Array-Comparative Genomic Hybridization Analysis in Fetuses with Major Congenital Malformations Reveals that 24% of Cases Have Pathogenic Deletions/Duplications

ORIGINAL ARTICLE

Array-CGH analysis in fetuses with major congenital malformations reveals that 24% of cases have pathogenic deletions/duplications.

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ABSTRACT

Karyotype and array Comparative Genomic Hybridization (a-CGH) are routinely used to identify genetic determinants of major congenital malformations (MCMs) in fetal deaths or terminations of pregnancy after prenatal diagnosis. Pathogenic rearrangements are found with a variable rate of 9 to 39% for a-CGH. We collected 33 fetuses, nine with a single MCM and 24 with MCMs involving two to four organ systems. Array-CGH revealed Copy Number Variants (CNVs) in 14 out of 33 (42 %) cases. Eight were classified as pathogenic: this equals a detection rate of 24% (8/33) considering fetuses with one or more MCMs, and 33% (8/24) taking into account fetuses with multiple malformations only. Three of the pathogenic variants were known microdeletion syndromes [22q11.21 deletion, central chromosome 22q11.21 deletion and Thrombocytopenia Absent Radius (TAR) syndrome] and five were large rearrangements, amounting up to >11 Mb per subject, and spanning strong phenotype-related genes. One of those was a *de novo* complex rearrangement. The remaining four duplications and two deletions were 130-900 kb in size, containing 1 to 7 genes, and were classified as variants of unknown clinical significance (VOUS). Our study confirms a-CGH as a powerful technique to ascertain the genetic etiology of fetal major congenital malformations.

INTRODUCTION

There is no consensus for defining major and minor Congenital Malformations (CMs). Commonly accepted definitions describe major anomalies (MCM) as “anatomic abnormalities that are severe enough to reduce life expectancy or compromise normal function”. Minor anomalies are “unusual morphologic features that are of no serious medical or cosmetic consequence to the patient”. They may be indicative of a more generalized altered morphogenesis or may represent a valuable indication for identifying a specific malformative syndromes (Kennet 2005; Kumar and Burton 2007).

Data from the EUROCAT network established a prevalence of MCMs in Europe of 2.6% between 2008 and 2012, including live births (2.1%), and fetal deaths/terminations of pregnancy after prenatal diagnosis (0.5%). Around 18% of MCMs were ascribed to known genetic conditions (http://www.eurocat-network.eu).

The genetic etiology of MCMs is widely variable: monogenic syndromes are estimated to account for 2-10% of cases, whereas chromosomal abnormalities are found in 10-15% of liveborn with MCMs (Stevenson 2006) and in 9-39% of fetuses with abnormal ultrasound findings, depending on the presence of a single anomaly or multiple malformations (Rizzo et al. 1996; Saldarriaga et al. 2015; Tseng et al. 2006; Wilson et al. 1992; Yashwanth et al. 2010). Subtelomeric deletions are present in around 5% of patients with multiple CMs associated with mental retardation (Koolen et al. 2004). Retrospective studies using array-Comparative Genomic Hybridization (a-CGH) in fetuses with multiple malformations report a detection rate of pathogenic chromosomal imbalances from 8 to 18% and a 10%-increase in the diagnostic yield of chromosomal microarray over karyotyping (95% confidence interval)(de Wit et al. 2014; Hillman et al. 2013; Le Caignec et al. 2005; Schaeffer et al. 2004; Tyreman et al. 2009; Vialard et al. 2009). It is estimated that ~50% of CMs have an unknown etiology (Rajangam and Nanjappa 2007).

In this work, we evaluated the detection rate of a-CGH analysis in identifying pathogenic chromosomal deletions/duplication in a group of fetuses with single or multiple CMs.

Materials and Methods

Cases and anatomopathological evaluation

Between January 2010 and September 2013 at the "Città della Salute e della Scienza di Torino" University Hospital, approximately 960 fetuses underwent autopsy following spontaneous abortions, intrauterine/neonatal deaths, and Termination Of Pregnancy for Fetal Anomalies (TOPFA). Fetal autopsy included evaluation of external appearance, measurement of morphometric parameters, examination of thorax and abdominal anatomy, and histological examination of main organs and pathological tissues. Skeletal X-ray analysis was performed on each fetus. Fetuses with suspicious infective causes of anomalies were excluded by placental examination. Excluding cases suggestive of a monogenic disease or a non-genetic origin, we selected 49 fetuses with MCMs. Sixteen a-CGH analyses failed due to the inadequate quality of genomic DNA. The remaining 33 analyses were performed on 9 fetuses with one major malformation and 24 with two or more major malformations. The study was approved by the Internal Review Board, and informed consent was obtained from parents of the analyzed fetuses.

