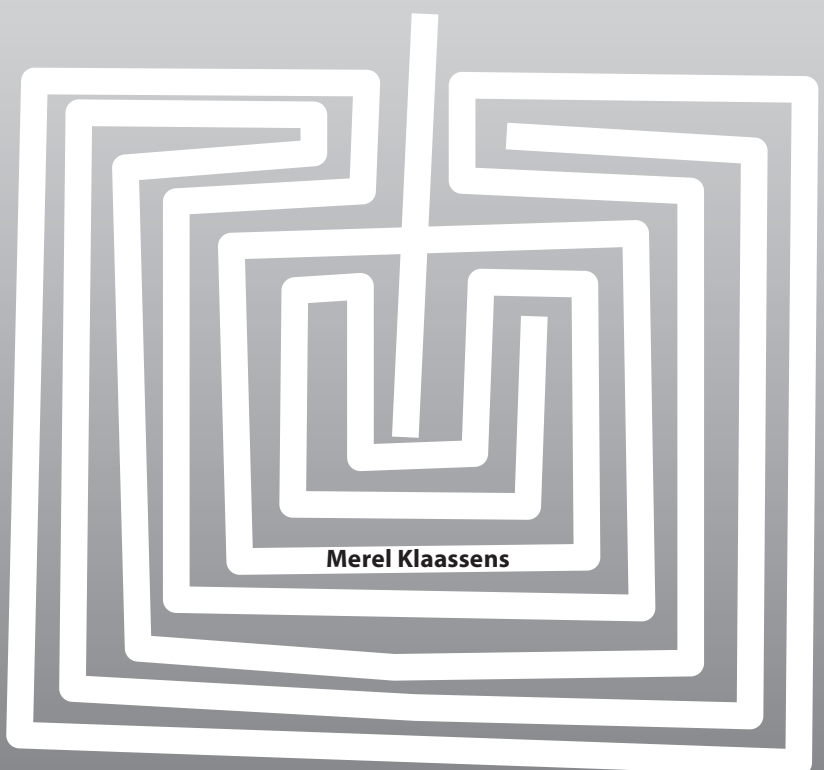
Genetic Factors in the Etiology of Congenital Diaphragmatic Hernia



Merel Klaassens

Cover: **Tápu'at** (Mother and Child).
A common symbol among Native American tribes, including the Hopi, representing progress and spiritual rebirth of one world into the succeeding one. The maze represents the earth as a womb, and the straight line represents the path of emergence from the stage of the unborn child to its birth. This symbol is also one of the oldest known images of a Labyrinth (maze).

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CONTENTS

CHAPTER 1

Introduction

Based on:

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INTRODUCTION

Major congenital malformations affect approximately 1 in 33 children, and nearly 10% of newborns with a birth defect die as a result of their congenital anomaly. Congenital Diaphragmatic Hernia (CDH, [MIM 142340]) is estimated to occur in 1 per 2,000 – 3,000 births and accounts for approximately 8% of all major congenital anomalies (Torfs et al. 1992; Moya and Lally 2005). In the Netherlands, each year approximately 60 children are born alive with CDH.

The first anatomical descriptions of this birth defect can be traced back to the mid 17th century, but it was not until the late 19th century that the first surgical procedures were described (Golombek 2002). A defect in formation of the diaphragm is still a key factor in CDH, although clinical symptoms are mainly determined by the associated pulmonary hypoplasia and the variable amount of pulmonary hypertension, the result of an abnormal vascular structure in the lungs. In the past it was believed that surgical repair of the diaphragm defect was the sole treatment for this anomaly. Nowadays, we know that the diaphragm defect itself is not the major cause of death due to CDH, apart from cases of complete agenesis of the diaphragm, but that pulmonary problems, such as hypoplasia and pulmonary hypertension, and the presence of other congenital anomalies are the major problems in these children (Lally et al. 2006). Advances in our understanding of the pathophysiology, in particular focused on ventilator management and the use of ECMO, has improved survival in the past years. Expert centers nowadays report survival rates of up to 85% (Boloker et al. 2002; Colvin et al. 2005; Yang et al. 2006). Nevertheless, depending on case selection criteria, the mortality remains high and long-term morbidity among survivors is substantial (Doyle and Lally 2004). The most reported problems are chronic oxygen depencency, a decreased exercise tolerance, feeding difficulties due to gastro-esophageal reflux, and problems due to complications from ECMO treatment, in the worst case causing mental retardation, deafness or blindness (Cortes et al. 2005).

Diaphragm development and anatomical characterization of diaphragm defects

There are three recognizable types of CDH:

1. A posterolateral defect. This type of CDH has been described for the first time by Bochdalek in 1848 and therefore is commonly referred to as Bochdalek-type CDH. It is the most common type of CDH and occurs in 90-95% of cases (Torfs et al. 1992; Robert et al. 1997).
2. An anterolateral defect, also referred to as Morgagni-type CDH. Only 2.1% of affected children have this type of CDH. This type is more common in patients with trisomy 21 (Down's syndrome [OMIM 190685]).
3. A central defect. This form of CDH occurs in 2.1% of all patients. It is almost solely seen as part of the Pentalogy of Cantrell (OMIM 313850).

In the majority of patients with posterolateral defects, this defect is located on the left side (90% of patients). Approximately 8% of patients have a right-sided defect and in 2% of the cases the defect is bilateral (Torfs et al. 1992). Familial occurrence has been described in CDH, providing more evidence for a genetic contribution (Crane 1979; Pober et al. 2005). Because these descriptions are limited within the population of CDH patients the recurrence risk for isolated cases of CDH is often quoted as <2% based on a mathematical model of multifactorial inheritance risk (Edwards 1960; Norio et al. 1984; Torfs et al. 1992). Although multifactorial inheritance may best explain most cases of human CDH, much has been

learned about the genetic factors that play a role in the development of CDH by studying patients with CDH caused by specific genetic syndromes and chromosome anomalies. Our understanding of CDH has also been aided through basic research using teratogen and knock-out animal models of CDH.

However, much is still unknown about development of the diaphragm and the pathogenesis of congenital diaphragmatic hernia. The development of the human diaphragm occurs between the fourth and twelfth week of gestation. Traditional views of diaphragm development suggest that the diaphragm arises from four different structures, as reviewed by Clugston *et al* (2006). It has always been thought that the septum transversum gives rise to the central portion of the diaphragm, the pleuroperitoneal folds (PPFs) to the posterolateral section of the diaphragm, the dorsal (esophageal) mesentery to a portion of the diaphragm posterior to the esophagus, and elements from the thoracic body wall contribute to a rim of musculature around the diaphragm's periphery.
In contrast to this traditional view, systematic examinations of diaphragm development in rodents have failed to identify contributions to the diaphragm musculature from the lateral body wall, septum transversum, esophageal mesenchyme, or the lateral body wall (Babiuk et al. 2003). Rather, myogenic cells and axons were shown to coalesce within the PPF and then expand to form the neuromuscular component of the diaphragm. If further investigation shows that this model provides an accurate depiction of diaphragm development in humans, the classic view of diaphragm development may need to be revised (Clugston et al. 2006).

Several theories have been proposed concerning the primary embryologic events that lead to the development of CDH. Events implicated in these theories have included: 1) abnormalities in (ipsilateral) lung development, 2) failure of closure of the pleuroperitoneal canals, 3) defective myoblast formation, and 4) abnormal phrenic nerve innervation (Iritani 1984; Skandalakis et al. 1994; Thebaud et al. 1999).

Although it is possible that each of these abnormalities may play a role in the development of some cases of CDH, there is growing evidence from animal models that CDH arises from malformation of the amuscular mesenchymal substratum of the PPF prior to pleuroperitoneal canal closure (Allan and Greer 1997; Babiuk and Greer 2002; Clugston et al. 2006). Critical findings that support this model over other theories include normal formation of the primordial diaphragm in *Fgf10*$^{-/-}$ mouse embryos with complete lung agenesis, and the ability to induce defects characteristic of CDH in *c-met*$^{-/-}$ mouse embryos that do not form diaphragm muscle fibers due to a defect in muscle precursor migration (Babiuk and Greer 2002). As mentioned before, pulmonary hypoplasia is one of the most serious clinical problems for patients with CDH. In many cases the degree of lung hypoplasia is not correlated with size or location of the diaphragmatic defect. This observation may be explained by the "double-hit hypothesis" which suggests that there is an early insult that directly affects lung development followed by further restriction in lung growth, later in gestation, secondary to diminished fetal breathing movements and competition for space as a result of the herniation of the abdominal contents into the thoracic cavity (Keijzer et al. 2000). It is possible that these two hits may be caused by defects within a single gene that affects both lung and diaphragm development. As genes involved in the development of CDH are identified it may be possible to test this hypothesis using conditional knock-out mice in which the lungs and the primordial diaphragm are targeted separately. These studies may also provide another means of testing whether diaphragmatic defects can be induced or altered by a primary pulmonary insult.

Genetic animal models in CDH

Important evidence, supporting the role of genetic factors in the etiology of CDH, is derived from animal models. Several of these models were not designed to study CDH and the diaphragm defects were a coincidental finding. Other models were developed specifically to study diaphragm (and lung) development, such as the *Fog2*, *Coup-tfII* and *Gata4* mice models. Interestingly, several of the genes targeted in animal studies are located in the recurrently altered chromosomal regions in human patients. In the future, combining this data will lead to useful insights into the etiology of CDH. An overview of all animal models related to CDH is given in Table 1. The most important animal models will be discussed in the following section.

Table 1. CDH animal models

Gene	Human locus	Phenotype	Reference
COUP-TFII	15q26.2	Posterolateral diaphragm defect, abnormal patterning of the stomach, asplenia	You *et al.* PNAS 2005
FOG2	8q23	Abnormal muscularization of the diaphragm, lunghypoplasia	Ackerman *et al.* PloS Genetics 2005
GATA4	8p23.1	Midline diaphragm defect, dilated distal airways, cardiac malformations	Jay *et al.* Dev Biol 2006
RARα/β2	9p24.2/6p21.32	Diaphragm defect	Mendelsohn *et al.* Development 1994
SLIT3	5q35	Central (septum transversum) diaphragm defect, enlarged right ventricle, kidney defects (e.g. unilateral or bilateral agenesis, hypoplasia)	Liu *et al.* Mech Dev 2003; Yuan et al. PNAS 2003
WT1	11p13	Left-sided posterolateral diaphragm defect, small heart, edema, lunghypoplasia, failure of kidney and gonad development	Kreidberg *et al.* Cell 1993 ; Clugston et al. Am J Physiol 2006
ROBO1	3p	Diaphragmatic hernias, lung hypoplasia	Xian *et al.* PNAS 2001
c-Met	7q31.2	No formation of diaphragm muscle fibers	Babiuk *et al.* Am J Physiol Lung Cell Mol Physiol 2002
MyoD	11p15	Reduced skeletal muscle compartment of diaphragm, intact mesenchymal compartment, lunghypoplasia	Inanlou *et al.* Dev Biol 2003
PAX3	2q36.1	Absence muscular diaphragm, intraventricular septum defects, triscuspid valve insufficiency, absent muscles limbs	Li *et al.* Dev 1999 ; Lagutina *et al.* Mol Cell Biol 2002
SIM2	21q22.3	Diaphragm hypoplasia (thinner), rib protrusions, abnormal intercostal muscle attachments, pleural mesothelium tearing	Goshu *et al.* Mol Cell Biol 2002
NEDD4	15q21.3	Diaphragm hypoplasia (thinner), central nervous system abnormalities	Shi *et al.* 55th Annual Meeting American Society of Human Genetics, poster #901
LOX	5q23.1	Cardiovascular instability (ruptured arterial aneurysms), diaphragmatic rupture	Hornstra *et al.* J Biol Chem 2003

Chick Ovalbumin Upstream Promoter-Transcription Factor II (COUP-TFII)

COUP-TFII (*NR2F2*), a transcription factor from the steroid/thyroid hormone receptor super family, is located on human chromosome 15q26 in a region recurrently deleted in individuals with CDH (Lurie 2003). In chapters 2 and 3 our studies on the determination of the minimally deleted region for CDH on chromosome 15q26 are described (Klaassens et al. 2005). In the general discussion (Chapter 7) the possible role of COUP-TFII in the etiology of CDH is discussed.

Friend of GATA2 (FOG2)

FOG2 (ZFPM2), a zinc finger containing protein that modulates the transcriptional activity of GATA proteins, is located on human chromosome 8q23. The first indication that *FOG2* might play a role in normal diaphragm development came with the discovery of an ENU mouse mutant with pulmonary hypoplasia and an abnormal diaphragm that lacked muscularization of the posterolateral and peripheral regions. Sequencing of the *Fog2* gene in this mouse revealed hypomorphic splice donor mutation. A *de novo* R112X heterozygous mutation was subsequently found in an infant who died shortly after birth with diaphragmatic eventration and severe pulmonary hypoplasia (Ackerman et al. 2005).

GATA-binding protein 4 (GATA4)

GATA4 is a member of a family of DNA-binding proteins that recognize a consensus sequence (the GATA motif), which is found in the promotor regions of many genes. GATA4 encodes for a transcription factor that interacts with FOG2 during the morphogenesis of the heart and testis and plays an important role in early embryogenesis. GATA4 is located on chromosome 8p23.1, a region recurrently deleted in individuals with CDH.
Recently, Jay *et al.* showed that heterozygous *Gata4*$^{+/\Delta ex2}$ mice on a C57B1/6 background have diaphragm defects, providing additional evidence that GATA4 is be important for lung and diaphragm development (Jay et al. 2006).

Wilms Tumor 1 (WT1)

WT1 is located on human chromosome 11p13, a region recurrently deleted in individuals with CDH, and encodes a zinc finger transcription factor that is expressed in the pleural and abdominal mesothelium that help to form the diaphragm (Gustavsson et al. 1984a; Pritchard-Jones et al. 1990; Scott et al. 2005). Mutations within *WT1* have been described in two patients with CDH one of whom had Denys-Drash syndrome ([MIM 194080], male pseudohermaphroditism, nephropathy, and Wilms tumor) and one who had Frasier syndrome ([MIM 136680], focal and segmental glomerulosclerosis, male pseudohermaphroditism, and gonadoblastoma) (Devriendt et al. 1995; D'Agostino 1997). Further evidence for the role of *WT1* in CDH comes from homozygous *Wt1* null-mouse embryos that develop diaphragmatic hernias (Kreidberg et al. 1993).

Homolog of Drosophila slit 3 (SLIT3)

SLIT3 is located on human chromosome 5q35.1. In mice, *Slit3* is expressed predominantly in the mesothelium of the diaphragm during embryonic development (Yuan et al. 2003). Homozygous *Slit*3 deficient mice have congenital diaphragmatic hernias on or near the ventral midline portion of the

central tendon that are similar to the central (septum transversum) type of diaphragmatic hernias seen in humans (Yuan et al. 2003). Although *SLIT3* seems to be a strong candidate gene for this relatively rare type of CDH, no *SLIT3* mutations have been identified in human cases of CDH to date.

Syndromes and chromosomal anomalies associated with CDH

CDH may occur either as an isolated birth defect or in association with other non-hernia-related anomalies (in general referred to as non-isolated CDH or CDH+). Some anomalies (including lung hypoplasia, abnormalities in cardiac position, intestinal malrotation, and patent ductus arteriosus) are typically considered secondary effects of CDH and are not considered grounds for classification as non-isolated CDH. Common findings associated with CDH include cardiovascular abnormalities, limb abnormalities, abnormalities of the CNS and geniotourinary/renal anomalies.

Some individuals with non-isolated CDH have patterns of anomalies that are strongly suggestive of a specific genetic syndrome. In CDH patients for whom a syndromic diagnosis can be provided, the most frequently diagnosed syndrome is Fryns syndrome [MIM 229850] (Slavotinek 2004; Slavotinek et al. 2005; Kantarci et al. 2006). In Fryns syndrome CDH is a frequent, if not obligatory, finding. However, there are many syndromes in which the rates of CDH are lower, but probably exceed the level seen in

Table 2. Syndromes associated with CDH

Syndrome	Locus / Gene	Associated anomalies
Fryns	unknown	Coarse facial features, cleft lip/palate, cardiac anomalies, cerebral anomalies, nail hypoplasia (fingers & toes)
Pallister-Killian	tetrasomy 12p	Coarse facial features, hypertelorism, sparse temporal hair, hypopigmentations, mental retardation
Cornelia de Lange	NIPBL (~50% of patients)	Distinctive facial features, microcephaly, hirsutism, malformations upper limbs, growth retardation
Donnai-Barrow	unknown	Hypertelorism, agenesis corpus callosum, omphalocele
Wolf-Hirschorn	deletion 4p	"Greek helmet" facial appearance, cleft lip/palate, cardiac anomalies, mental retardation, growth retardation
Denys-Drash	WT1	Male pseudohermaphroditism, genital anomalies, increased risk Wilms tumour
Simpson-Golabi-Behmel	GPC3	Macrosomia, coarse facial features, hypertelorism, macroglossia, abdominal wall defects, renal anomalies
Beckwith-Wiedemann	imprinted genes on 11p15	Macrosomia, macroglossia, visceromegaly, abdominal wall defects
CHARGE	CHD7	Coloboma, cardiac anomalies, choanal atresia, growth retardation, genital anomalies, ear anomalies
Craniofrontonasal	EFNB1	Craniosynostosis, hypertelorism, broad nasal tip, grooved nails hallux and thumb, syndactyly, skeletal anomalies
Perlman	unknown	Overgrowth, dysmorphic features, renal dysplasia/ tumours
PAGOD	unknown	Hypoplasia pulmonary arteries, agonadism, omphalocele, genital anomalies
(no name)	STRA6	Bilateral anophthalmia, pulmonary anomalies and/or CDH, cardiac anomalies, characteristic facial features

the general population. An example of such a syndrome is Cornelia de Lange Syndrome (CdLS [MIM 122470]). Fryns syndrome, Cornelia de Lange Syndrome and examples of other named syndromes associated with CDH are described in Table 2. Most of these syndromes are associated with a specific Mendelian inheritance pattern and, in some cases, the location and/or the identity of the causative gene(s) is known.
The existence of genetic syndromes associated with CDH provides one of the strongest lines of evidence that genetic factors play a role in the development of CDH. It is likely that much of our understanding of CDH will be shaped by studies that focus on understanding the genes that cause these forms of CDH.
Additional evidence pointing towards a genetic contribution to the etiology of CDH exists of chromosomal anomalies identified in human patients with CDH. Chromosomal anomalies have been identified as the etiology for numerous cases CDH, almost exclusively in patients with non-isolated CDH. Candidate genes can be identified by the so-called "positional candidate approach", which has been used by us to identify genes in patients with CDH. For CDH this approach is particularly useful, since other ways of analysis, such as linkage analysis, are almost impossible to perform due to the lack of large-enough affected families. In the majority of published cases, chromosome anomalies were identified using a combination of G-banded chromosome analysis and/or FISH. The use of new genomic technologies (like array-based comparative genomic hybridization) is likely to increase the number of chromosomal anomalies identified in individuals with CDH and may aid in the identification of CDH-related genes (Le Caignec et al. 2005; Slavotinek et al. 2005; Kantarci et al. 2006).

Trisomy 13, 18, and 21 and 45,X are the most common aneuplodies described in association with CDH (Tibboel and Gaag 1996). Structural abnormalities (including deletions, duplications, inversions, and translocations) of all chromosomes have also been described in association with CDH (Enns et al. 1998; Lurie 2003). An overview of all CDH-associated chromosomal anomalies presented in the literature can be found in Table 3 in the appendix. These chromosomal anomalies in patients with CDH are of particular interest to researchers since they are more likely to harbor genes that cause or predispose to the development of CDH than regions of the genome that are less commonly affected. When considering the likelihood that any particular region contains one or more CDH-related genes, it is important to note that many of the deletions and duplications described in the literature are the product of unbalanced translocations and it is possible that the diaphragmatic defects seen in these cases are caused by two or more genes affected by a combination of segmental aneuploidies. It should also be noted that, in most cases, CDH occurs in only a fraction of individuals with a particular chromosomal abnormality. This suggests that genetic background, environmental factors, and/or stochastic events may also play a role in determining whether an individual develops CDH. Chromosomal regions that have been associated with CDH in three or more individuals are shown in Figure 1. One can only presee that more structural anomalies will be described and that the regions identified will continu to narrow in size as newer high-resolution techniques are used in both clinical diagnosis and CDH research. The most important regions published up to now are described in the following section.

Deletion of 1q41-q42

This chromosomal abnormality has been reported in four cases of CDH (Youssoufian et al. 1988; Rogers et al. 1995; Kantarci et al. 2006; Slavotinek et al. 2006). Three cases have a larger deletion, identified by standard cytogenetic techniques. The smallest deletion was determined by Kantarci *et al.* using

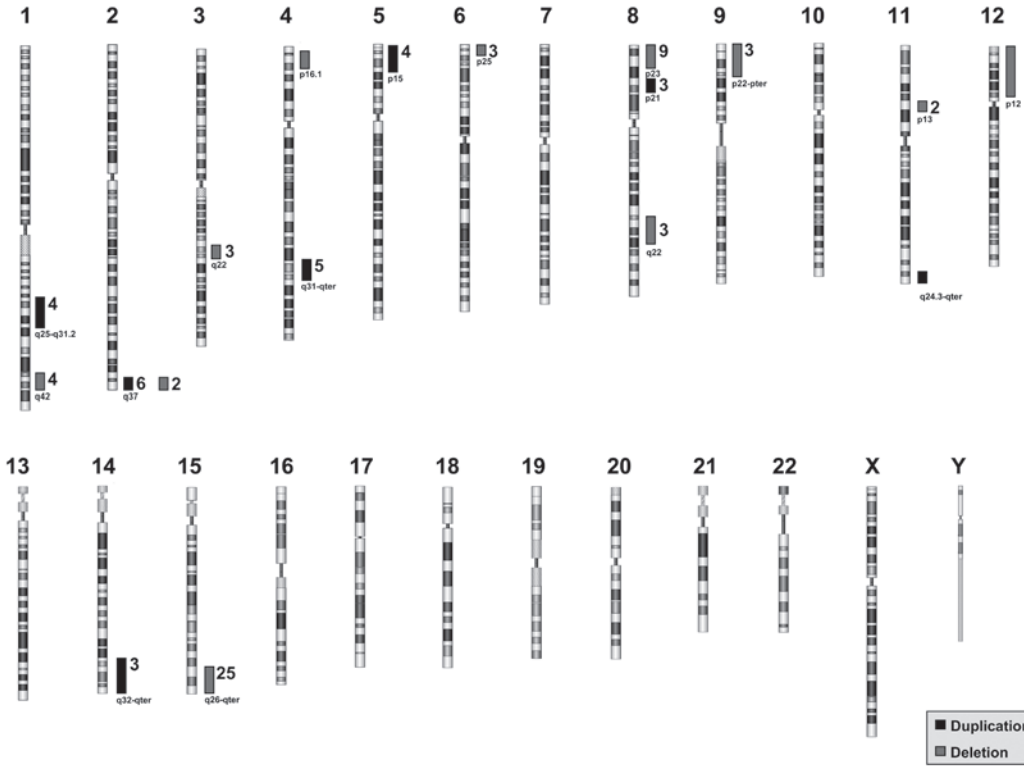

Fig.1. Recurrent chromosomal abnormalities associated with CDH are represented by colored bars. For each region, the number of patients described with that anomaly is listed. For an overview of all chromosomal anomalies described in patients with CDH, see Table 3 in the appendix. Color figure can be found in the appendix. See page 130.

high-resolution aCGH that refined the interval to an ~5 Mb region bounded by BACs RP11-553F10 and RP11-275O4 (Kantarci et al. 2006). Two individuals with balanced translocations involving 1q41 have also been described (Smith et al. 1994; Priolo et al. 2004).

Deletion/Duplication of 2q37

CDH has been reported in six patients with 2q37 deletions and two patients with 2q37 duplications (de la Fuente et al. 1988; Johnson et al. 1992; Enns et al. 1998; Reddy 1999; Casas et al. 2004; Tonks et al. 2004). In all these patients the duplication or deletion starts at band q37. No larger imbalances have been described in CDH patients. Patient 1 described in Chapter 4 has a similar sized duplication of 2q37, associated with a deletion of 15q.

Deletion of 3q22

This deletion has been reported in three individuals with CDH. Two of these patients had blepharophimosis and facial dysmorphism most likely attributable to deletions of *FOXL2* which is known to cause blepharophimosis, ptosis, and epicanthus inversus syndrome [MIM 110100] (Wolstenholme

et al. 1994; Dillon et al. 2000). The most promising CDH candidate genes located in this region are *RBP1* cellular retinol binding protein 1 (*RBP1*)[MIM 180260] and *RBP2* cellular retinol binding protein 2 (*RBP2*)[MIM 180280]. 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 (Ghyselinck et al. 1999; E et al. 2002). However, no mutations in *RBP1* or *RBP2* have been described in CDH patients to date.

Deletion of 4p16

Wolf-Hirschhorn syndrome [MIM 194190] is associated with deletions of 4p16 and is characterized by a "Greek helmet" facial appearance, growth retardation, mental retardation, seizures/epilepsy, cleft lip/palate and cardiac abnormalities. Although not a common finding in Wolf-Hirschhorn syndrome, CDH has been described in association with at least 13 cases of 4p16 deletions (Laziuk et al. 1979; Tachdjian et al. 1992; Kobori et al. 1993; Bird et al. 1994; Howe et al. 1996; Sergi et al. 1998; Tapper et al. 2002; Schinzel 2004; van Dooren et al. 2004; Pober et al. 2005; Casaccia et al. 2006).

Duplication of 8p21

Duplication of 8p21 has been described three times in patients with CDH (Moreno Fuenmayor et al. 1980; Ringer et al. 1995; Schinzel 2004). The patient described by Moreno-Fuenmayor *et al.* had a phenotype consistent with that of other patients with duplication 8p21 (Moog et al. 2000). The patient described by Ringer *et al.* had an inverted duplication (inv dup 8p). In some cases patients with an inv dup 8p also have a small deletion of 8p23.1, a region recurrently deleted in CDH, so therefore we might should see these two regions together as one candidate region for CDH. Unfortunately, it is unclear whether the patient described by Ringer *et al.* also carried this deletion (Moog et al. 2000).

Deletion of 8p23.1

This anomaly has been described in more than 30 individuals with abnormal phenotypes including nine times in patients with CDH (Pecile et al. 1990; Fraer et al. 1992; Howe et al. 1996; Faivre et al. 1998; Kousseff 2000; Borys and Taxy 2004; Shimokawa et al. 2005; Slavotinek et al. 2005; Lopez et al. 2006). More distal deletions of 8p23.1-p23.2 have also been found in unaffected individuals suggesting that more telomeric deletions may be a normal variant in the Caucasian population (Reddy 1999). *GATA-binding protein 4* (*GATA4*)[MIM 600576] resides within this region and has been proposed as a candidate gene for CDH. Of note, deletions and loss-of-function mutations of *GATA4* have been seen in individuals with cardiac defects involving the cardiac septum and the majority CDH patients with deletion of 8p23.1 also have cardiac anomalies (ASD, VSD or AVSD) (Devriendt et al. 1998; Garg et al. 2003; Okubo et al. 2004). *Gata4* heterozygous mutant mice of certain strains also display diaphragm defects in association with pulmonary and cardiac abnormalities (Jay et al. 2006).

Deletion of 8q22-q23

Three CDH patients with 8q deletions have been described (Harnsberger et al. 1983; Maerzke et al. 1993; Capellini et al. 1996). Each of these deletions included bands 8q22-q23 and all of these patients had dysmorphic features similar to other patients with 8q22-q23 deletions (Wilson et al. 1983). *Friend of GATA2* (*FOG2*)[MIM 60369] resides within this region and animal data support its role in the development of diaphragmatic hernia (Ackerman et al. 2005).

Deletion of 11p13

Although only two CDH patients have been described with a deletion of 11p13 this region is of particular interest, because this is where *WT1* is located (Gustavsson et al. 1984b; Scott et al. 2005). Animal studies and the occurrence of CDH in several WT1-associated syndromes point towards a role for this gene in the etiology of CDH.

Duplication of 11q24.3-qter

This duplication has been described numerous times in patients with CDH. In most cases, this duplication is the result of the more common chromosomal anomaly 47,XX or XY,+der(22)t(11;22) resulting from 3:1 meiotic segregation (Klaassens et al. 2006). Three patients in whom the duplication of 11q24-qter is the result of an unbalanced translocation with another autosome have been reported, one of which is described in detail in chapter 5 of this thesis (Park et al. 1993; Boycott 2006; Klaassens et al. 2006). No isolated duplications of this region have been described.

Duplication of 12p

Mosaic tetrasomy 12p, or Pallister Killian syndrome, is characterized by coarse facial features, sparse temporal hair, skin abnormalities, mental retardation, and high rate of CDH (Mowery-Rushton et al. 1997). This syndrome usually results from mosaicism for an isochromosome: i(12)(p10) (Peltomaki et al. 1987).

Deletion of 15q26

Deletions of the distal part of the long arm of chromosome 15 have been described in numerous patients with non-isolated CDH, making this anomaly one of the most reported structural chromosomal anomalies in CDH. The majority of patients with deletions of the long arm of 15q have a complex phenotype that includes cardiac abnormalities, limb abnormalities, and dysmorphic features that could be described as a Fryns-like appearance. A detailed description of our studies for the identification of this candidate region and determination of the phenotype associated with this deletion can be found in chapters 2, 3 and 4 of this thesis.

The "Rotterdam cohort" of CDH patients

The Sophia Children's Hospital, part of the Erasmus University Medical Center serves the southwestern part of the Netherlands, with a population of 4 million people and an annual birth rate of approximately 35,000 births. The Sophia Children's Hospital is a tertiary referral center for CDH and one of two centers in the Netherlands where ECMO-treatment is available. CDH has been the subject of scientific research within the department of pediatric surgery for a long time. Data on patients treated in the Sophia Children's Hospital since 1972 has been stored in a database. However, for these early years much data is not available anymore. Data on patients treated since 1988 is complete. The data that is stored includes general clinical data (e.g. gestational age, birthweight, etc), CDH-specific data (side of the defect, type of defect, etc) and family data (e.g. occurrence of congenital anomalies in parents and siblings, etc). After birth, parents are asked to participate in our study on etiological factors of CDH. When parents give consent, material (if possible chromosomes, DNA, cell lines, and parental DNA) is stored for future

analysis. In the last few years, karyotyping has become part of standardized diagnostic procedures in all patients with a congenital anomaly.

Since 1972 data on 402 patients is stored in the database. Of these patients 360 cases (89.6%) have been karyotyped. In 166 (46%) of the cases the CDH was accompanied by another congenital anomaly (not including patent ductus arteriosus, persistent foramen ovale, malrotation and mediastinal shift). In 25 (15%) of these MCA-cases a chromosomal anomaly was identified (see Table 4) and in another 25 a syndrome diagnosis could be made (see Figure 2). The overall mortality in the whole period was 42.8%.

Table 4. Chromosomal anomalies identified in patients from the Rotterdam cohort

Chromosomal anomaly	Number of patients
46,XX,r(15)(p11;q26.1)	1
46,XY,r(15)(p11;q26)	1
46,XY,inv(6),t(1;14),del(15)(q26)	1
46,XX,der(15)t(2;15)(q37.2 ;q26.2)	1
46,XX,inv(1)(p36.1q42)pat	1
46,XY,der(12)t(11;12)(q23.3;q24.3)mat	1
46,XY,der(3)t(3 ;8)(p23;p23.1)pat	1
46,XY.ish del(4)(p16.1)	1
47,XY,+der(22)t(11;22)(q23.3 ;q11.2)	1
47,XX,+i(12)(p)	1
47,XX,+13 / 47,XY,+13	2
47,XX,+18 / 47,XY,+18	9
47,XY,t(5;21),+21	1
47,XX,+21 / 47,XY,+21	3
Total	**25**

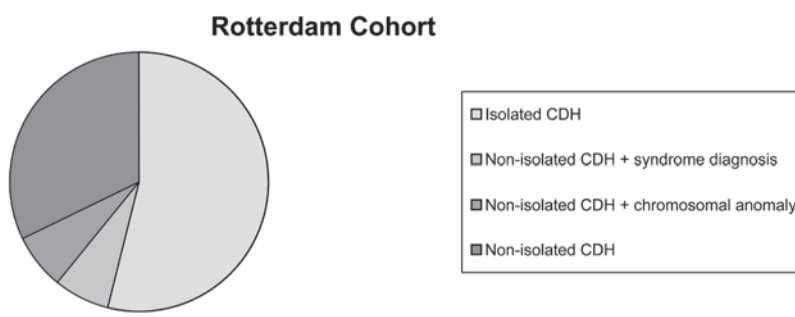

Fig. 2.
Patient groups within the Rotterdam cohort of CDH patients.

Objectives of the studies described in this thesis

The main objective of our study was to identify chromosomal loci that might play a role in the etiology of CDH using the "positional candidate approach". For this analysis we started with our own Rotterdam cohort of patients, but as time evolved, we were also able to include several patients from other centers.

In part 1 of this thesis the mapping of the CDH-critical region on chromosome 15q is described (Chapters 2, 3 and 4). By means of complementary (molecular) cytogenetic techniques we identified candidate genes in this region that might cause or predispose to the development of CDH. In part 2 of

this thesis, the identification and analysis of another CDH-associated locus and its candidate genes will be described (Chapter 5). The third part is focused on the use of relatively new molecular cytogenetic techniques, such as oligonucleotide-based array-CGH (Chapter 6).
The last part of this thesis will be devoted to a general discussion of the findings and the formulation of a hypothesis on the development of the lung- and diaphragm defects seen in children with congenital diaphragmatic hernia (Chapter 7). A guideline for prospective evaluation of patients diagnosed with CDH, either pre- or postnatal, is included.

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CHAPTER 2

Congenital Diaphragmatic Hernia and Chromosome 15q26: Determination of a Candidate Region by use of Fluorescent In Situ Hybridisation and Array-based Comparative Genomic Hybridisation.

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ABSTRACT

Congenital diaphragmatic hernia (CDH) has an incidence of 1 in 3000 births and a high mortality rate (33-58%). Multifactorial inheritance, teratogenic agents, and genetic abnormalities all have 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: deletion of 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 fluorescence in situ hybridization to determine the boundaries of the deletions and by including data from two individuals with terminal 15q deletion, 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 ~ 5Mb at chromosome 15q26.1-26.2. The region contains four known genes, of which two (NR2F2 and CHD2) are particularly intriguing candidates for CDH.

INTRODUCTION

Congenital Diaphragmatic Hernia (CDH, [OMIM 142340]) is a severe life-threatening congenital anomaly, characterized by a variable defect in the diaphragm, pulmonary hypoplasia and postnatal, sometimes therapy-resistant, pulmonary hypertension. There are four recognizable types of CDH of which the posterolateral (Bochdalek) and Morgagni hernia are the most common (Torfs et al. 1992). It is a relatively common anomaly, with an incidence of 1 in 3000 live births (Torfs et al. 1992; Langham et al. 1996). The mortality is high, varying between 33% and 58% (Beresford and Shaw 2000) and survival depends on the degree of pulmonary hypoplasia, the severity of pulmonary hypertension, and the co-existence of associated congenital anomalies (Howe et al. 1996).
Congenital diaphragmatic hernia can occur as an isolated defect, in combination with multiple congenital anomalies, or as part of a defined syndrome (Enns et al. 1998), for example Fryns syndrome (OMIM [229850]) (Fryns et al. 1979; Goddeeris et al. 1980). Little is known about the etiology of CDH. Multifactorial inheritance has been suggested, although environmental factors such as toxin exposure also have been proposed as possible etiological factors (Sutherland et al. 1989; Solomon et al. 2000). There is increasing evidence for a genetic cause of diaphragmatic hernia. Various chromosomal anomalies have been described in CDH, with numerical abnormalities, such as trisomy 13, 18 and 21 being the most common. Structural chromosomal anomalies, involving almost all chromosomes, have been reported, reviewed by Lurie (2003).

