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Introducing array comparative genomic hybridization into routine prenatal diagnosis practice: a prospective study on over 1000 consecutive clinical cases

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Objective To assess the feasibility of offering array-based comparative genomic hybridization testing for prenatal diagnosis as a first-line test, a prospective study was performed, comparing the results achieved from array comparative genomic hybridization (aCGH) with those obtained from conventional karyotype.

Method Women undergoing amniocentesis or chorionic villus sampling were offered aCGH analysis. A total of 1037 prenatal samples were processed in parallel using both aCGH and G-banding for standard karyotyping. Specimen types included amniotic fluid (89.0%), chorionic villus sampling (9.5%) and cultured amniocytes (1.5%).

Results Chromosomal abnormalities were identified in 34 (3.3%) samples; in 9 out of 34 cases (26.5%) aCGH detected pathogenic copy number variations that would not have been found if only a standard karyotype had been performed. aCGH was also able to detect chromosomal mosaicism at as low as a 10% level. There was complete concordance between the conventional karyotyping and aCGH results, except for 2 cases that were only correctly diagnosed by aCGH.

Conclusions This study demonstrates that aCGH represents an improved diagnostic tool for prenatal detection of chromosomal abnormalities. Although larger studies are needed, our results provide further evidence on the feasibility of introducing aCGH as a first-line diagnostic test in routine prenatal diagnosis practice. Copyright © 2011 John Wiley & Sons, Ltd.

Supporting information may be found in the online version of this article.

KEY WORDS: array comparative genomic hybridization; chromosomal abnormality; copy number variant; low level chromosomal mosaicism; prenatal diagnosis

INTRODUCTION

In recent years, array comparative genomic hybridization (aCGH) has been introduced into routine practice for clinical diagnosis of chromosome imbalances (Cheung *et al.*, 2005; Roa *et al.*, 2005; Lu *et al.*, 2007; Shaffer *et al.*, 2007a). Array CGH has the potential to deliver a higher resolution test compared with G-banded chromosome analysis, allowing detection and detailed characterization of submicroscopic copy number variants (CNVs). This category of rearrangements represents an increasingly recognized cause of genetic disorders and has been associated with up to 15% of syndromic and nonsyndromic mental retardation cases (Visser *et al.*, 2003; de Vries *et al.*, 2005). Array CGH also has the added advantage of high throughput analysis, minimal required amount of DNA, rapid turnaround time and avoidance of culturing fetal cells. It can objectively and simultaneously interrogate multiple clinically relevant genomic gains and losses that are associated with genetic disorders (Bejjani *et al.*, 2005; Emanuel and Saitta, 2007; Shaffer *et al.*, 2007a, 2007b). The above characteristics make aCGH an attractive alternative to current techniques for prenatal cytogenetic testing.

Array CGH is now widely used for the clinical evaluation of pediatric patients with congenital anomalies, cognitive deficits, developmental delays, growth abnormalities or behaviour problems (Bejjani *et al.*, 2005; Cheung *et al.*, 2005; Rauch *et al.*, 2006; Shaffer *et al.*, 2006; Lu *et al.*, 2007; Stankiewicz and Beaudet, 2007; Hochstenbach *et al.*, 2009). An international consensus statement has recently recommended the use of this assay as a first-line test in place of traditional karyotype analysis (Miller *et al.*, 2010).

While experience with diagnostic aCGH in the pediatric population is extensive, experience with its use for clinical prenatal diagnosis is still relatively limited (Hillman *et al.*, 2011). In the last few years, several retrospective (Le Caignec *et al.*, 2005; Rickman *et al.*, 2006) and prospective (Sahoo *et al.*, 2006; Shaffer *et al.*, 2008; Coppinger *et al.*, 2009; Van den Veyver *et al.*, 2009; Maya *et al.*, 2010) studies have been performed to explore the usefulness of aCGH in prenatal diagnosis. Despite the relatively small size of the cohorts, the above studies have ascertained that aCGH is able to detect clinically significant microscopic and submicroscopic chromosome abnormalities in prenatal samples, without an appreciable increase in results of unclear clinical relevance.

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Nevertheless, larger prospective trials, with samples processed for both aCGH and conventional cytogenetic analysis, are still necessary before aCGH can be recommended as a first-line test in routine clinical prenatal diagnosis for detection of chromosomal abnormalities in fetal samples (Vermeesch *et al*., 2007; ACOG Committee, 2009).

Here, we present the cytogenetic findings of a prospective study, performed on a cohort of 1037 consecutive prenatal samples. Comparisons of results obtained using a bacterial artificial chromosome (BAC)-based aCGH platform are made with those obtained from standard G-banded karyotyping. The main objective is to assess the feasibility of offering aCGH as a first-line test in the clinical prenatal diagnostic setting.

MATERIALS AND METHODS

Patient counselling

Array CGH analysis was offered as an option to couples considering an invasive prenatal genetic testing procedure, in addition to conventional karyotyping. Patients underwent pretest counselling as described elsewhere (Darilek *et al*., 2008), during which the issues that are encountered with aCGH testing were discussed. The couples who accepted evaluation by aCGH signed an informed consent form containing a summary of the testing process, potential benefits and limitations of testing, and possible testing outcomes. The study was approved by the Institutional Review Board of GENOMA laboratory.

Clinical indications

The indications for invasive prenatal testing included increased risk of fetal aneuploidy associated with advanced maternal age (AMA), that is, 35 years or older at time of conception, abnormal results of maternal serum screening tests (MSS), abnormal ultrasound findings (AUS), a known abnormal fetal karyotype (AFK), family history of a genetic condition or chromosome abnormality (FIS), parental anxiety (PA), cell culture failure (CCF) and multiple indications (MI) (Table 1).

Prenatal samples

Samples included in this dataset were received between 1 October 2010 and 30 April 2011 from healthcare providers in Italy. Specimen types submitted included amniotic fluid (AF), chorionic villus sampling (CVS), cultured amniocytes (CA), or DNA extracted from uncultured amniocytes isolated directly from AF. A summary of the prenatal specimens processed, grouped by indication for study, is reported in Table 1.

