Subtelomeric screening in Serbian children with dysmorphic features and unexplained developmental delay/intellectual disabilities

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Developmental delay and intellectual disabilities (DD/ID) are significant health problems affecting 3% of the human population. Submicroscopic chromosomal rearrangements involving subtelomeric regions are often considered to be the cause of unexplained DD/ID.

Screening of subtelomeric regions was performed in 80 unrelated patients with DD/ID and normal GTG-banded chromosomes using the MLPA method with two kits (SALSA P070-B1 and P036-E1). The MLPA screening revealed subtelomeric chromosome aberrations in four cases (5%). The aberrations detected were: 1p deletion, 1p deletion combined with 12q duplication, 4p deletion, and 9p deletion combined with 15q duplication. The deletions detected were classified as causative for the patients' observed phenotypes.

This study confirms the high frequency of subtelomeric rearrangements in unexplained DD/ID and reinforces the argument for routine subtelomeric screening in order to get a correct diagnosis, establish genotype-phenotype correlations and offer accurate genetic counseling.

Key words: MLPA method, subtelomeric chromosome screening, intellectual disabilities, developmental delay.

The incidence of developmental delay (DD) and intellectual disabilities (ID) is reported to be 1-3%. DD/IDs show a high degree of clinical and genetic heterogeneity[1]. Despite a rising number of known causes, a specific reason is detected in only 25% of patients, thus placing the majority of DD/ID cases in the unexplained etiology[2] category. Consequently, genetic counseling is difficult for these cases.

Chromosome rearrangements represent the most common single cause of DD/ID. Subtelomeric regions are gene-rich and often involved in chromosomal rearrangements[3]. It has been suggested that imbalances involving telomeres might be significant contributors to DD/ID. According to recent reports, 1.3-10.9% of patients with DD/ID have subtelomeric rearrangements[4], and these aberrations are an important cause of both sporadic and familial unexplained cases[5-8].

As is well known, initial testing for DD/ID includes conventional karyotype analysis and tests in order to rule out common inborn metabolic disorders. Chromosome fragments involved in rearrangements are below the resolution of conventional cytogenetic methods. Several molecular approaches have been successfully used to investigate the integrity

of subtelomeric regions, such as multiprobe telomere fluorescent in situ hybridization (T-FISH), multiallelic marker analysis, quantitative real-time PCR, comparative genomic hybridization (CGH) and multiplex ligation-dependent probe amplification (MLPA)[9]. As DD/IDs are common disorders with a significant impact on family planning, a large number of patients need to be tested routinely. However, due to the relative complexity and high cost of the screening methods used to this point, only preselected patients, preferentially including more severely affected and syndromic cases, have been screened. MLPA is a simple, fast, sensitive, specific and reliable screening method, potentially suitable for routine diagnostics[10,11].

The aim of the study was to screen patients in the Serbian population with unexplained DD/ID for subtelomeric aberrations using MLPA.

Material and Methods

The study involved 80 Serbian children (42 boys and 38 girls) from unrelated families with unexplained DD/ID diagnosed in the last four years at the Department of Medical Genetics and the Department of Neurology at the University Children's Hospital, Belgrade, Serbia. Patients had to meet the following criteria: 1) mild to severe ID (mild, IQ 50–70; moderate, IQ 30–50; severe, IQ <30) and at least one additional dysmorphic feature or congenital malformation; 2) definite exclusion of perinatal brain injury; 3) no history of toxication, hypoxia, central nervous system infection or cranial trauma; 4) normal/routine karyotypes on GTG-banded analysis at 400 to 550 resolution; 5) no evidence of recognizable inherited metabolic or specific neurodegenerative disorders on brain imaging or blood/urinary metabolic screening. All patients were scored according to the checklist for submicroscopic subtelomeric rearrangements[12]. In addition, samples from the parents of each patient with consistent positive results for subtelomeric rearrangement were also tested.

The Ethics Committee of the University of Belgrade Faculty of Medicine approved the research. Informed consent was obtained from each patient. Genomic DNA of the patients and patients' parents was extracted from peripheral blood using a standard salting-out method.

