Is Array-Comparative Genomic Hybridization Useful for Children with Apparently Isolated Congenital Anomalies?

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ABSTRACT

To consider the utility of array-comparative genomic hybridization patients with apparently isolated congenital anomalies, 109 patients whose indication for testing was a birth defect without other concerns about syndromes or intellectual disability were selected from 1766 analyses conducted from 2009 to 2017. Twenty-nine had copy number variants more likely to contribute to the birth defect and another 80 were potentially contributory. Defects of the central nervous (23 patients), cardiovascular (20), digestive (19), and pulmonary (9) systems predominated with some high prevalence variants, traditionally reported as benign, recurring in particular anomaly cases. This small number of anomaly-associated copy number variants does not provide a definitive answer to the title question. However, the value of further studies is supported by consideration of the polygenic inheritance/multifactorial determination of congenital malformations, their derivation from common developmental fields, and their possible prediction by determination of copy number variant profiles.

Key Words: Array-Comparative Genomic Hybridization, Congenital Anomalies, Copy Number Variants, Microdeletion/duplication, Chromosome Disorders

1. INTRODUCTION

Multiple congenital anomalies have been a cardinal indicator for chromosome studies, whether obvious in devastating 13/18 trisomies or more subtle in patterns of major and minor anomalies exemplified by Down syndrome.\(^1\) When DNA technology allowed detection of small aneuploid segments, first through targeted fluorescent in situ hybridization (FISH) and then by genomic array-comparative genomic hybridization-microarray analysis (aCGH), subtle chromosome changes were sometimes detected in apparently isolated congenital anomalies like costovertebral dysplasia.\(^2\) As genomic screening became routine and affordable, dysmorphology diagnoses based on defect pattern were
supplemented and substantially replaced by specifying particular microdeletions or duplications. In similar fashion, the minimal facial changes of many with DiGeorge/Shprintzen velocardiofacial syndrome, especially challenging for prenatal detection, were replaced by use of a prevalent major anomaly—the cardiac defect—as the indication for chromosome 22q11 deletion testing. Extension from targeted 22q11 FISH to aCGH for any child with a major cardiac anomaly can be justified by considerable yields of DNA and copy number variants (CNVs) when companion Next Generation/Whole Exome Sequencing (NGS/WES) and/or aCGH are employed.

Here, we evaluate aCGH findings in 109 children with apparently isolated congenital malformations selected from 1766 analyses performed over 9 years. We suggest that attention to all aCGH changes, including combinations of those deemed benign and/or pathogenic, may give insights into the evaluation and prevention of congenital anomalies.

2. METHODS

The Texas Tech Health Science Center Cytogenetic Laboratory follows Agilent Technologies protocols for aCGH consisting of DNA extraction from whole blood using the Maxwell 16 DNA extraction instrument and corresponding blood DNA extraction kit. DNA concentration is measured by the NanoDropND-1000 spectrophotometer and its quality analyzed by agarose gel electrophoresis to exclude degradation or RNA contamination. Genomic DNA (0.5 μg) from experimental and gender-matched reference samples are labeled using the SureTag Complete Genomic Labeling kit, each incubated at 37°C for 2 hours in the presence of cyanine 5-dCTP (for the experimental sample) or cyanine 3-dCTP (for the reference sample). Labeled experimental and reference DNAs are pooled and incubated with human Cot-1 DNA as blocking agent. The labeled samples were applied to the Cytochip 60K oligonucleotide array slide, placed in a microarray hybridization chamber, hybridized for at least 24 hours at 65°C in a rotating hybridization oven, and washed according to protocol. The Cytochip 60K oligonucleotide array combines targeted and genome-wide array analyses, including high density coverage for clinically relevant deletion/duplication syndromes and telomeric or pericentromeric regions.

Array slides are scanned into image files using an Agilent Microarray Scanner (PN G2565BA), then quantified using Agilent Feature Extraction software. The text file outputs from quantitative analysis are imported into the Agilent Genomic Workbench Software v7.0.4.0 for copy number analysis. CNVs detected by aCGH are systematically evaluated for clinical significance by comparison with the internal laboratory and publicly available databases. CNVs of potential clinical significance and of size >100 Kb are confirmed by FISH analysis. Relevant probes are obtained commercially and hybridized to metaphase and/or interphase preparations from patient peripheral lymphocytes; analysis of 500 interphase and/or 15 metaphase cells used a fluorescence inverted microscope and digital FISH images were captured by Cytovision Software.

