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Prevalence and penetrance of *ZFPM2* mutations and deletions causing congenital diaphragmatic hernia

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Abstract

Zinc finger protein, FOG2 family member 2 (*ZFPM2*) (previously named *FOG2*) gene defects result in the highly morbid congenital diaphragmatic hernia (CDH) in humans and animal models. In a cohort of 275 CDH patient exomes, we estimated the prevalence of damaging *ZFPM2* mutations to be almost 5%. Genetic analysis of a multigenerational family identified a heritable intragenic *ZFPM2* deletion with an estimated penetrance of 37.5%, which has important

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Conflict of interest

The authors have declared no conflicting interests.

Supporting Information

The following Supporting information is available for this article:

Appendix S1. Clinical reports.

Additional Supporting information may be found in the online version of this article.

implications for genetic counseling. Similarly, a low penetrance *ZFPM2* frameshift mutation was observed in a second multiplex family. Isolated CDH was the predominant phenotype observed in our *ZFPM2* mutation patients. Findings from the patients described herein indicate that *ZFPM2* point mutations or deletions are a recurring cause of CDH.

Keywords

congenital diaphragmatic hernia; DNA copy number variation; exome; FOG2; penetrance; ZFPM2

Congenital diaphragmatic hernia (CDH) is a common birth defect (1 in 3000 live births) associated with significant morbidity and mortality. Most CDH patients have a diaphragmatic defect as an isolated, left posterolateral defect, which often includes lung hypoplasia. The remainder have a complex phenotype with additional malformations, at times as part of a recognizable syndrome (1). Most CDH cases are sporadic, with a single affected individual in the family. However, rare CDH kindreds have proven instructive for gene discovery (1–3).

Point mutations and deletions in *ZFPM2* (zinc finger protein, FOG family member 2) have been reported in patients with diaphragmatic defects. *ZFPM2* was identified as a candidate based on a mouse model and a patient with a *de novo* non-sense mutation, and it is expressed diffusely in the developing mouse diaphragm before and after muscularization and in the pulmonary mesenchyme during branching morphogenesis (4). Subsequently, two isolated CDH patients with missense variants in conserved residues were reported; because parental samples were not available it was not possible to assess whether they were inherited (5). Mutations in *ZFPM2* have been identified in patients with tetralogy of fallot (TOF) or double outlet right ventricle (DORV), without diaphragmatic involvement (6).

More recently, *ZFPM2* deletions were identified in two unrelated patients with isolated CDH; in both cases the deletion was inherited from an unaffected parent, suggesting reduced penetrance (7). However, the prevalence of *ZFPM2* mutations and the degree of penetrance have never been systematically determined in CDH patients. We address the former by exome sequencing analysis in a cohort of sporadic unrelated CDH cases, while the latter is estimated by *ZFPM2* findings in familial cases.

Materials and methods

Patient recruitment

Informed consent was obtained according to Partners Human Research Committee and Children's Hospital Boston Clinical Investigation standards (Protocol 2000P000372 and 05-07-105R, respectively). All consented individuals underwent examination by a geneticist and/or review of medical records.

Sample collection and processing

Whole blood samples were collected for direct extraction (QIAamp DNA Blood Maxi kit, Qiagen, Valencia, CA) and Epstein-Barr virus (EBV) transformation (8). Primary fibroblast

cultures were established from mechanically dissociated skin biopsies, plated in Dulbecco's Modified Eagle's medium (DMEM) with 10% fetal bovine serum (Gibco®|Life Technologies™, Grand Island, NY).

Whole exome sequencing

Whole exome sequencing was performed on 93 CDH patients at the Northwest Genomics Center (Seattle, WA) (9); and on 182 patients at Yale Center for Genomic Analysis (New Haven, CT), using the Illumina Genome Analyzer II (San Diego, CA). Reads were aligned using MAQ (sourceforge.net) and variant calling was performed by Genome Analysis Toolkit (GATK). *ZFPM2* variants were reviewed by Ingenuity Variant Analysis™ (reference: NM_012082.3). 2-II-3 underwent clinical exome using Illumina HiSeq platform. The data were converted to FastQ by Illumina CASAVA 1.7 and mapped to BWA. Variant calling was performed using Atlas-SNP and Atlasindel (sourceforge.net).

