Genomic Microarray in Intellectual Disability: The Usefulness of Existing Systems in the Interpretation of Copy Number Variation

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Abstract

Keywords

- ▶ intellectual disability
- ▶ comparative genomic hybridization array
- ▶ copy number variants
- ▶ qPCR

Whole genome array technology is an essential tool for the detection of a large number of copy number variants (CNVs) in patients with ID and/or multiple congenital anomalies. However, the clinical significance of some microimbalances is not known. In this article, we succeeded to detect seven new variations of unknown significance (dup12p13.33, dup2p16.3, dupXq13.2, del12q24.33, dup16p13.11, trip4q22.1, and dup9p21.3), one CNV classified as known pathogenic syndrome (del22q13.31-q33), and one CNV classified as potentially pathogenic (del11q24.3). We emphasize the role of comparative genomic hybridization arrays in the investigation of intellectual disability and evaluate the usefulness of existing systems in the interpretation of CNVs.

Introduction

Intellectual disability (ID) is a variable heterogeneous manifestation of central nervous system dysfunctions occurring in 1 to 3% of the general population. ID represents one of the most frequently diagnosed disabling conditions in our society, characterized by impairment of cognitive and adaptive skills. The etiologies are very heterogeneous, and, unfortunately, in about one-half of cases, the cause of ID is still elusive.^{1,2}

Array comparative genomic hybridization (CGH) technology pointed a new important source of human genomic disorder variations. Analysis of normal control individuals revealed that copy number variants (CNVs) are abundantly present in the human genome. A catalog of this structural variation is provided by the Database of Genomic Variants (DGV; <http://projects.tcag.ca/variation/>), DECIPHER (<https://decipher.sanger.ac.uk/>) and ISCA (<https://www.iscaconsortium.org/>) databases. The clinical significance of these CNVs remains largely uncertain, and we face the challenge of the interpretation of variations of unknown

significance (VOUS).^{3,4} VOUS are usually unreported variants. They contain either genes of uncertain clinical significance or genes that may regulate nearby Online Mendelian Inheritance in Man (OMIM) genes. Various workflows, websites, and databases⁵ are being constantly updated with the aim of improving the interpretation of VOUS. In this article, we report on five patients with idiopathic ID and/or congenital anomalies and carrying submicroscopic chromosomal aberrations. These chromosomal aberrations included three deletions, five duplications, and one triplication. We discuss the challenges and accompany the use of existing score systems in terms of determining the clinical significance of unexpected genomic variations. Faced to these, VOUS-consequent clinician attitude is here discussed.

Material and Methods

Patients

This study includes five patients with ID and multiple congenital malformations. These patients are strictly selected according to

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Fig. 1 Face view of patient 1. The dysmorphic features include dolichocephaly, epicanthic folds, large dysplastic ears, and bulbous nasal tip.

the criteria of de Vries et al.⁶ Severe phenotypes are favored for the global screening by CGH array (► **Fig. 1**). A summary of the clinical and cytogenetic data of all patients is shown in ► **Table 1**.

Karyotype

Conventional cytogenetic analysis was performed on the peripheral blood lymphocytes at 450 to 550 Giemsa Reverse Banding (RHG). Chromosomal analysis of the patients and their families was performed according to standard procedures. Peripheral blood lymphocytes were cultured in RPMI-1640 medium (Gibco, Grand Island, New York, United States) enriched with 20% fetal calf serum, L-glutamine, antibiotics (penicillin and streptomycin), and phytohemagglutinin. Cells were cultured for 72 hours at 37°C in an incubator with 5% CO₂. The culture was stopped using Colcemid solution (0.05 mg/mL; KaryoMAX, Gibco, Grand Island, New York, United States) for 45 minutes. After harvesting, cells were exposed to hypotonic solution (0.075 mol/L KCl) and fixed with methanol/acetic acid (3:1). Slides were prepared and stained using R-banding. A minimum of 20 metaphases were analyzed from each sample, using the CytoVision Karyotyping System (Applied Imaging, Grand Island, New York, United States). Karyotypes were assigned according to the recommendations of the International System of Human Cytogenetic Nomenclature. We obtained a consent from the patient's families.