Karyotyping and array-CGH analysis

Karyotyping on GTG-banded chromosomes (≥ 400 bands) was performed on chorionic villus sampling, amniotic fluid, or fibroblasts. Genomic DNA was extracted from fetal frozen tissues using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Array-CGH was performed using a 60K whole-genome oligonucleotide microarray following the manufacturer's protocol (Agilent Technologies, Santa Clara, California, USA). Slides were scanned using a G2565BA scanner, and analyzed using Agilent CGH Analytics software ver.

4.0.81 (Agilent Technologies Inc.) with the statistical algorithm ADM-2 and a sensitivity threshold of 6.0. Significant copy-number changes were identified by at least three consecutive aberrant probes. Reference human genomic DNA was GRCh37/hg19. We followed pathogenicity criteria for CNVs as reported in the reference (Kearney et al. 2011).

Sequencing analysis

In patient DGT300197, we screened two SNPs in the 5-prime UTR (rs139428292: G>A) and in the first intron (rs201779890: G>C) of the RNA binding motif protein 8A (*RBM8A*) gene by Sanger sequencing. The following PCR conditions were used: 10 µMol primers (5'- gcctttgattggtcagcttg; 5'- aaggggcggaatctctaat), 200 µMol dNTPs, 60 ng of genomic DNA, and 0.5 unit of KAPA-fast 2G kit (Kapa Biosystems, Inc., MA, USA) in a 25 µl final volume under standard amplification conditions (56°C annealing temperature). PCR products were purified using Agencourt AMPure XP-PCR Purification (Beckman Coulter, Miami, FL, USA) and sequenced with the Big Dye v3.1 kit (Applied Biosystems, Foster City, CA, USA). Products were purified using Agencourt CleanSEQ-Dye Terminator Removal (Beckman Coulter) and run on an ABI-3730 platform, using the POP7 polymer (Applied Biosystems).

Results

Standard karyotype analysis was negative on fetal tissue samples, with the exception of nine in which the analysis was not possible due to culture failure. Using a-CGH, we identified one or more Copy Number Variants (CNVs) in 14 cases (14/33, 42%). Eight were classified as pathogenic: this equals to a detection rate of 24% (8/33) considering fetuses with one or more CMs, and 33% (8/24) taking in account fetuses with multiple malformations only. Among the eight pathogenic variants, three were associated with known microdeletion syndromes: a chromosome 22q11.21 deletion syndrome (MIM 188400), a central chromosome 22q11.21 deletion syndrome (Rump et al. 2014),

and a Thrombocytopenia Absent-Radius (TAR) syndrome (MIM 274000; Table 1, cases 1-3). The majority of TAR patients are compound heterozygous for a deletion on 1q21.1 encompassing the *RBM8A* causative gene, and a low-functioning SNP in either its 5'UTR or intron 1 (Albers et al. 2012). By sequencing analysis in our TAR case, we identified one of those variants, the hypomorphic "C" allele at SNP rs#201779890 on intron 1 of the non-deleted allele.

In the other five pathogenic CNVs, the extension of the total rearrangements per subject (11-41 Mb), and the number and the type of genes involved allowed the clear classification of the CNV as pathogenic (Table 1, subjects 4-8).

Cases 4 and 5 are already detailed in (Di Gregorio et al. 2014) as examples of cryptic large CNVs (>6 Mb) undetectable by conventional karyotyping.

Briefly, fetus 4 showed a complex phenotype, displaying partially overlapping features with Cornelia de Lange syndrome (MIM 122470). We found two large telomeric rearrangements: a 3q29 duplication (13.4 Mb) associated with the Cornelia de Lange phenotype (Dundar et al. 2011; Holder et al. 1994), and a 15q26.1q26.3 deletion (11.5 Mb) encompassing the minimal region of the 15q26-qter deletion syndrome (MIM 612626).

