VILNIUS UNIVERSITY

EGLĖ PREIKŠAITIENĖ

EVALUATION OF THE IMPACT OF GENOMIC STRUCTURAL ALTERATIONS ON THE PHENOME IN PATIENTS WITH INTELLECTUAL DISABILITY

Summary of doctoral dissertation

Biomedical Sciences, Medicine (06 B)

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VILNIAUS UNIVERSITETAS

EGLĖ PREIKŠAITIENĖ

ASMENŲ, TURINČIŲ INTELEKTINĘ NEGALIĄ, GENOMO STRUKTŪRINIŲ POKYČIŲ ĮTAKOS FENOMUI ĮVERTINIMAS

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INTRODUCTION

Intellectual disability is a broad diagnosis encompassing a wide variety of overlapping phenotypes and severities. It is characterised by significantly impaired cognitive functioning and deficits in two or more adaptive behaviours that appears before adulthood. Intellectual disability can range from mild to profound and can be associated with other clinical symptoms as part of a syndrome or can occur as an isolated phenotype. Taken together, syndromic and non-syndromic intellectual disability affects possibly more than 3 % of the general population worldwide, and this is the leading socio-economic problem of health care. Although non-genetic factors (as infection or trauma) can result in cognitive impairment, genetic aetiologies are found in approximately two-thirds of cases, with underlying mutations ranging from large cytogenetic aberrations to point mutations and even epigenetic alterations. The leading known cause of developmental delay/intellectual disability (DD/ID), explaining about 30 % of cases, is currently chromosomal alterations. These abnormalities are microscopically visible cytogenetic alterations and smaller copy number variants visible by genome-wide chromosomal microarray technologies (molecular karyotyping), such as array-based comparative genomic hybridisation (aCGH) and single nucleotide polymorphism (SNP) arrays. Point mutations and small insertions or deletions are found in an additional 10 % of patients with DD/ID, and multifactorial and epigenetic alterations can be detected in 10 % of cases. Currently the genetic aetiology of 50 % of patients with DD/ID remains unexplained. Despite the enormous technical progress that has been achieved in the area of molecular cytogenetics and molecular genetics over the last few years, the evaluation of the genetic causes of DD/ID is challenging because these conditions are genetically heterogeneous with many different genetic alterations that may manifest in clinically indistinguishable phenotypes.

The prevalence, poor curability and the lifelong severity emphasise the impact of ID and need for research (1). Genome-wide chromosomal microarray technologies have accelerated the identification of novel cytogenetic abnormalities (2, 3) and enabled the accurate diagnosis in a significant fraction of patients with previously idiopathic ID and

developmental disorders (4). The introduction of aCGH provided a unique possibility to identify novel microdeletion/microduplication syndromes, as well as to expand the phenotypes and to elucidate the genomic aetiology of previously well-known conditions. Increasing the resolution of array platforms further accelerated the pace of the discovery of genes that are implicated in the manifestation of both contiguous gene and monogenic conditions. The identification of Mendelian disease genes is still the main focus of human genetics. Based on the estimated 10−12 % of X-linked genes associated with intellectual disability, the number of autosomal intellectual disability genes might be as high as 800−850 (5), or even 2000 (6), but only a small fraction of them are known ID genes. Phenotypic characterisation of patients with DD/ID and chromosomal alterations, determination of novel recognisable syndromes, detailed characterisation of known microdeletion/microduplication syndromes, and identification of critical genes for ID is fundamental for effective genetic diagnostics. Understanding the cause of ID is important for many reasons, including prevention or earlier detection of pathogenic clinical signs, optimal management of a patient, effective genetic counselling for the affected individual and his/her family, and prenatal diagnosis (7).

Aim of the study:

To determine the phenotypic impact of pathogenic copy number variants and their role in disturbing the development and function of the central nervous system in patients with intellectual disability.

Main tasks of the study:

1. To evaluate the characteristics and clinical consequences of the pathogenic copy number variants in patients with DD/ID and to determine the diagnostic yield of genome-wide chromosomal microarray technologies.
2. To define the phenotypic features of patients with pathogenic copy number variants in comparison with patients without pathogenic chromosomal alterations.
3. To delineate the phenotypic features associated with unique chromosomal alterations and to determine candidate genes for ID and other clinical features on the basis of an

analysis of the relationship between the genotype and phenotype using literature and web-based, community resources.

4. To determine the role of pathogenic chromosomal alterations in determining the chromosomal regions significant for the expression of known genes.
5. To determine the possible clinical consequences of complex chromosomal alterations and their significance in genetic counselling.
6. To make evidence-based recommendations concerning the evaluation of patients with DD/ID.

Relevance and novelty of the research

In recent years the clinical implementation of genome-wide chromosomal microarray technologies has revolutionised the diagnosis of patients with syndromic and non-syndromic ID. These technologies allow for a high-resolution evaluation of DNA copy number variations and enable the genome-wide detection of submicroscopic imbalances. Microarray analysis can identify any segmental imbalance (aneuploidy, deletion, duplication) of the loci represented on the microarray (8). Molecular karyotyping therefore offers a number of advantages over conventional cytogenetic analysis, fluorescence *in situ* hybridisation (FISH), and multiplex ligation-dependent probe amplification (MLPA), as it is sensitive, fast and amenable to very high resolution (9). In recent years many studies have shown that molecular karyotyping is an effective diagnostic tool in patients with DD/ID, for whom previous conventional cytogenetic analysis has proven negative (10). The information about the nature of the copy number variant, size, and gene content in the chromosomal alteration allow for the identification of the known and new microdeletion/microduplication syndromes, narrowing the critical regions of known syndromes or even identifying critical genes for syndromes. The quantitative or structural alterations of these genes may cause clinical phenotype.

Application of genome-wide chromosomal microarray technologies has significantly changed the strategies for the identification and characterisation of new syndromes. Before the advent of molecular karyotyping, many genomic disorders were initially characterised according the common clinical features in a group of patients,

subsequently defining the genetic cause (a *phenotype first* approach). Molecular karyotyping technologies enabled the reverse model, when individuals with common chromosomal rearrangements are identified, and then the clinical features are compared. Chromosomal microarray technologies are therefore very effective not only in revealing molecular diagnosis, but also in identifying new microdeletion/microduplication syndromes.

The principles of identifying genes are also changing. Until 2000, most gene identification methods relied on linkage and positional cloning methodologies. Thus only large families with many affected individuals were amenable to the study. Autosomal gene mutation (dominant effect) cases with severe ID are usually sporadic due to lower reproductive function, however. Critical autosomal genes were identified only in exceptional cases, as balanced translocation events disrupting the critical gene or in cases of consanguineous marriages (recessive effect mutations). With the use of genome-wide chromosomal microarray technologies, the chromosomal cause of ID can be detected by testing a single patient. Further detailed analysis of the critical chromosomal region can be helpful in identifying the genes that are essential for the development and functioning of the central nervous system.

This research is based on the investigation of 211 patients with DD/ID by molecular karyotyping and detailed clinical characterisation, aiming to evaluate the clinical consequences of pathogenic copy number variants and their role in cognitive function. A large cohort of patients with DD/ID and random phenotypic features was investigated. For the first time in Lithuania, molecular karyotyping analysis of patients by aCGH and SNP arrays was performed. The results that were obtained increased the understanding of the aetiology of ID and filled the gaps in the knowledge of phenotypic characteristics of patients with chromosomal alterations. Analysis of the pathogenic copy number variants that were detected revealed the detailed clinical characteristics of the novel chromosomal alterations and new candidate genes for ID. A straightforward outcome of this study was the establishment of diagnosis in patients with previously unknown aetiology of DD/ID

(the basis of effective genetic counselling). The genetic diagnostic workflow for the patients with DD/ID was carried out according the results of the study and literature.

Statements to be defended:

1. The diagnostic yield of molecular karyotyping is more than 10 %.
2. Specific clinical features are common in patients with pathogenic copy number variants.
3. The detection of novel pathogenic copy number variants is essential for the characterisation of new microdeletion/microduplication syndromes.
4. The analysis of pathogenic copy number variants is significant for the identification of new candidate genes for ID and detection of regulatory regions of genes.
5. The use of molecular karyotyping in diagnostic investigation changes the diagnostic workflow of patients with DD/ID.

PATIENTS, MATERIALS AND METHODS

The study was conducted over a period from 2011 to 2013 on the basis of the scientific activities that took place as part of the CHERISH and PROGENET projects. Two hundred eleven patients with DD/ID were enrolled in the study. The main selection criterion for molecular karyotyping was unknown aetiology of DD/ID (Table 1). Both syndromic and non-syndromic cases were included. The DD/ID levels of patients ranged from mild to profound. Previously performed conventional karyotyping, targeted FISH, molecular tests, and investigations for metabolic disorders revealed no causative anomaly. Detailed clinical history and data of physical examinations were reviewed for all patients. Informed consent for genetic investigations was obtained from the participating families. The study was approved by the Vilnius Regional Biomedical Research Ethics Committee.

DNA from the patients was isolated using the phenol-chlorophorm extraction method. Diagnostic screening was performed using aCGH with 44K (n=10), 105K (n=102) and 400K (n=76) oligo chips (Agilent Technologies, Santa Clara, CA, USA) or

Table 1. The main criterion for recruitment of patients.