Comparative Genomic Hybridization Array

Oligonucleotide array CGH was performed using the Agilent Human Genome CGH Microarray180K (Agilent Technologies, Santa Clara, CA, United States). This microarray is constituted of more than 180,000 60-mer oligonucleotide probes that span both coding and noncoding regions. Coverage of the human genome is made with an average spatial resolution of 18,300 bp. The experiments were performed according to version 4.0 (June 2006) of the protocol provided by Agilent (Agilent Oligonucleotide Array-Based CGH for Genomic DNA Analysis).

Table 1 Cytogenetic, clinical, and CGH array data for all patients

Patients	Clinical features ^a	Karyotype	CGH array	Structural aberration	Imbalance start	Imbalance end	Size (bp)	Validation methods	Origin	Associated genes
P1	Familial ID, facial dysmorphism, psychomotor delay.	46,XY,del(22)	180K	Del 22q13.31-q33 Dup 12p13.33	44,406,540 628,213	51,219,009 835,424	6,812,469 207,211	FISH qPCR	de novo Paternal	22qter deletion syndrome SHANK 3 NINJ2
P2	Severe ID, epilepsy	Normal 46,XX	180K	Dup 2p16.3 Dup Xq13.2	50,138,031 73,160,794	50,194,246 73,409,149	56,215 137,602	qPCR	Paternal	NRXN1
P3	ID, growth retardation, neurologic regression	Normal 46,XX	180K	Del 11q24.3 Del 12q24.33	128,993,322 132,681,054	129,034,246 132,807,842	40,924 126,788	qPCR	n/a	RICS GALNT9
P4	ID, generalized seizures, hypotonia	Normal 46,XY	180K	Dup 16p13.11 Trip 4q22.1	15,101,948 88,705,632	16,267,306 88,838,442	1,165,358 132,810	n/c DGV qPCR	Parental	MEPE
P5	ID, aggressive behavior, growth retardation	Normal 46,XY	180K	Dup 9p21.3	22,246,765	22,555,566	308,801	qPCR	Maternal	DMRTA1

Abbreviations: CGH, comparative genomic hybridization; DGV, Database of Genomic Variants; FISH, fluorescent in situ hybridization; ID, intellectual disability; n/a, (one of the) parent not available; n/c, not confirmed and reported in DGV; qPCR, quantitative polymerase chain reaction.

^aExtended clinical information provided in results.

Microarrays were scanned using the Agilent scanner G2565BA. Images were analyzed using Agilent Feature Extraction Software version 9.1 (CGH-v4_91 protocol), permitting creation of QC reports for each patient where the value of the derivative log ratio spread, which is the spread of the derivative log ratio values, was used as a quality criterion. A graphical overview and analysis of the data were obtained by using the Agilent CGH Analytics software version 3.4.27 (statistical algorithm: z-score; sensitivity threshold: 2.5; moving average window: 5 points).

Only imbalances involving three or more adjacent probes were retained. Identification of probes with a significant gain or loss was based on the log2 ratio plot deviation from 0 with cutoff values of 0.5 to 1, and -0.5 to -1, respectively.

Validation of Copy Number Variants

The DGV (<http://dgv.tcag.ca/dgv/app/home>), UCSC Genome browser (<http://www.genome.ucsc.edu/>), and DECIPHER (<https://decipher.sanger.ac.uk/>) databases were used to identify known genomic variants, genes, and ID-associated regions, respectively.

The validation method was chosen depending on the imbalance type (deletion or duplication), size, and practical reasons (type of sample available: DNA and/or metaphase spreads).

Fluorescent In Situ Hybridization

Fluorescence in situ hybridization using commercial probes (MD DiGeorge “Tuplet” (22q11) 22qter) was performed according to the manufacturer’s instructions. Probe mixture (10 µL) was applied to metaphase slides and co-denaturized for 5 minutes at 72°C. After overnight hybridization at 37°C, slides were washed for 5 minutes in 2XSSC/NP40 (Vysis, Santa Clara, California, United States) at 72°C. Finally, the slides were mounted with 15 mL of 4, 6-diamidino-2- phenylindole (Vysis) and analyzed using a fluorescence in situ hybridization station (CytoVision, Applied Imaging).