Case 5 carried two duplications on chromosome 7p22.3p22.2 and 7p22.1p21.2, and a deletion of chromosome 11q24.1q25. The duplication of the short arm of chromosome 7 causes a characteristic pattern of malformations including developmental, craniofacial, skeletal and cardiovascular anomalies, the severity of which depends on the size of the duplication (Papadopoulou et al. 2006). The neuronal migration defect could be explained by the deletion of Cell Adhesion Molecule-Related/Downregulated By Oncogenes (*CDON*, MIM 608707) and Kin of IRRE-like 3 (*KIRREL3*, MIM 607761) on chromosome 11, which are two genes involved in the control of neuronal migration and axon guidance (Bhalla et al. 2008; Okada et al. 2006). The cardiac phenotype (hypoplastic left heart) is likely to be part of the Jacobsen syndrome spectrum (MIM 147791), due to the 11q23 deletion (Grossfeld et al. 2009).

In case 6, we found six *de novo* duplications ranging from 37 kb to 4.8 Mb, with a total of 15.9 Mb, involving six different chromosomes. Among these, the 2q31.1 duplication spanned the *HOXD* gene cluster, a family of highly conserved transcription factors involved in the antero-posterior development of the limb and in the specification of fingers (Johnson et al. 2003; Johnson et al. 1998; Kessel and Gruss 1990), and could explain the polydactyly and digital shape anomalies in the fetus. Over-expression of *HOXD10* in mice cause polydactyly, whereas the duplication of the *HOXD* gene cluster modifies finger-shape and number in mice (Sheth et al. 2007; Tarchini et al. 2006; Zakany et al. 2004). The other five identified rearrangements could not be clearly related to the remaining clinical features. Moreover, a-CGH analysis in parents showed a 1.8 Mb duplication on chromosome 2q12.3q13 in his father.

Case 7 carried a large 13.5 Mb deletion of chromosome 15q25.3q26.3, partially overlapping with the 15q26-qter deletion syndrome region (MIM 612626). This fetus and patients carrying 15q26-qter deletion show micrognathia, which is part of the Robin sequence (Roback et al. 1991; Tonnies et al. 2001). Furthermore, the same region is associated with diaphragmatic hernia, also present in our case (MIM 142340)(Castiglia et al. 2005; Klaassens et al. 2007; Klaassens et al. 2005; Mosca et al. 2011). *IGF1R* and *ARRDC4* have been identified as the strongest candidates for this phenotype, because both are expressed in the developing diaphragm. Nasal bone agenesis could be explained by genes in the centromeric segment non-overlapping15q26-qter deletion.

Case 8 showed a 11.4 Mb deletion on chromosome 3p14.2p13. One of his key features was the presence of a congenital heart defect (CHD, Table 1): assessing the pathogenicity of a CNV associated with CHD is not always straightforward, as many deletions/duplications encompass genes that are not clearly related to cardiac development (Richards and Garg 2010). A notable gene present in the deleted region was Forkhead Box P1 (*FOXP1*, MIM 605515), which is critical for heart development and was found to be deleted in a patient with an atrioventricular septal defect (Chang et al. 2013). Moreover, a rare contiguous gene syndrome has been described in patients

carrying a chromosome 3p14 deletion: key features of this syndrome include short stature, dysmorphisms, CHD, developmental delay, urogenital and neurological problems (Dimitrov et al. 2015). Interestingly, scrotal hypoplasia in the fetus might be explained by the deletion of the Prokineticin 2 gene (*PROK2,* MIM 607002), whose point mutations cause Kallmann syndrome type 4 (Dode et al. 2006), a disease featuring hypogonadotropic hypogonadism with testicular/scrotal atrophy (MIM 610628). A second candidate was the testis-expressed G-protein coupled receptor 27 (*GPR27*, MIM 605187), whose function is still poorly defined. Clenched hands in the fetus could be explained by the deletion of Forkhead Box Protein P1 (*FOXP1*) as hypothesized in a patient with limb contractures, speech delay, hypertonia and blepharophimosis carrying a 785-kb *de novo* 3p14 deletion: *FOXP1* haploinsufficiency may be involved in defects of motor neuron development resulting in limb contractures (Pariani et al. 2009).

Among the analyzed fetuses 6/33 (18%) carried CNVs ranging from 130-900 kb (four duplications and one deletion), classified as variants of unknown clinical significance (VOUS), which contained 1-7 genes. Segregation of the identified variants could not be assessed. In these regions, no obvious candidate gene could be associated with fetuses clinical features.

