MICROARRAY-BASED COMPARATIVE GENOMIC HYBRIDIZATION (aCGH) – BETWEEN BASIC RESEARCH AND CLINICAL DIAGNOSTIC

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Abstract: In the vast spectrum of human pathology, a significant proportion is represented by genetic disorders. To elucidate the mechanisms leading to disease, various approaches have been used: G-banded karyotyping and FISH allow the survey of the entire genome for large aberrations or analysis of pre-defined segments, while sequencing detects nucleotide alterations with the prior requirement of knowing which DNA segment to address. The last two decades have seen the rise of another generation of investigative methods, such as aCGH, which inquire the condition of the whole genome at sequence level; starting as a research instrument, aCGH is increasingly regarded as a powerful diagnostic tool for clinical use. As an example of its utility in the diagnostic of mental retardation, we present three cases where aCGH contributed to the identification and refinement of the precise genetic aberrations.

INTRODUCTION

Array comparative genomic hybridization (aCGH, also called molecular or virtual karyotyping) was first described in 1992 as a genome-wide screening method of copy number variations (CNVs) and was initially used as a tool for the investigation of human neoplasms (Shinawi and Cheung, 2008, Wicker *et al.*, 2007). Basically, in aCGH, equal amounts of fluorescently labeled, enzymatically digested genomic DNA from a test (patient) and, respectively, a reference sample are cohybridized to an array consisting of a predefined number of DNA probes distributed in spots onto a slide support. The reference is usually represented by pooled genomic DNA, collected from a variable number of individuals. The two samples are differentially labeled, in red and, respectively, green. The cohybridization is done in two steps: in the first one, test and reference samples are mixed and denatured together (in the presence of Cot I DNA, to block repetitive sequences); subsequently, the mix is distributed onto the array and subjected to hybridization with the probes. According to the amount of red or green labeled DNA which forms complexes with the probes, each spot will have different fluorescence intensities, which will be measured and quantified by a scanner; the numerical data thus obtained will be analyzed by specialized software, translating and associating the raw numbers into variations of the copy numbers of certain chromosomal region. In other word, the green:red ratio indicates a gain or a loss of the test genetic material, at a particular genomic region (Shinawi and Cheung, 2008).

The actual set of probes used in the technique varies according to the researcher's interest. Presently, there are several commercially-available types of grids, designed to investigate preponderantly certain genomic regions, while custom-made grids can be obtained as well. The probes are segments of human DNA in the form of different artificial chromosomes (e.g. BAC, P1), cosmids or fosmids, or simply oligonucleotides printed directly onto the slide support. The resolution of the technique depends on the type/length and the spacing of the probes within the genome and can reach up to 200 pb (Urban *et al.* 2006).

In addition to the relative simplicity of the technique, its potential for automation and its survey of the whole genome, the precise mapping of quantitative aberrations make it a powerful tool in the investigation of the amplifications and deletions of the genetic material that, if small enough, could be otherwise omitted by classic methods. For instance, high-resolution G-banding allows detection of alterations of 3 Mb in size, while surveying the whole genome; FISH techniques go beyond this limit, to detect microdeletions, but usually address only to specific regions. From this point of view, aCGH covers the whole genome, at resolutions which can reach hundreds of base pairs. However, there are several limitations of aCGH. The main drawback is its inability to identify balanced rearrangements such as inversions and translocations. Moreover, in cases where samples contain genetically heterogeneous cell populations, the analysis may be difficult. Sometimes, the results may be difficult to gauge in the light of clinical relevance. And at last, but not at least, its high cost may overshadow its performances.

In the present paper, we present three cases where aCGH contributed to the elucidation of the genetic defect beyond the diagnostic of mental retardation (MR).

MATERIALS AND METHODS

The three patients were recommended for genetic investigations consecutive to clinical examination showing MR and dysmorphic features. Informed consent was obtained from legal guardians. In a first stage, cytogenetic analysis was performed on phytohemagglutinin-stimulated peripheral blood culture followed by standard GTG-banding technique. Metaphase images were acquired with an Axio Z1 Zeiss microscope (*Carl Zeiss GmbH*) equipped with Metafer and Ikaros softwares for scanning, capture and karyotyping.

