Retinoid Pathway and Congenital Diaphragmatic Hernia: Hypothesis from the Analysis of Chromosomal Abnormalities

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Key Words

Congenital diaphragmatic hernia • Retinoic acid • Chromosome loci

Abstract

Background/Objectives: Although there is strong evidence implicating genetic factors in congenital diaphragmatic hernia (CDH) pathogenesis, few causal genes have been identified. Many studies suggest that early disruption of the retinoid signaling pathway during gestation may contribute to CDH etiology. Chromosome abnormalities are detected in 10–20% of CDH cases. Chromosomal regions that are involved in balanced translocations or are recurrently deleted or duplicated in patients with CDH are of particular interest to researchers because they are more likely to harbor genes that cause or predispose one to the development of CDH. The aim of this review was to select chromosome loci which have been shown to be associated with CDH and to investigate if these loci contain candidate genes involved in the retinoid signaling pathway. **Data Sources:** We have re-examined the known CDH-critical chromosomal loci and searched in available databases, such as the UCSC Genome Browser and OMIM, to see whether candidate genes related to the retinoid pathway were present within these loci. **Results:**

Twelve retinoid-related genes have been proposed as potential candidates. Among them, COUP-TFII, FOG2 and GATA4 have already been well studied, especially in animal models. We propose other candidates such as STRA6, LRAT, CRBP1, CRBP2 and CRABP1 are directly implicated in retinoic acid metabolism. **Conclusion:** The identification of CDH-related genes and pathways affecting a normal diaphragm will contribute to the understanding of the pathophysiology of this severe embryopathy and might help to facilitate prenatal management and devise more individual treatment strategies. Further studies are necessary to screen large cohorts of patients with CDH for microimbalances or de novo mutations in these candidate genes. Moreover, functional analyses are needed to establish their exact role in CDH etiology.

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Background/Objectives

Congenital diaphragmatic hernia (CDH) is a severe birth defect with an estimated prevalence of 1 in 3,000 [1–4]. Posterolateral defects, named Bochdalek hernias, account for approximately 95% of CDH, with more than 80% of cases being left-sided. The defect in diaphragm development leads to the herniation of abdominal viscera

Table 1. Monogenic syndromes in which CDH commonly occurs

Syndrome	OMIM	Gene	Chromosomal location
Simpson-Golabi-Behmel	312870	<i>GPC3</i> (glypican-3)	Xq26.1
Denys-Drash	194080	<i>WT1</i> (Wilms' tumor 1)	11p13
Donnai-Barrow	222448	<i>LRP2</i> (low-density lipoprotein-related protein 2)	2q31.1
Spondylocostal dysostosis	277300	<i>DLL3</i> (delta-like-3)*	19q13.2
Matthew-Wood	601186	<i>STRA6</i> (stimulated by retinoic acid gene 6 homolog)	15q24.1
Craniofrontal dysplasia	304110	<i>EFNB1</i> (ephrin B1)	Xq12
Cornelia de Lange	122470	<i>NIPBL</i> (nipped-B-like)	5p13.1
Marfan	154700	<i>FBN1</i> (fibrillin 1)	15q21.1
Ehlers-Danlos types IV and VII	130050	<i>COL3A1</i> (collagen type III)	2q31
	130060	<i>COL1A1</i> and <i>COL1A2</i>	17q21-q22, 7q22.1

* Most commonly mutated gene.

into the chest cavity during the early stages of lung development. Newborns with CDH often have severe respiratory distress resulting from pulmonary hypoplasia. CDH occurs as an isolated birth defect (isolated CDH) or is associated with additional malformations (non-isolated CDH), such as cardiovascular defects, abnormalities of the CNS and urogenital anomalies.

The development of the human diaphragm occurs between the 4th and 12th week of gestation. The primordial diaphragm development arises from four different structures: septum transversum, pleuroperitoneal folds, dorsal mesentery and elements from the thoracic body wall [5, 6]. Several theories have been proposed to explain primary embryologic events leading to CDH, including failure of closure of the pleuroperitoneal canals, defective myoblast formation or abnormal phrenic nerve innervation [7–9]. In animal models, CDH arises from a malformation of the amuscular mesenchymal substratum of the pleuroperitoneal folds before pleuroperitoneal canal closure [10, 11].