Discussion

In the recent years, about twenty studies have examined the potential impact/pathogenicity of CNVs in fetal malformations in prenatal diagnostic setting or after terminations of pregnancy (de Wit et al. 2014; Papoulidis et al. 2015; Srebniak et al. 2015). The proportion of clinically relevant findings is highly variable (from 4.3 to 18%) (de Wit et al. 2014; Papoulidis et al. 2015; Srebniak et al. 2015) reaching 24% with our study. Discrepancies among studies may be due to different technical platforms, different selection criteria, the size of the sample or the inclusion of VOUS among the identified CNVs. Based on the reported data, selection criteria are particular relevant: among our 24

cases with multisystem anomalies (2 to 4 organ systems), we found 13 cases with a positive a-CGH result (54%), and eight of these had true pathogenic variants (33%).

Notably, four of the eight cases carried at least one rearrangements above 10 Mb, which was undetected by conventional chromosomal analysis. Their loss may be due to the low karyotype resolution on chorionic villi or fetal fibroblasts, or to cryptic rearrangements as described by (Di Gregorio et al. 2014). Indeed, in a recent meta-analysis such large anomalies were excluded from the reported results, lowering the percentage of a-CGH detection (de Wit et al. 2014). Using these criteria, we would estimate a 15% of pathogenic copy number variations detected by a-CGH, in line with published data.

We classified as VOUS all the rearrangements without obvious biological effect, absent in the DGV database, and/or those for which it was not possible to perform segregation analysis.

Our results showed a higher percentage of VOUS in comparison to reported studies, where the detection rate ranged from 0.5 to 1.7% (Brady et al. 2014; Papoulidis et al. 2015). This discrepancy may be due to: 1) VOUS definition, considering that some group does not report rearrangements containing no genes (Brady et al. 2014); 2) array resolution (e.g., BAC array vs. oligonucleotide-array); 3) lack of knowledge about *de novo* vs. inherited VOUS, which are often considered benign if inherited from an healthy parent, especially if gene-devoid; 4) the presence of pathogenic copy number variants among VOUS: two of the detected VOUS were >500 kb, above an indicative limit of pathogenicity of 400 kb (Miller et al. 2010) and one contained 7 genes.

Genetic counseling to couples after termination of pregnancy due to a fetal malformation is particularly difficult. Phenotypic diagnosis is often impossible, and karyotype analysis has a limited detection rate.

Array-CGH data in our report demonstrate that the presence of pathogenic CNVs can allow a more precise evaluation of the recurrence risk. This was particularly important for cases in which the

derivative of a cryptic rearrangement present in one of the parents was identified (cases 4 and 5), or for the known 22q11.21 and TAR syndromes (cases 1, 2 and 3), which are associated with a significant recurrence risk, giving the possibility of an early prenatal test in subsequent pregnancies. Array-CGH using genomic DNA allows overcome tissue culture difficulties and the low resolution of prenatal/fetal karyotypes. Furthermore, balanced rearrangements undetectable at a-CGH (e.g., a translocations/insertions) are very unlikely causes of fetal malformations, suggesting this method to be suitable as a first tier analysis (Astbury et al. 2004). ~~However, given the high cost of the technique PCR-based Rapid Aneuploidy Detection should be recommended before a-CGH.~~

In conclusion, we confirm a-CGH as a powerful technique to identify pathogenic chromosomal rearrangements in fetuses with MCMs, with a higher detection rate proportional to MCMs severity.

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Table 1. Array-CGH analysis in 14 fetuses with major congenital malformations.

