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REVIEW ARTICLE

# ARRAY-BASED COMPARATIVE GENOMIC HYBRIDIZATION APPLICATION FOR REVEALING GENOMIC MICRO IMBALANCES IN CONGENITAL MALFORMATIONS

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## ABSTRACT

Birth defects affect 3-5% of live births and are a major cause of fetal, neonatal and infant morbidity and mortality in all industrialized countries. Some 40-60% of congenital physical anomalies in humans have no cause, 20% that seem to be multifactorial, 10-13% environmental and 12-25% genetic.

Classical cytogenetic or common comparative genomic hybridization (CGH) methods have limited use in investigation of the whole genome because of their low resolution (5-10 Mb). Fluorescence *in situ* hybridization (FISH) and quantitative fluorescence polymerase chain reaction (QF-PCR) have higher resolution but do not allow genome-wide screening and require some prior knowledge regarding the suspected chromosomal abnormality and its genomic location.

Because of these limitations, the impact of genetic micro imbalances as etiological factors for the development of congenital malformations (CM) is underestimated. Array-based techniques have enabled higher resolution screens for genomic imbalances in CM as they permit identification of micro aberrations with a size between 60 bp and several hundred kilobases. They make possible screening of the whole genome and detection of novel unbalanced micro structural rearrangements in a single reaction and also effective screening of new dose-dependent genes. In addition, the application of the aCGH technology has the potential to improve our understanding of the normal quantitative variants of the human genome.

**Key words:** Array comparative genomic hybridization (aCGH); Copy number variations (CNVs); Congenital malformations (CM); Micro imbalances

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## INTRODUCTION

**Prevalence.** Genetic disorders and congenital abnormalities (also called birth defects) affect between 3 and 5% of the live-births in Europe [1] and in the United States [2]. Congenital anomalies (CA) or birth defects are defined as the presence, at birth, of structural, functional and/or biochemical-molecular defects, irrespective of whether they have been detected at that time or not. Congenital malformations (CM), which comprise 60% of all CA, are the major group. A CM is a physical congenital anomaly that is deleterious, *i.e.,* a structural defect perceived as a problem. A typical combination of malformations affecting more than one body part is referred to as a malformation syndrome. Some of the congenital defects are genetic in origin and are referred to as "genetic disorders."

Congenital malformations are a major cause of fetal, neonatal and infant morbidity and mortality in all industrialized countries [3,4]. They are the fifth leading cause of mortality. Twenty percent of infant deaths are attributed to CM; in developed countries, the percentage has increased over time. The morbidity and disability experienced by surviving children also have a major impact on public health [3,4]. Ap-

proximately 25% of the number of pediatric hospital admissions and one-third of the total number of pediatric hospital days are attributed to different types of CM [5,6]. This is associated with enormous costs for medical care and creates heavy psychological and emotional burdens for the affected individuals and/or their families.

Congenital malformations involving the brain comprise the largest group with prevalence of 10/1,000 live births, compared to congenital heart disease (8/1,000), urinary tract (4/1,000) and limb anomalies (1/1,000). The remaining types of CM have a combined prevalence of 6/1,000 live births. Congenital heart anomalies are responsible for 28% of infant deaths related to CM; central nervous system malformations account for about 12% of infant deaths, while chromosomal and respiratory system abnormalities each account for 15% of infant deaths [1].

**Classification.** Congenital malformations can be divided into three groups: ***1) Lethal*** if the defects (such as anencephaly or hypoplastic left heart syndrome) cause still births (late fetal death), infant death or the pregnancy is terminated after prenatal diagnosis of fetal defects in more than 50% of cases. ***2) Severe*** if the defects (such as cleft lip or congenital pyloric stenosis) which, without medical intervention, cause handicap or death. ***3) Mild*** if defects (such as congenital dislocation of the hip or undescended testes) require medical intervention but life expectancy is good.

Lethal and severe defects together constitute major congenital abnormalities. Minor anomalies or morphological variants (such as epicanthal folds, ocular hypotelorism, preauricular tags and pits, low-set ears, simian crease, clino- and camptodactyly, partial syndactyly between toes 2 and 3, hydrocele, umbilical hernia, sacral dimple, *etc.*) without serious medical or cosmetic consequences, are excluded from the category of congenital malformations [7]. While minor anomalies in themselves do not greatly affect the child, they can be associated with major anomalies or be indications of certain syndromes [8,9].

**Etiological Classification of Congenital Malformations [10].** ***1)*** Microscopically visible, unbalanced chromosome abnormalities. ***2)*** Submicroscopic chromosome abnormalities including microdeletions, uniparental disomy and imprinting mutations. ***3)*** Teratogens and prenatal infections. ***4)*** New dominant mutations. ***5)*** Familial disorders not included as a new dominant mutation. ***6)*** Recognized non familial, non chromosomal syndromes. ***7)*** Isolated anomalies.