Intellectual disability (IQ<70, Wechsler scale) or developmental delay (for children under 6 years old) in cases of:
– congenital malformations of the central nervous system,
– multiple congenital anomalies/ID syndromes, not previously described,
– neurological syndromes of unknown cause (dystonia/ataxia/cerebral palsy),
– idiopathic severe epilepsy,
– autism,
– unspecific intellectual disability, sporadic case,
– apparently balanced chromosomal aberration,
– suspected known syndrome/condition with unknown genomic aetiology.

SNP arrays with 300K (n=12) and 700K (n=11) platforms (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. FISH and real time-PCR were used for the confirmation of gene-dose imbalances and investigation of parental samples. The pathogenicity of the copy number variants was assessed using the guidelines described by Lee et al. (11) and Rosenberg et al. (12).

Statistical analysis was performed using IBM SPSS Statistics 20 software (IBM, Armonk, NY, USA). Clinical data of patients with chromosomal alterations were compared with those having normal molecular karyotyping results using the χ^2 test. Fisher's exact test rather than the χ^2 test was used when one or more of the cells had an expected value less than 5. The statistical relationship between variables was estimated using the Spearman correlation coefficient. Multivariate logistic regression analysis was performed to determine the predictors of pathogenic copy number variants, using the result of molecular karyotyping as a dependent variable. A p value of <0.05 was considered statistically significant.

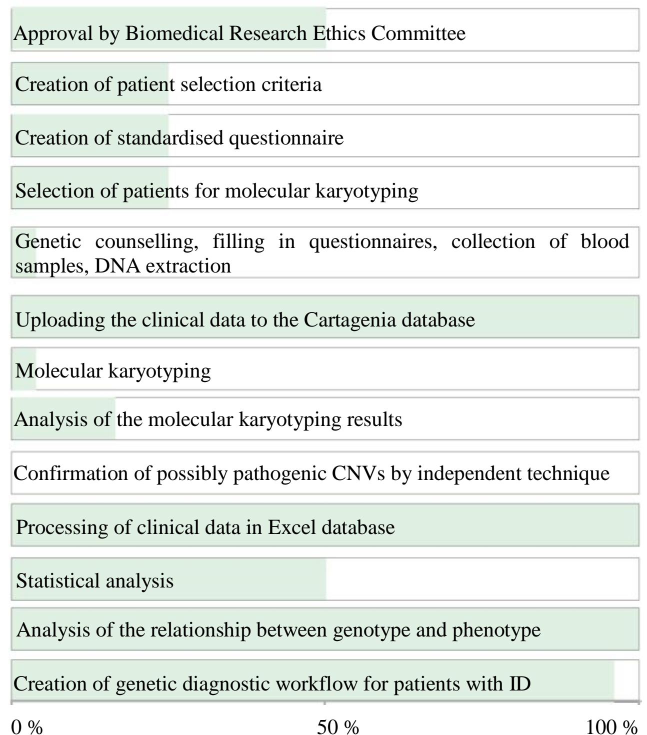

Figure 1. Scheme of work and contribution of the author (indicated in green bars).

Analysis of the relationship between genotype and phenotype in patients with

unique chromosomal alterations was performed using data from literature as well as web-based resources and aCGH databases. On the basis of literature and databases, the genes involved in the novel chromosomal alterations that were detected were analysed, and candidate genes for DD/ID and other clinical features were identified. Analysis of the complex chromosomal rearrangements that were detected was performed, and a possibly significant region for the known gene expression was determined by analysing unique chromosomal alterations.

The contribution of the author is shown in figure 1.

RESULTS AND DISCUSSION

1. The characteristics of pathogenic copy number variants in patients with DD/ID

In this study 36 pathogenic copy number variants in 29 of 211 patients (13.7 %) were identified, ranging in size from 0.26 Mb to 18.42 Mb, with median and mean sizes of 3.12 Mb and 5.11 Mb, respectively. Twenty-four of all detected pathogenic copy number variants were deletions, and 12 were duplications. Patients with pathogenic copy number variants were subdivided into three groups: known microdeletion/microduplication syndromes (9/29=31 %), novel pathogenic copy number variants (13/29=45 %), and unbalanced translocations or other complex chromosomal abnormalities (7/29=24 %). This subdivision was not used further for the statistical analysis. The clinical and genetic information of all patients with pathogenic copy number variants is presented in table 2.

Table 2. Genomic and clinical data of patients with pathogenic copy number variants.

Nomenclature according to ISCN 2009 (NCBI build 36/hg 18)	Size (Mb)	Age, sex, phenotype	Syndrome
Known microdeletion/ microduplication syndromes			
arr 2q37.3 (239,525,614-242,717,216)x1 dn	3.19	3 years, ♀. DD, epilepsy, hypotelorism, pointed chin, long toes.	2q37 monosomy s.
arr 5q35.3 (176,728,738-176,992,730)x3 dn	0.26	4 years, ♀. DD, hypotonia, internal hydrocephalus, tall stature, macrocephaly, long face, pointed chin, hypertelorism, torticollis.	Sotos s.

Nomenclature according to ISCN 2009 (NCBI build 36/hg 18)	Size (Mb)	Age, sex, phenotype	Syndrome
arr 11q23.3q25 (120,505,418-134,452,384)x1 mat Karyotype of the mother: 46,XX,t(11;13)(q23.3;q10)	13.9	11 months, ♂. DD, atopic dermatitis, anemia, thrombocytopenia, macrocephaly, short neck, hypertelorism, strabismus, epicanthus, palpebral fissures slant down, low set and dysplastic ears, retrognathia, brachydactyly, narrow feet, 1-2 toe syndactyly, dysplastic scrotum, deafness.	Jacobsen s.
arr15q11.2q13.1 (22,293,861-26,890,764)x1 dn	4.6	11 months, ♂. DD, stereotopy, microcephaly, low set and protruding ears, transverse palmar creases, hypopastic scrotum.	Angelman s.
arr 16p11.2 (29,563,985-30,106,254)x1 dn	0.89	6 years, ♂. ID, hypotonia, hypermetropia, dolichocephaly, low set ears, palpebral fissures slant down, thick lower lip, pectus carinatum, clinodactyly.	16p11.2 microdeletion s.
arr 16p11.2 (29,215,334-30,106,254)x1 mat	0.54	7 years, ♀. Mild ID, pectus excavatum, joint laxity.	16p11.2 microdeletion s.
arr 16p13.3 (2,589,524-3,911,387)x3 dn	1.32	16 years, ♀. Mild ID, late puberty, obesity, brachycephaly, coarse facial features, short forehead, low set ears, mild neck pterygia, broad thorax.	16p13.3 microduplicatio n s.
arr 22q13.31 q13.33 (45,834,903-49,529,400)x1 dn	3.86	2 years, ♂. DD, autistic behaviour, hypotonia, hydrocephalus, cerebellar hemisphere hypoplasia, prominent forehead, dysplastic ears, 1-2 toe syndactyly, large feet.	Phelan McDermid s.
arr 22q11.21 (17,018,751-19,795,282)x1 dn	2.78	20 years, ♂. Mild ID, sleep disturbances, narrow palpebral fissures, dysplastic ears, kyphosis, ventricular septal defect, chronic thyroiditis.	DiGeorge s.
Novel pathogenic copy number variants			
arr 1p36.11 (26,714,062-27,728,654)x1 dn	1.02	7 years, ♀. ID, deafness, agenesis of corpus callosum, internal hydrocephalus, congenital laryngeal stridor, bicornuate uterus, abnormality of the ureters, asymmetrical skull, low set ears, preauricular pit, thick lips, pectus excavatum, umbilical hernia, broad toes.	
arr 2p16.1p14 (58,656,378-68,790,747)x3 dn	10.13	9 years, ♂. Mild ID, behavioural problems, epilepsy, macrocephaly, acanthosis nigricans, hepatomegaly.	
arr 2p22.1p16.1 (40,059,584-57,546,352)x3 dn	17.49	13 years, ♀. Moderate ID, behavioural problems, atrial septum defect, macrocephaly, high and prominent forehead, arched eyebrows, hypertelorism, preauricular pit, dystrophic toenails, lumbar hyperlordosis, flexion deformity of knees, flat arches of feet.	
arr 2q34 (212,505,294-213,463,152)x1 dn	0.96	15 years, ♂. Moderate ID, tall stature, thick eyebrows, protruding ears, thick lips, clinodactyly of the 5th finger.	Walsh et al. (13), Backx et al. (14)
arr 2q24.2q24.3 (162,118,995-164,461,141)x1dn	2.34	3 years, ♂. DD, hypotonia in infancy, stereotopy, strabismus, torticollis, sacral dimple.	Magri et al.(15), Krepischi et al. (16)