Case no. 1 is that of a 6 year-old boy, the first child of non-consanguineous parents, born after uneventful pregnancy. His birth weight was small-for-date (2500 g) and he had nourishing difficulties in the first months of live, failure to thrive, and a retarded neuropsychomotor development. At presentation, he showed severe MR, hyperkinesia and autistic features, as well as dysmorphic features (large forehead, malformed ears, partial ptosis, open fish-shape mouth, micrognatia). Cytogenetic investigation by GTG-banding showed a normal karyotype.

Case no. 2 is a 5 year-old boy, the first child of non-consanguineous parents, born after an uneventful pregnancy and delivery, with a birth weight of 3500 g and an Apgar score of 9. Subsequently, he presented a delayed psychomotor development. At admittance in the Department of Neurology, he showed dysmorphic facial, limb, and body features (square facies, broad forehead, short palpebral fissures – especially at the left eye, epicanthic folds, broad nasal bridge, short philtrum, low set, malformed ears; short fingers; the 5th toe shorter and covered by the 4th toe; small penis and scrotal abnormalities, cryptorchidism; sacral sinus). In addition, he was severely retarded in the psychomotor and speech development, and exhibited deafness and bilateral pyramidal syndrome. Paraclinical investigations indicated a left cerebellum subarachnoidian cyst, large cisterna magna, moderate frontal atrophy, bilateral sclerosis of ear bone chain and atrial septal defect. GTG-banding indicated a deletion at 3q14 (Budisteanu M., personal communication). Nevertheless, the panel of clinical and genetic findings did not fit to the well-known 3p- syndrome implying a deletion at 3p25-pter (Phipps *et al.* 1994, Shuib *et al.* 2009), but rather to the more recently proposed 3p12 interstitial deletion syndrome (Lalli *et al.* 2007, Neri *et al.* 2009).

Case no. 3 is a 6 month-old girl, the 11th offspring of non-consanguineous parents. Her mother's age at birth was 42; pregnancy and birth were uneventful. The girl presented facial (hypertelorism, almond eyes, flattened nose, protruding tongue, small, malformed ears – especially the right ear) and limb abnormalities, severe psychomotor retardation and growth failure, muscular hypotonia, congenital cardiac malformation. The clinical diagnostic of Down syndrome was confirmed by cytogenetic analysis; however, a marker chromosome was also noted in her karyotype, smaller than the chromosomes of group F. FISH excluded the origin of the marker chromosome as follows: in 15, 19, and 21 pairs (by whole chromosome painting); in 17p13, 18p11.32, 22q11, 22qter regions (by hybridization with locus-specific probes); in centromeric regions of 1, 5, 18, 19, X, and Y chromosomes (by hybridization with α-satelite probes) (Lungeanu A., personal communication).

Subsequently, gDNA was isolated from hemolyzed peripheral blood collected on EDTA, using DNeasy Blood&Tissue Kit (*Qiagen*). Human DNA (*Promega*) of appropriate sex was used as control. Enzymatic labeling, hybridization, scanning and analysis were carried out according to Agilent Oligonucleotide Array-Based CGH for gDNA Analysis protocol (*Agilent Technologies*), following spectrophotometric and electrophoretic assessment of sample gDNA concentration and quality. Basically, double restriction digestion was carried out with *Alu*I and *Rsa*I (*Promega*) in standard conditions. Subsequently, the digested DNA was labeled with cyanine 5-dUTP (sample) and cyanine 3-dUTP (control), using Agilent Genomic DNA Enzymatic Labeling Kit. Clean-up of the labeled DNA was done with Microcon YM-30 filters (*Millipore*) and its concentration and fluorochrome incorporation were assessed with a Nanodrop ND-3300 UV-VIS Spectrophotometer (*Thermo Scientific*). Samples that met the yield and fluorochrome specific activity criteria were prepared for hybridization using Agilent Oligo aCGH Hybridization Kit and Human Cot-1 DNA (*Roche*). Next, the mixed sample and control DNA was cohybridized with the oligonucleotides printed in array, for 24 hrs, at 65°C. After thorough washing as recommended by the above-mentioned Agilent protocol, the arrays were scanned with Agilent DNA Microarray Scanner with Surescan and Agilent Scan Control Software A.8.1.3. The raw data were extracted with Feature Extraction v.10.7.3.1 and analyzed with Genomic Workbench v5.0 softwares (*Agilent Technologies*), using ADM-2 algorithm (other parameters: ADM-2 threshold 6.0, fuzzy zero on, centralization threshold 6.0, default aberration filters).

