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Report

Congenital Diaphragmatic Hernia and Chromosome 15q26: Determination of a Candidate Region by Use of Fluorescent In Situ Hybridization and Array-Based Comparative Genomic Hybridization

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Congenital diaphragmatic hernia (CDH) has an incidence of 1 in 3,000 births and a high mortality rate (33%–58%). Multifactorial inheritance, teratogenic agents, and genetic abnormalities have all been suggested as possible etiologic factors. To define candidate regions for CDH, we analyzed cytogenetic data collected on 200 CDH cases, of which 7% and 5% showed numerical and structural abnormalities, respectively. This study focused on the most frequent structural anomaly found: a deletion on chromosome 15q. We analyzed material from three of our patients and from four previously published patients with CDH and a 15q deletion. By using array-based comparative genomic hybridization and fluorescent in situ hybridization to determine the boundaries of the deletions and by including data from two individuals with terminal 15q deletions but without CDH, we were able to exclude a substantial portion of the telomeric region from the genetic etiology of this disorder. Moreover, one patient with CDH harbored a small interstitial deletion. Together, these findings allowed us to define a minimal deletion region of ~5 Mb at chromosome 15q26.1-26.2. The region contains four known genes, of which two—*NR2F2* and *CHD2*—are particularly intriguing gene candidates for CDH.

Congenital diaphragmatic hernia (CDH [MIM 142340]), a severe, life-threatening, congenital anomaly characterized by variable defect in the diaphragm, pulmonary hypoplasia, and postnatal pulmonary hypertension, is a relatively common anomaly (Torfs et al. 1992; Beresford and Shaw 2000). CDH can occur as an isolated defect, in combination with multiple congenital anomalies, or as part of a defined syndrome (Fryns et al. 1979; Enns et al. 1998). Little is known about the etiology of CDH. However, there is increasing evidence for a genetic cause of CDH. Various chromosomal anomalies have been described in CDH, with numerical abnormalities (such as trisomies 13, 18, and 21) as the most common type. Structural chromosomal anomalies, involving almost every chromosome, have been reported (Lurie 2003). Since 1988, clinical data from all Erasmus Medical Centre patients with CDH have been stored in a database. Cytogenetic data from 200 patients with CDH were available, and 24 patients (12%) showed an abnormality. Fourteen patients (7%) showed a numerical abnormality (trisomy 18 or 21). The remaining 10 patients (5%) had a structural anomaly, and 3 of those patients (1.5%) were shown to have a deletion of part of chromosome 15q. The present study focuses on this subset of patients with CDH and chromosome 15q deletions. Data from four previously published patients with CDH and small chromosome 15q deletions were generously made available to us (table 1) (de Jong et al. 1989; Rosenberg et al. 1992; Chen et al. 1998; Schlembach et al. 2001). Clinical evaluation of the seven patients revealed a left-sided diaphragmatic hernia of the Bochdalek type, in-

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Table 1

Summary of Clinical and Cytogenetic Data for Patients with Deletions Involving Chromosome 15

Patient	Karyotype	CDH[a]	Other Abnormalities[b]	Deletion Size (Mb)
1	46,XY,t(1;14),inv(6),del(15)(q26)	Yes	Genital anomalies; IUGR	6.3
2	46,XY,r(15)(p11q26)	Yes	Dysmorphic features; cardiac, renal, genital, and limb abnormalities; IUGR	19.9
3	46,XY,r(15)(p11q26)	Yes	Dysmorphic features; cardiac abnormalities; IUGR	23.3
4	46,XX,der(15)t(3;15)(q29;q26.1)	Yes	Dysmorphic features; cardiac and limb abnormalities; two-vessel umbilical cord; IUGR	22.8
5	46,XY,r(15)(p11q26.1)	Yes	Dysmorphic features; genital and limb abnormalities; IUGR	23
6	46,XX,der(15)t(8;15)(q24.1;q26.1)	Yes	Hydrocephaly; dysmorphic features; cardiac, renal, limb, and spine abnormalities; IUGR	16.8
7	46,XX,del(15)(q25q26.3)	Yes	Dysmorphic features; renal and limb abnormalities; IUGR	22.3
8	46,XX,r(15)(p11.1q26.3)	No	Mental retardation; mild dysmorphic features; IUGR	16.3
9	46,XY,del(15)(q26)	No	Mental retardation	15.6

NOTE.—Sources for patients 1–3 and 8, Erasmus Medical Centre, Rotterdam, The Netherlands; patient 4, Rosenberg et al. 1992 (case 2); patient 5, de Jong et al. 1989; patient 6, Chen et al. 1998; patient 7, Schlembach et al. 2001; patient 9, J. Wauters, University Hospital Antwerpen, Antwerpen, Belgium.

