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ARTICLE

Array comparative genomic hybridization in patients with congenital diaphragmatic hernia: mapping of four CDH-critical regions and sequencing of candidate genes at 15q26.1–15q26.2

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Congenital diaphragmatic hernia (CDH) is a common birth defect with a high mortality and morbidity. There have been few studies that have assessed copy number changes in CDH. We present array comparative genomic hybridization data for 29 CDH patients to identify and map chromosome aberrations in this disease. Three patients with 15q26.1–15q26.2 deletions had heterogeneous breakpoints that overlapped with the critical 4 Mb region previously delineated for CDH, confirming 15q26.1–15q26.2 as a critical region for CDH. The three other most compelling CDH-critical regions for genomic deletions based on these data and a literature review are located at chromosomes 8p23.1, 4p16.3–4pter, and 1q41–1q42.1. Based on these recurrent deletions at 15q26.1–15q26.2, we hypothesized that loss-of-function mutations in a gene or genes from this region could cause CDH and sequenced six candidate genes from this region in more than 100 patients with CDH. For three of these genes (*CHD2*, *ARRDC4*, and *RGMA*), we identified missense changes and that were not identified in normal controls; however, none of these alterations appeared unambiguously causal with CDH. These data suggest that CDH caused by chromosome deletions at 15q26.2 may arise because of a contiguous gene deletion syndrome or may have a multifactorial etiology. In addition, there is evidence for substantial genetic heterogeneity in CDH and diaphragmatic hernias can be non-penetrant in patients who have deletions involving CDH-critical regions.
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Introduction

Congenital diaphragmatic hernia (CDH) is estimated to occur in 1/2000–1/3000 live- and still-births and the mortality and morbidity remain high despite new

therapeutic interventions.[1,2] CDH can occur as an isolated diaphragm defect (referred to here as isolated CDH) or be present with other phenotypic anomalies (referred to here as CDH with anomalies). Genetic factors have been established as important in the etiology of both isolated CDH and CDH with anomalies,[3] and an increased rate of concordance in monozygotic twins with isolated CDH compared to dizygotic twins has been demonstrated. However, large pedigrees with CDH are rare, making the identification of disease-causing genes from pedigree studies difficult. Nevertheless, one apparent mutation in the *FOG2* gene has been described in an isolated patient with diaphragmatic eventration (thinning of the diaphragm),[4] supporting the expected genetic etiology of a proportion of CDH patients.

Chromosomal deletions and duplications have been reported to be present in up to 33% of individuals with CDH[5–7] and it has been postulated that these cytogenetic abnormalities can provide positional information about the genomic locations for CDH-causing genes.[7] More recent technologies such as array comparative genomic hybridization (array CGH) are beginning to prove powerful in the identification of submicroscopic and microscopic chromosome aberrations, which can be suggestive of regions harboring a disease-causing gene(s).[8,9] However, relatively few patients with CDH have been studied using higher resolution techniques such as array CGH. In one study by different authors, array CGH and fluorescence *in situ* hybridization (FISH) were used to delineate a 4–5 Mb critical interval for an apparent CDH-causing region at chromosome 15q26.2 in seven patients with CDH.[10,11] In three patients with a Fryns-like phenotype comprising CDH and other anomalies, a previous study by the authors of this paper described two 15q26.2 deletions as well as an 8p23.1 deletion.[12] A third study by a third group of authors reported a deletion of 1q41–1q42 in a patient with CDH and other anomalies.[13] In the present study, we sought to further explore array CGH for identifying and mapping chromosome aberrations in a large panel of CDH patients to provide additional genomic information on regions likely to contain genes of relevance to CDH etiology.

Materials and methods

Patient samples

DNA samples were obtained from probands and parents under Committee for Human Subjects Research (CHR) approved protocols at University of California, San Francisco (UCSF). DNA was extracted from peripheral blood lymphocytes, paraffin sections, or blood spots obtained from the California Birth Defects Monitoring Program by digestion with proteinase K and salting-out according to standard procedures.[14] Blood spot DNA was amplified using whole genome amplification (GenomiPhi™ GE Healthcare) and used for DNA sequencing only; we were unable to obtain satisfactory array CGH results from amplified DNA from the blood spots. However, we did use DNA extracted from paraffin for array CGH despite a lower success rate of satisfactory hybridizations, as paraffin was frequently the only source of DNA from deceased children with CDH and additional anomalies in this study.