Blood samples from both parents were requested with the fetal sample to test for possible maternal cell contamination and immediate characterization of potential familial CNVs, where necessary.

Cell culture and DNA extraction

Prenatal samples were processed in parallel using both aCGH and G-banding for standard karyotyping. Typically, 3–5 mg of CVS tissue or 15 mL of AF (at a gestational age of at least 15 weeks), was required.

High molecular weight DNA was extracted from 5 mL of AF and 1 mg of CVS using the QIAamp DNA Blood Mini Kit (Qiagen, Milan, Italy), according to the manufacturer's protocol.

In all cases, cell cultures were set up with the remainder of the fetal samples for conventional G-banded karyotypes, using standard protocols. The workflow of prenatal samples processed in the study is shown in Figure 1.

Table 1—Number and types of prenatal samples processed for study according to primary indication

	Amniotic fluid				
Indication	Direct AF	Cultured amniocytes	DNA from uncultured amniocytes	CVS	Total (%)
Advanced maternal age (≥35 years at conception)	376	3	1	64	444 (42.8)
Abnormal ultrasound findings	30	0	0	18	48 (4.6)
Known abnormal fetal karyotype	4	3	1	0	8 (0.8)
Abnormal results of maternal serum screening tests	11	0	0	2	13 (1.3)
Family history of a genetic condition or chromosome abnormality	6	0	0	5	11 (1.1)
Parental anxiety	476	8	0	0	484 (46.7)
Cell culture failure	1	1	2	0	4 (0.4)
Multiple indications	15	0	0	10	25 (2.4)
– AMA + AUS	9	0	0	8	17 (1.6)
– AMA + MSS	2	0	0	0	2 (0.2)
– AMA + FIS	3	0	0	2	5 (0.5)
– MSS + AUS	1	0	0	0	1 (0.1)
Total (%)	919 (88.6)	15 (1.5)	4 (0.4)	99 (9.5)	1037

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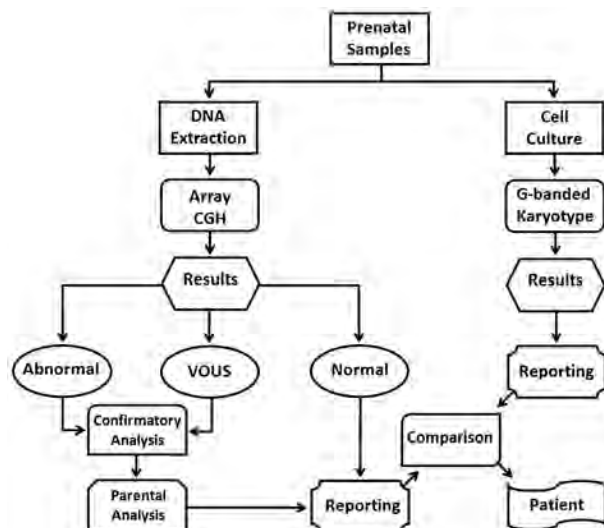

Figure 1—Workflow of prenatal samples in the prospective study. Prenatal samples were processed in parallel using both aCGH and G-banding for standard karyotyping. Cell cultures were set up for conventional G-banded karyotypes. The aCGH process involved DNA extraction from fetal cells, followed by hybridization to BAC microarrays. Detected copy number gains or losses were first assessed for the clinical significance of the variation. If the detected variation result is likely to be clinically significant or of uncertain clinical significance (VOUS), confirmatory studies are also performed. The final step before reporting is parental analysis to assess whether the aCGH findings are inherited or *de novo*. Finally, the aCGH results were then compared with those obtained from G-banded karyotypes

Gender determination and maternal cell contamination testing

Prior to labelling and hybridization, 10 ng of genomic DNA was used to determine the gender of the fetus using a PCR protocol involving amplification of the *Amelogenin* gene, as previously described (Iacobelli *et al*., 2003). All fetal DNA samples used for aCGH were also tested for maternal cell contamination using the PCR-based protocol including the short tandem repeat markers for chromosomes 13, 18 and 21 reported elsewhere (Fiorentino *et al*., 2010). The multiplex PCR reaction was performed as previously described (Fiorentino *et al*., 2003).

Array comparative genomic hybridization

Differently fluorescently labelled test and reference DNAs of the same gender were competitively hybridized to whole-genome BAC microarrays – CytoChip Focus Constitutional (BlueGnome, Cambridge, UK). DNA samples were processed according to the manufacturer's protocol (available at www.cytochip.com). The genomic coverage of these arrays is up to 1 Mb resolution across the genome and ~100 kb resolution in 139 regions associated with constitutional disorders.

A laser scanner InnoScan 710 AL (INNOPSYS, Carbonne, France) was used to excite the hybridized fluorophores, read and store the resulting images of the hybridization. Scanned image quantification, array quality control and aberration detection were performed by algorithm fixed settings in BLUEFUSE MULTI software (BlueGnome, Cambridge, UK).

Confirmatory analysis

Detected copy number gains or losses were compared with known CNVs in publicly available databases (e.g. Database of Genomic Variants – DGV; Decipher; etc.) and in our own database of results to ascertain the clinical significance of the variation. If copy number changes were clinically significant or of uncertain clinical significance, confirmatory studies were also performed (Figure 1). To confirm that the array findings were not artefactual, the identified CNVs were first confirmed by '*dye swap*' (hybridisation of patient DNA against control DNA, with a repeat assay but with labelling in opposite colours). Subsequently, array results were confirmed by fluorescence *in situ* hybridisation on metaphase spreads prepared from amniocytes or CVS cultures, using one or more BAC clones within the abnormal region, or by quantitative fluorescent PCR or short tandem repeat marker genotyping.

Classification of the results

The results were classified according to whether the detected CNV was clinically significant, likely benign, or of uncertain clinical significance (VOUS) (Miller *et al*., 2010).

Clinically significant CNVs are defined as those that are *de novo*, rare, relatively large, and/or contained clinically relevant genes or are related to well-established syndromes.

