.

NR2F2 is an orphan receptor and a member of the steroid/thyroid hormone receptor superfamily. Four known isoforms of *NR2F2* (*COUP-TFII*) exist due to various transcription initiation sites and alternative splicing (Figure 1d). The previously identified *NR2F2*

frameshift mutation p.Pro33Ala*fs*Ter77 (NM_021005.3) by High and others (2016) is located in a unique first exon of isoform 1 that contains the DNA binding domain, whereas the frameshift mutation p.Leu264Ser*fs*Ter55 (NM_021005.3) detected in this study is in an exon of four isoforms (Figure 1d). This exon contains the protein-protein interaction domains to form heterodimers with ZFPM2 (FOG2) which, in turn, modulates the transcriptional activity of GATA4, GATA5, and GATA6 (Holder and others, 2007; Pereira and others, 2000). We hypothesize that our frameshift mutation prevents interaction with ZFPM2 (FOG2) in all four protein isoforms, thus failing to modulate target genes. It is compelling that ZFPM2 (FOG2) mutations has been observed in CDH (Longoni and others, 2015).

The same patient carried an additional germline heterozygous *de novo* missense mutation in *XIRP2.* This variant was predicted as damaging by SIFT and FATHMM, and possibly damaging by Polyphen2 (see MATERIALS and METHODS, Supplementary Table 2). The same heterozygous mutation was reported only once in the ExAC exome database of over 60,000 unrelated individuals. *XIRP2*, also known as Myomaxin, is a transcriptional target of *MEF2A* which regulates the expression of muscle-specific genes and the differentiation of muscle cells from their precursors (Huang and others, 2006). Moreover, *XIRP2* is essential for the formation of the intercalated disc, a cardiac structure that plays important roles in communication and signaling among cardiomyocytes (Wang and others, 2013). While the functional relevance of this *XIRP2* missense mutation in CDH is unclear, both *MEF2A*, which regulates *XIRP2*, and *NR2F2* genes map to chromosome 15q26 where recurring *de novo* deletions and unbalanced translocations were observed in CDH (Klaassens and others, 2005). Detailed analysis with array CGH and FISH analysis defined the minimal deletion region to ~5 Mb that contain several genes including *NR2F2*, but not *MEF2A*. This region was also confirmed to be involved in human disease by our shared genetic segment analysis in extended CDH pedigrees (Arrington and others, 2012). Since *MEF2A* is located outside of the 15q26 minimal region, it limits the role of *MEF2A* as well as its target *XIRP2* in CDH. However, since these genes regulate muscle cell differentiation, it is possible that they may affect the disease types or severities, including cardiovascular defects.

We also tested the hypothesis that somatic mutations in diaphragm tissue might cause CDH. We performed WGS on DNA isolated from the matching diaphragm muscular tissues from 10 patients. However, we did not detect any damaging somatic mutations in the diaphragm, lowering the possibility of the somatic hypothesis. On the other hand, *de novo* functional mutations detected in blood samples from patients were all present in the matching diaphragm tissues (Table, Supplementary Data). These data do not however exclude that other sections of the diaphragm (for example diaphragmatic membrane) might harbor specific pathogenic variants or that testing larger numbers of patients might allow the identification of somatic defects in CDH. Overall, a limitation of our report is that it is based on the analysis of a limited number of patients (16 total patients and their parents, among which 10 patients also had analysis of diaphragm samples).

In summary, the *de novo* frameshift mutation in *NR2F2* found in one patient in this study provides further support for its role in the etiology of CDH and possibly comorbid cardiac

anomalies in humans, while the *de novo* missense mutation in *XIRP2* in the same patient may contribute to modify disease phenotypes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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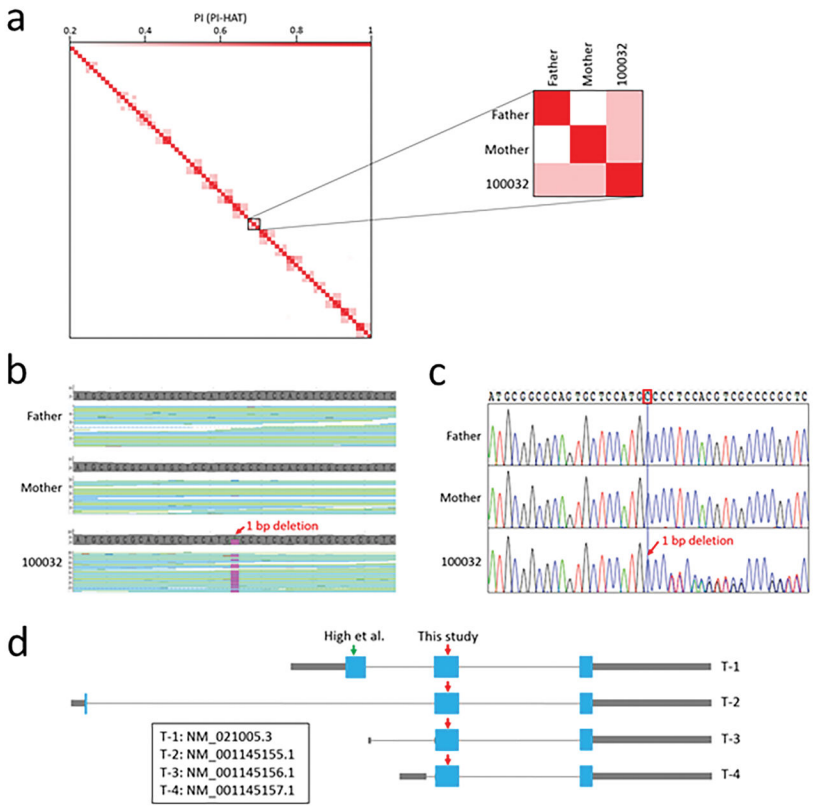

