Pre- and Postnatal Analysis of Chromosome 15q26.1 and 8p23.1 Deletions in Congenital Diaphragmatic Hernia

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Key Words

Congenital diaphragmatic hernia · Deletion 8p23.1 · Deletion 15q26.1 · FISH

Abstract

Congenital diaphragmatic hernia (CDH) is defined as a protrusion of abdominal content into the thoracic cavity through an abnormal opening in the diaphragm present at birth. It is a common birth defect with high mortality and morbidity. Sub-microscopic deletions of 15q26.1 and 8p23.1 have been reported in several cases of CDH. We studied a total of 17 cases with CDH in pre- and postnatal samples using FISH probes. Deletion 15q26.1 was seen in 1/17 prenatal samples. There was no deletion for 8p23.1 in all the samples analyzed. CDH has a genetic etiology, and deletion 15q26.1 increases the risk of CDH. Deletion 15q26.1 in a fetus with CDH is a predictor of poor prognosis. This deletion is also seen in a phenotype similar to Fryns syndrome. CDH identified pre- or postnatally should be investigated further to exclude a 15q26.1 deletion and enable appropriate parental counseling.

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Congenital diaphragmatic hernia (CDH) is a malformation of the developing diaphragm in which the abdominal organs are pushed into the thorax. CDH is characterized by a variable defect in the diaphragm, pulmo-

nary hypoplasia, and postnatal pulmonary hypertension. A diaphragmatic hernia is an emergency that requires surgery. Prevalence of CDH in newborns is ~1 in 3,000 [Holder et al., 2007]. The overall mortality rate for CDH remains high, despite advances in neonatal care. The development of the human diaphragm occurs between the 4th and 12th week of gestation. CDH can be detected prenatally by an ultrasound examination performed during the second trimester in most affected infants. CDH can occur as an isolated defect, in combination with multiple congenital anomalies or as a part of a defined syndrome. Isolated CDH or nonsyndromic CDH is the only birth defect in 60% of the cases. The additional features such as pulmonary hypoplasia, cardiac dextroposition and left heart hypoplasia are usually considered as a part of CDH. The mortality rate of isolated CDH is 10–20% lower than that of syndromic CDH [Pober, 2008]. Nonisolated or syndromic CDH accounts for 40% of CDH. The single gene disorders associated with CDH are Fryns syndrome, Donnai-Barrow syndrome and others. Chromosomal abnormalities have been implicated in ~15% of the cases. The most common chromosomal abnormalities are trisomy 18, isochromosome12p (Pallister-Killian syndrome), trisomy 13, trisomy 21, and 45,X. Chromosome structural abnormalities have also been described in association with CDH [Holder et al., 2007; Pober, 2008]. The deletions of 15q26.1 and 8p23.1 have been reported frequently in patients with CDH.

A del(15)(q26.1q26.2) (OMIM 142340, DIH1) has been reported in more than 2 dozen cases with the compelling evidence of the increased risk for abnormal diaphragm formation. Loss of this interval can result from one of several mechanisms such as de novo deletions, unbalanced translocations or formation of a ring chromosome 15. All patients had intellectual disability, growth retardation and/or additional birth defects such as craniofacial anomalies, cardiovascular malformations, hypoplastic genitalia or cryptorchidism, or an overlapping facial appearance with Fryns syndrome [Klaassens et al., 2007; Pober, 2008]. The *COUP-TFII* gene is located in chromosome 15q26 in a region recurrently deleted in individuals with CDH [Klaassens et al., 2005]. *COUP-TFII*, also known as NR2F2, is a transcription factor in the steroid/thyroid hormone receptor superfamily. It is expressed in the embryonic pleuroperitoneal folds in the early stage of development and in the diaphragm in the late days of gestation [Dingemann et al., 2012].

A del(8)(p23.1) (OMIM 222400, DIH2) is another CDH 'hot spot' revealed by cytogenetic and molecular cytogenetic studies [Shimokawa et al., 2005]. This region contains the candidate gene *GATA4*, known to be important for heart, lung and diaphragm development [Pober, 2008]. *GATA4* is located in chromosome 8p23.1, a region recurrently deleted in individuals with CDH. *GATA4* is important for lung and diaphragm development in humans. *GATA4* encodes a transcription factor that interacts with *FOG2* during the morphogenesis of the heart [Holder et al., 2007].

