



ORIGINAL ARTICLE

Genomic alterations that contribute to the development of isolated and non-isolated congenital diaphragmatic hernia

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ABSTRACT

Background Congenital diaphragmatic hernia (CDH) is a life threatening birth defect. Most of the genetic factors that contribute to the development of CDH remain unidentified.

Objective To identify genomic alterations that contribute to the development of diaphragmatic defects.

Methods A cohort of 45 unrelated patients with CDH or diaphragmatic eventrations was screened for genomic alterations by array comparative genomic hybridisation or single nucleotide polymorphism based copy number analysis.

Results Genomic alterations that were likely to have contributed to the development of CDH were identified in 8 patients. Inherited deletions of *ZFPM2* were identified in 2 patients with isolated diaphragmatic defects and a large de novo 8q deletion overlapping the same gene was found in a patient with non-isolated CDH. A de novo microdeletion of chromosome 1q41q42 and two de novo microdeletions on chromosome 16p11.2 were identified in patients with non-isolated CDH. Duplications of distal 11q and proximal 13q were found in a patient with non-isolated CDH and a de novo single gene deletion of *FZD2* was identified in a patient with a partial pentalogy of Cantrell phenotype.

Conclusions Haploinsufficiency of *ZFPM2* can cause dominantly inherited isolated diaphragmatic defects with incomplete penetrance. These data define a new minimal deleted region for CDH on 1q41q42, provide evidence for the existence of CDH related genes on chromosomes 16p11.2, 11q23-24 and 13q12, and suggest a possible role for *FZD2* and Wnt signalling in pentalogy of Cantrell phenotypes. These results demonstrate the clinical utility of screening for genomic alterations in individuals with both isolated and non-isolated diaphragmatic defects.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) is a life threatening birth defect that occurs in approximately 1/4000 live births; it is defined as a protrusion of abdominal viscera into the thorax through an abnormal opening or defect that is present at birth.^{1 2} In some cases the herniated viscera are covered by a membranous sac. This type of CDH can

be difficult to distinguish from a diaphragmatic eventration in which there is extreme elevation of a part of the diaphragm that is often atrophic and abnormally thin. The majority of CDH cases are sporadic, with the recurrence risk for isolated CDH typically quoted as <2% based on a model of multifactorial inheritance.³ However, there is a growing body of evidence that small chromosomal anomalies can predispose to the development of CDH.^{4–10}

The identification of a predisposing genomic change greatly enhances the ability of physicians to provide individualised genetic counselling and to formulate optimal treatment and surveillance plans. This would suggest that screening for genomic changes by array comparative genomic hybridisation (aCGH) or single nucleotide polymorphism (SNP) based copy number analysis should be performed on all patients with diaphragmatic defects.^{10 11} However, this practice has not been universally applied.

Efforts to map the locations of all reported chromosomal anomalies associated with CDH have revealed many regions of the genome that are recurrently deleted, duplicated, or translocated in individuals with CDH.^{12 13} Each of these genomic regions is likely to harbour one or more CDH related genes. For some of these regions, a specific gene has been implicated in the development of diaphragmatic defects. Chromosome 8q22q23, for example, is recurrently deleted and translocated in individuals with CDH. This region harbours the zinc finger protein, multitype 2 (*ZFPM2*) gene. *ZFPM2* has been implicated in the development of diaphragmatic defects, based on the identification of a de novo truncating mutation in a child with a severe diaphragmatic eventration and the development of similar diaphragmatic defects in mice that are homozygous for a hypomorphic allele of *Zfp2*.¹⁴ In contrast, chromosome 1q41q42—which is also recurrently deleted in individuals with CDH—harbours a number of genes, none of which have been clearly shown to cause diaphragmatic defects in humans or animal models.¹⁵

In this report we screened a cohort of 45 individuals with diaphragmatic defects for genomic alterations by high resolution aCGH or SNP based

copy number analysis. Chromosomal anomalies that are likely to have caused or contributed to the development of diaphragmatic defects were identified in eight patients. These findings allow us to define the inheritance pattern of diaphragmatic defects associated with haploinsufficiency of *ZFPM2* and delineate a new minimal deleted region for CDH on 1q41q42. They also provide evidence for the existence of CDH related genes on chromosomes 16p11.2, 11q23-24 and 13q12 and suggest a possible role for *FZD2* and Wnt signalling in the development of various phenotypes seen in pentalogy of Cantrell.¹⁶

METHODOLOGY

Patient accrual

For array studies, informed consent was obtained from a convenience sample of 45 patients with CDH or diaphragmatic eventrations and, when possible, their parents in accordance with institutional review board approved protocols. Clinical and molecular data from these patients have not been published with the exception of patients 5 and 7 whose clinical findings were summarised by Shinawi *et al* and Fruhman *et al*, respectively.^{17 18}

For sequence analysis of *ZFPM2*, a cohort of 52 patients with CDH and 10 patients with diaphragmatic eventrations were screened for deleterious changes. This cohort consisted of a subset of patients from the array cohort and similarly consented individuals. *ZFPM2* sequence analyses for these individuals have not been previously published.

aCGH and SNP based copy number analyses

In most cases, chromosomal deletions and duplications were identified or confirmed by aCGH on a research basis using Human Genome CGH 244K or SurePrint G3 Human CGH 1M Oligo Microarray Kits (G4411B, G4447; Agilent Technologies, Santa Clara, California, USA), prepared according to the manufacturer's protocols and analysed as previously described using individual sex matched controls with no personal or family history of CDH.⁷ Putative copy number changes were defined by two or more adjacent probes at 244 K resolution or three or more adjacent probes at 1 M resolution with \log_2 ratios suggestive of a deletion or duplication when compared to those of adjacent probes.

