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Copy number imbalances detected with a BAC-based array comparative genomic hybridization platform in congenital diaphragmatic hernia fetuses

I.N. Machado[1,2], J.K. Heinrich[2], R. Barini[1] and C.F.A. Peralta[1]

[1]Programa de Medicina Fetal, Departamento de Tocoginecologia, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP, Brasil
[2]Laboratório de Cultivo Celular e Citogenética, Centro de Atenção Integral à Saúde da Mulher, Universidade Estadual de Campinas, Campinas, SP, Brasil

Corresponding author: I.N. Machado
E-mail: imachado@fcm.unicamp.br

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ABSTRACT. Congenital diaphragmatic hernia (CDH) is a phenotypically and genetically heterogeneous disorder, with a complex inheritance pattern. Structural abnormalities of almost all chromosomes have been described in association with CDH. We made a molecular analysis through array comparative genomic hybridization (array CGH) of a group of fetuses with prenatal ultrasound diagnosis of CDH and normal G-banded karyotypes. A whole genome BAC-array CGH, composed of approximately 5000 BAC clones, was carried out on blood samples from fetuses with prenatal ultrasound diagnosis of CDH and a normal karyotype (500-band level). All potential cytogenetic alterations detected on the arrays were reported. The array CGH analysis showed copy number gains and losses in 10 of 12 cases. Eighty-five clones showed genomic imbalances, and 29 clones displayed described copy number variations. We

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identified a recurrent gain in 17q12 in two of 12 cases, which has not been previously described. Our results may contribute to determining the effectiveness and applicability of array CGH for prenatal diagnosis purposes, and also to elucidate the submicroscopic genomic instability of CDH fetuses.

Key words: Congenital diaphragmatic hernia; Genetic pathways; Array comparative genomic hybridization; Prenatal diagnosis

INTRODUCTION

Congenital diaphragmatic hernia (CDH, OMIM 142340) is a phenotypically and genetically heterogeneous disorder. It can occur as an isolated anomaly, associated with multiple defects or as part of a defined syndrome. Although the exact etiology of most cases of CDH remains unknown, there is increasing evidence that genetic factors play an important role in the development of CDH. Different chromosomal abnormalities are associated with CDH (Pober et al., 2005), and in about 10% of the prenatally detected cases, a chromosomal anomaly is identified, most often aneuploidy (Witters et al., 2001). With the advent of novel molecular cytogenetic techniques, an increasing number of structural submicroscopic chromosomal anomalies have been detected.

The aim of this study was to describe the molecular analysis through a whole genome array comparative genomic hybridization (array CGH) of a group of fetuses with prenatal ultrasound diagnosis of CDH.

PATIENTS AND METHODS

Patients and samples

This study was carried out prospectively during a 19-month period (from January 2008 to July 2009), after protocol approval by the Institutional Ethics Committee. The inclusion criteria consisted of fetuses with an ultrasound diagnosis of CDH and normal G-banding karyotype. The fetal and parental karyotype analysis was performed using G-banded metaphase chromosomes at approximately the 500-band level, and all parents gave informed consent.

Fetal samples were collected by cordocentesis at different weeks of pregnancy for karyotyping, according to the guidelines of the Fetal Medicine Program of the Center for Integral Assistance for Women's Health of the State University of Campinas (UNICAMP).

Clinical data were obtained from medical records. Besides the demographic characterization of the sample, the data included the complete findings described in the prenatal ultrasound records, the babies' features observed through clinical examination by neonatologists and geneticists after birth, and the cytogenetic results.

Molecular study

Genomic DNA was extracted and purified from fetal blood by means of the Wizard®

Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA), according to the manufacturer protocol for whole blood. Array comparative genomic hybridization was carried out using Constitutional Chip® 4.0 (PerkinElmer Inc., Turku, Finland), comprised of approximately 5000 BAC (bacterial artificial chromosome) clones.

For each experiment, a sex-mismatched normal reference DNA (Promega Corp.) was used. All experiments included dye reversal and two array hybridizations to obtain an accurate ratio. The labeling and hybridization steps involved reagents supplied by the array manufacturer (PerkinElmer Inc.). The labeled DNA was hybridized to Constitutional Chip® 4.0 at 37°C for 16-18 h. After post-hybridization washes, slides were scanned, and captured images were analyzed by either GenePix® Pro 6.0 (Molecular Devices Corp.) or ScanArray Express® (Microarray Analysis System 4.0.0.4) softwares.