Sanger sequencing

Primers were designed using PrimerBLAST (NCBI). Polymerase chain reaction (PCR) was performed using Qiagen Taq PCR Master Mix (Qiagen, Valencia, CA), sequenced by Taq DyeDeoxy Terminator cycle sequencing kit and resolved on the ABI 3730XL DNA analyzer (Applied Biosystems, Grand Island, NY).

Copy number variant (CNV) analysis

In Family 1, Affymetrix 6.0 chips and Agilent 1 M microarrays were hybridized as previously described (10) (Santa Clara, CA). Agilent 244 k arrays were performed on individual CDH12. Birdsuite (broadinstitute.org) and Agilent Feature Extraction (10.7.3.1), respectively, were used to generate CNV calls. Clinical microarrays were obtained on 2-II-1 [ClariSure CGH, Quest Diagnostics (Madison, NJ)], 2-II-2 and 2-II-3 [GenomeDx v5, GeneDx (Gaithersburg, MD)].

Expression of mutant *ZFPM2* allele

Lymphoblastoid lines were treated with retinoic acid (1×10^{-7} M) and dibutyryl-cyclic AMP (1 mM) (Sigma, St Louis, MO) for 24 h before RNA extraction, and retrotranscribed with SuperScript® III Reverse Transcriptase (Invitrogen Life Technologies, Grand Island, NY). PCR was performed with GoTaq® Green Master Mix (Promega Corporation, Madison, WI).

Results

Sporadic CDH cohort

From 275 CDH patient exomes, we identified 14 potentially damaging heterozygous *ZFPM2* sequence mutations in 13 unrelated CDH patients (5%) (Table 1; patients CDH1-CDH13). The majority of *ZFPM2* mutations were missense and mapped to highly conserved nucleotides (phyloP p-value $<10^{-4}$) (11) or known functional domains; p.E58X (CDH4) was a pre-mature stop codon, and p.N1062fs*23 (CDH13) was a frameshift expected to cause the loss of the fifth *ZFPM2* zinc finger domain. *ZFPM2* mutations were paternally or

maternally inherited, except one, which arose *de novo* (Table 1). Because of lack of parental samples, inheritance could not be determined for every mutation.

Patient CDH13 had a potential second hit with a p.R213C variant (phyloP p-value 7.228×10^{-7}) in *NR2F2*, also implicated in CDH (1). The *NR2F2* variant maps to the protein domain thought to bind ZFPM2 (UniProtKB, uniprot.org).

Every patient presented with isolated posterolateral CDH, except CDH12, who also had craniofacial abnormalities, TOF with an overriding aorta, a ventricular septal defect, and a narrow right ventricular outflow tract. It should be noted that CDH12 had also a 250 kb copy number gain of unknown significance in 8q24.21 (chr8:130,867,815-131,117,179; NCBI36/hg18), detected by Agilent 244 K aCGH and containing the *FAM49B* and *ASAP1* genes. Parental samples were not available, precluding determination whether this CNV was *de novo*. Additional possibly pathogenic mutations or CNVs were not detected in these 13 patients.

Family 1—We characterized a multigenerational family of European ancestry, in which four relatives (1-II-6; 1-III-1; 1-III-2; 1-III-4) displayed isolated diaphragmatic defects, inherited in an autosomal dominant manner with incomplete penetrance (Fig. 1a). Clinical descriptions are available in the Supporting Information.

A single copy number loss within the *ZFPM2* gene was detected in all three individuals with CDH from whom a DNA sample was available (Fig. 1a–c). This intragenic deletion, spanning chr8:106,417,969-106,704,253 (NCBI36/hg18), was also present in both unaffected obligate carriers (1-II-3, 1-II-8), and as well as in three additional unaffected family members (Fig. 1c). The mutated transcript is predicted to be 3076 nucleotides long (380 bases shorter than the normal transcript) because of a frameshift introducing a premature stop codon. In the theoretical resulting peptide, only 63 amino acids would be the same as normal *ZFPM2*.