Benign CNVs are defined as those that are common or observed in the normal population without known phenotypic signs or inherited from a healthy parent.

Copy number variants of uncertain clinical significance are defined as those for which phenotypic consequences may be difficult to predict. These results require parental analysis to aid in the final clinical interpretation.

If no copy-number changes, or if only benign CNVs are identified, the result is considered 'normal'. If a clinically significant CNV is detected, the result is considered 'abnormal'.

Finally, the aCGH results were compared with those obtained from G-banded karyotypes in a blinded fashion.

RESULTS

Prenatal samples and clinical indications

A total of 1037 prenatal samples were processed, 919 (88.6%) of which were AF, 99 (9.5%) CVS, 15 (1.5%) CA, 4 (0.4%) DNA extracted from uncultured amniocytes (Table 1). Hence, analyses were performed on uncultured material in 1022/1037 (98.5%) and on cultured cells in 15/1037 (1.5%) of prenatal samples.

The indications for performing invasive prenatal testing included AMA (n = 444; 42.8%), MSS (n = 13; 1.3%), AUS (n = 48; 4.6%), AFK (n = 8; 0.8%), FIS (n = 11; 1.1%), PA (n = 484: 46.8%) and cell culture failure (n = 4; 0.4%). For 25 (2.4%) patients, more than one indication was recorded (MI).

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Sufficient quantities of DNA were isolated from all the samples included in the study (Table 1, see Supporting Information). The average amount of DNA obtained per mL of amniotic fluid was 99 ± 98 ng (range 7–1694 ng), and 2894 ± 2420 ng (range 306–12807 ng) from CVS tissue. The average quantity of DNA used in the aCGH process was 264 ± 109 ng (range 28–510 ng).

The average turnaround time for aCGH results was 2.4 ± 0.5 (range 2–3) working days from sample's receipt if no abnormal results were found, and 6.3 ± 1.0 (range 2–7) working days in cases with a detected CNV needing confirmatory studies.

Array CGH findings

Detected copy number changes were categorized into one of the following groups: chromosome abnormalities of clinical significance, findings of uncertain clinical significance (VOUS), and benign CNVs. Results are summarized in Figure 2.

The majority of prenatal samples (1003/1037; 96.7%) had normal results, with no copy number changes or only benign CNVs identified. CNVs interpreted as likely benign and of no clinical significance were identified in 135 samples (13.0%) (Table 2, see Supporting Information). These CNVs had been previously seen multiple times in our internal database of previously analyzed clinical cases and phenotypically normal individuals, and/or represented in the DGV. Likely benign CNVs were recorded but not reported.

Clinically significant chromosome alterations were identified in 34 out of 1037 (3.3%) samples, 19 (55.9%) of which were AF, 14 (41.2%) were CVS and 1 (2.9%) was a sample of cultured amniocytes. Twenty-five (73.5%) clinically significant results were also identified by conventional karyotyping performed concurrently with aCGH (Table 2). Array CGH was also able to detect chromosomal mosaicism in four samples, with the lowest abnormal chromosome representation being at the 10% level.

In nine samples (26.5% of the chromosomal abnormalities detected and 0.9% of the samples included in the study), aCGH provided diagnosis of clinically significant chromosomal abnormality, not detected by conventional karyotyping, which would have otherwise been overlooked if only a G-banded karyotype had been performed (Table 3). Six of the nine were *de novo* CNVs identified in the fetal DNA but not in parental DNAs and not recorded as benign CNVs in the DGV database or in our own database of aCGH results; three CNV results were also detected in one of the parents so were inherited. Seven of the above CNVs were related to well-established syndromes described in Online Mendelian Inheritance in Man (OMIM) database. One of these (case 3) was a recurrent chromosomal rearrangement and one (Case 2) was classified as pathogenic CNV because it was characterized as a *de novo* complex aberration, involving relatively large chromosomal regions, containing clinically relevant genes and considering the abnormal ultrasound findings (Figure 3). Following parental studies, no findings of unclear significance remained. These results are summarized in Table 3.

G-banded karyotype results and comparative analysis

Conventional G-banded karyotype analysis was performed concurrently with aCGH in a blinded fashion. Traditional karyotyping was successful on 1030 of the samples (99.3%), which detected 24 (2.4%) chromosome abnormalities. In seven samples, a balanced translocation or inversion was identified. In these cases, because aCGH did not detect chromosomal imbalances, we were able to reassure the families that the rearrangements seen by karyotyping were unlikely to contain imbalances and therefore unlikely to be pathogenic.

There was complete concordance between the conventional karyotyping and the aCGH results, except for two cases (Table 2). The first concerned a sample of CA, referred because of a suspected 5q duplication, aCGH testing identified a duplication 15q24.1-qter (Figures 4 (H)–(J)). The second was an AF that appeared normal after aCGH, while G-banded karyotype revealed a mosaic trisomy 20 (84%). A DNA sample from the cultured amniocytes of the above case was then processed by aCGH, which showed trisomy 20, confirming the G-banding results. These results were consistent with an interpretation of an *in vitro* artefact caused by cell culture of amniocytes, a common finding for trisomy 20 mosaic.

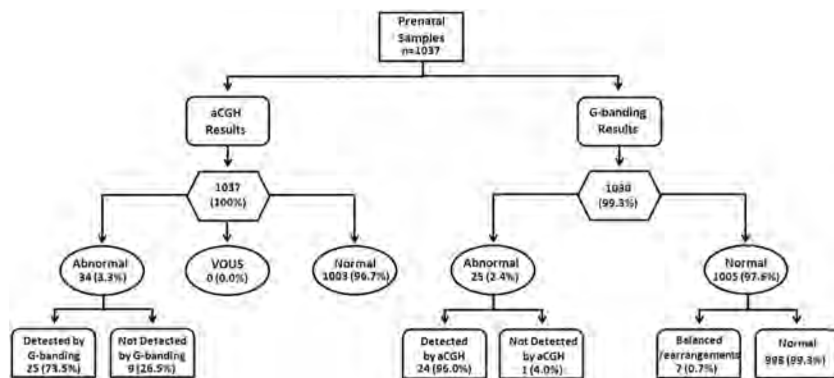

Figure 2—Karyotyping results from prenatal samples processed in parallel using both aCGH and G-banding

