Published in final edited form as:

Am J Med Genet A. 2016 September ; 170(9): 2457–2461. doi:10.1002/ajmg.a.37830.

De Novo Frameshift Mutation in *COUP-TFII* (*NR2F2*) in Human Congenital Diaphragmatic Hernia

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Abstract

COUP-TFII (*NR2F2*) is mapped to the 15q26 deletion hotspot associated with the common and highly morbid congenital diaphragmatic hernia (CDH). Conditional homozygous deletions of *COUP-TFII* in mice result in diaphragmatic defects analogous to the human Bochdalek-type hernia phenotype. Despite evidence from animal models however, mutations in the coding sequence of *COUP-TFII* have not been reported in patients, prompting the speculation that additional coding or non-coding sequences in the 15q26 locus are necessary for diaphragmatic hernias to develop. In this report, we describe a case of a patient with a heterozygous de novo *COUP-TFII* frameshift mutation, presenting with CDH and an atrial septal defect. The p.Pro33Ala/sTer77 mutation specifically disrupts protein isoform 1 which contains the DNA binding domain. In addition, we review other *COUP-TFII* sequence variations and deletions that have been described in cases of CDH. We conclude that *COUP-TFII* mutations can cause diaphragmatic hernias, and should be included in the differential diagnosis of CDH patients, particularly those with comorbid congenital heart defects.

Keywords

COUP-TFII; NR2F2; 15q26; congenital diaphragmatic hernia; atrial septal defect; de novo; trio; pleuroperitoneal folds

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SUPPORTING INFORMATION

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Conflict of interest: The authors declare that there are no conflicts of interest.

INTRODUCTION

De novo deletions and unbalanced translocations of chromosome 15q26 are a recurring cause of congenital diaphragmatic hernia (CDH) (OMIM # 142340) [Biggio et al., 2004; Klaassens et al., 2005; Longoni et al., 2014], a relatively common and severe birth defect characterized by abnormal formation of the diaphragm and lung hypoplasia, often complicated by pulmonary hypertension. Deletions at this locus are occasionally associated with hypoplastic left heart syndrome (HLHS), coarctation of the aorta, and dysmorphic features, while a few patients have been described as affected by Fryns syndrome [Slavotinek et al., 2005]. Several genes mapped to 15q26 have been suggested as possible CDH-related candidates, including the nuclear receptor subfamily 2, group F, member 2 (*NR2F2*, also known as chicken ovalbumin upstream promoter transcription factor two or *COUP-TFII*), chromodomain helicase DNA-binding protein 2 (*CHD2*), and MADS box transcription factor 2, polypeptide A (*MEF2A*), which regulates the expression of muscle-specific genes [Pollock and Treisman, 1991]. In fact, many 15q26 genes are expressed in the developing rodent diaphragm, and it has been suggested that more than one are required for proper diaphragm development [Clugston et al., 2008]. The critical region was refined to 5 Mb on 15q26.1-q26.2 using a combination of array-CGH and FISH on samples derived from two individuals with telomeric deletions and from a third, affected with CDH, showing a smaller interstitial deletion. The minimally deleted region contained multiple genes, including *COUP-TFII* and *CHD2* [Klaassens et al., 2005]. An unbalanced translocation involving 15q26 was later described in a patient with CDH, hypoplastic left heart syndrome, and hypoplastic nails. This region included *COUP-TFII* but not *CHD2* [Scott et al., 2007]. The region was narrowed further by the identification of a fetus with CDH and coarctation of the aorta with a smaller deletion encompassing the *COUP-TFII* and *SPATA8* genes [Brady et al., 2013].

The presence of *COUP-TFII* in the minimally deleted region of the 15q26 locus, combined with the observation of diaphragm defects in a mouse model [You et al., 2005] has prompted speculation that *COUP-TFII* is a critical candidate gene for CDH. Despite this, sequence variants in the *COUP-TFII* coding region have not yet been described in CDH patients [Slavotinek et al., 2006; Scott et al., 2007]. Consistent with these findings, it was proposed that non-coding sequences in or around the *COUP-TFII* locus were responsible for the diaphragmatic defect [Arrington et al., 2012]. Others have proposed that alternative genes within the 15q26 locus may play a role in CDH. For example, an unbalanced translocation between chromosome 2p16.3p25.3 and 15q26qter was described in a fetus with left-sided CDH, dysmorphic features, tetralogy of Fallot, growth retardation, and kidney and limb anomalies [Mosca et al., 2011]. The deletion in this case did not include *COUP-TFII*, suggesting the existence of an alternative critical region of 1.8 Mb containing two genes expressed in the embryonic diaphragm: *IGF1R* and *ARRDC4*.