Some individuals with non-isolated CDH have patterns of anomalies that are strongly suggestive of a specific genetic syndrome (table 1) [12–17]. The same rare mutation in *WT1* has been reported in three cases of CDH with clinical features of Denys-Drash syndrome [18–20]. This gene encodes a zinc-finger transcription factor expressed in the septum transversum and in the pleural and abdominal mesothelial tissues that form the diaphragm. Homozygous null mouse embryos for *WT1* develop diaphragmatic hernia [21]. Thus, *WT1* appeared as a good candidate for CDH in humans, even though no mutation was found in a screening study of 27 children [22]. Interestingly, this gene is located on chromosome

11p13, a region recurrently deleted in individuals with CDH [23].

Chromosomal abnormalities were identified in approximately 10–20% of CDH cases, the rate being higher in cases with associated malformations [24–28]. The existence of several chromosome ‘hot spots’ suggests the presence of genes that cause or predispose one to the development of CDH in these regions. Trisomy 18, more rarely trisomies 13 and 21, and structural chromosome abnormalities, such as the presence of a supernumerary derivative chromosome 22, have been described in association with CDH. CDH is also frequently present in the Pallister-Killian syndrome associated with a tetrasomy 12p [29–31]. Other chromosomal defects involving almost all the chromosome pairs have been described [25, 32].

In the majority of published cases, chromosome abnormalities were identified using R- or G-banded analysis and FISH. Recently, high resolution techniques such as array-based comparative genomic hybridization (aCGH) have revealed various small recurrent chromosome abnormalities in CDH patients and allowed a more precise breakpoint characterization facilitating the identification of CDH-related genes [33–38]. Several studies have suggested that 15q24–26 and 8p23.1 are critical for normal development of the diaphragm since recurrent deletions within these regions were associated with CDH [33–35, 39–42]. CDH has also been reported in several cases of monosomy 4p16pter associated with Wolf-Hirschhorn syndrome [43–45]. Other candidate regions such as 1q41–q42, 6p22–p25, or 22q11 have also been described [23, 36, 38]. Balanced reciprocal translocations were also described in CDH patients [23, 25]. These trans-

Fig. 1. A conceptual model describing RA synthesis and signaling in target cells' retinol transported in the plasma bound RBP4 (plasma retinol-binding protein). STRA6 binds RBP4, removes the retinol from RBP4 and transports it across the plasma membrane, where it can be metabolized. Within the target cell, retinol binds to CRBP (gene names *RBP1* and *RBP2*) that regulates the cellular metabolism of retinol by presenting it either to alcohol or retinol dehydrogenases (ADHs/RDHs) for conversion to retinal or to LRAT (lecithin:retinol acyltransferase) that esterifies the retinol to retinyl esters. Retinal is then oxidized to RA by retinal dehydrogenase (RALDH). Cellular retinoic-acid-binding protein (CRABP) assists RA entry into the nucleus and RA exerts its bio-

logical effects through binding to the nuclear receptors RAR and RXR. Excess of RA is catabolized in the cytoplasm by the CYP26 class of P450 enzymes. In the nuclei, RA binds to RAR and RXR, which themselves heterodimerize and bind to a short DNA sequence: the RA-response element (RARE). This binding activates the transcription of many target genes. COUP-TFII can act as a repressor of this pathway by directly sequestering RXR, thereby preventing the formation of RAR/RXR heterodimer and inhibiting gene transcription. COUP-TFII interacts with FOG2 that modulates the transcriptional activity of GATA4 proteins, which is a transcription factor playing an important role in early embryogenesis.

locations might cause CDH by disrupting or inactivating specific genes, and the characterization of breakpoints in such cases may be a valuable approach to identify candidate genes [46].