[a] Left-sided, Bochdalek-type CDH (if present).

[b] IUGR = intrauterine growth retardation (birth weight <3rd percentile).

trauterine growth retardation (all patients had birth weights <3rd percentile), and multiple other congenital anomalies, such as cardiac and renal abnormalities. In addition to the seven patients with CDH, we analyzed data from two patients with mental retardation and 15q deletions but without CDH manifestation. Genomic DNA was extracted from cultured cells (from patients 1, 2, 3, 4, and 9) or paraffin-embedded tissue (from patients 5 and 6).

To delineate the possible candidate region, array-based comparative genomic hybridization (array CGH) was performed using the 1-Mb Human BAC Array (Spectral Genomics) in accordance with the manufacturer's instructions. A dye-swap experimental strategy was used as an additional internal control. Fluorescent signals on the arrays were visualized using the ScanArray Express HT scanner. Images were analyzed with Spectral Ware 2 (Spectral Genomics). Results for patients 1, 3, 4, 7, and 8 are shown in figure 1.

To further delineate the deleted region and to deter-

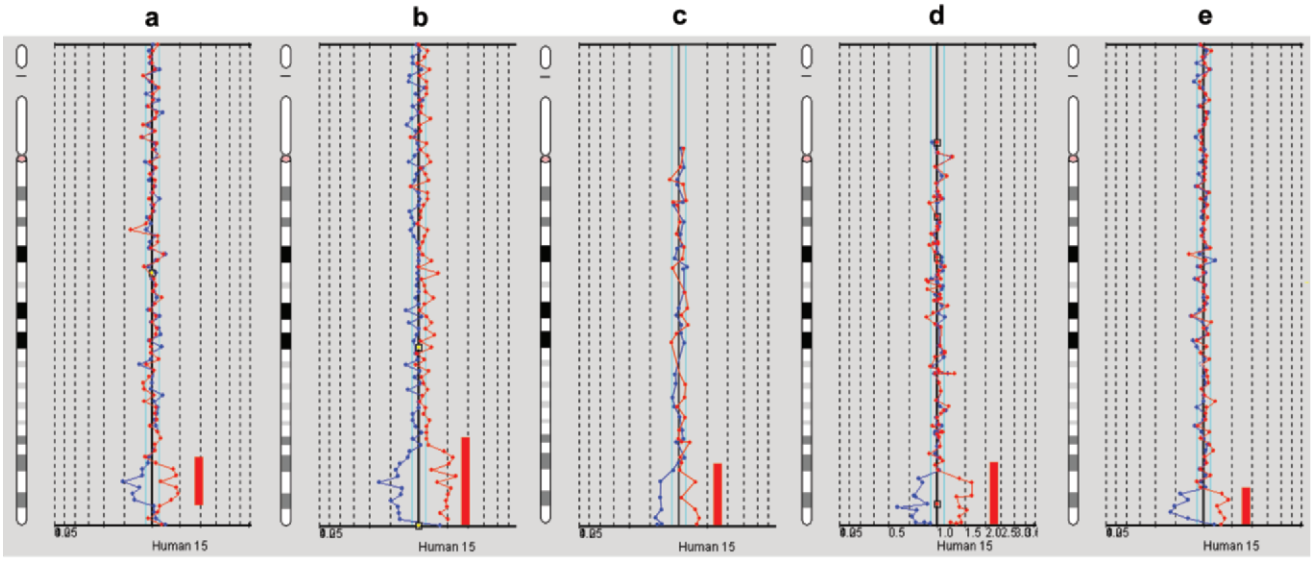

Figure 1 Array CGH results. *a*, Patient 1, with CDH and del(15) interstitial deletion. *b*, Patient 3, with CDH and r(15)(p11q26). *c*, Patient 4, with CDH and der(15)t(3;15)(q29;q26.1). *d*, Patient 7, with CDH and del(15)(q25q26.3). *e*, Patient 8, without CDH and with r(15)(p11.1q26.3).