Multiplex ligation-dependent probe amplification analysis was performed in line with the manufacturer's instructions. Two specifically designed sets of probes for testing subtelomeric imbalances (SALSA P070-B1 and P036-E1, MRC-Holland, Amsterdam, Netherlands; http://www.mrc-holland.com) were used. Amplification products were identified and quantified by capillary electrophoresis on an ABI 3130 genetic analyzer (Applied Biosystems). The data obtained were analyzed using Genemapper 4 software. The final analysis of the MLPA data was carried out using Coffalyser 8 software. For each patient, the normalized peak pattern of each subtelomeric region was divided by the average peak pattern of all samples (n>10) in the same experiment. The resulting values were approximately 1.0 for wild-type peaks, <0.75 for deletions, and >1.3 for duplications.

The advantage of the MLPA technique is that one kit serves to analyze all subtelomeric regions, and the other to confirm the abnormalities detected. That is, each of the detected deletions or duplications is confirmed by two different probes, because the sequences obtained by the two probe mixes were different from each other.

Results

Subtelomeric regions of 80 patients with unexplained DD/ID and dysmorphism or/and congenital malformations were analyzed by MLPA. All patients were diagnosed with a normal karyotype after standard GTG banding. The patients' age ranged from 2.5 months to 18 years (mean 5.88 years), and the male-to-female ratio was 1.10 (42:38). Patients under 3 years of age (n=21, 26.25%) could not be diagnosed with ID. These patients were diagnosed with DD if a delay in achieving developmental milestones was observed. All patients older than 3 years (n=59, 73.75%) were diagnosed with ID. The degree of intellectual disability was mild in 24/80 of the cases (30.00%), moderate in 23/80 (28.75%), and severe in 12/80 (15.00%). A positive family history of DD/ID was confirmed in 15.00% of all patients.

Facial dysmorphic features were observed in 39 (48.75%) and cleft palate in 3 (3.75%) patients. Seizure and epilepsy were recorded in 10 (12.5%) patients. Microcephaly, macrocephaly and congenital heart defects frequently

Table I. Frequency of Clinical Features in 80 Children with Unexplained Intellectual Disabilities and Developmental Delay

Clinical data	Number of cases (%)
Family history of ID/DD	12/80 (15.00)
Prenatal growth retardation	10/80 (12.50)
Postnatal growth retardation	7/80 (8.75)
Microcephaly	29/80 (36.25)
Macrocephaly	5/80 (6.25)
Dysmorphic facial features	39/80 (48.75)
Nonfacial dysmorphism and congenital abnormalities	16/80 (20.00)
Congenital heart defects (PDA, ASD, VSD)	19/80 (23.75)
Seizures and epilepsy	10/80 (12.50)

accompanied DD/ID (Table I).

All patients were scored according to the checklist for submicroscopic subtelomeric rearrangements[12]. In the group of patients with scores of 1 and 2 (n=23), submicrosopic chromosomal aberrations were not detected. Aberrations were detected only in 4 of the 57 patients with a clinical score ≥3.

Among the 80 patients analyzed, 4 (5.0%) were found to have cryptic subtelomeric chromosomal imbalances. Clinical features of those patients and the aberrations detected with MLPA were:

Patient 1

A 14-month-old girl is the first child of young, healthy and unrelated parents. Pregnancy history and delivery were unremarkable. Weight at birth was 2600 g (between the 10th and 25th centiles). Head circumference at the age of 9 months was 42 cm (between the 5th and 10th centiles). Initial physical examination revealed craniofacial dysmorphia with a prominent forehead, sunken eyes and a broad nasal bridge, as well as moderate generalized hypotonia. Brain magnetic resonance imaging (MRI) showed corpus callosum hypoplasia, while echocardiography identified muscular and membranous ventricular septal defect with a significant left to right shunt. Ventricular septal defects had been successfully closed surgically at the age of one year. Currently, at the age of three years, she presents with the same craniofacial dysmorphia, moderate developmental delay and the ability to sit unsupported but only to speak a few different words. In this patient, MLPA revealed 1p deletion (Fig. 1).