To determine if the mutant allele was transcribed, immortalized lymphoblastoid cell lines from two affected individuals (1-III-2, and 1-II-6), one unaffected deletion carrier (1-II-3), and one family member without the deletion (1-II-4) were studied. In all samples, we detected only a 672 bp band, corresponding to the wild type allele, suggesting the predicted mutant transcript was unstable and/or underwent non-sense mediated decay (Fig. 2).

Family 2—Phenotypically normal parents in a second multiplex family, also of European ancestry, had three children with left posterolateral CDH (2-II-1; 2-II-2; 2-II-3) (Fig. 3a). All siblings carried a p.Y467fs*23 mutation in *ZFPM2*, inherited from their mother (2-I-1), who did not have a clinical diagnosis of CDH (Fig. 3b). The p.Y467fs*23 mutation results in the truncation of over 50% of the protein, including the zinc finger domains (Fig. 3c). In addition to CDH, individual 2-II-1 had mild facial dysmorphism, hydrocephalus, and autistic behaviors, and individual 2-II-3 had reported developmental delays. Their mother (2-I-1) also had self-reported history of learning disabilities. These three individuals, but not 2-II-2, also harbor a chromosomal anomaly del(1)(q21.1q21.2)/dup(1)(q21.1), which appeared to segregate with the neurodevelopmental phenotype.

Discussion

In this study, we identified *ZFPM2* mutations in a cohort of CDH patients, as well as investigated two families with inherited *ZFPM2* mutations showing reduced penetrance. *ZFPM2* mutations, present in ~5% of our CDH cohort, were considered pathogenic either on the basis of *in silico* algorithms or because they were reported to cause heart defects (6, 12, 13), a common CDH comorbidity (14). Seven mutations were familial, while one was *de novo* [p.E30G (CDH1)]. Interestingly, CDH7 carried two *ZFPM2* missense variants, each inherited from a different parent, indicating compound heterozygosity. Although both had been previously associated with conotruncal defects (13), functional studies were never carried out to determine their pathogenicity, which raises the possibility that one is a benign variant. Even though p.E30G mutations have a frequency of up to 1% in individuals without a history of CDH (Table 1), this observation does not discount a role in CDH pathogenesis, as this and other studies have shown reduced penetrance or subclinical diaphragmatic defects in carriers of *ZFPM2* mutations (7).

Mutations predicted to cause *ZFPM2* because of pre mature stop codons leading to non-sense mediated decay constituted a major risk factor for CDH in two kindreds with apparent autosomal dominant inheritance and reduced penetrance. Because diaphragm morphology and function could not be studied in our phenotypically normal carriers, it is possible that these individuals could have a subclinical diaphragm abnormality. In Family 1, we estimate the penetrance for clinically relevant diaphragmatic defects to be around 37.5% of carriers.

Our case series indicates that patients with *ZFPM2* mutations most often present with an isolated CDH phenotype. *ZFPM2* mutations have been previously reported in patients with conotruncal heart defects, specifically TOF and DORV (6, 12, 13). Among the few reports to date, mutations associated with DORV were frequently *de novo* and occurred in exon 8 (13), but reduced penetrance was observed in at least two TOF patients (12). Interestingly, *ZFPM2* mutation M703L has been associated with DORV (13) and CDH (5), but not in the same individual. Only patient CDH12 displayed a more complex phenotype. In addition to CDH and TOF with a ventricular septal defect, he displayed left anophthalmos and cleft palate, which sometimes co-occur with CDH (1). We cannot determine whether these anomalies are because of his *ZFPM2* mutation or to the 8q24.21 copy number gain.

Several individuals from Family 2 were found to carry a chromosome 1q21 deletion/duplication in addition to the p.Y467fs*23 *ZFPM2* mutation. 1q21 abnormalities have been associated with a variable phenotype, from developmental delay, mental retardation, learning disability, microcephaly and autism to no clinically relevant findings (15). Therefore, it is possible that the mild learning impairments in 2-I-1 and 2-II-3, and hydrocephalus and autism in 2-II-1 are because of this chromosomal rearrangement. Sibling 2-II-2, on the other hand, had only the p.Y467fs*23 *ZFPM2* mutation and displayed isolated CDH with no apparent neurodevelopmental findings.