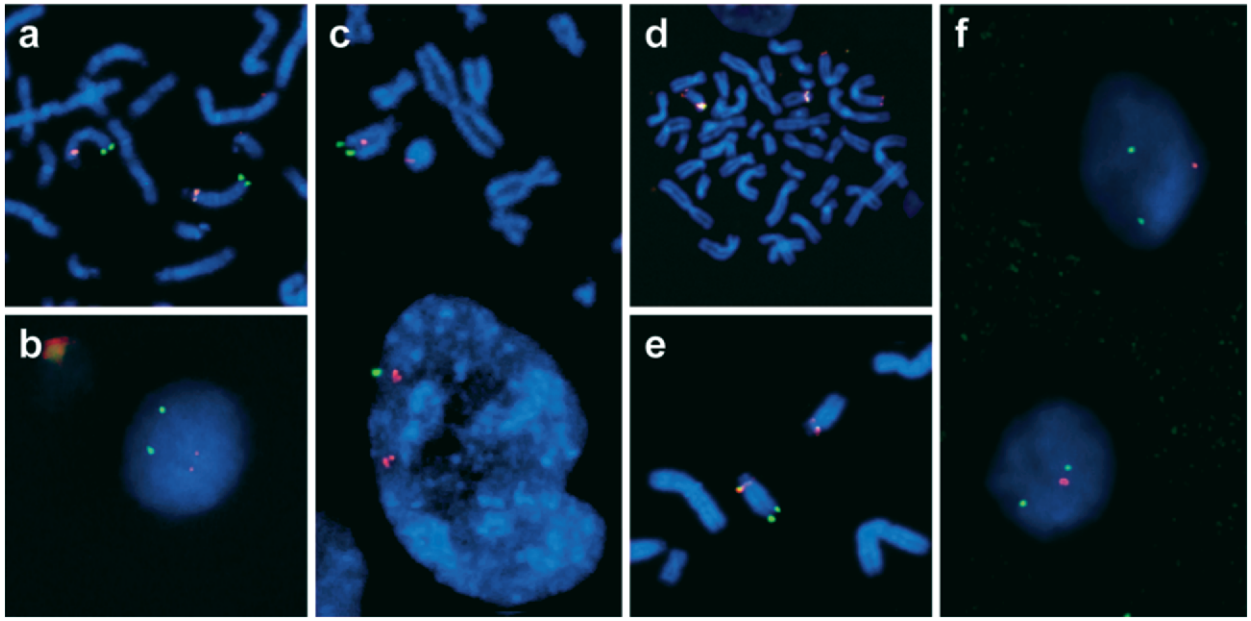

Figure 2 FISH results. *a,* Patient 1: partial metaphase, probe D15Z4 (*red signal*) at chromosome 15 centromeric locus and probe RP11-114I24 (*green signal*) at 15q26.3. *b,* Patient 2: interphase, probe RP11-369K8 (*red signal*) and RP11-253B9 (*green signal*) near the chromosome 5 centromeric region at 5p13.2. *c,* Patient 3: partial metaphase and interphase, deletion probe RP11-143C19 (*green signal*) and normal probe RP11-64K10 (*red signal*) at 15q23. *d,* Patient 4: metaphase spread, gain of chromosome 3q29; probe RP1-196F4 (*red signal*) (3qtel) present on der(15) and normal signal probe *D15Z4* (*yellow/red signal*) at the centromeric region of chromosome 15. The der(15) contains both signals. *e,* Patient 4: partial metaphase, deletion probe RP11-183E24 (*green signal*) at 15q26.2 and normal probe *D15Z4* (*yellow/red signal*). *f,* Patient 6: interphase, deletion probe RP11–57P19 (*red signal*) and normal probe *D15Z4* (*green signal*). Patients 1–6 all have CDH.