Quantitative Polymerase Chain Reaction

Real-time quantitative polymerase chain reaction (qPCR) was performed on QuantiTect SYBR Green PCR Kits (Eurogentec S.A., Seraing, Belgium). Gene-specific primers for target genes and endogenous control gene (*Adora2b*) were designed by Eurogentec Oligo Center (Angers Cedex, France) (–Table 2). Each sample was run in triplate for the quantification of the target genes as compared with the endogenous control gene. Data evaluation was performed using the Lightcycler Software version 3.4 (Roche Diagnostics, Meylan Cedex, France) and Microsoft Excel, using the comparative ΔΔ threshold cycle number (Ct) method.²

Results

Previously reported criteria of pathogenicity are established in a perspective study by Lee et al^{7,8} and are used to determine the clinical relevance of the observed CNVs. Pathogenic CNVs criteria are then listed. Indeed, a CNV is more likely to be pathogenic if it is de novo or inherited from an affected parent or observed in an affected relative. It should not overlap a polymorphism listed in CNV databases for healthy individuals, but rather it overlaps a genomic imbalance in CNV databases for affected individuals and/or contains morbid OMIM genes or are gene-rich. Other characteristics include the nature of the imbalance (deletion rather than duplication), the size (>3 Mb), and the existence of regulatory elements. De novo occurrence or inheritance from a healthy parent is one of the main points guiding toward pathogenic or benign characteristic of a CNV, respectively. Besides, we confront the Scoring System for Individual Copy Number Variants.⁸ In fact, CNVs were coded as follows:

- “Potentially” pathogenic if they “scored” 5 or 6: These were usually rare genic CNV, which overlapped with the critical region of recognized microdeletion/duplication syndromes, or contained candidate genes for ID (where change in gene dosage was predicted to be pathogenic), with supportive evidence in the literature of pathogenicity.

Table 2 List of primers used for qPCR experiments

Patients	Primers	Sequences	Temperature (°C)	qPCR product size (bp)
P1	NINJ2	5'ACCATTACGCCACCAAGAAG 3' 3'AATGACCACGAGCAGGACAC 5'	60	188
P2	NRXN1	5'GTCACCATTCTGCGTCCTC 3' 3'GGATGTCATCTGTGGTCTGC 5'	60	241
	SLC16A2	5'ACAAAGCCCTGATTGACTGC 3' 3'TGTCCCTCCTTGGACTTGAG 5'	60	228
P3	RICS	5'TGGGAACCCAGTCTCTTCTG 3' 3'CTGCACACAGTCATCAGG 5'	60	210
	GALNT9	5'CCTACTTCTGGCCTTGCA 3' 3'CGTACGTGAGGGTGTGTTG 5'	60	152
P4	MEPE	5'GCAAAGCTGTGTGGAAGAGC 3' 3'CCATTCAAAGTGCCCAAGAC 5'	60	130
P5	DMRTA1	5'GGAGGCACAAGGCATTAAC3' 3'GAGTCCCAAGTGGAAGTGC5'	60	238

Abbreviation: qPCR, quantitative polymerase chain reaction.

- Variants of uncertain clinical significance (score: 2–4): These were rare genic CNV currently lacking evidence of pathogenicity.
- (Likely to be) benign if they “scored” 0 or 1: These were generally nongenic CNVs or CNVs that were well recognized as benign variations (at least two concordant overlapping benign CNVs), with no literature evidence of potential pathogenicity.

For the five patients included in this study, 12 CNVs are observed and consist of gain/loss ranging size from 40 kb to 6 Mb (►Table 1). One imbalance de novo has a known pathogenic character (►Fig. 2). In the remaining patients, the imbalances were unknown (►Table 3 and ►Fig. 3).

Table 3 Analysis of qPCR ratios

Patients	Genes	Normalized ratio ^a		
		Patient	Father	Mother
P1	<i>NINJ2</i>	1.442	1.452	0.97
P2	<i>NRXN1</i>	2.666	2.196	1.177
	<i>SLC16A2</i>	1.525	0.903	0.943
P3	<i>RICS</i>	0.751	n/a	1.563
	<i>GALNT9</i>	0.476	n/a	1.027
P4	<i>MEPE</i>	2.438	1.716	1.701
P5	<i>DMRTA1</i>	1,562	n/a	1,461

Abbreviations: n/a, (one of the) parent not available; qPCR, quantitative polymerase chain reaction.
^aqPCR ratio > 1.2: Duplication, qPCR ratio < 0.8: Deletion, qPCR ratio > 1.8: triplication.