There are a number of evidences that show there are certain genes and pathways which play a role in the development of CDH. The retinoid acid signaling pathway and genes such as *COUP-TFII*, *FOG2*, *GATA4*, *WTI*, and *SLIT3* are thought to be involved in the development of CDH. Preliminary evidence came from a small study in which the levels of plasma retinol and retinol-binding protein in the cord blood of infants with CDH was found to be 50% lower than those in age-matched controls [Holder et al., 2007]. Vitamin A (retinol) and its derivatives such as retinoid are essential for the embryonic development of the diaphragm. *COUP-TFII* expression is upregulated by retinoid acid. Diaphragmatic hernia 1(DIH1) has been mapped to chromosome 15q26.1 (*COUP-TFII*), Diaphragmatic hernia 2(DIH2) to chromosome 8p23.1 (*GATA4*) and Diaphragmatic hernia 3 (DIH3) to chromosome 8q23 (*ZFPM2*). Submicroscopic deletions of 15q26.1 and 8p23.1 have been reported in several cases of CDH. We therefore studied a total of 17 cases with CDH in pre- and postnatal samples to comple-

Table 1. Type of samples

	Samples (n = 17)	Abnormal (n = 1)
Amniotic fluid	5	1
Cord blood	6	0
Chorionic villi	1	0
Peripheral blood	5	0

ment evidence and extend existing knowledge of the *COUP-II* and *GATA4* genes with respect to previous research.

Materials and Methods

Sample Details

We studied a total of 17 samples with CDH (12 prenatal and 5 postnatal samples; tables 1–3). Peripheral blood was used for analysis in postnatal samples. Chorionic villi, amniotic fluid and cord blood were used in prenatal samples. Fetuses and children with CDH were included in the study. Fetuses with normal ultrasound were excluded from the study. Fetuses and children with common autosomal trisomy were also excluded.

Karyotyping

All pre- and postnatal samples were cultured and harvested. The chromosome slides were prepared and banded using standard protocols [Hungerford, 1965; Seabright, 1971; Priest and Rao, 1997]. Twenty well-spread and well-banded metaphases were captured using Olympus BX51 (Japan), analyzed and karyotyped using Applied Spectral Imaging BandView ver. 5.0 software, Israel. The chromosome abnormalities were identified and designated as per ISCN (2013) nomenclature.

Fluorescence in situ Hybridization

FISH analysis was used to precisely define the 15q26.1 and 8p23.1 deletion break points. Cytocell UK designed the custom-made probe localized to 15q26.1 for the *NR2F2* gene with BAC clones RP11-327J17, CTD-2027G2 and RP11-143C19. The *SNRPN* gene was used as a control. The BAC clone localized to 8p23.1 was selected from the human DNA library in the UCSC genome browser and provided by BACPAC Resources (<http://bacpac.chori.org/home.htm>). The BAC clone RP11-235I5 (*GATA4*) ~180–200 kb was used. 8q11.1 was used as a control. DNA was isolated from cultured BACs, amplified and then labeled with digoxigenin 11-dUTP by nick translation according to standard protocols. FISH probes were hybridized onto the slides and detected using anti-DIG antibody tagged with rhodamine [Sambrook and Russell, 2001]. The probe and prepared slides were denatured, hybridized, washed, and counter stained. Slides were observed under an Olympus BX60, a fluorescence microscope using multi-bandpass filter [Pinkel et al., 1988; Ried et al., 1992]. Analysis of the slide was done using the Applied Spectral Imaging ver. 5.0. A minimum of 10–15 metaphases were analyzed, and a minimum of 3 metaphase pictures were captured.

Table 2. Prenatal sample details

Sample no.	Age		Sex	Consanguinity	Indications	Karyotype	FISH 15q	FISH 8p
	mother, years	gestational, weeks						
CDH1	28	20	M	no	CDH	normal	normal	normal
CDH2	28	18	M	no	CDH, IUGR	mos 46,idic(15)[12]/46,r(15)[6]/46[2]	abnormal	–
CDH3	21	20	M	no	CDH	normal	normal	normal
CDH4	25	21	F	no	CDH, pseudodextrocardia	normal	normal	normal
CDH5	22	23	F	yes	CDH, DWM	normal	normal	normal
CDH6	24	22	F	no	CDH, right-sided heterotaxy	normal	normal	normal
CDH7	26	22	M	no	CDH	normal	normal	normal
CDH8	22	24	M	yes	CDH, AVSD, DWM, ambiguous genitalia, micrognathia, CTEV	normal	normal	normal
CDH9	24	33	F	yes	CDH, ventriculomegaly, micrognathia	normal	normal	normal
CDH10	28	23	M	no	CDH	normal	normal	normal
CDH11	26	23	M	no	CDH	normal	normal	normal
CDH12	23	14	M	no	CDH	normal	normal	normal

AVSD = Atrioventricular septal defect; CTEV = congenital talipes equinovarus; DWM = Dandy-Walker malformation; IUGR = intrauterine growth retardation.