mine the deletions in patients 4, 5, and 9, ~110 BAC clones were selected from the University of California Santa Cruz (UCSC) and Ensembl genome browsers to cover the distal part of chromosome 15. Using the appropriate BAC clones, we performed FISH on metaphase chromosomes from patients 1, 3, and 4 (fig. 2*a* and 2*c*–2*e*). Interphase FISH was performed on nuclei extracted from paraffin-embedded tissues from patients 2, 5, and 6 (fig. 2*b* and 2*f*; data for patient 5 not shown). Only genomic DNA was available from patients 7 and 8, so the size of the deletion in these patients was determined using only array CGH. FISH slides were analyzed using the Axioplan 2 Imaging microscope (Zeiss), and images were collected using the Isis Software System (Metasystems). Combining FISH and array CGH data, we were able to approximately determine the breakpoints in all patients (fig. 3). In patient 1, the interstitial deletion found by array CGH was confirmed and was narrowed to a 6-Mb deletion between BAC clones RP11-79A7 and RP11-616M17. In patient 2, the deletion extended from a region distal to RP11-79A7; in patient 3, it extended from a region distal to RP11-300G22. The proximal breakpoint in patient 4 lies within BAC clone RP11-617F23. In patient 5, the break occurs distal to RP11-300G22. In patient 6, the most distal probe tested that was present on the deleted chromosome 15 was RP11-79A7. The terminal deletion in patient 7 occurs distal to RP11-360F18. The proximal breakpoints of the deletion interval in the two patients without CDH were similarly determined (fig. 3). In the first patient without CDH, who had a ring chromosome 15, the most distal BAC clone tested that was present on the ring chromosome was RP11-120N1. In the second patient without CDH, the most distal BAC clone present was RP11-262P8. Combining all data, we determined the smallest common deletion interval in patients with CDH to be at 15q26.1-26.2 (which we have termed the "CDH region"). This region is ~5 Mb in size and is bordered by BAC clones RP11-79A7 and RP11-80F4.

CDH is unlike many genetic disorders, for which candidate genes can be determined by using linkage analyses of familial cases, because the vast majority of CDH cases occur de novo. For this type of disorder, the best way to determine which genes are involved is by analyzing a large number of patients for common aberrations by use of different high-resolution genetic methodologies, such as FISH or array CGH. This strategy has already been used successfully to identify genes involved in CHARGE syndrome (MIM 214800) (Vissers et al. 2004) and Cornelia de Lange syndrome (CdLS [MIM 122470]) (Krantz et al. 2004; Tonkin et al. 2004).