In two cases—patients 2 and 6—causative chromosomal deletions were identified before accrual and further aCGH testing was unwarranted based on the molecular data already available. The deletion in patient 2 was identified using an Illumina CytoSNP bead version 12.2 (Illumina, Inc, San Diego, California, USA) and the deletion in patient 6 was identified and defined on a clinical basis using a 105 K Combimatrix Molecular Diagnostics array (Combimatrix Molecular Diagnostics, Irvine, California, USA) hybridised, extracted, and evaluated according to manufacturer's instructions.

Identification of previously reported copy number variants

To determine if putative changes identified by aCGH or SNP based copy number analysis had been described previously in normal controls, we searched for similar deletions or duplications in the Database of Genomic Variants (<http://projects.tcag.ca/variation/>).

Confirmation of genomic changes

Changes that were not identified in the Database of Genomic Variants were confirmed by real-time quantitative PCR, with the exception of causative changes identified in patients 3 and 7 which were confirmed by chromosome analysis, and patients 4 and 6 which were confirmed by fluorescence in situ hybridisation (FISH) analysis.

Quantitative real-time PCR

Quantitative real-time PCR (qPCR) analysis experiments were designed and carried out as previously described.⁷ For qPCR analysis within the *ZFPM2* gene, experiments were designed in a manner similar to the standard curve method described by Boehm *et al* with a region of the c14orf145 gene serving as a control locus.¹⁹

Chromosome analyses and FISH studies

Chromosome analyses were performed for patients 3 and 7 on a clinical basis by the Molecular Genetics Laboratory at Baylor College of Medicine. FISH analyses were performed for patient 4 by the Cytogenetic Laboratory at Texas Tech University Health Sciences Center School of Medicine and for patient 6 by Combimatrix Molecular Diagnostics.

Long range PCR amplification and sequencing

Long range PCR amplification was carried out using the TaKaRa long range PCR system (TaKaRa Bio, Otsu, Shiga, Japan) according to manufacturer's instructions. PCR products were gel purified, sequenced, and analysed using Sequencher 4.7 software (Gene Codes Corporation, Ann Arbor, Michigan, USA).

Identification of *ZFPM2* sequence changes

Primers were designed to amplify the coding sequence and the intron/exon boundaries of *ZFPM2* and used to amplify patient DNA. Sequence changes in PCR amplified products were identified by comparison with control DNA sequences using Sequencher 4.7 software (Gene Codes Corporation, Ann Arbor, Michigan, USA).

Hispanic control samples were obtained from the Baylor Polymorphism Resource, a collection of approximately 600 anonymous control samples from major ethnic and racial backgrounds.

RESULTS

Identification of genomic changes in patients with CDH or diaphragmatic eventrations

Forty-five subjects with congenital diaphragmatic hernias or diaphragmatic eventrations of varying severity were screened for chromosomal anomalies by aCGH or SNP based copy number analysis. Eight subjects carried genomic changes that likely contributed to the development of their diaphragmatic defects. Clinical and molecular data from these subjects are summarised in table 1.

Patient 1 is a male of mixed ancestry who was diagnosed at 9 days of age with intestinal malrotation and a large, left-sided diaphragmatic eventration (figure 1). Later in life, he was diagnosed with left-sided radioulnar synostosis. Despite normal motor development, at 24 months of age he spoke only two words. However, he made rapid progress with speech therapy and by 31 months of age he was speaking in four-word sentences.

aCGH analysis revealed an ~1 Mb deletion that included only the *ZFPM2* gene on chromosome 8q22.3-23.1 (figure 1). Quantitative and long range PCR analysis revealed that the patient's father carried the same deletion (data not shown). A review of his father's medical records did not reveal evidence of a diaphragmatic anomaly despite an extensive evaluation after a severe automobile accident which included an abdominal CT scan. We screened the coding sequence and associated intron/exon boundaries of patient 1's remaining *ZFPM2* allele looking for changes that might account for the difference in phenotype between him and his father, but no sequence changes were identified.

Patient 2 is a Caucasian male that was diagnosed prenatally with a left-sided CDH. His brother was similarly affected with

Table 1 Summary of data from subjects with chromosomal changes likely to have contributed to diaphragm defects