We studied 29 patients, 16 with CDH and additional anomalies and 13 with isolated CDH. Six of these patients had previously detected cytogenetic aberrations and three of these patients had previously been published.[12] However, all of these six patients were further mapped using array CGH or microsatellite markers for this paper. All of the DNA samples were obtained directly from peripheral blood lymphocytes or paraffin sections. The clinical summaries of the patients with CDH and chromosome aberrations are available as Supplementary Information at the European Journal of Human Genetics website.

Array CGH

The HumArray 2.0 and HumArray 3.1 bacterial artificial chromosome (BAC) arrays comprising 2464 BAC, PAC, and P1 clones were used from the UCSF Comprehensive Cancer Center Microarray Core (http://cc.ucsf.edu/microarrays/links/). The majority of the clones (2442/2464; 99%) on the array are single copy with an average resolution of 1.4 Mb for the HumArray 2.0. The array CGH methodology and analysis has previously been described.[8,9,12]

Fluorescence *in-situ* hybridization (FISH)

Slides with metaphase cells were made from lymphoblastoid or fibroblast cell lines using standard techniques. Probes were labeled by nick translation with CyDye 3-dUTP (d-UTP; Little Chalfont, Buckinghamshire, UK) or fluorescein12-dUTP, (FITC; PerkinElmer Life Sciences Inc., Boston, MA, USA) according to a modification of manufacturer's instructions. One microgram of labeled probe was combined with 10 μg of cot-1 DNA and hybridized according to standard techniques.

Microsatellite marker analysis

Microsatellite markers were selected from the UCSC Genome Browser (http://genome.ucsc. edu/) and primer pairs were labeled with 5′6-FAM (Integrated DNA Technologies. Coralville, IA, USA). The method has previously been described.[12] We performed microsatellite studies only when DNA samples were available from a proband and both parents.

Sequencing of genes in the 15q26.2 CDH-critical interval

Primer oligonucleotides for PCR on each gene were chosen on repeat-masked DNA using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). M13(−21) forward (TGT AAA ACG ACG GCC AGT) and M13 reverse (CAG GAA ACA GCT ATG AC) tails were added to each

primer for subsequent use in high-throughput DNA sequencing reactions. Each PCR reaction was performed in a 96-well plate format using a panel of the human CDH DNAs as template. Amplified PCR products from each individual were sequenced in both directions using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on an ABI 3730 machine. Sequence variants were identified using the PolyPhred program (http://droog.mbt.washington.edu/PolyPhred/html) and analyzed manually for accuracy. Sequence variants that were suggestive of mutations were verified by repeat sequencing or restriction enzyme digestion according to standard techniques.

Results

Array CGH data

To identify and map chromosome aberrations, we performed array CGH on 29 patients and these data are summarized in Table 1. We selected a copy number of +0.7 for duplications and a copy number of −1.0 for deletions as array CGH cutoffs as previously established for this array[9] and required aberrant copy number changes to be present for a minimum of two successive BAC clones. Patients 1–3 had known 15q26.1–15q26.2 deletions;[12,15] in patients 1 and 2, array CGH studies had previously been published.[12] These two patients were further mapped using microsatellite markers in this paper. Patient 3 had monosomy for 15q26 resulting from a chromosome translocation identified by G-banding and FISH (karyotype 46,XY,-der(15)t(8;15)(q24.2;q26.2))[15] and we mapped the 15q deletion and 8q duplication using array CGH in this paper (Figure 1). We find that the 15q deletions for these patients overlap the previously defined CDH-critical region at 15q26 (Table 2).[10–12] The array CGH results and marker studies suggested similar but not identical centromeric breakpoints for the first and third patients between clone CTD2272E1 at 15q26.1 and clone RP11-185D5 at 15q26.2. The deletions ranged between 8 and 11 Mb (Table 2) and deletions were of mixed paternal (patient 1) or maternal origin (patients 2 and 3).

Patient 2 had a smaller deletion in which microsatellite markers D15S533 and D15S985 only were deleted, with a maximum deletion size of 1–2 Mb (Table 2), which would refine the CDH-critical interval at 15q26.1–15q26.2. However, marker D15S533 is contained within BAC RP11-120N1, and deletion of this BAC clone has been reported in apparently normal individuals (Database of Genomic Variants; http://projects.tcag.ca/variation). It is therefore possible that this patient has a deletion at 15q26.2–3 that is unrelated to the CDH. However, array CGH in the patient's mother did confirm that the deletion was *de novo*