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Table 2—Clinically significant chromosomal abnormalities detected in prenatal samples by both conventional karyotyping and aCGH

Sample type	No. of samples	Indication	Chromosomal findings		Concordance	Final diagnosis	Outcome
			G-banding results[a]	aCGH result[a]			
AF-CVS	14	AMA, MSS, AUS	47, XX,+21 or 47, XY,+21	arr 21q11.2q22.3 (13,452,809-46, 844,477×3)	Y	Trisomy 21	TOP ($n = 14$)
AF-CVS	3	AMA, AUS	47, XX,+18 or 47, XY,+18	arr 18p11.32q23 (74,461-76,025, 499×3)	Y	Trisomy 18	TOP ($n = 3$)
CVS	1	AUS	47, XX,+13	arr 13q12.11q34 (18,425,650-114, 037,803×3)	Y	Trisomy 13	TOP
LA	1	AMA	46, XX[85]/45, X[15]	arr Xp22.33q28 (3,031,202-154, 782,695×1)	Y	Monosomy X mosaic	Continued
LA	1	AMA	46, XX[90]/45, X[10]	arr Xp22.33q28 (3,031,202-154, 782,695×1)	Y	Monosomy X mosaic	Continued
LA	1	AMA	47, XYY	arr Yp11.32q12 (386,805-57, 461,078×2)	Y	47, XYY	Continued
CVS	1	AMA	46, XX[80]/47, XX,+7[20]	arr 7p22.3q36.3 (168,315-158, 628,932×3)	Y	Trisomy 7 mosaic	Continued (46, XX after amniocentesis)
AF	1	PA	46, XX,[16]/47, XX,+20[84]	arr(1–22,X)×2	N	46, XX	Continued
CA	1	AMA, AFK	46, XY,dup(5)(q?)	arr 15q24.2q26.3 (73,240,751×2, 73,867,177-100, 171,678×3)	N	Duplication 15q24.1–qter	TOP
CVS	1	AMA	46, XX,[80]/46, XX,dup(5) (p15p12)[20]	arr 5p15.33p12 (109,395, 234, 837-43,988, 038×3, 45,195, 011×2)	Y	Trisomy 5p mosaic	amniocentesis

[a]International System for Human Cytogenetic Nomenclature (ISCN) 2009; TOP, termination of pregnancy.

DISCUSSION

In this study, we investigated the reliability and accuracy of aCGH technology for testing prenatal samples and compared it with standard prenatal karyotyping. We aimed to assess the feasibility of offering aCGH in prenatal diagnosis on a routine basis, trying to address the following issues: (1) if aCGH is accurate in the detection of common and submicroscopic chromosome abnormalities in prenatal samples; (2) if the technique improves the prenatal detection rate of genetic aberrations or, on the contrary, whether aCGH misses potential pathogenic chromosomal abnormalities, compared with conventional karyotyping; (3) if there is an appreciable increase in results of unclear clinical relevance that may cause difficulties in case management and parental anxiety; and (4) whether aCGH should be applied to all prenatal samples as first-line test or its use should be limited to specific indications (e.g. in cases of abnormal ultrasound findings but normal karyotype).

The results obtained from the prenatal samples included in this prospective study demonstrated the feasibility and benefits of prenatal diagnosis performed by whole-genome aCGH for direct analysis of amniocytes or CVS tissues, without culturing cells.

A major potential limitation with the use of the aCGH assay on prenatal samples could be an inability to isolate sufficient quantities of fetal DNA, especially from AF specimens. Furthermore, the quality of DNA isolated from such samples is often suboptimal because of the presence of dead cells, small degraded DNA fragments, and other unknown inhibiting factors. Our results show the viability of using DNA isolated from uncultured amniocytes or chorionic villi for aCGH. All prenatal samples that were processed in this study yielded sufficient DNA for successful aCGH analysis, providing high-quality profiles with as little as 28 ng of DNA, notably less than the amount of DNA (500 ng) generally suggested to process postnatal blood samples for an aCGH assay.

Array CGH using DNA directly extracted from prenatal samples also led to rapid turnaround time (2.5 ± 0.6 working days, on average); an important issue for prenatal diagnosis. In addition, culture artefacts are completely avoided, which makes it easier to interpret cytogenetic findings. We experienced one such problem in an AF sample, in which conventional karyotyping revealed a

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Table 3—Clinically significant array CGH findings in prenatal samples, not detected by conventional karyotyping

Case no.	Sample type	Patient's age	Indication	Chromosomal findings					Parental analysis	Interpretation	Final diagnosis	Pregnancy outcome
					aCGH result							
				G-banding results[a]	ISCN Formula[a]	Location	Gain / loss	Estimated size (Mb)				
1	AF	35	AMA + AUS (single umbilical artery)	46, XX	arr 17p12 (10,451,110×2, 12,023,651-15,415,749×1, 16,622,659×2)	17p12	Loss	3.4	Inherited (maternal)	Deletion of the chromosomal region including the PMP22 gene (OMIM: 601097) consistent with hereditary neuropathy with liability to pressure palsies	OMIM disease (162500), abnormal	Continued and delivered
2	CVS	39	AMA + AUS (Cystic Hygroma)	46, XY	arr 10q26.12q26.3 (120,459,257×2, 121,580,323-135,215,585×1);	10q26.12–10q26.3	Loss	13.6	*De novo*	Clinically significant CNV characterized by a *de novo* complex rearrangement involving relatively large chromosomal regions and containing clinically relevant genes	Abnormal	TOP
					arr 16q23.1q24.3 (71,387,225×2, 74,055,794-88,674,700×3)	16q23.1–q24.3	Gain	14.6				
3	CVS	33	AUS (abnormal nuchal translucency)	Cell culture failure	arr 8p23.3p23.1 (115,733-6,615,616×1, 8,235,335×2);	8p23.3–p23.1	Loss	6.5	*De novo*	Clinically significant CNV characterized by a *de novo* complex rearrangement consistent with inv dup del(8p)	Abnormal	TOP
					arr 8p22p21.1 (11,900,030×2, 12,718,648-27,369,929×3, 28,531,954×2)	8p22–p21.1	Gain	14.6				
4	AF	43	AMA	46, XY	arr 17p12 (13,313,672×2, 14,324,519-15,415,749×3, 16,622,659×2)	17p12	Gain	1.1	Inherited (maternal)	Duplication of the chromosomal region including the PMP22 gene (OMIM: 601097) consistent with Charcot–Marie–Tooth neuropathy type 1 A	OMIM disease (118220), abnormal	Continued

