

## ORIGINAL ARTICLE

**BENIGN, PATHOGENIC AND COPY NUMBER VARIATIONS OF UNKNOWN CLINICAL SIGNIFICANCE IN PATIENTS WITH CONGENITAL MALFORMATIONS AND DEVELOPMENTAL DELAY**Mihaylova M<sup>1,\*</sup>, Staneva R<sup>1,2</sup>, Toncheva D<sup>1</sup>, Pancheva M<sup>1,2</sup>, Hadjidekova S<sup>1,2</sup>

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**ABSTRACT**

The high frequency (3.0-5.0%) of congenital anomalies (CA) and intellectual disabilities (IDs), make them a serious problem, responsible for a high percentage (33.0%) of neonatal mortality. The genetic cause remains unclear in 40.0% of cases. Recently, molecular karyotyping has become the most powerful method for detection of pathogenic imbalances in patients with multiple CAs and IDs. This method is with high resolution and gives us the opportunity to investigate and identify candidate genes that could explain the genotype-phenotype correlations. This article describes the results from analysis of 81 patients with congenital malformations (CMs), developmental delay (DD) and ID, in which we utilized the CytoChip ISCA oligo microarray, 4 × 44 k, covering the whole genome with a resolution of 70 kb. In the selected group of patients with CAs, 280 copy number variations (CNVs) have been proven, 41 were pathogenic, 118 benign and 121 of unknown clinical significance (average number of variations 3.5). In six patients with established pathogenic variations, our data revealed eight pathogenic aberrations associated with the corresponding phenotype. The interpretation of the other CNVs was made on the basis of their frequency in the investigated group, the size of the variation, content of genes in the region and the type of the CNVs (deletion or duplication).

**Keywords:** Microarray comparative genomic hybridization (aCGH); Congenital anomalies (CAs); Copy number variations (CNVs).

**INTRODUCTION**

A high percentage of genetic diseases manifest themselves in the first 28 days after birth, but the full clinical symptoms may not be evident in newborns. Conventional diagnostic tests are with low resolution. They are also time-consuming, labor-intensive and are difficult to automate, therefore, the etiology of congenital anomalies (CAs) in 40.0-60.0% of the cases remains unclear. Molecular and molecular cytogenetic techniques such as FISH (fluorescent *in situ* hybridization), QF-PCR (quantitative fluorescence-polymerase chain reaction), MLPA (multiplex ligation-dependent probe amplification), have enhanced the diagnosis, but they require preliminary information on the suspected chromosomal abnormality and on the location. The new genomic technologies such as microarray comparative genomic hybridization (aCGH) and next-generation sequencing (NGS), combine the advantages of conventional technologies, enabling whole-genome screening in high resolution.

At present, great interest has arisen in a newly discovered variation known as copy number variations (CNVs), which, in 2007, was indicated to be the “breakthrough” of the year [1]. Currently, CNVs are defined as a ubiquitous segment of DNA longer than 1 kb, presented at a variable copy number, compared to the reference genome. The great interest in CNVs was aroused by the observation that they can affect transcriptional activity and translational levels of adjacent genes [2-8]. An association was found between certain CNVs and predisposition to multifactorial diseases [3,9-15]. Copy number variations differ in type and frequency in different populations and can be used as a valuable source of information for the study of genetic characteristics of the nations [16-20]. With the accumulation of huge amounts of data for different CNVs, certain phenotype-genotype correlations have been defined [21].

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It is therefore possible for CNVs that have been initially considered benign to later be proved as increasing sensitivity to multifactorial disease or causing genetic disease with late onset or incomplete penetrance. Therefore, the term CNVs is generally used to describe the CNVs both in patients and in healthy controls, as well as to describe the genomic imbalances, causing known microdeletion/micro-duplication syndromes. Consequently, CNVs can be divided into benign, pathogenic and CNVs of unknown clinical significance [22-25] and can be polymorphic with frequency greater than 1.0% or rare, less than 1.0%. They can also arise as *de novo* anomalies or be inherited, multiallelic or biallelic [26]. According to the data from two large studies in healthy populations, the human genome contains 12 CNVs on average [27,28]. Another study revealed that 12.0% of the human genome is represented by CNVs covering hundreds of genes, disease loci, functional elements and segmental duplications [29]. Sequencing of the human genome revealed that insertions and deletions in absolute terms are responsible for 22.0% of the variations and cover 74.0% of the affected nucleotides [30]. Recent data indicate that the genomes of two individuals may vary between 1.0 and 3.0% [31].