Nomenclature according to ISCN 2009 (NCBI build 36/hg 18)	Size (Mb)	Age, sex, phenotype	Syndrome
arr 4q28.3 (137,417,138-138,947,393)x1mat	1.53	3years, ♂. Severe DD, seizures, microcephaly, hypoplasia of corpus callosum, internal hydrocephalus, round face, large ears, joint laxity, tapering fingers, broad halluxes, sacral dimple.	
arr 4q21.22 (83,373,844-84,097,897)x1 dn	0.72	18 years, ♀. Severe ID, epilepsy, hypotonia in infancy, obesity, mandibular prognathia, small hands and feet, transverse palmar creases, brachydactyly.	Bonnet et al. (17)
arr 7p22.1 (5,337,072-6,316,915)x3 dn	0.98	14 years, ♀. Mild ID, obesity, macrocephaly, internal hydrocephalus, craniofacial dysmorphism, tapering fingers, flat arches of feet, scoliosis.	Chui et al. (18)
arr 7q35q36.1 (146,392,196-149,441,454)x1dn	3.05	11 years, ♂. Moderate ID, epilepsy, macrocephaly, low set and protruding ears, narrow palpebral fissures, thick lower lip, pectus carinatum, limited movements of knees and elbows.	Poot et al. (19)
arr 14q32.12q32.3 (90,907,243-105,866,436)x3	14.96	21 years, ♂. Mild ID, schizoaffective disorder (depressive type), obesity, transverse palmar creases, gap between first and second toes, joint laxity, osteochondrosis, lordosis, herniation of intervertebral nuclei.	
arr 15q22.21q24.1 (62,383,648-71,456,752)x3 dn	9.06	23 years, ♀. ID, congenital abnormality of the heart, strabismus, abnormality of the hip, coarse facial features, short forehead, retrognathia, high palate, single central incisor, brachydactyly, flat arches of feet, bilateral club feet.	
arr 16q22.3 (70,919,282-71,645,680)x1 dn	0.73	18 years, ♀. ID, hypoplasia of the uterus, long face, high forehead, thick lips, dysplastic ears.	
arr 17q21.33 (93497294 - 105866436)x1 dn	1.8	17 years, ♂. Mild ID, growth retardation, poor weight gain, microcephaly, long face, large beaked nose, thick lower lip, micrognathia, malocclusion of teeth, clinodactyly of the 5th fingers, gap between first and second toe, myopia.	
Unblalanced translocations and other complex chromosomal abnormalities			
arr 8 p23.3p23.1 (0-7,036,726)x1, 15q26.1q26.3 (90,472,251-100,338,915)x3dn	7.0 9.9	2 years, ♀. DD, hypotonia in infancy, ventriculomegaly, hypermetropia, strabismus, high forehead, temporal narrowing, palpebral fissures slant down, ptosis of eyelids, low set ears, tapering fingers, hypertrichosis.	
arr 21q22.3 (41,817,957-46,944,323)x1, 21q22.2q22.3 (39,774,417-41,817,957)x3 dn	5.03 2.04	14 years, ♂. Moderate ID, motor alalia, scoliosis, abnormal number of hair whorls, abnormal dermatoglyphic patterns, long toes.	
arr 4p16.3 (0-4,194,871)x1, 11p15.5p15.4 (0- 3,357,154)x3 pat	4.19 3.36	19 years, ♀. ID, hyperactivity, epilepsy, abnormality of the hip, atrial septum defect , pulmonary stenosis, short stature, microcephaly, prominent nasal bridge, hypertelorism, coloboma of eyelids, short philtrum, micrognathia, scoliosis, clinodactyly of the 5th finger, gap between first and second toes, abnormal dermatoglyphic patterns.	Deletion of the 4p Wolf-Hirschhorn s. region

Nomenclature according to ISCN 2009 (NCBI build 36/hg 18)	Size (Mb)	Age, sex, phenotype	Syndrome
arr 5p15.33p15.31 (0-8,157,370)x1, 12q24.21q24.33 (113,931,344-132,349,534)x3 mat	8.16 18.42	3 years, ♂. DD, epilepsy, hypoplasia of corpus callosum, high forehead , arched eyebrows, hypertelorism, short philtrum, micrognathia, proximal placement of thumb, sacral dimple.	
arr 5p14.3 p14.1 (23,025,478-26,938,536)x1 dn 46,XY,t(3;14)(6;20)(q12;q11.2)(q21;p11.2), *de novo*	3.91	4 years, ♂. Severe DD, seizures, microcephaly, large ears, sloping forehead, optic atrophy.	Deletion 5p - Cri du chat s. region
arr 5q14.3 (86,456,211-89,104,733)x1, 6q21 (104,995,949-105,493,496)x1, 16q23.1 (81,507,514-81,784,949)x1 dn 46,XY,t(6;16)(q25;q12.1), *de novo*	2.65 0.5 0.28	7 months, ♂. Severe ID, hypertonia, patent foramen ovale, brachycephaly, hypertelorism, strabismus, depressed nasal bridge, long philtrum, microstomia, low set ears, micropenis.	
arr 4q34.3q35.2 (179,503,254-191,162,351)x1, 8p23.3p23.1 (0-10,772,254)x3	11.66 10.77	12 years, ♂. Moderate ID, aggressive behavior, deafness, macrocephaly, high forehead, hypertelorism, strabismus, crease of ear lobule, depressed nasal bridge, supernumerary nipples, arachnodactyly.	

In summary, the estimated yield of molecular karyotyping of patients with DD/ID in this study is 13.7 %. This result is consistent with previously reported results (5–15 %) (20-21). Our total detection rate of pathogenic copy number variants other than associated with known microdeletion/microduplication syndromes is 9.5 % (20/29). Molecular karyotyping therefore provides a unique possibility in many of patients with DD/ID to formulate a genetic diagnosis, which could not be done by the *phenotype first* approach. The detection rate of pathogenic copy number variants can be influenced by the preselection criteria of the patients and the median spacing of the probes in the platform used. The recruitment of patients in this study was not selective (regarding the severity of DD/ID and presence of congenital malformations and minor anomalies) and likely represented a general population of patients with unknown causes of DD/ID. Unless several different platforms were used for molecular karyotyping, most patients

were investigated using high-resolution platforms (201/211). Since high resolution molecular karyotyping revealed small copy number variants (<500 kb in size) in 1 % of patients (2/201), the possibility of undetected small pathogenic copy number variants in the remaining 10 patients tested by low resolution arrays was about 0.1 % and was considered insignificant.

2. Phenomic clues to genomic variation

The purpose of this task was to determine whether patients with pathogenic copy number variants can confidently be assigned to a distinct clinical subgroup. The analysis of the main characteristics of genealogies and personal histories of patients with pathogenic copy number variants and without chromosomal alterations revealed no statistical significant differences between these two groups. In the total sample of 211 patients, 798 phenotypic features were registered, of them 192 (24.1 %) were congenital anomalies (classified according Q chapters with BPA extension, version 23 June 2008) and 606 (75.9 %) were minor anomalies (according EUROCAT list "Minor Anomalies for Exclusion"). The findings of statistical analysis indicate the increased frequency of pathogenic copy number variants in patients with syndromic DD/ID (p=0.018), at least one congenital anomaly (p=0.005), three and more minor anomalies (p=0.016), congenital malformations of CNS (p=0.026), congenital malformations of musculosceletal system (p=0.037), and minor anomalies of eye, ear, face and neck subgroup (p=0.003). Several statistically significant phenotypic traits such as hydrocephalus (p=0.023), congenital malformations of the corpus callosum (p=0.014), downward slanting palpebral fissures (p=0.008), minor anomalies of the ear (p=0.002), micrognathia (p=0.004), brachydactyly (p=0.005), and umbilical hernia (p=0.008) were more frequent in patients with pathogenic copy number variants. *Café au lait* spots were more frequently in patients without pathogenic copy number variants (p=0.023) (Figure 2).

Multivariate logistic regression analysis was performed to determine the predictors of pathogenic copy number variants. Three independent predictors of pathogenic