Since 1988, pre- and postnatal data from all Erasmus Medical Center patients with CDH have been stored in a database. Information on clinical findings and environmental exposures has also been collected. Karyotyping of each patient with a congenital anomaly has become standard practice. The purpose of this study was to define possible candidate regions for CDH by means of complementary, high resolution, molecular cytogenetic techniques.

MATERIALS & METHODS

Since 1988, we have collected data from patients with a diaphragmatic defect (including posterolateral and medial hernia, and eventration of the diaphragm) seen in the Sophia Children's Hospital (Erasmus MC, Rotterdam, The Netherlands), a tertiary care facility for children. The database includes clinical data, data on associated anomalies, therapy/surgery procedures, pathology data and, if available, karyotypes. According to standard practice in children with congenital anomalies, blood samples were collected from patients and used for routine cytogenetic analysis, DNA isolation and, whenever possible, fibroblast or EBV-transformed cell line development. Blood from parents was also stored to allow future analysis.
This study focused on the subset of patients with a congenital diaphragmatic hernia and a chromosome 15q deletion. Cell lines were available from two of the three Rotterdam patients in the study while fixed post-mortem cells from a third patient were available.
We contacted the authors who previously described patients with CDH and a chromosome 15q deletion (Kristoffersson et al. 1987; Jong de et al. 1989; Rosenberg et al. 1992; Howe et al. 1996; Bettelheim et al. 1998; Chen et al. 1998; Aviram-Goldring et al. 2000; Schlembach et al. 2001) and obtained material from four additional patients as either paraffin embedded tissue (Jong de et al. 1989; Chen et al. 1998), cell line (Rosenberg et al. 1992) or genomic DNA (Schlembach et al. 2001; Van Dooren 2004). In addition to these seven cases, we studied two unrelated patients with a 15q deletion but without CDH manifestation.

Chromosome analysis

Karyotyping was performed on 200 patients with CDH of which 80 had multiple congenital anomalies. Chromosome analysis was performed at the 550-band level according to standard procedures with GTG-banded chromosomes from cultured peripheral blood lymphocytes.

Array-CGH

Genomic DNA was extracted from cultured cells or paraffin embedded tissues using the DNeasy Tissue Kit (Qiagen). After EcoRI digestion the DNA fragments were labeled (Random Prime Labelling Kit, Invitrogen). Array-CGH was performed using the 1 Mb Human BAC Array (Spectral Genomics Inc., Houston, USA) according to the manufacturer's instructions. A dye-swap and sex-mismatch experimental strategy were used as additional internal controls. The subtelomeric probes on the 1 Mb Array sometimes gave unreliable signals and the 15q subtelomeric probes were used in confirmatory FISH assays. Fluorescent signals on the arrays were obtained using the ScanArray Express HT scanner. Images were analyzed with Spectral Ware 2 (Spectral Genomics Inc., Houston, USA).

Fluorescent in situ hybridization (FISH)

To cover the distal part of chromosome 15, approximately 110 BAC clones were selected from the University of California Santa Cruz (UCSC) and Ensembl browsers. The BAC clones were purchased from BACPAC Resources or Invitrogen. DNA from these clones was isolated, amplified and, after digestion with MboI, the amplified BAC DNAs were labeled with biotin-16-dUTP or digoxygenin-11-dUTP by a modified labeling protocol (Random Prime Labeling Kit, Invitrogen). Probes were first hybridized to metaphase cells from healthy individuals to confirm their cytogenetic positions. From patients 1, 3 and 4, metaphase chromosomes were available. From patients 2, 5 and 6, interphase nuclei, isolated from the paraffin embedded tissue were available. From patient 7, only genomic DNA was available. Material from the two patients without CDH was available as genomic DNA and metaphase chromosome preparations. FISH slides were analyzed using the Axioplan 2 Imaging microscope (Zeiss) and images were collected using the Isis Software System (Metasystems).

RESULTS

Since 1988, data on 338 patients with CDH have been collected and stored in a database. Results from GTG-banded karyotypes were available from 200 patients, of which 24 patients (12%) had abnormalities. Ten patients (5%) had a trisomy 18, four patients (2%) had a trisomy 21, the remaining 10 cases (5%), had a structural chromosomal anomaly. Three of the latter patients were found to have a deletion of the long arm of one chromosome 15. In two patients, this was due to loss of material during the formation of a ring chromosome 15 (patients 2 and 3: both 46,XY,r(15)(p11q26)). The third patient had a complex abnormal karyotype with loss of part of chromosome 15 (patient 1: 46,XY,t(1;14),inv(6),del(15)(q26)).
We acquired material from four additional CDH patients with a 15q deletion. In these patients, three kinds of chromosome 15 anomalies were found. Patient 5 had a ring chromosome 15, with a deletion of 15q (46,XY,r(15)(p11q26)). Patient 7 had an isolated 15q deletion (46,XX,del(15)(q25q26.2)). Patients 4 and 6 had a deletion of 15q resulting from unbalanced segregation of a balanced parental chromosomal translocation (46,XX,der(15)t(3;15)(q29;q26.1); 46,XX,der(15)t(8;15)(q24.1;q26.1)) with gain of additional

material from chromosomes 3 and 8, respectively. Of the two patients without a diaphragmatic hernia, the first one had a ring chromosome 15 (patient 8: 46,XX,r(15)(p11.1q26.3)). The second one had a de novo deletion of 15q (patient 9: 46,XY,del(15)(q26)) (Table 1).

Table 1. Clinical and cytogenetic data for patients with deletions involving chromosome 15

Patient	Karyotype	CDH[a]	Other abnormalities	Deletion size (Mb)
1	46,XY,t(1;14),inv(6),del(15)(q26)	Yes	Genital anomalies; IUGR[b]	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	23.0
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

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 = intra-uterine growth retardation

All patients had other congenital anomalies besides the diaphragmatic hernia; however, the CDH was left-sided in all cases. None of the patients survived beyond the neonatal period. Patients 3 and 6 died after termination of pregnancy because of multiple severe congenital abnormalities. Patient 5 was stillborn. All patients had intrauterine growth retardation (below the 3rd percentile) and dysmorphic features (Table 1). Other abnormalities that were present included cardiac, renal and genital abnormalities, limb deformities, and hydrocephalus. Both patients without CDH have mental retardation and mild dysmorphic features of which only the r(15) patient (patient 8) had growth-retardation.

To delineate the possible candidate region for CDH on chromosome 15, we performed array-CGH on patients 1,2,3,6,7, and one of the non-CDH patients (patient 8) (Figure 1). The interstitial deletion, found by karyotyping in patient 1 was confirmed with array-CGH. This deletion was shown to be flanked by BAC clones RP11-79A7 and RP11-90E5 on the array-CGH platform used (Figure 1a). The chromosome 15 deletions in patients 2 and 6 extended from RP11-79A7 towards the telomere. The chromosome 15 deletion in patient 3 extended from RP11-300G22 (Figure 1b) and in patient 4 from RP11-533L13 (Figure 1c). In patient 7 the deletion extended from RP11-360F18 (Figure 1d). The deletion

in the first patient without CDH (patient 8) extended from RP11-120N1 to the distal chromosome 15 terminus (Figure 1e).

To further define the deleted regions we selected 110 BAC clones spanning 15q. By using the appropriate BAC clones, we performed fluorescence in situ hybridization on metaphase chromosomes to refine the size of all deletions found on the array and to determine the deletions in patients 4, 5, and 9 (Figure 2). On the material extracted from the paraffin-blocks, we performed interphase-FISH (Figure 2). From patient 7 only genomic DNA was available and therefore the size of the 15q deletion in this patient was only determined by array-CGH.

In the six patients with a terminal deletion, we were able to approximately determine the proximal breakpoint. In patient 1, the interstitial deletion was found to be 6 Mb in size, between BAC clones RP11-79A7 and RP11-616M17. The deletion in patient 2, extended from a region distal to BAC clone RP11-79A7 and in patient 3 from a region distal to RP11-300G22. The breakpoint in patient 4 lies exactly within BAC clone RP11-617F23 which is partly present. In patient 5, the break occurs distal from 0RP11-300G22. In patient 6, the most distal probe present on the deleted chromosome 15 is RP11-79A7. The terminal deletion in patient 7 occurs distal to RP11-360F18. The proximal boundary of the deletion interval in the two non-CDH patients was similarly determined. In the first non-CDH patient with a ring chromosome 15, the most distal BAC clone present on the ring chromosome was RP11-120N1. In the second non-CDH patient, the most distal BAC clone present was RP11-262P8. By combining FISH and array-CGH data from all nine patients, we determined the smallest common deletion interval (Figure 3). This critical deletion interval at 15q26.1-26.2 in patients with CDH (which we have termed the CDH-region) is approximately 5 Mb in size and is bordered by BAC clones RP11-152L20 and RP11-262P8.

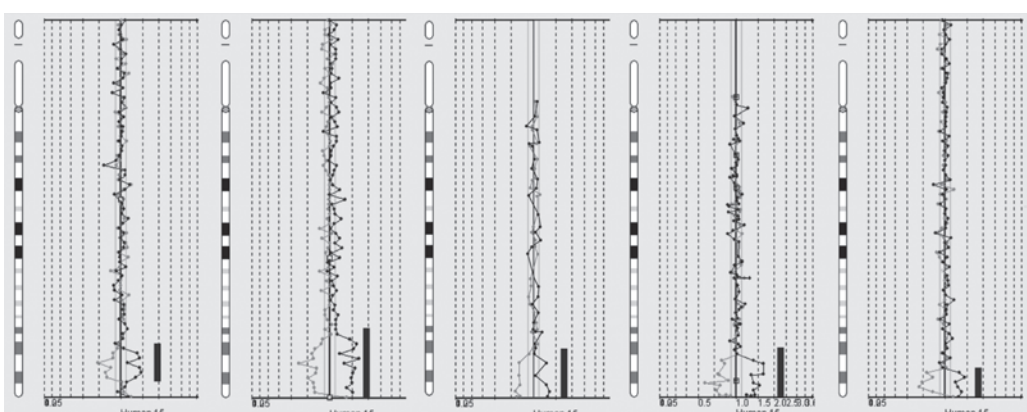

Fig.1. Array-CGH
a: Patient 1, with CDH and del(15) interstitial deletion. **b**: Patient 3, with CDH and r(15)(p11q26). **c**: Patient 4, with CDH and der(15)t(3 ;15)(q29 ;q26.1). **d**: Patient 7, with CDH and del(15)(q25q26.3). **e**: Patient 8, without CDH and with r(15)(p11.1q26.3).
Color figure can be found in the appendix. See page 130.

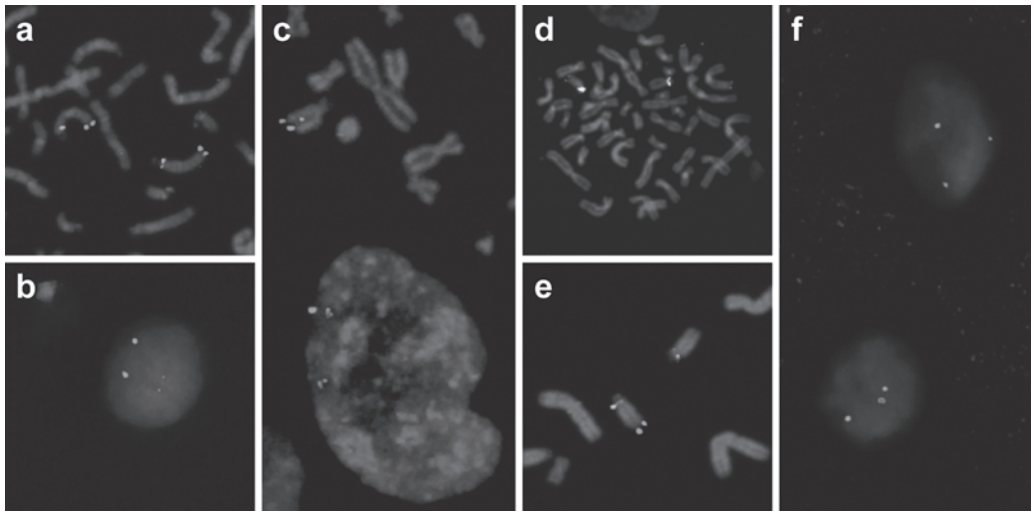

Fig. 2. FISH results.
a: Patient 1, partial metaphase, probe D15Z4 (*red signal*) at chromosome 15 centromeric locus and probe RP11-114I24 (*green signal*) at 15q26.3. **b**: Patient 2, interphase, probe RP11-369K8 (*red signal*) and RP11-253B9 (*green signal*) near the chromosome 5 centromeric region 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. Color figure can be found in the appendix. See page 131.

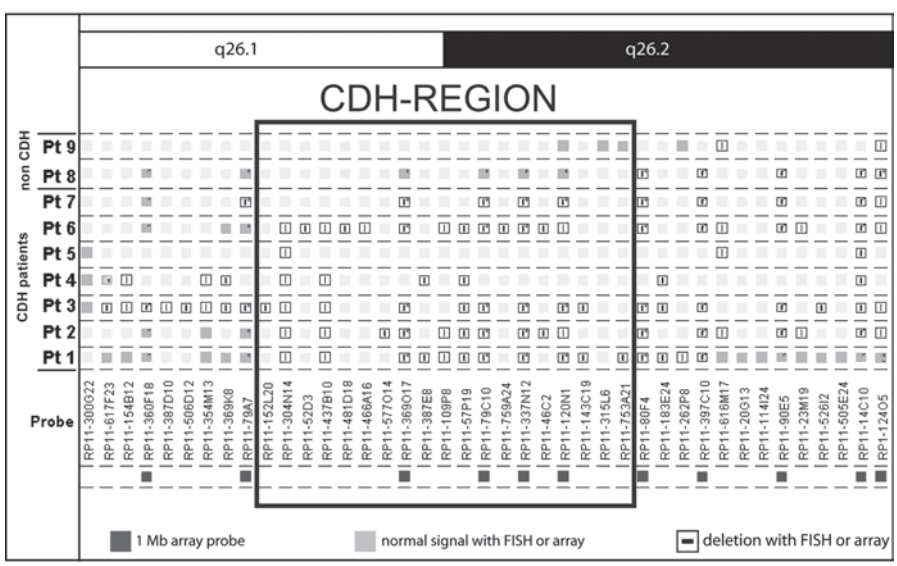

Fig. 3. CDH-region.
Schematic representation of the critical CDH region, with partial ideogram of chromosome 15q. Patients (Pt) 1 – 7 have CDH; patients 8 and 9 do not have CDH. BAC clones that were tested by array CGH and FISH are listed. The black dots inside boxes indicate that probes have been tested only on the array and not by FISH. The smallest common overlapping deletion interval involved in CDH is denoted by the large red square. Color figure can be found in the appendix. See page 131.

DISCUSSION

Unlike many genetic disorders in which candidate genes can be determined using linkage analyses on familial cases, the vast majority of congenital diaphragmatic hernia occurs *de novo*. For such disorders, the best way to determine which genes are involved is by analyzing a large number of patients for common aberrations with 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 (OMIM [214800]) (Vissers et al. 2004) and Cornelia de Lange syndrome (CdLS, OMIM [122470]) (Krantz et al. 2004; Tonkin et al. 2004).

Our study is the first to analyse data from multiple patients with CDH and with 15q deletions, using these complementary techniques. Isolated reports of distal chromosome 15 deletions have been described (Kristoffersson et al. 1987; Jong de et al. 1989; Rosenberg et al. 1992; Howe et al. 1996; Bettelheim et al. 1998; Chen et al. 1998; Aviram-Goldring et al. 2000; Schlembach et al. 2001), suggesting involvement of this region in the pathogenesis of CDH. Recently, Biggio et al. described a patient with a right-sided CDH and an associated coarctation of the aorta with a deletion of band 15q26, narrowing the possible CDH candidate region on this chromosome (Biggio et al. 2004). By using array-CGH, along with systematic FISH analysis, on 7 CDH cases with 15q deletions and including two 'control' patients with a 15q deletion but without CDH, we have refined the chromosome 15q26 critical region to a 5 Mb area within 15q26.1-26.2. Clinical evaluation of the seven patients with CDH revealed a left-sided diaphragmatic defect of the Bochdalek-type, intrauterine growth retardation (all patients had birth weights below the 3rd percentile) and other multiple congenital malformations (Table 1). In all patients the clinical abnormalities resemble the features described previously in other patients with a 15q deletion, with or without CDH (Roback et al. 1991), ring chromosome 15-syndrome (Wilson et al. 1985; Butler et al. 1988), and other syndromes such as Fryns syndrome (Fryns et al. 1979; Goddeeris et al. 1980; Moerman et al. 1988; Slavotinek 2004).

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 (interstitial) deletion, does not have many other defects in addition to the diaphragmatic hernia. All other patients have deletions that are similar in size with a similar spectrum of anatomical anomalies. In the two patients with an unbalanced translocation, this variation in phenotype could also be, in part, the result of partial duplication of another region. As can be expected, the r(15) patients, with or without CDH, had phenotypes similar to the ring chromosome 15-syndrome, as described previously (Butler et al. 1988; Roback et al. 1991; Rogan et al. 1996). To our knowledge, congenital diaphragmatic hernia has never been described previously in ring chromosome 15 syndrome. It would be interesting to study the data and perform analysis on more patients with a ring 15 chromosome.

Duplicons are known to promote pathogenic rearrangements (Stankiewicz and Lupski 2002). However, there are no known repeat clusters in or near the CDH critical region, which makes this proposed molecular mechanism less likely for these patients. In patients 3, 4, 6, and 7 the proximal breakpoints are at approximately the same location, but the deletions we have found do not resemble the ones found in other known syndromes, so a different mechanism may be involved.

The region of the smallest common deletion (the CDH-region) contains four known genes. None of these genes have been described previously in studies on diaphragm development or diaphragmatic hernia. With respect to their structure and function, two of these genes are very interesting in potential relation to CDH. The first gene, *NR2F2* (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). This gene has been suggested to play a role in pulmonary vascular development (Tuyl van 2004). A knockout mouse model of NR2F2 showed that NR2F2-/- mice are not viable and die at E9 in utero, due to 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 described in the original publication (Pereira et al. 1999; Cooney et al. 2001). The second interesting gene is the chromodomain helicase 2 (*CHD2*) gene, a member of the SNF2/RAD54 helicase family. Recently, mutations in another member of this family (*CHD7*) have been found to cause the CHARGE-syndrome (Vissers et al. 2004). Looking at the similar structure of the two genes of this family, in particular the presence of the helicase domain, it is possible that a similar mutation in the *CHD2* gene is responsible for causing CDH. The third gene in this region is the repulsive guidance molecule, *RGMa,* which is involved in the guidance of growth cones of 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 sialyltransferase 8B (*SIAT8B*), a gene that plays a role in the adhesive properties of neural cell adhesion molecules (Angata et al. 1997).

Haploinsufficiency due to loss of one 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. suggested that the myocyte enhancer factor 2A, *MEF2A*, could be involved in the pathogenesis of diaphragmatic defects (Biggio et al. 2004). *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 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 CDH critical region, which limits their possible role in CDH.

Very recently, two CDH patients with a 15q deletion were described, independent from each other (Hengstschlager et al. 2004; Tonks et al. 2004). It would be very interesting to see if these two patients have a deletion similar to the ones found in our group.

This study describes the molecular cytogenetic analyses of a group of patients with 15q deletions and CDH. In conclusion, we have mapped a potential CDH critical 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 mutation analysis 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 outcome and could provide more information for genetic counseling.

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Electronic-database information

Accession number and 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/

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CHAPTER 3

Letter to the editor: Reply to Castiglia et al.

M. Klaassens, D. Tibboel, B.A. Oostra and A. de Klein

American Journal of Human Genetics (2005) 77: 892 – 894

Written in respons to the following publication:
"Narrowing the candidate region for congenital diaphragmatic hernia in chromosome 15q26: contradictory results"
L. Castiglia, M. Fichera, C. Romano, O. Galesi, L. Grillo, M. Sturnio and P. Failla
American Journal of Human Genetics (2005) 77: 892 – 894
(this publication can be found in the appendix)

In response to our article in the May issue of the *Journal* (Klaassens et al. 2005), Castiglia *et al* (2005) address the strategy of including patients with a 15q deletion but without congenital diaphragmatic hernia (see original publication in the appendix). They defined a deletion on chromosome 15q26.1-26.2 in a girl with multiple congenital anomalies but without CDH. Combining data from this patient with previously published data from two patients with a 15q deletion but without CDH (Rogan et al. 1996; Tonnies et al. 2001), Castiglia *et al.* (2005) found a discrepancy between our data and the CDH locus that they determined. Of the two hypotheses postulated to explain these contradictory results, we support the first one which suggests that including patients without CDH in the analysis might be inappropriate because of the possibility that heterozygous deletion of a part of 15q (which results in haploinsufficiency for this locus) might not be completely penetrant. Incomplete penetrance could also explain, in part, the variability in phenotype of patients with CDH and a 15q deletion.

Since the publication of our article, we have been able to more precisely define the deletions in our patients with CDH. With CDH patients only, a 4Mb common CDH-region would be located between BAC clones RP11-44A22 (overlapping with RP11-641M8 & RP11-261M12; see Figure 1 in Castiglia *et al*) and RP11-616M17 (data not shown), with the telomeric boundary determined by the interstitial deletion of patient 1 (patient 8 in Figure 1 in Castiglia *et al*). We, therefore, excluded some genes from the region, including the SIAT8B gene (MIM 602546) suggested by Castiglia *et al* (2005) as one of the candidate genes for CDH. The remaining region still contains NR2F2, IGF1R and three hypothetical genes. We are in the process of screening, with mutation analysis, all genes in this deleted region in a large group of CDH patients and screening with FISH for deletions. Of the genes located in this region, we still consider NR2F2 to be the most likely candidate. The recent report by Tümer *et al* supports this hypothesis. They analyzed three ring carriers, one of which had a different phenotype than the other two patients (Tumer et al. 2004). This third patient had a CDH and other anomalies, and the deletion included the same genes as in the other two patients, except for the NR2F2 gene, which was deleted only in the patient with CDH. In contrast to the opinion of Castiglia *et* al (2005) we believe ring carriers can provide valuable clues in the search for chromosomal loci that could be involved in the etiology of congenital anomalies. Although ring chromosomes can be unstable we have not observed gain or loss of other genetic material. In addition, the new chromosomal telomeric DNA of derivative chromosomes in unbalanced translocations could be of influence.

In conclusion, we hypothesize that 15q26.1-26.2, a gene-poor region, plays an important role in the etiology of CDH. Haploinsufficiency of this region might not be completely penetrant. We still propose NR2F2 to be the most likely candidate, but disruption of a regulatory element or other gene in this region cannot be excluded as a cause of CDH.

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CHAPTER 4

Prenatal detection and outcome of congenital diaphragmatic hernia (CDH) associated with deletion of chromosome 15q26: two cases and review of the literature

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submitted

ABSTRACT

Congenital diaphragmatic hernia (CDH) is a severe birth defect characterized by a defect in the diaphragm with pulmonary hypoplasia and postnatal pulmonary hypertension. Approximately 50% of CDH cases are associated with other non-pulmonary congenital anomalies (so called non-isolated CDH) and in 5-10% of cases there is a chromosomal etiology. The majority of CDH cases are detected prenatally. In some cases prenatal chromosome analysis reveals a causative chromosomal anomaly, most often aneuploidy. Deletion of 15q26 is the most frequently described structural chromosomal aberration in patients with non-isolated CDH.

In this paper we report two patients with a deletion of 15q26 and phenotypes similar to other patients with CDH caused by 15q26 deletions. This phenotype consists of intra-uterine growth retardation, left-sided CDH, cardiac anomalies and characteristic facial features, similar to those seen in Fryns syndrome. We propose 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, since the diagnosis of this deletion will have major consequences for the prognosis and, therefore, can affect decision making.

INTRODUCTION

Congenital Diaphragmatic Hernia (CDH, MIM 142340) is a relatively common birth defect with an incidence of 1 in 3000 live births (Moya and Lally 2005). CDH is characterized by a defect in the diaphragm with associated pulmonary anomalies (hypoplasia and abnormal vascular structure) and postnatal pulmonary hypertension. The overall mortality for CDH has been reported as being around 50% (Torfs et al. 1992). With advances in treatment including improvements in ventilator-strategies, the survival of infants with isolated CDH has improved (Doyle and Lally 2004). However, mortality for children with non-isolated CDH still remains high.

Approximately 50% of isolated CDH cases are detected prenatally. When additional congenital anomalies are also present, this percentage increases to 84% (Tonks et al. 2004). In about 10-12% of the prenatally detected cases a chromosomal anomaly is identified (Witters et al. 2001). Most of these chromosomal anomalies are aneuploidies (60%), but, using high-resolution molecular cytogenetic techniques, an increasing number of structural submicroscopic chromosomal anomalies are detected.

In our hospital, from 1996 until 2006, 96 CDH cases were detected prenatally. Thirty-three of these diagnoses (~ 34%) were made before 24 weeks of gestation, before which a termination of pregnancy is allowed in the Netherlands. In total, 20 couples choose to terminate the pregnancy before a gestational age of 24 weeks and in one case a late termination was performed (at 32 weeks) because of anencephaly and CDH. In most cases, the presence of associated congenital anomalies and/or a chromosomal anomaly has a major impact on the decision making. It is known that when CDH is associated with other congenital anomalies and/or a chromosomal anomaly, this may affect treatment decisions, such as the decision to terminate the pregnancy or the decision not to intervene after birth (Witters et al. 2001).

In this letter we present two patients with a deletion of 15q26, one of the most common structural chromosomal anomalies in children with non-isolated CDH, and review the literature available on patients with 15q26 deletions and CDH. Our review suggests that these children share many phenotypic characteristics of which several can be diagnosed by advanced fetal ultrasound examination. We discuss the impact of this diagnosis on treatment decisions and outcome.

CLINICAL REPORTS

Patient 1

Patient 1 is a female child born to a healthy 25-year-old mother and a healthy 30-year-old father with a normal family history. Parents are unrelated. Mother was G1P0. At the gestational age of 33 weeks, routine prenatal ultrasound examination was performed because of the suspicion of intra-uterine growth retardation (IUGR), which was confirmed. In addition, an advanced ultrasound examination revealed an abnormally small cerebellum, a left-sided diaphragmatic hernia and a ventricular septal defect with an overriding aorta. Amniocentesis was performed for chromosomal analysis and a normal 46,XX karyotype was found. Routine prenatal and obstetric care was carried out, and the child was born after a spontaneous delivery at 42 1/7 weeks. She had severe growth retardation with a birth

weight of 1750 gram (<< 2.3rd percentile). According to standardized treatment for CDH she was intubated at birth and standard ventilator care for CDH was initiated. Additional congenital anomalies identified on physical examination were dysmorphic features - slightly coarse facies, broad nose with a flat nasal bridge and hypertelorism - and hypoplastic toenails. Based on these findings a diagnosis of Fryns syndrome was suggested. Unfortunately, no definite syndromic diagnosis could be made at that time. A cardiac ultrasound of this patient showed a double-outlet right ventricle with a small left ventricle, an atrial septal defect, a ventricular septal defect, a hypoplastic aorta and a persistent duct of Botalli. Despite intensive treatment it was not possible to stabilize the child. Extra-corporeal membrane oxygenation (ECMO) treatment was not initiated because of the poor prognosis due to CDH in combination with severe pulmonary hypertension and a complex cardiac anomaly. The patient died at the age of 7 hours. Although parents did not consent for postnatal photography, MRI, or autopsy, consent was obtained for further cytogenetic analysis.

Patient 2

Patient 2 is a female child born to a healthy 33-year-old mother and a healthy 34-year-old father with a normal family history. Parents are unrelated. Mother was G2P1. Their first child is a healthy 2-year old girl. A routine prenatal ultrasound examination in the second trimester showed intra-uterine growth retardation, a left-sided diaphragmatic hernia, cardiac abnormalities - VSD, single atrial/ventricular valve, and hypoplastic aorta - and club feet. Amniocentesis was performed and G-banded chromosome analysis revealed the presence of part of chromosome Y heterochromatin on the long arm of chromosome 15q. The subtelomeric molecular probe for 15q gave only 1 signal, so the karyotype is 46,XX,der(15)t(Y;15).

Patient 2 was delivered at 39 weeks of gestation. Because of the severe IUGR (1300 gram, which is << 2.3rd percentile) and therefore no eligibility for ECMO, parents agreed to the decision not to initiate treatment and the child died peacefully at the age of 1.5 hours. Postnatal clinical and radiological examination confirmed the presence of CDH, club feet (talipes) and the cardiac anomaly, and also showed the dysmorphic features of hypertelorism with wide nasal bridge, “pinched” appearance of the nose, down-turned corners of the mouth, micrognathia, slightly posteriorly rotated ears and widely spaced nipples (see Figure 1). Also noted were bilateral second and fifth finger clinodactyly and overlapping toes with the first toe overlapping the second and the fifth toe overlapping the fourth bilaterally. No postnatal MRI or autopsy were performed precluding the identification of other internal malformations. Postnatal pictures were taken (see Figure 1) and parental consent was obtained for further clinical and cytogenetic analysis.

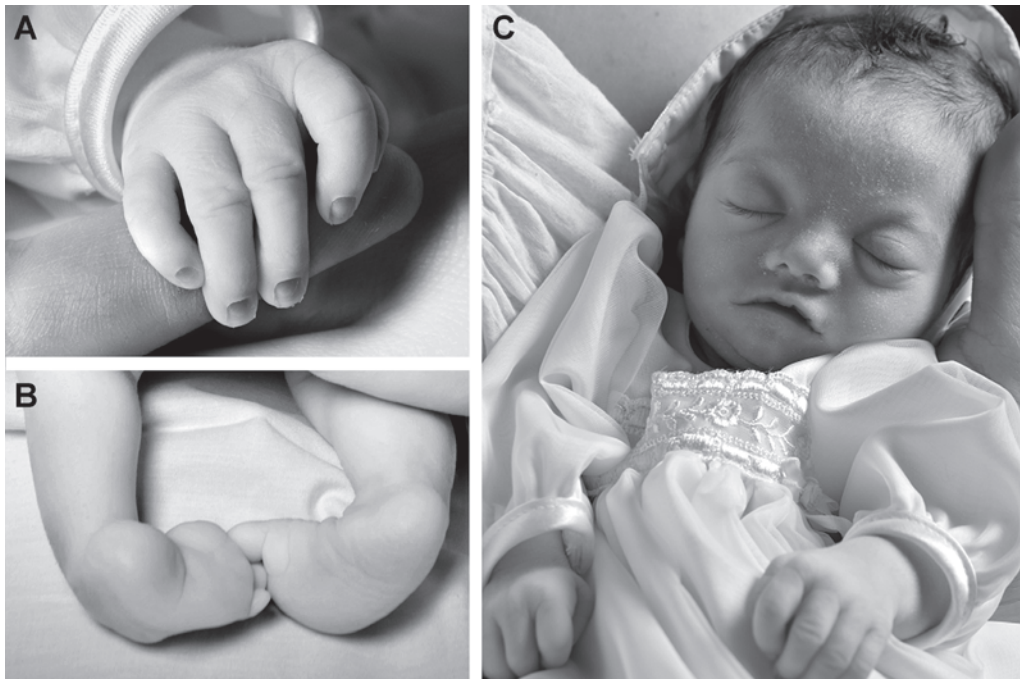

Fig. 1. Clinical features of Patient 2. A, clinodactyly second and fifth finger; B, bilateral club feet; C, facial features. Note the mild coarse facial features with hypertelorism with wide nasal bridge, "pinched" appearance of the nose, down turned mouth and mild micrognathia. Color figure can be found in the appendix. See page 132.

CYTOGENETIC ANALYSIS

Materials & Methods

G-banded chromosome analysis of cultured amniocytes was performed according to standard procedures. To confirm the results obtained by G-banding, and to map the breakpoints more accurately, BAC clones were selected from a database of BAC probes previously used in our study on the CDH critical region on chromosome 15 (Klaassens et al. 2005a; Klaassens et al. 2005b). For the regions on chromosome 2 and Y, BAC clones were selected from the University of Santa Cruz (UCSC) genome browser and ordered from BACPAC Resources. After confirmation of their cytogenetic locus they were used for Fluorescence *In Situ* Hybridization (FISH) on amniocytes. Patient 1 was also tested using the Human Genome CGH 44k Oligo Microarray Kit (Agilent), according to the manufacturer's instructions. The chromosomal anomalies identified by array-CGH in Patient 1 were also confirmed by quantitative-PCR using the LightCycler System (Roche Molecular Diagnostics), and by Multiplex Ligation-dependent Probe Amplification (MLPA)-analysis in an experiment using the P036B and P070 Salsa telomere kit (MRC Holland). In Patient 2 the presence of Y chromosome material was confirmed by PCR of the AZF regions (normally used to detect Y deletions in infertile men) using the method that has been described by Simoni *et al.* (Simoni et al. 1999).

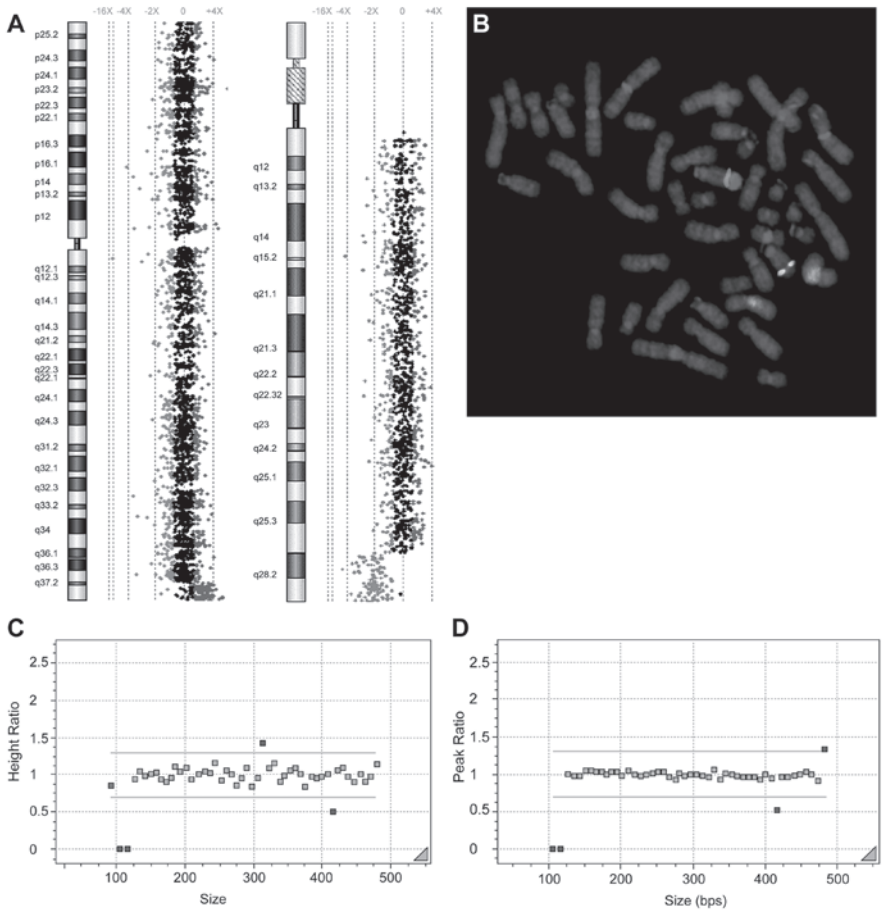

Fig. 2. Results of molecular cytogenetic analysis. A: Oligonucleotide-based ArrayCGH results for Patient 1: gain of 2q37 (left panel) and loss of 15q26 (right panel)(Scott et al. 2007); B: Fluorescent In Situ Hybridization results for Patient 2. Loss of RP11-154B12 on chromosome 15q26.1 (green signal) and presence of RP11-99M1 on chromosome Yq22 (red signal) with aspecific signals on chromosome X (light red patches); C: Multiplex Ligation-dependent Probe Amplification (MLPA) results for Patient 1 (gain of CAPN10 on 2q37 and loss of ALDH1A3 on 15q26); D: MLPA results for Patient 2 (gain of SYBL1 on Yq and loss of ALDH1A3 on 15q26). Color figure can be found in the appendix. See page 133.