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5	AF	34	PA	46, XY	arr Xp21.2p21.1 (31,101,821×1, 31,149,156-31,598,354×2, 31,604,241×1)	Xp21.2–p21.1	Gain	0.45	*De novo*	Duplication of the chromosomal region including exons 56–77 of the Dystrophin gene (OMIM: 300377) consistent with Duchenne muscular dystrophy	OMIM disease (310200), abnormal	TOP
6	AF	37	AMA	46, XX	arr 15q13.1q13.3 (27,819,621×2, 27,927,504-30,870,822×1, 30,928,648×2)	15q13.1–q13.3	Loss	2.9	*De novo*	Deletion of a 2.9 Mb region including the cytogenetic band 15q13.1-q13.3 consistent with 15q13.3 microdeletion syndrome	OMIM disease (612001), abnormal	TOP
7	AF	41	AMA + AUS (tetralogy of Fallot)	46, XX	arr 22q11.21 (16,038,711×2, 17,552,769-18,223,647×1, 21,021,548×2)	22q11.21	Loss	0.67	*De novo*	Deletion of a 670 Kb region including the cytogenetic band 22q11.21 consistent with 22q11.2 deletion syndrome	OMIM disease (188400), Abnormal	TOP
8	CVS	38	AMA + AUS (abnormal nuchal translucency)	46, XY	arr 5q35.2q35.3 (174,837,770×2, 175,499,944-177,240,560×1, 177,681,483×2)	5q35.2–q35.3	Loss	1.7	*De novo*	Deletion of the chromosomal region including the NSD1 gene (OMIM: 606681) consistent with Sotos syndrome	OMIM disease (117550), abnormal	TOP
9	AF	35	AMA	46, XX	arr 22q11.21 (16,038,711×2, 17,552,769-18,223,647×3, 21,021,548×2)	22q11.21	Gain	0.67	Inherited (maternal)[b]	Duplication of a 670 Kb region including the cytogenetic band 22q11.21 consistent with 22q11.2 microduplication syndrome	OMIM disease (608363), abnormal	TOP

[a]According to International System for Human Cytogenetic Nomenclature (ISCN) 2009; TOP, termination of pregnancy.
[b]Parental analysis revealed the duplication in the mother, who had no clinical signs, while the two children of the couple resulted not carrying the chromosomal abnormality.

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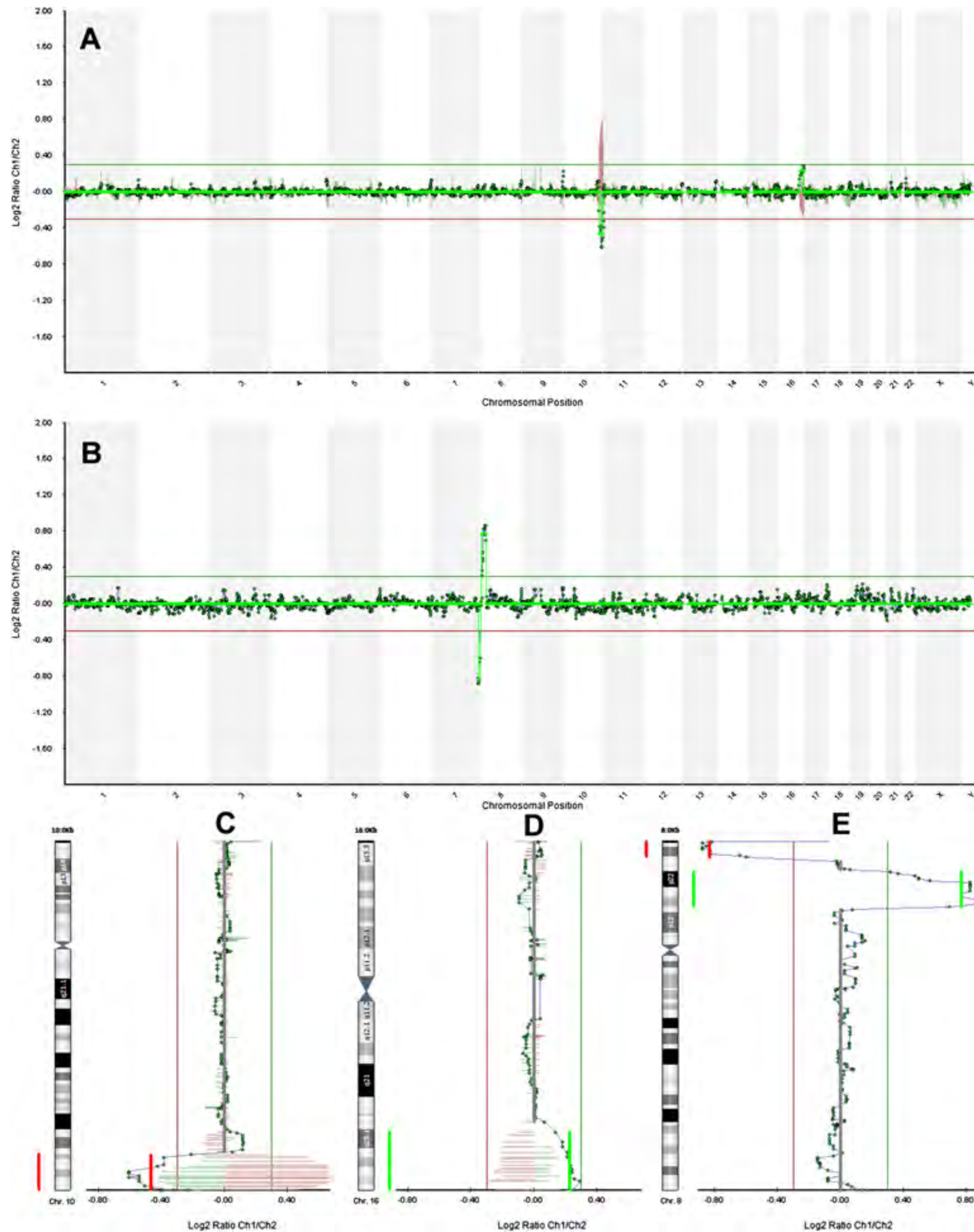