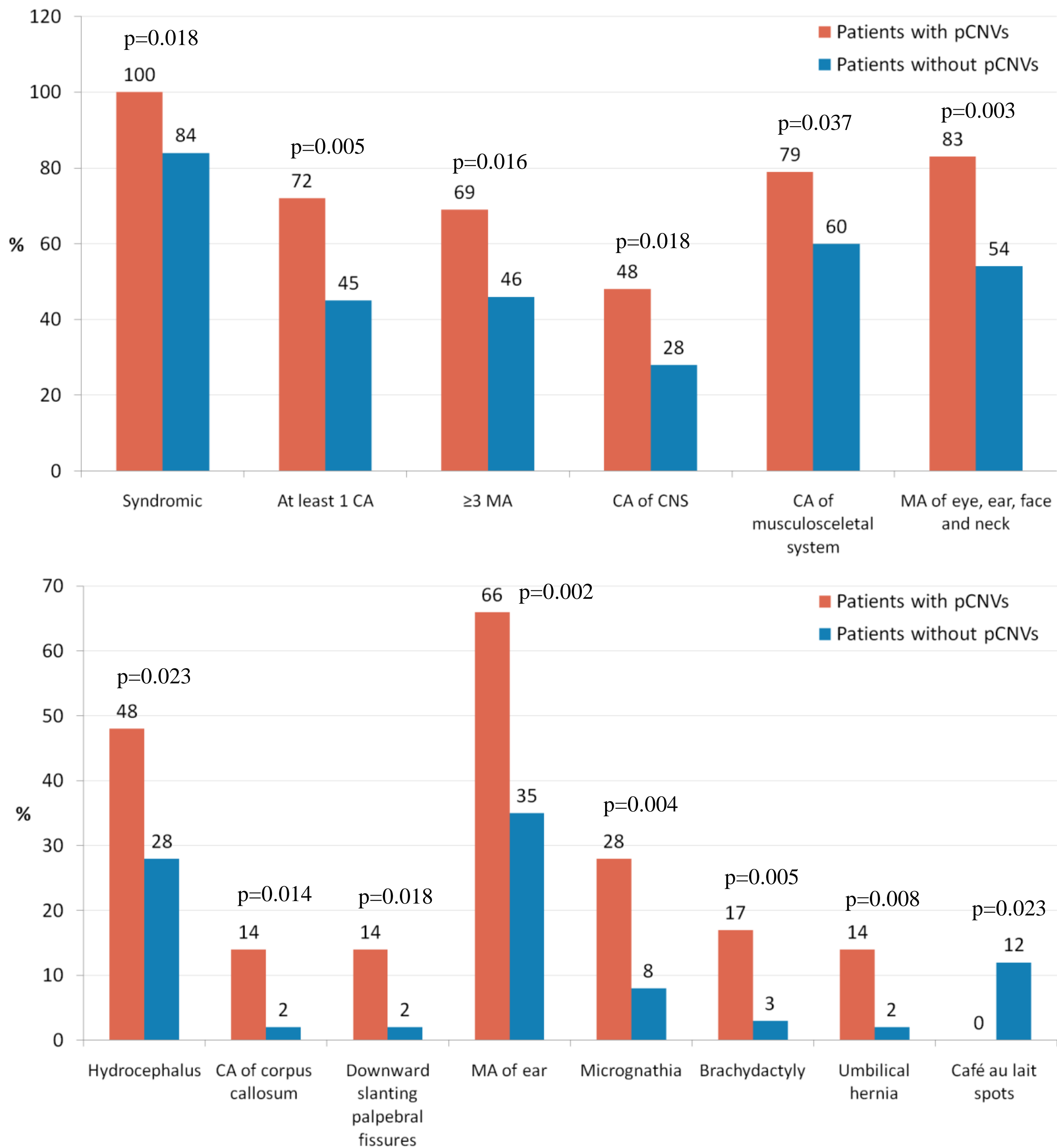

Figure 2. Evaluation of clinical data of patients with pathogenic copy number variants in comparison with patients with normal molecular karyotyping results (CA – congenital anomaly, MA – minor anomaly, pCNVs – pathogenic copy number variants).

Table 3. Results of multivariate logistic regression analysis.

Independent predictors	p value	Exp(B)	Odds ratio (95 % CI)
Congenital malformations of the corpus callosum	0.006	8.450	1.828-39.070
Minor anomalies of the ear	0.006	3.489	1.469-8.286
Brachydactyly	0.004	7.399	1.860-29.429

copy number variants were determined: congenital malformations of the corpus callosum, minor anomalies of the ear and brachydactyly, increasing the risk of pathogenic copy number variants by 8.5, 3.5 and 7.4 times respectively (Table 3).

A classification tree plot (dendrogram) revealed well-separated clusters, when all the significantly different anomalies between the groups of patients that were analysed were included (Figure 3). The dendrogram shows the similarity between patients, where individuals in the same cluster are more similar regarding their phenotypic features to each other than to patients in other clusters. Consequently, the cohort of patients with DD/ID in the study was not homogeneous phenotypically.

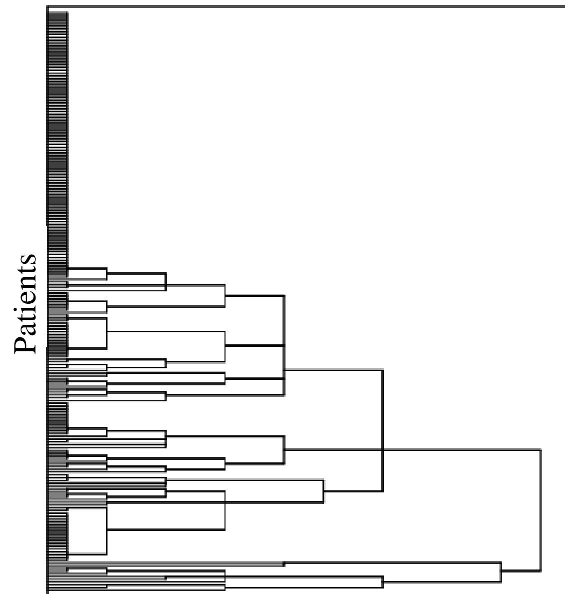

Figure 3. Distribution of patients with chromosomal alterations and without pathogenic copy number variants according phenotypic features (Furthest neighbour cluster analysis).

In summary, the detailed analysis of clinical data of patients with DD/ID revealed phenotypic clues leading to the detection of pathogenic copy number variants. Since the introduction of high-resolution microarray technologies it has become apparent that structural chromosomal rearrangements can lead to wide variety of clinical manifestations, including ID/DD, congenital anomalies, and dysmorphic features. It has been recommended that an array-based screen for genomic deletions and duplications should be the first-tier cytogenetic diagnostic test for patients with DD/ID, autism spectrum disorders, or multiple congenital anomalies (22). Though many diagnostic genetic laboratories have started to implement this technology into the routine diagnostic

setting, still not all laboratories have the possibility to perform this analysis on every patient. The results of this study enhanced knowledge about the phenotypic features of patients with pathogenic copy number variants. The information about the specific spectrum of minor anomalies and congenital malformations in patients with pathogenic copy number variants may be helpful in selecting patients for molecular karyotyping and increasing the diagnostic yield of this investigation.

3. Analysis of genotype-phenotype relationship in patients with unique pathogenic copy number variants and detection of candidate genes for DD/ID and other clinical features

Clinical and molecular characterisation of 7p22.1 microduplication

7p22.1 microduplication was detected in a patient with mild ID, craniofacial dysmorphism and skeletal abnormalities. One patient with overlapping microduplication was previously reported in the literature. The genomic and clinical data of the other three patients was obtained through the DECIPHER database (Table 4, Figure 4).

Table 4. Genomic and clinical data of patients with 7p22.1 microduplication.

Patient No, references	Genomic data	Clinical data
I (our patient)	arr 7p22.1 (5,337,072-6,316,915)x3 dn (hg18)	14 years, ♀. Mild ID, truncual obesity, tall stature, macrocephaly, internal hydrocephalus, mid-face hypoplasia, facial asymmetry, low set and protruding ears, hypertelorism, palpebral fissures slant down, short nose, anteverted nares, wide-spaced teeth, severe microretrognathia, high and narrow palate, microstomia, thin lips, midline pseudo-cleft upper lip, abnormal palmar dermatoglyphic patterns, tapering fingers, flat arches of feet, scoliosis.
II (Chui et al. 2011)	arr7p22.1(5,092,748-6,797,449)x3 (hg18)	28 months, ♂. DD, macrocephaly, open anterior frontanel, frontal bossing, flat and broad nasal bridge, anteverted nares, ocular hypertelorism, low set and posteriorly rotated ears, left preauricular pit, wide-spaced and pegged teeth, mild kyphosis, bilateral bridged palmar creases, broad thumbs, undescended left testis.
III (DECIPHER 266923)	arr7p22.1(5,475,440-6,659,432)x3 dn (hg19)	6 years, ♂. DD, autistic behaviour, hypotonia, truncual obesity, frontal angioma, mid-face hypoplasia, low set and protruding ears, hypertelorism, short palpebral fissures, wide-spaced teeth, retrognathia, umbilical hernia, tapering fingers.
IV (DECIPHER 262563)	arr7p22.1(6,036,431-6,870,943)x3 (hg19)	2 years, ♂. DD, autism, stereotopy, perimembraneous ventricular septal defect, growth retardation, microcephaly, epicanthic folds, hypertelorism, long palpebral fissures, slight synophris, strabismus, long and flat philtrum, thin lips,unilateral single palmar crease.
V (DECIPHER 254379)	arr7p22.1(6,026,434-6,777,442)x3 (hg19)	2 years, ♂. DD, microcephaly, short stature, no obvious dysmorphism.