The development of the diaphragm strongly depends on the role of proteins associated with the metabolism and binding of retinoids [47, 48]. The nitrofen rat model has particularly highlighted the importance of retinoic acid (RA) in the diaphragm development, but this model has also provided limited insights into understanding the genetic basis of CDH [9]. Retinoids play a central role in many biological processes, particularly during embryo-

genesis and lung development [49–53]. The RA signaling pathway is complex, but recent studies in several species have increased our understanding of the role of RA as a signaling molecule during vertebrate development [54, 55].

Figure 1 represents a schematic overview of the RA signaling pathway. Numerous studies have revealed the role of a retinoid signaling pathway disruption in the pathogenesis of CDH [47, 48, 56]. In rodents, the first evidence linking retinoids with CDH comes from the observation that 25–40% of the offspring of rat dams that were fed a diet deficient in vitamin A developed CDH [57,

58]. The number of affected pups decreased when vitamin A was reintroduced into the diet in mid-gestation [59]. A proportion of RAR double mutants in mice lacking both α - and β -subtypes exhibited a posterolateral diaphragmatic defect which is similar to that seen in humans [60]. In utero exposure to the nitrofen herbicide, which inhibits the enzymatic activity of RALDH2 – a key molecule responsible for the conversion of retinal in RA – was shown to cause CDH and primary lung defects [61]. Recently, Clugston et al. [62] have shown that a blockage of RAR signaling with the pan-RAR antagonist BMS493 induced a very high degree of CDH with a marked left-right sidedness that depended on the timing of drug delivery. In humans, preliminary evidence that retinoids may play a role in CDH development comes from a small study in which retinol and retinol-binding-protein plasma levels were found to be decreased by around 50% in newborns with CDH compared with healthy newborns [63]. Recently, the retinoid hypothesis has been reinforced by the identification of mutations in *STRA6* (stimulated by RA 6), a membrane receptor for the retinol-binding protein that mediates cellular uptake of vitamin A, in a pleiotropic malformation syndrome including CDH [15].

The aim of this review was to select chromosome loci which have been shown to be associated with CDH and to investigate if these loci contain candidate genes involved in the retinoic signaling pathway. This overview of chromosomal hot spots and associated candidate genes could have future diagnostic and therapeutic interests in terms of clinical management of CDH.

Data Sources

This work was performed thanks to the collaboration between a cytogenetic laboratory and a research team working on the developmental implications of the active derivatives of retinoids for mammalian species over the last few years.

We first identified chromosomal loci recurrently affected in the CDH context using an extensive review of the literature with the PubMed database (<http://www.ncbi.nlm.nih.gov/pubmed>). Both Lurie [32] and Enns et al. [25] have published useful reviews of chromosomal anomalies associated with CDH. Using these reviews as a foundation, we have compiled an updated list of the CDH-associated chromosomal anomalies. Then we searched in available databases such as UCSC Genome Browser (<http://genome.ucsc.edu>) and OMIM (<http://www.ncbi.nlm.nih.gov/omim>) to see whether candidate genes related to the retinoid pathway were present within the selected CDH-critical chromosomal loci.

More than twenty RA metabolic pathway genes are currently known. These genes are involved in vitamin A binding and transport (*RBP4*, transthyretin, *STRA6*, *CRBP1-2*, *CRABP1-2*), storage (*LRAT*, diacylglycerol acyltransferase), intracellular RA synthesis [*ADH3-4*, *dehydrogenase/reductase* (SDR family) members 4 and 9, retinol dehydrogenase 4, epimerase, *RALDH1-4*, aldo-keto reductase family 1, members *B1* and *B10*] and RA degradation (*CYP26A1*, *B1* and *C1*). In addition, six genes encoding nuclear receptors are involved in the RA signal transduction (3 *RARs* and 3 *RXRs*) and many other genes such as *GATA4*, *FOG2* and *COUP-TFII* act as regulators of this pathway. Among all these genes, we identified 12 candidates in the selected chromosome regions.

Results

CDH-Associated Chromosomal Hot Spots and Candidate Genes Involved in the RA Pathway or RA Regulated

Chromosome 1

1q41: DISP1 [MIM 607502]

There is emerging evidence that loss of one or more genes in the 1q41–q42 region predisposes one to CDH [36, 41, 64–67]. Combining cytogenetic results from all published CDH cases, the smallest region of overlap is approximately 1.2 Mb. The *DISP1* (dispatched 1) gene could be the prime candidate gene in this region due to its interaction with Sonic hedgehog (Shh), a crucial protein for the patterning of the early respiratory system in the mouse embryo [67–69].