Figure 3—Clinically significant complex rearrangements identified in prenatal samples. (A) Microarray plot for a *de novo* unbalanced translocation t(10;16) (q26.12;q23), identified in a CVS sample referred for AMA and cystic hygroma indications (Case 2), resulting in a 13.6 Mb deletion of 10q26.12–10q26.3 and a 14.6 Mb gain of 16q23.1–q24.3, detected as a shift of the BAC clones located in the above regions towards the red line (loss) and the green line (gain), respectively. (B) Microarray plot for a *de novo* double segmental imbalance involving chromosome 8, identified in a CVS sample referred because of abnormal nuchal translucency (Case 3), characterized by a 6.5 Mb deletion of 8p23.3–p23.1 and a 14.6 Mb gain of 8p22–p21.1, consistent with inv dup del (8p). (C) and (D) Chromosomal details for segmental imbalances from (A), and (E) segmental imbalances from (B)

mosaic trisomy 20 (84% mosaic) that was not detected by aCGH from the equivalent uncultured amniocytes, demonstrating this to be an *in vitro* artefact of cell culture.

Another potential limitation of using aCGH on prenatal samples is the fact that low level mosaicism (LLM) occurrences may remain undetected. Although cases exhibiting chromosomal mosaicism identified by aCGH have been reported (Cheung *et al*., 2007; Menten *et al*., 2006; Shinawi *et al*., 2008; Ballif *et al*., 2006, 2007; Stankiewicz and Beaudet, 2007), the ability of aCGH to detect LLM in prenatal samples is not yet well defined. Several studies performed in post-natal samples demonstrated that aCGH may detect as low as 10% mosaicism (Ballif *et al*., 2006; Menten *et al*., 2006; Xiang *et al*., 2008; Scott *et al*., 2010). In this study we detected four occurrences of mosaicism, three of which involving a whole chromosome and one concerning a partial duplication (Figure 5). The above results indicate that a BAC-array can accurately detect LLM down to 10% also in prenatal samples.

The use of aCGH notably increased the sensitivity and accuracy of the prenatal analysis allowing for identification of submicroscopic chromosome abnormalities with clinical significance that were not detectable by conventional karyotyping, in addition to the microscopic imbalances that

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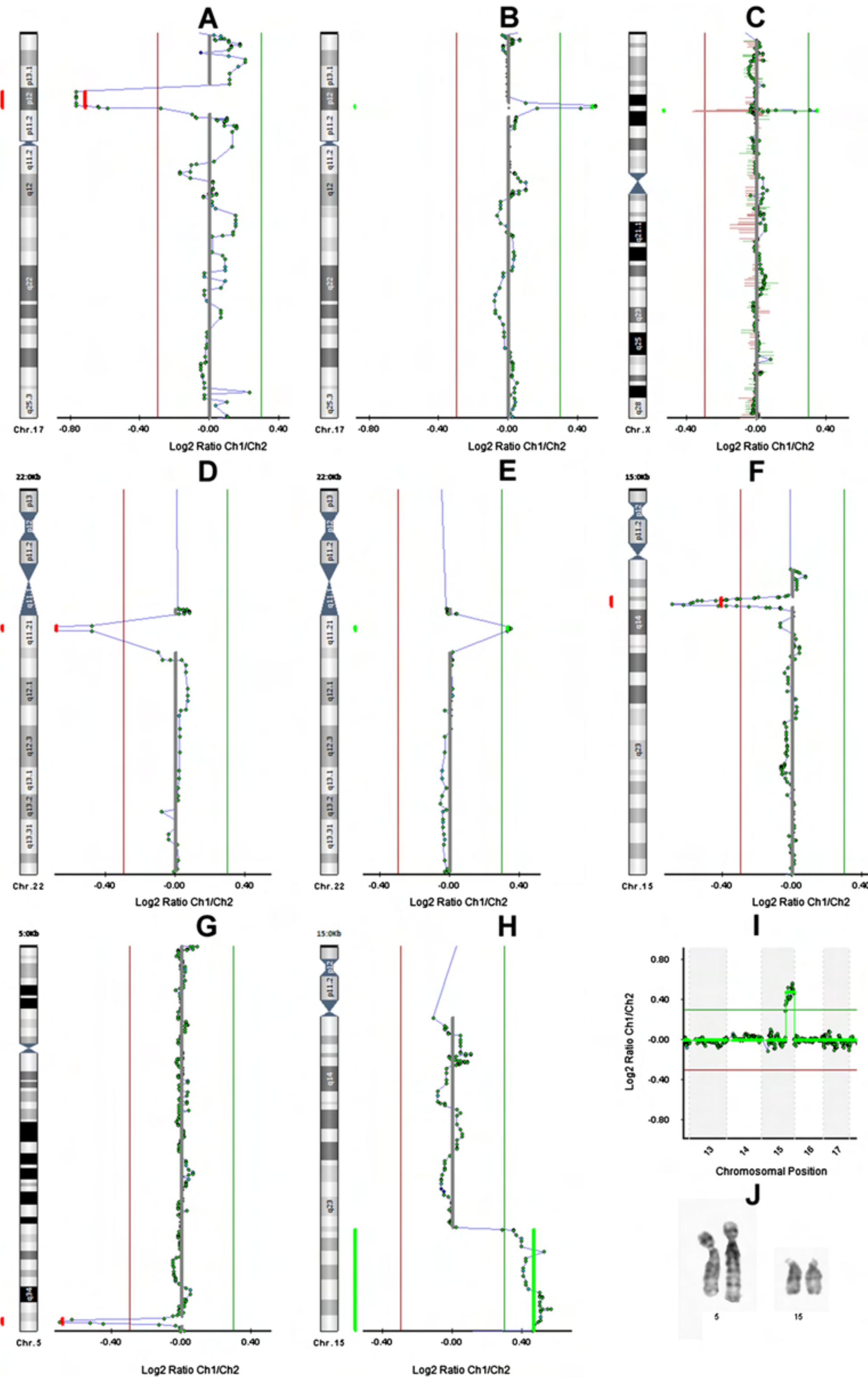