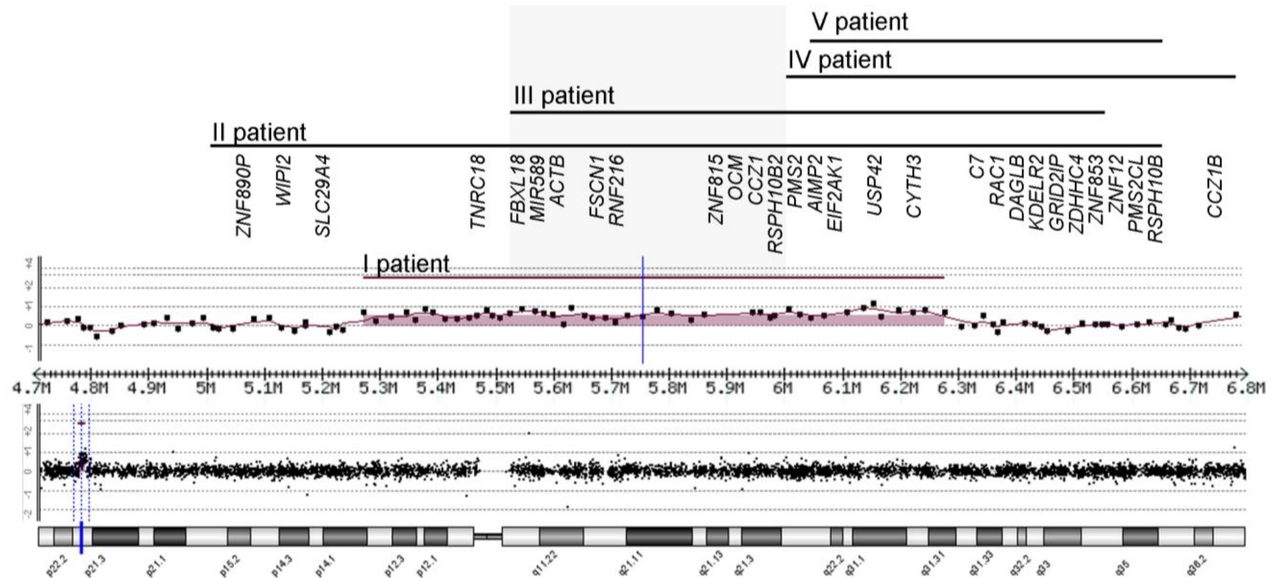

Figure 4. Ideogram of chromosome 7 and array CGH results of the patients with 7p22.1 microduplication. The region possibly associated with craniofacial dysmorphism is highlighted in grey.

The analysis of five cases of dup7p22.1 allowed for more precise genotype-phenotype relationship analysis. Patients I, II and III share several major features, including speech delay, macrocephaly, ocular hypertelorism, low set ears, micrognathia, and skeletal anomalies. Since no skeletal abnormalities were detected in the other two patients with overlapping microduplications (IV, V) and no dysmorphic features were reported in patient V, the critical region for craniofacial and skeletal abnormalities was restricted by the 7p22.1 region, which contains nine genes (*FBXL18, MIR589, ACTB, FSCN1, RNF216, ZNF815, OCM, CCZ1* and *RSPH10B2*). The candidate gene for craniofacial dysmorphism and skeletal abnormalities could be the disease gene *ACTB* (β-actin, OMIM 102630), which encodes β-actin, is an essential component of the cystosceleton, and plays a critical role in cell migration (23). Head morphogenesis is an intricate process that involves all three germ layers, and correct development depends on precise spatiotemporal interactions among all these structures and their derivatives (24). Cell motility is therefore an important process throughout the entire life span of vertebrates and is especially critical during many stages of craniofacial development.

The findings of this study suggest that the region 5,337,072-6,316,915 (NCBI build 36) on chromosome 7p22.1 is possibly the critical region of this rare, clinically recognisable condition characterised by DD/ID, craniofacial dysmorphism and skeletal abnormalities. Further cases with similar duplications will contribute to the delineation of the potentially new microduplication syndrome of 7p22.1.

Clinical and molecular characterisation of 17q21.33 microdeletion

17q21.33 microdeletion (1.8 Mb in size), previously not reported, was detected in a patient with mild intellectual disability and dysmorphic features. A search for further patients revealed three individuals with informative overlapping deletions through the DECIPHER and ECARUCA databases (Table 5, Figure 5). Two duplications overlapping the 17q21.33 region are reported in the literature (25-26), suggesting the presence of dosage-sensitive genes that are critical to the neurodevelopmental processes in this gene-rich chromosomal region.

Table 5. Genomic and clinical data of patients with 17q21.33 related chromosomal alterations.

Patient No, references	Genomic data	Main clinical phenotype
I (our patient)	arr 17q21.33 (93,497,294 – 105,866,436)x1 dn (hg 18)	17 years, ♂. Mild ID, growth retardation, poor weight gain, microcephaly, long face, large beaked nose, thick lower lip, micrognathia, malocclusion of teeth, clinodactyly of the 5th fingers, gap between first and second toe, myopia.
II (DECIPHER 878)	arr17q21.33 (48,965,790-54,817,786)x1 (hg 19)	4 years, ♂. DD, poor weight gain, normal height, microcephaly, brachycephaly, round face, micrognathia, fleshy and relatively large ears, aplasia cutis congenita, partial cutaneous syndactyly of 2-3 toes, abnormal flexion of the thumb.
III (DECIPHER 255632)	arr17q21.33 (47,554,863-49,471,989)x1 dn (hg 19)	♀. ID, general abnormalities of face, oesophageal atresia/stenosis.
IV (ECARUCA)	Del17q21.3q23	♂. ID, tracheo-oesophageal fistula, club foot varus, low birth-weight, short stature, brachycephaly, plagiocephaly, microcephaly, dystopia canthorum, hypertelorism, short palpebral fissures, palpebral fissures slant up, round face, micrognathia, down-turned corners of the mouth, long philtrum, cleft uvula, lose skin in neck, synostosis of fingers, proximal placement of thumb.
V (Zahir et al.2009)	arr17q21.33 (45,093,544-46,196,038)x3dn (hg 18)	♀, 14 years. Mental retardation, microcephaly, deafness, vision abnormalities, epicanthic folds, palpebral fissures slant up, short palpebral fissures, high palate, auricular tags, clinodactyly, pes cavus, syndactyly 2-3 of toes, tapering fingers.
VI (Leana-Cox et al. 1993)	Dup17q21.3 q22	♂, 3 years. Palpebral fissures slant up, colobomata, large beaked nose, prominent nasal tip, microghathia, wide-spaced nipples, syndactyly, hypoplastic nails, coronal hypospadias.

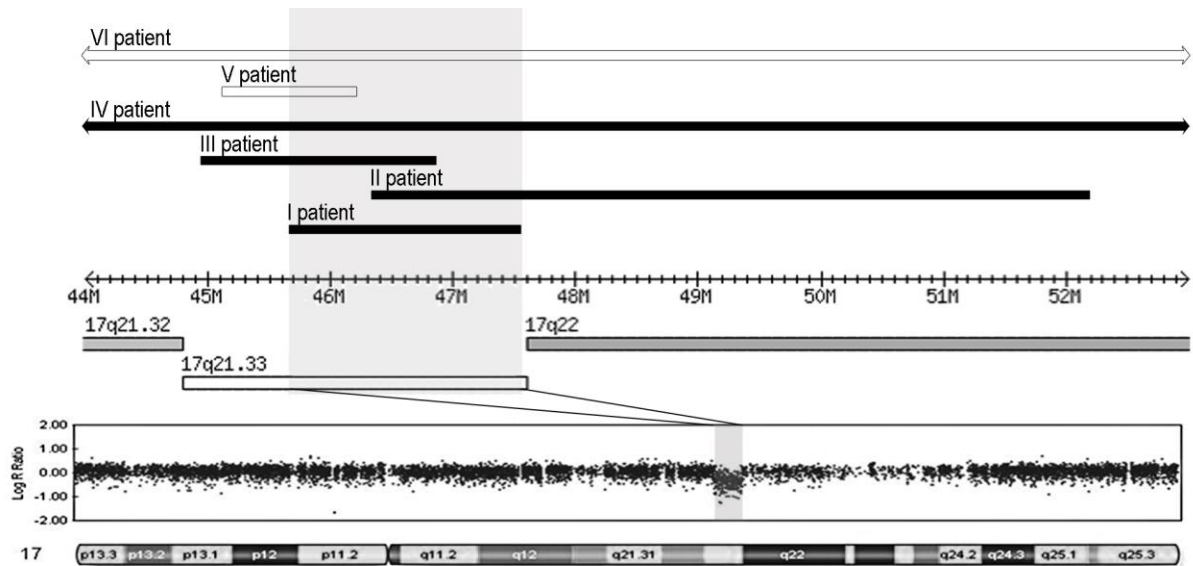

Figure 5. Ideogram of chromosome 17 and the map of 17q21.33 related chromosomal alterations (deletions in black and duplications in white lines).

The deletion encompasses up to 24 known genes, 21 protein-coding genes, and 3 non-coding RNA genes (Human NCBI36 Assembly, hg18). Seventeen of the known genes have been shown to be expressed in the brain (*CACNA1G, WFIKKN2, EME1, ANKRD40, LUC7L3, RSAD1, CA10, NME1-NME2, MRPL27, SPAG9, LRRC59, ACSF2, UTP18, TOB1, MYCBPAP, MBTD1* and *EPN3*). These genes are therefore functional candidates contributing to the impairment of the central nervous system in our patient. Among them is the *CACNA1G* gene encoding a voltage-dependent calcium channel that is thought to be involved in neuronal oscillations and resonance, in pacemaker activity in central neurons, and in neurotransmission. *CACNA1G* gene mutations have not been reported in humans. In recent studies, its association with autism (27) and epilepsy (28) has been implicated, however. These studies support this gene as a good candidate gene for intellectual disability. The other gene which haploinsufficiency may be related to our patient's impaired cognitive functioning is the *CA10* gene. *CA10* is highly expressed in the human brain (cerebellum, frontal cortex, parietal cortex and midbrain), testes, salivary glands, and kidneys (29) and is thought to play a significant role in the central nervous system, especially in brain development. A possible candidate gene for prenatal and postnatal growth retardation may be the *CHAD* gene, the product of which,

chondroadherin, is a cartilage protein with cell-binding properties. The expression of chondroadherin was studied in rat tissue and during postnatal femoral head development. The high expression of this cell binding protein in a dynamic region of cartilage suggests an important role for chondroadherin in the regulation of chondrocyte growth and proliferation (30).