Despite reported monogenic forms, isolated CDH is believed to be mostly multifactorial/polygenic. There is precedence for non-Mendelian genetics in disorders such as cleft lip and palate and congenital heart defects, where the phenotype is affected by unknown risk

factors, modifier genes, and environmental triggers, all of which must be kept in mind during genetic counseling (16). We propose that the *ZFPM2* mutations described above, in light of their reduced penetrance, should be considered risk factors. Our findings imply that *ZFPM2* may require additional hits, absence of a protective gene, or environmental factor to cause CDH with all of its clinical implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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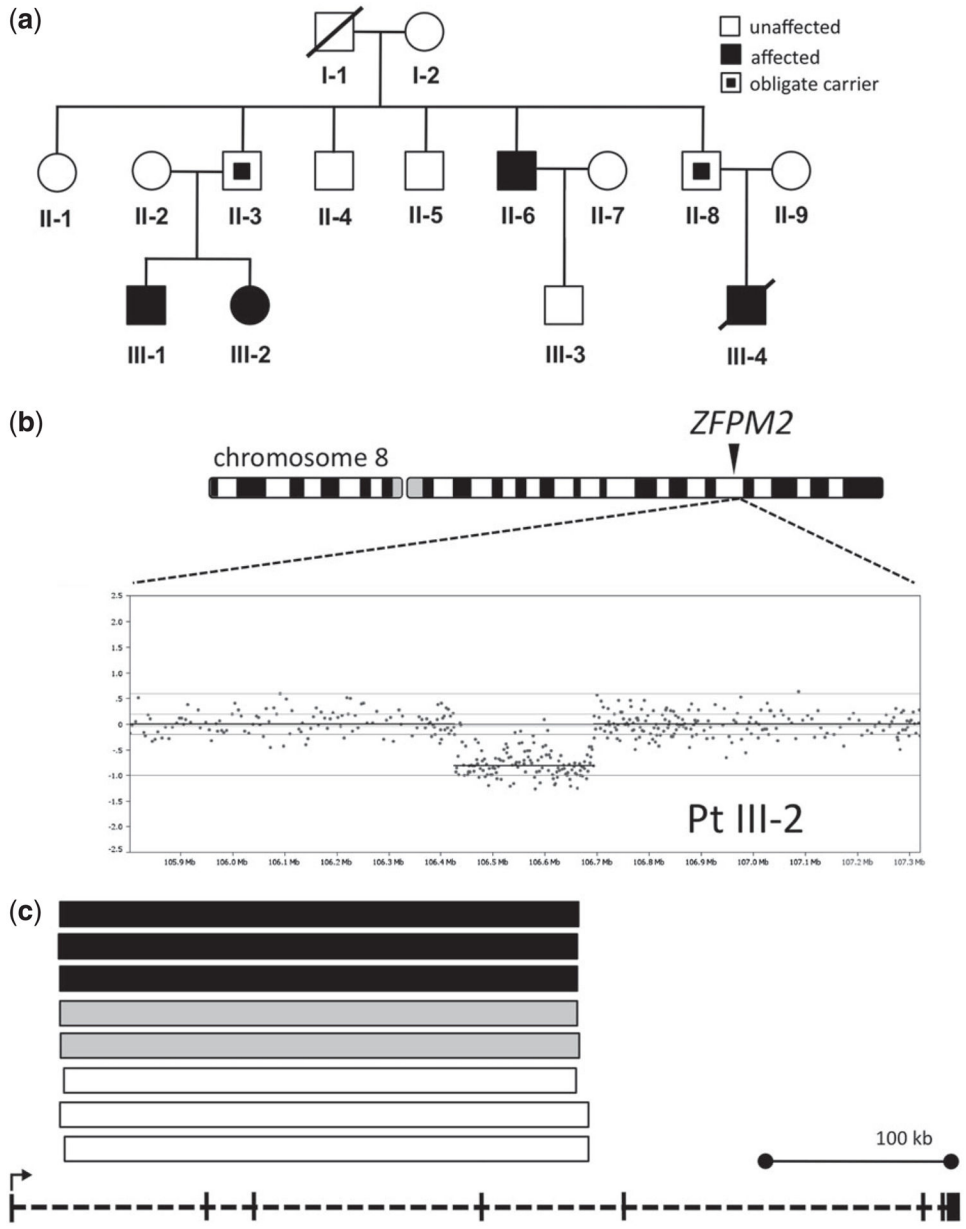

