Prenatal diagnosis of genomic disorders and chromosome abnormalities using array-based comparative genomic hybridization

Francesca Gullotta[1]
Michela Biancolella[1]
Elena Costa[1]
Isabella Colapietro[1]
Anna Maria Nardone[1]
Paolo Molinaro[1]
Adalgisa Pietropolli[2]
Marianovella Narcisi[2]
Cristiana Di Rosa[1]
Giuseppe Novelli[1]

[1] Dipartimento di Biopatologia e Diagnostica per Immagini, Università di Roma Tor Vergata and Azienda Ospedaliera Universitaria Policlinico Tor Vergata, Roma, Italy
[2] Sezione di Clinica Ginecologica e Ostetrica A.O.U. Policlinico Tor Vergata, Roma, Italy

Reprint requests to: Prof. Giuseppe Novelli
Laboratorio di Genetica Medica
Azienda Ospedaliera Universitaria
Policlinico Tor Vergata, Roma
Viale Oxford, 81 - 00133 Roma
Fax: +39/06/20900669
E.mail: novelli@med.uniroma2.it

Summary

Cytogenetic analysis is a crucial tool of prenatal diagnosis. The ability to rapidly detect aneuploidy and identify small structural abnormalities of foetal chromosomes has been greatly improved by the use of molecular cytogenetic technologies. Microarray-based Comparative Genomic Hybridization (aCGH) has been recently employed in postnatal diagnosis of cryptic chromosomal aberrations, but use in prenatal diagnosis is still limited.
We set-up a diagnostic protocol which uses aCGH technology on genomic DNA isolated from uncultured chorionic villus sampled at 11-12 week's gestation. We used a commercially targeted microarray (MDTelArray, Technogenetics Srl – Bouty Group, Sesto S. Giovanni, Milan, Italy) constituted by 167 genomic clones corresponding to 34 critical regions frequently involved in microdeletions and microduplications and 126 subtelomeric clones. Array validation has been carried-out via retrospective analysis of DNA isolated from a series of cytogenetically normal chorionic villus samples (CVS) and of DNA isolated from cytogenetically abnormal cultured amniocytes, CVS or peripheral blood. A pilot prospective study was undertaken analyzing 25 CVS obtained from foetuses at risk for chromosomal aberrations. aCGH results both for retrospective and prospective studies were in agreement with data obtained using "classical" cytogenetic analysis, and/or FISH analysis or DNA testing. Although these preliminary data support the usefulness of aCGH in prenatal diagnosis, further prospective studies are required to verify the feasibility of introducing this technique as part of the diagnostic armamentarium for identify affected foetuses.

KEY WORDS: prenatal diagnosis, array-based comparative genomic hybridization (aCGH), aneuploidy, cryptic chromosomal aberrations, chorionic villus samples (CVS).

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Introduction

A potentially lethal or handicapping major defect occurs in 2-3% of liveborn infants (1). Congenital malformations have become the main cause of infant mortality during the first years of life (2, 3). Approximately 10-15% of stillborn and liveborn infants with malformations have chromosomal imbalances (4, 5). Since the development of chromosome banding techniques in the late 1960's (6), microscopic karyotype analysis has been applied to prenatal testing and is still today considered the gold standard for prenatal diagnosis. This procedure results to be highly reliable for identifying chromosome copy number abnormalities (aneuploidy) and large structural rearrangements in foetal cells obtained invasively by either amniocentesis or chorionic villus sampling (CVS). However, even if this procedure results highly reliable, a number of limitations frequently occur. The resolution appear to be inadequate to detect deletions or duplications <10 Mb. In addition, the technique requires cells culture and a long time for definitive results generating frequently anxiety for parents during a pregnancy. Studies have demonstrated the ability of molecular techniques to detect aneuploidy and submicroscopic chromosomal anomalies within 24 hrs. These include, fluorescence *in situ* hybridization (FISH), quantitative fluorescence polymerase chain reaction (QF-PCR), and multiplex ligation-dependent probe amplification (MLPA) (7-9). However, all these techniques seems to be inadequate to perform a genomewide screening. Recent studies have demonstrated the ability of aCGH to detect submicroscopic chromosomal anomalies in individuals with learning and developmental disability providing evidence for a genomewide screening strategy in detecting DNA copy number imbalances in a rapid and less labour-intensive manner (10, 11). This technique is similar in principle to conventional metaphase CGH (12, 13), but uses arrayed DNA sequences instead of metaphase