Table 1 Summary of array CGH results in 29 patients with CDH

Patient	*Phenotype*	*Source*	*Result*	*Method of confirmation*
1	CDH+/15q−	Paraffin	15q deletion	Microsatellites
2	CDH+/15q−	Paraffin	15q deletion	Microsatellites
3	CDH+/15q−	Fibroblasts	15q deletion	FISH
4	CDH+/8p−	Paraffin	8p deletion	Microsatellites
5	CDH+/4p−	Blood	4p deletion	FISH
6	CDH+/1q−	Paraffin	1q deletion	Microsatellites
7	CDH	Paraffin	8p CNV	Not done
8	CDH	Blood	8p CNV	FISH
9	CDH	Blood	8p CNV	FISH
10	CDH	Blood	8p CNV	Not done
11	CDH+	Blood	8p CNV	Not done
12	CDH+	Blood	Apparently normal	NA
13	CDH+	Paraffin	Apparently normal	NA
14	CDH+	Blood	Apparently normal	NA
15	CDH+	Blood	Apparently normal	NA
16	CDH+	Blood	Apparently normal	NA
17	CDH+	Blood	Apparently normal	NA
18	CDH+	Paraffin	Apparently normal	NA
19	CDH+	Paraffin	Apparently normal	NA
20	CDH+	Paraffin	Apparently normal	NA
21	CDH	Blood	Apparently normal	NA
22	CDH	Blood	Apparently normal	NA
23	CDH	Blood	Apparently normal	NA
24	CDH	Blood	Apparently normal	NA
25	CDH	Blood	Apparently normal	NA
26	CDH	Blood	Apparently normal	NA
27	CDH	Blood	Apparently normal	NA
28	CDH	Blood	Apparently normal	NA
29	CDH	Blood	Apparently normal	NA

CDH+ = CDH with anomalies; CDH = isolated CDH; CNV = copy number variant; FISH = fluorescence *in-situ* hybridization; NA = not applicable.

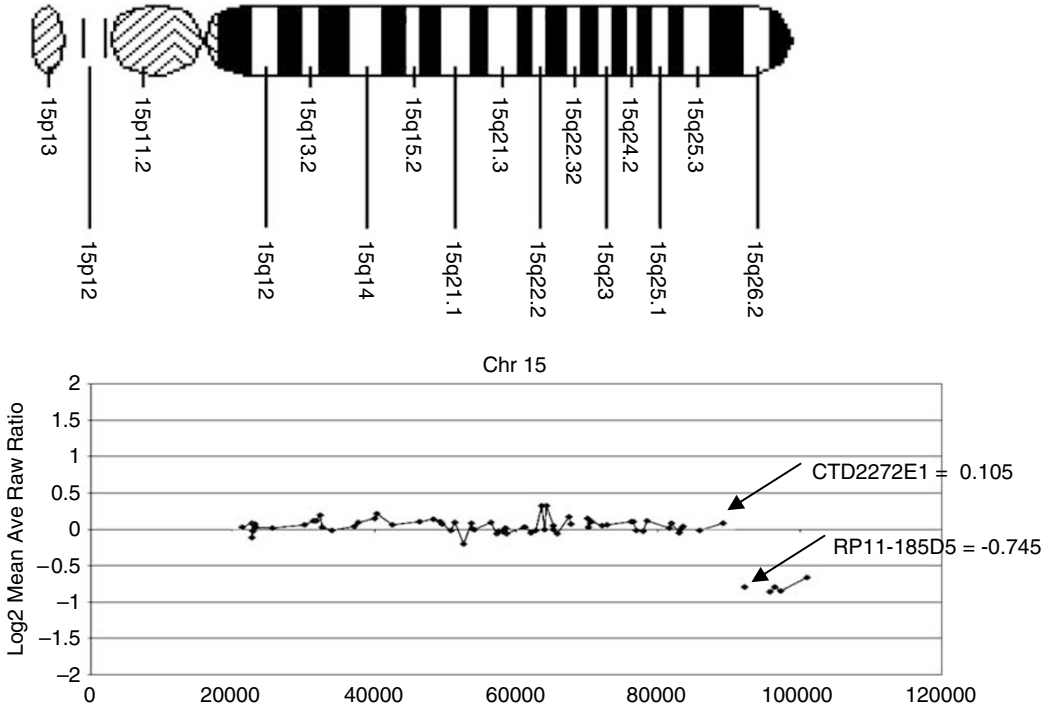

Figure 1 Array CGH in a male with a diaphragmatic hernia and a 15q26.2 deletion resulting from an unbalanced translocation (karyotype 46,XY,der(15)t(8;15)(q24.2;q26.2)). Each dot corresponds to a genomic region on chromosome 15q, with each spot being 1.4 Mb apart and covering 150 kb on average. The *Y*-axis shows the $\log_2$ ratios of the total integrated Cy3 and Cy5 intensities for each clone from a hybridization of patient and control DNA. A value of −1.0 is suggestive of reduced copy number for patient DNA caused by a chromosome deletion. BAC clone RP11-185D5 has a copy number of −0.745, suggestive of a 15q deletion from this clone that extends to the 15q telomere.