Fig. 1.

ZFPM2 deletion in Family 1. (A) Pedigree. Black inner square, unaffected carrier. (B) Chromosome 8 (schematic) and Agilent 1 M aCGH findings in III-2. (C) *ZFPM2* deletion in other family members: affected (black), obligate carriers (gray), and unaffected individuals (white). Deletion intervals plotted against a diagram of the *ZFPM2* gene (bottom). *ZFPM2* deletions were not present in 7000 neuropsychiatric patients and 6000 controls.

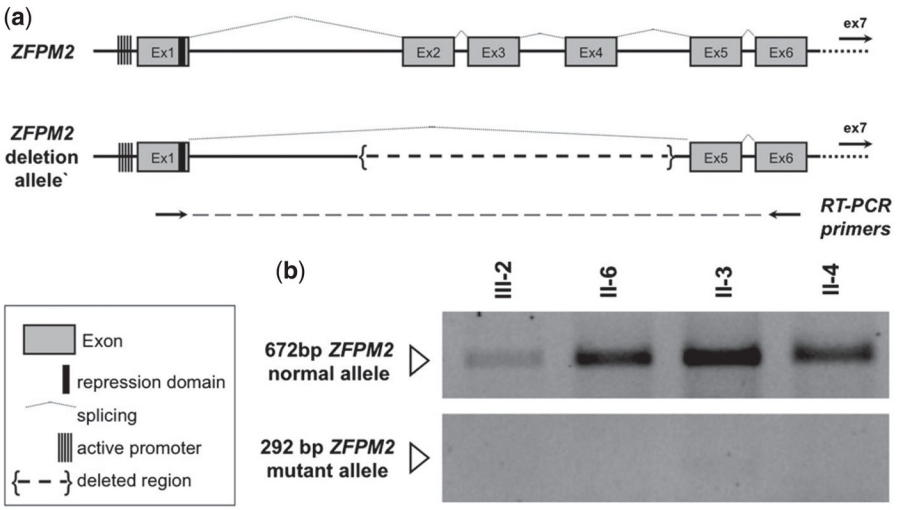

Fig. 2.

Expression of *ZFPM2* mutant transcript. (**a**) Predicted splicing of normal and mutant *ZFPM2* allele. Retro-transcription (RT) PCR primers were designed in exons 1 and 6 to amplify and discriminate full length and mutant transcripts. (**b**) RT-PCR on lymphoblastoid line cDNAs from III-2, II-6 (deletion carriers, affected), II-3 (deletion carrier, unaffected), and II-4 (non-carrier, unaffected). ZPMF2 promoter methylation was not different in affected or unaffected deletion carriers.