For 20% of the CM there seems to be a "multifactorial" cause, meaning a complex interaction of multiple minor genetic abnormalities with environmental risk factors. Another 10-13% of CM have a purely environmental cause (*e.g.,* infection, illness, medication or drug abuse in the mother). Around 12-25% of CM have a genetic cause. The etiology of CM is not always clear. Some 40-60% of CM have no known cause.[11-13].

**Genetic Methods for Determining the Etiology of Congenital Malformations.** The information provided by methods such as fluorescence *in situ* hybridization (FISH), quantitative fluorescence polymerase chain reaction (QF-PCR), MLPA (multiplex ligation-dependent probe amplification) or classical cytogenetics on etiology of CM is limited, since the majority of these cases do not.detect micro structural genomic imbalances [14]. Array-based techniques have enabled higher resolution screens for genomic imbalances and permit identification of micro structural aberrations between 60 bp and several hundred kilobases in size that are identified only by the size and density of the sequences spotted on the microarray. Whole genome screening and detection of novel unbalanced micro structural rearrangements are possible in a single reaction [15].

In the array comparative genomic hybridization (aCGH) method, hybridization of DNA takes place on an array of mapped DNA clones rather than metaphase chromosomes and leads to "molecular karyotyping" rather than conventional karyotyping [16,17]. It can be carried out on DNA from single cells, from chorionic villus cells and from amniocytes. Molecular karyotyping has doubled the detection rate of pathogenic chromosomal imbalances by increasing the resolution level from 5 Mb (with conventional karyotyping) to as low as 100 kb.

In a study of spontaneous miscarriages, aCGH detected all abnormalities previously identified by microscopic karyotype analysis and additional abnormalities in some 10% of cases [18]. In 98 pregnancies (56 amniotic fluid and 42 chorionic villus sample specimens) complete concordance of array results was found for direct and cultured cell analyses in 57 cases tested by both methods [19]. Bar-Shira *et al.* [20] screened eight patients with multiple CA, mental

deficiency and dysmorphic features by aCGH. In two previously undiagnosed cases, they detected chromosomal micro alterations. Thienpont *et al.* [21] found by aCGH that 30% of patients with congenital heart defects had imbalances that were not described in phenotypically normal individuals. Menten *et al.* [22] reported 20% submicroscopic chromosomal imbalances, detected by aCGH, in a series of 140 patients with idiopathic multiple congenital malformations and mental retardation having normal karyotypes.

Array CGH appears to be more sensitive for detecting mosaicism than conventional cytogenetic methodologies. One expalanation is that if mosaicism is not suspected on the basis of the clinical findings, the number of metaphases counted may be insufficient to detect the mosaicism. Another explanation is that since the chromosome analysis relies on stimulated cells, the aneuploid cells may be under represented in the cell population [23]. In a study of 2,585 samples, chromosomal mosaicism was detected by aCGH in 12 patients, 10 of whom were reported to have normal chromosomes in blood cells [24]. Ballif *et al.* [25] reported 18 cases of mosaicism detected by aCGH in a routine diagnostic setting. In all cases, FISH confirmed the mosaic chromosome abnormalities, showing that the percentage of abnormal cells in unstimulated cultures was, in some cases, different from that found in PHA-stimulated cells. Thus, aCGH based on direct extraction of genomic DNA from uncultured peripheral blood, may be more likely to detect low-level mosaicism than traditional cytogenetic techniques [25].

We have used aCGH to screen for micro structural whole genome copy number changes in five patients with CM and normal karyotypes. Underlying unbalanced micro structural aberrations were found in two patients with CM, in one a low level mosaicism form of the deletion 18q21.1-q23 and in the other a 1p36 monosomy [26].

It was found that over 12% of the human genome includes submicroscopic benign copy number variable regions [27]. Array CGH has revealed frequent imbalances associated with clinical syndromes, but also a large number of copy number variations (CNVs) - large segments of DNA, ranging in size from thousands to millions of DNA bases with variations in the copy number. Some of these variations may represent risk factors for particular clinical anomalies. A CNV is operationally defined as a DNA segment, longer than 1 kb, with a variable copy number compared with a reference genome. This definition may not be useful in deciding the clinical impact of certain genomic imbalances. Copy number variations may be categorized into those that are likely to be benign (polymorphic), those that are likely to be pathogenic and those of unknown clinical significance [28-30].