In the nitrofen rat model of CDH and in the hypoplastic lung of human fetuses with CDH, it has been shown that Shh was downregulated [70]. It has been shown that *COUP-TFII*, a repressor of the retinoid pathway (fig. 1), is a target gene of Shh [71]. Therefore, it is tempting to speculate that a deregulation of the Shh pathway, through an alteration of *DISP1*, might disrupt the RA pathway by deregulating *COUP-TFII* and lead to CDH.

Recently, *HLX* [MIM 142995] has been proposed as a new candidate gene in this region because sequence variants have been identified in patients with isolated CDH [72].

Chromosome 3

3q23: *CRBP1/RBP1* [MIM 180260] and *CRBP2/RBP2* [MIM 180280]

Wolstenholme et al. [73] reported an association of blepharophimosis sequence and CDH in a child with a del(3)(q21q23). Dillon et al. [74] also reported CDH and del(3)(q22) in two patients. Lurie [32] suggested that 3q22 may harbor a gene that when deleted could lead to CDH. *RBP1* (retinol binding protein 1) and *RBP2* (retinol binding protein 2) map at the distal end of the 3q23 band flanking 3q22. *RBP1* and *RBP2* encode cellular RBPs (CRBPs) involved in the intracellular movement of retinol [75] (fig. 1). These genes are part of the retinol signaling pathway and have been shown to play a role in vitamin A homeostasis and lung maturation in mice [10, 76].

Chromosome 4

4q32.1: *LRAT* [MIM 604863]

CDH has been described in four individuals with 4q31 deletion and four individuals with duplication of this region [23]. The *LRAT* gene maps to 4q32.1 flanking 4q31. The protein encoded by this gene is a microsomal enzyme that catalyzes the esterification of retinol into retinyl esters, an essential reaction for the retinoid homeostasis [77, 78] (fig. 1).

Recently, Nakazawa et al. [79] studied the effects of nitrofen on the retinoid-signaling pathway in hypoplastic lungs. They demonstrated that *LRAT* was downregulated, causing a shift of retinol from storage to conversion in RA. This suggests that nitrofen disturbs retinoid signaling at an early stage of this pathway rather than by blocking RALDH as mentioned by Mey et al. [61]. Kim et al. [80] also confirmed recently that retinyl ester formation by *LRAT* is a key regulator of retinoid homeostasis in mouse embryogenesis and that, in contrast, the pathway of RA synthesis does not contribute significantly to the regulation of retinoid homeostasis during mammalian development. Interestingly, RA receptors and GATA transcription factors activate the transcription of the human *LRAT* gene [81].

Chromosome 6

6q23.3: *ALDH8A1/RALDH4* [MIM606467]

Two reports describe a del(6)(q23) associated with CDH [32]. Lurie [32] suggested that the distal part of 6q may contain a locus whose deletion leads to CDH. Furthermore, Howe et al. [43] showed a balanced t(6;8)(q24;q23) translocation in a CDH fetus. One candidate gene in this region could be *RALDH4* (retinal dehydrogenase 4) encoding a protein belonging to the aldehyde de-

hydrogenases family of proteins. This protein plays a role in the in vivo pathway of 9-cis-RA biosynthesis (fig. 1). This enzyme converts 9-cis-retinal into the retinoid X receptor ligand 9-cis-RA, and has approximately 40-fold higher activity with 9-cis-retinal than with all-trans-retinal.

Chromosome 8

8p23.1: *GATA4* [MIM 600576]

The human *GATA4* gene is located on 8p23.1 where microdeletions are recurrent abnormalities in patients with CDH [32, 37, 82, 83]. Faivre et al. [42] suggested that cases with a combination of CDH and cardiac defect should be analyzed for the presence of an 8p23.1 deletion. *GATA4* is a zinc-finger transcription factor expressed in mesenchymal cells of the developing diaphragm, lung and heart. The expression and activity of *GATA4* are influenced by retinoids [84, 85]. Jay et al. [86] described a novel mouse model of CDH based on heterozygosity of a *GATA4* deletion mutation. This *GATA4*^{+/ Δ ex2} mouse developed midline diaphragmatic hernia, dilated distal airways, thickened pulmonary mesenchyme and cardiac malformations.