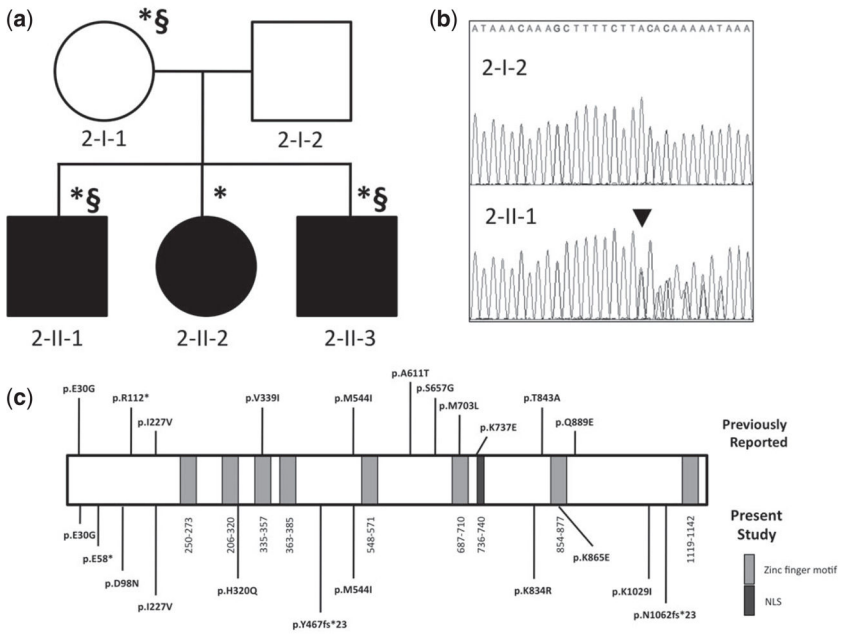

Fig. 3.

ZFPM2 mutation in Family 2. (**a**) Pedigree. *, p.Y467fs*23. §, 1q21 del/dup. (**b**) *ZFPM2* reference and c.1396_1399dup (p.Y467fs*23, black arrowhead) in 2-II-1. (**c**) Familial mutation compared to sporadic cases (below) and previously reported *ZFPM2* variants (above).

Table 1

Exonic *ZFPM2* variants in 13 CDH study cohort patients. Reported associations, phenotypes associated to this mutation in the medical literature

		Mutation nomenclature		Prediction algorithms			Frequency (%) in unaffected individuals					
Sample ID	**hg19 location**	**cDNA**	**Protein**	**SIfT**	**PolyPhen**	**dbSNP ID**	**1000 genomes**	**CG public genomes**	**NHLBIESP**	**phyloP ($\times 10^{-4}$)**	**Inheritance**	**Reported associations**
CDH1	106431420	c.89A > G	p.E30G	Damaging	Possibly damaging	121908601	0.26	0.92	0.45	0.23	*de novo*	TOF
CDH2	106431420	c.89A > G	p.E30G	Damaging	Possibly damaging	121908601	0.26	0.92	0.45	0.23	Unknown	TOF
CDH3	106431420	c.89A > G	p.E30G	Damaging	Possibly damaging	121908601	0.26	0.92	0.45	0.23	Maternal	TOF
CDH4	106431503	c.172G > T	p.E58*	NA	NA	–	0	0	0	0.02	Paternal	–
CDH5	106456600	c.292G > A	p.D98N	Tolerated	Possibly damaging	202217256	0.14	0	0.37	1.22	Unknown	–
CDH6	106456600	c.292G > A	p.D98N	Tolerated	Possibly damaging	202217256	0.14	0	0.37	1.22	Maternal	–
CDH7	106801092	c.679A > G	p.I227V	Tolerated	Probably damaging	202204708	0	0	0.08	0.13	Paternal	DORV
CDH7	106813942	c.1632G > A	p.M544I	Tolerated	Benign	187043152	0.24	0	0.35	0.32	Maternal	TOF
CDH8	106801092	c.679A > G	p.I227V	Tolerated	Probably damaging	202204708	0	0	0.08	0.13	Paternal	DORV
CDH9	106811172	c.960C > G	p.H320Q	Tolerated	Possibly damaging	–	0	0	0	0.63	Paternal	-
CDH10	106814811	c.2501A > G	p.K834R	Damaging	Probably damaging	113289249	0.05	0	0.25	0.66	Unknown	-
CDH11	106814903	c.2593A > G	p.K865E	Tolerated	Probably damaging	367893066	0	0	0.02	2.6	Unknown	-
CDH12	106815396	c.3086A > T	p.K1029I	Tolerated	Probably damaging	201729935	0	0	0.02	0.13	Unknown	-
CDH13	106815496	c.3186delC	p.N1062fs*23	NA	NA	–	0	0	0	NA	Unknown	-

CDH, congenital diaphragmatic hernia; NA, not applicable.