(data not shown). We did not perform array CGH in the father, as the microsatellite marker studies demonstrated two paternal alleles at both deleted loci (data not shown), arguing against an inherited paternal deletion. The refined deleted interval contains only the *ARRDC4* gene. The clinical features of these patients are shown together with other reported patients with 15q26 deletions in Table 3.

In patient 4, chromosome analysis on amniocytes had shown a normal male karyotype (46, XY) but array CGH-detected copy number changes were consistent with a deletion for five BAC clones at chromosome 8p22–23.1; the array CGH study has been published.[12] In this paper, we mapped this *de novo*, maternal interstitial deletion of 5–6 Mb to between telomeric marker D8S1706 and centromeric marker D8S552 at chromosome 8p23.1 (data not shown). This deletion is similar to a reported 8p23.1 deletion found in male infant with CDH and cardiac anomalies[16] and thus strongly suggest that these two patients define a new CDH-critical region at 8p23.1.

We used array CGH to map a deletion of chromosome 4p16.3 in a male with CDH and Wolf–Hirschhorn syndrome (WHS) who had *de novo* deletion of 4p16 (karyotype 46,XY,del(4)(p16)) on G-banding at 500 band resolution and with FISH who had not been studied with array CGH. Array CGH localized the breakpoint to between BAC clones RP11-97H19, which was deleted, and RP11-101J14, which was not deleted, with an estimated deletion size of 8 Mb at 4p16.1 (Figure 2). These results were confirmed using FISH with labeled probes for these BACs (data not shown). Deletions of 4p16.3 and WHS have previously been associated with CDH, but no critical region had been identified. However, the deleted region in this patient is larger than at least one previously reported patient with WHS and CDH.[17]

A previously unstudied male with syndromic CDH had been identified as having a *de novo*, interstitial deletion of chromosome 1q on G-banded chromosome analysis (karyotype 46,XY,del(1)(q32.3q42.2)). Array CGH on DNA extracted from a paraffin section from this patient showed reduced copy number changes for clones CTD-2235K13 (−0.992) and RP11-192M1 (−1.029) at 1q41–1q42 (data not shown). Microsatellite markers were used to refine the breakpoints and defined an interstitial deletion on the paternal chromosome of 10–12 Mb in size, including centromeric marker D1S1626 (UCSC Genome browser map position 217,677,120) and telomeric marker D1S2860 (UCSC Genome browser map position 227,054,747) at chromosome 1q41–1q42 (data not shown). This patient is the second of two patients with CDH and overlapping

Table 2 Mapping data for patients 1–3 with CDH and 15q26 deletions

1	*2*	*3*	*BAC/Microsatellite marker*	*Band*	*UCSC*[a]	*Gene*
+	+	+	D15S127	15q26.1	89,198,604	–
		+	CTD-2272E1	15q26.1	89,274,936	–
–		+	D15S652	15q26.1	90,318,339	–
			RP11-79A7	**15q26.1**	**90,582,758**	–
			RP11-304N14	15q26.1	90,740,137	*SIAT8B*
			RP11-52D3	15q26.1	91,066,106	*CHD2*
		–	RP11-369O17	15q26.1	91,385,740	*RGMA/UNQ9370*
UI	+	UI	D15S1004	15q26.1	92,132,022	–
		–	RP11-185D5	15q26.2	92,238,363	–
UI	+	UI	D15S130	15q26.2	92,512,174	–
			RP11-109P8	15q26.2	92,491,964	*MCTP2*
			RP11-261M12	**15q26.2**	**93,900,834**	
–	+	–	D15S207	15q26.2	94,011,773	–
			RP11-337N12	15q26.2	94,516,962	*NR2F2*
	UI	–	D15S657	15q26.2	94,505,791	–
			–	15q26.2	94,629,301	*AK128633/NR2F2*
			RP11-46C2	15q26.2	94,590,469	*NR2F2*
			–	15q26.2	94,703,896	*AK090480*
			–	15q26.2	95,127,683	*SRP8/AY489187*
–	+		D15S1014	15q26.2	95,803,741	–
	–		D15S533	15q26.2	95,883,860	–
–	UI		D15S212	15q26.2	95,936,666	–
			RP11-308P12	15q26.2-3	96,148,975	*ARRDC4*
			RP11-80F4	**15q26.3**	**96,495,497**	–
	–		D15S985	15q26.3	96,766,282	–
UI	+		D15S966	15q26.3	96,684,023	–
			–	15q26.3	96,797,913	*FLJ39743*
UI	+		D15S120	15q26.3	97,409,503	–
		–	**RP11-397C10**	**15q26.3**	**97,234,641**	**IGF1R**
			–	15q26.3	97,462,808	*DMN*
			AC03749.9	**15q26.3**	**97,575,624**	–
			RP11-616M17			
			–	15q26.3	97 923 678	*MEF2A*
			RP11-90E5	15q26.3	98 388 044	*ADAMTS17*
			RP11-66B24	15q26.3	99 149 459	*ALDH1A3*
(+)			RP11-530H6	15q26.3	99 746 878	*PACE4/PCSK6*
		–	15qtel	15qter		–