RESULTS AND DISCUSSION

The three cases were referred for aCGH in order to further investigate their genetic make-up after the primary diagnosis by GTG-banding.

In case no. 1, the DNA array indicated a normal karyotype except for a duplication of the long arm subtelomeric region (q28) of chromsome X (152824755-153262507), less than 1.5 Mb in size, therefore well below the limit of detection of GTG-banding (Fig. 1A). The deletion at 3p in case no. 2 spanned between 65317864-77902945 (p12.3-p14.1, 12.5 Mb) and it was the single alteration detected in the karyotype (Fig. 1B). In case no. 3, the trisomy 21 was confirmed and, in addition, a duplication of 0.2 Mb at 17q21.31 (41566340-41700962) was detected; however, the size of the duplication did not match the size of the observed marker chromosome (Fig. 1C). The loci detected as copy number variations (CNVs) in the investigated subjects are listed in table 1.

Table 1. CNVs detected in the investigated subjects.

Case no.	Genes
1	*AVPR2, ARHGAP4, ARD1A, RENBP, HCFC1, TMEM187, IRAK1, MECP2, OPN1LW, OPN1MW, OPN1MW2, TEX28, TKTL1, FLNA, EMD*
2	*MAGI1, SLC25A26, LRIG1, KBTBD8, SUCLG2, FAM19A1, FAM19A4, C3orf64, TMF1, UBA3, ARL6IP5, LMOD3, FRMD4B, MITF, FOXP1, EIF4E3, GPR27, PROK2, RYBP, SHQ1, GLT8D4, PPP4R2, FLJ10213, PDZRN3, CNTN3, FAM86D, FRG2C, ZNF717, ROBO2*
3	*KIAA1267*

When comparing these data with those in two public databases (Database of Genomic Variants (DGV), Copy Number Variation project at Children’s Hospital of Philadelphia (CNV-CHOP)) which report CNVs detected in healthy subjects, part of the loci listed for case no. 1, namely *EMD, FLNA, IRAK1, OPN1LW, OPN1MW, OPN1MW2, TEX28, TKTL1* were found both as gains and losses (additions and deletions of genetic material, respectively); therefore, these genes may not be involved in the MR-related pathology. However, the duplications at Xq28 are associated with autistic features and non-syndromic MR; particularly, alterations in *MECP2* gene are described in Rett syndrome or as a specific phenotype (Lugtenberg *et al.* 2009, Meins *et al.* 2005). In our case, the clinical differential diagnosis excluded Rett syndrome, while other *MECP2*-related phenotypes are yet to be systematized. Considering the duplication of *MECP2* sequence, beside several other loci with still unknown potential, it can be concluded that the alteration occurring at Xq28 band may responsible for this patient’s phenotype.

Regarding the case no. 2, the 3p14 region is known to contain a fragile site and multiple genes that may be involved in oncogenesis. Nevertheless, an interstitial deletion of the proximal 3p was proposed recently as a syndrome (Lalli *et al.* 2007, Neri *et al.* 2009) and there are several reports citing this aberration in connection with MR. The reported phenotypes include square facies, broad forehead, broad nasal bridge, low set ears, clinodactyly, hearing impairment, heart defect, and genital hypoplasia (Morales *et al.* 2009, Naritomi *et al.* 2007, Wieczorek *et al.* 1998) which we encountered in our subject as well, together with the MR. Moreover, the deleted region precisely includes genes that may play a role in neurodevelopment (*ROBO2, PDZRN3, CNTN3*) and that, due to deletion, may be a cause of the observed delay in the patient. Hearing impairment might be linked to the heterozygotic loss of *MITF* gene, encoding a transcription factor that ultimately regulates inner ear development, among several developmental processes (NCBI database).