RESULTS

Patient 1

Initial G-banding of cultured amniocytes showed no chromosomal abnormalities. Parents decided to participate in our CDH study and material from Patient 1 was screened by oligonucleotide array-CGH. This analysis revealed a ~7.2 Mb gain of the distal part of chromosome 2q and a ~7.8 Mb loss of the distal part of chromosome 15q, suggestive for an unbalanced translocation of chromosomes 2 and 15 (see Figure 2). Because of a limited amount of amniocytes available only one FISH experiment was performed to confirm the duplication and deletion. One copy of BAC clone RP11-45L20 on chromosome

2q37 was duplicated and one copy of BAC clone RP11-57P19 on chromosome 15q was shown to be deleted, thereby confirming the array-CGH results (data not shown). A more specific mapping of the breakpoints was performed by quantitative-PCR. This part of the molecular (cyto)genetic analysis of this patient has been recently described (patient N9 from Scott *et al.*(2007)). MLPA-analysis confirmed the abnormality (see Figure 2).

Patient 2

G-banding of cultured amniocytes revealed the presence of heterochromatin from the long arm of chromosome Y on one copy of chromosome 15q. FISH analysis showed the presence of a ~40.3 Mb portion of the long arm of chromosome Y on a derivative chromosome 15. The most centromeric probes present were RP11-99M1 and RP11-224C16 located at Yq11.22. The most telomeric region present was the Y heterochromatin. The terminal deletion of chromosome 15q26 started at BAC clone RP11-154B12, located on band 15q26.1. The most telomeric probe tested, RP1-154P1, was deleted. PCR of the AZF regions confirmed the presence of the breakpoint on chromosome Y. The ZFX/ZFY region was tested as an internal control and gave a unique band. As expected, the SRY gene (marker sY14) was absent. The AZFb (markers sY127 and sY134) and AZFc (markers sY254 and sY255) regions were both present, but the AZFa (markers sY84 and sY86) region was absent. Chromosome analysis of both parents showed no abnormalities, resulting in the following karyotype for this patient: 46,XX,der(15)t(Y;15)(q11.22;q26.1)*de novo*

DISCUSSION

Generally, the prognosis of children with multiple congenital anomalies (MCA) changes dramatically whenever a chromosomal anomaly is detected. In this paper we report two patients with a terminal deletion of chromosome 15q26 and non-isolated CDH. Deletion of 15q26 is the most commonly described unbalanced structural anomaly (25 cases) in patients with CDH and has a dramatic impact on mortality (see Table 1) (Kristoffersson et al. 1987; de Jong et al. 1989; Rosenberg et al. 1992; Howe et al. 1996; Bettelheim et al. 1998; Chen et al. 1998; Aviram-Goldring et al. 2000; Schlembach et al. 2001; Biggio et al. 2004; Hengstschlager et al. 2004; Tonks et al. 2004; Tumer et al. 2004; Klaassens et al. 2005b; Pober et al. 2005; Slavotinek et al. 2005; Elghezal 2006; Lopez et al. 2006; Slavotinek et al. 2006).

Because of striking similarities between the two children described in this paper, we reviewed the literature to determine a possible phenotype-genotype relationship for 15q26 deletions. In those cases in which CDH is accompanied by a 15q26 deletion, multiple associated anomalies are described and it is clear that this deletion leads to some degree of phenotypic heterogeneity as has been described for other deletions, such as deletions of 22q11 (Momma 2007). The heterogeneity among the patients with a deletion 15q26 cannot completely be explained by the size of the deleted segment alone, since some individuals with small deletions have a greater number of associated anomalies than individuals with larger deletions. As most deletions of 15q26 are not “pure” monosomies, but the result of unbalanced translocations or the formation of a ring chromosome, we cannot exclude the possibility that part of the phenotypic heterogeneity is caused by the influence of associated segmental aneuploidies. Nevertheless, to some degree it is possible to describe a phenotype-genotype relationship for 15q26 deletions since the majority of the patients share several phenotypic characteristics (for an overview see Table 1).

Table 1. Clinical features patients with deletion 15q26

Author	Size deletion	Trisomy(*)	Side CDH	Lung hypoplasia	Percentile birth weight
"Pure monosomy"					
Klaassens (2005) / 1	6.3	n.r.	L	+	< P2.3
Slavotinek (2005 JMG) / 1	~ 8.0	n.r.	L	+	< P10
Slavotinek (2005 JMG) / 2	~ 8.0	n.r.	L	+	90
Slavotinek (2006 EJHG) / case 3	~ 8.0	n.r.	L	+	NA
Biggio (2004)	~13.0	n.r.	R	+	< P2.3
Tonks (2004)	~13.0	n.r.	NA	NA	NA
Schlembach (2001)	22.3	n.r.	L	+	< P2.3
Bettelheim (1998) / 1	> 25	n.r.	L	+	< P2.3
Bettelheim (1998) / 2	> 25	n.r.	L	+	< P2.3
Hengstschläger (2004)	> 25	n.r.	L	+	< P2.3
Total for this subgroup				*9/9*	*7/8 IUGR*
Monosomy 15q resulting from an unbalanced translocation					
This publication / 1	7.8	2q37.2	L	+	< P2.3
This publication / 2	11.2	Yq11	L	+	< P2.3
Chen (1998)	16.8	8q24.1	L	+	< P5
Rosenberg (1992)	22.8	3q29	L	+	< P2.3
Howe (1996)	~ 25	17q23.3	L	NA	NA
Kristofferson (1987) / 1	> 25	6p25	L	+	< P2.3
Kristofferson (1987) / 2	> 25	6p25	L	+	+ (Pval NA)
AviramGoldring (2000) / 1	> 25	5p15.3	L	+	NA
AviramGoldring (2001) / 2	> 25	5p15.3	L	+	NA
Total for this subgroup				*8/8*	*6/6 IUGR*
Ring chromosome 15					
Tümer (2004)	~ 6.4	n.r.	L	+	< P2.3
Lopez (2006)	~ 13.0	n.r.	L	+	+ (Pval NA)
Elghezal (2006)	~ 15.0	n.r.	L	+	< P2.3
Klaassens (2005) / 2	19.9	n.r.	L	+	< P2.3
De Jong (1989)	23.0	n.r.	L	+	< P2.3
Klaassens (2005) / 3	23.3	n.r.	L	+	< P2.3
Total for this subgroup				*6/6*	*6/6 IUGR*
Total			**23/24**	**23/23**	**19/20 IUGR**

*, in case of unbalanced translocation; n.r., not relevant; NA, data not available or not mentioned in the original publication; L, left; R, right; P2.3, 2.3th percentile; P10, 10th percentile; IUGR, Intra-Uterine Growth Retardation; SUA, single umbilical artery; F, female; NND, neonatal death; TOP, termination of pregnancy; ^, alive but ventilator dependant; †, deceased

Cerebral anomalies	Dysmorphic features	Cardiac anomalies	Kidney anomalies	SUA	Cryptor-chidism	Limb anomalies	Outcome
-	-	-	-	-	+	-	NND
-	-	-	-	+	F	-	Stillbirth
-	-	-	-	+	F	-	NND
-	+	+	-	+	NA	+	NA
-	+	+	-	-	F	+	14 mo^
NA	NA	NA	NA	NA	NA	NA	NA
NA	+	+	+	-	F	+	NND
-	NA	-	-	+	NA	-	Stillbirth
-	NA	-	-	+	F	-	NND
-	+	+	+	NA	F	+	NND
0/9	*4/7*	*4/9*	*2/9*	*5/8*	*1/1*	*4/9*	***† 7/8***
NA	+	+	NA	-	F	+	NND
NA	+	+	+	-	F	+	NND
+	+	+	+	-	F	+	TOP
NA	+	+	NA	+	F	+	NND
NA	NA	NA	NA	NA	F	NA	TOP
+	+	-	-	-	+	+	NND
-	+	-	+	+	-	-	TOP
NA	+	NA	+	NA	NA	NA	TOP
NA	+	NA	-	NA	NA	NA	TOP
2/3	*8/8*	*4/6*	*4/6*	*2/6*	*1/2*	*5/6*	***† 9/9***
NA	-	+	+	+	-	+	Stillbirth
NA	NA	+	NA	NA	F	+	TOP
-	+	-	+	-	F	-	TOP
+	+	+	-	-	+	+	NND
-	+	-	+	-	+	+	Stillbirth
-	+	+	-	-	F	-	TOP
1/4	*4/5*	*4/6*	*3/5*	*1/5*	*2/3*	*4/6*	***† 6/6***
3/15	**16/20**	**12/21**	**9/20**	**8/19**	**4/6**	**13/21**	**† 24/25**

In the majority of the cases where CDH is caused by deletion of 15q26, the diaphragm defect is located on the left-side (92%, slightly higher than reported in other studies) (Harmath et al. 2006). The majority (19/20, see Table 1) of the children with CDH and a 15q26 deletion have intra-uterine growth retardation (IUGR), usually with a birth weight far below the 2.3rd percentile. The majority of the children that are born with a congenital anomaly are growth retarded at birth, but in the case of 15q26 deletion this severe growth retardation is most likely the result of loss of one copy of the IGF1-receptor gene (*IGF1R*). It is known that there is a dosage-effect for the *IGF1R* gene and that haploinsufficiency for *IGF1R* results in severe pre- and postnatal growth retardation (Harada et al. 2002).

The two new cases we report here, and most of the other 15q26 deletion patients, have dysmorphic features (16/21, see Table 1). Although this appears to be more frequent in the group of patients with an unbalanced translocation, the abnormal facial characteristics that are described, appear to be similar between all patients and include coarse facial features with hypertelorism and posteriorly rotated abnormal shaped ears. The facial features of the patients described here could be classified as 'Fryns-like'. Fryns Syndrome is a CDH-related syndrome for which no gene has been identified. However, several genetic loci, including 15q26, have been suggested as possible loci for this syndrome (Slavotinek et al. 2005; Kantarci et al. 2006).

Limb defects are seen in several (13/21, see Table 1) patients with 15q26 deletions, regardless of whether they have a "pure" monosomy 15q26 or an unbalanced translocation. These defects are mainly talipes (club feet) or rockerbottom feet. Some patients have nail hypoplasia or distal digit hypoplasia, a characteristic also described in Fryns syndrome patients. It is difficult to establish the exact percentage of nail hypoplasia in the cohort of 15q26 deletion patients since most authors do not specifically mention the presence or absence of this feature. The occurrence of limb defects is higher (~ 62%) than in the total CDH population in which an incidence of 10-18 % is reported (van Dooren et al. 2003). With regards to the etiology of limb defects no candidate genes within the region on 15q26 have been described so far.

In approximately 57% (12/21) of the cases an associated cardiac anomaly was present (see Table 2), compared to ~23% in the total population of patients with non-isolated CDH (Witters et al. 2001). There are no major differences between the types of cardiac defects seen in the three groups of patients, although cardiac anomalies in general appear to be a bit less frequent in children with "pure" monosomy 15q26 compared to the other two groups (44% of the patients with "pure" monosomy vs. 67% of the patients with an unbalanced translocation or a ring chromosome 15). The combination of a diaphragm defect and a cardiac anomaly has been described in several CDH animal models, such as the Rarα/β-knockout mouse, the Coup-tfII knock-out mouse and in the nitrofen rat model (Mendelsohn et al. 1994; Pereira et al. 1999; You et al. 2005). *COUP-TFII* is located in the common deleted region for all patients (see Figure 3).

Single umbilical artery (SUA) appears to be more frequent in patients with a "pure monosomy" of 15q26 (63% of the patients with "pure" monosomy and 33% and 20%, respectively, in the other two groups). SUA occurs in approximately 0.3-2% of all pregnancies, but is known to be more frequent in children with aneuploidies if the SUA is associated with other structural anomalies detected by ultrasound

(Cristina et al. 2005). No known major malformation or structural chromosomal anomaly is invariably associated with SUA.

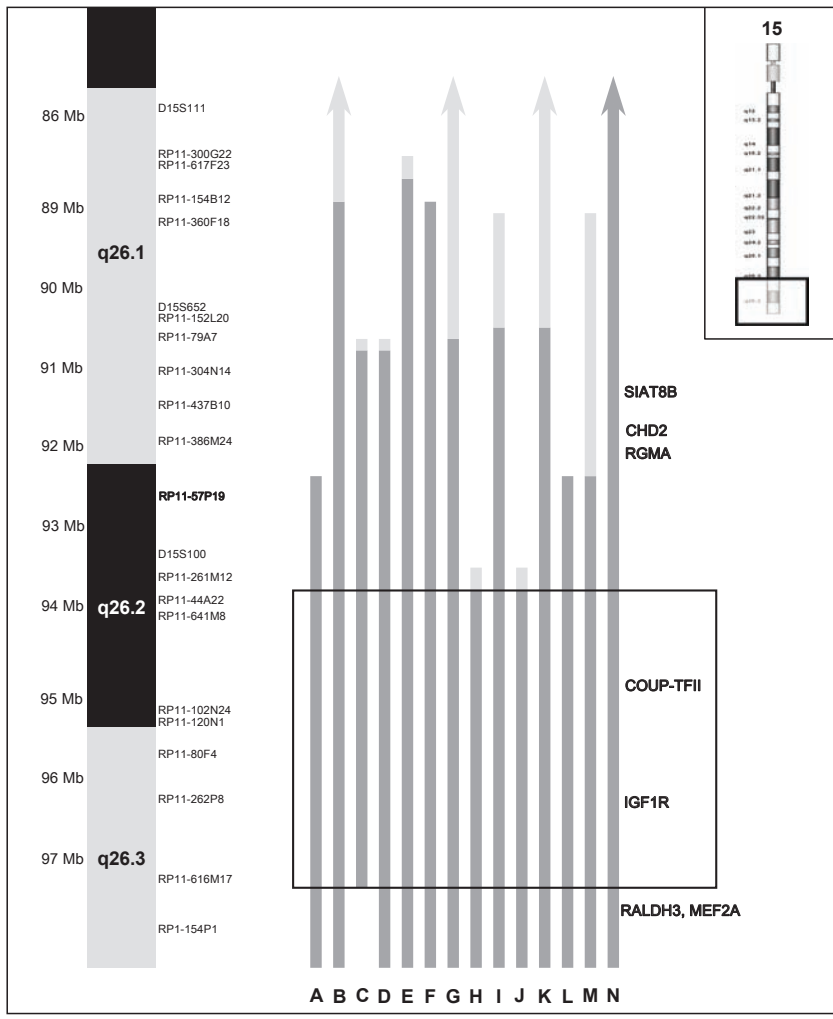

Fig. 3. Schematic view of all deletions 15q26 described in patients with non-isolated CDH, modified and updated from a figure presented by Castiglia *et al.*(Castiglia et al. 2005). On the left bands 15q26.1-q26.3 are shown with BAC clones. On the right all deletions are shown. Candidate genes are indicated. The black square box indicates the smallest region of overlap.
A. patient 1; **B**. patient 2; **C**. patient 1 Klaassens *et al.* 2005; **D**. patient 2 Klaassens *et al.* 2005; **E**. patient 3 Klaassens *et al.* 2005; **F**. patient 4 Klaassens *et al.* 2005; **G**. patient 5 Klaassens *et al.* 2005; **H**. patient 6 Klaassens *et al.* 2005; **I**. patient 7 Klaassens *et al.* 2005; **J**. Tümer *et al.* 2004; **K**. Elghezal *et al.* 2006; **L**. patients 1 and 2 Slavotinek *et al.* 2005; **M**. patient 3 Slavotinek *et al.* 2006; **N**. patients desribed by Kristofferson *et al.* 1987 (2x), Howe *et al.* 1996, Bettelheim *et al.* 1998 (2x), Aviram-Goldring *et al.* 2000 (2x), Biggio *et al.* 2004, Hengstschläger *et al.* 2004, Tonks *et al.* 2004, Lopez *et al.* 2005 and Pober *et al.* 2005 (all have deletions much larger than the other ones described).

Table 2. Cardiac anomalies in patients with CDH and 15q26 deletions

Type of defect	Pure monosomy	Unbal. translocation	Ring chromosome
Bicuspid aortic valve	1	-	-
Mitral valve stenosis	1	-	-
Mitral valve atresia	-	-	1
Bicuspid aortic valve	1	-	-
Common AV valve	-	1	1
Coarctation aortae / hypoplastic aorta	2	2	2
Single ventricle	-	-	1
Hypoplastic Left Heart	1	-	-
Hypoplastic Left Ventricle	1	1	-
Hypoplastic Left Atrium	-	-	1
Atrial Septal defect (ASD)	-	1	-
Ventricular Septal defect (VSD) / multiple VSDs	2	3	2
Double Outlet Right Ventricle (DORV)	-	1	-
Other (cardiomegaly, arrythmia)	-	1	-
Complex cardiac anomaly, not further specified	-	3	1

Many of the anomalies seen in the two patients presented here, and of those reported in the literature, are also characteristic of children with Fryns-syndrome, which was also proposed as a possible diagnosis by Slavotinek *et al* for some of the published patients (Slavotinek 2004). In the case of patient 1, this syndrome diagnosis was considered as a very strong possibility. This provides more evidence that 15q26 is a candidate locus for Fryns Syndrome. Because of its relative homogeneous phenotype and the occurrence of Fryns syndrome in siblings and in children born to consanguineous parents, it was believed that this syndrome is a monogenic disorder with an autosomal recessive inheritance (Enns et al. 1998). Thus far, no causative gene has been identified for this syndrome. However, several chromosomal loci have been suggested for Fryns syndrome, such as 1q42 and 8p23.1, and 15q26. Given the phenotypic characteristics of deletion 15q26 patients, this locus is the most likely candidate for true Fryns syndrome. However, this would not explain the recessive inheritance of some cases of Fryns syndrome which may best be explained by recessive gene-mutations.

Nearly all patients (24/25) reported in the literature with non-isolated CDH and chromosome 15q26 deletions have died, either after termination of pregnancy because of the severity of the anomalies, or due to early neonatal death, despite intensive treatment. One patient was reported to be alive at the age of 14 months but was still ventilator-dependent (Biggio et al. 2004). In the first case described in this paper, treatment was withheld and the patient died at 7 hours of age. In the second case the parents and physicians decided not to initiate treatment after birth because of the severity of the anomalies and the associated poor prognosis.

In general one can conclude that the phenotype that is associated with a deletion of 15q26 consists of characteristic dysmorphic features (coarse facial features, hypertelorism, abnormally placed and shaped ears), left-sided CDH, a (complex) cardiac anomaly and limb defects. Therefore, in cases where CDH is accompanied by any of these anomalies, diagnostic procedures aimed at identifying or excluding a deletion of the long arm of 15q, should be performed. The diagnosis of this deletion can affect clinical decision making including decisions regarding the termination of pregnancy or decisions against aggressive intervention. In cases where aggressive treatment is initiated, parents and physicians should be aware of the poor prognosis associated with this combination of birth defects and the deletion 15q26.

Acknowledgements

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CHAPTER 5

Congenital Diaphragmatic Hernia Associated with Duplication of 11q23-qter

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ABSTRACT

Congenital Diaphragmatic Hernia (CDH) is a relatively common birth defect with a high mortality. Although little is known about its etiology, there is increasing evidence for a strong genetic contribution. Both numerical and structural chromosomal abnormalities have been described in patients with CDH. Partial trisomy 11q and partial trisomy 22q associated with the common t(11;22) has been reported in several cases of CDH. It has been assumed that the diaphragmatic defect seen in these individuals was primarily due to duplication of material from chromosome 22q11. However, in this report we describe a family with a t(11;12) in which one of two brothers with partial trisomy 11q has a left sided posterolateral CDH. This is the second case of CDH in partial trisomy 11q due to an unbalanced translocation other than t(11;22). Using array-based comparative genomic hybridization and fluorescent *in situ* hybridization we mapped the breakpoints in both brothers and their mother who is a balanced translocation carrier. Our results suggest that duplication of one or more genes on a ~19 Mb region of 11q23.3-qter predisposes to the development of CDH. These effects may be the primary cause of CDH in individuals t(11;22) or may be additive to effects from the duplication of chromosome 22 material. We also conclude that the partial trisomy 11q syndrome has a variable phenotype and that CDH should be added to the spectrum of anomalies that can be present in this syndrome.

INTRODUCTION

Congenital diaphragmatic hernia (CDH, [OMIM142340]) is a relatively common anomaly with an incidence of 1 in 3,000 births (Torfs et al. 1992). CDH is characterized by a variable defect in the diaphragm, lung hypoplasia, and postnatal pulmonary hypertension that is often resistant to therapy (Torfs et al. 1992; Beresford and Shaw 2000). It has a high mortality. CDH can occur as an isolated defect, in combination with multiple congenital anomalies or as part of a defined syndrome. There is increasing evidence for a genetic cause of CDH. Trisomy 18 and tetrasomy 12p (Pallister-Killian), are the most common identifiable chromosomal cause of CDH (Pober et al. 2006) Structural chromosomal abnormalities encompassing almost every chromosome have been described (Lurie 2003). Deletions/duplications of some chromosomal regions have been described in multiple individuals with CDH. Copy number alterations of one or more genes in these regions are likely to predispose to the development of CDH. We have recently defined a minimally deleted region for CDH on chromosome 15q26 (Klaassens et al. 2005).

A second structural chromosomal anomaly repeatedly implicated in CDH is the t(11;22). The only viable type of unbalanced segregation of this translocation is 3:1 meiotic segregation resulting in a 47,XX or XY,+der(22)t(11;22)(q23.3;q11.2) karyotype which results in trisomy for a portion of 11q and 22q (Fraccaro et al. 1980; Lurie 2003). The diaphragmatic defects associated with this abnormality have been attributed primarily to duplication of material from chromosome 22. This was based on several reports of diaphragmatic hernia in individuals with trisomy 22 and the existence of only a single report of CDH associated with partial duplication 11q due to a t(11;13) resulting in 47,XY,+der(13)t(11;13)(q21;q 14) (Park et al. 1993).

The phenotype accompanying t(11;22) was first described as "trisomy 22" but this constellation of findings is now known as "partial trisomy 11q syndrome" since most of the features are thought to be caused by the duplication of genes on 11q (Francke et al. 1977; Pihko et al. 1981). Translocations of 11q with autosomes other than 22 have also been described (Vianello et al. 1986) (Francke et al. 1977; de France et al. 1984; Van Opstal et al. 1993; Zhao et al. 2003). These translocations have been associated with loss or gain of material from the other homologous autosome. Chromosomal breaks on chromosome 11q occur most often at band q23.3, that contains a fragile site that predisposes to breaks and recombination between chromosomes (Pfeiffer and Schutz 1993).

In this report we describe two siblings, born to a mother with a t(11;12). This family came to our attention when the youngest child was diagnosed prenatally with CDH. Both siblings were found to carry a partial duplication of chromosome 11q and a partial deletion of 12q. This is the second time that CDH has been reported in and individual with partial trisomy 11q due to an unbalanced translocation other than t(11;22) (Park et al. 1993). Using complementary cytogenetic techniques, we mapped the breakpoints in three family members carrying either balanced or unbalanced forms of this translocation. Our results suggest that duplication of one or more genes on an ~19 Mb region of 11q23.3-qter is likely to predispose to the development of CDH. These effects may be the primary cause of CDH in individuals t(11;22) or may be additive to effects from the duplication of chromosome 22 material. We also conclude that partial trisomy 11q syndrome has a variable phenotype which may include CDH.

Clinical Reports

Patient 1

Patient 1 (IV:2, Figure 1) is a male child born at term to a 40-year old G2P0 mother with a history of a single spontaneous abortion. After birth, multiple congenital anomalies were noted including a severe Pierre-Robin sequence (cleft palate, micrognathia and glossoptosis) requiring tracheostomy after closure of the cleft palate. There were mild dysmorphic features (high anterior hairline, hypotelorism, metopic ridge, upslant of the eyes with telecanthic folds, short palpebral fissures, broad, flattened upturned nose, prominent philtrum, open mouth appearance), prominent heels and a micropenis. Because of these abnormalities and marked delay in psycho- and motor-development, he was referred to the clinical genetics department for evaluation at the age of 1 year and 3 months. Cytogenetic analysis revealed an unbalanced translocation: 46,XY,der(12)t(11;12)(q23;q24).
Re-evaluation of Patient 1 at 24 months of age revealed marked delay in his psychomotordevelopment and hypotonia. His upper airway compromise had diminished and he no longer required his tracheostomy. The dysmorphic features were still present.

Patient 2

At the time of diagnosis, Patient 1's mother was already pregnant with Patient 2 (IV:3, Figure 1). Prenatal ultrasound examination during this pregnancy revealed a male fetus with a left-sided CDH, unilateral mild hydronephrosis, prominent heels and a micropenis. Amniocentesis was performed and a G-banded chromosome analysis revealed a 46,XY,der(12)t(11;12)(q23;q24)mat chromosomal complement.
Patient 2 was delivered at 40 weeks of gestation and had a birth weight of ~3,000 g (~ 50th centile). Accordingly to standardized treatment for CDH he was intubated at birth and standard ventilator care for CDH was initiated. Physical and radiological examination confirmed the presence of a left-sided diaphragmatic hernia with severe pulmonary hypoplasia, seen on prenatal ultrasound. Additional anomalies identified at the time of birth included mild dysmorphic features (low-set, slightly dysplastic ears and a short, broad neck), remarkably loose skin and subcutaneous tissue, hypoplastic toenails, prominent heels, and a micropenis. Cardiac ultrasound revealed a ventricular septal defect. No other signs of Fryns's syndrome, besides hypoplastic nails, were present. Patient 2's perinatal course was complicated by severe, therapy-resistant, pulmonary hypertension and recurrent pneumothorax. The patient was not considered a candidate for surgical repair because of instability of the clinical situation. Extra-corporeal membrane oxygenation (ECMO) therapy was not initiated because of his unbalanced chromosomal abnormality. The patient died at the age of 7 days as the result of therapy-resistant pulmonary hypertension and circulatory failure. Although parents did not grant permission for autopsy, consent was obtained for further clinical and cytogenetic analysis.

Cytogenetic Analysis

G-banded chromosome analysis of peripheral blood lymphocytes from Patient 1 initially showed no chromosomal abnormalities. A repeat investigation at the age of 15 months revealed a chromosomal translocation with a 46,XY,der(12)t(11;12)(q23.3;q24.3)mat chromosome complement resulting in partial trisomy for 11q and partial monosomy for 12q. Prenatal chromosome analysis of patient 2 revealed the same unbalanced translocation. This was confirmed on peripheral blood lymphocytes by GTG banding, by whole chromosome painting of chromosomes 11 and 12 (figure 2), and by Multiplex Ligation-dependent Probe Amplification (MLPA, results not shown).

Conventional G-banded chromosome analysis performed on parental peripheral blood lymphocytes revealed a maternal reciprocal translocation resulting in a 46,XX,t(11;12)(q23;q24.3) karyotype (figure 2). Paternal chromosomes were normal. No biological material was available from the mother's first spontaneous abortion. All other family members of the mother are living abroad and were unavailable for investigation.

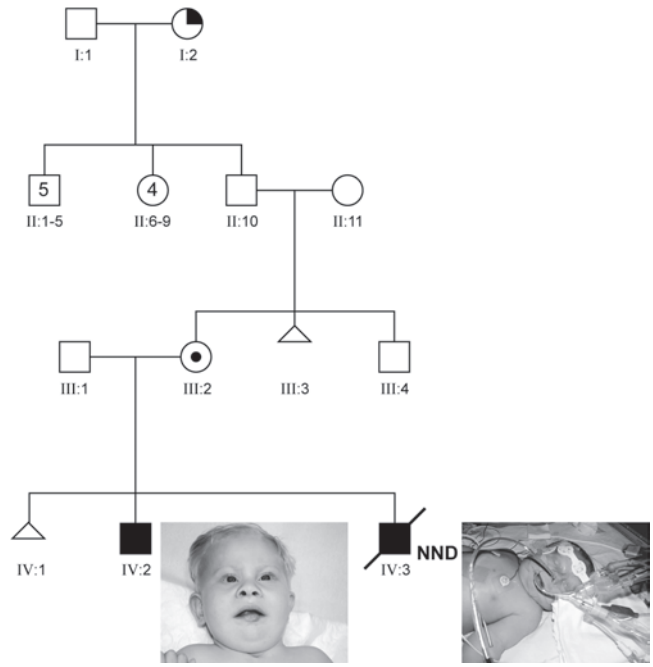

Fig. 1. Pedigree of family with t(11;12).
III:2 Healthy carrier mother; IV:1 spontaneous abortion; IV:2 Patient 1, note the dysmorphic features such as short nose, micrognathia and prominent upper lip; IV:3 Patient 2, dysmorphic features difficult to see due to presence of tubes and facial edema. Pictures of Patient 1 (IV:2) and 2 (IV:3) are shown with parental permission. Color figure can be found in the appendix. See page 134.

To delineate the exact size of the deletion and duplication present in Patient 2, array-based comparative genomic hybridization (array-CGH) was performed using a whole-genome tiling BAC array with a resolution of ~300 kb (Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, USA) (Li et al. 2003). This analysis revealed an ~19 Mb duplication of chromosome 11q23.3-qter, but no abnormality of chromosome 12 was detected (Table 1).

To validate results found by array-CGH and to map the breakpoints more precisely, BAC clones were selected from the University of California Santa Cruz (UCSC) and Ensembl genome browsers and ordered from BACPAC Resources, or obtained from the Department of Molecular and Human Genetics at Baylor College of Medicine (Houston, USA). We used these BAC clones for FISH on metaphase chromosomes (Figure 2). These results confirmed the ~19 Mb duplication of 11q23.3-qter and mapped the breakpoint to a region located within BAC clones RP11-728G20 and RP11-1147E8 (partially overlapping clones). Using FISH a terminal deletion of chromosome 12q24.33 was found, proximally bordered by BAC clone RP11-1038O14 and extending towards the telomere. Identical results were found for Patient 1.

A whole chromosome paint of chromosomes 11 and 12 performed on maternal lymphocytes revealed a translocation of chromosome 11 material to chromosome 12. No translocation of chromosome 12 material could be detected using this technique. FISH analysis, however, revealed the same breakpoint locations identified in Patient 1 and 2 with more distally located clones on both chromosomes translocating to the corresponding derivative chromosome, confirming the reciprocal translocation.

Table 1. Results cytogenetic analysis

Probe Name	Location	Patient 1 (FISH)	Patient 2 (Array-CGH)	Patient 2 (FISH)	Mother (FISH)
Chromosome 11					
RP1-44H16	11pter	N		N	N
RP11-42L18	11q22			N	
RP11-235P12	11q23.3		N	N	
RP11-114K7	11q23.3		N	N	
RP11-840K20	11q23.3	N	N	N	
RP11-1058J6	11q23.3	N	N	N	
RP11-728G20	11q23.3	N / Dup [a]	Dup	N / Dup [a]	N / M [b]
RP11-1147E8	11q23.3	N / Dup [a]	Dup	N / Dup [a]	N / M [b]
RP11-2O20	11q23.3	Dup	Dup	Dup	M
RP11-890O20	11q23.3		Dup	Dup	M
RP11-786F19	11q23.3		Dup	Dup	M
RP11-664F8	11q23.3	Dup	Dup	Dup	M
RP11-778O17	11q23.3		Dup	Dup	
RP11-158K18	11q23.3	Dup	Dup	Dup	
RP11-77K9	11q24.1		Dup	Dup	
RP11-102M23	11q25			Dup	
RP1-26N8	11qter	Dup		Dup	M
Chromosome 12					
CTB-124K20	12pter	N		N	N
RP11-29G23	12q12			N	
RP11-269C10	12q24		N	N	
RP11-813G9	12q24		N		
RP11-91M21	12q24	N	N	N	
RP11-19F21	12q24.21	N		N	
RP11-641K13	12q24.32		N	N	
RP11-1023E10	12q24.32	N	N	N	
RP11-503G7	12q24.32	N	N	N	N
RP11-1038O14	12q24.33	N	N	N	N
RP11-452D11	12q24.33	Del	N	Del	M
RP11-962N24	12q24.33	Del		Del	M
RP1-221K18	12qter	Del		Del	M

N = normal signal, Dup = duplication, Del = deletion, M = moved to derivative chromosome
blank space = not tested
[a] Lower signal intensity of duplicated signal on derivative chromosome.
[b] Lower signal intensity of signal on derivative chromosome 11 and 12 ("split-spot")

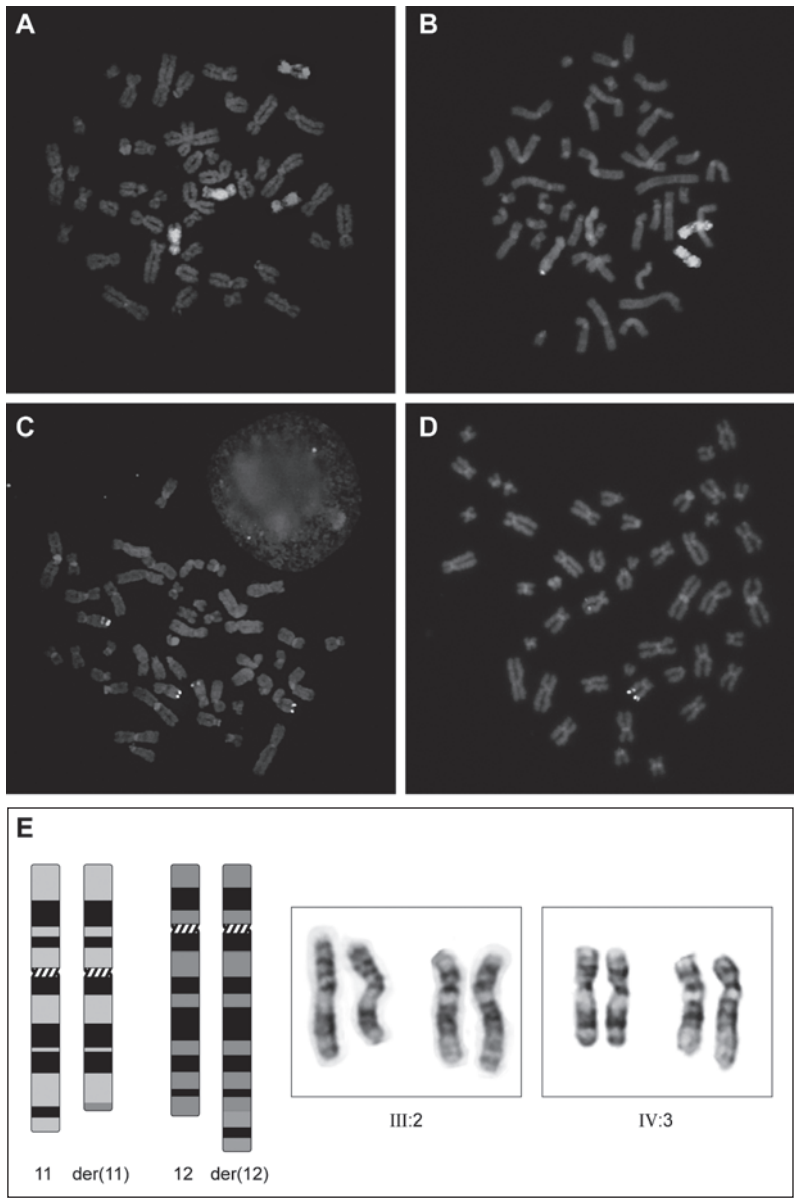

Fig. 2. a-**d.** Fluorescence in situ Hybridization experiments:
WCP11 (red) & WCP12 (green) paint of chromosomes from Patient 1 showing additional chromosome 11 material a on the derivative chromosome 12 (**a**), WCP11 (green) & WCP12 (red) paint of maternal chromosomes showing balanced translocation of chromosome 11 material to chromosome 12. (**b**), RP11-19F21 (red signal) & RP1-26N8 (green signal) showing the presence of three copies of the terminal region of 11q in Patient 1 (**c**), RP11-19F21 (red signal) & RP1-221K18 (green signal) showing only a single signal from the terminal region of 12q in Patient 2 (**d**).
e: Ideograms and partial karyotypes of mother (III:2) and Patient 2 (IV:3).
On the left a schematic representation of the balanced translocation is shown. Chromosome 11 (green) and chromosome 12 (pink) are shown. On the right pictures of chromosomes 11 and 12 of both III:2 (balanced translocation carrier) and IV:3.
Color figure can be found in the appendix. See page 135.