Table 3. Postnatal samples with normal karyotypes and normal FISH results

Sample No.	Age		Sex	Indications
	patient	mother		
CDH13	1 day	29 years	M	CDH
CDH14	2 years, 1 month	28 years	F	CDH
CDH15	1 day	26 years	F	CDH
CDH16	20 months	35 years	M	CDH, ASD, developmental delay
CDH17	2 years, 1 month	26 years	M	CDH

ASD = Atrial septal defect. No consanguinity existent.

Results

One of the prenatal samples showed a mosaic pattern, dicentric chromosome 15 in 12 metaphases, ring chromosome 15 in 6 metaphases, and normal chromosomes in 2 metaphases analyzed (fig. 1). FISH study for 15q26.1 also showed a mosaic pattern, deletion in 80% and normal chromosomes in 20% of the metaphases (fig. 2). The

rest of the samples did not show the deletion. A deletion 15q26.1 was seen in one (8.33%) of the prenatal samples and overall 1/17 (5.88%). No deletion was seen for 8p23.1 (fig. 3) in all 17 samples analyzed. In this study, 70% of the samples were prenatal and the remaining 30% were postnatal. In the prenatal cases, cord blood samples formed the majority with 50%, followed by 42% of amniotic fluid and 8% of chorionic villi samples. Isolated CDH was seen in 10/17 (59%) patients, while 7/17 (41%) had additional malformations. Of these patients, a heart defect was seen in 4/7(57%). All CDH were observed to be left-sided hernias. Consanguinity was seen in ~17.65% of CDH patients. None of the mothers with advanced maternal age had a baby with CDH. In fact, the majority (94%) of mothers were in the age group of 21–30 years.

In the 15q26.1 deleted case, amniocentesis was done at 18 weeks of gestation for CDH, intrauterine growth restriction and triple test positive for Patau syndrome. The mother was primigravida in a nonconsanguineous marriage. The karyotype of the parents was normal. In her next pregnancy, nuchal translucency, anomaly and growth scan were normal, and no invasive procedure was performed. She delivered a normal healthy baby girl. All prenatal cases were terminated and all postnatal cases were operated and were doing fine. To the best of our

Fig. 1. a Karyotype showing the dicentric chromosome 15. **b** Comparison of chromosome 15 showing ring and dicentric chromosomes.

Fig. 2. FISH study showing a mosaic pattern of deletion 15q26.1. **a** Normal signal. **b** Deletion 15q26.1 (Spectrum Orange: RP11-327J17, CTD-2027G2 and RP11-143C19, *COUP-II*; Spectrum Green: 15q11, *SNRPN* control).

Fig. 3. FISH study showing a normal signal pattern for 8p23.1 (Spectrum Orange RP11-235I5, *GATA4* and Spectrum Green for 8q11.1, control).

knowledge, this is the first prenatal or postnatal submicroscopic deletion study for CDH from India and the first case of a prenatally diagnosed deletion 15q26.1 from India.

Discussion

In this study, we analyzed 17 patients with CDH and identified one case with a 15q26.1 deletion. Klaassens et al. [2005] studied 200 patients with CDH and identified

3 patients with a 15q deletion. They defined ~5 Mb at chromosome 15q26.1q26.2, a minimally deleted region for CDH by use of FISH and array CGH data. One of their patients had a ring chromosome 15. CDH has not yet been described as a main feature in ring chromosome 15 syndrome. The key features of ring chromosome 15 include growth retardation, microcephaly, variable mental retardation, and delayed bone age [Butler et al., 1988]. In the ring chromosome 15 in this present study, the fetus had CDH and intrauterine growth retardation. Britto et al. [2014] and Hatem et al. [2007] each reported

a case of prenatal diagnosis of CDH and intrauterine growth retardation in a fetus with ring chromosome 15 similar to our study. Klaassens et al. [2007] reported 2 patients with a deletion of 15q26 and the phenotype consists of intrauterine growth retardation, left-sided CDH, cardiac anomalies, and characteristic facial features, similar to those seen in Fryns syndrome. They proposed that when this combination of birth defects is identified, either pre- or postnatally, further investigations to confirm or exclude a deletion of 15q26 are indicated. Slavotinek et al. [2005] identified submicroscopic chromosome deletions in 3 probands by array CGH who had previously been diagnosed with Fryns syndrome and had normal karyotypes. Two female infants were found to have microdeletions involving 15q26.2, and one male infant had a deletion in band 8p23.1. Several recent reports describe nonisolated CDH cases with apparently normal karyotypes but abnormalities were detected by array CGH. Array CGH should be considered in prenatally diagnosed cases of CDH [Holder et al., 2007]. Brady et al. [2013] studied copy number variations using microarray in 75 fetuses with CDH and found haploinsufficiency of *NR2F2*.