chromosomes as targets for hybridization, thus providing a direct link between detected aberrations and the physical and genetic maps of the human genome. Patient and reference genomic DNAs labelled with two different fluorochromes are co-hybridized to an array of mapped DNA fragments immobilized on slides (12, 14). The genomic resolution depends on the physical distance between two clones and the sizes of individual clones. This technique is able to detect, in a single experiment, any dosage imbalances including aneuploidies, deletions or duplications, but it cannot detect balanced rearrangements such as reciprocal and Robertsonian translocations or inversions. aCGH shows a number of advantages compared to conventional techniques in terms of clinical practice and cost implications.

Here we present our experience in validation of an innovative aCGH (MDTelArray, Technogenetics Srl – Bouty Group, Sesto S. Giovanni, Milan, Italy) for prenatal diagnosis. For this purpose, we established an original protocol based on genomic DNA extracted from CVS during the first trimester of gestation. MDTelArray was arranged by 167 genomic clones corresponding to 34 critical genomic regions frequently involved in microdeletions and microduplications and 126 subtelomeric clones. The aCGH was tested in a series of six retrospective unbalanced cases, and 16 normal DNA samples. In addition, 25 prospective samples of chorionic villi obtained from foetuses at high risk for chromosomal aberrations were studied.

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Methods

Retrospective series

A series of 6 pathological DNA obtained from CVS or cultured amniotic cells of samples affected foetuses (n. 3 respectively, trisomy 21, Klinefelter syndrome, and Duchenne Type Muscular Dystrophy, DMD) or from affected children (n. 3 respectively, Turner syndrome, Prader-Willi syndrome and Smith-Magenis syndrome) and 16 normal DNA samples (CVS) obtained from unaffected foetuses, were studied. All retrospective series of samples were investigated by routine cytogenetics analysis and/or FISH, or DNA testing (DMD).

Prospective series

Twenty-five CVS obtained from foetuses at risk for chromosomal aberrations due to advanced maternal age, were obtained by the subjects' obstetricians using their standard clinical procedures. All CVS were prepared for standard G-band karyotype for clinical laboratory protocols and, in some cases, for the molecular genetic analysis as well. The remainder of the foetal sample was collected for aCGH analysis. For each experiment, we used at least 2 µg of DNA extracted both from fresh chorionic villus samples and corionic villus samples preserved at -20°C even for as long as one month. Test DNA was extracted using a Wizard Genomic DNA Purification Kit (Promega, Madison, WI), according to the manufacturer's protocols. Reference genomic DNA was derived from peripheral blood of phenotypically normal male and female control individuals (Promega, Madison, WI).