Table 2. Clinical Features.

Features of "partial trisomy 11q syndrome"	Patient 1	Patient 2
Mental retardation	+	?
Motor retardation	+	?
IUGR	-	-
Postnatal growth deficiency	-	-
Hypotonia	+	+
Microcephaly	-	-
Retro / micrognathia	+[a]	+
Craniofacial asymmetry	-	-
Deformity ears	-	+
Short nose	+	+
Prominent upper lip	+	-
Retracted lower lip	-	-
Cleft palate / high arched palate / bifid uvula	+	+
Glossoptosis	+	-
Cardiac abnormality	-	+
CDH	-	+
Renal abnormality	-	+
Small / hypoplastic nails	-	+
Dislocated hip joints	-	?
Clavicle defect	-	-
Cutis laxa	-	+
Short / broad neck	-	+
Other abnormalities	-	-

IUGR, intra-uterine growth retardation; CDH, Congenital Diaphragmatic Hernia; +, present; -, not present; ?, no data available

[a] micrognathia more pronounced at birth. No longer obvious as the age of 2 8/12 years.

DISCUSSION

We describe two siblings with the same unbalanced translocation of chromosomes 11 and 12. Although these siblings are concordant for most of the features and share a phenotype that is consistent with partial trisomy 11q syndrome, they are discordant for several anomalies including CDH (Table 2). This leads us to conclude that partial trisomy 11q syndrome has a variable phenotype and that CDH should be added to the spectrum of anomalies that can be present in this syndrome.

By means of complementary cytogenetic techniques, we mapped the breakpoints in both patients and their mother who carries the corresponding reciprocal translocation. The unbalanced chromosomal anomaly was detected by conventional G-banded chromosome analysis. The duplication of the ~19 Mb distal portion of 11q was easily detected by array-CGH and confirmed by FISH. The smaller ~0.5 Mb deletion of 12qter was not detected by array-CGH but was confirmed by FISH studies. The failure of the array to detect this deletion is most likely due to low representation of material distal to the breakpoint (Table 1). This region contains a cluster of zinc finger genes and a repeat cluster. RP11-452D11, the only BAC representing this region on the array partially overlapped this repeat cluster.

Due to the unbalanced translocation there is a duplication of part of the long arm of chromosome 11, an abnormality that has been described several times in the literature as "partial trisomy 11q syndrome" or "duplication 11(q21/q23→qter) syndrome" (Francke et al. 1977; Pihko et al. 1981). The clinical features of this syndrome and the phenotype of Patients 1 and 2 are summarized in Table 2.

Congenital diaphragmatic hernia has been described in several patients with partial duplication of 11q due to a t(11;22) (Biederman et al. 1980; Kadir et al. 1997; Borys and Taxy 2004). To our knowledge CDH has only been described once in a child with partial duplication 11q due to an unbalanced translocation with another chromosome than 22 (Park et al. 1993). Patient 2 represents the second case of an unbalanced translocation other than the t(11;22) resulting in partial duplication 11q associated with CDH, in this case associated with a small partial monosomy 12q. Although we cannot exclude a potential role for the genes deleted on 12q or that the CDH in patient 2 only occurred by coincidence, we believe that it is more likely that one or more genes in the duplicated region on 11q predispose to the development of CDH. There have been two case-reports on monosomy 12q24, but in none of these CDH has been described (Sathya et al. 1999; Plotner et al. 2003). Ultimately, environmental factors and the genetic background determine whether an individual eventually develops CDH. The discordance in these two siblings might be the result of epigenetic differences between the two brothers, such as different methylation status of genes in the duplicated region.

The duplicated region on 11q23-qter contains almost a hundred known genes and multiple unknown transcripts. Although none of the genes in this region have been implicated in the etiology of CDH, there are several genes that could play a role in the development of CDH based on their known functions. ROBO3 (roundabout, axon guidance receptor, homolog 3 [OMIM 607630]), located at 11q24.2, regulates axon guidance across the midline in the brain (Sabatier et al. 2004) and might be involved in non-neuronal morphogenesis (Anselmo et al. 2003). ROBO genes encode the receptors for the Slit-family of genes in both Drosophila and vertebrates, and *Slit3* (OMIM 603745) binds to ROBO3 in vertebrates. Although it is unclear at this time whether central- and posterolateral-type CDHs are caused by related genes, it is interesting to note that *Slit3* knockout mice display a septum transversum CDH associated with kidney agenesis and cardiac defects (Liu et al. 2003). Another member of the roundabout-gene-family, ROBO4 (roundabout, axon guidance receptor, homolog 4 [OMIM 607528]), also located on

11q24.2, is also duplicated in our patients. This gene is mainly expressed at sites of active angiogenesis, especially under hypoxic conditions (Huminiecki et al. 2002). A third duplicated gene is CDON (Cell Adhesion Molecule-related/Downregulated by Oncogenes [OMIM 608707]), located at 11q24. In complex with BOC (Brother of CDON [OMIM 608708]) this gene regulates myogenic differentiation (Kang et al. 2002). Further delineation of breakpoints in patients with smaller partial duplications of 11q and CDH, functional studies, and transgenic animal experiments may help focus attention on one or more of these genes.

Recently, Zhao *et al* (2003) described four patients with partial trisomy 11q and severe upper airway malformations. They attributed the upper airway malformations in these patients to duplication of the 11q21-q23.2 region. This conclusion was based on the proximal location of the breakpoints in patients with the upper airway anomalies (which started at q21) in comparison to patients without upper airway malformations, in which the breakpoints were located more distally at q23.2. The phenotype of Patient 1, who presented with severe Pierre-Robin sequence, would suggest that the 11q23.2-qter region is involved in upper airway formation and that incomplete penetrance may account for those individuals with 11q23-qter duplications who lack this phenotype.

In conclusion, the "partial trisomy 11q syndrome" has a highly variable phenotype. Our findings implicate that CDH should be added to the spectrum of abnormalities that can be present in this syndrome.

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Electronic-database Information

Accession number and URLs for data presented herein are as follows:
Ensembl Genome Browser, http://www.ensemb..org/Homo_Sapiens/
Online Mendelian Inheritance in Man (OMIM),
http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?.db=OMIM
Unigene, http://www.ncbi.nlm.nih.gov/Genomes/index.html
University of California Santa Cruz (UCSC) Genome Browser, http://genome.cse.ucsc.edu/

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CHAPTER 6

Genome-Wide Oligonucleotide-Based Array Comparative Genome Hybridization Analysis of Non-Isolated Congenital Diaphragmatic Hernia

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ABSTRACT

Non-isolated congenital diaphragmatic hernia (CDH+) is a severe birth defect that is often caused by *de novo* chromosomal anomalies. In this report we use genome-wide oligonucleotide-based array comparative genome hybridization (array-CGH) followed by rapid real-time quantitative PCR analysis to identify, confirm, and map chromosomal anomalies in a cohort of 26 CDH+ patients. 105 putative copy number changes were identified by array-CGH in our cohort of CDH+ patients. Sixty-one of these changes (58%) had been previously described in normal controls. Twenty of the remaining 44 changes (45%) were confirmed by quantitative real-time PCR or standard cytogenetic techniques. These changes included *de novo* chromosomal abnormalities in five of the 26 patients (19%), two of whom had previously normal G-banded chromosome analyses. Data from these patients provide evidence for the existence of CDH-related genes on chromosomes 2q37, 6p22-25, and 14q, and refine the CDH minimal deleted region on 15q26 to an interval that contains *COUP-TFII* and only eight other known genes. Although *COUP-TFII* is likely to play a role in the development of CDH in patients with 15q26 deletions, we did not find *COUP-TFII* mutations in 73 CDH samples. We conclude that the combination of oligonucleotide-based aCGH and quantitative real-time PCR is an effective method of identifying, confirming, and mapping clinically relevant copy number changes in patients with CDH+. This method is more sensitive than G-banded chromosome analysis and may find wide application in screening patients with congenital anomalies.

INTRODUCTION

Congenital diaphragmatic hernia (CDH) [OMIM 142340] is a common birth defect with an estimated incidence of 1 in 2,500 births, and accounts for 8% of all major congenital anomalies (Torfs et al. 1992; Langham et al. 1996). Mortality remains high among severely affected children and long-term complications in survivors are common (Harrison et al. 1978; Nobuhara and Wilson 1996; Conforti and Losty 2006). CDH can present as an isolated birth defect or in combination with other non-hernia-related anomalies (CDH+). Chromosomal anomalies are common in CDH+ and are typically screened for using G-banded chromosome analysis (Howe et al. 1996; Enns et al. 1998; Lurie 2003). Recent reports suggest that some causative chromosomal anomalies may go undetected in infants with CDH+ screened using standard cytogenetic techniques (Slavotinek et al. 2005; Kantarci et al. 2006). These reports emphasize the need for screening methods with greater sensitivity since the detection of a chromosome anomaly can impact prognosis, the selection of additional screening tests, treatment decisions, and recurrence risk counseling (Howe et al. 1996; Tonks et al. 2004). Identifying cryptic chromosomal abnormalities in patients with CDH+ may also help to focus research efforts on regions of the genome that are likely to harbor one or more genes that play a role in the development of CDH.

Array comparative genomic hybridization (array-CGH) is a powerful technique for identifying deletions and duplications in genomic DNA (Pinkel and Albertson 2005). Recently, regional BAC-based array-CGH analysis has become available on a clinical basis (Bejjani et al. 2005; Cheung et al. 2005). BAC-based arrays can provide improved resolution over G-banding but their maximal resolution is limited by the relatively large size of BAC clones (~100 to 200kb). Although oligonucleotide-based arrays (with probe sizes typically ranging from 45 to 80 bp) have the potential for much higher resolution, they have not been used extensively in the clinical setting (Ylstra et al. 2006).

One obstacle to wider use of oligonucleotide-based arrays as a diagnostic tool is the need for an independent method for evaluating small copy number changes below the resolution of FISH. In this report we describe the first use of a combination of high-resolution, genome-wide oligonucleotide array-CGH and quantitative real-time PCR as a screening method for identifying, confirming, and mapping copy number changes in patients with CDH+. Our results suggest that this combination of techniques is more sensitive than G-banded chromosome analysis and may find wide application in screening for copy number variations in patients with congenital anomalies.

MATERIALS & METHODS

Subject Accrual for Array CGH Studies

Seventy-five subjects with CDH were ascertained from patients seen at tertiary care centers in Houston, Texas, and Rotterdam, The Netherlands in accordance with IRB approved protocols. Thirty-five of the 75 patients (47%) were identified as having non-isolated CDH based on documentation of CDH and at least one other non-hernia-related anomaly. Lung hypoplasia, abnormalities in cardiac position, intestinal malrotation, and patent ductus arteriosus are examples of hernia-related defects and were not considered grounds for a diagnosis of non-isolated CDH. Twenty-six of the 35 patients with CDH+ (74%) were selected for further study based on the availability of a sufficient quantity of high quality DNA (~4 micrograms). Three of the 26 patients (12%) had chromosome anomalies that were previously identified by G-banded chromosome analysis or FISH and acted as positive controls.

Oligonucleotide-Based Array CGH

Array CGH was performed using Human Genome CGH 44B Oligo Microarray Kits (Agilent) according to the manufacturer's protocol version 2.0. Arrays were scanned using an Agilent DNA Microarray Scanner. Data was extracted using Feature Extraction Software 8.1 (Agilent) and analyzed using CGHAnalytics3.2.32 Software (Agilent). Control DNA consisted of commercially available pooled control DNA (Promega) or DNA from a healthy male and female control with no personal or family history of CDH. These individual control samples proved particularly useful since each contained small, rare, copy number variations which served as internal positive controls.
Putative copy number changes were defined by intervals of two or more adjacent probes with log2 ratios suggestive of a deletion or duplication when compared to the log2 ratios of adjacent probes. These changes were identified with the assistance of the Aberration Detection Method 1 algorithm contained within the CGHAnalytics 3.2.32 Software.

Quantitative Real-Time PCR

Primer pairs for quantitative real-time PCR were designed from unique sequences within the minimal deleted/duplicated region of each putative copy number change using Oligo 6.0 software (Molecular Biology Insights). For regions <1kb, primers were chosen from a region that included 5kb of flanking sequence. Quantitative PCR confirmed array findings in each of these cases. The nucleotide-nucleotide BLAST algorithm at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/) was used to confirm that each PCR amplification product was unique and did not contain sequences that were significantly similar to those contained in Alu repeats. The presence of primer dimmers and non-specific amplification products in PCR reactions was minimized by careful primer design and annealing temperature optimization. Quantitative PCR analyses were performed using a LightCycler 1.1 instrument in combination with LightCycler FastStart DNA Master SYBR Green I kits (Roche Molecular Diagnostics). Experiments were designed in a manner similar to the standard curve method described by Boehm et al. with a region of the 3' portion of the *TCOF1* gene serving as a control locus (Boehm et al. 2004). All experimental values were determined in triplicate. At the locus of interest, copy number values between 0.8 and 1.2 were considered normal while values ≥1.3 and ≤0.7 were considered evidence of duplication and deletion respectively.

Identification of Previously Reported Changes

To determine if a putative change identified by array-CGH had been described previously in normal controls, we searched for similar deletion/duplications in the Database of Genomic Variants hosted by the Center for Applied Genomics (http://projects.tcag.ca/variation/). To be considered as previously reported, a putative change had to be of the same type (deletion or duplication) and involve the same approximate interval.

COUP-TFII Mutation Screening

Seventy-three DNA samples from patients with CDH were screened for mutation in the coding sequence of *COUP-TFII* and the surrounding intronic splice donor and splice receptor sequences by direct sequencing of purified PCR amplification products. The sequences for primers used in this

screen are available on request. Sequence changes in patient samples were identified by comparison to control DNA sequences using Sequencher 4.6 software (Gene Codes Corporation).

RESULTS

Twenty-six subjects with CDH were screened for chromosomal anomalies by array-CGH. Three of the 26 patients (12%) had chromosome anomalies previously identified by G-banded chromosome analysis or FISH including patient N4 whose *de novo* chromosomal anomalies have been published previously (Table 1). These chromosomal anomalies served as positive controls for the array analysis.

Table 1. Characteristics of CDH+ patients with *de novo* chromosomal anomalies

Patient	CDH type	Ass. anomalies	array-CGH	Known?	Confirmed by	Breakpoint positions
TX19	Posterolateral, left	Microcephaly, dysmorphic features, bilateral ptosis, developmental delay, failure to thrive	Partial duplication of 6p	Yes, G-banding 46,XX,inv dup (6) (p25.2p22.2)	qPCR, FISH	tel: 2,977,284 – 3,010,031 cent: 25,000,597 – 25,041,232
TX20	Posterolateral, left	Microtia, microphthalmia, corneal clouding, congenital cataract, cleft palate, ASD, thickened aortic & pulmonary valves, bilateral kidney malrotation, ulnar deviation hands, long thin fingernails, 5th finger clinodactyly	Duplication of 14q	No. Recounting cells after aCGH analysis: isochromosome 14q in 2% of cells	FISH, on EBV transformed cells used for DNA isolation for aCGH revealed three signals of 14q in all cells	N/A
TX21	Posterolateral, right	Ear anomalies, wide-spaced nipples, ASD	Deletion of 1 copy of X chromosome	Yes, G-banding 45,XO	N/A	N/A
N4	Posterolateral, left	Dysmorphic features, VSD, micropenis, hypoplastic toe nails, loose skin	Partial duplication of 11q & partial deletion of 12q	Yes, G-banding 46,XY,der(12)t(11;12) (q23.3;q24.3)	FISH	Previously described (Klaassens *et al.* AJMG 2006)
N9	Posterolateral, left	Dysmorphic features, double outlet right ventricle, hypoplastic nails	Partial duplication of 2q & partial deletion of 15q	No.	qPCR, FISH	2q: 235,322,898 – 235,351,819 15q: 92,219,239 – 92,231,673

One hundred and five (105) putative copy number changes were identified in these patients including all changes previously detected by G-banding or FISH (Figure 1).

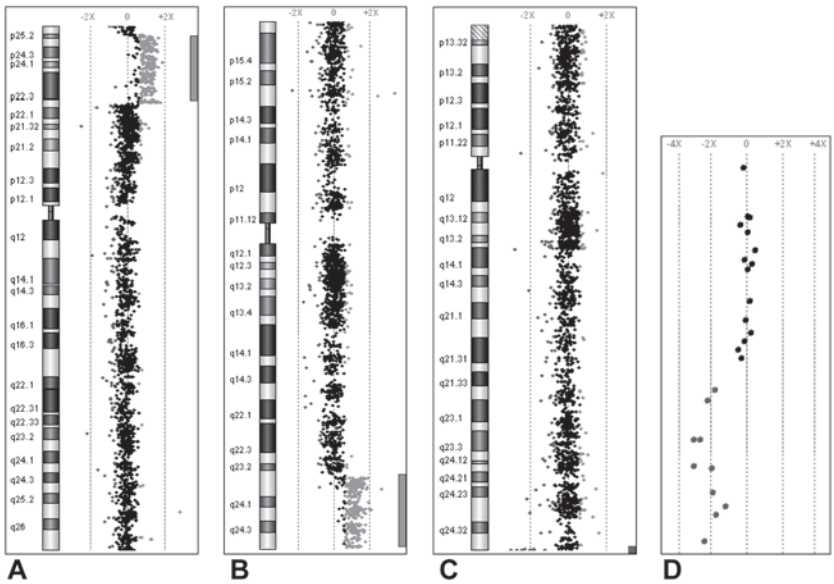

Fig. 1. Examples of copy number changes identified by aCGH in patients with CDH+. Colored bars on the right hand side of panels *A-C* mark affected regions. (**A**) An ~22 Mb duplication of chromosome 6p in patient TX19. (**B**) an ~18 Mb duplication of chromosome 11q in patient N4. (**C**) An ~0.5 Mb deletion of chromosome 12q in patient N4. Previously published aCGH studies using a BAC-based array CGH failed to identify this deletion due to incomplete coverage of the telomere region (16). (**D**) A more detailed view of the 12q aCGH results in patient N4. Multiple deleted probes mark the affected region of 12q. Color figure can be found in the appendix. See page 136.

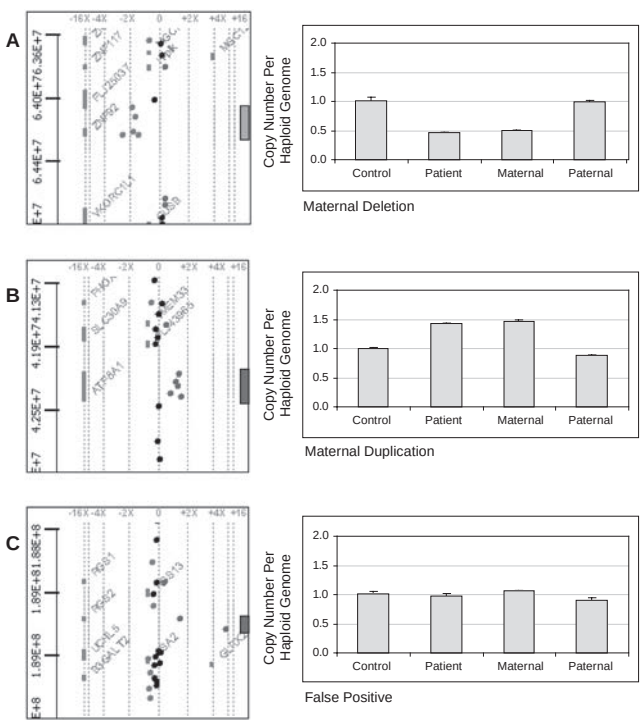

Fig. 2. Quantitative real-time PCR can be used to interrogate putative deletions/duplications identified by oligonucleotide-based aCGH and can simultaneously determine inheritance patterns. The left hand portions of panels show aCGH data from the region around each putative deletion/duplication. The approximate location of each region of interest is marked with a colored bar. The right hand portion of each panel shows quantitative PCR results for each region of interest. (**A**) A maternally inherited deletion. (**B**) A maternally inherited duplication. (**C**) A false positive result as indicated by a normal copy number in the patient sample. Color figure can be found in the appendix. See page 136.

To determine if these putative changes had been described previously in normal controls, we searched for similar deletion/duplications in the Database of Genomic Variants hosted by the Center for Applied Genomics. Of the 105 putative changes, 61 (58%) had been previously described in normal control individuals and were, therefore, unlikely to represent *de novo* CDH+ causing changes.

Of the 44 remaining changes, three (7%) were chromosomal anomalies previously identified by G-banded chromosome analysis and/or FISH (Table 1). To determine if the remaining 40 putative changes represented true deletions/duplication, we used quantitative real-time PCR as an independent evaluation of copy number (Figure 2). Sixteen of the 40 putative changes (40%) were confirmed, 23 (58%) were found to be associated with a normal copy number (false positives), and one change on chromosome 22p11 (2%) could not be interrogated using this method due to high levels of homology to other chromosomes throughout the minimally deleted region.

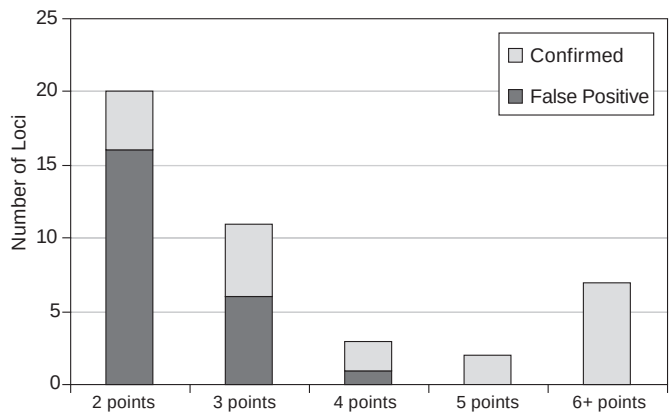

Fig. 3. Confirmed and false positive loci as determined by quantitative real-time PCR. The total numbers of confirmed and false positive loci are shown based on the number of putatively deleted/duplicated aCGH probes with the region of interest. As expected, the total number and percentage of false positive results drops with increasing numbers of putatively deleted/duplicated probes identified by oligonucleotide-based aCGH.

As expected, the percentage of false positive results decreased with increasing numbers of putatively deleted or duplicated probes within a region of interest (Figure 3).

To further determine if failure to confirm putative changes was due to a true lack of copy number change (false positive result on array) or a lack of sensitivity on the part of the quantitative PCR assay (false negative result on quantitative PCR), we performed a second round quantitative PCR using new PCR primer pairs on ten of the false positive loci. In each case results from the second round of quantitative PCR were in agreement with the original results (false positive result on array). This suggests that although the sensitivity of quantitative real-time PCR might, in some cases, be improved by use of multiple primer pairs, the interrogation of most loci can be accurately completed using a single carefully selected primer pair.

When parental DNA samples were available, we used quantitative PCR or standard cytogenetic techniques to determine the inheritance pattern of the confirmed deletion/duplication (Figure 2). We were able to determine the inheritance pattern of 13 of the 16 confirmed anomalies (81%) of which three (23%) represented *de novo* changes. These changes represent the likely etiology of CDH+ in two patients, both of whom had previously normal G-banded chromosome analyses.

Patient N9 was found to carry an unbalanced translocation resulting in a duplication of chromosome 2q and a deletion of chromosome 15q (Table 1). Patient TX20 was found to have a duplication of chromosome 14q by array-CGH. Scoring of additional cells from the original chromosome analysis

confirmed mosaicism for an isochromosome 14q in 2% of cells. However, FISH analysis performed on cells from the EBV transformed lymphocyte culture used as a DNA source for the array-CGH studies revealed that 100% of cells carried the isochromosome. This suggests a transformation or survival bias for cells containing the isochromosome 14q.

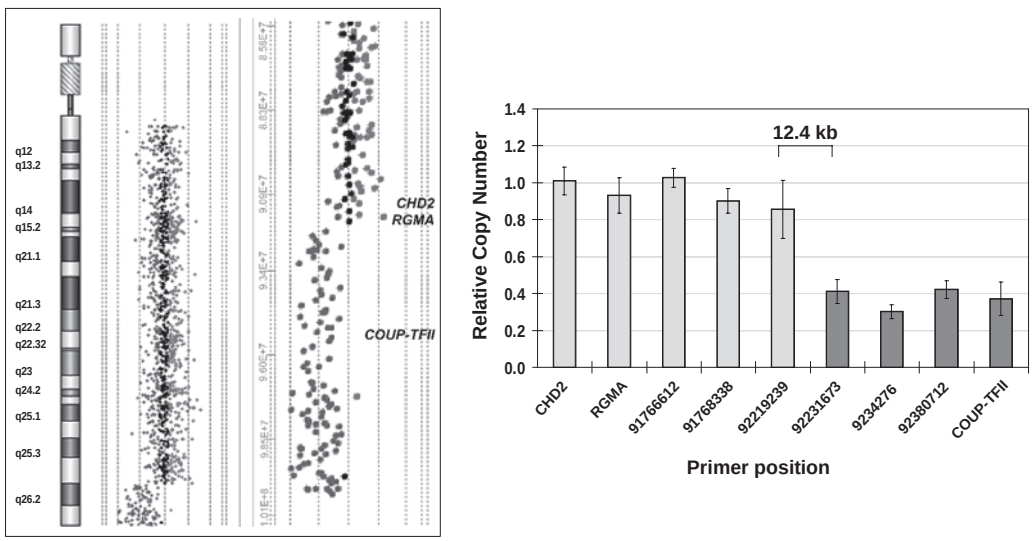

Fig.4. Rapid localization of breakpoints using oligonucleotide-based aCGH and real-time quantitative PCR. (**A**) aCGH data demonstrating a deletion of chromosome 15q in patient N9. (**B**) An enlarged view of the deleted region with the approximate location of candidate genes *CHD2*, *RGMA*, and *COUP-TFII* indicated. (**C**) Quantitative real-time PCR data demonstrating a reduction in copy number for *COUP-TFII*, indicating a deletion, and preservation of normal copy number for *CHD2* and *RGMA*. Data presented were used to localize the 15q breakpoint in patient N9 to an ~12.4 kb interval. The position of each real-time quantitative PCR primer set is given based on NCBI build 35. Color figure can be found in the appendix. See page 137.

FISH is commonly used to define the boundaries of chromosomal anomalies identified by G-banded chromosome analysis. In an alternative method we used quantitative PCR in combination with array-CGH data to rapidly map the location of the key breakpoints involved in the 6p duplication identified in patient TX19, and the 2q duplication and 15q deletion identified in patient N9 (Table 1, Figure 4).

To determine if *de novo* mutations in the *COUP-TFII* are a common cause of congenital diaphragmatic hernia we screened 73 patient samples (35 with isolated CDH and 38 with non-isolated CDH) for *COUP-TFII* mutations. No sequence changes were identified within the coding sequence and the surrounding intronic splice donor and splice receptor sequences. Assuming an 80% mutation detection rate, this sample set would be sufficiently large to give a 95% probability of detecting a causative mutation if mutations in *COUP-TFII* were responsible for 5% of all CDH cases.

DISCUSSION

This study marks the first time that the combination of oligonucleotide-based array-CGH and quantitative real-time PCR has been used to identify and confirm copy number changes responsible for non-isolated congenital diaphragmatic hernia. Using this combination of techniques, we identified a total of 81 copy number changes in our cohort of 26 CDH+ patients. These changes included *de*

novo changes in five patients, two of whom had previous normal G-banded chromosome analyses. We conclude that the combination of oligonucleotide-based array-CGH and quantitative real-time PCR is an effective method of identifying and independently confirming clinically relevant copy number changes in patients with CDH+. This combined method is more sensitive than G-banded chromosome analysis and may find wider application in screening for copy number variations in patients with congenital anomalies.

In addition, we have demonstrated that the combination of oligonucleotide-based array-CGH and quantitative real-time PCR can be used to rapidly determine the location of the breakpoints surrounding a chromosomal copy number anomaly. As more is learned about the function of individual genes (and the biological consequences of altering their copy number) accurately defining the location of breakpoints will have increasing clinical importance and may ultimately allow counseling, therapy, and surveillance to be individualized based on the genes and regulatory regions affected.

Mapping the boundaries of chromosomal anomalies can also be an important step towards identifying genes responsible for various clinical phenotypes. Recently, Klaassens et al. defined a minimally deleted region for CDH on chromosome 15q26 using FISH and array CGH data from patients with CDH+ (Klaassens et al. 2005). This interval was based on a CDH patient with an interstitial deletion of chromosome 15q26 and an individual without CDH who had a terminal deletion starting at 15q26.2 (Figure 5). Of the genes in this region, *COUP-TFII* was considered a particularly strong candidate since it is regulated by the retinoid signaling pathway which has long been implicated in the development of CDH (Major et al. 1998; Greer et al. 2003; Babiuk et al. 2004). Following this report, Castiglia et al. presented data from several patients with relatively large 15q terminal deletions without CDH and suggested that defining the CDH minimally deleted region using data from patients without CDH could be unwise since haploinsufficiency of the CDH locus may be characterized by reduced penetrance (Castiglia et al. 2005).

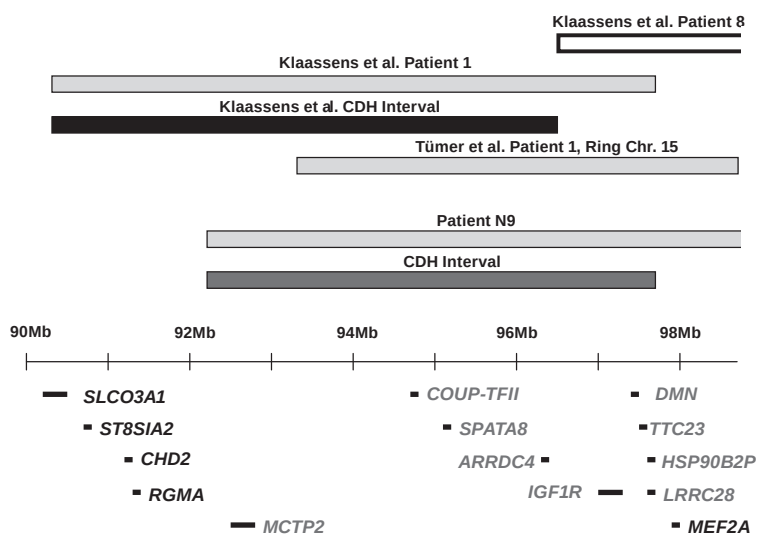

Fig. 5. Defining the CDH minimal deleted region on chromosome 15q26. Deletions in individuals with CHD are shown in grey and deletions in individuals without CDH are shown in non-filled rectangles (17,22). The minimal deleted interval defined by Klaassens et al. is shown in black and the new minimal deleted region defined by patient N9 and Patient 1 described by Klaassens et al. is shown in red. The approximate locations of genes within this region are shown with genes residing inside the new interval depicted in red. Color figure can be found in the appendix. See page 137.

We agree that the most conservative approach to defining a CDH minimal region would be to use data only from patients with CDH. With this in mind, data from patient N9 can be combined with data from Patient 8 presented by Klaassens et al. to define a new minimally deleted region on 15q26 that contains *COUP-TFII* and only eight other known genes (Figure 5).

This interval could be further refined by a CDH patient carrying a ring chromosome 15 described by Tümer et al (Tumer et al. 2004). However, establishing a clear genotype/phenotype relationship in a ring chromosome carrier can be difficult due to the potential instability of ring chromosomes which may result gain or loss of genetic material in different tissues (Tumer et al. 2004; Castiglia et al. 2005). In either case, *COUP-TFII* would be located within the minimal deleted region and the proposed candidate genes *CHD2* and *RGMA* would be located outside the interval (Slavotinek et al. 2006). It is impossible, however, to exclude the possibility that the minimal deleted interval contains control elements required for the normal function of these genes.

Additional *in vivo* evidence of the role of *COUP-TFII* in the development of CDH comes from the targeted ablation of *Coup-TFII* in mice. You et al. recently showed that homozygous ablation of *Coup-TFII* in the foregut mesenchyme results in a left-sided, posterolateral CDH similar to that seen in patients with 15q26 deletions (You et al. 2005). Taken together, these data strongly suggest that *COUP-TFII* plays a role in the development of CDH in individuals with 15q26 deletions.

As previously mentioned, CDH is not seen in all individuals with 15q26 deletions involving *COUP-TFII* (Castiglia *et al.* 2005). It is likely, therefore, that other genetic and/or environmental influences either raise or lower the threshold for CDH development in individuals with this deletion. Given that Patient N9 has both a 15q26 deletion and a 2q37 duplication it is possible that overexpression of genes on 2q37 also played a role in the development of CDH in this patient. The hypothesis that overexpression of one or more genes on 2q37 may influence diaphragm development is supported by three other reported cases of CDH involving duplications of 2q37 (Johnson et al. 1992; Brackley et al. 1999; Tonks et al. 2004).

Although it is likely that *COUP-TFII* plays an important role in the development of CDH in individuals with 15q26 deletions, we did not identify CDH-related mutations in the *COUP-TFII* coding region and surrounding splice donor/splice acceptor sequences in our screen of 73 CDH patients. Although this suggests that mutations in *COUP-TFII* are unlikely to be associated with >5% of all CDH cases, it is possible that mutations in this gene are more common in a specific subset of CDH patients such as those with heart defects or other anomalies seen in patients with 15q26 deletions.

Literature reviews have identified several other chromosomal regions that are recurrently duplicated or deleted in patients with non-isolated CDH and are likely to harbor one or more CDH-related genes (Howe et al. 1996; Enns et al. 1998; Lurie 2003). *FOG2*, for example, is located on chromosome 8q23.1 (a region recurrently deleted in CDH patients) and has been shown by Ackerman et al. to cause diaphragmatic eventrations in mice and humans (Ackerman et al. 2005). Data from our patients provide additional evidence for the existence of CGH-related regions on chromosomes 6p22-25 and 14q. Patient TX19 is the first CDH patient to be described with an isolated duplication of 6p but CDH has also been reported previously in a patient with partial trisomy 6p and partial trisomy 22 resulting from 3:1 meiotic disjunction of maternal (6p;22q) translocation (Scarbrough et al. 1986). Although patient TX20 is, to our knowledge, only the second CDH+ patient described with mosaic trisomy 14, partial duplications involving 14q32 have been previously described in two other patients with CDH (de la Fuente et al. 1988; Masada et al. 1989; Howe et al. 1996). These data suggest that over expression of one or more genes in these regions may predispose to the development of CDH.