Caramaschi *et al.* [32] carried out a survey of 116 patients. The detected CNVs were analyzed for genotype/phenotype correlations with the clinical features of the patients. Pathogenic CNVs (21 deletions, three duplications and three cases with both of them) were observed in 27 patients (23.3%). The analysis showed a significant association between pathogenic CNVs and the first appearance of the symptoms before the age of 1 year and the presence of malformations [32]. According to another study on CNVs in 2500 individuals, it was found that in 65.0 to 80.0% of the cases, CNVs were greater than 100 kb, in 5.0-10.0% greater than 500 kb, and in 1.0-2.0% greater than 1 Mb [19]. In addition, the biggest part of the genomic variations were found with a frequency of 0.02 to 1.0%, comprising 6.0% of our genome. At the same time, polymorphic CNVs represented 0.09%. Another study conducted by Pinto *et al.* [20] on a healthy population detected that 160 Mb (~5.0%) was represented by CNVs, from which 96.0% were rare, less than 2.0%, while others were common. In this study, we present our findings with regard to the distribution, frequency and clinical significance of the detected CNVs.

## MATERIALS AND METHODS

The present study focuses on 81 patients of both sexes with dysmorphic features, with or without intellectual disability, behavioral problems, failure to thrive, neurological

disorders. All of them presented a normal karyotype. The blood was taken in compliance with the standard procedures for good laboratory practice. The genomic screening, array-based comparative genomic hybridization (aCGH) was performed by standard protocol using the CytoChip ISCA oligo microarray, 4 × 44 k (BlueGnome Ltd., Cambridge, Cambridgeshire, UK), resolution 70 kb. Data were analyzed with the BluefuseMulti v.4.2 software (BlueGnome Ltd.).

## RESULTS

In the investigated group, pathogenic CNVs were found in 31 patients. In addition, in the majority of all surveyed individuals, we identified benign CNVs (58/81 patients) and variations of unknown clinical significance (66/81 patients). There were 280 CNVs detected, of which 41 were pathogenic (28 deletions, 13 duplications), 118 benign (91 deletions, 27 duplications) and 121 of unknown clinical significance (50 deletions, 71 duplications). Copy number variations have not been established in six of the patients. The size of all identified CNVs was from 100,021 to 13,881,527 bp. There were 169 deletions and 111 duplications. The largest duplication covered 13,881,527 bp, and the smallest included 100,021 bp. The region of the smallest deletion covered 102,202 bp, and the size of the largest in four (q34.3q35.2) region: arr 4q34.3q35.2 (178,213,959-190,896,645) ×1, was 12,682,687 bp. We revealed eight pathogenic variations associated with the phenotype in six patients from the total group. Variations were distributed over eight chromosomes (chromosomes 2, 4, 5, 10, 12, 15, 17, 22). In two of the analyzed patients, a combination of two pathogenic CNVs on different chromosomes was found, and four of the patients had a single pathogenic variation. Six of the aberrations were deletions and two of them were duplications. The amount of detected genomic pathogenic changes ranges from 300 kb to 13 Mb. In five patients of the total group, we discovered five pathogenic CNVs that were not associated with the observed phenotype (Table 1).

As well as pathogenic variations, benign CNVs and CNVs of unknown clinical significance were found. There were 239 (an average of 2.9 per patient variations of the total group). These CNVs were distributed over 75 patients. All established benign and unknown CNVs ranged in size from 106,847 to 1,348,283 bp. From the total number of identified CNVs (239 without pathogenic CNVs), 179 (74.9%) covered 100-500 Kb, 52 (21.8%), from 500 Kb to 1 Mb and eight (3.3%) were larger than 1 Mb. There were 141 deletions and 98 duplications. One hundred and eighteen of the CNVs were benign. They were divided by

**Table 1.** Summarized results with pathogenic findings identified after microarray comparative genomic hybridization and associated with the phenotype of the patient.