| | Patient 1 | Patient 2 | Patient 3 | Patient 4 | Patient 5 | Patient 6 | Patient 7 | Patient 8 |
|---|---|--|--|---|---|--|--|--|
| Final Karyotype | 46,XY,del(8)(q22.3q23.1) | 46,XY,del(8)(q23.1q23.1) | 46,XX,del(8)(q22.3q24.23) | 46,XY,del(1)(q41q42.12) | 46,XY,del(16)(p11.2p11.2) | 46,XY,del(16)(p11.2p11.2) | 47,XX,+der(13)(t(11;13)(q23.2;q12.3) | 46,XY,del(17)(q12.2-q12.2) |
| Minimal/ maximal affected region (hg19) | Chromosome 8 deletion 105 914 640-106 907 764 (~993 kb)/ 105 880 441-106 922 626 (~1.04 Mb) | Chromosome 8 deletion 106 812 277-107 420 029 (~608 kb)/ 106 800 200-107 511 467 (~711 kb) | Chromosome 8 deletion 104 516 737-136 746 871 (~32.2 Mb)/ 104 508 082-136 809 150 (~32.3 Mb) | Chromosome 1 deletion 223 076 895-225 311 293 (2.2 Mb)/ 223 073 839-225 318 623 (2.2 Mb) | Chromosome 16 deletion 29 652 999-30 199 351 (~554 kb)/ 29 350 831-30 332 522 (~982 kb) | Chromosome 16 deletion 29 502 653-30 274 073 (~771 kb) defined clinically with no minimum or maximum region specified. | Chromosome 11 duplication 112 355 952-134 927 114 (26.6 Mb)/ 112 347 267-134 927 114 (26.6 Mb) | Chromosome 17 deletion 42 633 066-42 650 463 (~17.4 kb)/ 42 622 151-42 657 504 (~35.4 kb) |
| Confirmation method | Real-time qPCR | Real-time qPCR | Chromosome analysis | FISH | Real-time qPCR | FISH | Chromosome analysis | Real-time qPCR |
| Inheritance | Paternal | Maternal | De novo | De novo | De novo | De novo | Maternal balanced translocation | De novo |
| Age | 4 years old | 2 days old | 1 day old | 2 years old | 2 years old | 17 days old | 8 days old | 2 years old |
| Gender | Male | Male | Female | Male | Male | Male | Female | Male |
| Ethnicity | Mixed | Caucasian | Hispanic | Caucasian | Mixed | Caucasian | Hispanic | Caucasian |
| Prenatally identified abnormalities, exposures, and prenatal karyotype | No abnormalities identified | CDH, intrauterine growth retardation, single umbilical artery, 46,XY | CDH, short extremities, oedema/ascites, polyhydramnios,46,XX,del(8)(q22q24) | No abnormalities identified on two prenatal ultrasounds | CDH, maternal smoking in first trimester | CDH, 46,XY | CDH, polyhydramnios and intrauterine growth retardation | CDH and omphalocele detected, 46,XY |
| Birth history | Vaginal delivery at term, vomiting 2 days after birth | Caesarean section at 36 3/7 weeks for abnormal fetal heart rate and decreased movement | Spontaneous vaginal delivery at 34 5/7 weeks | Spontaneous vaginal delivery at 37 weeks | Delivery at 37 weeks, after birth he decompensated and required 20 min of CPR | Repeat caesarean section at 39 6/7 weeks | Spontaneous vaginal delivery at 37 3/7 weeks | Scheduled caesarean section at 37 1/7 weeks |
| Birth weight, length and OFC | Weight 3.8 kg | Weight: 1.72 kg, length 42 cm; OFC 29.5 cm | No information available | Weight 3.2 kg; length 49.5 cm | Weight estimated at 2.5 kg; length 49 cm; OFC 34 cm | Weight ~3.5 kg; length 50 cm; OFC 35.5 cm | No information available | Weight 2.2 kg; length 45 cm, OFC 33 cm |
| Diaphragm | Diaphragmatic eventration | Left-sided CDH | Large left-sided CDH | CDH identified at 11 months | Right-sided CDH | Left-sided CDH | Large left-sided CDH | Anterior medial CDH |
| Cardiac | No known abnormalities | No known abnormalities | No known abnormalities | No known abnormalities | No structural abnormalities noted on echo | No structural abnormalities noted on echo | Small perimembranous VSD, secundum ASD | Large perimembranous VSD, PFO versus ASD |
| Additional anomalies | Intestinal malrotation, radioulnar synostosis | None | All extremities were symmetrically short with fixed flexion of the distal upper extremities. | Right cerebral volume loss with ex vacuo dilatation of the right lateral ventricle, diffuse thin cortical mantle, atrophy of right hippocampus, thin corpus callosum, left-sided cryptorchidism with non-attachment to the epididymis | Micro-retrognathia, cleft palate, right inguinal hernia and bilateral post-axial polydactyly (paternal) | Hypoplastic, non-articulating thumbs, extra thoracic vertebra, 13 pairs of ribs | Clouded corneas, cupped optic nerves, cleft palate, small anterior anus, prominent forehead, large anterior fontanel, posteriorly rotated low set ears, micro-retrognathia, wide spaced hypoplastic nipples, right single palmar crease, tapering fingers, prominent heels | Coronal craniosynostosis, diminished CNS white matter with thinning of the corpus callosum, omphalocele, bilateral inguinal hernias, left-sided cryptorchidism |
| Clinical course/development | Only two words at age 2 years. Responded well to speech therapy. Now normal | Died on the 2nd day of life with respiratory insufficiency and pulmonary hypertension | Died shortly after delivery | Persistent hypoglycaemia after birth, gross motor, fine motor and language delay, seizures starting at 20 months | No oxygen requirement at 24 months but still receiving supplemental nutrition via gastric tube | Died on the 17th day of life with severe respiratory insufficiency and pulmonary hypertension | Support was withdrawn at 8 days of age. Findings at autopsy included abnormal lung fissures and coronary artery anomalies | At 28 months could walk with a walker, nutritional support via gastric tube, ventilator support with acute illness |

ASD, atrial septal defect; CDH, congenital diaphragmatic hernia; CNS, central nervous system; CPR, cardiopulmonary resuscitation; echo, echocardiogram; FISH, fluorescence in situ hybridisation; OFC, occipital frontal circumference; PFO, patent foramen ovale; VSD, ventricular septal defect.