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CHAPTER 7

General Discussion

GENERAL DISCUSSION

Many years of research that have been put into the subject have yielded interesting results, which will help to unravel the exact mechanisms of lung- and diaphragm development and the etiology of congenital diaphragmatic hernia. Nevertheless, many questions still need to be answered. In the last few years extensive progress has been made in the care for patients with congenital diaphragmatic hernia and much more is known on factors that are likely to play a role in the etiology of CDH. As shown in this thesis and in many other publications, one of the major players in the etiology of CDH are genetic factors. But whether these genetic factors are the sole cause of CDH, or that they merely provide a specific background in which environmental factors can disrupt diaphragm- and lung development in susceptible individuals, remains to be proven. In this chapter I will summarize the data from this thesis, propose some ideas on the mechanism by which these genetic factors can cause CDH, and make recommendations for future diagnostic and research strategies.

Strategies to identify genetic factors in CDH

One of the major difficulties in studying the etiology of CDH is the substantial variability in clinical expression of "the congenital anomaly CDH". The phenotype is highly variable and there are many likely causative factors. This observed clinical variability could be due to environmental factors and modifier genes affecting monogenic inheritance, but perhaps it is more realistic to consider CDH to be a complex multigenic disease. This would explain, in part, why in identifying the genetic factors involved in the etiology of CDH, it is difficult to use approaches that are also used to identify causes for simple Mendelian disorders. Nevertheless, researchers have used several approaches to identify candidate genes for complex disorders. The ones used most often are linkage-based approaches, mutation screens of candidate genes, the study of syndromic forms of CDH and a "positional-candidate" approach.

Linkage-based approaches

Linkage-based analysis is the basis for positional cloning and has turned out to be very useful in studies of monogenic disorders, such as cystic fibrosis (Lander and Botstein 1986). In this type of studies haplotypes shared among affected family-members are determined to identify candidate regions and genes (Dean 2003). A prerequisite for linkage analysis is the availability of a set of families with several affected members. As CDH most often occurs as a *de novo* event within a family there are almost no CDH-families available for this type of analysis. In the Netherlands only three families are known that have more than one affected child and even combining these three families would only lead to low LOD scores. Within the frame-work of large (inter)national collaborations, such as the Congenital Diaphragmatic Hernia (CDH) Study Group, it may be possible to combine data from multiple families to try to reach an acceptable level of significance, but it is doubtful whether this approach will be successful because of phenotypical heterogeneity.

Homozygosity mapping is a linkage-based approach that can be useful in the study of some syndromic forms of CDH that are believed to be caused by recessive inheritance (e.g. Fryns syndrome [OMIM 229850], Donnai-Barrow syndrome [OMIM 222448]) (Langer et al. 1994; Gripp et al. 1997). This approach has been used to study syndromic forms of CDH. Parvari *et al.* (1994) used linkage analysis to map a

syndromic form of Morgagni-type CDH to Xq25-q26. Two syndromic forms of Bochdalek-type CDH were mapped by Kantarci *et al.* (2006b) and Pasutto *et al.*(2007). Kantarci *et al.* used homozygosity mapping in a highly inbred family with Donnai-Barrow syndrome (agenesis of the corpus callosum, hypertelorism, CDH and omphalocele), using SNP genotyping and microsatellite marker analysis. They mapped the locus for this syndrome to chromosome 2q23.3-q31.1 and are in the process of performing mutation analysis of the candidate genes in this region (Kantarci et al. 2006b). Recently, Pasutto *et al.* successfully performed a linkage screen in two unrelated consanguineous families with multiple affected children displaying characteristic facial features in combination with bilateral anophthalmia, pulmonary anomalies, diaphragmatic hernia and cardiac anomalies. They identified homozygous mutations in a gene on chromosome 15q24.1, STRA6 (Stimulated by Retinoic Acid 6), a protein involved in the retinoic acid pathway (Pasutto et al. 2007). Nevertheless, due to genetic heterogeneity of other cases of CDH and low LOD scores caused hereby, linkage analysis has limited use in the search for CDH-candidate genes. However, with the recent development of SNP-genotyping approaches this may change in the future and the technique may find widespread use in the study of CDH and other complex congenital anomalies.

Mutation screen of candidate genes

Another approach that is used in several centers that study CDH, is the screening of candidate genes for mutations in affected individuals. These candidate genes are chosen based on animal studies (for an overview, see Chapter 1), their location in recurrently deleted or duplicated regions in humans, or based on their function in pathways suggested to be involved in the etiology of CDH. Over the last few years several candidate genes for CDH have been screened this way (such as COUP-TFII, SHH, GLI2, GLI3, SLIT3, ROBO1, RALDH2, RALDH3, RLBP1 etc), but no mutations have been identified. With the increasing knowledge on possible involvement of pathways, and findings from other studies, in the future, many more genes (e.g. STRA6, MEF2A) will be analysed by mutation screening.

Studying syndromic forms of CDH

The study of human syndromes has allowed identification of developmental genes and may provide valuable information on the mechanisms by which these genes may cause a specific birth defect (Donnai and Read 2003).

Several syndromes have CDH as one of their clinical features. The most frequently diagnosed syndromes are Edwards' syndrome (trisomy 18) and Patau syndrome (trisomy 13). It is still not known what mechanisms underlie the occurrence of birth defects in trisomies, although downregulation of cholesterol synthesis has been suggested, at least in case of trisomy 18 (Lam et al. 2006).

For several of the syndromes that can have CDH as a feature, the genetic locus is known (Slavotinek 2005). Unfortunately, since CDH is only seen in a minority of the patients affected by these syndromes (e.g. Beckwith-Wiedemann syndrome [OMIM 130650], Cornelia de Lange syndrome [OMIM 122470]), this has not yet resulted in the identification of the causative mechanism for CDH in these patients.

Several other syndromes have CDH as a more frequent (or even cardinal) feature (Goddeeris et al. 1980; Donnai and Barrow 1993; Slavotinek 2004) and many efforts are put into studying these syndromes to identify the locus and/or gene(s) involved in their etiology. For one of the most frequently diagnosed and most studied CDH-syndromes, Fryns syndrome, several candidate loci have been identified, such as 1q42.1, 8q22-q23 and 15q26.2 (Slavotinek et al. 2005; Kantarci et al. 2006a). Because of its relative

homogeneous phenotype and the occurrence of Fryns syndrome in siblings and in children born to consanguineous parents, it was believed that this syndrome is a monogenic disorder with an autosomal recessive inheritance (Manni et al. 1994; Enns et al. 1998). Thus far, no causative gene has been identified for this syndrome. However, it has become clear that the phenotype might be more heterogeneous than was initially believed and some authors have debated whether CDH is truly an obligatory feature of this syndrome (Bamforth et al. 1989; Hanssen et al. 1992; Alessandri et al. 2005). Once the gene for this syndrome is identified, the phenotype will be determined more accurately, but we can never exclude the influence of modifier genes and environmental factors that can lead to phenotypic variability (Brunner and van Driel 2004). It is possible that mutations in different genes give rise to Fryns syndrome, as has been described for other congenital anomalies, such as post-axial polydactyly type A (IMIM 174200), for which at least three loci are known (Biesecker 1998).

In Chapter 4 we describe two patients with a recently identified deletion of chromosome 15q26 and review the literature on this subject. We hypothesize that several of these patients have Fryns syndrome. Although this cannot be said with great certainty for all patients described, several others have a "Fryns-like" appearance. This provides further evidence for a genetic locus for Fryns syndrome on chromosome 15q26. The most likely candidate gene is COUP-TFII, as in animal models disruption of this gene can cause cardiac, cerebral and diaphragm defects similar to Fryns syndrome (Pereira et al. 1999; You et al. 2005). Whether a deletion of the 15q26 region results in true Fryns syndrome and that other chromosomal anomalies (e.g. deletion 1q42.1, deletion 8p23.1) result in "Fryns-like" phenotypes remains to be proven.

In the future, identification of candidate genes and their function will lead to a further understanding of the mechanism that causes this and other CDH syndromes. One of the approaches that will be used in these studies is a "positional-candidate" approach in which chromosomal anomalies are studied in affected patients.

"Positional-candidate" Approach

This method is based on the identification of the smallest region of overlap (SRO) in patients with similar phenotypes that have segmental aneuploidies of the same chromosomal region. This SRO is very likely to contain the gene or genes responsible for development of the disorder that is studied. The positional candidate approach has been used successfully in the identification of CHD7 as the causative gene for CHARGE syndrome (OMIM 214800), and the identification of NIPBL for Cornelia de Lange Syndrome, both syndromes that have CDH as an occasional feature (Krantz et al. 2004; Tonkin et al. 2004; Vissers et al. 2004).

We have used this method to identify the critical region for CDH on chromosome 15q26, as described in Chapters 2 and 3. With improvements in techniques that are used for this approach, such as high-resolution array-CGH, we can only foresee that many more candidate regions and genes for CDH (and other congenital anomalies such as esophageal atresia and different cardiac anomalies) will be identified, and known regions will continue to narrow in size. The ultimate proof that a candidate gene is indeed causative for CDH is the identification of mutations in more than two patients with similar CDH phenotypes. Additional proof can be gained from functional studies to determine the mechanism by which these genes cause diaphragm- and lung developmental defects. In humans this will prove difficult, since it is not feasible to study human diaphragm- and lung development during its critical time-point in development. Therefore, animal models will be essential to study these mechanisms,

complemented by human studies at time-points as close to the critical period as possible, for example in embryonic and fetal tissue, following termination or pregnancy or intra-uterine fetal death.

Methodological considerations: Advantages and disadvantages of the techniques used for the "positional-candidate" approach

Until a few years ago, not many techniques were available for the detection of chromosomal anomalies. In general, only G-banded chromosome analysis was performed, but because of the low resolution of this technique it is likely that cryptic deletions and duplications will not be found. Whenever a distinct phenotype is present, G-banded chromosome analysis is complemented by fluorescence *in situ* hybridization (FISH) to assess a specific locus, but obviously this approach requires prior knowledge on the locus of interest. In some cases also Comparative Genomic Hybridisation (CGH) or Spectral Karyotyping (SKY) are used. Over the last few years, high-resolution whole-genome screening techniques have been developed of which different types of array-based comparative genomic hybridization (array-CGH) have found the most widespread use (for an overview see Table 1) (Pinkel et al. 1998; Speicher and Carter 2005; Ylstra et al. 2006). Array-CGH has proven a reliable and fast technique in several studies, but still many issues exist.

Table 1. Overview of techniques used in (molecular) cytogenetics*

Method	Resolution	Detection of balanced anomalies?	Detection of unbalanced anomalies?
G-banding	~ 5 – 10 Mb	Yes	Yes
FISH	~ 100 – 200 kb	Yes	Yes
CGH	~ 5 – 20 Mb	No	Yes
MLPA	~ 1 – 40 kb	No	Yes
Array-CGH	~ 10 kb – 1 Mb **	No	Yes

* adapted from Feenstra *et al* and Van Dijk *et al.* ** depending on type (BAC, oligo or SNP) and number of probes and their distribution.

Validation of array-CGH results

One issue is how to interpret the many results of these high-resolution arrays, because the numerous calls per patient that will be identified all need validation. Several of these calls will turn out to be false-positives, as we have shown in Chapter 6, in which we found an average of 4 calls per patient. It proved difficult to say whether a change was real by only eyeballing these results and many validation experiments were needed. This is generally no problem in research, but might not be very practical when using these types of array for diagnostic purposes. As expected, the percentage of false-positive results decreased with increasing numbers of putative deleted or duplicated probes. Even if a change is real, it needs to be determined if such a change could just be normal variation in the human population, a subject which will be addressed later in this chapter.

Differences in coverage of the genome between arrays

A second issue is the coverage of different regions of the genome, which differs between array-types,

as illustrated by our analysis of the duplication 11q in CDH (Chapter 5). The telomeric deletion of 12q in both siblings was not seen on the 32k BAC-array we used, probably due to limited coverage of the telomeres on this array, as is the case for many other BAC-arrays as well. Since telomeric rearrangements are among the most frequently identified chromosomal aberrations in patients with CDH or other congenital anomalies, it is imperative to diagnose these aberrations, which can be achieved by using arrays with a good coverage of the telomeric region. There are many other types of arrays available, several of which do have a higher coverage of the whole genome, including the telomeres (Veltman 2006). As these types of array are still relatively expensive, at the moment we first perform Multiplex Ligation-dependent Probe Analysis (MLPA) to screen a CDH patient for subtelomeric imbalances (Schouten et al. 2002). If this analysis is normal, we perform a high-resolution screening by array-CGH.

Copy Number Variation (CNV)

Many deletions and duplications in phenotypically normal individuals have been identified using high-resolution array-CGH. These large-scale copy-number variations (CNVs), distributed throughout the human genome, provide another level of influence on human variation (Feuk et al. 2006; Redon et al. 2006). CNVs are widely distributed throughout the human genome (Iafrate et al. 2004; Sebat et al. 2004; Sharp et al. 2005). Their existence has implications for our studies into copy number changes and their role in the development of CDH and other congenital anomalies, because a majority of the changes that will be identified in studies using a "positional-candidate" approach are likely to be CNVs, instead of disease-causing deletions or duplications.

In Chapter 6 we identified a total of 81 changes in 26 patients. Only 7 turned out to have occured *de novo,* and thus are more likely to be causative. A large number of the inherited changes had been described in the Database of Genomic Variants (http://projects.tcag.ca/variation/) so we assumed these were CNVs. This might not have been completely correct. Recently it has been shown that deletion of 17q21, flanked by two low copy repeats (LCRs) can lead to a distinct phenotype of characteristic facial features, mental retardation and severe hypotonia (Koolen et al. 2006; Shaw-Smith et al. 2006). In the past, the region of 17q21 was only known as an inversion polymorphism. If this inversion is present it positions two LCRs in a tandem position, predisposing to deletion of this region. In the past, phenotypic descriptions of microdeletion syndromes have always preceded the identification of the causative chromosomal aberration, for example the DiGeorge/Velocardiofacial syndrome (OMIM 188400) caused by a microdeletion of 22q11.2. But the introduction of high-resolution techniques now allows us to identify cytogenetic anomalies in advance of clinical recognition of the associated phenotype, as illustrated by the deletion 17q21.31 findings, in which the molecular cytogenetic screening was carried out before detailed information on the phenotype was known. The search for causative chromosomal anomalies and genes can thus also be carried out "the other way around" and patients with rare phenotypes will become more and more interesting. We, therefore, may be able to discriminate between different groups of CDH patients, initially believed to be similar, by identifying their chromosomal anomaly first. This may provide more detailed information on causative mechanisms. International collaborations are needed to collect groups of patients that are large enough to make identification of such phenotypes possible. This also emphasizes the need for accurate phenotypic descriptions of patients, and the sharing of these clinical and cytogenetic data, for which several online databases have been established, such as the ECARUCA (http://www.ecaruca.net/) database and the DECIPHER (http://www.sanger.ac.uk/PostGenomics/decipher/) database.

Inherited changes identified in patients with CDH

In addition to the *de novo* changes and known polymorphisms, we identified several changes that were shown to be inherited from a phenotypically normal parent (see Chapter 6). We assumed that these changes were copy number variations that are present in the normal population. Since a deletion or duplication inherited from an unaffected parent is not necessarily a polymorphism, this strategy may not have been entirely correct. Through different mechanisms, inherited copy number changes can play a role in the development of CDH or other congenital anomalies. Firstly, reduced penetrance can be present, which is known be the case for other genetic anomalies, such as the microdeletion that has recently been discovered in patients with Thrombocytopenia-Absent Radius Syndrome (TAR syndrome) which was also present in several phenotypically unaffected family members (Klopocki et al. 2007). At this point, for most of the chromosomal anomalies identified in CDH, we do not know whether these changes are 100% penetrant. Therefore, we might need to refine our conclusion from chapter 2. If a deletion of 15q26.2 is not completely penetrant, excluding a part of 15q26 from the critical region by including patients without CDH in our analysis, may not have been correct, a point also made by Castiglia *et al* (2005) (see chapter 3 and a copy of their publication in the appendix). If we exclude the patients without CDH from the analysis, the CDH critical region is located on chromosome 15q26.2-q26.3 (see Chapter 4) and contains several additional candidate genes. Reduced penetrance could explain the variability in phenotype seen in patients with deletions of 15q26 and would therefore support the hypothesized role of this locus in the etiology of CDH. An additional level of variation may be added by environmental factors (e.g. toxins, or nutritional factors such as vitamin A) acting on genetically susceptible individuals.

Another reason why an inherited change could be causative, is that a second 'hit' may be needed before CDH develops and that the parents each only carry 1 of these "hits", explaining their normal phenotypes. Such a second hit can be a deletion or mutation disrupting another gene or disrupting the other allele of the same gene, a phenomenon known as "compound heterozygosity" (Wijmenga et al. 2000; Verstraeten et al. 2006). A similar mechanism has been recently described for Peters Plus syndrome (OMIM 261570), an autosomal recessive disorder characterised by eye-anomalies, short stature and developmental delay. Lesnik Oberstein *et al*. (2006) identified a family in which two affected brothers had a maternally inherited deletion associated with a paternally inherited mutation of the B3GALTL gene. Since both parents had only one affected allele they were phenotypically normal. Homozygous mutations of B3GALTL were shown to cause Peters Plus syndrome in all additional patients analysed. Another example is the, previously mentioned, TAR Syndrome. Klopocki et al. (2007) identified a microdeletion of 1q21 in 30 patients with this syndrome and found that in 75% of the cases this deletion was inherited from an phenotypically normal parent, suggesting that a second affected locus has to be present in an individual to cause the disease phenotype, a mechanism known as double or compound heterozygosity. The occurrence of compound heterozygosity could be an explanation for the phenotype seen in patients with 15q26 deletions, as this region contains several genes.

The occurrence of multiple congenital anomalies in patients with 15q26 deletions may also be explained by the possibility that monosomy 15q26 is a contiguous gene syndrome in which haploinsufficiency of several functionally unrelated genes within the same region is responsible for the clinical features, as has been described for, for example, monosomy 1p36 and deletion 22q11.2 (DiGeorge/Velocardiofacial syndrome) (Wu et al. 1999; Momma 2007). It is possible that deletion of two or more genes together is responsible for the disease phenotype.

Possible mechanisms of defective lung- and diaphragm development

Given the phenotypic ánd genetic heterogeneity is it likely that CDH has a multifactorial inheritance mode. The genetic heterogeneity might reflect interactions of several candidate genes at the protein level, such as proteins that function at different steps of a pathway, and there are many levels at which it can go wrong, causing a specific disease phenotype. One pathway that for long has been hypothesized to play a role in CDH is the Retinoic Acid Signaling (RA) Pathway.

In chapters 2 to 6 our characterization of some of these CDH candidate loci is described. In the following part of this discussion I will show that these loci harbour several interesting candidate genes. These candidate genes and others, identified in animal studies, have been shown to disrupt diaphragm- and lung development. The development of lung and diaphragm cannot be regarded as two separate processes, since both humans and animals with CDH have similar lung phenotypes, in addition to a diaphragm defect. The exact mechanism of diaphragm development is still unknown, but more is known on pulmonary development, which is regulated by many pathways and genes. It is outside the scope of this thesis to address all these pathways, but the most important ones will be mentioned.

Pathways and genes involved in lung development

Human lung development can be divided into six stages, based on morphological changes: 1, the *embryonic period*, in which the main bronchi of the lungs are formed; 2, the *pseudoglandular period*, in which branching morphogenesis occurs and the terminal bronchioli are formed; 3, the *canalicular period*, in which respiratory bronchioli appear and vascularization of the perifpheral mesenchyme increases; 4, the *saccular period*, when the peripheral airspaces expand and become sacculi; 5, the *alveolar period*, in which secondary septa appear on the existing primary septa in the saccular lung to form alveoli; and 6, *microvascular maturation* (for review see Groenman et al. 2005).

Several growth factor- and signalling pathways play a role in lung development (see Figure 1). One of the most important growth factor pathways is the Fibroblast Growth Factor (FGF)-pathway, in particular mesenchymal FGF-10 and its epithelial receptor FGFR2. Epithelial-mesenchymal signalling is necessary for the development of several organs, such as the lungs in which the epithelial-mesenchymal FGF-pathway is involved in the formation of airway branches. FGF-10, amongst others, influences the expression of Bone Morphogenetic Protein (BMP) 4, which regulates epithelial proliferation and proximal-distal differentiation. BMP4 expression is actived by several genes, such as GATA4, which is one of the candidate genes for CDH (Nemer and Nemer 2003). GATA4 may mediate the indirect effect of retinoic acid on BMP4 and as retinoic acid also shows a proximal-distal gradient during certain stages of lung development, these expression pattern may be functionally related to each other (see Figure 1) (Helvering et al. 2000; Malpel et al. 2000).

Another important pathway involved in lung-development, is the Sonic Hedgehog (SHH) Signaling-pathway. It has been proposed that SHH is a target of FGF-10/FGFR2 signalling. Mesenchymal FGF-10 up-regulates the epithelial expression of SHH, which signals back to FGF-10 via a negative regulatory pathway, so that the epithelium can control the extent of its outgrowth. This feedback loop may be controlled by T-box genes (TBX) 4 and 5 (Groenman et al. 2005). During lung development a vascular network surrounds the emerging lung bud (Parera et al. 2005). Vascular Endothelial Growth Factor (VEGF) is an important angiogenic factor, regulated by the Hypoxia Inducible Factor 1α (HIF-1α), which in turn is controlled by the tumour suppressor gene Von Hippel-Lindau factor (VHL). Mutations in VHL can cause neural crest cell-derived tumours such as neuroblastoma and pheochromocytoma

(Dannenberg et al. 2003; Hoebeeck et al. 2006). Neural crest cells are responsive to numerous factors, such as signals from the SHH – and FGF-pathways (Sandell and Trainor 2006). The expression of FGF-10, FGFR2 and VEGF was shown to be downregulated in the lungs of rats exposed to nitrofen (Teramoto et al. 2003; Chang et al. 2004). In humans, lungs of CDH patients show differences in expression patterns of VHL and HIF-1α, and delayed expression of the SHH pathway, which peaks in the canalicular/saccular period as opposed to the pseudoglandular period in normal lung development (Unger et al. 2003; de Rooij et al. 2004). This suggests a persistence of the fetal stage of pulmonary development in patients with CDH due to aberrant signalling in lung morphogenesis (Yu et al. 2001; Groenman et al. 2005).
Although not much is known about the exact role of CDH candidate genes in pulmonary development, in almost all animal models for CDH there is also abnormal development of the lungs. The lung abnormalities seen in the nitrofen rat-model precede the visceral herniation and it is suggested that lung hypoplasia and aberrant pulmonary vasculature development precede the actual occurrence of the diaphragm defect. This is consistent with the findings of Babiuk and Greer (2002), who showed that FGF-10$^{-/-}$ mice, which lack formation of the lungs, do develop a diaphragm. A defective development of the diaphragm may aggravate disruption of lung development (Keijzer et al. 2000). Several genes suggested to be involved in the etiology of CDH have been shown to play a role in epithelial-mesenchymal signalling (e.g. COUP-TFII and the RARs), corroborating the link between diaphragm- and lung development.

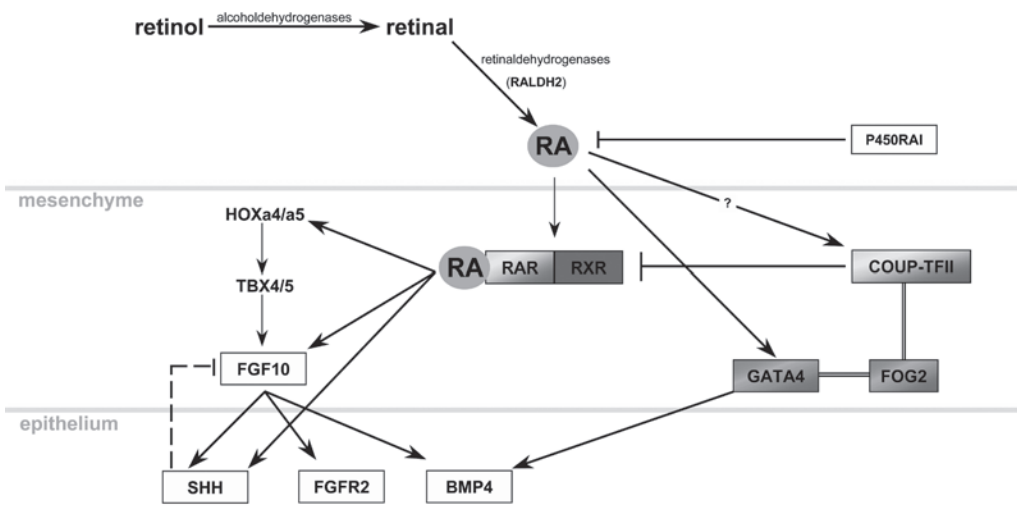

Fig. 1.
Cellular interactions in lung development. Genes that play a role in epithelial-mesenchymal signalling are shown. Influence of retinoic acid on these genes, if present, is indicated.
■ cardiac anomaly in animal models or in humans when mutated; ■ "true" diaphragm defect in animal models;
■ muscularization defect in animal models;
⊣ inhibition; → proven (in)direct regulation by retinoic acid; **?**, intermediate, unknown, gene; - - -⊣ possible inhibition;
═ binds to; **RA**, retinoic acid; **(C)RBP**, (cellular) retinol binding protein; **RAR**, retinoic acid receptor; **RXR**, retinoid X receptor; **RARE**, retinoic acid response element. Color figure can be found in the appendix. See page 138.

Chromosome 15q26 candidate genes and their role in CDH

The most important region described in this thesis is the CDH critical region on chromosome 15q26, which was proposed as a CDH candidate region for the first time a few years ago (Biggio et al. 2004; Tonnies 2004). The first patient with CDH and a 15q deletion was described in 1987, but it was not until 2004 when 8 patients had been described, that Biggio *et al.* suggested that the distal part of chromosome 15q might play a crucial role in the development of CDH (Kristoffersson et al. 1987; Biggio et al. 2004). The distal part of chromosome 15q26 harbours several CDH candidate genes:

COUP-TFII (NR2F2)

The most promising candidate gene located within the 15q critical region is the Chicken Ovalbumin Upstream Promotor-Transcription Factor II (COUP-TFII; Nuclear Receptor Subfamily 2, group F, member 2 or NR2F2), an orphan member of the steroid receptor superfamily (Cooney et al. 2001). This gene has been implicated in mesenchymal-epithelial interaction during organogenesis of different organs, such as the lung (see Figure 1) (Tsai and Tsai 1997; Malpel et al. 2000). In 1999, a group at Baylor College of Medicine in Houston, published the first results on their *Coup-tfII* null mutant mouse in which they showed that *Coup-tfII* is essential for cardiac and vascular development (Pereira et al. 1999). Homozygous null mice die at E9.0 due to stagnation of atrial development. Six years later, a few months after our first publication on the CDH critical region on chromosome 15q26, the same group showed that targeted ablation of *Coup-tfII* in the foregut mesenchyme and post-hepatic mesenchymal plate (the tissues that will eventually form the diaphragm and lungs) results in posterolateral diaphragm defects and lung hypoplasia, similar to humans (You et al. 2005). The expression of COUP-TFII has previously been shown to be regulated by retinoids, and COUP-TFII itself regulates gene transcription by influencing RAR/RXR heterodimerization to limit the availability of RA to prevent teratogenic effects (see Figure 2) (Qiu et al. 1996; Malpel et al. 2000; Wang et al. 2006). The role of this pathway in the etiology of CDH will be discussed later.

As COUP-TFII has numerous roles in embryonic development it is very likely that disruption of COUP-TFII causes a multisystem malformation syndrome that includes lung-, diaphragm- and cardiac defects. Whether disruption of COUP-TFII is also responsible for cases of isolated CDH remains questionable.

RALDH2 & RALDH3

Chromosome 15q contains two important genes involved in the retinoic acid signalling pathway: RALDH2 and RALDH3. RALDH2 (15q22.1) is a key enzyme in the RA pathway and has been shown to be inhibited in the nitrofen rat model for CDH (see later in this chapter) (Mey et al. 2003). RALDH3 (15q26.3), another retinaldehydrogenase, is deleted in almost all CDH patients with 15q26 deletions (see Chapter 1). Compound heterozygosity for one or more of these genes in association with a disruption of COUP-TFII, could be responsible for the development of CDH. Mutations in these genes might also be responsible for the development of CDH in patients without 15q deletions, although no mutations have been identified in large mutation screens by us and collaborating research groups.

STRA6

Pasutto *et al.* (2007) described two unrelated consanguineous families with several children affected by bilateral anophthalmia and distinct facial features. In addition, most of the affected children had

pulmonary anomalies (alveolar capillary dysplasia, lung hypoplasia), diaphragm defects and cardiac anomalies. Mutations in STRA6 (Stimulated by Retinoic Acid 6), located on 15q24.1, were found in the affected family members and in three additional, unrelated, cases. Although STRA6 is located outside the CDH critical region, it is possible that disruption of this gene is also responsible for other cases of CDH.

CHD2

Another gene, CHD2 (chromodomain helicase 2), is a member of the SNF2/RAD54 helicase family. Mutations in another member of this family (CHD7) have been shown to cause CHARGE-syndrome (Vissers et al. 2004). It is possible that a disruption of the function of CHD2 is involved in causing CDH. However, since this gene is located approximately 1Mb proximal of the CDH critical region, such a disruption could only be explained by another mechanism than deletion of the gene itself, for example deletion of a downstream located regulatory element or alterations in the localization of CHD2 in the nucleus.

MEF2A

Biggio *et al.* suggested that the myocyte-enhancer factor 2A (MEF2A) was the best candidate gene for the development of CDH in humans with this deletion, since MEF2A plays a critical role in muscle differentiation and development, also in the developing diaphragm (Naya et al. 2002). However, by determining the CDH critical region on chromosome 15q26.2 we showed that this gene is located outside this critical region. It needs to be noted that this gene is only excluded from the region by the distal breakpoint in just one patient (case 1 in chapter 2) and that all other patients described are haploinsufficient for this gene. One of the hypotheses regarding the etiology of CDH is a defect in muscularization of the diaphragm and therefore MEF2A should not completely be excluded as a candidate gene for CDH, at least for some subtypes of CDH.

Other possible mechanisms of disruption of COUP-TFII and other candidate genes

These data make a role for COUP-TFII and other genes of the RA-pathway in the development of CDH likely, but whether abnormalities in COUP-TFII are responsible for cases of CDH not associated with 15q26 deletions remains to be proven, still leaving more than 90% of cases with an unknown etiology. We, in collaboration with other research groups (in Houston, Boston and San Francisco), have screened approximately 150 patients with isolated and non-isolated CDH, but to date, no CDH-causing mutations within the coding region of COUP-TFII have been identified. However, it is possible that disruption of the function of COUP-TFII is caused by another mechanism than a missense or nonsense coding mutation in the coding region, such as disruption of a distant regulatory element of COUP-TFII, the influence of micro-RNAs or distortion of the 3-dimensional structure of the chromosomes.

Gene deserts and distant regulatory elements

COUP-TFII is located in a gene-poor region on chromosome 15q, possibly between a pair of gene deserts, that are known to cluster in pairs surrounding a small number of genes (Ovcharenko et al. 2005). The long loci with minimum gene density that arise this way are usually well preserved throughout the evolution of vertebrates, as is true for the region surrounding COUP-TFII. The majority of genes in these

regions are involved in core biochemical processes, such as regulation of transcription and these gene deserts are known to harbour distant gene-regulatory elements in the human genome (Ovcharenko et al. 2005). It could very well be that, instead of an alteration in the coding region of COUP-TFII, a mutation in such a regulatory element is present, even though such alterations have only been identified in less than 1% of mutations (Botstein and Risch 2003). A well-known example is preaxial polydactyly which can be caused by mutations in the Sonic Hedgehog Enhancer Element (Lettice et al. 2003). Several regulatory factors can influence the expression of a gene during different stages of embryonic development or in different structures and if such a specific regulatory factor would be disrupted, instead of the gene itself, this would explain the fact that patients with 15q deletions survive to birth, since complete haploinsufficiency of COUP-TFII in the developing embryo is very likely to be lethal early in development. However, such regulatory elements for COUP-TFII have not yet been identified.

Micro-RNAs (miRNAs)

Besides structural and regulatory genes that control lung- and diaphragm development, there may also be small non-coding RNAs, such as micro-RNAs (miRNAs) that are expressed, which target mRNA of genes involved in translational inhibition or degradation, thereby influencing the expression of genes involved in lung- and diaphragm development. Several miRNAs, in particular miR1, are known to influence cardiac- and skeletal muscle regulation, via SRF1, MEF2 and MYO D (Srivastava 2006). The understanding of the role that these and other miRNAs play in this development, and the etiology of CDH, is an important subject for future research.

3D configuration

It is known that the three-dimensional (3D) organization of chromosomes in the nucleus influences gene expression through physical interaction of genes separated by large stretches of DNA or located on different chromosomes (Parada et al. 2004). Therefore, another mechanism by which disruption of the function of one of the 15q genes (e.g. COUP-TFII, CHD2, RALDH2) causes CDH, is that deletion of a region distal from this gene disrupts the 3D organization. This may lead to changes in the physical interaction between the candidate gene and other genes necessary for correct regulation of embryonic diaphragm- and lung development (Kleinjan and van Heyningen 1998). The field of 3D organization of the nucleus and its role in gene expression is arising as a new topic in cellular biology. In the future, techniques aimed at unravelling this 3D structure and organization, and the mechanisms by which it influences gene regulation, will yield many intriguing results (Simonis et al. 2006).

Retinoic Acid and CDH

The retinoid receptors RAR and RXR are ubiquitously expressed in the developing lung and expression patterns of different subtypes (mainly RARα, RXRα and RXRγ) change with increasing gestational age (Kimura et al. 2002; Rajatapiti et al. 2005). During lung development there is a dynamic pattern of expression of components of the RA-pathway that can be summarized in the following phases: 1, highly active and ubiquitous expression during the initial stage of lung formation; 2, a proximal-distal gradient of RA activation during the appearance of lateral buds, with less RA response in the distal mesenchyme near sites of budding; and 3, continued RA synthesis (by the pleura) but little RA usage. COUP-TFII plays an important role in the second and third phases of this gradient. In the distal

lung mesenchyme it negatively regulates RA synthesis by inhibiting RAR/RXR heterodimerization. In the epithelium RA synthesis is regulated by P450RAI (P450 Retinoic Acid Inactivating) mediated degradation. Both mechanisms prevent an excess of RA in the developing lung (Malpel et al. 2000). Vitamin A (retinol) and its derivatives (in particular the active forms *all-trans* and *9-cis* retinoic acid) are essential for embryonic development. The RA-signaling pathway has been shown to be involved in most morphogenetic and patterning processes, and abnormalities in the retinoic acid signalling pathway, and its downstream targets, have long been hypothesized to lead to the development of CDH. The first evidence for the role of vitamin A in the etiology of CDH arose already 60 years ago when it was shown that the offspring of rat dams fed a diet deficient in vitamin A developed CDH, and that the proportion of affected offspring diminished when vitamin A was reintroduced into the diet (Anderson 1941; Anderson 1949; Wilson et al. 1953). Additional evidence for the role of the RA-pathway came when it was shown that *in utero* exposure to the herbicide nitrofen, bisdiamine (a spermatogenesis inhibitor), SB-210661 (a 5-lipoxygenase inhibitor) or BPCA (a thromboxane-A_2 receptor antagonist), were shown to cause diaphragm defects and abnormal pulmonary development in rodents. These teratogens were shown to inhibit one of the genes implicated in the etiology of CDH, retinaldehyde-dehydroxygenase 2, a key enzyme in the RA pathway (RALDH2, see Figures 2) (Mey et al. 2003).
Both an excess of RA and RA deficiency are harmful and may cause CDH. Mammalian embryos are protected against vitamin A deficiency by maternal retinoid homeostasis until these storages are depleted (Morriss-Kay and Sokolova 1996). Major *et al.*(Major et al. 1998) published data showing that children with CDH at birth had significantly lower levels of retinol and retinol-binding-protein (RBP) than controls. Their mothers, however, had significantly higher levels of retinol and RBP. This could indicate a problem in placental transport.