Patient	Chromosome	Type of Aberration	Position (bp)		Size (bp)	Cytoband	
			Start	End		Start	End
79	2	deletion	50,982,143	51,314,401	332,259	2p16.3	2p16.3
		deletion	175,470,501	177,136,261	1,665,761	5q35.2	5q35.3
52	10	deletion	122,804,780	135,434,149	12,629,370	10q26.12	10q26.3
46	4	deletion	178,213,959	190,896,645	12,682,687	4q34.3	4q35.2
		duplication	230,451	14,111,977	13,881,527	12p13.33	12p13.1
41	17	deletion	34,450,435	36,248,889	1,798,455	17q12	17q12
35	22	deletion	21,561,492	22,905,039	1,343,548	22q11.21	22q11.22
30	15	duplication	22,765,658	29,030,488	6,264,831	15q11.2	15q13.1

**Table 2.** Number and size of detected benign copy number variations and copy number variations of unknown clinical significance.

Parameters	Total Number	100-500 kb	500 kb - 1 Mb	>1 Mb
Benign and CNVs of unknown clinical significance	239	179	52	8
Benign CNVs	118	90	21	7
CNVs of unknown clinical significance	121	89	31	1

CNVs: copy number variations.

size as follows: 90 (76.3%) covered 100-500 Kb; 21 (17.8%) were from 500 Kb to 1 Mb; seven (5.9%) were larger than 1 Mb. There were 27 duplications and 91 deletions (Table 2). The CNVs of unknown clinical significance numbered 121 and were of the following size: 89 (73.5%) from 100 to 500 Kb; 31 (25.6%) were from 500 Kb to 1 Mb; one (0.8%) was a size larger than 1 Mb. Copy number variations (excluding the pathogenic ones) were present in 74 patients. In 70 persons, the size of CNVs ranged from 100 to 500 Kb; in 39 CNVs were from 500 Kb to 1 Mb, and in seven larger than 1 Mb. In the majority of the cases, a combination of benign CNVs and CNVs of unknown clinical significance was defined. It is noteworthy that in our sample, CNVs ranging in size between 100 and 500 kb were of greater frequency.

## DISCUSSION

In 31 patients, 41 pathogenic variations were found. In six of these patients, eight pathogenic aberrations associated with the corresponding phenotype were defined. The interpretation of other variations was made on the basis of the frequency in the studied group, the size of the variations, content of genes in the region and the type of the CNVs (deletion or duplication).

The CNVs with a 1.0-3.0% frequency in the population are accepted as benign polymorphisms. This gave us reason to consider as probably benign, those varia-

tions that occurred at frequencies higher than 1.2% in the studied group. Twenty-four of the CNVs conform with these criteria and were therefore considered to probably be benign. Abnormal variations with a frequency higher than 1.2%, were found in the following chromosomal loci: 2q13 (2.5%), 3q29 (2.5%), 7q11.23 (3.7%), 8p23.1 (8.6%) and Xp22.33 (18.5%).

The deletion in the (2)(p16.3) region, found in patient 79, was pathogenic and covered one *HGNC* gene (*NRXN1*) and one *OMIM* gene (*NRXN1*). It covered the OMIM loci associated with autism. The detected deletion in the (5) (q35.2q35.3) region in the same patient was also pathogenic and covered 40 *HGNC* and 24 *OMIM* genes among which was the *NSD1*, whose loss of function was considered a major cause of Sotos syndrome. The NSD1 protein controls the activity of genes related to normal growth and development, although many of these genes have not been identified [33]. The described pathogenic CNVs in this gene were more than 100. Sotos syndrome is characterized by facial dysmorphism, dolichocephaly, cognitive decline, mild-to-severe intellectual disability and overgrowth. In the second patient (#52), aCGH revealed a pathogenic deletion, del(10)(q26.12q26.3), covering 85 *HGNC* and 49 *OMIM* genes. This type of chromosomal aberration leads to phenotypic manifestations such as facial dysmorphism, postnatal growth retardation, developmental delay, intellectual disability, hypotonia in newborns, feeding disorders, microcephaly, digital anomalies, heart

defects and defects of the genitourinary system. The common feature of the patients with these aberrations is that they have behavioral problems. Some sources considered four candidate genes: *CTBP2*, *ADRB1*, *DPYSL4*, *DRD1IP*, associated with neural development and function [34].