In the case no. 3, while the trisomy 21 was confirmed, the small duplication at 17q21.31 including only the locus *KIAA1267* was found among the CNVs presented in healthy subjects (DGV, CNV-CHOP). Despite the association of both microdeletions and duplications of the 17q21.31 region with autistic-spectrum disorders (Grisart *et al.* 2009, Kirchhoff *et al.* 2007), the discrete loci involved were different (*MAPT, CRHR*) than in our subject. Consequently, here, this particular polymorphism did not play any particular role in MR. However, it may account for the

supplementary genetic material on the small marker chromosome (SMC); aCGH failed to supply further information regarding the origin of the SMC, probably due to its heterochromatic content. Supplemental C-banding and FISH analyses may uncover its origin.

CONCLUSIONS

These three cases illustrate the use and utility of the modern technology of aCGH in the investigation of the genetic causes of MR. The first case shows how screening the genome at high resolution may uncover defects that classical methods overlook, thus providing a way to clarify diagnosis. In the second case, aCGH brought supplemental information concerning the deleted fragment, which completes the clinical panel for a newly proposed syndrome – proximal 3p deletion. Nevertheless, in the third case, while completing the information regarding the patient, aCGH could not fully elucidate the genetic make-up. It may be concluded that, despite inherent limitations, aCGH is a useful instrument of genetic analysis, especially in non-syndromic MR. It not only provides details for clinical research, but also is an extremely powerful diagnostic tool.

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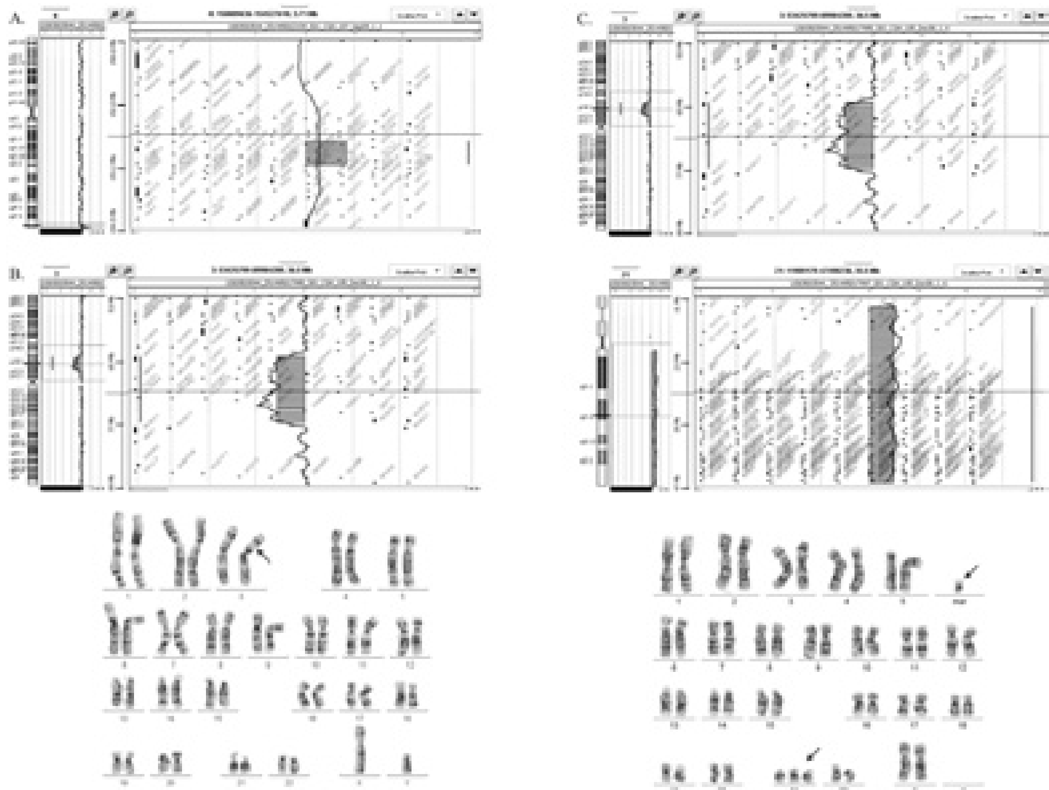

Fig. 1. aCGH profiles and karyotypes of the investigated patients. A. aCGH profile of case no. 1. B. aCGH profile and karyotype of case no. 2 (arrow: deletion at p12.3-p14.1). C. aCGH profiles (chromosomes 17 and 21, respectively) and karyotype of case no. 3 (arrows: SMC, trisomy 21).