Microarray constitution, hybridization and data analysis

aCGH analysis was performed using a targeted microarray (MDTelArray, Technogenetics Srl – Bouty Group, Sesto S. Giovanni, Milan, Italy) constituted by 167 genomic clones corresponding to 34 critical regions frequently involved in microdeletions and microduplications (Table I) and 126 subtelomeric clones. Each BAC clone was spotted in triplicate and positive (pool of human BAC clone) and negative (rice DNA) control clones were also printed. This array enables the simultaneous analysis of all the regions above via a single hybridization, therefore saving both time and sample material. The array's high resolution, allows for more reliable and precise data. Moreover, two different hybridization areas were spotted on the array, consenting to perform the experiment in dye-reversal (Fig. 1). This permits to achieve high quality data and provides additional confirmation of true copy number alterations (15, 16). In dye-reversal technique, for each patient sample, two experiments were performed with reversal of the dye labels for the control and the test samples (17), followed by integration of the data from both dye-reversed hybridizations to determine interferences for each case (Fig. 2). After purification (Zymo Research's Clean and Concentrator™ -25 , Orange, CA), an equal amount (1 µg) of both test and reference DNA was labelled with Cy3-dCTP and Cy5-dCTP (Amersham Biosciences, Little Chalfont, UK), respectively, by random priming (Bioprime® Array CGH Genomic Labelling Module, Invitrogen, Carlsbad, CA), purified with CyScribe™ GFX™ Purification Kit (Amersham Biosciences, Little Chalfont, UK), precipitated and hybridized following the microarray manufacturer's protocols (Technogenetics Srl – Bouty Group, Sesto S. Giovanni, Milan, Italy). A dye-reversal experiment was performed for each patient sample. Images were acquired using GenePix 4000B dual-laser scanner and GenePix Pro 6.0 software (Axon Instruments, Sunnyvale, CA). The average ratios of the six spots for each patient were analyzed and plotted using BlueFuse for Microarrays 3.3 (5164) software (BlueGenome Ltd, Cambridge, UK). Increases (gains) and decreases (losses) in DNA sequence copy number were defined by test/reference ratios above 1.2 and below 0.8, respectively, based upon previous reports in which 1.2 ($\log_2$ 1.23=0.3) and 0.8 ($\log_2$ 0.81=-0.3) were selected as cut-off values based on experimental results of normal variation observed when two normal reference DNAs were co-hybridized to genomic microarrays (18, 10). To ensure detection of any sex chromosomes abnormalities if the sex of the foetus was different from the sex of the DNA reference, during array validation chromosomes mismatch experiments, using male vs. female DNA of cytogenetically normal individuals, were performed.

Table I - Microdeletion syndromes represented in the MDTelArray.

Monosomy 1p36 Syndrome [(OMIM #607872) (1p36)]
Van der Woude Syndrome [(OMIM #119300) (1q32-q41)]
Nephronophthisis 1 [(OMIM #256100) (2q13)]
Brachydactyly-Mental Retardation Syndrome [(OMIM %600430) (2q37)]
Wolf-Hirschhorn Syndrome [(OMIM #194190) (4p16.3)]
Cri-du-Chat Syndrome [(OMIM #123450) (5p15.2)]
Adenomatous Polyposis of the Colon [(OMIM +175100) (5q21-q22)]
Sotos Syndrome [(OMIM #117550) (5q35)]
Saethre-Chotzen Syndrome [(OMIM #101400) (7p21.1)]
Williams-Beuren Syndrome [(OMIM #194050) (7q11.2)]
Kallmann Syndrome 2 [(OMIM #147950) (8p11.2-p11.1)]
Langer-Giedion Syndrome [(OMIM #150230) (8q24.11-q24.13)]
Monosomy 9p Syndrome [(OMIM #158170)]
HDR Syndrome [(OMIM #146255) (10p15)]
DiGeorge Syndrome/Velocardiofacial Syndrome Spectrum of Malformation 2 [(OMIM %601362) (10p14-10p13)]
Wagr Syndrome [(OMIM #194072) (11p13)]
Potocki-Shaffer Syndrome [(OMIM #601224) (11p11.2)]
Prader-Willi Syndrome / Angelman Syndrome [(OMIM #176270/#105830) (15q11-q13)]
ATR-16 Syndrome [(OMIM #141750) (16pter-p13.3)]
Miller-Dieker Lissencephaly Syndrome [(OMIM #247200) (17p13.3)]
Charcot-Marie-Tooth Disease Neuropathy, Type 1A [(OMIM #118220) (17p11.2)]
Neuropathy, Hereditary, with Liability to Pressure Palsies [(OMIM #162500) (17p11.2)]
Smith-Magenis Syndrome [(OMIM #182290) (17p11.2)]
Neurofibromatosis, Familial Spinal (OMIM #162210) (17q11.2)]
Alagille Syndrome 1 [(OMIM #118450) (20p12)]
DiGeorge Syndrome [(OMIM #188400) (22q11.2)]
Neurofibromatosis, Type II [(OMIM #101000) (22q12.2)]
Short Stature, Idiopathic, Autosomal [(OMIM #604271) (Xpter-p22.32, Ypter-p11.2)]
Steroid Sulfatase Deficiency [(OMIM +308100) (Xp22.32)]
Kallmann Syndrome 1 [(OMIM +308700) (Xp22.3)]
Muscular Dystrophy, Duchenne Type [(OMIM #310200) (Xp21.2)]
ATR-X Syndrome [(OMIM #301040) (Xq13)]
Pelizaeus-Merzbacher Disease [(OMIM #312080) (Xq22)]
XX Male Syndrome [(OMIM 278850)]