The pathogenic deletion in patient 46, del(4)(q34.3q35.2), included 49 *HGNC* and 21 *OMIM* genes and the duplication, also pathogenic, on chromosome 12, dup(12)(p13.33p13.1), covered 226 *HGNC* and 156 *OMIM* genes. The presence of terminal aberrations is very often a result of family rearrangements, but due to lack of samples from the parents, the origin of the detected aberrations was not set. The clinical features included facial dysmorphism, obesity, increased appetite and other symptoms.

Patient 41 was revealed to carry pathogenic deletion 17, del(17)(q12q12), covering 24 *HGNC* and 24 *OMIM* genes, which are associated with renal cysts, diabetic syndrome, developmental delay, autism and schizophrenia, seizures and less common phenotypes with dysmorphic features, transitory neonatal hypercalcaemia, Müller aplasia and congenital diaphragmatic hernia. An important candidate gene was *HNF1B* associated with the development of the kidneys and pancreas.

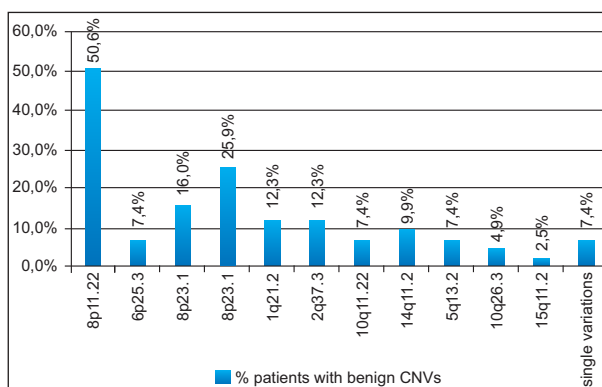
The detected deletion on chromosome 22, del(22)(q11.21q11.22), in patient 35, was also pathogenic covering 68 *HGNC* and 12 *OMIM* genes and affecting many organs and systems. It was assumed that the majority of the symptoms (heart defects, cleft palate, facial dysmorphism, hearing loss, hypocalcaemia), were caused by deletion of the *TBX1* gene.

The last detected pathogenic aberration was a duplication in chromosome 15, dup(15)(q11.2q13.1), which included 111 *HGNC* and 16 *OMIM* genes. This syndrome is represented by autism, intellectual disability, muscular hypotonia, ataxia, seizures, developmental delay and behavioral problems [35]. According to Tan *et al.* [36], at least three genes (*NIPAI*, *NIPA2*, *CYFIP1*), located in this region were associated with the development of CNS (central nervous system). A duplication of the copy of the *SNRPN* gene obtained from the mother is associated with autism. Other important genes associated with the development and neurological disorders are *GABRA5*, *GABRA3*, *GABRG3*, *MAGEL2*, *MKRN3*, *NDN*, *SNRPN* and *UBE3A*. The first three encode subunits of the GABA-receptors that mediate the main inhibitory neurotransmitter in the brain (GABA) [36].

The total number of established benign CNVs and CNVs of unknown clinical significance, amounted to 239, 141 of which were deletions and 98 were duplications. The total number of variations (with pathogenic ones) was

280, an average of 3.5 variations per patient. Two large studies in 2004 revealed that the genome of each person contains approximately 12 CNVs [27,28]. Later, in 2009, Itsara *et al.* [19] analyzed 2500 controls and found that the average number of CNVs for one person is between three and seven variations. Our data is very similar to that of Itsara *et al.* [19].

In more than one patient, benign CNVs were identified in the following cytogenetic loci: 8p11.22, 6p25.3, 1q21.2, 2q37.3, 10q11.22, 14q11.2, 5q13.2, 10q26.3, 15q11.2, 19q13.31, and two different CNVs were detected in region 8p23.1. In six patients, six single variations in the loci were found: 14q32.33, 10q11.22, 5p15.33, 16p13.11, 15q14, 14q21.2 (Figure 1).



**Figure 1.** Frequency of various benign CNVs in the studied group.

The most common benign CNVs were found in over 10.0% of the tested patients and were at the following locations: 8p11.22 and 1q21.2, and two different variations in the 8p23.1 locus. Deletions were identified in some of the cases, while in others, duplications, which indicated that these areas contained a significant number of variations. To establish the actual frequency of the detected unique benign CNVs, it was better to perform a genomic screening of a larger group of patients. According to Kooy [37], polymorphic CNVs are part of the natural genetic differences in humans, while rare variants can be related to certain disorders. A large study found that polymorphic CNVs are probably not closely related to the genetic etiology of multifactorial diseases, and it is possible that they are part of the benign genetic variations between individuals [38].