Genes and their associated diaphragm defects

As mentioned in chapter 1, several animal models in which diaphragm defects are part of the spectrum of anomalies have been developed (see also Table 1 in Chapter 1). These models can be roughly divided into two groups:

1. disruption of diaphragm development resulting in 'true' diaphragm defects where there is a (partial) absence of all structures of the diaphragm at that location, resulting in a hole in the diaphragm. These models include the tissue specific *Coup-tfII*, the double *Rar*α and β, the *Wt1*, the *Robo1* and the *Slit3* knock-out mice, and the nitrofen rat model.
2. disruption of diaphragm development resulting in muscularization defects. There is still some tissue present, but this is merely a thin layer of connective tissue without the normal layers of muscle tissue, and much weaker than a normal diaphragm. These models include the *Fog2* ENU-mutant, the *Gata4*, the *Pax3*, the *c-Met*, the *Lysyl Oxidase* (*Lox*), the *Nedd4* and the *Sim2* knock-out mice.

This second group of diaphragm defects resembles a particular type of CDH seen in humans in which there is a remaining sac of diaphragm tissue covers the abdominal contents, an "eventration of the diaphragm", and these contents herniate into the thoracic cavity. Although patients with an eventration may have similar postnatal problems resulting from the associated pulmonary hypoplasia and abnormal lung vasculature, it has been thought for a long time that this eventration of the diaphragm and other diaphragm defects, such as the posterolateral CDH, are two distinct entities. Ackerman *et al.* (2005)

suggested that FOG2 mutations might not only cause muscularization defects of the diaphragm, but also play a role in the development of posterolateral diaphragm defects. To date no mutations in any other CDH patient have been identified. However, it is interesting to note that human FOG2 is located on chromosome 8q23 in a region commonly deleted in individuals with CDH, and that FOG2 interacts physically with COUP-TFII (Huggins et al. 2001). It is possible that these proteins work together to regulate down-stream target genes that play a role in the development of CDH. FOG2 can also interact physically with the GATA genes, in particular GATA4 (Cantor and Orkin 2005). Binding of COUP-TFII and GATA4 to FOG2 can occur at the same time, because both genes bind preferentially to different zinc fingers of FOG2 (see Figure 2) (Huggins et al. 2001). The heterozygous *Gata4*$^{+/\Delta ex2}$ mice, described

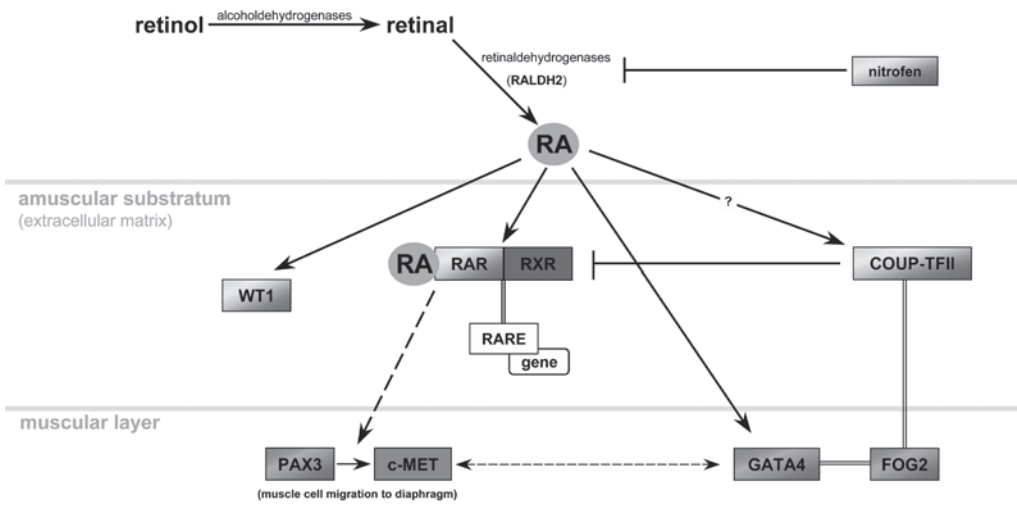

Fig. 2.
Cellular interactions in diaphragm development. Genes that cause diaphragm defects in animal models are shown. Genes in the region of the "amuscular substratum" cause "true" diaphragm defects when disrupted in mice. Genes in the muscular layer cause eventration of the diaphragm in mutant mice.
cardiac anomaly in animal models or in humans when mutated; "true" diaphragm defect in animal models; muscularization defect in animal models;
inhibition; possible regulation or interaction, not proven; (in)direct regulation; **?**, intermediate, unknown, gene; binds to; **RA**, retinoic acid; **(C)RBP**, (cellular) retinol binding protein; **RAR**, retinoic acid receptor; **RXR**, retinoid X receptor; **RARE**, retinoic acid response element. Color figure can be found in the appendix. See page 138.

by Jay *et al.* (2007), displayed cardiac, lung, or diaphragm defects. The diaphragmatic defects, which affected approximately 30% of mice, were located in the ventral midline and were covered by a sac that was continuous with the diaphragm, resembling both the *Fog2* animal model (eventration of the diaphragm) and *Slit3* animal model (central localization of the defect). However, to date no mutations in GATA4 have been identified in patients with CDH.

Possible role for disruption of neural crest development in the etiology of CDH

Several of the candidate genes for CDH have been shown to be involved in RA-signaling in one or another way. Remarkably, most of these genes cause a "true" diaphragm defect (hole) when disrupted

in animals. In contrast, the genes that cause muscularization defects in animal models of CDH have not yet been shown to be involved in the RA pathway (see Figure 2). Exceptions within group 1 ("true" defects) are SLIT3 and its receptor ROBO1 (Xian et al. 2001; Liu et al. 2003; Yuan et al. 2003). *Slit3* deficient mice have diaphragm defects and it is possible that central defects are caused by a different mechanism than posterolateral defects (Xian et al. 2001). An exception within group 2 (muscularization defects) is GATA4 which can be regulated by retinoic acid (Kostetskii et al. 1999).

These differences automatically raise the question whether true diaphragm defects (a hole) and muscularization defects (an eventration) are distinct entities after all, and caused by disruption of different pathways.

This explanation is less likely, because patients with "true" diaphragm defects as well as patients with an eventration have similar lung phenotypes, and genes from both groups do interact at some level (GATA4 can be regulated by RA, FOG2 interacts with COUP-TFII).

Therefore, it is more likely that both types of diaphragm defects share a common factor. A possibility for such a common factor might be present in Neural Crest Cell (NCC) development (see Figure 3), which was suggested to be involved in CDH already a decade ago (Alles et al. 1995).

In 1995, Alles *et al.* showed excessive cell death patterns in somites 2 - 4 in the nitrofen rat model, 24 hours after administration of nitrofen [Alles 1995]. Later in development cell death was apparent in the mesoderm adjacent to the somites in the septum transversum. Cervical somites 3, 4 and 5 contribute to the primordial diaphragm and form the major portion of the diaphragm muscle (Moore and Persaud 1993). Alles *et al.* (1995) suggested the possibility that damage of the mesodermal cell populations in cervical somites 2 – 4 and in the mesenchyme adjacent to the septum transversum reduces the number of progenitor cells that are necessary to populate the primordial diaphragm. Several of the CDH candidate genes (GATA4, ROBO1, SLIT3, PAX3 and c-MET) have been shown to be involved in neural crest development as have several of the lung development genes (Kostetskii et al. 1999; Lang et al. 2004; Jia et al. 2005).

NCC development has been shown to be regulated by many signalling pathways, one of which is the RA-pathway. RAR$\alpha\beta$-null mutants and mice exposed to nitrofen display a complex of anomalies similar to the human DiGeorge/Velocardiofacial syndrome (DG/VCF syndrome) with thymic, parathyroid, craniofacial and cardiac anomalies, that are also seen in a number of human patients with CDH (Wickman et al. 1993; Mark et al. 2004; Jones 2005). Disruption of cervical neural crest development is believed to be the basis for DG/VCF syndrome (Momma 2007).

Cardiac anomalies are seen in almost all CDH animal models and in a large percentage of human patients with CDH (~10-25%) (Graziano 2005; Yang et al. 2006). The major target of RA in cardiac development is the neural crest. Yu *et al.* (2001) showed that the malformations seen in rats exposed to nitrofen were affecting the structures derived from the pharyngeal arches and thus are neural crest-related. Abnormally high and low levels of RA induce similar cardiac anomalies which suggests a critical balance in RA signalling. Also, in eye-, craniofacial- and forebrain development the regulation of NCC development by RA is very important (Matt et al. 2005; Ribes et al. 2006).

Based on these data it is very well possible that both the RA pathway and the neural crest play an important role in lung- and diaphragm development, and the etiology of CDH. It is too early to decide which of these two pathways is the most important one, or whether both are equally important but each cause a different type of diaphragm defect.

Nevertheless, in conclusion we can say that several evolutionary conserved transcription factors (e.g. GATA4, TBX4/5, COUP-TFII and possibly MEF2A) regulate lung- and diaphragm development. It may be that this group of genes constitutes a "core regulatory network" which controls cell fate, the expression of different genes and morphogenesis of the lung and diaphragm. Such a "core regulatory network" has been identified for cardiac development and may serve as a framework for other organ systems as well (Olson 2006). Each of the genes in this network has several family members (e.g. the different GATA genes) that have functional redundancy. Different organ systems may arise, based on differences in expression of these different family members. The overlap in gene function and regulatory networks may explain the co-occurrence of CDH and cardiac defects in human patients and animal models, as several genes are involved in both organ systems (see Figures 1 – 3). The "core regulatory network" is influenced by different inductive signals, of which most have not yet been identified. Retinoic acid

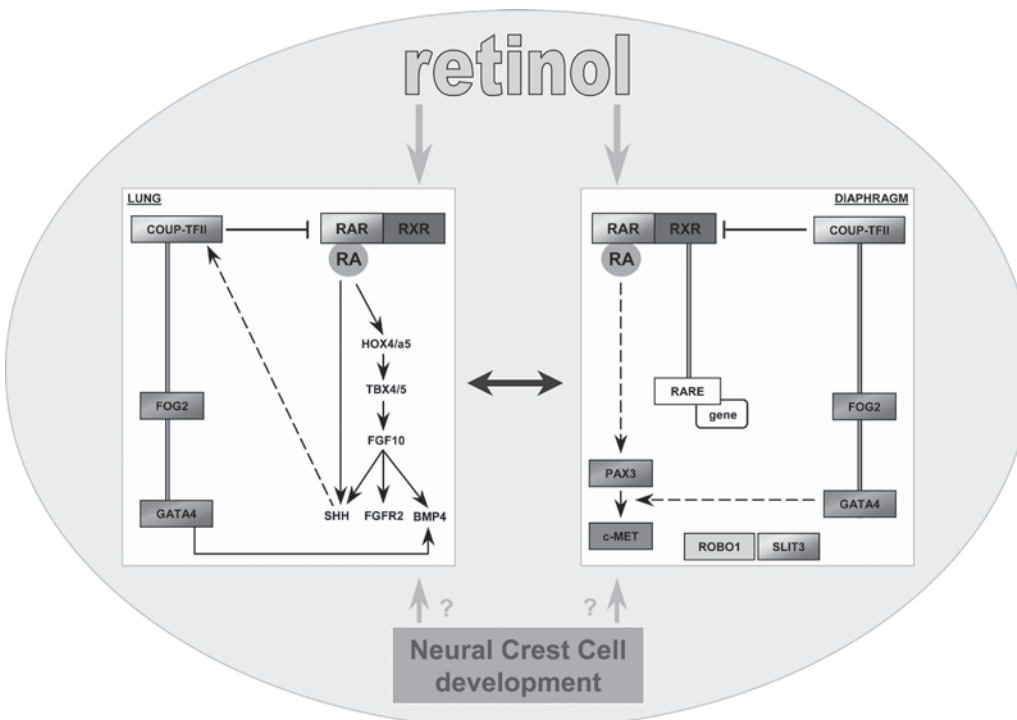

Fig. 3.
Possible cellular interactions involved in the etiology of Congenital Diaphragmatic Hernia. Both lung- and diaphragm development are under the influence of retinal and its active metabolite retinoic acid, a diffusible factor in embryonic development. Neural Crest Cell development may be a common factor, involved in all types of diaphragm defects and lung development, is also influenced by retinoic acid.
■ cardiac anomaly in animal models or in humans when mutated; ■ "true" diaphragm defect in animal models; ■ muscularization defect in animal models; ○ retinol (and retinoic acid), an important diffusible factor; ⊣ inhibition; - - ➤ possible regulation or interaction, not proven; ⟶ (in)direct regulation; **?**, intermediate, unknown, gene; ═ binds to; **RA**, retinoic acid; **(C)RBP**, (cellular) retinol binding protein; **RAR**, retinoic acid receptor; **RXR**, retinoid X receptor; **RARE**, retinoic acid response element. Color figure can be found in the appendix. See page 139.

has been suggested to be one of these signals and perhaps other signals arise from the neural crest. But since the translation of animal findings to the human situation is difficult, as there are differences in redundancy of members of a gene family between mouse and humans, many factors need to be investigated before the core regulatory network for CDH can be identified.

Future perspectives

Research into the underlying causes of CDH has the potential to positively affect the clinical management of individuals with CDH and their families. The description of multiple genetic syndromes associated with CDH highlights the importance of a careful evaluation of patients with CDH. Such an evaluation may have an influence on medical decision making including decisions about possible termination of a pregnancy or the decision to change or withhold treatment.

In most clinical settings newborns with CDH will not be examined by a clinical geneticist, except in those cases where there are strong indications that a syndrome or chromosomal anomaly may be present (e.g. the presence of dysmorphic features, associated major birth defects or a positive family history). Usually only in these cases, a clinical geneticist will be asked to assess the child. However, important clues to a diagnosis might be missed with this approach, because subtle anomalies are easily overlooked. Therefore, we recommend that all children with a diaphragm defect (or any other congenital anomaly) should be routinely evaluated by an experienced clinical geneticist/dysmorphologist, even if it seems that no other anomalies are present. This approach will not only be beneficial for diagnosis, but will also be potentially helpful in therapy, counselling and research.

Whenever a syndrome is diagnosed or when the phenotype is highly susceptive for a particular chromosomal anomaly, diagnostics (G-banding and / or FISH) to confirm such a diagnosis can be carried out. This strategy will only be applicable to a subset of patients with CDH, because the majority of patients indeed turn out to have isolated CDH, or are MCA patients in whom it is not possible to make a specific diagnosis. We believe that these groups of patients should be screened by a high-resolution whole-genome tool to identify cryptic unbalanced chromosomal rearrangements that otherwise could go undetected. Nowadays, with improvements in the field of genotyping, very small genomic deletions and duplications can be detected, and breakpoint mapping has become increasingly less labour-intensive. Therefore, DNA, chromosomes, cell lines and parental DNA should be routinely stored for diagnostic and research purposes, because we are now at the point where it has become necessary to screen large cohorts of patients for *de novo* mutations in the known candidate genes and their regulatory elements, to prove their role in human CDH. The chance of identifying such an event may be low, considering that CDH patients likely represent a heterogeneous population in which *de novo* mutations in many different genes can result in CDH. Such mutation screens require both the recruitment and screening of large numbers of CDH patients, which can be achieved within the framework of international collaborations, such as the Congenital Diaphragmatic Hernia Study Group, whose database already contains data of over 3500 patients.

As our knowledge on structural rearrangements of the human genome increases, these chromosomal regions will assist researchers in identifying clinically relevant regions and candidate genes to investigate further the etiology of CDH. However, we should not only look for unbalanced rearrangements, because a single clinical observation of a patient with a rare syndrome and a balanced chromosome translocation can lead to the identification of the gene involved (Donnai and Read 2003). If a balanced

translocation disrupting a gene within the 15q26 critical region would be found in a child with CDH (and possibly other congenital anomalies), this would provide us with essential information regarding the causative gene within this region. So far, no such translocation has been identified, nor for the region on 15q26 nor for any of the other CDH candidate regions.

Treatment of children with CDH still is, for a large part, trial-and-error. Despite advances made in therapy, including permissive hypercapnia, the use of inhaled nitric oxide and ECMO-treatment, it is still not known why some children respond well to therapy and others, who seem to have the same clinical characteristics, fail to respond to the same treatment-strategies. Our understanding of the genetic factors associated with CDH may make it possible to devise preventative strategies or improve therapeutic interventions for CDH patients. It is important to keep in mind that measures aimed at improving clinical outcome may not require the prevention/correction of the diaphragmatic defect itself since the size of the diaphragmatic defect is not correlated to survival, except in those cases where there is complete diaphragm agenesis (Lally et al. 2006). It may become possible to determine a "profile" for every patient with CDH, which can be used, in retrospect, to determine which group of patients responded well to therapy and which group of patients did not. In the future this might help us to devise more individual treatment strategies, which will lead to a decrease in mortality and morbidity and, potentially, even will lead to individual strategies following prenatal diagnosis.

Besides benefits for diagnosis, treatment and counselling, a precise description of the phenotypic characteristics (and syndrome diagnosis) will also provide more clues as to what factors might cause defective diaphragm development, and therefore will aid researchers in their search for CDH-causing factors. Future research efforts will improve our understanding of mechanisms involved in CDH. New candidate regions will be identified and known regions will continue to shrink in size, providing us with more clues as to which genes are important in the etiology of CDH. Additional effort must be put in determining the role these genes play in lung- and diaphragm development and we have to provide the ultimate proof that disruption of these candidate genes indeed causes CDH.
In part this can be achieved by using knowledge we already have on pathways that are likely to be involved in CDH, such as the RA-pathway, because this knowledge can be used to help the gene-finding process. One way to study these pathways is by using pathway analysis programs, such as Ingenuity. For such analyses it is necessary to work together with many different departments (e.g. bio-informatics, cell biology, etc.).
In addition to the identification of *de novo* mutations in CDH patients, research needs to be focused on functional studies of candidate genes. Many clues have come from animal studies over the last few years, but most of them are only single descriptions of null-mutant mice. It is clear, however, that creating a single knockout mouse model will rarely be sufficient to determine the function of a gene in development (Brunner and van Driel 2004). Studying expression and function of these candidate genes in human (fetal) lung- and diaphragm tissue will provide additional evidence for an etiological role of these genes in human CDH. Such studies can be complemented by transgenic mice models in which these genes are disrupted in a tissue-specific or time-point specific manner.

Protocol for the CDH patient

To expand existing research projects and to develop new projects, (inter)national collaborations are needed. And, maybe even more important, solid collaborations between different disciplines within a treatment center (e.g. pediatric surgery, clinical genetics) are needed to establish an infrastructure for excellent clinical care, counselling and research. Within the framework of these local collaborations, the following protocol can be used by everyone involved in the clinical care and research of CDH patients:

1. Assessment by a trained clinical geneticist / dysmorphologist
2. Clinical pictures
3. G-banded chromosome analysis (karyotyping)
4. Screening by MLPA for (sub)telomeric rearrangements
5. if MLPA is normal: screening by high-resolution array-CGH
6. Storage of chromosomes, DNA, cell line and parental DNA
7. Registration in an (inter)national database
8. Autopsy. If no consent by parents: ask for post-mortem MRI
9. Storage of diaphragm- and lung tissue for future functional analysis
10. Storage of blood for biochemical analysis

If such a protocol is established, this will provide an essential pool of information and material for future research. We can only foresee that many intriguing discoveries will be made within the next few years in this expanding field of research.

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SUMMARY

Congenital Diaphragmatic Hernia (CDH) is a severe birth defect, characterized by a defect in formation of the diaphragm, lung hypoplasia and postnatal pulmonary hyptertension. With an incidence of approximately 1 per 3000 births it is a relatively common anomaly. In approximately 50% of the cases CDH occurs as an isolated defect (isolated CDH). In the other 50% of the cases CDH is accompanied by other congenital anomalies (non-isolated CDH or CDH+). In some of these cases a distinct syndrome can be diagnosed, such as Fryns syndrome. The most common associated anomalies in children with CDH are cardiovascular anomalies, limb anomalies, renal anomalies and dysmorphic features.
Not much is known about the etiology of CDH. It is believed that it is a multifactorial disease in which both environmental factors (e.g. toxins, maternal environment) and genetic factors are involved. The most important environmental factor that has been proven to play a role in animal models of CDH is Retinoic Acid (RA). Disturbance of the RA Signaling pathway by teratogens (e.g. nitrofen) or gene knock-outs (e.g. RAR genes) can lead to the development of CDH and other congenital anomalies. The evidence for the role of genetic factors comes from animal models (in particular knock-out mice and teratogen models) and from the occurrence of chromosomal anomalies in patients with non-isolated CDH. Interestingly, many of the recurrently deleted or duplicated chromosomal regions contain genes that are involved in the RA signaling pathway.
This thesis focuses on the identification of genetic factors that might play a role in CDH. For this study we used patient material from the patients in our Rotterdam cohort (~ 400 patients) and material send to us by collaborating (inter)national centers.

In the first part of this thesis we describe the mapping of one of the most important CDH candidate loci, the critical region on chromosome 15q26. In our Rotterdam cohort we identified three patients with a deletion of the distal part of the long arm of chromosome 15. At that time, 8 other cases had been described in the literature and we received material from 4 of these patients. Using complementary molecular (cyto)genetic techniques, such as Fluorescent *In Situ* Hybridization (FISH) and Array-based Comparative Genomic Hybridization (array-CGH), we determined the smallest region of overlap in these patients, the "CDH critical region". In addition, we included 3 patients without CDH but with a 15q deletion to exclude part of chromosome 15q from the critical region. However, since we do not know whether 15q deletions are a 100% penetrant, this strategy might not have been correct, as was pointed out by Castiglia *et al.* in their comment in the AJHG. In the two years following our publication of the results on the 15q CDH critical region, many more patients with CDH and 15q deletions have been described. In Chapter 4 of this thesis we describe two of these additional patients, that we analysed in our lab, and we review the literature. All patients share a common phenotype, consisting of characteristic dysmorphic features, left-sided Bochdalek CDH and intra-uterine growth retardation. In most cases there is also a cardiac defect and/or a limb defect. This phenotype resembles Fryns syndrome and therefore we propose that 15q26 may be the locus for true Fryns syndrome and that deletions of other chromosomal regions (e.g. 1q42, 8p23) lead to Fryns-like phenotypes.
The CDH critical region on chromosome 15q26 contains an interesting candidate gene, the transcription factor COUP-TFII. In 2005 it was shown that targeted ablation of this gene in mice causes Bochdalek-type CDH, providing more evidence that this gene also plays a role in human CDH. COUP-TFII plays a major role in the Retinoic Acid Signaling Pathway and interacts with other CDH candidate genes, such as FOG2. No mutations in COUP-TFII have been identified in human patients with CDH and a normal karyotype.

In the second part of this thesis we describe another chromosomal region which might be involved in human CDH. Duplication of 11q23-qter due to the recurrent t(11;22) has been described numerous times in patients with CDH, but only one patient with duplication 11q23-qter due to an unbalanced translocation with another chromosome had been described. In Chapter 5 we describe the breakpoint mapping of a duplication 11q23-qter and deletion 12q24 in two brothers with an unbalanced t(11;12) of which one brother had CDH. This region on chromosome 11q contains severeal interesting candidate genes, of which ROBO3 and ROBO4 are the two most interesting ones.

In the third part of this thesis we focus on the use of relatively new techniques for the screening of CDH patients for cryptic chromosomal anomalies. We screened a cohort of 26 patients with non-isolated CDH by oligonucleotide-based array-CGH. Results found by this method were validated using quantitative-PCR on genomic DNA of the child and its parents to confirm the deletion or duplication and to determine inheritance. In total, 7 *de novo* changes were identified, all residing in chromosomal regions previously described in patients with CDH. The combination of oligo-arrayCGH and q-PCR has proven a fast and reliable method to screen for copy number changes in patients with CDH and can be used for all patients with congenital anomalies and/or mental retardation.

Finally, in the last part of this thesis we discuss the findings from our research in view of the literature and we conclude that CDH not only has phenotypic heterogeneity, but also has a great degree of genotypic heterogeneity. Many recurrent chromosomal deletions and duplications have been described of which several involve genes that play a role in the RA signaling pathway. Interestingly, several of the genes involved in muscularization defects of the diaphragm are also important in Neural Crest Cell migration. Therfore, we believe that defective Retinoic Acid Signaling is a major player in the etiology of CDH, possibly in relation to Neural Crest Cell migration.

SAMENVATTING

Congenitale Hernia Diafragmatica (CDH) is een ernstige aangeboren afwijkingen waarbij er een defect is in de vorming van het middenrif (diafragma), onderontwikkeling van de longen (longhypoplasie) en na de geboorte een verhoogde bloeddruk in de longvaten (pulmonale hypertensie). Met een incidentie van 1 per 3000 geboortes is CDH een relatief veel voorkomende aangeboren afwijking. In ongeveer de helft van de gevallen is CDH de enige aangeboren afwijking bij het kind (geïsoleerde CDH). In de andere gevallen gaat CDH gepaard met andere aangeboren afwijkingen (niet-geïsoleerde CDH of CDH+). In sommige van die gevallen kan een syndroom worden gediagnosticeerd, bv. Fryns syndroom. De meest voorkomende geassocieerde aangeboren afwijkingen zijn hartafwijkingen, ledemaatsafwijkingen, nierafwijkingen en dysmorfe kenmerken.
Er is nog veel onbekend over de etiologie van CDH. Er wordt gedacht dat zowel omgevingsfactoren (bijvoorbeeld toxinen, maternale factoren) als genetische factoren een rol spelen. De belangrijkste omgevingsfactor die geïdentificeerd is in dieronderzoeken is vitamine A (of Retinoic Acid, RA). In diermodellen kan verstoring van de RA stofwisseling door teratogenen (bv. nitrofen) of door het uitschakelen van bepaalde genen (bv. de RAR genen) leiden tot CDH en andere aangeboren afwijkingen. Het bewijs voor de rol van genetische factoren komt eveneens van dierexperimenten (met name knock-out muizen en teratogene modellen), maar ook van het voorkomen van chromosoomafwijkingen bij kinderen met niet-geïsoleerde CDH. Opvallend genoeg bevatten de chromosoomregio's die gedeleteerd zijn in deze CDH+ patiënten vaak genen die een rol spelen in de RA stofwisseling.
Dit proefschrift richt zich op de identificatie van genetische factoren die een rol kunnen spelen in het ontstaan van CDH. Voor dit onderzoek hebben we gebruik gemaakt van materiaal van de patiënten uit het Rotterdam cohort (~ 400 patiënten) en van materiaal dat naar ons werd verzonden vanuit andere (inter)nationale centra.

In het eerste deel van dit proefschrift beschrijven we de identificatie van een van de CDH kritische regio's, een regio op chromosoom 15q26. Het Rotterdamse cohort bevat drie patiënten met een deletie van het distale deel van de lange arm van chromosoom 15. In de literatuur waren 8 patiënten beschreven met CDH en een 15q deletie en van 4 van hen hebben wij materiaal ontvangen. Met behulp van moleculair (cyto)genetische technieken, zoals Fluorescentie *In Situ* Hybridisatie (FISH) en Array-CGH, hebben we de kleinste gemeenschappelijke deletie bepaald, de CDH kritische regio. Tevens hebben we in de analyse ook 3 patiënten geïncludeerd met een 15q deletie maar zonder CDH om een deel van chromosoom 15q uit te kunnen sluiten van de CDH regio. Echter, omdat we niet weten of 15q deleties wel 100% penetrant zijn, was deze strategie misschien niet geheel correct, zoals gesuggereerd door Castiglia *et al.* in hun publicatie in het AJHG. Gedurende de 2 jaren na onze publicatie zijn meerdere patiënten beschreven met 15q deleties en CDH. In hoofdstuk 4 van dit proefschrift beschrijven wij 2 nieuwe patiënten en een overzicht van de literatuur. Al deze patiënten delen een karakteristiek fenotype, gekenmerkt door karakterstieke dysmorfe kenmerken, linkszijdige Bochdalek CDH en intra-uteriene groeiretardatie. Daarnaast heeft een groot deel van deze patiënten een hartafwijking en/of een ledemaatstafwijking. Hetzelfde fenotype wordt ook beschreven bij Fryns syndroom. Chromosoom 15q26 zou dus het locus kunnen zijn voor Fryns syndroom en deleties van andere loci (bv. chromosoom 1q42 of 8p23) leiden mogelijk tot fenotypes gelijkend op Fryns syndrome (zogenoemde "Fryns-like phenotypes").

De CDH kritische regio op chromosoom 15q26 bevat een interessant kandidaat gen, de transcriptiefactor COUP-TFII. In 2005 werd bewezen dat uitschakeling van dit gen in bepaalde embryonale weefsels leidt tot Bochdalek-CDH in de muis. Het is dus zeer waarschijnlijk dat COUP-TFII bij mensen een rol speelt in het ontstaan van CDH wanneer een 15q deletie aanwezig is. COUP-TFII speelt een belangrijke rol in de RA stofwisseling en gaat interacties aan met andere CDH kandidaatgenen, zoals FOG2. Tot op heden zijn er geen mutaties in COUP-TFII aangetoond in patiënten met CDH en een normaal karyotype.

Het tweede deel van dit proefschrift richt zich op een tweede locus: chromosoom 11qter. Duplicatie van 11q23-qter is meerdere keren beschreven in patiënten met een t(11;22) translocatie, maar maar 1 keer in een patiënt met duplicatie 11q23-qter ten gevolge van een andere ongebalanceerde translocatie. In hoofdstuk 5 beschrijven wij de breukpuntbepaling in twee broers met een t(11;12) waarvan 1 een CDH heeft. Deze regio op chromosoom 11q bevat meerdere kandidaatgenen voor CDH, waarvan ROBO3 en ROBO4 de meest interessante genen zijn.

In het derde deel van dit proefschrift beschrijven we het gebruik van een relatief nieuwe techniek voor de screening van CDH patiënten op cryptische chromosoomafwijkingen. In totaal hebben we 26 patiënten gescreend met behulp van oligonucleotide array-CGH (oligo-arrayCGH). De resultaten die we hierbij vonden werden bevestigd door middel van quantitatieve PCR (q-PCR) op genomisch DNA voor het vaststellen van copy number changes. In totaal werden 105 veranderingen gevonden. De helft hiervan is reeds beschreven als normal variatie in de populatie. In totaal werden 7 *de novo* chromosoomafwijkingen gevonden, allemaal in regio's die eerder beschreven zijn in CDH patiënten. De combinatie van oligo-arrayCGH en q-PCR is een snelle en betrouwbare methode om te zoeken naar copy number changes in patiënten met CDH en kan worden gebruikt voor alle patiënten met congenitale afwijkingen en/of mentale retardatie.

Tot slot bediscussiëren we in het laatste deel van dit proefschrift onze bevindingen in vergelijking met de literatuur. We concluderen dat CDH niet allen fenotypisch heterogeen is, maar ook een grote genetische heterogeniciteit heeft. Meerdere recurrente deleties en duplicaties zijn beschreven en velen hiervan omvatten genen die een rol spelen in de RA stofwisseling. Opvallend is dat verschillende genen die een rol spelen in muscularisatiedefecten van het diafragma ook een rol spelen in migratie van Neurale Lijst cellen. Abnormale RA stofwisseling en signaling speelt een belangrijke rol in het ontstaan van CDH, mogelijk in relatie tot migratie van Neurale Lijst cellen.

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BIOGRAPHY

Merel Klaassens was born on June 19th, 1977 in Helmond, the Netherlands. After secondary school (VWO) at the Merletcollege Land van Cuijk, she started her medical training in 1995 at Maastricht University in Maastricht. During her medical training she dedicated her elective courses to pediatrics. In addition, she was a student member of the Ambassador Team and of the Faculty Board. After obtaining her medical degree in 2001 she worked with great enthousiasm as a pediatric resident in the Elkerliek Hospital in Helmond (January – December 2002) and the Canisius Wilhelmina Hospital in Nijmegen (January 2003 – April 2004). In April 2004 she started working as a research-physician at the Departments of Pediatric Surgery and Clinical Genetics under the supervision of Prof. Dr. D. Tibboel and Dr. A. de Klein. During this time she worked on the research presented in this thesis. In Spring 2006 she spent a period of three months at the Department of Molecular and Human Genetics of Baylor College of Medicine (Houston, USA), under the supervision of Dr. B. Lee and Dr. D.A. Scott.
In February 2007 she started her pediatric clinical training at the University Hospital Maastricht (AzM) (head: Prof. Dr. L.J.I. Zimmerman).