Results of aCGH are entered into a password-protected MS Excel database with IRB approval using laboratory numbers as patient identifiers (demographics and numbers are linked only in a separate, password-protected database). Prior analyses of Laboratory data compiled from 2009 to 2014 are supplemented here by data from 2014 to 2017.
3. RESULTS

Among aCGH results on 1766 patients were 109 listed in Table 1 with a single congenital anomaly as the indication for testing. The majority were infants (38%) or of age 1-2 years (52%) with 7.1% being prepubertal and 2.9% between 12 and 20 years. All patients had normal routine karyotypes and males (51%) slightly outnumbered females.

Each patient result is indicated by copy number variants (CNV) described by chromosome and band number, deletion (-) or deletion (+), and estimated size of aneuploid segment in kilobases—thus the 6p25.1(-)80 entry at the top of Table 1 indicates microdeletion at band 6p25.1 with a calculated maximal size of 80,000 base pairs or 80 Kb. The 1210 consecutive aCGH analysis tabulated previously⁴ provides a good sample size for identifying common variants that are likely benign, and these are indicated in italics in Table 1 (right column). For example, deletions at band 8p23.1 ranging from 39 to 1200 Kb were found in 290 of the 1210 patients, while duplications at that band—8p23.1(+-)- ranged from 39 to 1128 Kb in 64 patients; these respective 24% and 5.4% frequencies support data built in to the analysis software¹⁰ that classifies them as benign variants from the perspective of syndrome/intellectual disability diagnosis.

The CNVs listed in regular print either have a low prevalence in the test population or are of atypical size for variants at that locus. These 29 patients (middle column, Table 1) had CNVs that are more likely contributors to birth defect predisposition and include 8 with combinations of benign CNVs (from the syndrome perspective) that through joint interaction might also be more likely to contribute to developmental anomalies. These individually benign variants are listed in italics with a “/” separator, their potential impact in combination fitting with the polygenic/multifactorial determination of birth defects emphasized in the Discussion.

Above each organ system or region are listed the total number of patients with aCGH results and those with variants that are more likely to cause birth defect predisposition. Added to the 29 patients with less common variants (middle panel Table 1) are 80 (right column) with variants of prevalence greater than 1%.⁴ The predominance of central nervous (23 patients), cardiovascular (20 patients), digestive (19 patients), and pulmonary (9 patients) system birth defects is not surprising given the medical and/or surgical significance of these anomalies, also true of patients with defects affecting the skeleton (9 patients) or facial region (9 patients). It is important to realize that many other patients with major anomalies and microarray studies are not listed in Table 1 because their indications for testing were to exclude a syndrome—thus patients with heart defects tested because of possible velocardiofacial syndrome or those with duodenal atresia with the indication of confirming Down syndrome are not listed here. As an example, among the 1766 patients were 11 who had the standard 22q11(-) microdeletion⁵ after aCGH because of possible velocardiofacial syndrome, 8 of them with documented heart defects.
Table 1. Microarray changes associated with apparently isolated congenital anomalies

<table>
<thead>
<tr>
<th>Central nervous system (23 patients, 5 more likely contributing variants)</th>
<th>Encephalocoele</th>
<th>Holoprosencephaly</th>
<th>Hydrocephalus</th>
<th>Microencephaly</th>
<th>Spina bifida</th>
<th>Miscellaneous*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6p25.3(-)80</td>
<td>8p23.1(+)/+46</td>
<td>16p11.2.1(+)246</td>
<td>8p23.1(-)/-164</td>
<td>16p11.2.1(-)/-712</td>
<td>15q11.2(+)/+448</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Digestive system (19 patients 5 more likely contributing variants)</th>
<th>Anal atresia</th>
<th>Duodenal atresia</th>
<th>Esophageal Atresia</th>
<th>Gastrochisis</th>
<th>Omphalocoele</th>
<th>Miscellaneous*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2q37.3(-)/-77</td>
<td>5q11.2.1(+)367</td>
<td>8p23.1(-)/-768</td>
<td>6p25.3(+)/+30</td>
<td>6p25.3(-)/-37</td>
<td>6p25.3(+)/+34</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Face (9 patients, 2 more likely contributing variants)</th>
<th>Cleft lip/palate</th>
<th>Micrognathia</th>
<th>Robin sequence</th>
<th>Miscellaneous*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1p36.21(+)/-642</td>
<td>10q11.22(-)/-230</td>
<td>1q21.1(-)/-192</td>
<td>1q21.1(+)/+92</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Genital system (12 patients, 5 more likely contributing variants)</th>
<th>Ambiguous genitalia</th>
<th>Cryptorchidism</th>
<th>Microopen</th>
<th>Miscellaneous*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1q36.33(-)/-300</td>
<td>1p36.21(-)/-703</td>
<td>1p36.33(+)/+659</td>
<td>1q21.1(-)/-192</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ocular system (3 patients, 0 more likely contributing variants)</th>
<th>Congenital cataract</th>
<th>Unilateral coloboma</th>
<th>Cystic kidney</th>
<th>Prune belly anomaly</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10q11.22(+)/+515</td>
<td>8p23.1(-)/-1077</td>
<td>4p16.1(+)/+329</td>
<td>19q13.43(+)/+30</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pulmonary system (9 patients 2 more likely contributing variant combinations)</th>
<th>Diaphragm hernia</th>
<th>TEH</th>
<th>Miscellaneous*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4p16.3(-)/+322</td>
<td>4p16.3(-)/-210</td>
<td>6p25.3(+)/+80</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Skeletal (9 patients, 3 more likely contributing variants or combinations)</th>
<th>Club foot</th>
<th>Craniosynostosis</th>
<th>Limb-other*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5p13.33(-)/-185</td>
<td>3q34.3(+)/+0.197</td>
<td>8p23.1(-)/-32</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cardiovascular system (20 patients, 5 more likely contributing variants or combinations)</th>
<th>Coarctation of aorta</th>
<th>Septal defect</th>
<th>Miscellaneous*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6p25.3(-)/-144</td>
<td>1p36.21(+)/+643</td>
<td>6p25.3(-)/-30</td>
</tr>
</tbody>
</table>