This observation supports the role of *CA10* and *CACNA1G* genes in cognitive development. Detailed clinical examination of additional patients with a similar microdeletion is needed for a complete description of the phenotype and for the elucidation of the influences of *CA10, CACNA1G* and *CHAD* haploinsufficiency on that phenotype.

Clinical and molecular characterisation of 2q34 microdeletion

In a patient with intellectual disability, 2q34 microdeletion, which disrupts the *ERBB4* gene, was detected. Two cases of *ERBB4* gene disruption, one caused by deletion and one by the intragenic breakpoint in case of translocation event, have been reported previously (Table 6, Figure 6).

The *ERBB4* gene spans 1.16 Mb on chromosome 2q34 and codes one of four members of the mammalian ERBB family of transmembrane tyrosine kinases (31). Among the ERBB proteins, ERBB4 is important in the neurodevelopmental processes. The major ligands of the ERBB4 receptor are neuroregulins (NRG1-4), belonging to a family of growth factors. The *NRG1-ERBB4* signalling pathway is essential for many neurobiological processes. It has a developmental role in the generation of interneurons and their migration and differentiation in the neocortex and hippocampus and is involved

Table 6. Genomic and clinical data of patients with ERBB4 gene disruption events.

Patient No, references	Genomic data	Main clinical phenotype
I (our patient)	arr 2q34 (212,505,294-213,463,152) x1 dn	15 years, ♂. Moderate ID, tall stature, thick eyebrows, protruding ears, thick lips, clinodactyly of the 5th finger.
II (Walsh et al. 2008)	arr2q34(211,792,494-212,191,651)x1	13 years, ♂. Schizophrenia.
III (Backx et al. 2009)	t(2;6)(q33.1; p23)	4 years, ♀. Severe DD, seizures, hypotonia, polycystic kidney, microcephaly.

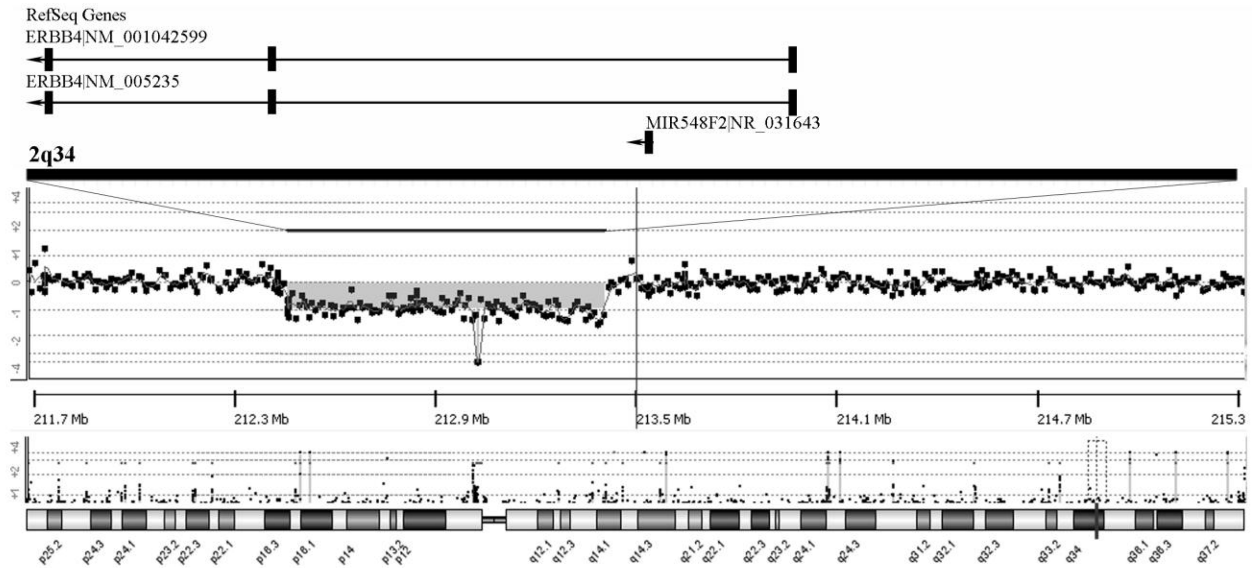

Figure 6. Ideogram of chromosome 2; array CGH results arr2q34(212,505,294-213,463,152)x1are shown in the lower protion, the RefSeq genes are presented above.

in neuronal signal processing in the adult brain by modulating synaptic plasticity and oscillations (32). Steffanson et al. provided the first evidence for the association of *NRG1* with schizophrenia in an Icelandic population (33). Various schizophrenia-associated SNPs in *ErbB4* have subsequently been identified in many populations (34). Neddens et al. (2011) provided evidence for conserved expression patterns of *ErbB4* across different rodent and primate species and also in humans. Several transgenic lines of *NRG1* and *ErbB4* have been developed. According to Golub et al. (2004), heterozygous null mice were additionally found to have cognitive deficit and delayed motor development, suggesting a delay in cerebellar development that could be related to the role of neuroregulin/*ErbB4* in neuronal migration (35). It is a challenge to relate the results of studies with transgenic animals directly to the phenotypic manifestation of a similar genetic variant in patients, but obviously the clinical consequences of *ERBB4* haploinsufficiency in our patient are compatible with reported hyperactivity and cognitive dysfunction in animal models.

This is the third reported case of *ERBB4* disruption and probably the first case of the pure haploinsufficiency of the *ERBB4* gene in a human so far. Unless various SNPs of the

NRG1-ERBB4 system are associated with schizophrenia, the haploinsufficiency of *ERBB4* is crucial for neurodevelopment and intellectual/cognitive function. These observations are compatible with previously reported results.

4. Identification of the regulatory region of a known gene

Duplication 5q35.3, not involving the *NSD1* gene, was detected in a girl with the clinical features of Sotos syndrome. The patient was a 4-year-old girl with developmental delay and dysmorphic features. Her physical examination was remarkable because of her tall stature, macrocephaly, long face, high forehead, sparse hair in the temporal regions, downslanting palpebral fissures, epicantus, large, soft and low-set ears, hypertelorism, small nose, anteverted nostrils, microstomia, micrognathia, high and narrow palate, broad alveolar ridges, open mouth appearance, and pointed chin (Figure 7). The girl's height and weight parameters have always been on the higher end of the growth chart (Figure 8).

The four previously published cases of 5q35.3 duplication (known as the critical region of Sotos syndrome), in which two cases were identified by aCGH (36), one by MLPA (37) and one by FISH (38), showed a phenotype opposite to that of Sotos syndrome (Figure 9). They were all characterised mainly by microcephaly, short stature,

Figure 7. Front facial view of the patient with characteristic features of Sotos syndrome.

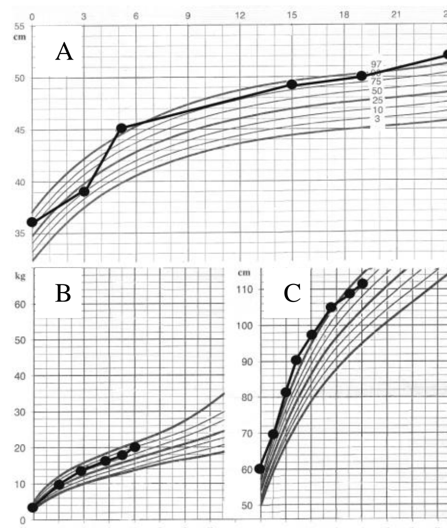

Figure 8. Growth curves of head circumference (A), weight (B) and height (C) of the patient from birth to 4 years of age.