Recently, somatic mutations have been detected in *GATA4* and other 'cardiac' transcription factors in the hearts of patients who died of congenital heart disease [87]. Thus, somatic mutations that arise during cardiogenesis may be a novel molecular cause of congenital heart disease and it is conceivable that somatic mutations in *GATA4* might contribute to the pathogenesis of CDH.

8q23.1: *FOG2/ZFPM2* [MIM 603693]

Three patients with CDH and 8q deletions have been reported in the review by Holder et al. [23]. Temple et al. [88] described two CDH patients with a balanced translocation involving the 8q22.3 region and CDH. Howe et al. [43] also showed a balanced t(6;8)(q24;q23) in a patient with CDH. *FOG2* (Friend of *GATA2*) is located at the proximal region of the 8q23.1 band flanking the 8q22.3 band. We also suggest that the truncation of *FOG2* or a positional effect affecting the transcription regulatory of this gene could be responsible for a CDH in these three patients. *FOG2* is a multi-zinc-finger transcriptional protein that binds to members of the family of transcription factors as *GATA4*. *FOG2* is expressed in mesodermal tissues, including pulmonary mesenchyme, mesothelium and pleuroperitoneal fold tissue [89]. It has been demonstrated that this protein can activate or downregulate expression of *GATA*-target genes via the formation of a heterodimer with transcription factors of the *GATA* family

(GATA4, GATA5 and GATA6), suggesting different modulation depending on the cell and promoter context.

Huggins et al. [90] demonstrated that FOG-2 can serve as a corepressor protein for both COUP-TFII and GATA4 proteins. Jay et al. [86] suggested that a concerted action of FOG2 and GATA4 is required to regulate mesenchymal cell function in the developing diaphragm and lung. In a screen of fetal mice carrying chemically induced genetic mutations, Ackerman et al. [91] found that a mutation in the gene *FOG2* causes abnormal diaphragm development and pulmonary hypoplasia. Based on this result, the authors identified a de novo *R112X* heterozygous mutation in an infant who died shortly after birth with diaphragmatic defect and severe pulmonary hypoplasia. More recently, Bleyl et al. [92] have identified two novel sequence alterations in *FOG2* in two patients with isolated CDH, reinforcing the hypothesis that FOG2 is critical for normal development of the diaphragm.

Chromosome 12

CDH is one of the most frequent abnormalities described in tetrasomy 12p cases, also known as Pallister-Killian syndrome. Likewise, Tonks et al. [28] described a t(3;12)(q21.1;p13.3) balanced translocation associated with CDH. We identified two candidate genes related to retinoids on the chromosome 12p13.

12p13.31: *RBP5/CRBP1* [MIM 611866]

RBP5 (retinol binding protein 5) is a new family member of RBPs and is predominantly expressed in the liver [93]. RBP5 binds all-trans retinol with a specific interaction similar to that observed in the retinol-RBP1 complex. RBP5 is a direct target of PPAR- γ , a member of the peroxisome proliferator-activated receptor (PPAR, MIM 601487) subfamily of nuclear receptors. PPARs form heterodimers with retinoid X receptors (RXRs) and these heterodimers regulate transcription of various genes [94]. Alteration of 9-cis RA generation could modify the PPAR/RXR activation and, therefore, RBP5 expression. It is not known to date whether the *RBP5* gene is expressed and whether it plays a role during embryogenesis.

12p13.1: *RAIG1/RAI3* [MIM 604138]

The *RAIG1* (retinoic acid-inducible gene 1) gene encodes a member of the type 3 G protein-coupling receptor family, characterized by the '7-transmembrane domain motif' signature [95]. This G-protein-coupled receptor could be involved in modulating differentiation and maintaining homeostasis of epithelial cells. The comparable expression level in fetal lung and kidney with adult

tissues suggests a possible role in embryonic development and maturation of these organs. *RAIG1* expression is induced by all-trans-RA via its receptors [96]. The encoded protein may be involved in the interaction between RA and G protein cellular signaling pathways.