Microdeletions (-) or duplications (+) are listed by chromosome and band number followed by size of aneuploid segment in kilobases; italicized changes are considered benign, while combined variants are separated by ;. Miscellaneous changes: cortical dysplasia, Dandy-Walker malformation, schizencephaly, septo-optic dysplasia; 'macroGLOSSia, microtia; congenital chest mass, pulmonary airway malformation, pulmonary hypoplasia; 'arthrogryposis, Madelung deformity, rhizomelia, polydactyly; 'dextrocardia, dilated cardiomyopathy, hypoplastic left heart syndrome, transposition of the great vessels.

4. DISCUSSION

Scanning the genome for altered DNA dosage (aCGH), augmented by additional or separate screening for DNA sequence change, is providing myriad new insights for disease cause and predisposition. This revolution is particularly obvious when...
testing children with autism and/or intellectual disability, for these combined genome-scanning techniques now provide a cause for over 80% of such patients.\textsuperscript{4} Even greater progress has been made for multiple congenital anomaly/intellectual disability syndromes, with 124 microdeletion or duplication syndromes accepted by listings in the Online Mendelian Inheritance in Man database and another 112 emerging syndromes based on several literature reports.\textsuperscript{4} Less progress has been made in defining genetic causes for isolated congenital malformations, a difficulty understandable because of the need to find multiple predisposing genes as expected from the polygenic/multifactorial determination with threshold model for birth defects.\textsuperscript{14} We suggest that the use of aCGH can add to gene association and NGS/WES sequencing studies in defining genes that predispose to congenital anomalies, but emphasize the need for changes in perspective from that guiding traditional karyotype/phenotype correlation.

Usual characterization of CNVs as pathogenic has been based on several criteria\textsuperscript{15}: a) recurring association with a distinctive clinical pattern or syndrome, as for velocardiofacial syndrome with 22q11 microdeletion,\textsuperscript{5} b) size above 500 Kb, c) presence of genes within the aneuploid interval, particularly when they have known functions that relate to patient findings, d) presence in the affected child but not in normal relatives, and, extrapolating beyond families, low frequencies in normal populations. Since isolated birth defects may not present in obvious patterns, and because the risk for transmission from parent to child for common multifactorial malformations is usually around 3-5%, the criteria for recurrent pattern association and parent-child correspondence cannot apply. Furthermore, 18 of the italicized CNVs listed in Table 1, traditionally characterized as benign because of high prevalence, are over 500 Kb.

The one remaining criterion of pathogenesis, the presence of candidate genes within the aneuploid segment, is also difficult to apply because congenital anomalies, as expected from the multifactorial model and illustrated by cardiac defects, have predisposing genes in almost all chromosome regions. Another problem with focus on a single causative gene within the aneuploid segment is the extreme variability even among established syndromes. For example, many patients with 5q14.3q21.3(-) have been defined, with the $MEF2C$ gene in that interval highlighted as the deciding cause of severe intellectual disability and epilepsy. Yet we reported a girl with a large deletion in that interval that included the $MEF2C$ gene who had a very mild phenotype.\textsuperscript{16} Such patients emphasize that all phenotypes caused by altered chromosome dosage are polygenic, influenced by several genes within or outside of the aneuploid segment.\textsuperscript{17} Attention must be paid to the genetic background as well as other genes within the interval to understand pathogenesis of 5q14q21 deletions, an approach especially necessary when considering multifactorial birth defects.