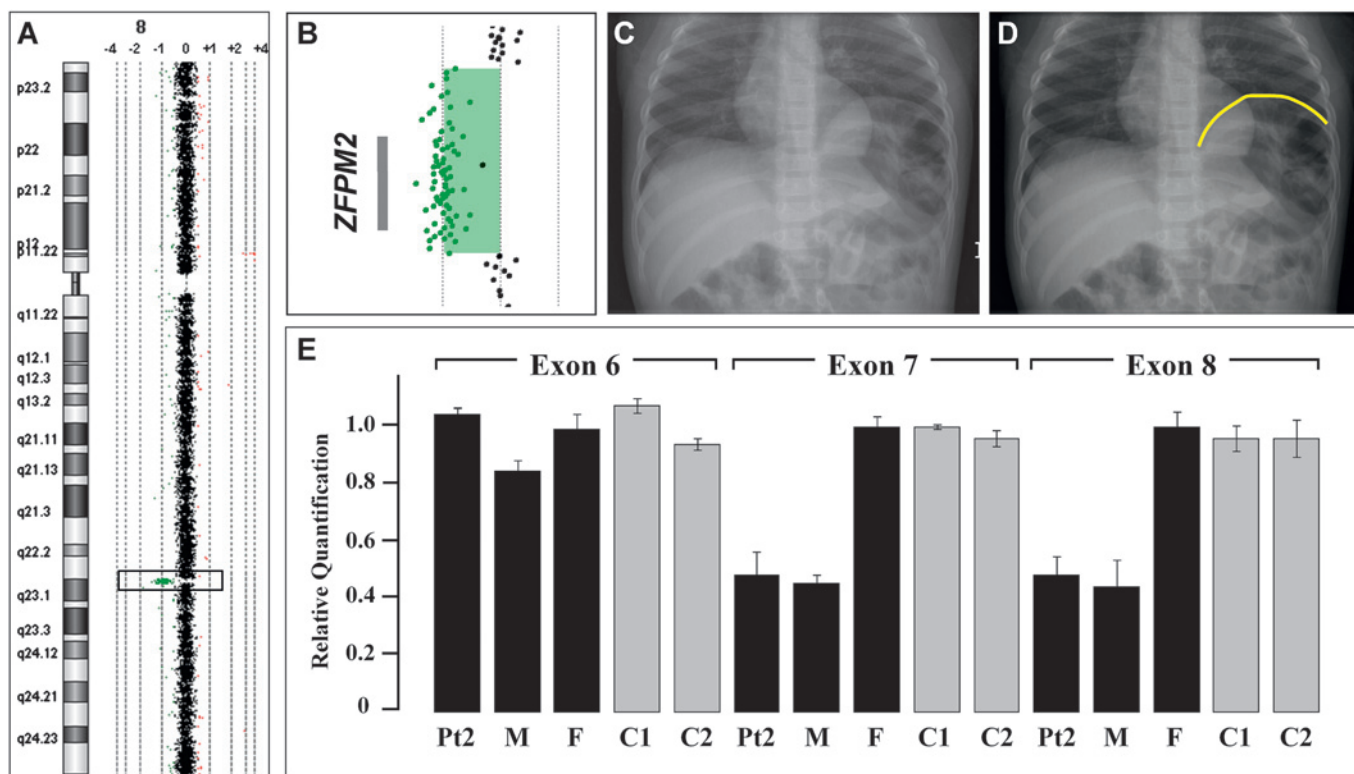


Figure 1 (A) Array comparative genomic hybridisation (aCGH) data from patient 1 showing an 8q22.3q23.1 single gene deletion of *ZFPM2*. (B) The relative location of the *ZFPM2* gene in relation to aCGH data from the deletion region in patient 1 is represented by a grey bar. (C) Chest radiograph of patient 1 demonstrating a severe diaphragmatic eventration containing loops of bowel. (D) The same radiograph shown in panel C with the limits of the diaphragmatic eventration outlined in yellow. (E) Quantitative PCR analysis demonstrates a normal copy number for *ZFPM2* exon 6 but a reduced copy number for exons 7 and 8 in DNA from patient 2 (Pt2) and his mother (M). Normal copy number values are seen in DNA from patient 2's father (F) and DNA from two unrelated controls (C1, C2).

CDH and died of complications related to his hernia. Except for a single umbilical artery, no additional anomalies were identified after birth. He died at 2 days of age from respiratory insufficiency and pulmonary hypertension. SNP based copy number analysis revealed a maternally inherited deletion on chromosome 8q23.1 that included a portion of the 3' coding region of *ZFPM2* and the first coding exon of oxidation resistance gene 1 (*OXR1*) based on mRNA transcript variant 1. Quantitative PCR analysis revealed that exons 7 and 8 of *ZFPM2* were included in the deletion (figure 1). No sequence changes were identified in the coding sequence and intron/exon boundaries of the remaining allele.

Patient 3 had a de novo ~32 Mb deletion from 8q22.3 to 8q24.23 that overlapped the *ZFPM2* deletions seen in patients 1 and 2 and also included the *EXT1* and *TRPS1* genes that are associated with Langer–Giedion syndrome (OMIM #150230). This Hispanic female was diagnosed prenatally with a large left-sided posterolateral CDH. Fetal MRI at 34 5/7 weeks also revealed an omphalocele and symmetrically short extremities. Due to severe pulmonary hypoplasia she was placed in neonatal hospice care immediately after birth and died shortly thereafter.

Patient 4 is a Caucasian male who had a normal prenatal course. He was born at 37 weeks gestation and his perinatal course was complicated by persistent hypoglycaemia. A diaphragmatic hernia was identified incidentally on a chest x-ray at 11 months of age during an investigation for failure to thrive. His motor and language development were delayed and he developed seizures at 20 months of age. Central nervous system (CNS) studies revealed right hemisphere cerebral volume loss with ex vacuo dilatation of the right lateral ventricle with diffuse thin cortical mantle, atrophy in the right hippocampus,

and a thin corpus callosum. aCGH analysis revealed a de novo 2.2 Mb interstitial deletion of 1q41–1q42.12 (figure 2).

Patients 5 and 6 were found to have de novo interstitial deletions of 16p11.2—a recurrent microdeletion region flanked by low copy repeats. Patient 5 is a 2-year-old male of mixed ancestry who had a severe right-sided posterolateral CDH, micrognathia, a U-shaped palatal cleft, paternally inherited autosomal dominant polydactyly, and dysmorphic features consistent with a 16p11.2 deletion which were previously described.¹⁷ He continues to need dietary supplementation via G-tube and supplemental oxygen when ill.

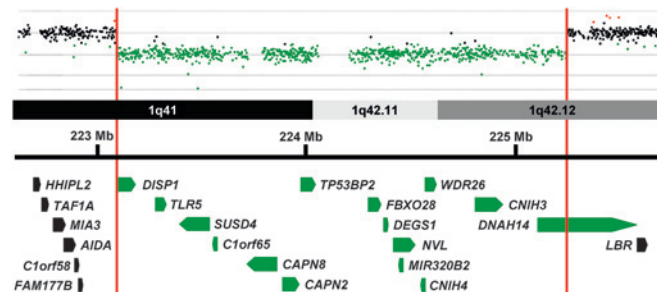


Figure 2 Array comparative genomic hybridisation data from patient 4 is shown above a schematic representation of a portion of the 1q41–1q42 region. Genes in this region are represented by block arrows. Red vertical bars mark the limits of the deletion in patient 4 and delineate the minimal deleted region for congenital diaphragmatic hernia (CDH) in this region of the genome. Genes with all or a portion of their coding sequence inside the minimal deleted region are shown in green.