[a]UCSC Genome Browser website. + = not deleted; – = deleted; (+) = not deleted on array CGH; UI = uninformative. The markers in bold define the CDH-critical regions described by Klaassens *et al*.[10,11]

deletions of 1q41–1q42 to be reported and the critical region is 5 Mb in size.[13] In the 15 patients with non-syndromic CDH, no other copy number changes consistent with chromosome aberrations were identified.

Sequencing data

We have summarized detected nucleotide alterations in six candidate genes for CDH from 15q26 in Table 4. In *ARRDC4*, we detected a nucleotide substitution, c.C1171T, that predicted p.R391W in both an Mexican American proband with CDH and his phenotypically normal father. This alteration was not present in more than 100 Mexican American control chromosomes. We found three alterations, c.C5128T, predicting p.R1710W in *CHD2* and c.G196A, predicting p.A66T, and c.C362G, predicting p.T121S, both in *RGMA*, in different Mexican American patients in whom parental samples were unavailable. None of these alterations were present in more than 100 Mexican American control chromosomes. In *SIAT8B*, we noted one alteration, c.A674G predicting p.K225R, but this gene has since been excluded from the CDH-critical region by further mapping studies.[11] We were unable to conclude that the alteration in any of the sequenced genes is disease-causing.

Discussion

We have performed array CGH in 29 probands with isolated CDH and CDH with anomalies and mapped four CDH-critical regions on chromosomes 15q26.2, 8p23.1, 4p16.3, and 1q41–42. Deletions of these four CDH-critical regions have previously been shown to be associated with abnormal diaphragm development and these regions are not novel.[10,11,13,16,17] However, our detection of further patients with CDH and deletions of these regions strengthens the concept that haploinsufficiency for a gene or genes in these regions leads to diaphragm defects. We hypothesize that loss of function of a gene(s) in these regions

Table 3 Clinical features of patients 1–3 with monosomy 15q24–15qter and CDH

	Patient 1	*Patient 2*	*Patient 3*	*Literature*[a]
CDH	+	+	+	19 out of 21
Pulmonary hypoplasia	+	+	+	8 out of 16
Intra-uterine growth retardation	+		+	16 out of 17
Cardiac defects				
Transposition of arteries		+		0 out of 19
Aortic stenosis				4 out of 19
Dextrocardia		+		1 out of 19
Hypoplastic left heart	+		+	2 out of 19
Atrial septal defect		+		1 out of 19
Ventricular septal defect		+	+	8 out of 19
Other				
Facial dysmorphism	+	+		8 out of 16
Cleft palate		+		2 out of 16
Nuchal webbing		+		1out of 16
Renal hypoplasia		+		5 out of 16
Double uterus/vagina	+	+		0
Single umbilical artery	+	+	+	5 out of 16
Talipes/rockerbottom feet		+	+	6 out of 16
Nail hypoplasia			+	2 out of 16
Prognosis	Died day 1	Died day 1	NA	

[a]Literature review includes patients reported by[5,10,28–40], and not all patients were included in every category as the clinical descriptions varied.