After quantification, the cyanine 5 and cyanine 3 average ratio fluorescence intensities for each BAC clone on each of the duplicate arrays (gpr files) were uploaded into the web-based SpectralWare® v2.3.3 software (PerkinElmer Inc.), normalized with linear regression algorithms (on a $\log_2$ scale) and plotted according to the BAC chromosomal location. The raw data from dye-reversed pairs were combined, and threshold values were ascertained to make inferences according to a clone-by-clone classification procedure to determine the gain, loss and no change status of each clone for each subject, relative to the diploid reference DNA. The threshold values were determined by the software using the 'Iterative 2.5X Sigmas' algorithm. Subsequent normalization of the data with the 'Block Lowess' method was performed for verification of copy number changes. The P values for each probe were also calculated, furnishing additional objective statistical criteria to determine whether deviation of each probe from zero was a significant change (Ng et al., 2006). The quality criteria adopted included standard deviation of the intensity ratios among the duplicates less than 10% and more than 97.5% of spots with adequate intensity ratio values for analysis (Vermeesch et al., 2005). For each analysis, all quality control metrics were noted to be optimal. Clone-by-clone changes were reviewed and only those aberrations detected in both hybridizations were studied further.

All potential cytogenetic alterations detected on the arrays were matched against the known online databases to determine whether they encompassed described copy number variation (CNV) regions.

RESULTS

Twelve unrelated fetuses were included in this study, 11 as an isolated malformation and one associated with omphalocele, cardiac anomaly and intra-uterine growth restriction. For all families, both women and their husbands were healthy and not consanguineous and showed normal chromosomes on G-banding analysis of peripheral blood. There was no family history of congenital malformations or genetic disorders. The side of the diaphragm defect, maternal age, fetal material source, fetal karyotype result, the number of genomic imbalances, and total number of CNV observed in each case are shown in Table 1.

Array CGH analysis showed copy number gains and losses in 10 of the 12 cases. All abnormal clones are listed in Table 2. A total of 85 clones displayed genomic imbalances, and 29 clones showed described CNV encompassing their loci.

Table 1. Maternal age, side of the diaphragm defect, fetal source for G-banding, karyotype results, total number of abnormal clones, and total number of abnormal clones encompassing described copy number variations (CNVs) found in 12 fetuses with congenital diaphragmatic hernia (CDH).

Case	Maternal age (years)	CDH side	Fetal material	Karyotype	Number of clones with changes	Number of described CNVs
1	32	PLR	Blood	46,XX	5	2
2	28	PLL	Blood	46,XY	0	0
3	36	PLL	Blood	46,XY	24	9
4	26	PLL	Blood	46,XX	2	0
5	30	PLL	Blood	46,XY	3	0
6	21	PLL	Blood	46,XX	1	1
7	20	PLL	AF	46,XY	6	2
8	26	PLR	Blood	46,XX	32	12
9	30	PLL	Blood	46,XY	0	0
10	36	PLL	Blood	46,XX	5	2
11	35	PLL	AF	46,XX	2	0
12*	19	PLL	Blood	46,XX	5	1

PLR = posterolateral right; PLL = posterolateral left; *Non-isolated CDH; AF = amniotic fluid.

Table 2. List of clones with genomic imbalances using a BAC-based array CGH.

Case	Gain			Loss
1	RP11-555E9*			RP11-239E10 RP1-103M22 RP11-257P3* RP11-22F2
3	RP11-1197E19 RP4-628J24 RP11-547D24 RP1-163M9* RP11-73D11 RP1-133H11	RP4-580L5 RP11-213G6 RP11-216L13 GS-908-H22* CTD-2009H2 RP11-303I17	RP11-616M22* RP11-252A24* RP5-59D14* RP11-62C7 RP3-437O22 RP3-402G11*	RP11-352A18 RP11-10O3 RP1-273P12* RP1-128O3* RP11-537K8 RP11-300G13*
4	RP11-79M19 RP11-744L17*			
5	RP11-625N16 RP11-52G4 RP11-79O9			
6	RP11-13C13*			
7	RP11-34I24 RP11-90A15 RP11-69I22 RP11-311A12*			RP11-344A5* RP11-765C10
8	RP11-121C9* RP11-477N3 RP11-140B20* RP11-34N13 RP11-91M18 RP11-352E6* RP11-29F10* RP11-5K24 RP11-636B14	RP11-22L21 RP11-164G6 RP11-957J11 RP11-170D7 RP11-184M21* CMB9-92L10 RP11-555G19 RP11-81G12* RP11-511H9	RP11-114P16 RP11-79B13 RP11-61A21* GS-227-L7* RP6-90M1 RP11-186N21* RP3-454M7 RP11-533L19*	RP11-91M6* RP11-63P12* RP11-56H7 RP11-81L17 RP5-961O8 RP3-424J12
10	RP11-90G17			RP11-1N7* RP11-21A22* RP11-720L3 RP11-354F21
11	RP11-63K6			RP11-80H6
12	RP3-395M20 CITB-51J22 RP11-18M11*	RP11-304L19 RP11-744L17*		