Figure 4—Clinically significant submicroscopic chromosome aberrations concerning well-established syndromes (A–G) and inconsistency between G-banding and aCGH findings (H–J), detected in prenatal samples. (A) An inherited 3.4 Mb deletion of 17p12, including the *PMP22* gene, associated with hereditary neuropathy with liability to pressure palsies disease (Case 1). (B) An inherited 1.1 Mb duplication at 17p12, including the *PMP22* gene, consistent with Charcot–Marie–Tooth neuropathy type 1 A disease (Case 4). (C) A male fetus with a *de novo* clinically significant 450 Kb duplication at Xp21.2–p21.1, encompassing exons 56–77 of the Dystrophin gene, consistent with a diagnosis of male affected by Duchenne muscular dystrophy (Case 5). (D) A *de novo* clinically significant 670 Kb deletion at 22q11.21, consistent with 22q11.2 deletion syndrome (Case 7). (E) An inherited 670 Kb duplication at 22q11.21, consistent with 22q11.2 microduplication syndrome (Case 9). (F) A *de novo* clinically significant 2.9 Mb deletion at 15q13.1–q13.3, consistent with 15q13.3 microdeletion syndrome (Case 6). (G) A *de novo* clinically significant 1.7 Mb deletion at 5q35.2–q35.3, consistent with Sotos syndrome (Case 8). (H) Chromosomal details for a sample of cultured amniocytes referred because of suspected duplication 5q, that after aCGH testing was detected as a duplication 15q24.1–qter [arr 15q24.2q26.3(73,240,751×2, 73,867,177-100,171,678×3]. (I) Microarray plot from (H). (J) G-banded karyotype from (H)(only chromosome 5 and 15 are shown)

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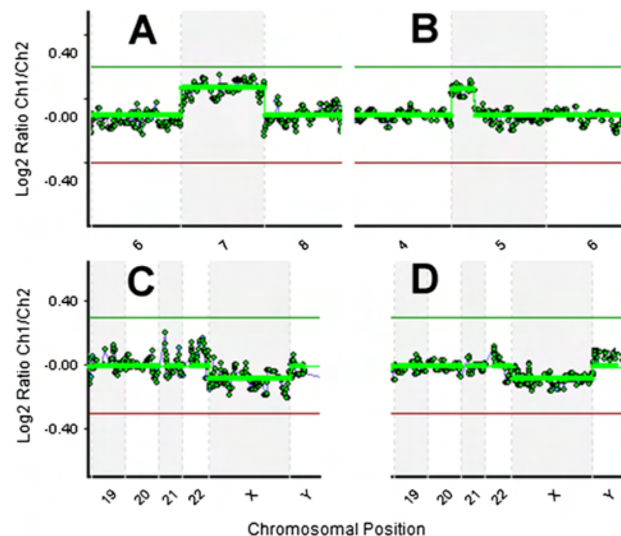

Figure 5—Microarray plots for prenatal samples exhibiting chromosomal mosaicism. (A) 46, XX[80]/47, XX,+7[20], identified in a CVS sample. (B) 46, XX,[80]/46, XX,dup(5)(p15p12)[20], detected in a CVS sample. (C) AF 46, XX[90]/45,X[10]. D) AF 46, XX[85]/45, X[15]

karyotyping identified. With this aCGH also offers rapid and precise characterization of the chromosome alterations. In our prospective series of 1037 prenatal cases, we found that aCGH yielded clinically relevant abnormal results in 34 (3.3%) samples. In nine (0.9%) of these cases (26.5% of the clinically relevant findings), aCGH detected chromosome abnormalities that would not have been found if only a conventional karyotype had been performed. Comparing the results obtained in the present study to other previous prospective aCGH studies (Sahoo *et al*., 2006; Shaffer *et al*., 2008; Kleeman *et al*., 2009; Coppinger *et al*., 2009; Van den Veyver *et al*., 2009; Maya *et al*., 2010), the detection rates are comparable except for Coppinger *et al*. (2009) and Kleeman *et al*. (2009), reporting a higher detection rate (Table 4). The above difference could be explained by the selected population included in their studies, compared with the present study.

Our study indicated that the aCGH approach was robust, with no false positive findings when followed up with different methodologies, or false negative findings when samples were tested in concomitance with conventional karyotyping, suggesting that the technique has the potential to replace the traditional cytogenetic analysis without missing significant results. On the contrary, in one case where the initial chromosome studies incorrectly identified a duplication of 5q, aCGH rapidly came to the correct diagnosis as duplication 15q24.1-qter (Figures 4(H)–(J)).

As expected, aCGH did not detect balanced rearrangements, such as reciprocal or Robertsonian translocations and inversions, in seven (0.7%) of the prenatal samples, that were identified using standard karyotyping. These changes would have gone undetected if aCGH was used alone. This represents a limitation of the technique because it may miss a clinically significant karyotype where a *de novo* balanced rearrangement may be disrupting gene function, although this represents a very rare event (0.0001%)(Ahn *et al*. 2010). Furthermore, carriers of balanced Robertsonian translocations are at risk from uniparental disomy (UPD), not detectable by BAC arrays. Inherited translocations, instead, would be considered incidental findings, which would not be relevant for the prenatal diagnosis purpose because of no phenotypic consequence to the current fetus, although this information is potentially of value for reproductive counselling for the parents.