Patient Decipher code	Prenatal Echography / Autopsy	Karyotype	a-CGH result	Size	MIM genes / phenotypes	Clinical significance
1 DGT308537	No echography / Pericerebellar and intraventricular hemorrhage, Situs ambiguous, Complex cardiopathy	46,XX	arr 22q11.21(18,919,942-21,440,514)x1	2.5 Mb	40/7	Known syndrome
2 DGT308539	Oligohydramnios, Right renal agenesis, Multicystic left kidney dysplasia / Potter facies, Right renal agenesis, Multicystic left kidney dysplasia	46,XY	arr 22q11.21(20,754,422-21,440,514)x1	0.8 Mb	11/3	Known syndrome
3 DGT300197	Bilateral upper limb bone malformations with agenesis of radius and ulna, Hands hyperflexion / Brain micro-hemorrhagic foci, Dysmorphic features, Bilateral upper limb bone malformations (radius agenesis, dysplastic and shorten ulna), Scoliosis, Supernumerary ribs	46,XY	arr 1q21.1(145,413,388-145,747,269)x1	0.3 Mb	8/2	Known syndrome
4 DGT283329	Intrauterine growth retardation, Abnormality of the ventricular septum, Cardiomegaly, Hyperechogenic bowel / Possible lissencephaly, Lower lip protrusion, Micrognathia, Abnormality of the ventricular septum, Ventriculomegaly, Arm hypoplasia	46,XX	arr 3q27.1q29(184,428,168 - 197,840,339)x3	13.4 Mb	75/18	Pathogenic
			arr 15q26.1q26.3(90,857,664 - 102,383,473)x1	11.5 Mb	27/10	
5 DGT290945	No echography / Agenesis of corpus callosum, Abnormality of neuronal migration, Abnormality of vertebrae, Flat nose, Low set ears, Hypertelorism, Aortic arch hypoplasia, Hypoplastic left heart, Hypoplastic long bones	46,XX	arr 7p22.3p22.2 (92,532-4,176,031)x3	4 Mb	26/5	Pathogenic
			arr 7p22.1p21.2 (7,044,310-15,709,683)x3	8.6 Mb	16/2	
			arr 11q24.1q25(122,467,330-134,868,407)x1	12.4 Mb	57/18	
6 DGT308533	Ambiguous genitalia, Penoscrotal hypospadias / Hyploplasia of the mandible, Hand polydactyly, Kidney dysplasia, Ambiguous genitalia with male gonads, Periportal fibrosis, Pancreatic hyperplasia, Anal atresia	46,XY	arr 2q31.1(176,958,852-176,996,671)x3	37 Kb	-	Pathogenic
			arr 8q24.3(142,205,751-145,747,468)x3	3.5 Mb	58/13	
			arr 10q26.3(133,749,950-135,276,555)x3	1.5 Mb	16/2	
			arr 11p15.5p15.4(218,365-3,243,604)x3	3 Mb	68/19	
			arr 16p13.3(106,271-4,902,543)x3	4.8 Mb	131/24	
			arr 20q13.3(60,112,364-62,893,189)x3	2.7 Mb	51/7	
7 DGT308538	No echography / Pierre-Robin sequence, Agenesis of the nasal bone, Left diaphragmatic hernia	46,XX	arr 15q25.3q26.3(87,344,281-100,872,951) x1	13.5 Mb	46/12	Pathogenic

8 DGT308536	Truncus arteriosus, Abnormality of the ventricular septum / Truncus arteriosus, Abnormality of the ventricular septum, Hand clenching, Scrotal hypoplasia	46,XY	arr 3p14.2p13(61,067,122-72,423,962)x1	11.4 Mb	23/6	Pathogenic
9 DGT301136	Intrauterine growth retardation / Dysmorphic features, Abnormality of vertebrae, Pulmonary hypoplasia, Bilateral renal agenesis, Aplasia of the uterus, Talipes equinovarus	No (culture failure)	arr 1p31.1(77,444,894-77,578,846)x3	0.13 Mb	2/0	VOUS
10 DGT301192	No echography / Potter facies, Patent ductus arteriosus, Vertebral fusion, Anal atresia	46,XY	arr 2p22.2(38,523,015-38,570,650)x3	0.47 Mb	1/0	VOUS
11 DGT301190	No echography / Potter facies, Lower limbs hypoplasia, Tibial torsion, Fibular aplasia, Talipes equinovarus and oligodactyly	46,XY	arr 2p23,1(30,814,684-30,832,151)x1	0.18 Mb	1/0	VOUS
12 DGT301184	Anhydramnios, Bilateral renal agenesis / Bilateral renal agenesis, Patent ductus arteriosus, Abnormality of the ventricular septum, Lower limbs hyperextension	46,XX	arr 3q25.1(150,651,708-151,542,568)x3	0.9 Mb	7/2	VOUS
13 DGT308540	No Echography / Dysmorphic features, Complex cardiac malformation, Polysplenia, Unilateral renal agenesis, Aplasia of the bladder, Ambiguous genitalia, Arms malformations, Anal atresia, Limb malformations, Oligodactyly, Syndactyly	46,XY	arr 5p11q11.1 (49,690,172-50,123,913)x3	0.5 Mb	2/0	VOUS
14 DGT312082	No Echography / Cerebellar vermis hypoplasia, Rhombencephalosynapsis, Subcutaneous edema, Aortic arch and aortic ductus defect	46,XY	arr 22q11.23(25.664.618-25.799.582)x1	0.14 Mb	2/0	VOUS