*Clones with described copy number variation regions; cases #2 and #9 showed no abnormal clones.

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We identified a recurrent gain at 17q12 (clone RP11-744L17 in cases #4 and #12) in 2 of 12 cases analyzed. Based on the physical mapping positions as obtained from the March 2006 Assembly of the UCSC Genome Browser, the size of the region was determined to be 66.3 kb (31,592,200-31,658,488) (Figure 1), and no CNV were found in the tested databases.

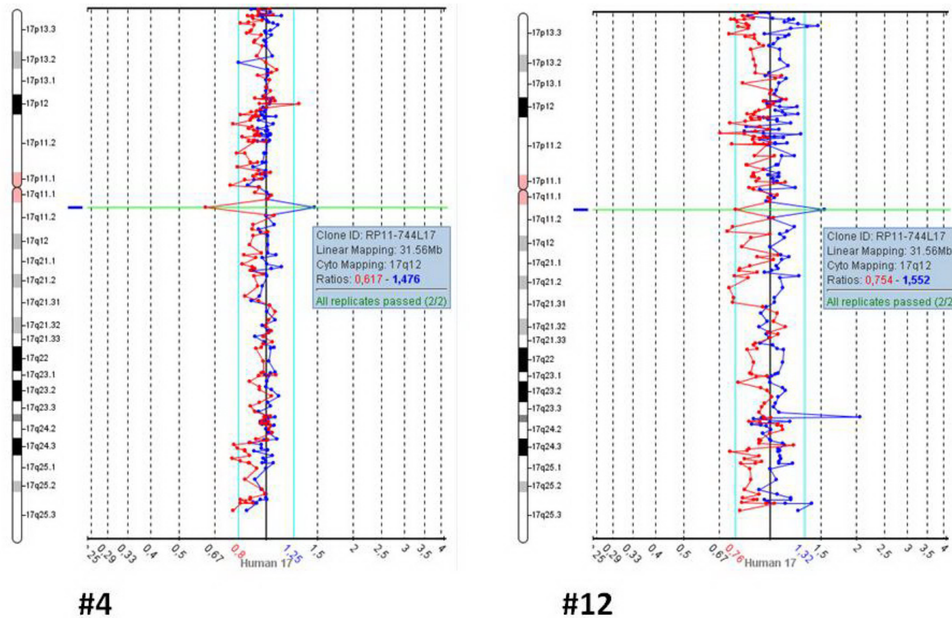

Figure 1. Spectral view of chromosome 17 showing the recurrent gain at 17q12.

We also observed a copy number gain at the regions 8p23.3 (clone RP11-555E9 in case #1 and clone RP4-580L5 in case #3) and 16p13.3 (clone RP11-616M22 in case #3 and clone RP11-304L19 in case #12) in 2 of the 13 fetuses involving clones in close chromosomal regions.

DISCUSSION

Molecular studies including array CGH to define CDH critical chromosomal regions have been relatively few and recent. Here, we report the BAC-array CGH findings of a group of fetuses with normal karyotype and an antenatal ultrasound diagnosis of congenital diaphragmatic hernia.

There are known candidate genes associated with human CDH, which include genes for transcription factors, molecules involved in cell migration, and extracellular matrix components (Kantarci and Donahoe, 2007). They are shown on almost every chromosome (Holder et al., 2007). In the present study, the unique chromosomes that did not show any copy number gains or losses were chromosomes 19 and 20, reinforcing the finding in a recent review (Holder et al., 2007), where the sole chromosome without any description of structural abnormality was chromosome 19.