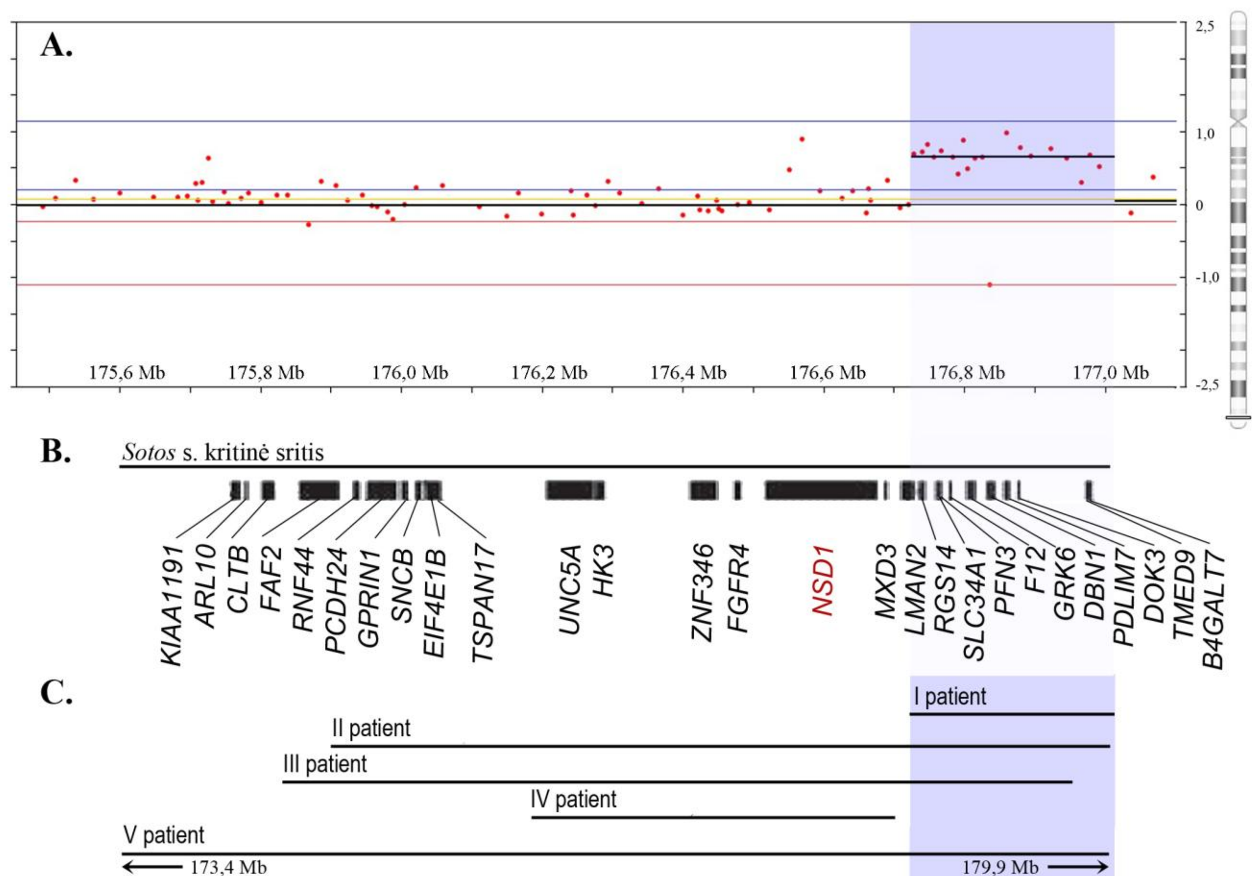

Figure 9. A. Scheme of the duplicated region in the distal long arm of chromosome 5 (5q35.3). B. Location of RefSeq genes in the region. C. Schematic view of the duplications detected in patients I-V.

and developmental delay. The duplicated region in our case (in the critical region of Sotos syndrome) is located from 176,728,738 to 176,992,730 (size of 264 kb), downstream from *NSD1* gene, but not including it. The distance from the *NSD1* gene to the first duplicated probe as detected by aCGH is 69 kb. Therefore, in this case there is no overdosage of the critical Sotos gene, *NSD1*. In light of the obvious Sotos syndrome phenotype of our patient and the fact that the duplication is located very near the critical gene of this syndrome, the expression of the *NSD1* gene could be disturbed, thus resulting in haploinsufficiency. Because the duplication is right downstream from the *NSD1* gene, regulatory elements critical to the gene expression sequence might be disrupted or the expression of the gene might otherwise be affected because of the duplication close to this gene.

Thus, when evaluating copy number variants that have been identified, the possible influence of the aberration on distant genes that could add additional diversity to genomic disorders should be also considered. This case demonstrates that evaluation of the size of the chromosomal alteration and gene content are not sufficient for assessing the pathogenicity of copy number variants and the context of adjacent genes should be considered.

5. Clinical consequences of complex chromosomal rearrangements and peculiarities in genetic counselling

A complex chromosomal rearrangement involving a *de novo* double t(3;14) and t(6;20) apparently balanced chromosomal translocations, and a 3.9 Mb interstitial *de novo* deletion at 5p14.1–p14.3 (Figure 10) was detected in a patient with severe DD, seizures, microcephaly, and dysmorphic features.

Deletion on chromosome 5p leads to a variety of developmental defects, with most cases classified as Cri du chat syndrome (MIM 123450) [Niebuhr, 1978]. Zhang *et al.* studied 94 patients with Cri du chat syndrome using aCGH and proposed the presence of three chromosomal regions (MRI-MRIII) at 5p that affect the manifestation of the level of mental retardation. The authors assigned a dominant role to MRI, while the deletions restricted to other two regions, MRII and MRIII, usually result in a much milder clinical effect or even no discernible phenotype (39). The deletion in our case is located within the MRIII region, and therefore based on the published genotype and phenotype relationship data, mild mental retardation or even normal phenotype should be expected in our patient. It is possible that the additional clinical effect of one or more of the four breakpoints in the chromosomes involved in the double balanced translocations (chromosomes 3, 6, 14 and 20) could also significantly influence our patient's phenotype.

This case describes a situation when clinical diagnosis could not be based on the results of aCGH only, as clinical manifestation was obviously too severe for the typical Cri du chat syndrome and could be exacerbated by other chromosomal balanced rearrangements in the genome. Both conventional karyotype and aCGH analyses are

therefore necessary in cases with inconsistency between genotype and phenotype. Such an approach may contribute to more accurate diagnosis and genetic counselling.

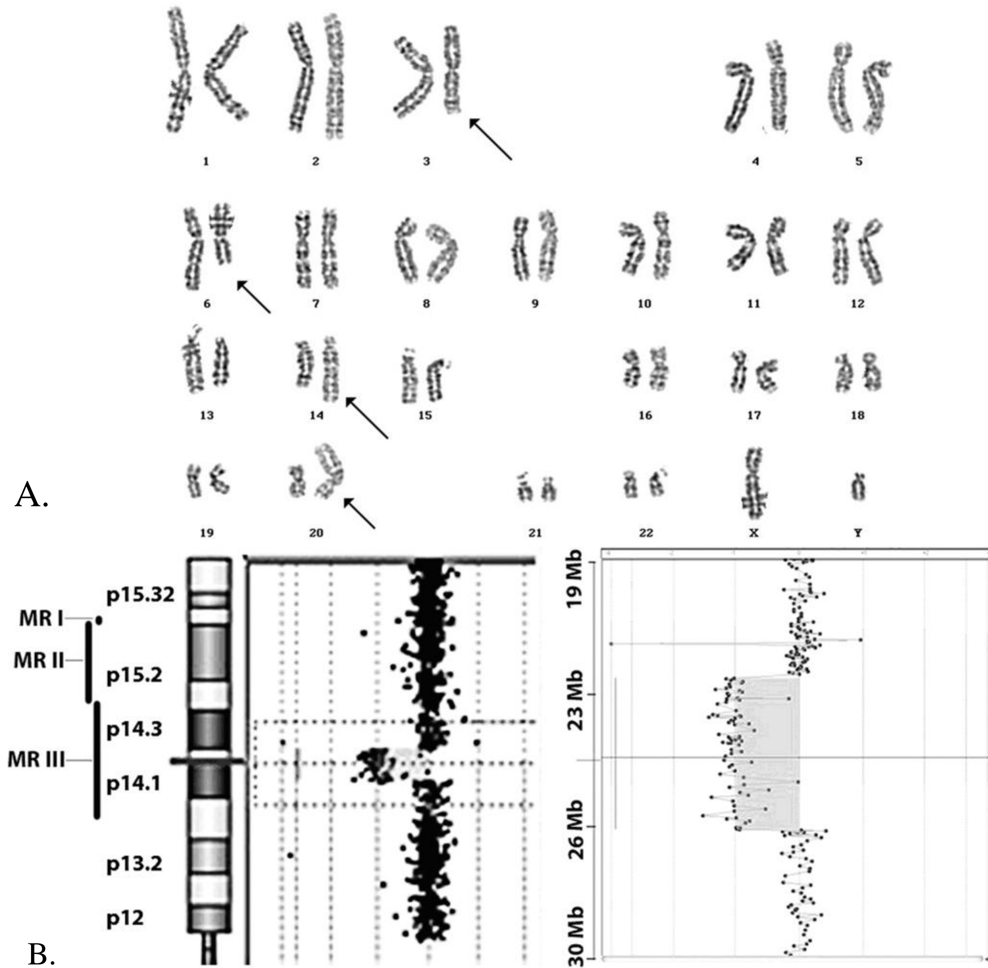

Figure 10. Karyotype showing double translocation, 46,XY,t(3;14)(q12;q11.2), t(6;20)(q21;p11.2). B. Array-CGH results showing a deletion, 3.9 Mb in size, which lies between nucleotides 23,025,478-26,938,536 (5p14.3-14.1), hg18. The location of MR I-III regions is shown in the left side of the figure.