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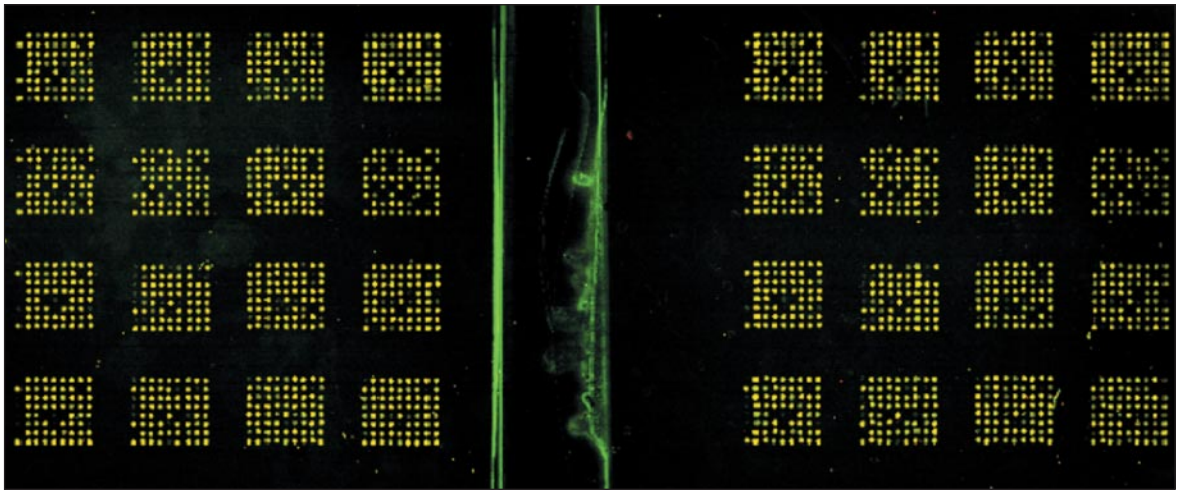

Figure 1 - MDTelArray imaging showing two different hybridization areas to perform the experiment in dye-reversal.