The main critical chromosomal regions involved with CDH cases are described at

15q26.2, 1q41-q42, 8p23.1, and 4p16.3 (Slavotinek et al., 2006). In the present study, there were three fetuses with some imbalances in these critical regions: one fetus with a deletion at 1q41-q42.13 (clone RP11-239E10), one fetus with a gain at 1q41 (clone RP11-121C9), and one fetus with a gain at 4p16.3 (clone RP11-1197E19).

The most cited CDH critical region is 15q26, encompassing many candidate genes, in a well-defined region of approximately 4 to 5 Mb (Biggio Jr. et al., 2004; Klaassens et al., 2005; Slavotinek et al., 2006). Other possible chromosome regions are 15q24 and 15q22, encompassing genes that contribute to an altered retinoic acid signaling pathway involved in lung and diaphragm development. A total of 24 cases of diaphragm abnormalities associated with deletions of chromosome 15q24-qter have now been reported and have been estimated to account for up to 1% of patients with CDH (Klaassens et al., 2005). We failed to identify genomic imbalances either at 15q26 or at 15q24 in any of the 12 fetuses analyzed. However, nearby, we identified in one case a deletion at 15q25.2 (clone RP11-81L17) and in another case a deletion in 15q22 (clone RP11-537K8).

We also observed gains at 8p23.3 and 8p24.2 regions, involving different but close clones. Interstitial and terminal deletions at 8p23.1 had been reported as a candidate genomic change for cardiac and CDH genetic etiology (Holder et al., 2007; Wat et al., 2009). At least ten previously reported cases of CDH with deletions encompassing this region had already been evaluated at the molecular level, nine of them having left-sided CDH and one right-sided (Wat et al., 2009). Among our cases, one of 2 cases with 8p23.3 deletion and one with deletion at 8p24.2 region had a right-sided CDH. Caution must be used in the interpretation of these findings because chromosome 8p contains many copy number variant regions whose potential contribution to the development of birth defects has not been adequately studied, making it difficult to determine if this deletion is indeed causal.

We could also find a recurrent gain at 17q12 (clone RP11-744L17) in 2 of 12 cases analyzed. The BAC clone in question is located over a region of segmental duplication (a low copy repeat region) and a Medline search could not find any citation about the relationship between CDH and this chromosomal region. Additional research is needed to confirm this abnormality and to further establish the role of genes from this chromosome region in lung and diaphragm development and to determine the prevalence of copy number gain in the 17q12 region among CDH patients.

There is evidence that CDH may have multiple molecular defects contributing to this same phenotype, which explains why the defects could vary in severity (Kantarci and Donahoe, 2007). In our study, there does not appear to be a direct relationship between the number of genomic imbalances and the presence of other structural abnormalities. The sole fetus with multiple congenital abnormalities showed 5 abnormal clones, while another fetus with isolated CDH showed even 32 detectable abnormalities.

Microarray-based CGH is a powerful method to detect and analyze genomic imbalances that are well below the level of detection on banded karyotype analysis, but it has some limitations. The high cost is an important limitation for its immediate application. In Brazil, this technique is only available in private and research laboratories. Besides, other confirmation studies are needed. On an oligonucleotide array CGH platform, more than 50% of the identified genomic changes in non-isolated CDH cases failed to be confirmed by quantitative polymerase chain reaction (PCR) (Scott et al., 2007). Although we considered the genomic changes as gains or losses only when they were observed in both Cy3 and Cy5 hybridizations

(dye swap), we did not use an independent method such as PCR or fluorescent *in situ* hybridization for confirmative studies, as our objective was more to report all the molecular findings than to point them out as true abnormalities.

Congenital diaphragmatic hernia is assumed to be a genetically heterogeneous disorder, and candidate genes can also be determined using linkage analysis of familial cases. For this type of disorder, the best way to determine which genes are involved is by analyzing a large number of patients for common aberrations by the use of high resolution genetic methodologies, such as array CGH. Novel molecular analysis of CDH-critical chromosomal regions may ultimately define smaller regions and identify genes responsible for CDH. Subsequently, sequence analysis of genes mapped to these critical regions may reveal gene mutations and contribute to the identification of causative genes for CDH in humans.

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