Evidence regarding the increased diagnostic yield of aCGH technique with respect to conventional karyotype makes its use attractive in a routine prenatal diagnosis practice. Although the debate on possible pitfalls of this approach is still ongoing, essentially concerning the possible detection of CNVs that are of unclear clinical significance, for which phenotypic consequences and penetrance may be difficult to predict. Considering the above mentioned prospective studies, combined with data from the present study, the probability of detecting such findings in prenatal samples is around 0.3% (Table 4).

There is understandable concern that VOUS might pose added complexities to counselling and case management; in addition, this may cause parental anxiety and potential termination of normal pregnancies (Shuster, 2007; Friedman, 2009). This issue can be adequately addressed through parental studies to determine whether the 'unclear' CNV detected in the fetus is *de novo* or inherited. *De novo* abnormalities are generally considered likely to be pathogenic, while it has been suggested that inherited imbalances should be classified as likely benign findings, although familial variants may not always be benign because of incomplete or variable penetrance (Lee *et al*., 2007).

In this study, we identified one sample with unclear clinical significance (case 2, Table 3). Although the phenotypic consequences were not fully predictable, after parental analysis this variant was in the end classified as pathogenic CNV, following the decision criteria reported by Miller *et al*. (2010) in their review of aCGH tests performed in postnatal cases.

Although detection of regions of unclear clinical relevance cannot be excluded with aCGH testing, the

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Table 4—Incidence of pathogenic and benign CNVs in prospective aCGH prenatal studies

Chromosome abnormality type	Sahoo *et al.* (2006) $n = 98$ (%)[a]	Shaffer *et al.* (2008) $n = 151$ (%)[a]	Kleeman *et al.* (2009) $n = 24$[b] +26[a] (%)	Coppinger *et al.* (2009)		Van de Veyver *et al.* (2009) $n = 190$[b] +110[a] (%)	Maya *et al.* (2010) $n = 269$ (%)[b]	Current study $n = 1037$ (%)	Combined $n = 2149$ (%)
				$n = 182$ (%)[b]	$n = 62$ (%)[a]				
No alteration	51 (52.0)	136 (90.1)	46 (92.0)	158 (86.8)	57 (91.9)	242 (80.7)	229 (85.1)	868 (83.7)	1787 (83.2)
Microscopic aberrations of clinical significance	5 (5.1)	0 (0.0)	0 (0.0)	2 (1.1)	0 (0.0)	13 (4.3)	4 (1.5)	25 (2.4)	49 (2.3)
Clinically significant submicroscopic aberrations	0 (0.0)	2 (1.3)	1 (2.0)	5 (2.7)	0 (0.0)	2 (0.7)	3 (1.1)	9 (0.9)	22 (1.0)
CNVs of unclear significance	2 (2.0)	1 (0.7)	0	1 (0.5)	0 (0.0)	3 (1.0)	0 (0.0)	0 (0.0)	7 (0.3)
Benign CNVs	40 (40.8)	12 (7.9)	3 (6.0)	16 (8.8)	5 (8.1)	40 (13.3)	33 (12.0)	135 (13.0)	284 (13.2)

[a]Targeted arrays.
[b]Whole-genome arrays.

increasing availability of shared databases of information regarding CNVs, together with experience and parental analysis, most alterations can be classified and interpreted. Furthermore, if parental samples could be submitted with the prenatal samples, parental testing can be performed as soon as a fetal chromosomal alteration is identified, without causing anxiety to the patients. In addition, results of unclear significance are not unfamiliar in prenatal diagnosis because unclear diagnostic results or findings with unclear clinical consequences are occasionally encountered even with conventional karyotyping. Thus, VOUS identified by prenatal aCGH might be approached in a similar manner and managed by providing the patients with thorough pretest and post-test counselling (Darilek *et al*., 2008).

Another confounding factor in data interpretation may arise from CNVs associated with recurrent microdeletion and microduplication syndromes, which are characterized by an incomplete penetrance and variable expressivity. An example of this is represented by the 22q11.2 microduplication syndrome (MIM: 608363), a disorder with a highly variable phenotype, ranging from apparently normal phenotype to mental retardation, learning disability, delayed psychomotor development, growth retardation, and/or hypotonia (Ensenauer *et al*., 2003; Yobb *et al*., 2005; Ou *et al.,* 2008; Wentzel *et al*., 2008; Portnoï *et al*., 2009).

The marked clinical variability of the phenotypes associated with this syndrome and the high rate of familial cases with reported seemingly normal parents, challenge the ability to draw meaningful genotype-phenotype correlations. This leads to difficulty in counselling because of the impossibility to predict the exact phenotypic outcome. We identified the 22q11.2 microduplication in an AF referred patient as a result of AMA indication (Case 9) (Table 3). Parental analysis revealed the duplication in the mother, who had apparently no clinical phenotype, while the other two children of the couple were found not to be carrying this chromosomal abnormality. After proper post-test counselling, the patients decided to terminate the pregnancy, because they were not willing to accept the underlying risk of a possible disease in the offspring.

Whether array CGH should be used in prenatal diagnosis as a first-line test has been widely debated (Pergament, 2007; Friedman, 2009; Bui *et al*., 2011). The results of this study, together with the previous reported experiences, indicate that it could be already acceptable to offer aCGH testing to women who are currently undergoing amniocentesis or CVS for routine examinations, at least concurrently with conventional karyotyping.

Further prospective studies in this area, with a large cohort of samples analysed, will further elucidate the role that this technique will come to play in prenatal diagnosis, including whether it may replace the use of standard karyotyping.

CONCLUSION

This study demonstrates that aCGH represents an improved diagnostic tool for prenatal detection of chromosomal abnormalities, allowing identification of submicroscopic clinically significant imbalances that are not detectable by

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conventional karyotyping. Although larger studies are needed, our findings provide further evidence on the feasibility of introducing aCGH into routine prenatal diagnosis practice as a first-line diagnostic test to detect chromosomal abnormalities in prenatal samples.

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