6. Genetic diagnostic workflow for patients with DD/ID

Over the past few years, enormous technical progress has been achieved in the area of molecular cytogenetics and molecular genetics, changing the recommendations for the evaluation of children with intellectual disability. Diagnostic success often depends on

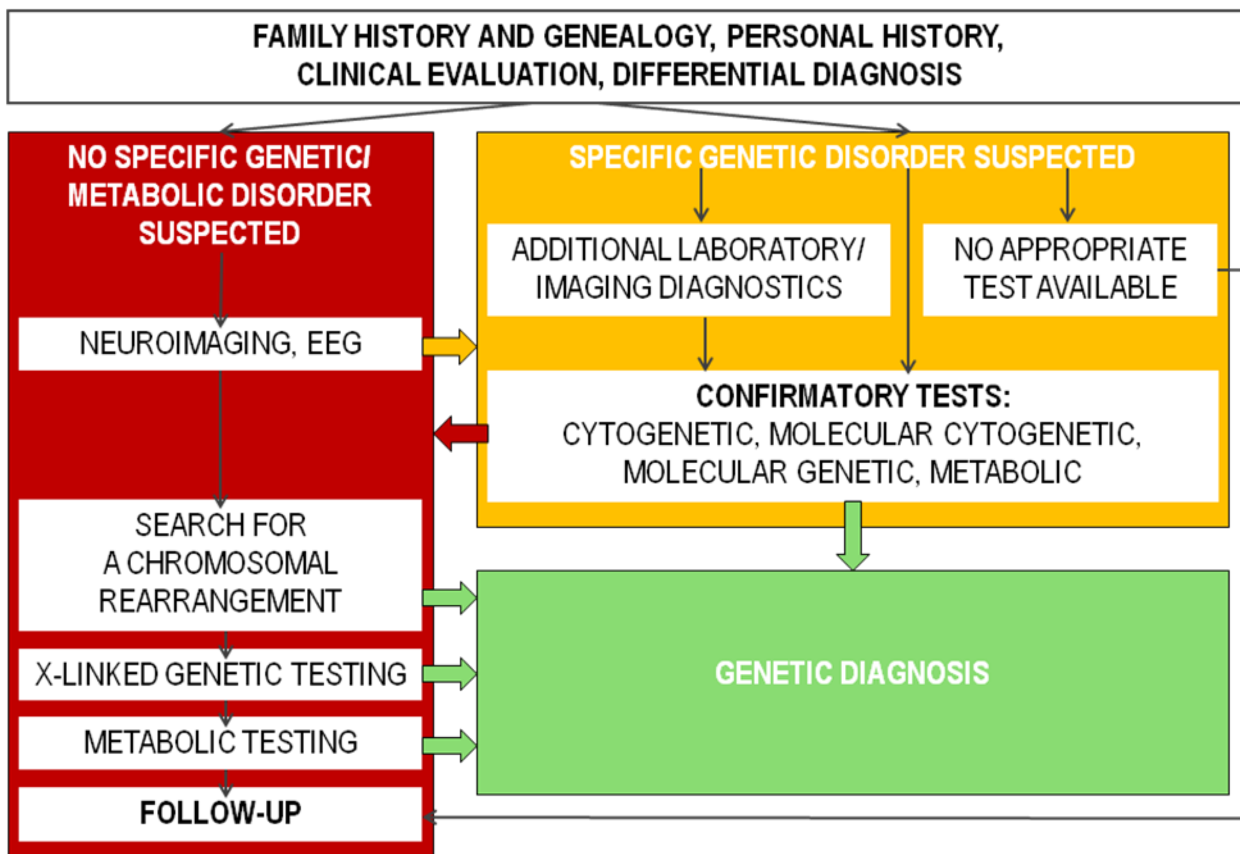

Figure 11. The scheme of the genetic diagnostic workflow for patients with DD/ID.

the diagnostic workflow of the patient. The stepwise approach of diagnostic evaluation increases the diagnostic yield and makes the use of expensive tools more rational.

An optimal diagnostic evaluation starts with family history and genealogy, personal history, and clinical evaluation, followed by differential diagnosis (Figure 11). When a specific genetic disorder is suspected, the confirmatory tests must be performed. But, according to the literature, in 65–85% of patients with ID no specific genetic/metabolic disorder is suspected after the first step of evaluation or the suspected diagnosis cannot be confirmed (40-41). In that case it is always important to search for additional clinical findings (including neuroimaging). If the specific genetic disorder remains unknown, molecular karyotyping is recommended (42). Since not all laboratories have the possibility to perform this test to every patient with DD/ID, the selection of patients can be based on the clinical features (according to this study, congenital malformations of the

corpus callosum, minor anomalies of the ear, and brachydactyly significantly increase the risk of pathogenic copy number variants). In case of inconsistency between genotype and phenotype, conventional karyotyping should be performed to search for balanced rearrangements. If molecular karyotyping is not possible, karyotype and subtelomeric FISH or MLPA are recommended. Finally, X-linked genetic testing followed by screening for inborn errors of metabolism is indicated (43).

Even if all currently available diagnostic methods were applied, there would remain patients with intellectual disability of undetermined cause, and the appropriate tests for the suspected genetic disorders are not always available. In all these cases, it is important when possible to store the patient's cell line or DNA. Systematic follow-up of the patient should be carried out routinely. Further technical advances are necessary to enable investigation of other genetic mechanisms, including somatic mutation, epigenetic dysregulation and polygenic disruption, each of which is likely to account for a significant proportion of cases (44-45).

CONCLUSIONS

1. The diagnostic yield of molecular karyotyping when testing Lithuanian patients with unknown aetiology of DD/ID is 13.7 %. When the cases of known microdeletion/microduplication syndromes are excluded, the diagnostic yield in revealing cases with novel pathogenic copy number variants by molecular karyotyping is 9.5 %.

2. Congenital malformations of the corpus callosum, minor anomalies of the ear, and brachydactyly are the phenotypic features that help to predict chromosomal alterations (p=0.006, p=0.006, p=0.004, respectively). The evaluation of specific phenotypic features in patients with DD/ID could be useful for more precise selection of patients for effective molecular karyotyping.

3.1. 7p22.1 microduplication is responsible for ID, craniofacial dysmorphism and skeletal abnormalities. 17q21.22 microdeletion is associated with ID, growth retardation, and dysmorphic facial features. Additional molecular and clinical data of patients with overlapping chromosomal alterations are required for the confirmation of these possibly new microdeletion/ microduplication syndromes.

3.2. The *ACTB* gene is a strong candidate gene for the disturbance of craniofacial development. The haploinsufficiency of *ERBB4*, *CAD10* and *CACNA1G* genes can be significant for the disturbance of cognitive function.

4. The region located 63 kb upstream of the *NSD1* gene might be important for the expression of the *NSD1* gene.

5. In case of inconsistency between genotype and phenotype after molecular karyotyping, cytogenetic investigation for the detection of balanced chromosomal rearrangements is indicated.

6. In light of the high diagnostic yield of molecular karyotyping and the observation that most of the pathogenic copy number variants detected were not related to known microdeletion/microduplication syndromes, molecular karyotyping is recommended as the first-tier test in the genetic evaluation of patients with DD/ID.

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SANTRAUKA

ASMENŲ, TURINČIŲ INTELEKTINĘ NEGALIĄ,

GENOMO STRUKTŪRINIŲ POKYČIŲ ĮTAKOS FENOMUI ĮVERTINIMAS

Intelektinė negalia (INN) yra įvairiai pasireiškianti būklė, dažnai siejama su didelės įvairovės ir sunkumo fenotipiniais požymiais. Chromosominiai pokyčiai yra dažniausia šiuo metu žinoma INN priežastis. Mokslo žinių gilinimo INN srityje reikšmė yra prioritetinė dėl šios būklės paplitimo populiacijoje masto, ilgalaikių socialinių ir ekonominių priežasčių bei ribotų gydymo galimybių. Šio darbo tikslas - nustatyti patogeninių DNR kopijų skaičiaus pokyčių (KSP) įtaką asmenų, turinčių INN, fenotipui ir centrinės nervų sistemos raidos bei funkcijos sutrikimams. Mokslinio darbo metu atrinkta 211 pacientų, jiems atliktas molekulinio kariotipavimo tyrimas. 36 patogeniniai KSP buvo nustatyti 29 iš 211 tiriamųjų. Taigi molekulinio kariotipavimo diagnostinis efektyvumas – 13,7 %. Atmetus žinomus mikrodelecinių/ mikroduplikacinių sindromų atvejus, molekulinio kariotipavimo efektyvumas, nustatant naujus patogeninius KSP, yra 9,5 %. Palyginus pacientų su patogeniniais KSP klinikinius duomenis su pacientų be patogeninių KSP, nustatyta, kad įgimta didžiosios smegenų jungties formavimosi yda, ausų mikroanomalijos ir brachidaktilija yra chromosominius pokyčius prognozuojančios fenotipinės anomalijos. Analizuojant unikalius patogeninius KSP, nustatyti 7p22.1 mikroduplikacijai ir 17q21.22 mikrodelecijai būdingi fenotipiniai požymiai, bei genai kandidatai INN (*ERBB4 CAD10* ir *CACNA1G* genai) ir kraniofacialinės raidos sutrikimui (*ACTB* genas). 264 kb dydžio 5q35.3 duplikacijos nustatymas buvo svarbus identifikuojant sritį, galimai reikšmingą *NSD1* geno raiškai, esančią 63 kb už *NSD1* geno. Analizuoti sudėtingi chromosominiai persitvarkymai bei sudarytos pacientų su INN genetinio ištyrimo gairės.

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