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Klaassens M, Van Dooren M, Eussen HJ, Douben H, Den Dekker AT, Lee C, Donahoe PK, Galjaard RJ, Goemaere N, De Krijger RR, Wouters C, Wauters J, Oostra BA, Tibboel D and De Klein A
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"Linking Animal Models to Human Congenital Diaphragmatic Hernia"
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APPENDIX

Chapter 1, Table 3. Overview of published chromosomal anomalies occuring in CDH patients

Chromosome 1		
balanced translocation		
Punnett et al.	AJMG 1994; 50:931-933	46,XX,t(1;X)
Kousseff et al.	2000 Proc. Greenwood Gen. Center	t(1;9)
Priolo et al.	Clin Dysmorphol 2004; 13:45-46	46,XY,t(1;15)(q41;q21.2)
Ahmed et al.	Clin Dysmorphol 2004; 13:111-112	46,XX,t(1;22)(q12;p12)
Smith (S.A.) et al.	Clin Dysmorphol 1994; 3:287-291	46,XY,t(1;15)(q41;q21.1)
Howe et al.	Prenat Diagn 1996; 16: 1003-1009	46,XY,t(1;21)(q32;q22)
Klaassens et al.	AJHG 2005; 76:877-882	46,XY,t(1;14),inv(6),del(15)(q26qter)
inversion		
Tonks et al.	Prenat Diagn 2004; 24: 596-604	46,XY,inv(1)(q41q44)mat
duplication		
Ahn et al.	Semin Perinat 2005; 20:895-898	46,XY / 46,XY,der(Y)t(1;Y)(q12;q12)
Zeng et al.	AJMG 2003; 120A:464-469	46,XY / 46,XY,der(Y)t(1;Y)(q12;q12)
Koussef et al.	2000 Proc.Greenwood Gen.Center	dup(1)
Schneider et al.	AJHG 1991; 49: Supplement 275	dup(1)(q22q32)
Schinzel	database 2004	dup(1)(q22q32)mosaicism
Clark & Fenner - G.	AJMG 1989; 34:422-426	46,XY / 46,XY,dup(1)(q24.2q31.2)
Mehraein et al.	Med Genet. 2000; 12:96	dup(1)(q25q31.2)
deletion		
Philip et al.	Eur J Pediatr 1991; 150:726-729	der(1)t(1;21)
Benjamin et al.	J Ped Surg 1988; 23:899-903	der(1)
Youssoufian et al.	Hum Genet. 1988; 78:267-270	46,XX,del(1)(q32q42)
Kantarci et al.	AJMG 2005; 140:17-23	46,XY,del(1)(q41q42.12)
Rogers et al.	AJHG 1995; 57: Suppl A125	del(1)(q41.11q42.3)

Chromosome 2		
duplication		
Grevengood et al.	AJHG 1993; 53: suppl 1796	dup(2)(p13q12)
Sarda et al.	Ann Genet 1992; 35:117-120	der(X)t(2;X)(p13;q27)
Schinzel	database	dup(2)(p13p25)
Schinzel	database	dup(2)(p21p25)
Bender et al.	Humangenetik 1969; 8:94-104	der(6)t(2;6)(p23;p25)
Fineman et al.	AJMG 1983; 15:451-456	dup(2)(p13p25)
Heathcote et al.	Can J Ophthalmol 1991; 26:35-43	dup(2)(p21p25)
Enns et al.	AJMG 1998; 79:215-225	dup(2)(p25.3pter)
Johnson et al.	AJHG 1992; 51: suppl A290	dup(2)(q33q37)
Brackley et al.	Pren Diagn 1999; 19:570-574	46,XX,der(2)t(2;7)(q36;q37)
Tonks et al.	Pren Diagn 2004; 24:596-604	der(7)t(2;7)(q37;q…)
deletion		
Enns et al.	AJMG 1998; 79:215-225	der(2)t(2;7)(p25.3;q34)

Tonks et al.	Pren Diagn 2004; 24:596-604	46,XY,del(2)(q33q35 or q35q37)*de novo*
Tonks et al.	Pren Diagn 2004; 24:596-604	46,XY,der(2)t(2;8)(q37;p11.2)pat
Schinzel	database	46,XX,der(2)t(2;14)(q37.1;q31.2)
De La Fuente et al.	Ann Genet 1988; 1:254-257	der(2)t(2;14)(q37;q31.2)
Casas et al.	AJMG 2004; 136A:331-339	46,XX,del(2)(q37.1)
Reddy et al.	AJMG 1999; 84:460-468	46,XY,del(2)(q37.3)

Chromosome 3

balanced translocation		
Tonks et al.	Prenat Diagn 2004; 24:596-604	46,XY,t(3;12)(p21.2;p13.1)*de novo*
duplication		
Pettigrew et al.	AJHG 1992; 51: suppl	der(21)t(3;21)(p24.3;q11.2)
Rosenberg et al.	AJHG 1992; 50:700-705	der(15)t(3;15)(q29;q24)
deletion		
Tibboel & Gaag	Clin Perinatol 1996; 23:689-699	del(3)
Steinhorn et al.	Arch Ped Adolesc Med 1994; 148:626	del(3)(p)
Pfeiffer et al.	Ann Genet 1998; 41:17-21	del(3)(p12p21)
Tibboel & Gaag	Clin Perinatol 1996; 23:689-699	46,XY,der(3)t(3;8)(p23;p23.1)
Brennan et al.	J Med Genet 2001; 38:556-558	del(3)(q11.1q13.2)
Wolstenholme et al.	J Med Genet 1994; 31:647-648	46,XY,del(3)(q21q23)
Dillon et al.	Br J Radiol 2000; 73:360-365	del(3)(q22)
Kristeshavilli et al.	Database chr. Anomalies	der(3)t(3;5)(q27;q31)

Chromosome 4

translocation		
Reiss et al.	abstract ASHG 1990: p1920	t(4;20)
ring chromosome (always with deletion)		
Kocks etal	J Med Genet 2002; 39: 23	46,XX / 45,XX,+r(4)(p16p33)
duplication		
Kobori et al.	1993; poster ASHG	del(4)(p15.2pter) + dup(4)(q25qter)
Frints et al.	Genet Couns 1996; 7:135-142	dup(4)(q26q32) + del(4)(q32qter)
Celle et al.	AJMG 2000; 94: 125-140	der(22)t(4;22)(q28.3;p13)
Yunis et al.	Ann Genet 1977; 20:243-248	der(18)t(4;18)(q31;q23)
Schinzel	database 2004	dup(4)(q25q31)
deletion		
van Dooren et al.	AJMG 2004; 127A: 194-196	46,XY,del(4)(p16pter)
Howe et al.	Pren Diagn 1993; 16:1003-1009	4p-
Bird et al.	AJMG 1994; 53: 33-38	4p-
Tachdjian et al.	Clin Genet 1992; 42:281-287	del(4)(p16)
Pober et al.	AJMG 2005; 138A: 81-88	del(4)(p16)
Casaccia etal	Birth Def Res A 2006; 76:210-213	del(4)(p16)

Kobori et al.	1993; poster ASHG	del(4)(p15.2pter) + dup(4)(q25qter)
Laziuk et al.	Arkh Patol 1986; 48:20-25	del(4)(p16)
Sergi et al.	Pathologica 1998; 90:285-293	del(4)(p16)
Tapper et al.	Fetal Diagn Ther 2002; 17:347-351	del(4)(p16)
Del Campo et al.	1997 Proc Greenwood Gen. Center	del(4)(p16)
Park Y. et al.	J Korean Med Sci 1993; 8:471-475	46,XX,del(4)(q)
Schinzel	database	del(4)(q31)
Wakui et al.	Hum Genet 1999; 44:85-90	del(4)(q31.3)
Young et al.	AJMG 1982; 12:103-107	del(4)(q31qter)
Frints et al.	Genet Couns 1996; 7:135-142	dup(4)(q26q32) + del(4)(q32qter)
Pober et al.	AJMG 2005; 138A: 81-88	der(4)t(4;20)(q34.2;q13.1)pat
Reiss et al.	AJHG 1996; 65: suppl A340	der(4)t(4;20)(q34.2;q13.1)pat

Chromosome 5

balanced translocation		
Masuno et al.	AJMG 1991; 41:32-34	dup 5p15-pter, del 13q10-q21
Torfs et al.	Teratology 1992; 46:555-565	46,XY,-9,+t(5;9)
duplication		
Bollmann et al.	Fetal Diagn Ther 1995; 10:52-59	partial trisomy 5
Korner et al.	Z Klin Med 1991; 46:427-429	partial trisomy 5
Aviram-Goldring et al.	AJMG 2000; 90:120-122	der(15)t(5;15)(p15.3;q26.2)
Liberfarb et al.	Ann Genet 1980; 23:26-30	der(9)t(5;9)(p13;p22)
Schinzel	database 2004	dup(5)(p15) + dup(13)(pterq21)
Kristeshavilli et al.	Database chr. Anomalies	der(3)t(3;5)(q27;q31)
Kousseff et al.	2000 Proc. Greenwood Gen. Center	del(5)(q15q22)
Kristeshavilli et al.	Database chr. Anomalies	der(3)t(3;5)(q27;q31)
Kousseff et al.	2000 Proc. Greenwood Gen. Center	del(5)(q15q22)

Chromosome 6

balanced translocation		
Howe et al.	Prenat Diagn 1996; 16:1003-1009	46,XY,t(6;8)(q24;q23)
inversion		
Klaassens et al.	AJHG 2005; 76:877-882	46,XY,t(1;14),inv(6),del(15)(q26qter)
duplication		
Scarbrough et al.	J Med Genet 1986; 23:185-187	47,XY,+6p,+22q,t(6;22)
Tonks et al.	Prenat Diagn 2004; 24:596-604	46,XX,dup(6)(q23 or q25)
deletion		
Bender et al.	Humangenetik 1969; 8:94-104	der(6)t(2;6)(p23;p25)
Batanian et al.	Clin Genet 2001; 59:52-57	der(6)t(6;X)(p25;p21.2)
Baruch & Erickson	AJMG 2001; 100:187-190	der(6)t(6;8)(p25.1;q24.23)
Yu & Bock	1997 Proc. Greenwood Gen. Center	del(6)(q15q21)

Shen-Schwarz et al.	AJMG 1989; 32:81-86	del(6)(q23)
Schinzel	database 2004	del(6)(q23)
Krassikoff & Sekhon	AJMG 1990; 36:363-364	del(6)(q25.3qter)
Le Caignec et al.	J Med Genet 2005; 42:121-128	del(6)(qter)

Chromosome 7

duplication		
Herrmann et al.	J Med Genet 1999; 11:166-169	dup(7)(p15p22)
Brackley et al.	Prenat Diagn 1999; 19:570-574	46,XX,der(2)t(2;7)(q36;q37)
Habedank & Trost-Binkhues	J Med Genet 1983; 20:377-379	der(18)t(7;18)(qter;p11.1)
deletion		
Schinzel	database 2004	del(7)(p21)
Fauza & Wilson	J Ped Surg 1994; 29:1113-1117	7q-
Klep-de Pater et al.	J Med Genet 1979; 16:151-154	del(7)(q11q22)
Tonks et al.	Prenat Diagn 2004; 24:596-604	del(7)q26qter)
Torfs et al.	Teratology 1992; 46:555-565	46,XY,del(7)(q32qter)
Dott et al.	Birth Def Res A 2003; 67:261-267	del(7)(q32)
Kjaer et al.	J Med Genet 1991; 28:846-855	der(7)t(7;20)(q33.2;p13)
Enns et al.	AJMG 1998; 79:215-225	46,XY,-7,+der(7)t(2;7)(p25.3;q34)mat

Chromosome 8

balanced translocation		
Howe et al.	Prenat Diagn 1996; 16:1003-1009	46,XY,t(6;8)(q24;q23)
Philip et al.	Eur J Ped 1991; 150:726-729	t(8;14)(q24;q21)
Temple et al.	J Med Genet 1994; 31:735-737	46,XX,t(8;13)(q22.2q22)mat
Temple et al.	J Med Genet 1994; 31:735-737	46,XX,t(8;13)(q22.3q15)
duplication		
Pober et al.	AJMG 2005; 138A:81-88	trisomy 8 mosaicism
Tonks et al.	Prenat Diagn 2004; 24:596-604	46,XY,der(2)t(2;8)(q37;p11.2)pat
Ringer et al.	1995; poster ASHG	dup(8)(p11.22p23.1)
Schinzel	database 2004	dup(8)(p21)
Moreno Fuenmayor et al.	AJMG 1980; 7:361-368	der(12)t(8;12)(p21;p13)
Chen et al.	Prenat Dian 1998; 18:1289-1293	46,XX,der(15)t(8;15)(q24.1;q26.1)
Hilfiker et al.	J Ped Surg 1998; 13:550-552	46,XY,dup(8)(q)
Baruch & Erickson	AJMG 2001; 100:187-190	der(6)t(6;8)(p25.1;q24.23)
deletion		
Thorpe-Beeston et al.	Fetal Ther 1989; 4:21-28	del(8)
Pober et al.	AJMG 2005; 138A:81-88	del(8)(p)
Kousseff et al.	2000 Proc. Greenwood Gen. Center	del(8)(p22pter)
Howe et al.	Prenat Diagn 1996; 16:1003-1009	46,XY,del(8)(p23)
Faivre et al.	Prenat Diagn 1998; 18:1055-1060	del(8)(p23.1)

Shimokawa et al.	AJMG 2005; 136A:49-51	del(8)(p23.1)
Borys & Taxy	Ped Dev Pathol 2004; 7:35-37	del(8)(p23.1)
Lopez et al.	Prenat Diagn 2006; 26: 577-580	46,XY,del(8)p23.1).ishdel(8)(p23.1p23.1)
Pecile et al.	Clin Genet 1990; 37:271-278	del(8)(p23.1)
Fraer et al.	AJHG 1992; 51: suppl A408	del(8)(p23.1)
Slavotinek et al.	J Med Genet 2005; 42:730-736	del(8)(p23.1)
Maerzke et al.	J Med Genet 1993; 5:121-125	del(8)(q21.2q22)
Harnsberger et al.	1982 Birth Def Conf Birmingham	del(8)(q22q24.1)
Capellini et al.	EJHG 1996; 4: suppl 1	del(8)(q22)

Chromosome 9

balanced translocation		
Kousseff et al.	2000 Proc. Greenwood Gen. Center	t(1;9)
ring chromosome (always with deletion)		
Dillon et al.	Br J Radiol 2000; 73:360-365	r(9)
duplication		
Chen et al.	Prenat Diagn 2004; 29:455-462	trisomy 9
Suzomori et al.	Prenat Diagn 2003; 23:866-868	trisomy 9
Sepulveda et al.	Ultras Obstet Gyn 2003; 22:479-483	trisomy 9
Frohlich et al.	J Med Genet 1982; 19:316-317	trisomy 9
Robert et al.	Eur J Epidem 1997; 13:665-673	trisomy 9
Dott et al.	Birth Def Res A 2003; 67:261-267	trisomy 9
Henriques-Coelho et al.	Ped Surg 2005; 40:e29-31	tetrasomy 9 (47,XX,+i(9p))
deletion		
Alfi et al.	Ann Genet 1976; 19:11-16	der(9)t(9;16)(p22;q24)
Liberfarb et al.	Ann Genet 1980; 23:26-30	der(9)t(5;9)(p13;p22)
Donnenfeld et al.	Am J Obstet Gynaecol 1993; 169:1017	46,XY,-9,+der(9;1)(p24;p12)pat
Torfs et al.	Teratology 1992; 46:555-565	46,XY,-9,+t(5q;9p)
Ferrera et al.	AJMG 2006; 140A:892-894	46,XY,der(9)t(9;16)(q34.3;q24.3)

Chromosome 10

balanced translocation		
Cunniff et al.	J Pediatr 1990; 116:258-261	t(10;X)
duplication		
Yunis et al.	Ann Genet 1976; 19:57-60	der(21)t(10;21)(p11;p11)
Lurie et al.	Humangenetik 1978; 41:235-241	der(20)t(10;20)(p12;p12)
Tonks et al.	Prenat Diagn 2004; 24:596-604	46,XY,add(10)(q?q24)*de novo*

Chromosome 11		
balanced translocation		
Donnenfeld et al.	Am J Obstet Gynaecol 1993; 169:1017	46,XY,-9,+der(9;1)p24;p12)pat
duplication		
Several authors	Iselius et al. (18x), Fraccaro et al., Phelan et al., Azancot et al., De Beaufort et al., Aurias et al., Noel et al., Dean et al., Kousseff et al., Hickman et al., Tonks et al.	47,XX or XY, +der(22)t(11;22)(q24.3;q23)
Park et al.	AJMG 1993; 45:163-165	47,XX,+der(11)t(11;13)(q24;q…)
Klaassens et al.	AJMG 2006; 140: 1580-1586	46,XY,der(12)t(11;12)(q24.3;q24)mat
deletion		
Scott et al.	AJMG 2005; 134A:430-433	del(11)(p12p15.1)
Gustavson et al.	Clin Genet 1984:247-249	del(11)(p13)
Decker-Philips et al.	AJHG 1995; 57: suppl A309	der(11)t(11;12)(q24;p11.2)

Chromosome 12		
balanced translocation		
Fauza & Wilson	J Ped Surg 1994; 29:1113-1117	t(12;15)
Tonks et al.	Prenat Diagn 2004; 24:596-604	46,XY,t(3;12)(q21.1;p13.1)*de novo*
duplication		
Several authors	Bergoffen et al. (12x), Coming et al., Rodriguez et al., Dott et al.2003, Betremieux et al. 2002, Veldman et al. 2002, Witters et al. 2001, Tonks et al. 2004, Takakawa et al., Pober et al. 2005 (3x),	tetrasomy 12p
Donnenfeld et al.	Am J Obstet Gynaecol 1993; 169:1017	isochromosome 12p
Borys & Taxy	Ped Dev Pathol 2004; 7:35-38	dup(12)(p10pter)
Decker-Philips et al.	AJHG 1995; 57: suppl A309	der(11)t(11;12)(q24;p11.2)
Pober et al.	AJMG 2005; 138A:81-88	der(15)t(12;15)
deletion		
Moreno Fuenmayor et al.	AJMG 1980; 7:361-368	der(12)t(8;12)(p21;p13)
Howe et al.	Prenat Diagn 1996; 16:1003-1009	46,XY,del(12)
Jardine et al.	Dysmorphol 1993; 2:269-273	der(6)t(6;12)(q…;q…)
Klaassens et al.	AJMG 2006; 140: 1580-1586	46,XY,der(12)t(11;12)(q24.2;q24)mat

Chromosome 13		
balanced translocation		
Masuno et al.	AJMG 1991; 41:32-34	t(5;13)
Temple et al.	J Med Genet 1994; 31:735-737	46,XX,t(8;13)(q22.3;q22)

ring chromosome (always with deletion)		
Schinzel	database 2004	r(13)
duplication		
Warburton et al.	AJHG 2000; 66:1796-1806	tetrasomy 13p31pter
Tohma et al.	ASHG 1998; suppl A862	tetrasomy 13p31pter
Lockhart et al.	ASHG; suppl A862	der(4)t(4;13)(p16;q32)
Park et al.	AJMG 1993; 45:163-165	47,XX,+der(11)t(11;13)(q24;q…)
deletion		
Benjamin et al.	J Pediatr Surg 1988; 23:899-903	13q-

Chromosome 14

balanced translocation		
Philip et al.	Eur J Ped 1991; 150:726-729	t(8;14)(q24;q21)
Klaassens et al.	AJHG 2005; 76:877-882	46,XY,t(1;14),inv(6),del(15)(q26qter)
duplication		
Schinzel	database 2004	dup(14)(q24q32)
Masada et al.	AJMG 1989; 34:524-528	dup(14)(q32.11qter)
De La Fuente et al.	Ann Genet 1988; 31:254-257	der(2)t(2;14)(q37;q31.2)
Howe et al.	Prenat Diagn 1996; 16:1003-1009	46,XY / 47,XY,+14

Chromosome 15

balanced translocation		
Smith (S.A.) et al.	Clin Dysmorphol 1994; 3:287-291	46,XY,t(1;15)(q41;q21.1)
Fauza & Wilson	J Ped Surg 1994; 29:1113-1117	t(12;15)
duplication		
Schinzel	database 2004	inv dup(15)
Hou et al.	Eur J Pediatr 1998; 157:122-127	inv dup(15)
Schinzel	database 2004	dup(15)(q15q26)
Schinzel	database 2004	dup(15)(q15q26) + del(X)(p22)
Zabel & Baumann	Ann Genet 1977; 20:285-289	der(X)t(15;X)(q15;p22)
Boyar et al.	Clin Genet 2001; 60:421-430	dup(15)(q11q13)
deletion		
de Jong et al.	J Med Genet 1989; 26:469-470	r(15)(p11q26)
Klaassens et al.	AJHG 2005; 76:877-882	r(15) with del(15)(q26.2qter)
Elghezal et al.	2006 ESHG, poster 477	r(15)(q25.3)
Kristofferson et al.	Clin Genet 1987; 32:169-171	del(15)(q24qter)
Rosenberg et al.	AJHG 1992; 50:700-705	der(15)t(3;15)(q29;q24)
Howe et al.	Prenat Diagn 1996; 16:1003-1009	der(15)t(15;17)(q24.3;q23.3)
Bettelheim et al.	Clin Genet 1998; 53:319-320	del(15)(q24qter)
Chen et al.	Prenat Diagn 1998; 18:1289-1293	46,XX,der(15)t(8;15)(q24.1;q26.1)
Reiss et al.	1999 ASHG; poster 1920	46,XY,der(15)t(15;20)(q26.3;q13.1)

Aviram-Goldring et al.	AJMG 2000; 90:120-122	der(15)t(5;15)(p15.2;q26.2)
Schlembach et al.	Prenat Diagn 2001; 21:289-292	46,XY,del(15)(q26.2qter)
Biggio et al.	AJMG 2004; 131:224	del(15)(q26.1qter)
Hengstschlager et al.	Fetal Diagn Ther 2004; 19: 510-512	del(15)(q26.1qter)
Tonks et al.	Prenat Diagn 2004; 24:596-604	46,XY,del(15)(q26.1)*de novo*
Tumer et al.	AJMG 2004; 130A: 340-344	46,XY,r(15)(p..;q26.2)
Klaassens et al.	AJHG 2005; 76:877-882	46,XY,t(1;14),inv(6),del(15)(q26qter)
Pober et al.	AJMG 2005; 138A:81-88	del(15)(q26qter)
Pober et al.	AJMG 2005; 138A:81-88	der(15)t(12;15)
Slavotinek et al.	J Med Genet 2005; 42:730-736	del(15)(q26.2) & (q26.2qter)
Slavotinek et al.	Eur J Hum Genet 2006; 14: 999-1008	del(15q)(q26.2qter)
Lopez et al.	Prenat Diagn 2006; 26: 577-580	del(15)(q26.1)ish(tel15q-)

Chromosome 16

translocation		
Giardino et al.	Eur J Hum Genet 2001; 9:881-885	(2;16)
duplication		
Howe et al.	Prenat Diagn 1996; 16:1003-1009	dup(16)
Alfi et al.	Ann Genet 1976; 19:11-16	der(9)t(9;16)(p22;q24)
Alfi et al.	Ann Genet 1973; 16: 11-16	der(9)t(9;16)(p22;q24)
Ferrera et al.	AJMG 2006; 140A:892-894	46,XY,der(9)t(9;16)(q34.3;q24.3)
Chen et al.	Prenat Diagn 2004; 24:455-462	46,XX [15] / 47,XX,+16 [3]
Johnson et al.	Prenat Diagn 2000; 20:417-421	47,XX,+16

Chromosome 17

ring chromosome (always with deletion)		
Baldermann et al.	AJHG 2000; 67: suppl 2	r(17)
duplication		
Howe et al.	Prenat Diagn 1996; 16:1003-1009	der(15)t(15;17)(q24.3;q23.3)

Chromosome 18

duplication		
Le Caignec et al.	J Med Genet 2005; 42:121-128	dup(18)(q)
Hayashi etal	Jpn J Hum Genet 1997	dup(18)(cen-qter) + del(18)(pter -cen)
deletion		
Hayashi etal	Jpn J Hum Genet 1997	dup(18)(cen-qter) + del(18)(pter -cen)
Habedank & Trost-Binkhues	J Med Genet 1983; 20:377-379	der(18)t(7;18)(qter;p11.1)
Yunis et al.	Ann Genet 1977; 20:243-248	der(18)t(4;18)(q31;q23)
Geneix et al.	Genet Couns 2001; 12:19-171	del(18)(qter) + del(22)(cen-q11)

Chromosome 19
No chromosomal abnormalities have been described in CDH patients

Chromosome 20		
duplication		
Reiss et al.	ASHG 1999; poster 1920	t(4;20)
Kjaer et al.	J Med Genet 1991; 28:846-855	der(7)t(7;20)(q33.2;p13)
Reiss et al.	ASHG 1999; poster 1920	46,XY,der(15)t(15;20)(q26.3;q13.1)
Pober et al.	AJMG 2005; 138A: 81-88	der(4)t(4;20)(q34.2;q13.1)pat
deletion		
Lurie et al.	Humangenetik 1978; 41:235-241	der(20)t(10;20)(p12;p12)

Chromosome 21		
translocation		
Howe et al.	Prenat Diagn 1996; 16: 1003-1009	46,XY,t(1;21)(q32;q22)
duplication		
Smith et al.	J Med Genet 1992; 29:503-506	der(X)t(21;X)(p11.1;cen-qter)
Philip et al.	Eur J Pediatr 1991; 150:726-729	der(1)t(1;21)
Pober et al.	AJMG 2005; 138A: 81-88	tetrasomy 21
deletion		
Yunis et al.	Ann Genet 1976; 19:57-60	der(21)t(10;21)(p11;p11)
Pettigrew et al.	AJHG 1992; 51: suppl	der(21)t(3;21)(p24.3;q11.2)

Chromosome 22		
translocation		
Ahmed et al.	Clin Dysmorphol 2004; 13:111-112	46,XX,t(1;22)(q12;p12)
duplication		
Several authors	Iselius et al. (18x), Fraccaro et al., Phelan et al., Azancot et al., De Beaufort et al., Aurias et al., Noel et al., Dean et al., Kousseff et al., Hickman et al., Tonks et al.	47,XX or XY, +der(22)t(11;22)(q24.3;q23)
deletion		
Geneix et al.	Genet Couns 2001; 12:19-171	del(18)(qter) + del(22)(cen-q11)
Betremieux et al.	Prenat Diagn 2002; 24:487-493	del(22)(q11)
Celle et al.	AJMG 2000; 94: 125-140	der(22)t(4;22)(q28.3;p13)

Chromosome X		
translocation		
Sarda et al.	Ann Genet 1992; 35:117-120	der(X)t(2;X)(p13;q27)
Zabel & Baumann	Ann Genet 1977; 20:285-289	der(X)t(15;X)(q15;p22)
Punnett et al.	AJMG 1994; 50:931-933	46,XX,t(1;X)
monosomy (45,XO)		
Several authors	David & Illingworth, Benjamin etal, Bollman et al.,	
	Tibboel & Gaag, Cunniff et al., Robert et al.,	
	Plaja et al., Dawani et al.	
duplication		
Batanian et al.	Clin Genet 2001; 59:52-57	der(6)t(6;X)(p25;p21.2)
deletion		
Pober et al.	AJMG 2005; 138A:81-88	der(X)t(X;Y)(p22.3;q11.2)
Smith et al.	J Med Genet 1992; 29:503-506	der(X)t(21;X)(p11.1;cen-qter)

Chromosome Y		
duplication		
Pober et al.	AJMG 2005; 138A:81-88	der(X)t(X;Y)(p22.3;q11.2)
deletion		
Ahn et al.	Semin Perinat 2005; 20:895-898	46,XY / 46,XY,der(Y)t(1;Y)(q12;q12)
Zeng et al.	AJMG 2003; 120A:464-469	46,XY / 46,XY,der(Y)t(1;Y)(q12;q12)

Narrowing the Candidate Region for Congenital Diaphragmatic Hernia in Chromosome 15q26: Contradictory Results

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To the Editor, Subtelomeric screening and FISH analysis of a 13-year-old girl with severe mental retardation, IUGR, microcephaly, facial dysmorphisms, hypoplastic kidney, short hands and feet but without congenital diaphragmatic hernia (CDH [MIM 142340]) , allowed us to find a de novo deletion in 15q26.1-26.2. That region, as shown by Klaassens et al. in a study published in the May 2005 issue of The American Journal of Human Genetics, contains a candidate region for CDH, a condition occurring about in 1 out of 3000 newborns, associated with a mortality rate ranging from 30 to 60%, with a significant morbidity among survivors (Harrison et al. 1994; Nobuhara et al. 1996). The etiology of this condition is barely known, and in most cases is considered idiopathic whereas approximately 15% of cases with CDH show chromosomal abnormalities. Recently, Biggio et al. (2004) reported on a child with a 15q26.1 deletion showing CDH, coartation of the aorta and dysmorphic features, suggesting such region as the possible candidate locus for CDH. Furthermore, the authors proposed myocyte-specific enhancer factor-2 A (MEF2A [MIM 600660]) as a candidate gene for CDH, coding for a protein playing a critical role in the control of muscle differentiation and development. Klaassens et al. (2005) found 7% numerical and 5% structural chromosome abnormalities out of 200 CDH patients. The most frequent chromosome abnormality was 15q deletion. Eventually, they determined the deletions' size in seven patients with CDH and incorporated data from two patients with terminal 15q deletions without CDH and one patient with a small 15q interstitial deletion and CDH. A minimal deletion region spanning about 5 Megabases (Mb) at chromosome bands 15q26.1-15q26.2 has been suggested by these authors. Two out of the known genes of this region, namely NR2F2 (MIM 107773) and CDH2 (MIM 602119), were considered the best candidates for CDH.

In order to better define the deletion in our patient, FISH experiments were carried out with a set of linearly ordered bacterial artificial chromosomes (BACs) selected by human NCBI Map Viewer (http://www.ncbi.nlm.nih.gov) (build 35.1) and provided by Sanger Institute. This analysis showed that the BAC RP11-386M24 localised to chromosome band 15q26.1 (< 9.0 Mb from the end of the chromosome) was the closest to the telomere that hybridized on both chromosomes in all examined metaphases. The immediately more centromeric CTD– 2313J17 BAC showed signals of different intensities on the 15q telomeres, suggesting that the breakpoint lay within this BAC whereas the overlapping RP11-437B10 BAC and all the distally placed BACs, showed no hybridisation signal (data not shown). We thus compared our results with the most significant previously characterized 15qter deletions, including ring chromosome 15, unbalanced translocations and pure 15qter monosomies, either associated with the CDH phenotype or not. As shown in figure 1, no clear critical region can be drawn from these data. This is essentially due to case 12 with CDH carrying a ring(15) resulting in a smaller deletion than cases 1, 2, and 3 without CDH. At least two hypotheses can be done to explain these contradictory data. First of

all, it is possible that haploinsufficiency of the CDH locus has a reduced penetrance and then data from patients without CDH could be useless in establishing the critical region. If this is true, the candidate region is restricted to ~ 3.5 Mb (fig. 1), includes the NR2F2 gene but its telomeric limit is more distal than that defined by Klaassens et al. (2005) which was derived from a deletion of a patient without CDH (fig. 1, case 13). On the other hand, drawing genotype-phenotype relationships may be difficult in ring carriers due to the potential instability of ring chromosomes which can be associated with gain or loss of genetic material in other tissues (Tümer et al. 2004). If we omit ring cases from the analysis, then the critical region would be narrowed to a 0.7 Mb genomic portion (fig. 1). The NR2F2 gene in this case would be located outside this putative critical region.

The ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 2 gene (ST8SIA2 [MIM 602546]) is the unique known gene in this region and encodes for a type II membrane protein that catalyzes the transfer of sialic acid from CMP-sialic acid to the neural cell adhesion molecules NCAMs (Ong et al. 1998). The ST8SIA2 gene is expressed in many tissues during development (Angata et al. 1997) and many evidences suggest that polysialylated NCAMs promote cell migration and thus it is thought to play a critical role in development. More specifically, it has been shown that during diaphragmatic morphogenesis the expression of polysialylated NCAMs is tightly modulated along each stage of myogenes (Allan et al. 1998). Finally, although less likely, we cannot exclude that both the mentioned hypotheses are true. In this case, the critical region would be represented by the extent of the deletion of patient 8 in figure 1. Additional findings are needed to refine the search for a CDH gene in 15q chromosome. However, it seems likely that NR2F2 and ST8SIA2 are the best candidates.

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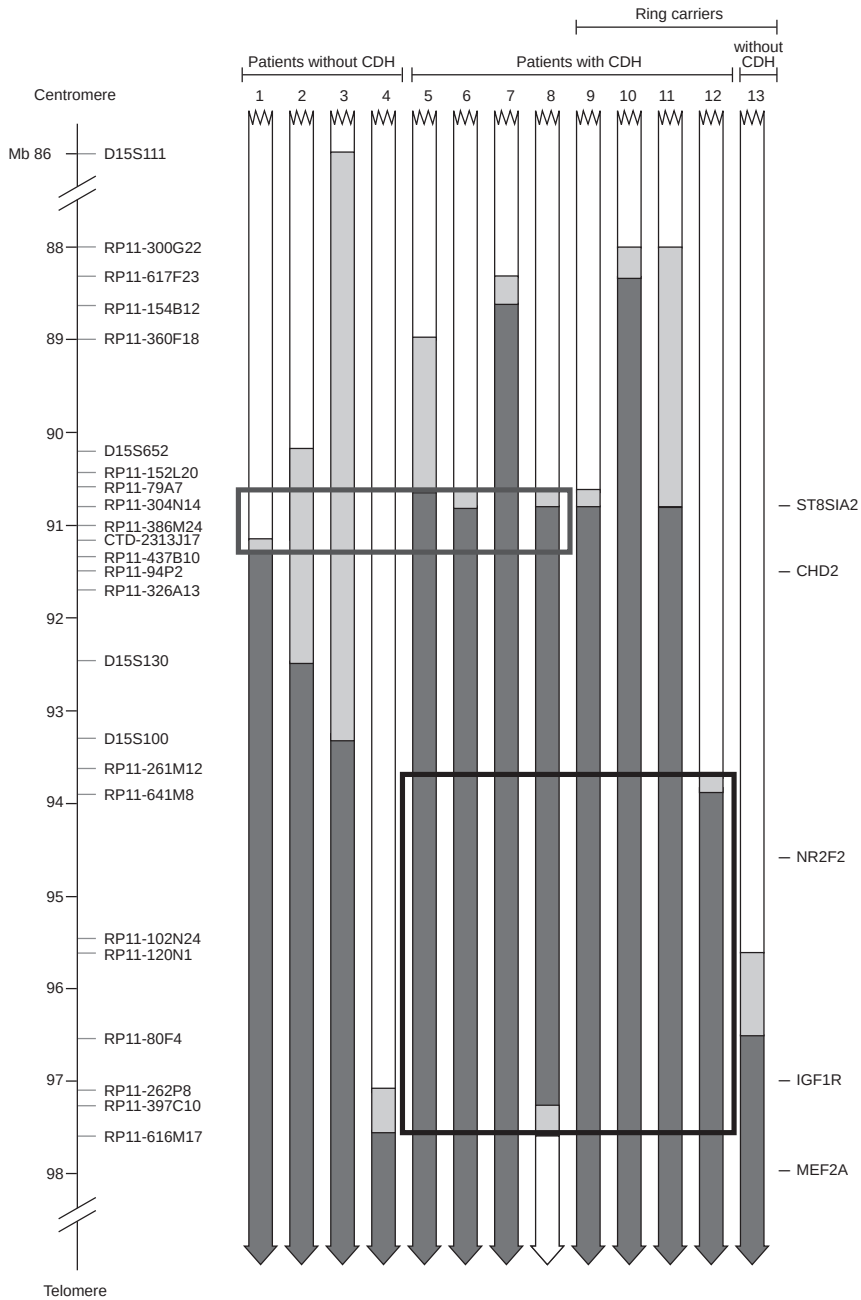

Fig. 1 Graphical representation of 15q deletions in patients with and without CDH The most significant BAC clones analyzed are shown on the left. Solid boxes represent deleted regions; hatched boxes indicate the uncertainty of the breakpoints, and open boxes reveal the normal chromosomal regions. The green rectangle includes the narrowed candidate CDH region taking into account only cases with CDH, whereas the red one indicates the critical region resulting when the ring cases are omitted from the analysis. Patient 1, our case; patient 2, Tönnies et al. 2001; patient 3, Rogan et al. 1996, (patient K); patient 4-11 and 13, Klaassens et al. 2005 (cases 9, 7, 6, 4, 1, 2, 3, 5 and 8, respectively), patient 12, Tümer et al. 2004, (case 1).

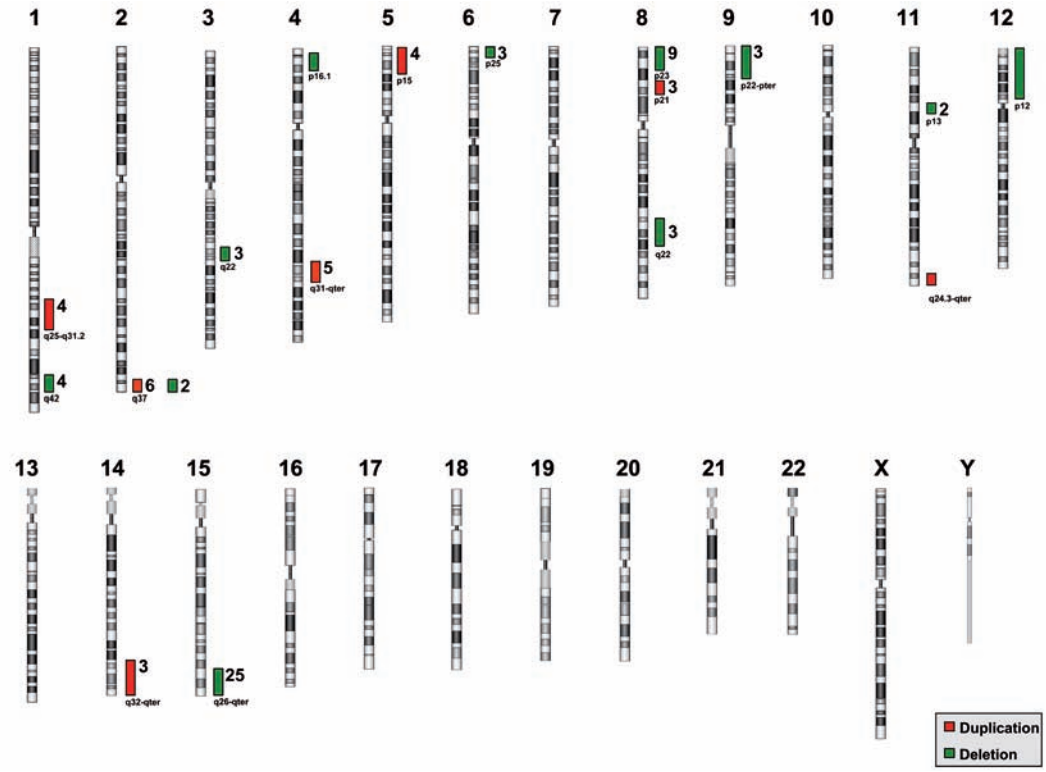

Fig.1. Recurrent chromosomal abnormalities associated with CDH are represented by colored bars. For each region, the number of patients described with that anomaly is listed. For an overview of all chromosomal anomalies described in patients with CDH, see Table 3 page 116.