In this report, we describe a de novo frameshift mutation in *COUP-TFII* in a patient with CDH and an atrial septal defect. Furthermore, we describe two other novel *COUP-TFII* sequence variants identified in a large cohort of CDH patients. These findings suggest that mutations in the coding region of this gene may be sufficient to cause CDH in humans.

CLINICAL REPORT

Patient 1 is a female who was diagnosed with left-sided CDH and a large atrial septal defect (ASD) by prenatal ultrasound at 38 weeks gestation. She was delivered vaginally at 39 weeks gestation and had a birth weight of 2790 g. Apgar scores were two at 1 min and nine at 5 min. She was intubated at birth. The diaphragmatic hernia was left posterolateral and small-to-moderate in size, with both liver and stomach remaining in the abdominal cavity. Surgical repair was done thorascopically at 4 days of age. The neonatal course was complicated by pulmonary hypertension, which had resolved by 2 months of age. She did not require extracorporeal membrane oxygenation. The ASD was followed clinically and closed spontaneously by 2 years of age. Postnatal echocardiograms also showed a patent foramen ovale (PFO), which was still present at cardiology follow-up at 4 years of age. She had gastroesophageal reflux, feeding intolerance, and aspiration requiring G-tube placement at 4 months of age. G-tube feeds were maintained for approximately 1 year and she had closure of a persistent gastrocutaneous fistula at 17 months of age. She also had exotropia that was treated with patching. Physical examination by a clinical geneticist at 2 years of age did not identify any distinctive dysmorphic features. She did well clinically and at routine follow-up at 5 years of age she was noted to have mild developmental delays but no other ongoing morbidities and was taking no medications. Array-CGH data are not available. This study was performed in accordance to the Partners Human Research Committee (protocol 2000P000372) and the Boston Children's Hospital clinical investigations standards (protocol 05-07-105R).

MATERIALS AND METHODS

Genomic DNA was extracted from peripheral blood samples in EDTA using QIAamp DNA Blood Mini Kit (Qiagen). Shotgun library construction was performed on 1 µg of genomic DNA after successful fragmentation through acoustic sonication (Covaris), end-polishing and A-tailing, ligation of sequencing adaptors and PCR amplification with 8 bp barcodes for multiplexing. Whole Exome Sequencing (WES) was performed on the three family members (Fig. 1A) with exome capture on the Roche/Nimblegen SeqCap EZ v2.0. The library concentration was determined by qPCR, and the molecular weight distributions verified on the Agilent Bioanalyzer. Sequencing was performed on the Illumina-HiSeq 2000 platform (Illumina). BWA-mem was employed to map sequencing reads to the hg19 assembly of the human genome [Li and Durbin, 2010]. GATK was then used for multiple realignment, base recalibration, and variant calling [McKenna et al., 2010].

Variant calls were analyzed using xBrowse (xbrowse.broadinstitute.org) under a de novo dominant inheritance model with an expected frequency of causative mutations <0.01 in the 1000 Genomes Phase 3 release [1000 Genomes Project Consortium et al., 2015] and in the Exome Aggregation Consortium (ExAC), Cambridge, MA (<http://exac.broadinstitute.org>). Automated functional annotations were used to filter variant calls. The search for high impact (nonsense, frameshift, essential splice site mutations) and missense variants predicted to be pathogenic by in silico algorithms returned a seven nucleotide c.92_98delGCCCCGC deletion in the *COUP-TFII* coding region (NM_021005.3) (Fig. 1B). This variant is novel and is not described in the 1000 Genomes, NHLBI ESP exomes, dbSNP, or ExAc databases.