Chromosome 15

15q24.1: *STRA6* [MIM 610745]

Sharp et al. [97] described a patient with a de novo 15q24 microdeletion associated with diaphragmatic hernia. Aviram-Goldring et al. [98] studied a family in which two fetuses had CDH associated with an apparently balanced t(5;15)(p15.3;q24), also present in the mother and in a normal child, suggesting that the CDH in these fetuses may have been caused by a cryptic imbalance at one of the breakpoints during meiosis. More recently, Van Esch et al. [99] described a 15q24 microdeletion of 3.1 Mb including the *STRA6* gene in a patient with severe mental retardation, facial dysmorphisms and CDH. *STRA6* encodes a specific receptor for RBP4-retinol located on cell membranes in the target tissues (fig. 1). It removes the retinol from RBP4 and mediates retinol uptake by cells [100–102]. The transcription of *STRA6* is directly regulated by RA levels. During embryogenesis, *STRA6* is expressed in respiratory mesenchyme and in respiratory/bronchial epithelium [103]. Consistent with various roles of vitamin A and the wide tissue expression pattern of *STRA6*, mutations in *STRA6* are associated with severe pathological phenotypes in humans. CDH is an important component of the phenotype observed in cases of *STRA6* mutations [15, 104].

15q24: *CRABP1* [MIM 180230]

CRABP1 (cellular retinoic acid binding protein 1) belongs to a superfamily of lipid-binding proteins that are thought to act by maintaining tolerable concentrations of intracellular RA as modulators of RA catabolism and as intracellular transporters for RA from the cytoplasm to nuclear receptors [105–107]. CRABP1 is supposed to play an important role in RA-mediated differentiation and proliferation processes.

15q26.1–q26.2: *COUP-TFII/NR2F2* [MIM 107773]

A minimally recurrently deleted region has been identified using FISH and aCGH on chromosome 15q26.1–q26.2 in patients with non-isolated CDH [32–34, 38, 40, 108].

COUP-TFII encodes a transcriptional factor from the steroid/thyroid hormone receptor superfamily. This gene is a nuclear orphan receptor expressed during embryonic

development in a variety of tissues, including mesodermal derivatives in the diaphragm, lung and heart [109]. Homozygous tissue-specific deletion of *COUP-TFII* in mice causes posterolateral CDH similar to the Bochdalek-type CDH seen in humans [110].

COUP-TFII appears to be a good candidate in the 15q26 region because (i) its expression is regulated by retinoids and (ii) *COUP-TFII* regulates gene transcription by influencing RAR/RXR heterodimerization [111, 112]. *COUP-TFII* is able to sequester RXR in a functionally inactive complex and to reduce the available nuclear concentrations of RXR. Thus, *COUP-TFII* can act as a repressor of the retinoid pathway by preventing RAR/RXR heterodimer formation and inhibiting target gene transcription [112]. In the nitrofen rat model, the repression of retinoid signaling pathway by upregulation of *COUP-TFII* may cause hypoplastic lung [18]. This process may be a negative feedback system that precisely balances the transcription of relevant genes during diaphragm development. *COUP-TFII* has been shown to interact physically with *FOG2*, implying that these two factors may cooperate during diaphragm morphogenesis [90] (fig. 1).

Recently, Clugston et al. [113] reinforced this hypothesis showing that 15q26 contains a cluster of genes, including *COUP-TFII*, which are expressed in the developing rodent diaphragm.

Taken together, these data suggest that *COUP-TFII* is likely to play a key role in diaphragm development, even though no mutations were found in 73 CDH samples tested by Scott et al. [38] and in more than 100 samples tested by Slavotinek et al. [41].