Isolated reports of distal chromosome 15 deletions

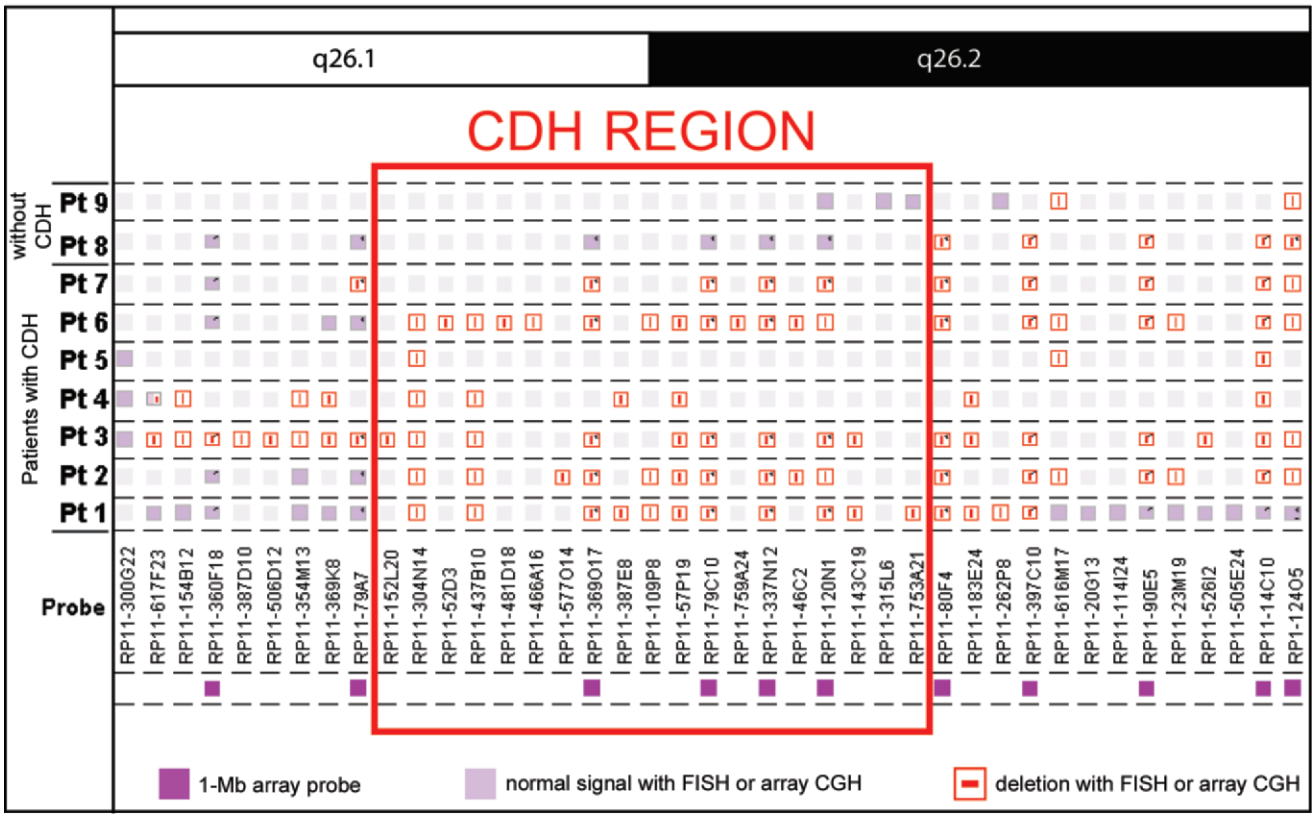

Figure 3 Schematic representation of the critical CDH region, with partial ideogram of chromosome 15q. Patients (Pt) 1–7 have CDH; patients 8 and 9 do not have CDH. BAC clones that were tested by array CGH and FISH are listed. The black dots inside boxes indicate that probes have been tested only on the array and not by FISH. The smallest common overlapping deletion interval involved in CDH is denoted by the large red square.

in patients with CDH have been described elsewhere (reviewed by Hengstschlager et al. [2004] and Schlembach et al. [2001]), suggesting involvement of this region in the pathogenesis of CDH. In the past year, three new cases of CDH have been reported (Biggio et al. 2004; Hengstschlager et al. 2004; Tonks et al. 2004). However, detailed molecular cytogenetic analyses of these cases are currently unavailable. To find other possible cases to study, we performed systematic FISH analysis on data from 25 patients with CDH, with the use of BAC clones spanning the CDH region. We identified no additional 15q deletions.

With array CGH and systematic FISH analysis performed on the seven patients with CDH and 15q deletions and the two patients without CDH but with 15q deletions, our study is the first to analyze data from multiple patients with CDH in detail and to refine the chromosome 15q critical CDH region to an ~5-Mb area within 15q26.1-26.2. In all patients, clinical abnormalities resembled the features described for other patients with a 15q deletion or with related syndromes, such as Fryns syndrome (MIM 229850) (Fryns et al. 1979). As can be expected, the r(15) patients, with or without CDH, had phenotypes similar to those seen with ring chromosome 15 syndrome (Butler et al. 1988; Rogan et al. 1996). To our knowledge, CDH has not yet been described as a main feature in ring chromosome 15 syndrome.

In our study, there appears to be a relationship between the number of abnormalities present and the size of the deletion. For example, the first patient, who had the smallest deletion, has few additional congenital defects. All other patients have deletions that are similar in size and have a similar spectrum of anatomical anomalies. In the two patients with an unbalanced translocation, the variation in phenotype could also be due, in part, to the presence of extra material from another chromosome.