Another approach to deciding if a genetic change is paramount in causing a congenital anomaly is to note the association of other defects and particular minor anomalies that can signal a syndrome—e. g., the single palmar crease or epidermal folds that in association with duodenal atresia would increase suspicion of Down syndrome.\textsuperscript{18} This approach could not be employed in the present study, most samples being sent from neonatal nurseries or pediatric clinics, and would not be relevant to the majority of aCGH tests since most pediatric patients will not have a sophisticated clinical genetic evaluation. Although decisions about
syndrome versus single defect are crucial for management, it is important to identify CNVs found in children with isolated birth defects as well as in those found in children with syndromes, documenting both pathogenic and accessory CNVs in the latter disorders. One can then identify CNVs that act in networks to produce apparently isolated defects and as well as those that act in the presence of a pathogenic, syndrome-causing gene and/or CNV to produce the defect in a subset of patients. A high-prevalence CNV that is benign in usual contexts might in some contexts interact with others to produce a heart defect \(^6,7\) and, in the presence of 22q11 deletion,\(^5\) be a determining factor in whether that particular deletion patient has a cardiac anomaly.

A final perspective needed to match predisposing CNVs with birth defects is to recognize the importance of developmental fields, the early embryologic pathways that can be perturbed to produce seemingly unrelated anomalies in their derived organs. An example is the relationship of omphalocele and gastroschisis, the former viewed as often genetic because of its association with numerous genetic disorders, the latter viewed as often environmental because of its recent increase in prevalence and association with maternal factors.\(^19\) Common patterns of anomalies are associated with both gastrointestinal defects, indicating a common developmental pathway that precedes appearance of omphalocele as a failure of normal gut retraction and gastroschisis as a vascular-mediated rending of abdominal wall.\(^20\) Looking at CNVs seen in patients with either anomaly may highlight chromosome regions that, when present in altered dosage, will predispose to either anomaly.

The few CNVs presented here do not demonstrate any obvious correlations with loci associated with particular birth defects. Ultimately one would like to match recurring CNVs with loci identified by association through advances in whole genome studies. An example for gastroschisis is the association of the nitric oxide synthase 3 NOS3 gene at 7q36.1, the adducin1 ADD1 gene at 4p16.3, the guanine nucleotide-binding protein beta-3 GNB3 gene at 12p13.31, and the intracellular adhesion molecule ICAM gene cluster at 19p13.2.\(^21\) None of these loci correspond to CNVs listed in Table 1 for omphalocele or gastroschisis. However, CNVs at 5q13.2 are seen for both anomalies along with other correlations that include 8p23.1 CNVs for holoprosencephaly and encephalocele, 6p25.3 CNVs for microencephaly and other brain anomalies, 6p25.3 CNVs in esophageal and duodenal atresias, 19p13.3/.4 CNVs in renal defects, 6p25.3 CNVs with genital defects, 7q34 CNVs with skeletal defects, 1p36.21, 6p25.3, and 8p23 CNVs with heart defects.

The presence of 6p25.3 (-) CNVs of various sizes may be viewed as an expected occurrence since this CNV ranging in size from 25 to 181 Kb is seen in 15% of patients in the 2009-2014 population.\(^4\) However, another interpretation is that this microdeletion slightly perturbs early developmental processes, causing some patients with particular accessory mutations or CNVs to cross the threshold for a birth defect. Common CNVs like those at 6q25.3 or 8p23.1 often occur with others, shown by the fact that only 46% of the 2009-2014 cases had one CNV, with 30% having two, 13% three, 5% four, and 3% five or more.\(^4\) It is thus possible that a simple increase in number of CNVs, regardless of locus and size, could cause predisposition to birth defects. If so, then determining both the number and types of CNVs by aCGH would be a vehicle for preconception counsel and enhanced prenatal monitoring in all pregnancies.
In answer to the title question of whether aCGH can be useful for apparently isolated congenital anomalies, we can only answer perhaps at this time. We do suggest that comprehensive tallying of all CNVs in patients with birth defects, whether or not they are judged to be pathogenic, is worth considering as an approach toward better understanding of malformation pathogenesis and genomic disease.  

REFERENCES


10. Illumina/Bluegnome Inc.--www.illumina.com, formerly cambridgebluegnome.com, accessed May, 2018