Chromosome X

Xp22.3: *TBL1X* [MIM 300196]

The Xp22.3 region is frequently affected in CDH [23]. This region carries the *TBL1X* (transducin β -like 1X) gene which encodes a protein that plays an essential role in transcriptional activation mediated by nuclear receptors [114]. *TBL1X* is found as a subunit in corepressor SMRT (silencing mediator for retinoid and thyroid receptors) complex along with histone deacetylase 3 protein, which is known to modulate the nuclear retinoid signaling pathway [115].

Discussion

A major challenge of CDH research is to characterize genes and signaling pathways that are critical for early mesenchymal cell function during morphogenesis of the

diaphragm. Although several genes have been clearly shown to underlie abnormal diaphragm development in mice, few CDH-related mutations have been identified in the corresponding genes in humans. In this review, we focused on genes involved in retinoid metabolism or regulated by retinoids, which are located within chromosomal regions recurrently affected in CDH patients.

The analysis of chromosomal aberrations may help in the mapping of disease loci and isolation of disease genes by positional cloning strategy [46, 116]. The principal pitfall in this chromosomal approach is that the localization of breakpoints may not be accurate since it is generally based on standard karyotyping. Recent aCGH technology provides a more precise characterization of chromosomal abnormalities, which helps to define the minimally affected region in patients with CDH and identify candidate genes within this region [38, 40, 41]. With this, *de novo* microdeletions in the regions 1q41–q42, 4p16.3, 8p23.1 and 15q26.1–q26.2 have been reported. These deleted chromosomal regions may be assumed to contain genes necessary for normal diaphragm development and these genes can subsequently be selected for sequencing in CDH patients. Among the retinoid-related genes included in these regions, *COUP-TFII* and *FOG2* were sequenced in CDH patients. To date, no mutation could be identified in *COUP-TFII* [38, 41]. A *de novo* mutation and sequence alterations in *FOG2* were found in three patients, reinforcing the hypothesis that *FOG2* is critical in diaphragmatic and lung development in humans [91, 92].

The *STRA6* gene located at chromosome 15q24.1 is also a promising candidate since CDH is an important component of the polymalformative syndrome observed in cases with *STRA6* mutations. Recently, Isken et al. [117] have shown that *STRA6* is essential in maintaining embryonic RA homeostasis and that *STRA6*-dependent transfer of retinol from RBP4 depends on LRAT. LRAT activity is required, like those of *STRA6* and RBP4 protein, for uptake of appropriate amounts of retinol into cells. As a result, LRAT is also likely to play a role in the development of CDH in individuals with 4q32.1–q31 rearrangements.

Other CDH-associated chromosomal hot spots such as 2q37, 6p25 and 22q11 do not contain genes related to the retinoid pathway. These regions might carry genes involved in pathways regulating differentiation of mesenchymal cells or cell migration, which are important for diaphragm development. For example, the *COL6A3* gene [MIM 120250] is located on chromosome 2q37, a region frequently deleted in CDH. Impaired formation of the extracellular matrix, caused by disruptions in either col-

lagen or elastic fibers, can lead to developmental defects in a wide range of organs including the diaphragm. Of note, CDH has been linked to several subtypes of Ehlers-Danlos syndrome caused by mutations in genes belonging to the collagen family (table 1), which is expressed during embryonic development in several organs [17]. In the same way, the 6p25 region repeatedly deleted in CDH patients contains the *FOXF2* gene (forkhead box F2), one of the human homologues of the *Drosophila melanogaster* transcription factor forkhead, which could be a good candidate [23, 38]. *FOXF2* is expressed in lung and placenta and was shown to activate transcription of several lung-specific genes [118, 119].

Systematic screening for mutations in CDH patients has been reported only for *WT1*, *COUP-TFII* and *FOG2*.

The same approach could be used to search for mutations in related genes from the RA metabolic and molecular signaling pathway. In addition, a simultaneous analysis of several loci by quantitative multiplex PCR of short fluorescent fragments in a cohort of CDH patients may be used to estimate the frequency of microdeletions or microduplications of candidate genes, which may help to establish their role in CDH etiology. The identification of CDH-related genes and pathways affecting normal diaphragm and lung development will contribute to the understanding of the pathophysiology of this severe embryopathy. Given the substantial mortality and morbidity associated with this developmental abnormality, advances in this area are critical.

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