In general, frameshift variants in *COUP-TFII* are not present in ExAC (<http://exac.broadinstitute.org/gene/ENSG00000185551>, last accession on April 27, 2016). A de novo premature termination codon in the Glutamate Receptor, Metabotropic 1 (*GRM1*) gene (p.Trp110Ter) was considered an incidental finding based on the patient phenotype.

The *COUP-TFII* region containing the variant was PCR amplified (PCR Master Mix, Promega, Madison, WI), cloned into a pCRTM4-TOPO[®] TA Vector and transformed into One Shot[®] TOP10 Chemically Competent Cells (ThermoFisher, Life Technologies Corporation, Grand Island, NY). Sanger sequencing of the T7 primed insert confirmed the deletion and that it was not inherited from either parental sample. The mutation is predicted to generate a grossly abnormal and non-functional peptide resulting from an early frameshift and premature truncation (p.Pro33Ala/sTer77).

Transcript variant 1 (NM_021005.3) contains exon 1b which encodes the DNA binding domain, and is the only isoform predicted to be affected by the c.92_98delGCCCCGCC mutation (Fig. 1C and D). To confirm that *COUP-TFII* variant 1 is expressed in the developing diaphragm, we obtained RNA from the pleuroperitoneal folds (PPF) of the embryonic diaphragm of E11.5 C57BL/6J mouse embryos, which had been embedded in OCT (Tissue-Tek) and rapidly frozen in an isopentane slurry to maintain their morphology (Jackson Laboratory Institutional Animal Care and Use Committee). Laser capture microdissection with Arcturus XT on CapSure HS LCM Caps (LCM0215) (Applied Biosystems), RNA purification, amplification, and retrotranscription were performed as previously described [Russell et al., 2012]. Primer pairs specific to murine isoform 1 (NM_009697.3) were designed using Primer-BLAST [Ye et al., 2012]. End-point PCR indicated that *COUP-TFII* variant 1 is expressed in the PPFs of laboratory mice at the time when the insult leading to diaphragmatic hernia is believed to occur (Fig. 1E).

Two additional variants in the *COUP-TFII* gene were present in a WES cohort of 275 patients with CDH, deposited in the database of Genotypes and Phenotypes (dbGAP) (accession no. phs000783.v1.p1) (Online Supplemental Table SI). Patient 2 is a male with isolated right-sided CDH who was found to have a single nucleotide variant within the 5' UTR of the *COUP-TFII* gene (c.-60C>T). This nucleotide is conserved across mammalian species and this variant is not present in the 1000 Genomes, NHLBI ESP exomes, or ExAC databases. Data from the ENCODE database shows that this region is a binding site for the E2F1 transcription factor, suggesting that this is a potential regulatory region. We have also identified another novel coding variant in *COUP-TFII* in a patient with isolated CDH (c.1096C>T; p.R213C). The latter variant, which we have previously published [Longoni et al., 2015], is predicted to be damaging by SIFT and Polyphen-2 algorithms. The inheritance of both of these variants is unknown due to the lack of available parental samples.

DISCUSSION

COUP-TFII is an orphan receptor and a member of the steroid/thyroid hormone receptor superfamily [Qiu et al., 1996]. Several *COUP-TFII* isoforms have been described due to different transcription initiation sites and alternative splicing, and they have been characterized extensively in human embryonic stem cells (hESC) during differentiation into

embryoid bodies (EB) [Rosa and Brivanlou, 2011]. The splicing variant 1 (NM_021005.3), the only isoform affected by the sequence variant identified in Patient 1, encodes the DNA binding domain. The alternative isoforms contain only the protein-protein interaction domains allowing them to form heterodimers, whereas they cannot bind DNA directly [Pereira et al., 2000]. We hypothesize that, in Patient 1, isoforms 2–4 are not affected by the mutation and can bind ZFPM2 (FOG2) but this interaction does not lead to an effect on target genes. Interestingly, a balanced translocation with a breakpoint between exons 1b and 2 of *COUP-TFII* has been reported in a patient with coarctation of the aorta without a diaphragmatic defect [Al Turki et al., 2014].