Fig. 2 Cytogenetic and molecular results for patient 1. (A) Partial karyotype showing chromosome 22. (B) Comparative genomic hybridization (CGH) array 180K showing 22q13 deletion. (C) CGH array 180K showing 12p13.3 duplication. (D) Confirmed 22qter deletion by FISH.

Fig. 3 Genomic imbalances array comparative genomic hybridization profiles. Corresponding patients are indicated below each profile: dotted lines represent log2 ratios, and copy number losses shift the ratio to the left and copy number gains to the right. Patient 2: (A) arr Xq13.2 (73,160,794–73,662,254)X3 and (B) arr 2p16.3 (50,138,031–50,194,246)X3. Patient 3: (C) arr 11q24.3 (128,993,322–129,034,246)X1 and (D) arr 12q24.33(132,681,054–132,807,842)X1.

Patient 1

The patient was a 3-year-old -oy suffering from ID, behavior problems, hypotonia, psychomotor delay, and dysmorphic features including dolichocephaly, epicanthic folds, large dysplastic ears, and bulbous nasal tip (–Fig. 1). Conventional karyotyping showed an asymmetry of the long arm of chromosome 22. CGH array 180K analysis revealed de novo 22qter deletion: arr 22q13.31-q13.33(44,406,540–51,219,009)X1 (hg19) of 6,812,469 bp. The presence of this deletion was confirmed by fluorescent in situ hybridization (FISH) using commercial probe targeting *SHANK3* gene (–Fig. 2). In asso-

ciation with this terminal deletion, an interstitial inherited duplication at 12p13.3 region: arr 12p13.33 (628,213–835,365)X3(hg19) of 207,211 bp, is revealed and confirmed by qPCR encompassing *NIN2* gene (–Fig. 2 and –Table 3).

Patient 2

The proband was a 15-year-old mentally delayed girl. She had severe behavior disorders, speech problems, and epilepsy (–Fig. 1). The magnetic resonance imaging (MRI) profile was normal. CGH array screening demonstrated duplications at 2p16.3 and Xq13.2 regions encompassing the *NRXN1* and

SLC16A2 genes, respectively: arr 2p16.3 (50,138,031–50,194,246)X3(hg19) of 56,215 bp and arr Xq13.2 (73,160,794–73,662,254)X3(hg19) of 137,602 bp. QPCR analysis confirmed these CNVs and revealed their parental origin (►Fig. 3 and ►Table 3).

Patient 3

The patient was a 9-year-old, single daughter of nonconsanguineous parents, diagnosed with ID, epilepsy, absence of speech, growth retardation, and neurologic regression (►Fig. 1). MRI analysis was normal as the conventional karyotyping. Additional CGH array screening identified two interstitial microdeletions at 11q24.3 and 12q24.33 regions: arr 11q24.3 (128,993,322–129,034,246)X1(hg19) of 40,924 bp and arr12q24.33 (132,681,054–132,807,842)X1(hg19) of 126,788 bp, confirmed by qPCR. The patient's mother showed a normal CGH array. The father was not available for testing (►Fig. 3 and ►Table 3).

Patient 4

The proband was a 10-year-old boy with severe and familial ID. He was diagnosed with generalized seizures, hypotonia, with quadruped walking (►Fig. 1).

CGH array 180K analysis demonstrated a 16p13.11 duplication and a 4q22.1 triplication: arr 4q22.1 (88,705,632–88,838,442)X4(hg19) of 132,810 bp and arr 16p13.11 (15,101,948–16,267,306)X3(hg19) of 1,165,358 bp (►Table 4). The qPCR analysis confirmed the CNV of 4q22.1 region and its inheritance from healthy parents who had duplicated copies of this region (►Fig. 4).