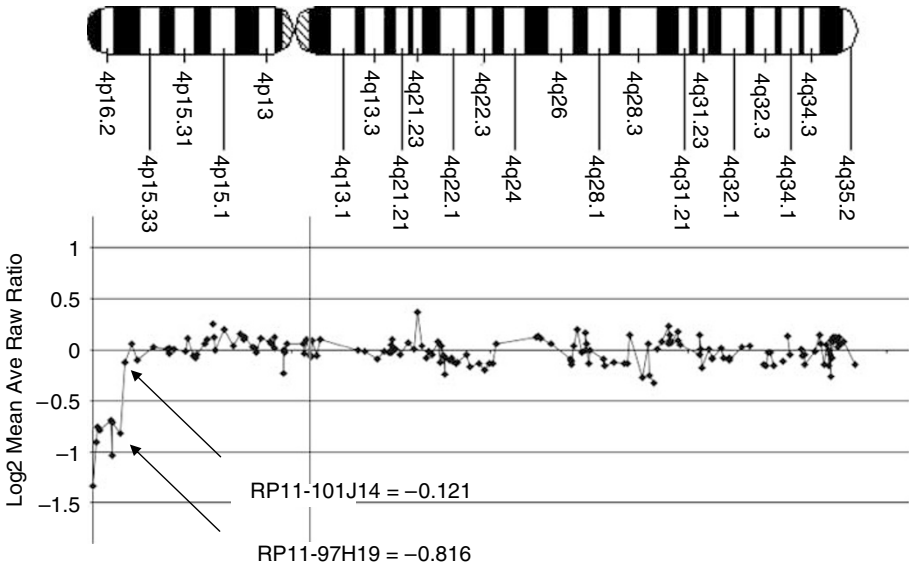

Figure 2 Array CGH in a male with a diaphragmatic hernia and a 4p16 deletion (karyotype 46,XY,del(4)(p16)). Each dot corresponds to a genomic region on chromosome 15q, with each spot being 1.4 Mb apart and covering 150 kb on average. The *Y*-axis shows the $\log_2$ ratios of the total integrated Cy3 and Cy5 intensities for each clone from a hybridization of patient and control DNA. A value of −1.0 is suggestive of reduced copy number for patient DNA caused by a chromosome deletion. BAC RP11-97H19 has a copy number of – 0.816, suggestive of a 4p16 deletion that extends to the 4p telomere.

can cause CDH, either through chromosome deletion or through possible gene mutation. The CDH-critical regions therefore provide a useful starting point for the selection of candidate genes for sequencing studies in patients with CDH without chromosome deletions.

Molecular or cytogenetic studies including array CGH to define CDH-critical regions have been relatively few. One group demarcated a 5 Mb critical region for CDH at chromosome 15q26.1–15q26.2 in seven patients with CDH and two patients without CDH using array CGH and FISH.[10] The critical region included BAC clones RP11-152L20 and RP11-753A21 and was bounded by BAC RP11-79A7 at 15q26.1 at the centromeric border and by BAC RP11-80F4 at 15q26.3 at the telomeric border (Table 2).[10]

Table 4 Sequencing data for genes *SIAT8B, CHD2, MCTP2, NR2F2, ARRDC4*, and *RGMA* at 15q26

Gene	*Nucleotide*	*Amino acid*	*Phenotype*	*Interpretation*
SIAT8B				
SIAT8B	c.C538T	p.D176D	NA	Silent Aa
SIAT8B	c.C538T	p.D176D	NA	Silent Aa
SIAT8B	c.C621G	p.P207P	NA	SNP
SIAT8B	**c.A674G**	**p.K225R**	**CDH**	**Unknown**
CHD2				
CHD2	c.C959T	p.T320T	NA	Silent Aa
CHD2	c.G1215A	p.P450P	NA	Silent Aa
CHD2	c.A2048G	p.E683E	NA	SNP
CHD2	c.A2546C	p.A849A	NA	Silent Aa
CHD2	c.A2717G	p.Q906R	NA	SNP
CHD2	c.C3564T	p.Y1188Y	NA	SNP
CHD2	c.C4527T	p.I1509I	NA	Silent Aa
CHD2	c.G4721C	p.G1574A	L CDH+	SNP
CHD2	**c.C5128T**	**p.R1710W**	**CDH**	**Unknown**
MCTP2				
MCTP2	c.G197A	p.R66Q	NA	Likely SNP
MCTP2	c.G579A	p.A193A	NA	Silent Aa
MCTP2	c.G608A	p.G203D	CDH	SNP
MCTP2	c.C924G	p.V308V	NA	Silent Aa
MCTP2	c.A1404G	p.T468T	L CDH	Silent Aa
MCTP2	c.A1587C	p.A519A	L CDH+	Silent Aa
MCTP2	c.C1798T	p.S596S	L CDH	Silent Aa
MCTP2	c.C2472T	p.G824G	NA	Silent Aa
NR2F2				
NR2F2	**c.G733T**	**p.L245F**	**CDH/15q-**	**Unknown**
NR2F2	c.C801T	p.A267A	CDH/15q-	Silent Aa
ARRDC4				
ARRDC4	c.C9G	p.G3G	NA	Silent Aa
ARRDC4	c.G138A	p.A46A	L CDH	Silent Aa
ARRDC4	c.C145T	p.P49S	CDH/CHD	SNP
ARRDC4	c.C145T	p.P49S	CDH/renal	SNP
ARRDC4	c.C231T	p.A77A	NA	Silent Aa
ARRDC4	c.G235A	p.A79T	NA	Likely SNP
ARRDC4	c.C1072T	p.P358S	NA	SNP
ARRDC4	**c.C1171T**	**p.R391W**	**CDH**	**Unknown**
RGMA				
RGMA	**c.G196A**	**p.A66T**	**CDH**	**Unknown**
RGMA	c.C210T	p.D70D	NA	Silent Aa
RGMA	**c.C362G**	**p.T121S**	**CDH+**	**Unknown**
RGMA	c.C498T	p.F166F	NA	Silent Aa
RGMA	c.C525T	p.T175T	NA	Silent Aa
RGMA	c.G564A	p.P188P	NA	Silent Aa
RGMA	c.T576C	p.N192N	NA	Silent Aa
RGMA	c.T576C	p.N192N	NA	Silent Aa
RGMA	c.G615A	p.L205L	NA	Likely SNP
RGMA	c.G1272C	p.A424A	NA	Silent Aa
RGMA	c.C1292T	p.A442V	NA	SNP
RGMA	c.C1297T	p.R444W	NA	SNP
RGMA	c.C1317G	p.L439L	NA	Silent Aa