The region of the smallest common deletion contains four known genes, none of which have been previously shown to be involved in diaphragm development or diaphragmatic hernia. On the basis of their structure and function, two of these genes are very interesting with respect to a potential relationship with CDH. The first gene, *NR2F2* (MIM 107773; also known as "*COUP-*

TFII"), is a member of the steroid/thyroid hormone receptor subfamily and is involved in retinoic acid metabolism (Kimura et al. 2002). A knockout mouse model of *NR2F2* showed that *Nr2f2*$^{-/-}$ mice are not viable and die at E9 in utero because of arrest of cardiac development (Pereira et al. 1999). Heterozygous knockout mice have poor viability in the neonatal period and are smaller than wild-type mice. However, the exact cause of death in these mice is not clear. The second interesting gene is the chromodomain helicase 2 gene (*CHD2* [MIM 602119]), a member of the SNF2/RAD54 helicase gene family. Recently, mutations in another member of this family (*CHD7*) have been found to cause CHARGE syndrome (Vissers et al. 2004). The third gene in the CDH region is the repulsive guidance molecule gene, *RGMA* (MIM 607362), which is involved in the guidance of growth cones in developing neurons (Brinks et al. 2004). This gene is not known to play a role in diaphragm development, nor has it been described in muscle or lung development. The fourth gene located in the smallest region of overlap is the sialyltransferase 8B gene (*SIAT8B* [MIM 602546]), which plays a role in the adhesive properties of neural cell adhesion molecules (Angata et al. 1997).

Haploinsufficiency due to the loss of a copy of one of these genes may be enough to result in a diaphragmatic defect. At the present time, the precise mechanism by which a deletion of or within one of these genes or a related gene mutation causes this developmental defect can only be speculated.

Elsewhere, other genes on chromosome 15q have been suggested as being involved in the pathogenesis of diaphragmatic defects. Biggio et al. (2004) suggested that the myocyte enhancer factor 2A gene, *MEF2A* (MIM 600660), could be involved in the pathogenesis of diaphragmatic defects. *MEF2A* maps to 15q26.3 and is involved in the differentiation of muscle cells from their precursors. Some genes involved in vitamin A metabolism—for example, *RALDH2* (MIM 603687), which maps to 15q21—have also been implicated in the pathogenesis of CDH (Greer et al. 2003). Both *MEF2A* and *RALDH2* are located outside our candidate critical CDH region, which limits their possible role in CDH in our subgroup of patients.

In conclusion, we have mapped a potential critical CDH region to 5 Mb at chromosome 15q26.1-26.2, a region that contains four genes, of which two are especially intriguing candidates in the etiology of diaphragmatic defects. Further research is needed to confirm their exact role in CDH and to determine the pathogenic mechanism. As a first step, we are performing FISH and mutation analyses of a large group of patients with CDH who have normal karyotypes. In the future, prenatal screening for 15q abnormalities when a diaphragmatic hernia is detected could give better clues for predicting the outcome and could provide more information for genetic counseling.

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Electronic-Database Information

The URLs for data presented herein are as follows:

Ensembl Genome Browser, http://www.ensembl.org/Homo_Sapiens/

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for CDH, CHARGE syndrome, CdLS, Fryns syndrome, *NR2F2, CHD2, RGMA, SIAT8B, MEF2A,* and *RALDH2*)

UCSC Genome Browser, http://genome.cse.ucsc.edu/

References

Angata K, Nakayama J, Fredette B, Chong K, Ranscht B, Fukuda M (1997) Human STX polysialyltransferase forms the embryonic form of the neural cell adhesion molecule: tissue-specific expression, neurite outgrowth, and chromosomal localization in comparison with another polysialyltransferase, PST. J Biol Chem 272:7182–7190

Beresford MW, Shaw NJ (2000) Outcome of congenital diaphragmatic hernia. Pediatr Pulmonol 30:249–256

Biggio JR Jr, Descartes MD, Carroll AJ, Holt RL (2004) Congenital diaphragmatic hernia: is 15q26.1-26.2 a candidate locus? Am J Med Genet A 126:183–185

Brinks H, Conrad S, Vogt J, Oldekamp J, Sierra A, Deitinghoff L, Bechmann I, Alvarez-Bolado G, Heimrich B, Monnier PP, Mueller BK, Skutella T (2004) The repulsive guidance molecule RGMa is involved in the formation of afferent connections in the dentate gyrus. J Neurosci 24:3862–3869

Butler MG, Fogo AB, Fuchs DA, Collins FS, Dev VG, Phillips JA III (1988) Two patients with ring chromosome 15 syndrome. Am J Med Genet 29:149–154