Patient 5

Our proband was a 13-year-old boy from unrelated parents. At the clinical examination, he presented severe ID with aggressiveness and growth retardation (he walked and spoke

at the age of 5). No epilepsy was described. CGH array 180K revealed a duplication at 9p21.3 region: arr 9p21.3 (22,246,765–22,555,566)X3(hg19) of 308,801 bp (►Fig. 4) encompassing *DMRTA1* gene. QPCR analysis has confirmed the gain and showed its maternal origin. The mother presented good health, but the father was not available for the investigation.

Discussion

Our study further demonstrates the efficiency of whole genome array in the detection of chromosomal microimbalances and highlights the complexity involved in the phenotypic effects of CNVs. We then discuss the usefulness of previous guidelines in the interpretation of CNVs and their contribution in the clinical and genetic counseling.

Patient 1 was consistent with a known syndrome, Phelan-McDermid syndrome. This recurrent monosomy is associated with mild-to-severe global developmental delay, absent or delayed speech, generalized hypotonia, and minor anomalies including dolichocephaly, ptosis, abnormal ears, relatively large hands, and behavior within the autistic spectrum.⁹

In patient 2, the DECIPHER database has a very small number of partial and intragenic *NRXN1* gene and *SLC16A2* gene duplications, but phenotypic information on most of these patients is not available.¹⁰ Since we are not able to investigate the father of patient 3, the causal effect of deletions 11q24.3 and 12q24.3 remains uncertain.¹¹ However, haploinsufficiency of *RICS* and *GALT9* genes may contribute to the patient's phenotype regarding their major role in the neurologic development and their high expression in the brain.⁴ In patients 4 and 5, the significance of CNVs in these regions has not been determined. It is unknown if the *MEPE* duplication or *DMRTA1* genes represent a variable phenotypic

Table 4 Scoring for novel copy number variants revealed by oligo-CGH 180K

CNVs	Contains genes	Contains candidate ID genes	Overlaps with critical region of known microdeletion/duplication syndrome	Partially overlapping with concordant CNV in DGV	Partial overlap with de novo patients on pathogenic databases	Score ^a	Classification of the CNV
Del22q13.31-q33	1	2	2	0	1	6	Known pathogenic syndrome
Dup12p13.33	1	0	0	1	1	3	VOUS
Dup2p16.3	1	2	0	0	1	4	VOUS
DupXq13.2	1	0	0	0	1	2	VOUS
Del11q24.3	1	2	2	0	1	6	Potentially pathogenic
Del12q24.33	1	0	2	0	1	4	VOUS
Dup16p13.11	1	0	0	1	1	3	VOUS
Trip4q22.1	1	0	0	0	1	2	VOUS
Dup 9p21.3	1	0	2	0	1	4	VOUS

Abbreviations: CGH, comparative genomic hybridization; CNV, copy number variants, DGV, Database of Genomic Variants; ID, intellectual disability; VOUS, variations of unknown significance.

^aThe score is based on Palmer et al.⁸

Fig. 4 Patient 4: (A) arr16p13.11 (15,101,948–16,267,306)X3 and (B) arr 4q22.1 (88,705,632–88,838,442)X4. Patient 5: arr 9p21.3 (22, 246,765–22,555,566)X3.

spectrum but a positive correlation with the ID and/or neurologic disorder.

In general, with the advent and application of microarrays screening, it was demonstrated that man is more genetically variable than previously considered. Each individual (healthy or not) presents multiple CNVs in its genome.¹² The existing system adopted here stresses on the difficulty to conclude once and for all the clinical relevance of some CNVs. Moreover, the incomplete penetrance and variable expression, exemplified by the inheritance of the VOUS encompassing disease genes from apparently unaffected parents, has further blurred the boundary between benign and pathogenic

variations.¹³ However, it can be expected that CNVs that are currently classified as VOUS will turn out to be causal in the near future, especially with continued gene discovery by next-generation sequencing (NGS) in a large cohort of patients.¹⁴ Therefore, functions of neurologic genes will be clearly elucidated. Here we emphasize the role of CGH arrays as a good genetic tool in ID investigations. It can clarify some ambiguous situations where standard genetic examinations were normal but rather may cause a real dilemma in genetic counseling. But with the advance of arrays and NGS guidelines and consortium in genetic counseling, many VOUS could be well elucidated in the near future.

Conflict of Interest

None.

Acknowledgments

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