Aa = amino acid; NA = not applicable; CDH = isolated CDH; CDH+ = CDH with anomalies; SNP = single nucleotide polymorphism.

However, patients with deletions encompassing this entire chromosome region have been reported without CDH, and the strategy of using patients without CDH to define the critical region was later questioned because of the possibility of non-penetrance of the diaphragmatic phenotype in those with deletions.[11] The same group repeated their analysis using only patients with CDH and refined the CDH-critical interval to a 4 Mb region between BAC clones RP11-44A22 and RP11-261M12 at 15q26.2.[11] The deletions in our first and third patients include this critical interval.

In our second patient, the deletion is much smaller and includes at least one BAC clone that has reportedly been deleted in normal individuals. If this deletion is truly causative, it implicates the *ARRDC4* gene in the pathogenesis of CDH (Table 2). We sequenced *ARRDC4* in more than 100 individuals with CDH and detected a nucleotide substitution, c.C1171T, that predicted p.R391W in both a proband with CDH and his phenotypically normal father. The altered amino acid was conserved in several species (Table 5), but the significance remains unclear as the alteration is present in both father and child. ARRDC4 has an N- and a C-terminal arrestin domain and the precise function of this gene and protein has not yet been determined. It remains still possible that mutations or large deletions involving this gene are rare, but capable of perturbing normal diaphragm development.

A total of 24 cases of diaphragm abnormalities associated with deletions of chromosome 15q24–15qter have now been reported (Table 3) and have been estimated to account for up to 1% of patients with CDH.[10] The phenotype resulting from 15q24–15qter monosomy is recognizable and comprises CDH or diaphragm hypoplasia, pulmonary hypoplasia, cardiac defects including hypoplastic left heart syndrome and coarctation of the aorta, severe growth retardation, mild facial dysmorphism, talipes and/or rockerbottom feet, and a single umbilical artery (Table 3).[12] Recognition of this phenotype with or without CDH should be a clear indication for molecular cytogenetic studies for 15q26.2 deletions, even if a G-banded karyotype is normal.

We also sequenced the *SIAT8B, RGMA, CHD2, MCTP2*, and *NR2F2* from the first critical interval described by Klaassens *et al*[10] in a minimum of 100 CDH patients per gene (Table 4) and detected several sequence alterations that result in missense amino-acid substitutions that were not present in more than 100 ethnically matched control chromosomes. We only targeted one CDH-critical region for sequencing. An alternative strategy could have been to sequence genes whose function is likely to be perturbed by haploinsufficiency, such as transcription factors, from more than one CDH-critical region, or to sequence candidate genes from animal models of CDH.[18]

In the 15q26 interval, for the *CHD2* gene, we found c.C5128T, predicting p.R1710W, in an Mexican American patient with CDH from whom parental DNA samples were unavailable. CHD2 is a DNA-binding protein belonging to the SNF2/RAD54 helicase family and contains two chromodomains. Although *CHD2* is not associated with a human phenotype, a related gene, *CHD7*, has been shown to cause a multiple congenital anomaly syndrome, CHARGE syndrome.[19]

In *RGMA*, two alterations were present, c.G196A, predicting p.A66T, and c.C362G, predicting p.T121S (Table 4) in two Mexican American children from whom parental samples were unavailable. *RGMA* is one of three vertebrate homologues of a gene family involved in neural cell guidance[20] but a homozygous null mouse for *Rgma* did not show any evidence of diaphragmatic defects (Dr Arber, personal communication).