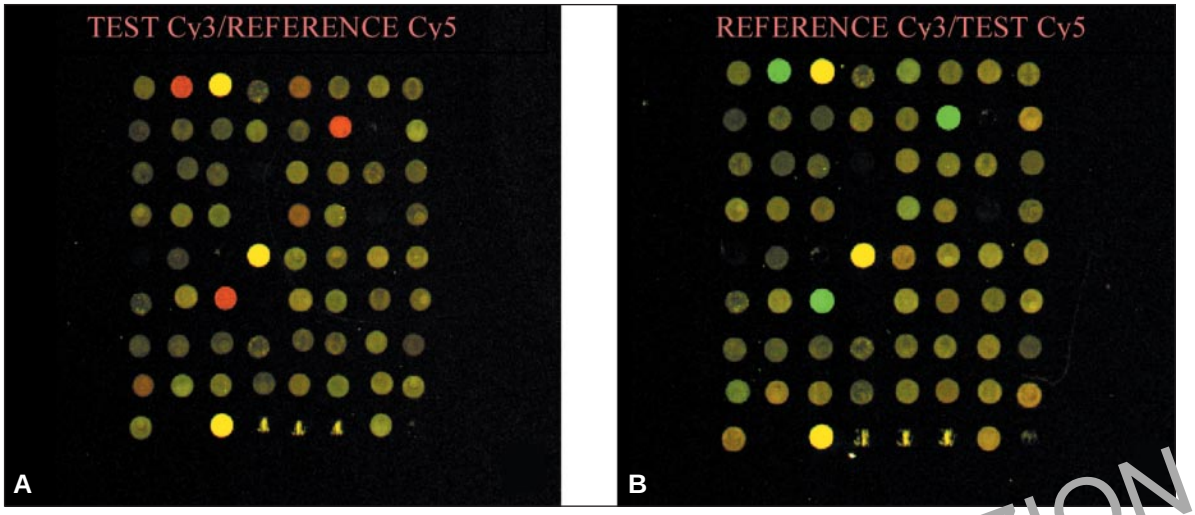

Figure 2 - An illustrative example of a microdeletion detected by MDTelArray using a dye-reversal experiment. For each patient sample, two experiments were performed with reversal of the dye labelling for the test and the reference samples (A and B). In A test (patient) DNA was labelled in Cy3 (green) and reference (control) DNA in Cy5 (red). In B, the same test DNA was labelled in Cy5 (red) and the reference DNA in Cy3 (green).

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Results and discussion

Initially we evaluated if the DNA extraction method influenced the aCGH results. For this purpose, we compared hybridization results obtained using aliquots of the same sample, immediately treated or stored at -20°C for up to one month. No significant differences were scored between samples processed immediately respect to those stored.

The aCGH analytical validity was carried-out via retrospective analysis of DNA isolated from a series of cytogenetically normal CVS and cytogenetically abnormal DNA obtained from cultured amniocytes, CVS or peripheral blood. Chromosomal abnormalities included aneuploidies (trisomy 21, 47,XXY, 45,X) and microdeletions [del (X)(p21.2), del(15)(q11.2-q13) and del(17)(p11.2)] respectively associated to DMD, Prader-Willi syndrome, and Smith-Magenis syndrome. aCHG analysis of the sample with trisomy 21 had a single gain in copy number of clones on chromosome 21 that was represented by 3 BAC clones with a ratio = 1.38 ± 0.03. DNA sample of kariotype 47,XXY also exhibited single copy gain of clones on chromosome Y as represented by 7 BAC clones with a ratio = 1.29 ± 0.04 (using a reference female DNA). aCGH analysis of 45,X DNA sample showed a single copy number loss of all BAC clones on chromosome X (ratio = 0.77 ± 0.012) (using a reference female DNA). DMD DNA sample had a copy number loss on chromosome X as showed by 3 BAC clones with a ratio = 0.58 ± 0.05. In Prader-Willi sample was evident the loss of copy number of 10 BAC clones (ratio = 0.71 ± 0.02) on chromosome 15. aCGH analysis of Smith-Magenis DNA showed a single copy loss of 5 BAC clones on chromosome 17 with a ratio = 0.78 ± 0.012. The aCGH results were in agreement with those obtained by the classical cytogenetic analysis, FISH analysis and DNA testing achieving an analytical sensitivity and specificity of 100% in the examined samples.