COUP-TFII is expressed in the main components of early embryonic diaphragm: the previously mentioned PPF, as well as the post-hepatic mesenchymal plate (PHMP), and the septum transversum [You et al., 2005; Clugston et al., 2008]. In the PPF, *COUP-TFII* is colocalized with the mesenchymal marker Wt1 and it is not expressed in the Pax3 positive pre-muscle cells [Clugston et al., 2006].

The function of this orphan receptor in the developing diaphragm has not been completely elucidated; however, conditional deletion of *COUP-TFII* with *Nkx3-2 Cre* in the foregut mesenchyme is sufficient to create a diaphragmatic anomaly mimicking Bochdalek-type CDH [You et al., 2005]. *COUP-TFII* is also expressed in the developing lung mesenchyme, which could explain the severe lung hypoplasia very frequently associated with diaphragmatic defects [You et al., 2005], and in a number of endoderm-derived tissues [Zhang et al., 2002]. Retinoic acid was found to provide partial rescue of the pulmonary phenotype in rats treated with the CDH-inducing teratogen Nitrofen, specifically by accelerating type one alveolar cell proliferation [Sugimoto et al., 2008] and promoting alveologenesis [Montedonico et al., 2008]. Increased expression of *COUP-TFII* in this rescue model suggests that this gene mediates the beneficial effects of retinoic acid [Doi et al., 2009]. Both variant 1 and variant 2 are expressed in P19 cells, however, only variant 1 is upregulated after retinoic acid treatment [Pickens et al., 2013].

Inherited and de novo missense variants in *COUP-TFII* have been reported in rare individuals with non-syndromic atrio-ventricular septal defects identified by WES of parent-offspring trios [Al Turki et al., 2014]. Among patients with *COUP-TFII* pathogenic variants, heart phenotypes included Tetralogy of Fallot, atrio-ventricular septal defects, and hypoplastic left heart syndrome. None of these patients presented with CDH, consistent with the previously held hypothesis that non-coding sequences may be responsible for 15q26-associated CDH [Arrington et al., 2012]. In fact, a number of antisense transcripts (*NR2F2-AS1*, Gene ID: 644192) are mapped in close proximity to the *COUP-TFII* gene. The possible role of these non-coding RNAs in the regulation of *COUP-TFII* or in CDH pathogenesis is not known.

In the Online Supplemental Table SI, we have summarized the three novel sequence variations in *COUP-TFII* that we have identified in our cohort of CDH patients, as well as published deletions at 15q26 with available array CGH data confirming involvement of the *COUP-TFII* gene. Based on these observations, we conclude that mutations in the coding region of *COUP-TFII*, alone or in combination with other contributing factors, could be a

rare cause of CDH. Patients with larger deletions appear to have complex phenotypes, while those with smaller deletions or *COUP-TFII* sequence variants have either CDH plus a congenital heart defect or isolated CDH. Therefore, we suggest that mutations in *COUP-TFII* should be included in the differential diagnosis in patients CDH, especially those with comorbid congenital heart disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding was provided by the National Institute of Child Health and Human Development (NICHD) P01HD068250. Whole exome sequencing services were provided through the RS&G Service by the Northwest Genomics Center at the University of Washington, Department of Genome Sciences, under U.S. Federal Government contract number HHSN268201100037C from the National Heart, Lung, and Blood Institute.

Grant sponsor: National Institute of Child Health and Human Development (NICHD); Grant number: P01HD068250.

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FIG. 1.

(A) Pedigree (B) Sanger sequencing of c.92_98delGCCCCGCC allele in the proband (C) The sequence variant, indicated by the arrow, plotted against *NR2F2* transcript 1 (NM_021005.3). Exon 1B, encoding the DNA binding domain of COUP-TFII and containing the de novo mutation discovered in the proband, is colored red. The site of the premature termination codon in the new reading frame is indicated by the black vertical bar. (D) The frameshift variant does not map to the coding regions of transcripts 2 (NM_001145155.1), 3 (NM_001145156.1), and 4 (NM_001145157.1). (E) Transcript specific RT-PCR of three laser captured PPF samples from two different litters, indicating that *COUP-TFII* variant 1 is expressed in mouse embryonic diaphragm at E11.5. Legend: T, Transcript. [Color figure can be seen in the online version of this article, available at <http://wileyonlinelibrary.com/journal/ajmga>].