Chen CP, Lee CC, Pan CW, Kir TY, Chen BF (1998) Partial trisomy 8q and partial monosomy 15q associated with congenital hydrocephalus, diaphragmatic hernia, urinary tract anomalies, congenital heart defect and kyphoscoliosis. Prenat Diagn 18:1289–1293

de Jong G, Rossouw RA, Retief AE (1989) Ring chromosome 15 in a patient with features of Fryns' syndrome. J Med Genet 26:469–470

Enns GM, Cox VA, Goldstein RB, Gibbs DL, Harrison MR, Golabi M (1998) Congenital diaphragmatic defects and

associated syndromes, malformations, and chromosome anomalies: a retrospective study of 60 patients and literature review. Am J Med Genet 79:215–225

Fryns JP, Moerman F, Goddeeris P, Bossuyt C, Van den Berghe H (1979) A new lethal syndrome with cloudy corneae, diaphragmatic defects and distal limb deformities. Hum Genet 50:65–70

Greer JJ, Babiuk RP, Thebaud B (2003) Etiology of congenital diaphragmatic hernia: the retinoid hypothesis. Pediatr Res 53:726–730

Hengstschlager M, Mittermayer C, Repa C, Drahonsky R, Deutinger J, Bernaschek G (2004) 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

Kimura Y, Suzuki T, Kaneko C, Darnel AD, Moriya T, Suzuki S, Handa M, Ebina M, Nukiwa T, Sasano H (2002) Retinoid receptors in the developing human lung. Clin Sci (Lond) 103: 613–621

Krantz ID, McCallum J, DeScipio C, Kaur M, Gillis LA, Yaeger D, Jukofsky L, Wasserman N, Bottani A, Morris CA, Nowaczyk MJ, Toriello H, Bamshad MJ, Carey JC, Rappaport E, Kawauchi S, Lander AD, Calof AL, Li HH, Devoto M, Jackson LG (2004) Cornelia de Lange syndrome is caused by mutations in *NIPBL,* the human homolog of *Drosophila melanogaster Nipped-B*. Nat Genet 36:631–635

Lurie IW (2003) Where to look for the genes related to diaphragmatic hernia? Genet Couns 14:75–93

Pereira FA, Qiu Y, Zhou G, Tsai MJ, Tsai SY (1999) The orphan nuclear receptor COUP-TFII is required for angiogenesis and heart development. Genes Dev 13:1037–1049

Rogan PK, Seip JR, Driscoll DJ, Papenhausen PR, Johnson VP, Raskin S, Woodward AL, Butler MG (1996) Distinct 15q genotypes in Russell-Silver and ring 15 syndromes. Am J Med Genet 62:10–15

Rosenberg C, Blakemore KJ, Kearns WG, Giraldez RA, Escallon CS, Pearson PL, Stetten G (1992) Analysis of reciprocal translocations by chromosome painting: applications and limitations of the technique. Am J Hum Genet 50:700–705

Schlembach D, Zenker M, Trautmann U, Ulmer R, Beinder E (2001) Deletion 15q24-26 in prenatally detected diaphragmatic hernia: increasing evidence of a candidate region for diaphragmatic development. Prenat Diagn 21:289–292

Tonkin ET, Wang TJ, Lisgo S, Bamshad MJ, Strachan T (2004) *NIPBL,* encoding a homolog of fungal Scc2-type sister chromatid cohesion proteins and fly Nipped-B, is mutated in Cornelia de Lange syndrome. Nat Genet 36:636–641

Tonks A, Wyldes M, Somerset DA, Dent K, Abhyankar A, Bagchi I, Lander A, Roberts E, Kilby MD (2004) Congenital malformations of the diaphragm: findings of the West Midlands Congenital Anomaly Register 1995 to 2000. Prenat Diagn 24:596–604

Torfs CP, Curry CJ, Bateson TF, Honore LH (1992) A population-based study of congenital diaphragmatic hernia. Teratology 46:555–565

Vissers LE, Van Ravenswaaij CM, Admiraal R, Hurst JA, De Vries BB, Janssen IM, Van Der Vliet WA, Huys EH, De Jong PJ, Hamel BC, Schoenmakers EF, Brunner HG, Veltman JA, Van Kessel AG (2004) Mutations in a new member of the chromodomain gene family cause CHARGE syndrome. Nat Genet 36:955–957