After array validation, 25 uncultured chorionic villus samples obtained from foetuses at risk for chromosomal aberrations were analyzed. 24 out of 25 examined samples did not show any chromosomal abnormalities in the analyzed regions (Fig. 3). In one case, with abnormal ultrasound findings, a trisomy 18 was detected (Fig. 4). aCHG analysis of the sample had a single gain in copy number of clones on chromosome 18 that was represented by 7 BAC clones with a ratio = 1.35 ± 0.04. The presence of this aneuploidy was confirmed by karyotype analysis. Cytogenetic analysis is an important component of prenatal diagnosis allowing the identification of aneuploidy and unbalanced structural rearrangements in foetuses including and/or one of the following: advanced maternal age, abnormal serum screening results, a high-risk for cytogenetic abnormalaties based on family history, abnormal ultrasound findings. Conventional karyotype analysis of banded chromosomes is still considered the gold standard method in prenatal diagnosis. Although highly reliable for identifying aneuploidies as well as large chromosomal rearrangements, its resolution size (5-10 Mb) significantly limits this technique. (reviewed in 19). In addition, the chromosomal origin of extra structurally abnormal chromosomes (ESACs) could not be revealed and microscopic rearrangements of subtelomeric regions cannot be detected (20, 21). Furthermore, the average time to receive the analysis results (approximately 14 days) represents another significant limitation. Molecular cytogenetic techniques were developed to overcome the resolution limitations of karyotype analysis, as well as reducing the average reporting time for a rapid screening of common aneuploidies (i.e. trisomy 13, 18 and 21, triploidy and aneuploidy of the sex chromosomes) (FISH and QF-PCR analyses) and MLPA (7-9). Although these techniques do not provide a genome wide screen, they are not likely to replace conventional karyotyping. In order to investigate structural chromosomal rearrangements associated with copy number variation in a genome wide scale, comparative genomic hybridization (CGH) has

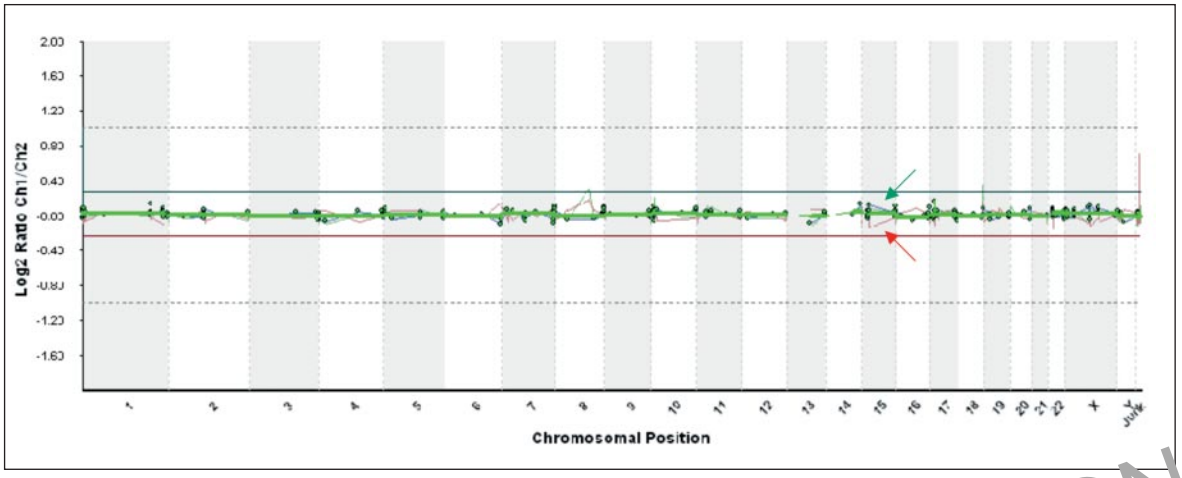

Figure 3 - MDTelArray linear plot showing normal results for all chromosomes. The thin green line represents the fluorescence intensity ratios between unaffected foetus (labbelled with Cy3) and control (labelled with Cy5) (green arrow). The thin red line represents the fluorescence intensity ratios obtained from a second hybridization in which the dyes have been reversed (control Cy3: unaffected foetus Cy5).