Patient 6 was a male infant conceived by intrauterine insemination with non-consanguineous Caucasian parents as donors. A left-sided diaphragmatic hernia was diagnosed prenatally. After birth, both of his thumbs were found to be proximally placed, hypoplastic and non-articulating, with the left thumb having a pedunculated appearance. Additional findings included an extra thoracic vertebra and 13 pairs of ribs. He required extracorporeal membrane oxygenation starting on the first day of life, developed severe diffuse oedema, and died on the 17th day of life as a result of his severe respiratory insufficiency and pulmonary hypertension.

Patient 7 was born at 37 6/7 weeks to non-consanguineous Hispanic parents. She had persistent respiratory distress at birth and intubation was complicated by micrognathia and a tongue anomaly. Further evaluation revealed a left-sided CDH, a ventricular septal defect (VSD) and atrial septal defect (ASD), partial cleft palate, small anteriorly placed anus, and dysmorphic features. A marker chromosome was identified by G-banded chromosome analysis and was shown to be the result of an unbalanced maternal translocation between chromosome 13q and 11q, resulting in a 47,XX,+der(13)t(11;13)(q23;q12.3) chromosome complement.¹⁸ Her prognosis was deemed poor in light of her multiple anomalies, and she died shortly after support was withdrawn on day 8 of life. Additional findings at autopsy included abnormal lung fissures and coronary artery anomalies.

Patient 8 is a 2-year old Caucasian male who was diagnosed prenatally with a left-sided anterior CDH, and a large omphalocele. Postnatal cardiac evaluation revealed a large perimembranous VSD and a patent foramen ovale versus ASD. Additional anomalies included bilateral inguinal hernias, left-sided cryptorchidism, and premature fusion of the coronal sutures evident on head CT performed at 7 and 12 months of age. An MRI obtained at 11 months of age revealed diminished bifrontal and bitemporal parenchyma white matter associated with thinning of the corpus callosum, delayed myelination, and generous extra-axial cerebrospinal fluid (CSF) spaces.

aCGH analysis revealed a de novo 17.4 kb deletion that involved only the frizzled homologue 2 gene (*FZD2*) on chromosome 17q12.2. Amplification of the breakpoint by long range PCR followed by sequencing revealed that the deletion had occurred between two Alu repeats; an Alu-Sb proximally and an Alu-Sq distally. Matsuzaki *et al* has reported deletions of this gene in 3/90 normal Yoruban individuals from Ibadan, Nigeria.²⁰ However, these changes were not identified by McCarroll *et al* who analysed the same cohort.²¹

To determine if the mutations in patient 8's remaining *FZD2* allele may have contributed to his phenotype, we sequenced the coding region of his remaining *FZD2* allele but did not identify any sequence changes.

Twenty changes not reported in the Database of Genomic Variants were identified in this cohort by aCGH and confirmed by either qPCR and/or FISH analysis (table 2). In two cases the extent of the copy number variation was larger than that seen in the parents. Patient TX-28 appeared to have four copies of a region on 11q13.5, while her parents each had three copies compared to control. Patient TX-50 had no copies of a region on 17q25.1, while his parents each had one copy compared to control. TX-50 also had two copies of a region on Xp27.1, similar to his mother, which could represent a de novo event.

Identifying *ZFPM2* sequence changes in patients with CDH and diaphragmatic eventrations

To determine if detrimental sequence changes in *ZFPM2* were a common cause of CDH or diaphragmatic eventrations, we

screened the coding region and intron/exon boundaries of this gene in 52 patients with CDH (table 3) and 10 patients with diaphragmatic eventrations (table 4). Three non-synonymous changes were identified which had not been previously described in the 1000 Genomes project or dbSNP. A D98N change was identified in a male with an isolated posterior left-sided hernia with a large sac. His mother did not carry a D98N allele, but the father was unavailable for analysis. This change was predicted to be possibly damaging by PolyPhen (<http://genetics.bwh.harvard.edu/pph/>) but non-deleterious by SNPs3D (<http://www.snps3d.org/>).^{22 23} The same change was reported by Bleyl *et al* in an individual with bilateral CDH.²⁴ However, they concluded that the change was likely to be a benign SNP since it was also identified in control samples.

A Q889E change was identified in a Hispanic female with a right-sided CDH, ambiguous genitalia, and a double vagina. Her mother did not carry the change, but the father was unavailable for analysis. This change was predicted to be benign by PolyPhen, non-deleterious by SNPs3D, and was subsequently identified in five of 152 (3.3%) Hispanic control chromosomes.

A paternally inherited S210T mutation was identified in an African American male with a left-sided CDH and dysmorphic features. This change was predicted to be benign by PolyPhen and non-deleterious by SNPs3D.

DISCUSSION

aCGH and SNP based copy number analyses are commonly used as a primary screening tool to identify genomic aetiologies in patients with congenital anomalies. Identification of these changes can provide information that physicians can use to hone the prognostic and recurrence risk information given to families and to create individualised therapeutic and surveillance plans for patients.