In *SIAT8B*, we noted one alteration, c.A674G predicting p.K225R, but this gene has since been excluded from the CDH-critical region by further mapping studies.[11] One exonic alteration of uncertain significance was noted in *NR2F2*, c.G733T, predicting p.L245F, which was found in patient 1 with a known 15q26 deletion encompassing this gene.

We are currently sequencing transcripts of unknown function in this interval as our screening has not yet been exhaustive. However, it is possible that the diaphragmatic defects result from a contiguous gene deletion syndrome in which deletions or abnormalities in two or more genes are required for the phenotype. An alternative explanation is that there is substantial genetic heterogeneity for diaphragmatic defects and that the contribution of gene mutations at 15q26.2 to the etiologies of CDH, in particular isolated CDH, is low.

Deletions of chromosome 8p23.1 have been associated with a phenotype that includes congenital heart defects, CDH, developmental and growth retardation, genitourinary anomalies, and facial dysmorphism.[12,16,21] Deletions of this chromosome region are most strongly associated with congenital heart defects and CDH has been reported in only 4/18 cases with 8p23.1 monosomy.[21] The CDH-critical region at 8p23.1 has been mapped in only one other infant besides our patient 4.[16] In our patient, the breakpoints are almost identical to those in this previously reported male with left-sided CDH, growth retardation, and bilateral cryptorchidism who had a paternally derived, *de novo* interstitial deletion at 8p23.1.[16] The 8p23.1 chromosome region is well known to harbor low-copy repeats, and it is highly likely that the chromosome deletions in the CDH patients have resulted from non-homologous recombination.

In patient 5 with WHS, we characterized the 4p16.3 deletion as being 8 Mb in size using array CGH and FISH.

Table 5 Conservation of altered amino-acid residues in *CHD2, ARRDC4*, and *RGMA* genes

Species	*CHD2/ p.R1710W*	*ARRDC4/ p.R391W*	*RGMA/ p.T121S*
Mus musculus	C	C	–
Fugu rubripes	C	–	–
Dario rario	C	–	C
Xenopus tropicalis	C	C	C
Gallus gallus	–	C	C
Caenorhabditis elegans	–	–	–
Drosophila melanogaster	C	–	–
Saccharomyces cerevisiae	–	–	–

C = altered amino acid is conserved in this species; – = not known.

CDH has been previously described in at least 12 infants with WHS, although the finding of CDH in WHS is rare. The extrathoracic clinical features of infants with WHS and CDH do not appear different from children with WHS who do not have CDH.[22–24] The smallest characterized 4p16 deletion associated with CDH had a breakpoint between BAC clones RP3-513G18 and RP11-489M13, located 4.0 Mb from the 4p telomere and the maximum size of the smallest deletion was 4.29 Mb as marker D4S3023 was not deleted.[17]

We also performed array CGH to map a 1q32.3–1q42.2 interstitial deletion detected by G-banding in a male with CDH and pulmonary hypoplasia, a double outlet right ventricle and a VSD, bilateral cleft lip and palate, a cystic hygroma, facial dysmorphism, bilateral hydronephrosis, cryptorchidism, and talipes equinovarus (karytoype 46,XY,del(1) (q32.3q42.2)). There is one other recent report of a patient with a similar phenotype studied with array CGH who was found to have a smaller submicroscopic deletion of 1q41–1q42.[13] G-banded chromosome studies have also reported additional overlapping 1q42 deletions in CDH patients. A female with CDH and multiple malformations had a karytoype of 46,XX,del(1)(pter→42.11::q42.3→qter) *de novo,* with normal FISH studies for the 1q telomeres.[25] A newborn male with CDH and pulmonary hypoplasia had a *de novo*, maternal interstitial 1q deletion (karytyope 46, XY,del(1) (pter→q32.31::q42.3→qter)).[26] However, CDH is not a common feature of 1q42 monosomy.[27]

Conclusion

We have performed array CGH for submicroscopic chromosome deletion identification and mapping in 29 patients with isolated CDH and CDH with anomalies. Our work has mapped CDH-critical regions at chromosomes 15q26.2, 8p23.1, 4p16.3, and 1q41–42 that are all situated in chromosome regions that have been previously associated with CDH from G-banded karyotyping studies. We did not detect novel chromosome aberrations in areas of the genome that have not previously been identified as putative loci for CDH. We conclude that the use of array CGH is necessary before providing a recurrence risk in patients with CDH with anomalies, but that in isolated CDH, its utility has not been fully established.

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