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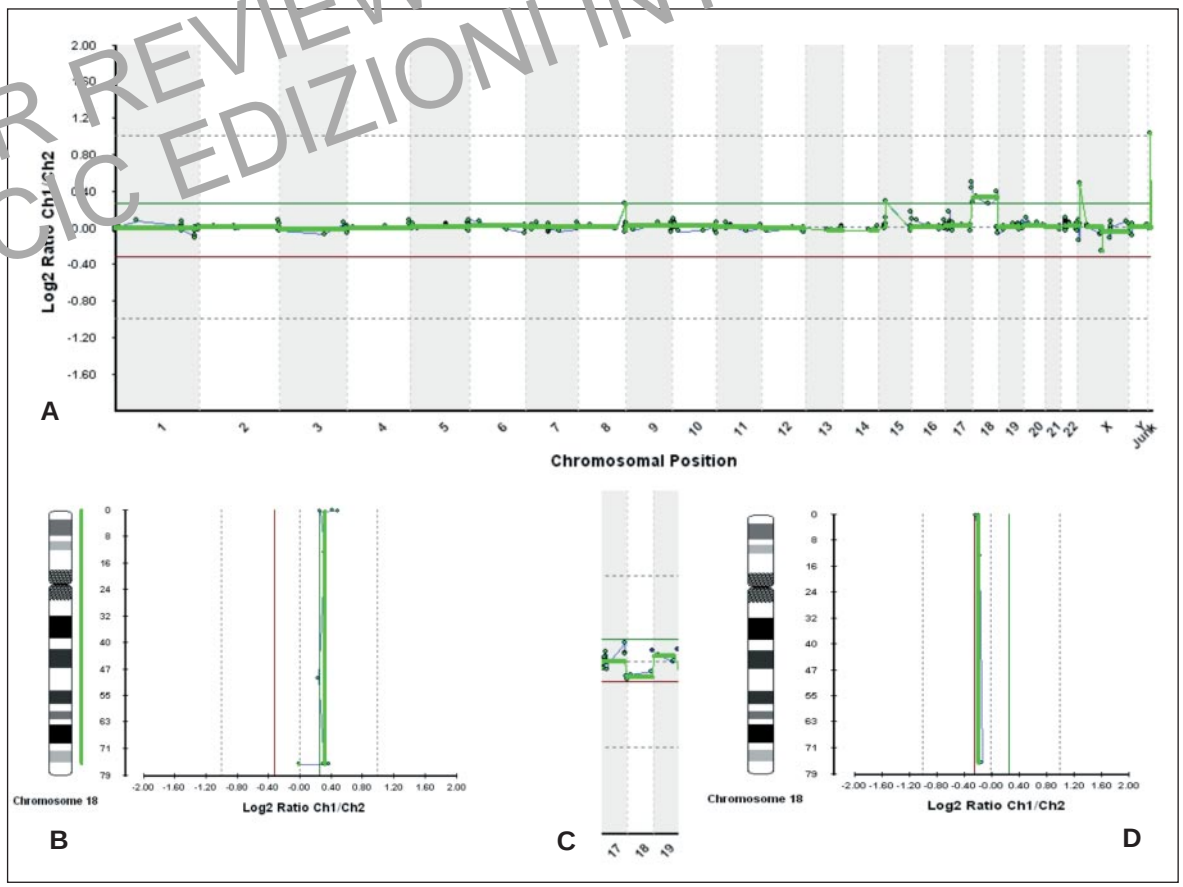

Figure 4 - (A) MDTelArray linear plot showing the trisomy of chromosome 18 (affected foetus DNA Cy3: control Cy5). Duplication is evident as a deviation from a $\log_2$ ratio of 0. (B) Chromosome 18 analysis showing fluorescence intensity ratios between affected foetus DNA (labbelled with Cy3) and control DNA (labelled with Cy5). (C) Partial of linear plot of the reverse experiment showing fluorescence intensity ratios between control DNA (labbelled with Cy3) and affected foetus DNA (labelled with Cy5). (D) Chromosome 18 analysis showing fluorescence intensity ratios between control DNA (labbelled with Cy3) and affected foetus DNA (labelled with Cy5).

been introduced to assist prenatal diagnosis (22-24). However, CGH still requires metaphase chromosomes as targets for hybridization, limiting its resolution to approximately 5 Mb, like microscopic karyotype analysis (25).