Haploinsufficiency of *ZFPM2* causes autosomal dominant isolated diaphragmatic defects with incomplete penetrance

ZFPM2 is a transcriptional co-factor that forms heterodimers with GATA transcription factors to regulate gene expression during development.²⁵ The role of *ZFPM2* in development of diaphragmatic defects was first demonstrated by Ackerman *et al* who showed that mice homozygous for a hypomorphic mutation in *Zfp2* develop diaphragmatic eventrations.¹⁴ In the same publication, they reported a de novo heterozygous R112X mutation in an infant with severe diaphragmatic eventrations and pulmonary hypoplasia who died shortly after birth.¹⁴

Patient 1 is the second child in which haploinsufficiency for *ZFPM2* has been seen in association with a severe diaphragmatic eventration. Although patient 2's deletion affects both the 3' end of *ZFPM2* and the first coding exon of *OXR1*, it is likely that disruption of *ZFPM2* underlies the development of CDH in this patient since *OXR1* appears to be involved in the prevention of oxidative damage—a process that has not been implicated in the development of diaphragmatic defects.²⁶ Although DNA is not available on patient 2's brother, it is likely that he inherited the same deletion from his mother which contributed to his CDH. Alterations in *ZFPM2* activity may also be the major, if not the sole, genetic factor that contributed to the development of CDH in patient 3 and previously reported individuals with CDH with deletions and translocation involving 8q22q23 (supplemental table 2).¹⁵ Since both patients 1 and 2 inherited their deletions from unaffected parents we conclude that loss-of-function alleles of *ZFPM2* are associated with autosomal dominant

Table 2 Rare inherited changes identified in patients with congenital diaphragmatic hernia

| Patient number | Copy number change | Inheritance pattern | Chromosome | Genes involved | Minimum/maximum hg19 | Clinical synopsis |
|----------------|--------------------|---|------------|--|---|--|
| TX-02 | Increase | Paternal | 15q24.2 | <i>COMMD4, NEIL1</i> | 75 605 889-75 644 263/ 75 600 108-75 648 132 | Female, left-sided CDH, large bronchopulmonary sequestration |
| TX-04 | Increase | Paternal and maternal increased copy number | 3q26.33 | <i>SOX2, SOX20T</i> | 181 430 003-181 431 902/ 181 425 656-181 432 270 | Male, isolated right-sided CDH |
| | Increase | Maternal | 5q11.2 | <i>ARL15, HSPB3</i> | 53 450 417-53 759 016/ 53 443 541-53 765 448 | |
| | Increase | Paternal and maternal increased copy number | 18p11.21 | <i>GNAL, MPPE1*</i> | 11 872 270-11 878 083/ 11 863 962-11 884 082 | |
| TX-12 | Decrease | Paternal | 7q32.1 | <i>AHCYL2</i> | 128 988 273-128 996 545/ 128 978 921-129 002 744 | Male, isolated left-sided CDH |
| TX-14 | Increase | Paternal | 13q21.33 | <i>KLHL1, SCA8, ATXN8OS</i> | 70 673 470-71 577 278/ 70 666 491-71 594 524 | Male, isolated left-sided CDH |
| TX-23 | Increase | Maternal | 1p31.3 | <i>CACHD1, RAVER2</i> | 65 144 120-65 210 203/ 65 137 345-65 218 480 | Female, right-sided CDH, ASD, missshaped kidney |
| TX-27 | Decrease | Parents not available | 14q21.1 | <i>FBXO33</i> | 39 884 781-40 262 115/ 39 875 206-40 301 762 | Female, isolated left-sided |
| TX-28 | Increase | Patient n=4 Mother and father n=3 | 11q13.5 | <i>AQP11, CLNS1A, RSF1</i> | 77 297 657-77 424 591/ 77 270 540-77 431 584 | Female, left-sided CDH, double outlet right ventricle, VSD |
| TX-36 | Decrease | Not maternal, father not available | 1p12 | <i>HSD3B2</i> | 119 962 240-119 983 891/ 119 957 963-119 994 447 | Male, isolated left-sided CDH |
| TX-39 | Decrease | Maternal | 16p13.3 | <i>DNASE1, TRAP1</i> | 3 684 132-3 716 095/ 3 672 598-3 726 606 | Female, left-sided posterior CDH, bicommisural aortic valve |
| TX-42 | Increase | Paternal and maternal increased copy number | 1q24.3 | <i>DNM3</i> | 171 944 462-171 958 347/ 171 936 289-171 970 634 | Male, isolated left-sided posterior CDH |
| | Decrease | Paternal | 15q21.2 | <i>USP8*, USP50</i> | 50 802 672-50 822 065/ 50 791 758-50 833 347 | |
| TX-50 | Decrease | Patient n=0 Parents n=1 | 17q25.1 | <i>TRIM4*</i> | 73 875 459-73 878 627/ 73 870 461-73 882 792 | Male, isolated left-sided CDH |
| | Increase | Maternal n=2 | Xq27.1 | <i>F9, MCF2, ATP11C, MIR505, CXorf66</i> | 138 594 571-139 089 290/ 138 556 308-139 102 322 | |
| TX-52 | Decrease | Paternal | 15q23 | <i>AAGAB*, IQCH, C15orf61, MAP2K5</i> | 67 548 229-68 040 596/ 67 540 042-68 049 656 | Female, right-sided CDH, ASD |
| TX-56 | Increase | Maternal | Xp11.1 | <i>ZXDA</i> | 57 704 902-57 987 522/ 57 673 005-58 051 706 | Male, right-sided CDH, left-sided inguinal hernia |
| TX-65 | Decrease | Maternal | 7q31.1 | <i>DOCK4</i> | 111 668 580-111 784 369/ 111 653 357-111 789 489 | Male, left-sided CDH, extra-lobar pulmonary sequestration |
| TX-72 | Increase | Paternal and maternal increased copy number | 3q13.31 | <i>GRAMD1C*, ZDHHC23</i> | 113 668 345-113 672 579/ 113 664 314-113 679 817 | Male, left-sided posterior CDH |
| TX-73 | Increase | Paternal | 20p12.2 | <i>SNAP25, MKKS, C20orf94, JAG1</i> | 10 223 539-10 902 949/ 10 213 446-10 937 659 | Male, left-sided CDH, ASD; sibling died shortly after birth with CDH |

*Genes located outside the minimal deleted region but are located, at least partially, inside the maximal deleted region. ASD, atrial septal defect; CDH, congenital diaphragmatic hernia; VSD, ventricular septal defect.

diaphragmatic defects—CDH or diaphragmatic eventrations—with incomplete penetrance.