The number of variants of unknown clinical significance was 121. They were distributed by size as follows: 89 (73.6%) 100-500 kb; 31 (25.6%) 500 kb-1 Mb; one (0.8%) larger than 1 Mb. There were 50 deletions and 71 duplications. From the identified 121 variations of unknown clinical significance, in the following cytogenetic loci: 8p11.22, 8p23.1, 14q32.33, Xp22.33, 17q21.31, 16p11.2,



2q37.3, 14q11.2, 22q11.23, 11q25, 12p13.31, Xq22.3, 2p11.1, CNVs were found in more than one patient. There were 33 single CNVs detected in 27 patients.

Our results revealed a high rate of CNVs of unknown clinical significance. It is very likely that some of these variations are benign for the Bulgarian population. It is well known that the Bulgarian nation has a great genetic heterogeneity. Thirteen types of CNVs of unknown clinical significance were defined. They were located as follows: 8p11.22, 8p23.1, 14q32.33, Xp22.33, 17q21.31, 16p11.2, 2q37.3, 14q11.2, 22q11.23, 11q25, 12p13.31, Xq22.3, 2p11.1 (88 of the total number), and occurred in over 1.2% of the patients. That gave us reason to assume that they had no pathogenic nature. The most common, probably benign CNVs in our study, were in loci 14q32.33, 17q21.31 and 16p11.12, occurring in over 15.0% of the patients. Thirteen of the CNVs detected in loci 14q32.33, 16p11.2, 16p12.3, 17q21.31, 1q21.2, 20p12.1, 22q11.22, 2q37.3, 4q35.2, 8p23.1 were found in patients with already defined pathogenic aberrations of major size, and associated with the corresponding phenotype. According to some sources, in the presence of large chromosomal aberrations associated with a particular phenotype, it is unlikely for the identified smaller rearrangement to be essential for the development of the disease [23]. Therefore we assumed that it was possible for these 13 variants to have no relation with the clinical phenotype. In patient 30 a duplication of unknown clinical significance was identified in the 1q21.2 region, which contained 15 *HGNC* and 1 *OMIM* genes. Among them was the *NBPF23* gene, which belongs to a gene family, characterized by tandem repeats of the DUF1220 protein domains. Several developmental problems and neurogenetic disorders such as microcephaly, macrocephaly, autism, schizophrenia, learning disorders, congenital heart disease, neuroblastoma, congenital anomalies of the kidney and urinary tract [39], are associated with identified CNVs located in the region with more DUF1220 domains. Based on this information, we could not exclude the potential pathogenic effect of the CNVs in this region. Nineteen of the rest of the CNVs of unknown clinical significance could not be interpreted as probably benign. We need more complementary studies to clarify the phenotypic effect.

Five types of the pathogenic CNVs, located in chromosomal loci 2q13, 3q29, 8p23.1, 7q11.23, Xp22.33 (24 variations) were found in a high percentage (over 1.2%) of the patients. Their high frequencies gave us reason to suppose that despite their pathogenic nature, these variations might be considered to be CNVs of unknown clinical significance or probably benign. In four patients, we identified four pathogenic variations with no clinical significance, in the

following chromosomal loci: 22q11.22, 8p23.2, 2q13, 4q12. To determine participation in the variation genes, we made a reference to the Database of Genomic Variants (DGV). The *OMIM* genes responsible for the clinical phenotype were found in none of the four CNVs.

In the selected group of patients with congenital anomalies, 280 CNVs have been proven, respectively 41 pathogenic, 118 benign and 121 of unknown clinical significance. We found 13.6% incidence of pathogenic CNVs by aCGH. Eight pathogenic variations corresponding to the clinical phenotype of the patients, were identified. We revealed genotype-phenotype correlations between many genes and the manifested clinical features. During the interpretation of the CNVs of unknown clinical significance and the pathogenic ones, an additional category was defined, "probably benign variations." It was concluded that there is a need to apply new genomic technologies with greater resolution, as a significant proportion of the patients were left with an unclear diagnosis. This would facilitate the detection and interpretation of the genomic aberrations in order to make an accurate diagnosis and to optimize therapeutic approaches.

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**Declaration of Interest.** The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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