Microarray-based comparative genomic hybridization (aCGH) is a recently developed technology that evolved from standard CGH on metaphase spreads. aCGH uses arrayed DNA sequences instead of metaphase chromosomes as target of hybridization, thus providing a direct link between detected aberrations and the physical and genetic maps of the human genome. Its resolution is limited only by the size of the target and the density of these clones. This technique is able to detect any dosage imbalances including aneuploidies, deletions or duplications, but it can not detect balanced rearrangements such as reciprocal and Robertsonian translocations or inversions. The primary advantage of aCGH over conventional cytogenetics and FISH analysis is its ability to detect DNA copy number changes simultaneously at multiple discrete loci in a genome. Moreover, aCGH offers rapid, high throughput analysis on minimal amounts of DNA, two prerequisites for any platform applied to prenatal diagnosis. In fact, its utility to identify chromosomal imbalances in prenatal samples has been recently reported (26-29). However whole-genome arrays could generate data that are difficult to interpret and that are subject to multiple FISH verifications per patient. Furthermore, the array content must be well considered before its application in prenatal testing. Copy number variations (CNVs) of genome regions not previously associated to "chromosomal phenotype" may be difficult to interpret. In addition, recent studies have shown that familiar DNA gains and losses across the genome are numerous and common (30, 31). CNVs may have no clinical significance if equally detected in phenotipically normal and abnormal individuals or have clinical importance, especially if identified as de novo in a person with abnormal phenotype. The remaining CNVs may have some clinical implications, but it will be necessary to observe the same alterations in more abnormal cases to understand their clinical relevance (31-33). Depending on the resolution and representation of the genome of the microarray, CNVs may be frequently found, thus complicating the interpretation of results. Thus, microarray design is very important to obtain high quality results.

In contrast to whole-genome aCGH, targeted arrays constructed with clones mapping within chromosome regions frequently involved in genomic disorders or subchromosomal anomalies have been developed (34, 15, 35). These arrays have been used in the clinical diagnosis of chromosome abnormalities in children with birth defects, mental retardation, or developmental disabilities (16, 36, 37), however prenatal uses are limited at present (38).

We present a preliminary study on validation and utilization in prenatal diagnosis, of a targeted array able to reveal specifically 34 critical chromosomal regions involved in microdeletions and/or microduplications that cause well known syndromes. The array contained also 41 subtelomeric regions target of mental retardation and learning and developmental disability. All aCGH results agreed with those obtained by karyotyping or FISH analysis or DNA testing, detecting both aneuploidies and chromosomal microdeletions. Even if we analyzed only a restricted number of samples, our results confirmed the possibility to use this kind of aCGH in prenatal diagnosis, minimalizing the difficulties of good interpretation of results. Furthermore, applying aCGH technology on genomic DNA isolated from uncultured chorionic villus samples we obtained the same results as obtained by DNA isolated from cultured cells. Thus, the time taken to report results back to patients could be significantly reduced. We are confident that this targeted array improves the analysis of segmental aneuploidy in uterus and suggests that it may prove feasible to introduce aCGH as part of the diagnostic armamentarium for detecting chromosomal rearrangements which would not be otherwise investigated in routine prenatal cytogenetic analysis.

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Acknowledgements

We thank Dr. G. Galluzzi for the gift of DMD DNA sample, Dr. Antonio Novelli for his help and suggestions, the Technogenetics Srl, Bolty Group, Sesto S. Giovanni, Milan, Italy, for the opportunity to use the MDTelArray. This work was supported in part by a grant from the Italian Ministry of Health and Italian Ministry of University and Research.

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