The identification of the causative genomic changes in patients 1 and 2, both of whom had only minor anomalies—radioulnar

synostosis diagnosed at 2 ½ years of age and a single umbilical artery, respectively—provide further evidence that such studies should be considered in individuals who appear to have isolated diaphragmatic defects.

Table 3 *ZFPM2* sequence changes in patients with congenital diaphragmatic hernia

| Observed change | Number of patients (inheritance pattern) | Exon | 1000 genomes*/dbSNP (heterozygosity) | Predicted effect (PolyPhen/SNPs3D) |
|------------------------|--|------|---|------------------------------------|
| Non-synonymous changes | | | | |
| D98N | 1 (Non-maternal, father not available) | 3 | No/no | Possibly damaging/non-deleterious |
| S210T | 1 (Paternal) | 6 | No/no | Benign/non-deleterious |
| A403G | 11 | 8 | Yes/yes (0.341) | Benign/non-deleterious |
| E782D | 10 | 8 | Yes/yes (0.078) | Benign/non-deleterious |
| Q889E | 1 (Non-maternal father not available) | 8 | No/no—3.3% Hispanic control chromosomes | Benign/non-deleterious |
| A1055V | 1 (Non-maternal, father not available) | 8 | Yes/yes (0.146) | Benign/non-deleterious |
| Synonymous changes | | | | |
| P454P | 4 | 8 | Yes/yes (0.234) | — |
| P592P | 3 | 8 | Yes/yes (0.101) | — |
| V795V | 2 | 8 | Yes/yes (0.120) | — |
| Y992Y | 3 | 8 | Yes/yes (0.104) | — |
| H1069H | 4 | 8 | Yes/yes (0.234) | — |
| L1123L | 3 | 8 | Yes/yes (0.156) | — |

*Data accessed on 8 March 2011.

Table 4 *ZFPM2* sequence changes in patients with diaphragmatic eventrations

| Observed change | Number of patients | Exon | 1000 genomes*/dbSNP (heterozygosity) | Predicted effect (PolyPhen) |
|------------------------|--------------------|------|--------------------------------------|-----------------------------|
| Non-synonymous changes | | | | |
| A403G | 1 | 8 | Yes/yes (0.341) | Benign/non-deleterious |
| E782D | 2 | 8 | Yes/yes (0.078) | Benign/non-deleterious |
| Synonymous changes | | | | |
| P454P | 1 | 8 | Yes/yes (0.234) | — |
| P592P | 1 | 8 | Yes/yes (0.101) | — |
| V795V | 2† | 8 | Yes/yes (0.120) | — |
| H1069H | 1 | 8 | Yes/yes (0.234) | — |

*Data accessed on 8 March 2011.

†Homozygous in one of the patients.

Alterations in *ZFPM2* underlie a small fraction of congenital diaphragmatic defects

Screening of the *ZFPM2* gene in 52 patients with CDH and 10 patients with diaphragmatic eventrations failed to identify any clearly deleterious changes in the *ZFPM2* gene. Similarly, Ackerman *et al* did not find deleterious *ZFPM2* sequence changes in 29 additional postmortem samples from children with diaphragmatic defects.¹⁴ However, Bleyl *et al* screened 96 CDH patients—53 with isolated CDH, 36 with CDH and additional anomalies, and seven with CDH and known chromosomal anomalies—and identified a rare amino acid change, M703L, in a patient with isolated CDH that is predicted to be possibly damaging by PolyPhen and deleterious by SNPs3D (supplemental table 3).²⁴ These data suggest that severe, deleterious alterations in *ZFPM2* account for only a small portion of diaphragmatic defects. It is unclear, however, whether individual changes, like M703L, increase the risk for CDH and, if so, to what degree.

Delineation of new minimal deleted region for CDH on 1q41q42

Chromosome 1q41q42 has been shown to be recurrently deleted in individuals with CDH. The 1q41q42 minimal deleted region for CDH was previously defined by the maximal region of overlap between a patient reported by Kantarci *et al* and a patient described by Van Hove *et al* who were subsequently referred to as patient 7 and patient 4, respectively, by Shaffer *et al*.^{6 15 27} This interval spanned ~6.3 Mb (220 120 384–226 397 002 hg19) and contained 48 RefSeq genes.

The maximal proximal and distal breakpoints of our patient 4's deletion are located inside the CDH region defined by Shaffer *et al*.¹⁵ These breakpoints can be used to define a new minimal deleted region for CDH on chromosome 1q41q42 which spans ~2.2 Mb (223 073 839–225 318 623 hg19) and contains only 15 RefSeq genes (figure 2).

This refined minimal deleted region for CDH does not contain the H2.0-like homeobox (*HLX*) gene which has been considered a candidate gene for CDH based on its expression in the murine diaphragm and the diaphragmatic defects identified in *Hlx*^{-/-} mice.^{28–30} Although it is possible that haploinsufficiency of *HLX* can contribute to the formation of diaphragmatic defects in humans, its exclusion from the minimal deleted region for CDH suggests that alterations in *HLX* function are not required for the development of CDH in association with 1q41q42 deletions.