Figure 1.

(a) Heat map of pair-wise IBD (Identity by Descent) estimate. A total of 78 samples with WGS data was analyzed. PI. 78 × 78 pairs of individuals with a relatedness measure PI (PI-HAT) are displayed on the left. PI near 0.5 indicates first degree relatives and PI near 1 indicates duplicate. Confirmation of family relatedness in a trio with *de novo* mutations in *NR2F2* and *XIRP2* is displayed on the right.

(b) An example of visual inspection of WGS coverage and pile-up. A trio with *de novo NR2F2* frameshift mutation is displayed. Coverage and pile-up for all 14 *de novo* mutations can be found in the supplementary data: WGS_Alignments.

(c) Sanger sequencing histograms of *de novo NR2F2* frameshift mutation in the same trio. Histograms of all 14 *de novo* mutations can be found in the supplementary data: Sanger_Sequencing_Histograms

(d) Four isoforms of *NR2F2*. Exons are shown in blue squares, untranslated regions are shown in thick grey lines, and introns are shown in thin grey lines. Our *NR2F2* frameshift mutation site is indicated with a red arrow, and frameshift mutation site discovered by High et al. (2016) is indicated with a green arrow.

Table 1

List of *de novo* mutations identified in this study

Patient	Gender	Type of CDH	Other Malformations	Examined Patient Tissue	Variant Position (GRCh37)	Reference Allele	Alternate Allele	Gene Name	Variant Type	Variation	ExAC Alt Allele Freq
95334	M	Bochdalek	None	Blood	chr7:100850181	C	G	PLOD3	Missense	p.Arg647Pro (NM_001084.4)	N/A
95377	F	Agenesis hemidiaphragm	None	Blood, Diaphragm	chr14:104395016	C	T	TDRD9	Missense	p.Pro57Leu (NM_153046.2)	N/A
98873	M	Bochdalek	None	Blood, Diaphragm	chr16:14345831	G	A	MKL2	Missense	p.Arg839Lys (NM_001308142.1)	N/A
99168	M	Bochdalek	Hypospadias	Blood, Diaphragm	chr11:129727277	T	C	TMEM45B	Missense	p.Phe214Leu (NM_138788.4)	N/A
100032	F	Left CDH	Severe coarctation of aorta, hypoplastic mitral valve	Blood	chr2:167760330	C	T	XIRP2	Missense	p.Ala113Val (NM_152381.5)	0.000008297 (1/120526)
					chr15:96877649	C	-	NR2F2	LoF - Frameshift	p.Leu264Ser*fs*Ter55 (NM_021005.3)	N/A
101260	F	Bochdalek	Duplicated pituitary gland, patent cranio-pharyngeal canal with large mass along the hard palate and nasopharynx, oropharyngeal mass, hypoplastic pons, mildly hypoplastic cerebellar vermis, T1 butterfly vertebrae with no posterior cervical dysraphic defect	Blood	chr6:76072494	A	G	FILIP1	Missense	p.Ile142Thr (NM_001289987.2)	0.00002471 (3/121392)
102081	M	Bochdalek	None	Blood	chr1:39788323	C	T	MACF1	Missense	p.Ser1363Leu (NM_012090.5)	0.00001648 (2/121366)
					chr2:100209827	G	A	AFF3	Missense	p.Leu791Phe (NM_001025108.1)	N/A
					chr3:63898372	A	C	ATXN7	Missense	p.Gln33Pro (NM_001177387.1)	0.001235 (1/810)
103680	M	Bochdalek	VSD, abnormal frontotemporal region	Blood, Diaphragm	chr8:24346763	T	-	ADAM7	LoF - Frameshift	p.Pro396Leu*fs*Ter41 (NM_003817.3)	N/A
					chr19:41307313	G	A	EGLN2	Missense	p.Arg279His (NM_053046.3)	N/A
108859	F	Agenesis hemidiaphragm	None	Blood, Diaphragm	chr9:4741087	T	C	AK3	LoF - Start Lost	p.Met1Val (NM_016282.3)	N/A
					chrX:108718807	G	A	GUCY2F	Missense	p.Ala120Val (NM_001522.2)	N/A