Wat et al. [2009] described a monozygotic twin with interstitial deletion of 8p23.1 by array CGH in the amniotic fluid. One twin had CDH and a heart defect, while the other twin only had a heart defect. They suggested that haploinsufficiency of other genes, in addition to *GATA4*, may play a role in severe heart defects and CDH associated with 8p23.1 deletions. Deletions involving 8p23.1 have been described in 9 patients with CDH [Pober, 2008]. In our study, we did not detect an 8p23.1 deletion, although we had 4 patients with CDH and heart defects. Yu et al. [2014] used whole exome sequencing in 2 families with CDH and congenital heart disease and identified mutations in *GATA6*. The use of new technologies will enhance our understanding of the heterogeneous molecular etiology of CDH [Longoni et al., 2014].

In their prospective study, Harrison et al. [1994] observed 58% mortality among CDH diagnosed before 24 weeks' gestation despite optimal postnatal care. Prenatally detected cases had a reduced survival to surgery rate compared to postnatally detected rates [Bhat et al., 2008]. Colvin et al. [2005] reported that prenatal diagnosis itself was an important predictor of mortality rates for live-born infants. Nearly half of the prenatally diagnosed fetuses underwent elective termination of pregnancy. This large number of fetal terminations confounds the accurate assessment of the true outcomes of this condition. In

our study, all prenatally diagnosed cases had also opted for termination of pregnancy. It is worthwhile to search for a 15q26.1 deletion in fetuses with CDH as this has a high mortality rate. This will enable appropriate parental counseling and, hence, decision making [Schlembach et al., 2001; Hengstschläger et al., 2004; Klaassens et al., 2007; Pober, 2008].

The challenge in the present study was to detect samples that met our criteria. In the antenatal period, it was difficult to recognize samples after ruling out common autosomal trisomy. As CDH was detected before birth, all patients opted for termination of pregnancy. Thus, acquiring postnatal samples was also difficult. The description of multiple genetic syndromes associated with CDH highlights the importance of a careful evaluation of patients with CDH [Holder et al., 2007; Pober, 2008]. Such an evaluation may have an influence on the couple's decision, including the possibility of terminating pregnancy. Understanding the genetic factors associated with CDH may make it possible to devise preventive strategies or to improve therapeutic interventions [Pober, 2008]. Every child with CDH and additional malformations or whenever a CDH is detected by prenatal ultrasound should be karyotyped and examined to exclude a 15q26.1 deletion. In case of a normal karyotype and no deletion of 15q26.1, CDH can be corrected by percutaneous fetoscopic endoluminal tracheal occlusion prenatally, or delivery should be planned at a tertiary care center where CDH can be managed postnatally.

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Statement of Ethics

The study was approved by the Institutional Ethics Committee. Informed consent was obtained from each family.

Disclosure Statement

The authors have no conflicts of interest to declare.