The dispatched homologue 1 (*DISP1*) gene has also been considered a candidate gene for CDH and is located within the new minimal deleted interval for CDH. *DISP1* is a membrane spanning protein that plays an essential role in SHH signalling.³¹ The potential role of *DISP1* in the formation of CDH is supported by evidence that murine *Disp1* is

expressed in the pleuroperitoneal fold (PPF), a structure that is often considered to be the primordial mouse diaphragm and the identification of a somatic Ala1471Gly mutation—predicted to be benign by PolyPhen but 'not tolerated' by SIFT (<http://blocks.fhcrc.org/sift/SIFT.html>)—in a patient with CDH and other anomalies.³² However, abnormalities in other SHH signalling proteins have yet to be clearly shown to cause CDH. Truncating mutations (W475X and Y734X) in *DISP1* have been found in two individuals with microforms of holoprosencephaly and their unaffected mothers—consistent with *DISP1*'s role in SHH signalling—but not in individuals with diaphragmatic defects.^{32 33} This suggests that consideration should be given to the possible role of other 1q41q42 genes in the development of CDH. The tumour protein p53 binding protein, 2 (*TP53BP2*) gene could play a role in CDH development by modulating apoptosis and cell proliferation through interactions with other regulatory molecules and has been shown to play a critical role in heart and CNS development in mice.^{34 35} The WD repeat domain 26 (*WDR26*) gene is another candidate gene that is widely expressed and could play a role in the development of CDH by regulating cell signalling pathways and modulating cell proliferation.^{36 37}

CDH is recurrently seen in 16p11.2 microdeletion syndrome

Recurrent 16p11.2 microdeletions result in a constellation of defects including developmental delay, cognitive impairment, behavioural problems, seizures, and congenital anomalies.¹⁷ Both patients 5 and 6 have de novo deletions of this region, suggesting that CDH should be added to the list of congenital anomalies associated with this microdeletion syndrome.

The T-box 6 gene (*TBX6*) is located on 16p11.2 and encodes a transcription factor that has been shown to play a critical role in important developmental processes including paraxial mesoderm differentiation and left–right patterning.^{38 39} As a result, *TBX6* has been hypothesised to contribute to many of the congenital anomalies associated with 16p11.2.¹⁷ It is possible that haploinsufficiency of *TBX6* also contributes to the development of CDH.

Evidence of CDH related genes on distal chromosome 11q and proximal chromosome 13q

Patient 7 carried an extra derivative chromosome 13 making her trisomic for a portion of chromosome 13 extending to 13q12.3 and a portion of chromosome 11 extending from 11q23 to the telomere—47,XX,+der(13)t(11;13)(q23.2;q12.3). A similar chromosomal complement 47,XY,+der(13)t(11;13)(q21;q14) was seen in a boy with CDH described by Park *et al*, suggesting that upregulation of genes on distal 11q and/or proximal 13q may play a role in the development of CDH.⁴⁰ In support of this hypothesis, duplications of 11q have been recurrently seen in patients with CDH with many cases being associated with an unbalanced 3:1 meiotic segregation of the common t(11;22) translocation—resulting in a 47,XX or XY,+der(22)t(11;22)(q23.3;q11.2) chromosomal complement.^{13 41} Multiple cases of CDH associated with trisomy 13 have also been described providing support for the existence of one or more CDH related genes on chromosome 13.¹³

Role of *FZD2* and Wnt signalling in the development of CDH and defects associated with pentalogy of Cantrell

The cardiac, diaphragmatic and anterior wall defects seen in patient 8 are similar to those described in pentalogy of Cantrell—a constellation of defects described by Cantrell *et al* that includes a supraumbilical omphalocele, a lower sternal cleft, a defect in the central tendon of the diaphragm, a defect in the pericardium, and an intracardiac anomaly.¹⁶ A de novo deletion

of *FZD2* was identified in patient 8, but similar deletions have been reported in normal individuals from Ibadan, Nigeria.²⁰ Although this suggests that haploinsufficiency of *FZD2* is unlikely to be the sole cause of this patient's phenotype, several lines of evidence suggest that decreased *FZD2* expression may have contributed to this child's phenotype.

Frizzled genes encode a family of seven transmembrane domain proteins that act as receptors for Wnt signalling proteins.⁴² *FZD2* is expressed in a variety of organs during development (supplemental table 1) and its protein product has been shown to interact with *WNT3A* and *WNT5A* and to signal through both the canonical (Wnt/ β -catenin) and non-canonical (Wnt/Ca⁺⁺ and cyclic GMP) Wnt pathways.⁴³ In chick embryos, *Wnt3a* and *Wnt5a* modulate the migration of mesodermal precursors and *Wnt3a* has been shown to control the movement patterns of cardiac progenitor cells.⁴⁴ ⁴⁵ Wnt signalling has also been shown to play a role in the development of CDH and omphalocele in humans. X linked dominant mutations in *PORCN*, a gene that encodes a protein that modifies Wnt proteins—including *WNT3A*—for membrane targeting and secretion, have been shown to cause CDH and omphalocele as part of focal dermal hypoplasia (Goltz syndrome; OMIM #305600).⁴⁶ ⁴⁷

It is possible that deletion of *FZD2*, in combination with other genetic and/or environmental factors, caused dysregulation of these Wnt signalling pathways, leading to the development of the cardiac defects, CDH and omphalocele seen in patient 8. The potential role of frizzled signalling in developmental processes that require directional tissue movements followed by tissue fusion is supported by the work of Yu *et al* who recently showed that mouse frizzled 1 (*Fzd1*) and frizzled 2 (*Fzd2*) genes play an essential and partially redundant role in the correct positioning of the cardiac outflow tract and closure of the palate and ventricular septum.⁴⁸ Genetic changes that disrupt these processes may ultimately be found to contribute to other cases of partial or complete pentalogy of Cantrell.

Genomic changes of unknown significance

The majority of the rare genomic changes catalogued in table 2 are not located within genomic regions that have been shown to be recurrently deleted or duplicated in individuals with CDH and do not affect genes/pathways that have been clearly implicated in the development of diaphragmatic defects. Although the significance of these changes is presently unknown, it is likely that future studies, in humans and animal models, will help to clarify whether these changes increase the risk of developing diaphragm defects.

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