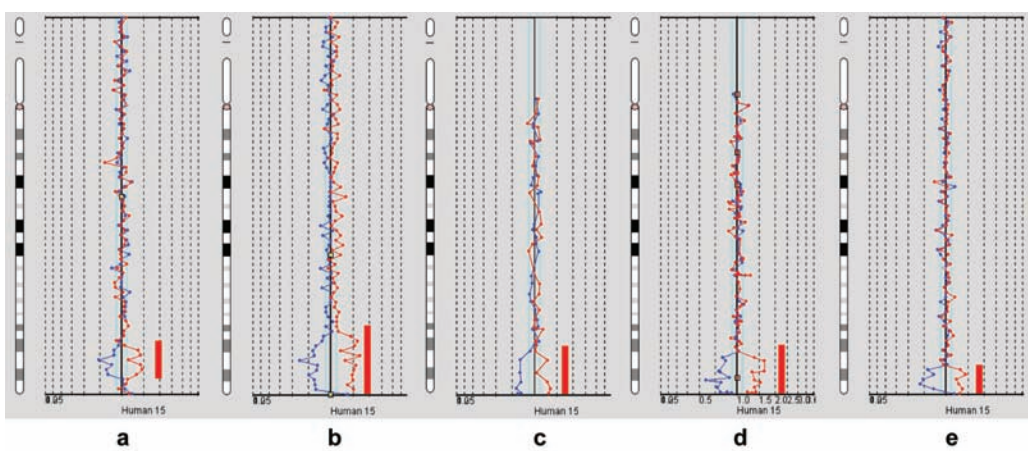

Fig.1. Array-CGH
a: Patient 1, with CDH and del(15) interstitial deletion. **b**: Patient 3, with CDH and r(15)(p11q26). **c**: Patient 4, with CDH and der(15)t(3 ;15)(q29 ;q26.1). **d**: Patient 7, with CDH and del(15)(q25q26.3). **e**: Patient 8, without CDH and with r(15)(p11.1q26.3).

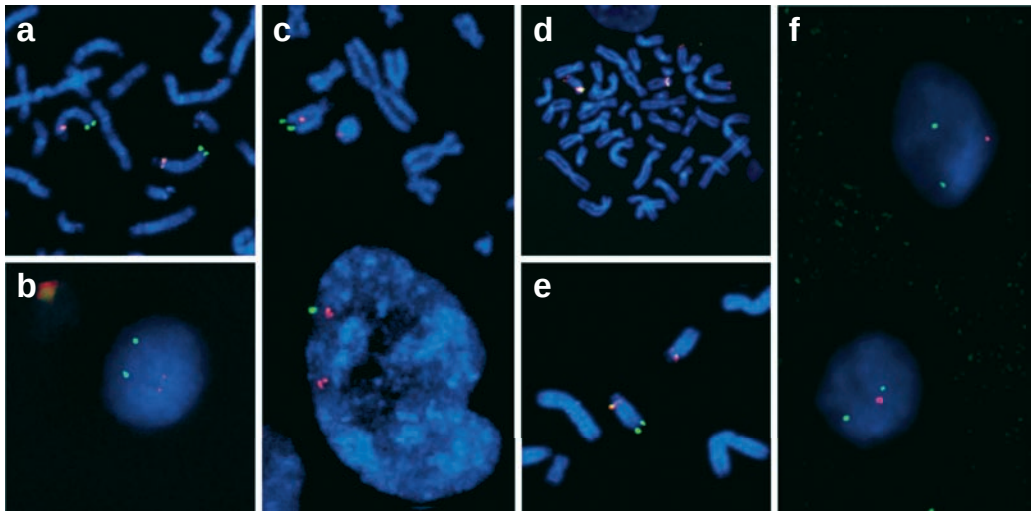

Fig. 2. FISH results.
a: Patient 1, partial metaphase, probe D15Z4 (*red signal*) at chromosome 15 centromeric locus and probe RP11-114I24 (*green signal*) at 15q26.3. **b**: Patient 2, interphase, probe RP11-369K8 (*red signal*) and RP11-253B9 (*green signal*) near the chromosome 5 centromeric region 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.

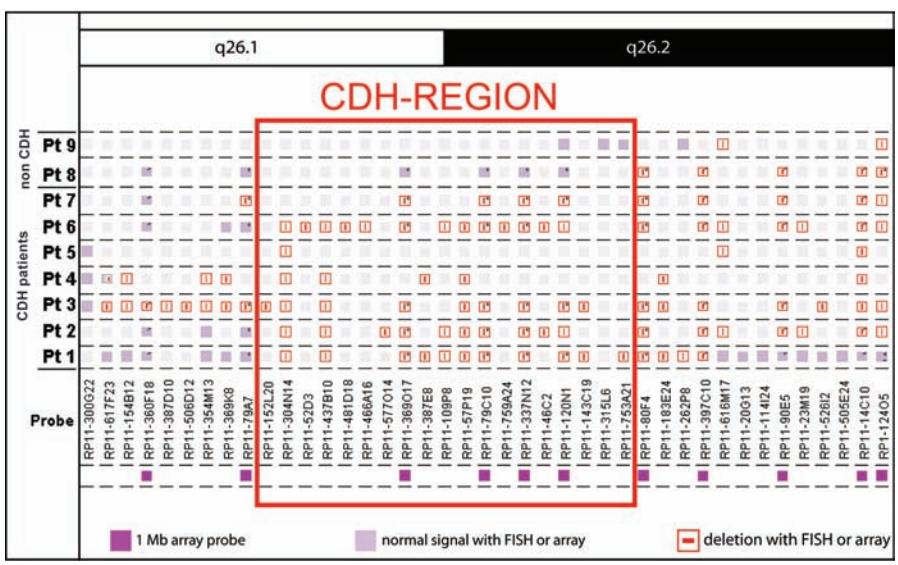

Fig. 3. CDH-region.
Schematic representation of the critical CDH region, with partial ideogram of chromosome 15q. Patients (Pt) 1 – 7 have CDH; patients 8 and 9 do not have CDH. BAC clones that were tested by array CGH and FISH are listed. The black dots inside boxes indicate that probes have been tested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

Fig. 1. Clinical features of Patient 2. A, clinodactyly second and fifth finger; B, bilateral club feet; C, facial features. Note the mild coarse facial features with hypertelorism with wide nasal bridge, "pinched" appearance of the nose, down turned mouth and mild micrognathia.

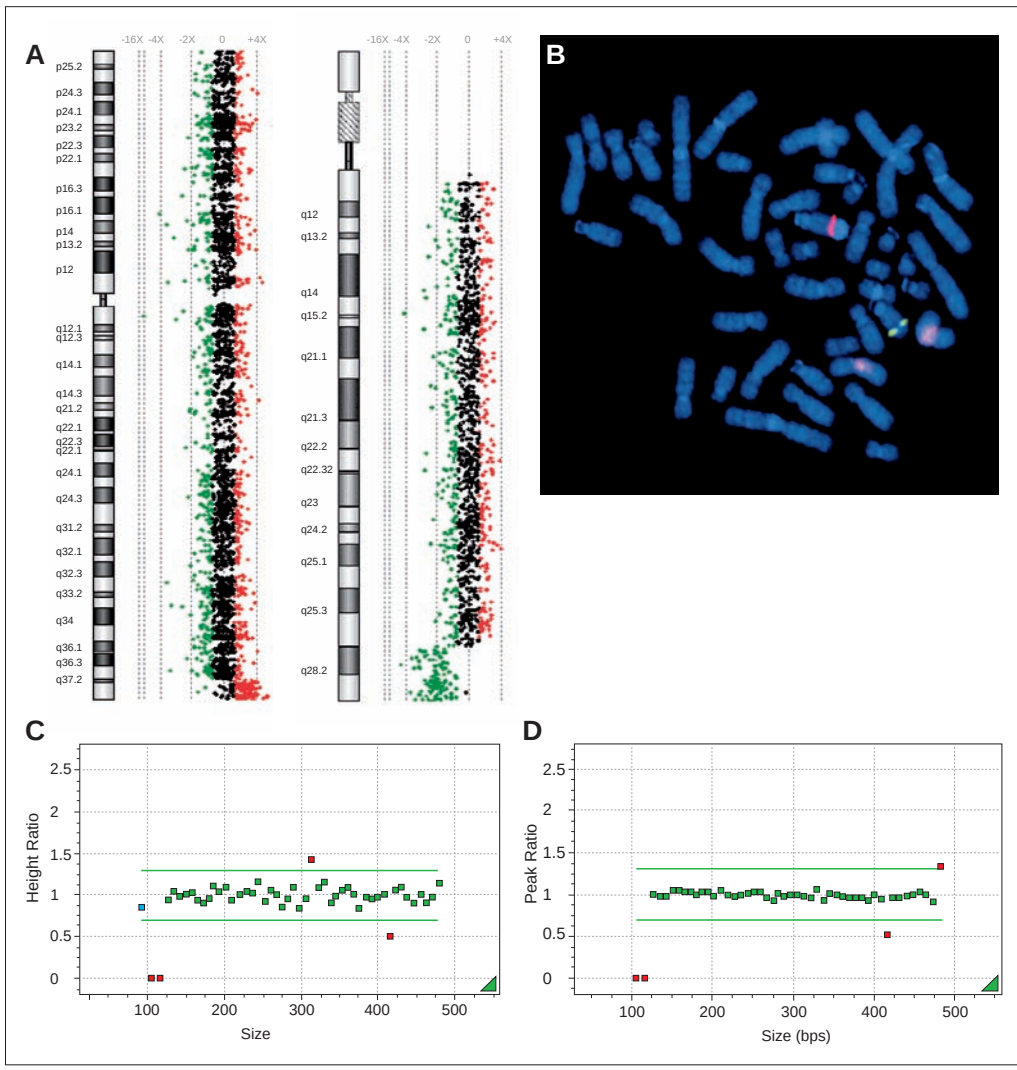

Fig. 2. Results of molecular cytogenetic analysis. A: Oligonucleotide-based ArrayCGH results for Patient 1: gain of 2q37 (left panel) and loss of 15q26 (right panel)(Scott et al. 2007); B: Fluorescent In Situ Hybridization results for Patient 2. Loss of RP11-154B12 on chromosome 15q26.1 (green signal) and presence of RP11-99M1 on chromosome Yq22 (red signal) with aspecific signals on chromosome X (light red patches); C: Multiplex Ligation-dependent Probe Amplification (MLPA) results for Patient 1 (gain of CAPN10 on 2q37 and loss of ALDH1A3 on 15q26); D: MLPA results for Patient 2 (gain of SYBL1 on Yq and loss of ALDH1A3 on 15q26).

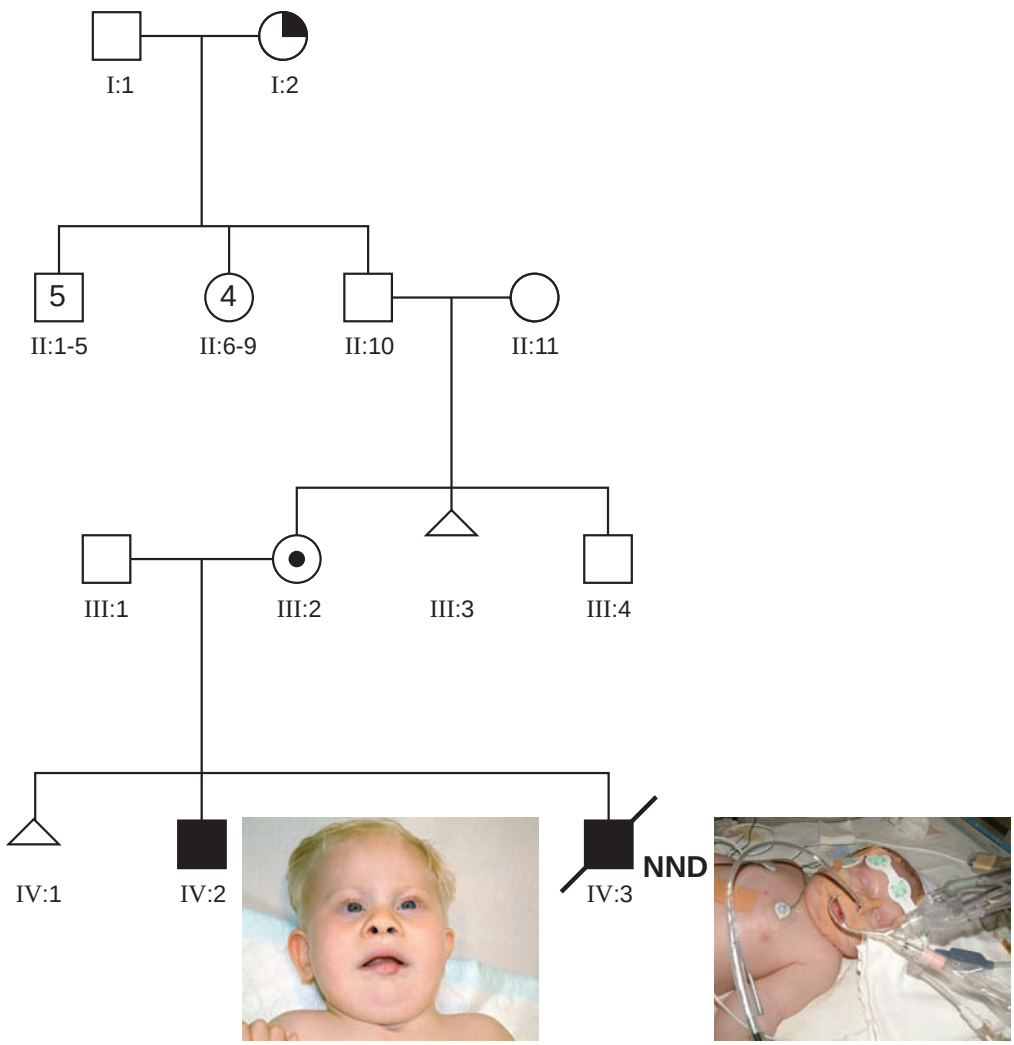

Fig. 1. Pedigree of family with t(11;12).
III:2 Healthy carrier mother; IV:1 spontaneous abortion; IV:2 Patient 1, note the dysmorphic features such as short nose, micrognathia and prominent upper lip; IV:3 Patient 2, dysmorphic features difficult to see due to presence of tubes and facial edema. Pictures of Patient 1 (IV:2) and 2 (IV:3) are shown with parental permission.

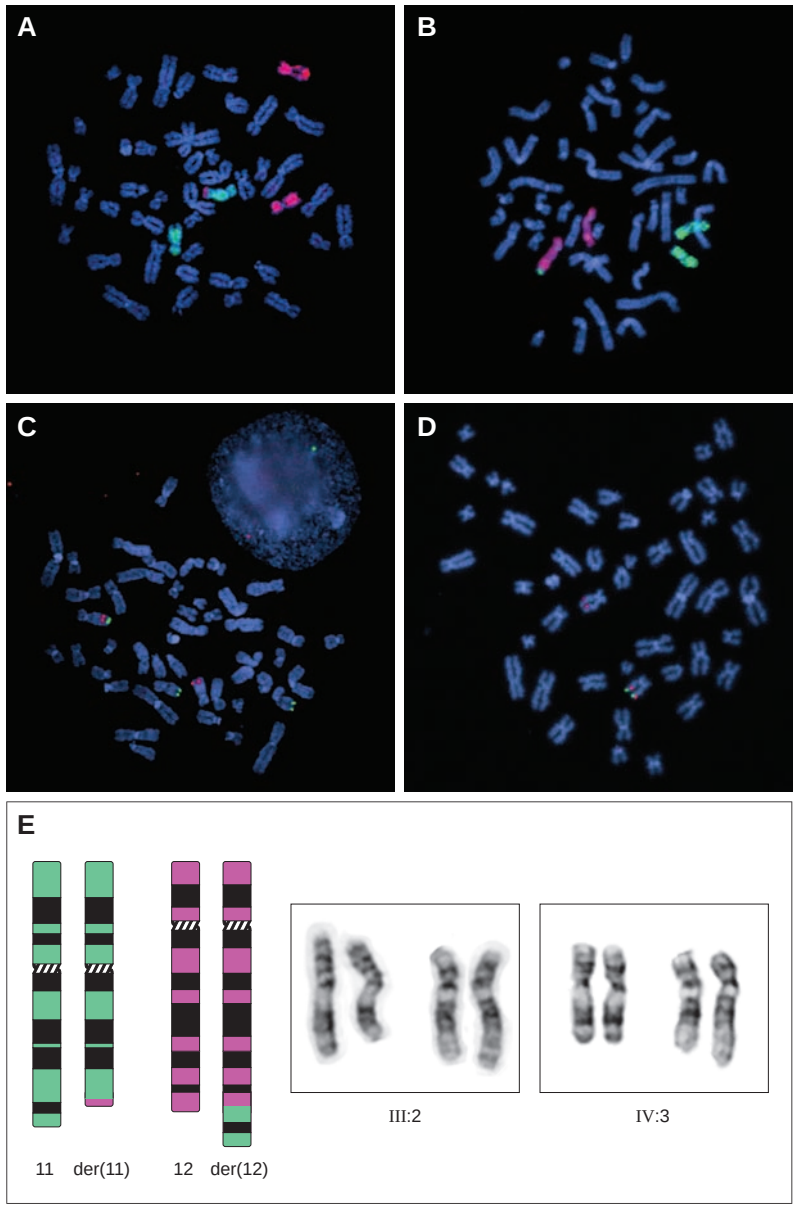

Fig. 2. a-**d.** Fluorescence in situ Hybridization experiments:
WCP11 (red) & WCP12 (green) paint of chromosomes from Patient 1 showing additional chromosome 11 material a on the derivative chromosome 12 (**a**), WCP11 (green) & WCP12 (red) paint of maternal chromosomes showing balanced translocation of chromosome 11 material to chromosome 12. (**b**), RP11-19F21 (red signal) & RP1-26N8 (green signal) showing the presence of three copies of the terminal region of 11q in Patient 1 (**c**), RP11-19F21 (red signal) & RP1-221K18 (green signal) showing only a single signal from the terminal region of 12q in Patient 2 (**d**).
e: Ideograms and partial karyotypes of mother (III:2) and Patient 2 (IV:3).
On the left a schematic representation of the balanced translocation is shown. Chromosome 11 (green) and chromosome 12 (pink) are shown. On the right pictures of chromosomes 11 and 12 of both III:2 (balanced translocation carrier) and IV:3.

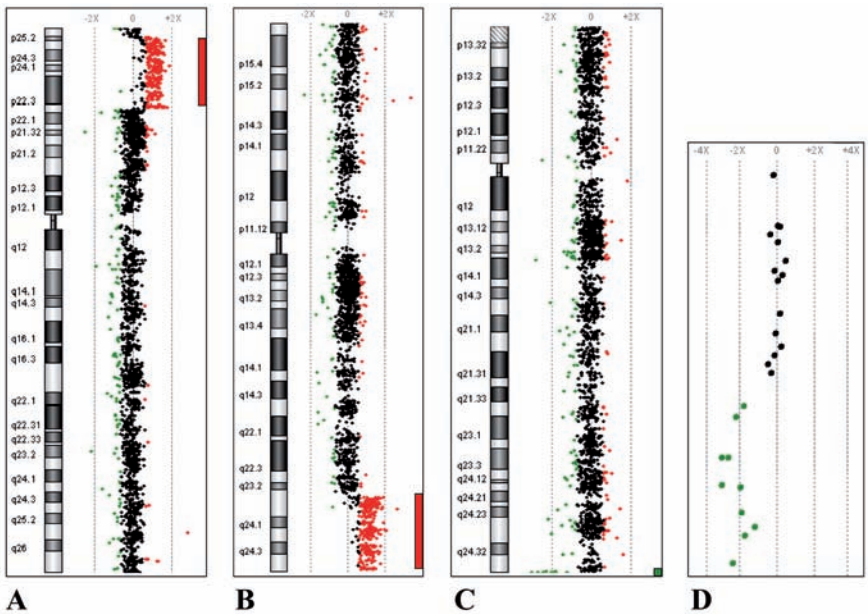

Fig. 1. Examples of copy number changes identified by aCGH in patients with CDH+. Colored bars on the right hand side of panels *A-C* mark affected regions. (**A**) An ~22 Mb duplication of chromosome 6p in patient TX19. (**B**) an ~18 Mb duplication of chromosome 11q in patient N4. (**C**) An ~0.5 Mb deletion of chromosome 12q in patient N4. Previously published aCGH studies using a BAC-based array CGH failed to identify this deletion due to incomplete coverage of the telomere region (16). (**D**) A more detailed view of the 12q aCGH results in patient N4. Multiple deleted probes mark the affected region of 12q.

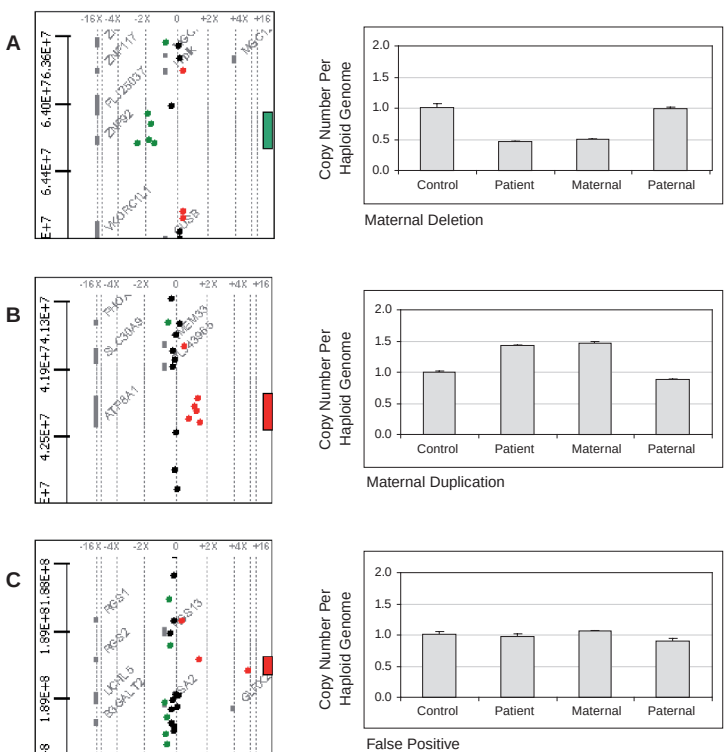

Fig. 2. Quantitative real-time PCR can be used to interrogate putative deletions/duplications identified by oligonucleotide-based aCGH and can simultaneously determine inheritance patterns. The left hand portions of panels show aCGH data from the region around each putative deletion/duplication. The approximate location of each region of interest is marked with a colored bar. The right hand portion of each panel shows quantitative PCR results for each region of interest. (**A**) A maternally inherited deletion. (**B**) A maternally inherited duplication. (**C**) A false positive result as indicated by a normal copy number in the patient sample.

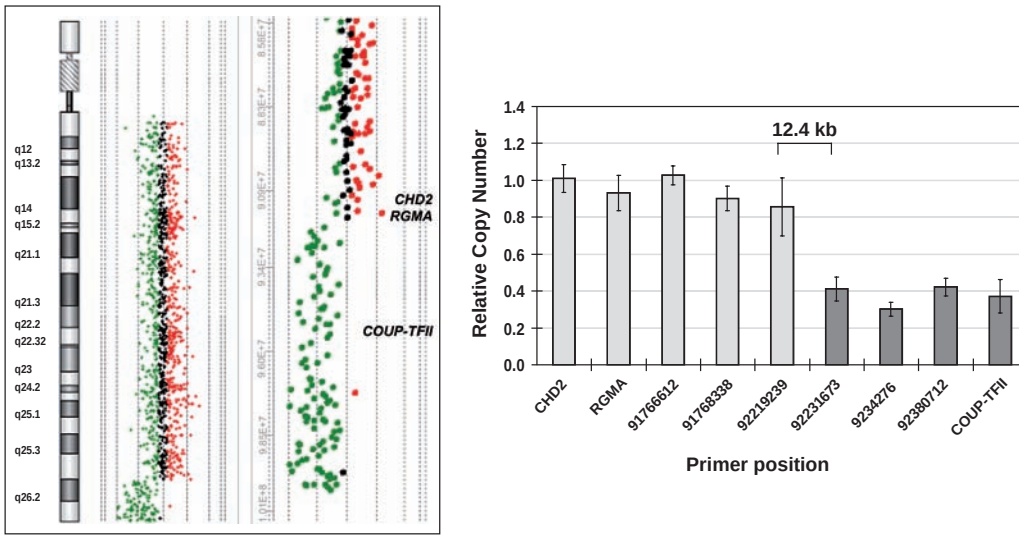

Fig.4. Rapid localization of breakpoints using oligonucleotide-based aCGH and real-time quantitative PCR. (**A**) aCGH data demonstrating a deletion of chromosome 15q in patient N9. (**B**) An enlarged view of the deleted region with the approximate location of candidate genes *CHD2*, *RGMA*, and *COUP-TFII* indicated. (**C**) Quantitative real-time PCR data demonstrating a reduction in copy number for *COUP-TFII*, indicating a deletion, and preservation of normal copy number for *CHD2* and *RGMA*. Data presented were used to localize the 15q breakpoint in patient N9 to an ~12.4 kb interval. The position of each real-time quantitative PCR primer set is given based on NCBI build 35.

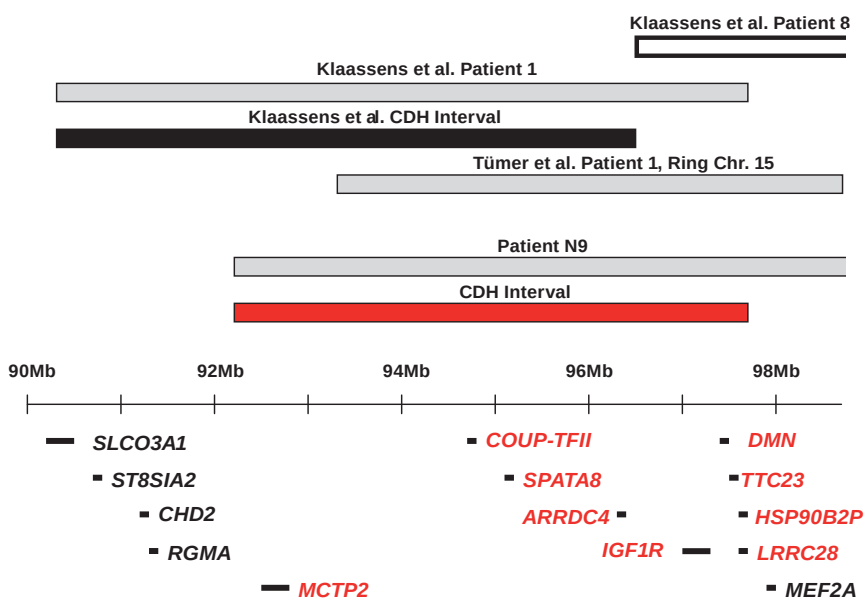

Fig. 5. Defining the CDH minimal deleted region on chromosome 15q26. Deletions in individuals with CHD are shown in grey and deletions in individuals without CDH are shown in non-filled rectangles (17,22). The minimal deleted interval defined by Klaassens et al. is shown in black and the new minimal deleted region defined by patient N9 and Patient 1 described by Klaassens et al. is shown in red. The approximate locations of genes within this region are shown with genes residing inside the new interval depicted in red.

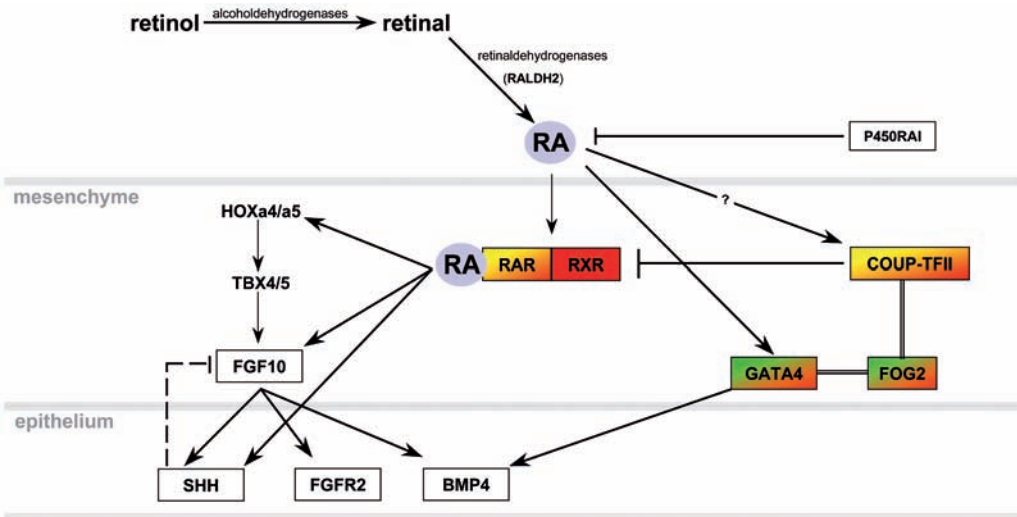

Fig. 1.
Cellular interactions in lung development. Genes that play a role in epithelial-mesenchymal signalling are shown. Influence of retinoic acid on these genes, if present, is indicated.
■ cardiac anomaly in animal models or in humans when mutated; ■ "true" diaphragm defect in animal models;
■ muscularization defect in animal models;
⊣ inhibition; ⟶ proven (in)direct regulation by retinoic acid; **?**, intermediate, unknown, gene; ⊣ - - possible inhibition;
═ binds to; **RA**, retinoic acid; **(C)RBP**, (cellular) retinol binding protein; **RAR**, retinoic acid receptor; **RXR**, retinoid X receptor; **RARE**, retinoic acid response element.

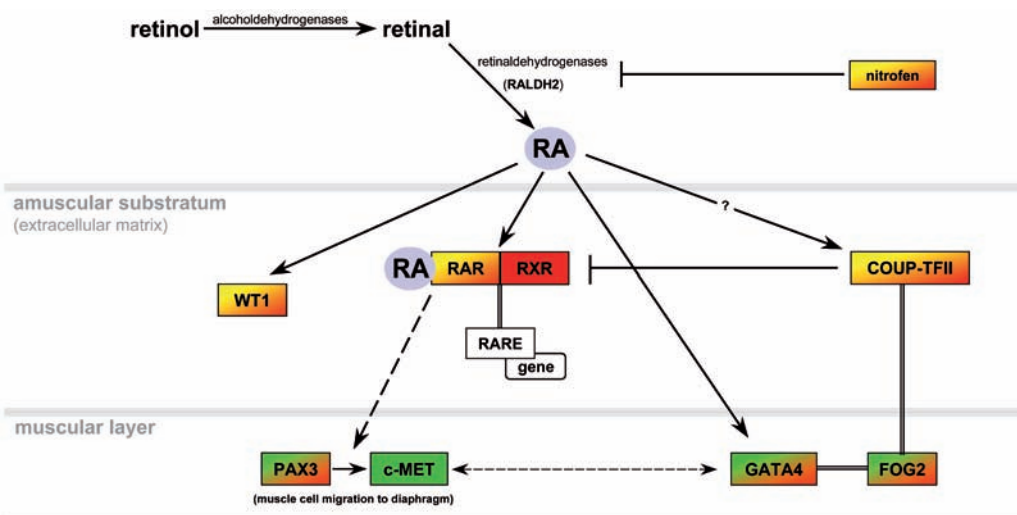

Fig. 2.
Cellular interactions in diaphragm development. Genes that cause diaphragm defects in animal models are shown. Genes in the region of the "amuscular substratum" cause "true" diaphragm defects when disrupted in mice. Genes in the muscular layer cause eventration of the diaphragm in mutant mice.
■ cardiac anomaly in animal models or in humans when mutated; ■ "true" diaphragm defect in animal models;
■ muscularization defect in animal models;
⊣ inhibition; - - ➔ possible regulation or interaction, not proven; ⟶ (in)direct regulation; **?**, intermediate, unknown, gene;
═ binds to; **RA**, retinoic acid; **(C)RBP**, (cellular) retinol binding protein; **RAR**, retinoic acid receptor; **RXR**, retinoid X receptor; **RARE**, retinoic acid response element..

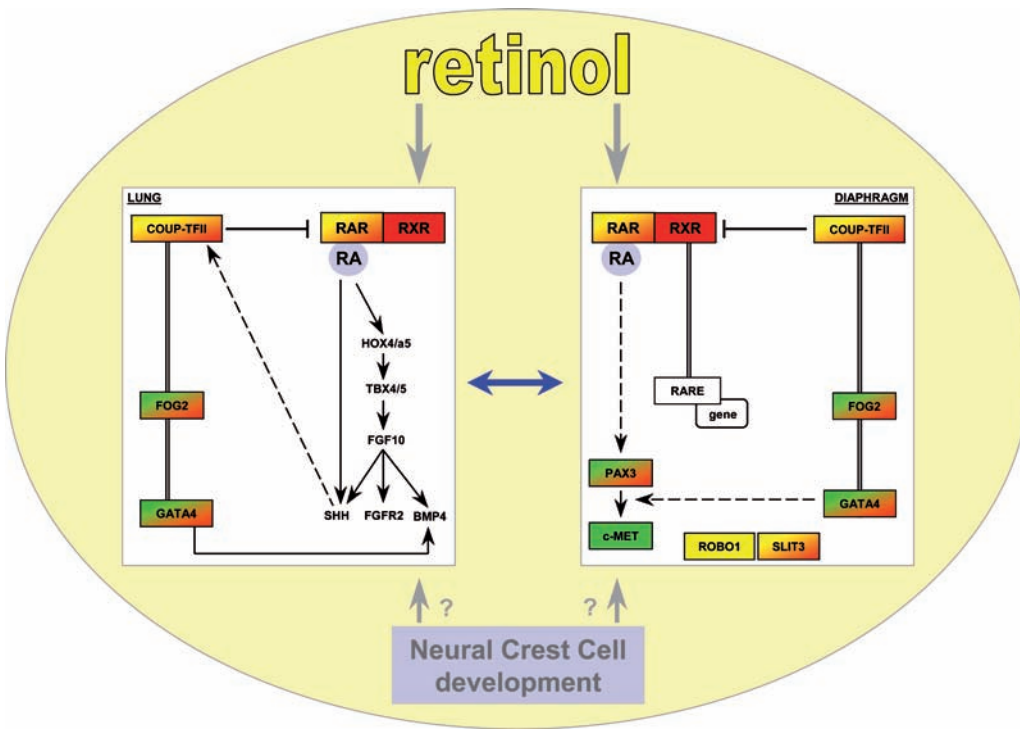

Fig. 3.
Possible cellular interactions involved in the etiology of Congenital Diaphragmatic Hernia. Both lung- and diaphragm development are under the influence of retinal and its active metabolite retinoic acid, a diffusible factor in embryonic development. Neural Crest Cell development may be a common factor, involved in all types of diaphragm defects and lung development, is also influenced by retinoic acid.
cardiac anomaly in animal models or in humans when mutated; "true" diaphragm defect in animal models; muscularization defect in animal models; retinol (and retinoic acid), an important diffusible factor; inhibition; possible regulation or interaction, not proven; (in)direct regulation; **?**, intermediate, unknown, gene; binds to; **RA**, retinoic acid; **(C)RBP**, (cellular) retinol binding protein; **RAR**, retinoic acid receptor; **RXR**, retinoid X receptor; **RARE**, retinoic acid response element.