Factors that influence the risk contribution of a CNV: if the genomic imbalance is found in the affected individual and in a healthy parent, it is more likely to be a benign CNV. To determine which imbalances are pathogenic, one could simply tabulate all those observed in an individual's aCGH test, disregard CNVs found in normal individuals and consider the remaining copy number changes as potentially pathogenic. The potential clinical relevance of a CNV increases in proportion to the number of genes within the region of genomic imbalance. As it is generally thought that duplications are better tolerated in the genome than deletions, deletion CNVs have a higher likelihood of being pathogenic.

Of 14 patients with a syndrome of aplasia of the Müllerian ducts, urinary tract anomalies, cardiac and skeletal defects, hearing impairment, and mental retardation, four had cryptic genomic alterations; all of which were independently ascertained and did not overlap. There were two duplications in one and three different deletions in the other three patients [31].

Of 49 fetuses with multiple malformation and normal karyotypes investigated by aCGH, eight had genomic rearrangements (16.3%). These included: subtelomeric deletions, interstitial deletions, submicroscopic duplications and multiplex genomic imbalance [32].

Array CGH is often used as a primary genetic screening method for diagnosis and research. The technique can detect pathogenic submicroscopic chromosomal imbalances in patients with developmental disorders. Most patients carry different chromosomal anomalies, which may be spread across the whole genome. These imbalances locate genes that are involved in human development. This is important for phenotype/genotype correlations and for the identification of genes. Since most imbalances encompass regions harboring multiple genes, the challenge is: ***1)*** to identify those genes responsible for the specific phenotype, and ***2)*** to disentangle the role of the different genes located in an imbalanced region. The high resolution of aCGH makes it a basic research

instrument. It helps in defining and refining the critical regions for a disease or a phenotype. This has led to a dramatic increase in gene identification through molecular karyotyping; it is likely that the function of many more genes will be identified in this way.

The ascertainment of unbalanced genomic micro aberrations through aCGH in syndromic patients may lead to the description of new syndromes and to the recognition of a broader spectrum of features for already described syndromes [22,28,33-38]. Array CGH is a rapid and reproducible procedure that provides reliable results in 5 days. It may develop into an excellent tool also for prenatal genetic diagnosis and holds promise for more accurate genetic counseling and reproductive risk assessment.

Hereditary diseases and CA occupy the third place in the morbidity structure of newborns in the neonatal period (11.4%). Congenital anomalies are ranked first in the infant mortality structure (40% of cases). In all industrialized countries, large scale programs for prevention of congenital anomalies have been developed.

Prevention approaches are often classified into three levels: ***1) Primary*** prevention: avoiding the cause(s) of congenital abnormalities, *e.g.,* rubella vaccination or periconceptional folic acid/multivitamin supplementation. ***2) Secondary*** prevention: early detection followed by effective early treatment, *e.g.,* congenital dislocation of the hip, also undescended testes. Previously, selective abortion/termination of pregnancy following the prenatal diagnosis of severe fetal defects, was also referred to as secondary prevention. Recently, the World Health Organization and other international bodies have excluded this approach from the term "prevention." ***3) Tertiary*** prevention: complete recovery of CM by early surgical intervention without residual defects or minimal after effects. Tertiary prevention allows the achievement of complete recovery in 33.5% of cases with CM.

The major proportion of CM (85.3%) are preventable; however, no single strategy for their prevention exists [7]. For these and for other reasons, prenatal diagnosis has long been recognized as an essential facet in the clinical management of the pregnancy itself, as well as a critical step toward the detection, prevention, and, eventually, treatment of genetic disorders. Array CGH offers new possibilities for prevention. It makes possible the genetic analysis of single cells; thus, it might give future opportunities for aneuploidy screening and detection of unbalanced translocations in preimplantation embryos [39].

## CONCLUSIONS

Investigations of individuals with multiple CM has shown that about 40% of these have apparently balanced chromosomal rearrangements and suggests that micro imbalances may be a common finding. The introduction of aCGH has doubled the detection rate of genomic micro imbalances in individuals with multiple CM or dysmorphism. Copy number variation in the human genome is now better understood and its implications in diagnosis and in genetic counseling are being rapidly uncovered. Submicroscopic chromosomal imbalances have been detected by aCGH in 10 to 30% of patients with otherwise unexplained CM. This method can also be used to make cytogenetic diagnosis more accurate and precise, since the size of a deletion and even the break points can be determined. This is important for the understanding of phenotype/genotype correlations and for the identification of disease-related genes. The application of aCGH in cases that are considered to have "balanced"translocations, could detect whether, in the vicinity of the breakpoints, the chromosomal material is deleted or duplicated (*i.e.,* is in an unbalanced form), or is truly balanced. In addition, the application of aCGH technology has the potential to improve our understanding of the normal quantitative variants of the human genome. Array CGH will have a major impact on pre- and postnatal diagnoses, genetic counseling and healthcare.

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