References

- Bhat YR, Kumar V, Rao A: Congenital diaphragmatic hernia in a developing country. *Singapore Med J* 49:715–718 (2008).
- Brady PD, DeKoninck P, Fryns JP, Devriendt K, Deprest JA, Vermeesch JR: Identification of dosage-sensitive genes in fetuses referred with severe isolated congenital diaphragmatic hernia. *Prenat Diagn* 33:1283–1292 (2013).
- Britto IS, Regina Silva Herbst S, Tedesco GD, Drummond CL, Bussamra LC, et al: Prenatal diagnosis of a fetus with ring chromosomal 15 by two- and three-dimensional ultrasonography. *Case Rep Obstet Gynecol* 2014:495702 (2014).
- Butler MG, Fogo AB, Fuchs DA, Collins FS, Dev VG, Phillips JA 3rd: Two patients with ring chromosome 15 syndrome. *Am J Med Genet* 29:149–154 (1988).
- Colvin J, Bower C, Dickinson JE, Sokol J: Outcomes of congenital diaphragmatic hernia: a population-based study in Western Australia. *Pediatrics* 116:e356–363 (2005).
- Dingemann J, Doi T, Rutenstock EM, Gosemann JH, Puri P: *COUP-TFII* gene expression is upregulated in embryonic pleuroperitoneal folds in the nitrofen-induced congenital diaphragmatic hernia rat model. *Eur J Pediatr Surg* 22:21–25 (2012).
- Harrison MR, Adzick NS, Estes JM, Howell LJ: A prospective study of the outcome for fetuses with diaphragmatic hernia. *JAMA* 271:382–384 (1994).
- Hatem E, Meriam BR, Walid D, Adenem M, Moez G, Ali S: Molecular characterization of a ring chromosome 15 in a fetus with intra uterine growth retardation and diaphragmatic hernia. *Prenat Diagn* 27:471–474 (2007).
- Hengstschläger M, Mittermayer C, Repa C, Dra-honsky R, Deutinger J, Bernaschek G: Association of deletions of the chromosomal region 15q24-ter and diaphragmatic hernia: a new case and discussion of the literature. *Fetal Diagn Ther* 19:510–512 (2004).
- Holder AM, Klaassens M, Tibboel D, de Klein A, Lee B, Scott DA: Genetic factors in congenital diaphragmatic hernia. *Am J Hum Genet* 80:825–845 (2007).
- Hungerford DA: Leukocytes cultured from small inocula of whole blood and the preparation of metaphase chromosomes by treatment with hypotonic KCl. *Stain Technol* 40:333–338 (1965).
- ISCN (2013): An International System for Human Cytogenetic Nomenclature, Shaffer LG, McGowan-Jordan J, Schmid M (eds) (Karger, Basel 2013).
- Klaassens M, Dooren M, Eussen HJ, Douben H, den Dekker AT, et al: Congenital diaphragmatic hernia and chromosome 15q26: determination of a candidate region by use of fluorescent in situ hybridization and array-based comparative genomic hybridization. *Am J Hum Genet* 76:877–882 (2005).
- Klaassens M, Galjaard RJ, Scott DA, Brüggewirth HT, van Opstal D, et al: Prenatal detection and outcome of congenital diaphragmatic hernia (CDH) associated with deletion of chromosome 15q26: two patients and review of the literature. *Am J Med Genet A* 143A:2204–2212 (2007).
- Longoni M, High FA, Russell MK, Kashani A, Tracy AA, et al: Molecular pathogenesis of congenital diaphragmatic hernia revealed by exome sequencing, developmental data, and bioinformatics. *Proc Natl Acad Sci USA* 111:12450–12455 (2014).
- Pinkel D, Landegent J, Collins C, Fuscoe J, Seg-raves R, et al: Fluorescence in situ hybridization with human chromosome-specific libraries: detection of trisomy 21 and translocations of chromosome 4. *Proc Natl Acad Sci USA* 85:9138–9142 (1988).
- Pober BR: Genetic aspects of human congenital diaphragmatic hernia. *Clin Genet* 74:1–15 (2008).
- Priest JH, Rao KW: Prenatal chromosome diagnosis, in Barch MJ, Knutsen T, Spurbeckeds JL (eds): *The AGT Cytogenetics Laboratory Manual*, ed 3, pp 199–258 (Lippincott-Raven Publisher, Philadelphia 1987).
- Ried T, Baldini AR, Ward DC: Simultaneous visualization of seven different DNA probes by in situ hybridization using combinatorial fluorescence and digital imaging microscopy. *Proc Natl Acad Sci USA* 89:1388–1392 (1992).
- Sambrook J, Russell DW: Working with bacterial artificial chromosomes; in *Molecular Cloning: A Laboratory Manual*, ed 3, pp 4.48–4.57 (CSHL Press, New York 2001).
- Schlembach D, Zenker M, Trautmann U, Ulmer R, Beinder E: Deletion 15q24–26 in prenatally detected diaphragmatic hernia: increasing evidence of a candidate region for diaphragmatic development. *Prenat Diagn* 21:289–292 (2001).
- Seabright M: A rapid banding technique for human chromosomes. *Lancet* 2:971–972 (1971).
- Shimokawa O, Miyake N, Yoshimura T, Sosonkina N, Harada N, et al: Molecular characterization of del(8)(p23.1p23.1) in a case of congenital diaphragmatic hernia. *Am J Med Genet A* 136:49–51 (2005).
- Slavotinek A, Lee SS, Davis R, Shrit A, Leppig KA, et al: Fryns syndrome phenotype caused by chromosome microdeletions at 15q26.2 and 8p23.1. *J Med Genet* 42:730–736 (2005).
- Wat MJ, Shchelochkov OA, Holder AM, Breman AM, Dagli A, et al: Chromosome 8p23.1 deletions as a cause of complex congenital heart defects and diaphragmatic hernia. *Am J Med Genet A* 149A:1661–1677 (2009).
- Yu L, Bennett JT, Wynn J, Carvill GL, Cheung YH, et al: Whole exome sequencing identifies de novo mutations in *GATA6* associated with congenital diaphragmatic hernia. *J Med Genet* 51:197–202 (2014).