Patient 2

A 4-month-old female infant was referred for genetic counseling due to restriction in growth and development and dysmorphic features. She is the first child of young, healthy and unrelated parents. Pregnancy and delivery were uneventful. Birth weight was 2850 g (between the 10th and 25th centiles), length was 50

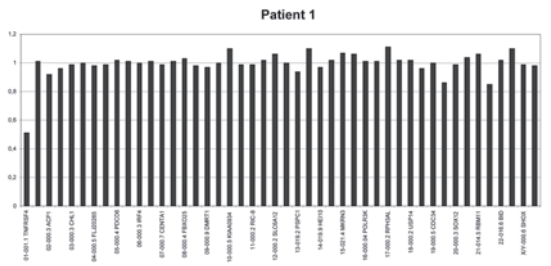


Fig. 1. 1p deletion detected in Patient 1

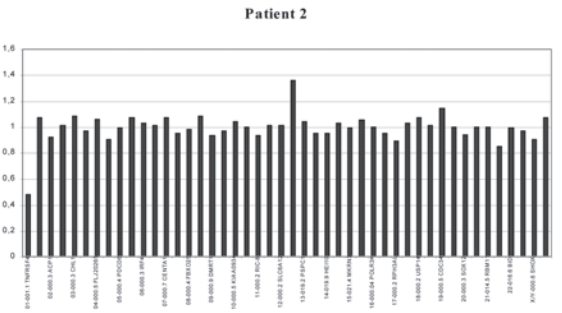


Fig. 2. 1p deletion and 12q duplication detected in Patient 2

cm (between the 25th and 50th centiles), and head circumference was 32 cm (5th centile). Her infancy was characterized by a severe deceleration of head growth and microcephaly, as well as pronounced generalized hypotonia and epilepsy. The most prominent facial features were a flat face, a short nose with a depressed nasal root, mild ptosis and downturned corners of the mouth. Brain MRI showed microcephaly without other brain malformations, while cardiac and abdominal ultrasound did not reveal any associated anomalies. At present, at the age of 3, all of the facial dysmorphisms persist, as well as the microcephalic aspect. Speech has remained underdeveloped, a part of overall intellectual delay. She sits without support but does not walk. In this patient, MLPA revealed 1p deletion and 12q duplication (Fig. 2).

Patient 3

A 5-year-old girl with severe growth and intellectual retardation, discrete facial dysmorphia and refractory epilepsy was described in our previous report[13]. In this patient, MLPA analysis revealed 4p deletion (Fig. 3).

Patient 4

A 30-month-old female infant, born after an uneventful pregnancy, of young, healthy and unrelated parents. Initial physical examination revealed mild facial dysmorphism, which presented with hypertelorism, epicanthal folds, concomitant convergent strabismus and a short nose with a depressed nasal root. Craniostenosis with coronal and metopic synostoses resulted in microcephaly. After surgery at the age of 6 months, head growth was relatively good (head circumference between the 10th and 25th centiles). Her developmental delay was apparent. At the age of 21 months she was unable to walk independently, and her developmental level was as expected for 13 months of age. In this patient, MLPA revealed 9p deletion and 15q duplication (Fig. 4).

The MLPA method did not identify in the parents the chromosomal rearrangements present in the patients. The deletions detected were classified as causative for the patients' observed phenotypes.

Discussion

Many different practical guidelines for testing patients with DD/ID suggest that the initial steps in diagnostics be: cytogenetic analysis, to exclude visible chromosomal anomalies, and metabolic tests, to exclude inborn errors of metabolism. In all cases with normal karyotypes and no evidence of recognizable inherited metabolic or specific neurodegenerative disorders[14,15], screening for subtelomeric chromosomal rearrangements should be performed. Detection of submicroscopic rearrangements requires sensitive techniques such as M-FISH, MLPA or CGH microarray. Multiplex ligation-dependent probe amplification (MLPA) is a technique based on the polymerase chain reaction (PCR) amplification of specific probes, which allows relative quantification of 46 to 50 different target DNA sequences in a single reaction, using only one PCR primer pair. A copy number variation of the target sequence for a MLPA probe results in a lower or higher relative amount of the probe amplification product. MLPA reactions are used to detect heterozygous deletions or duplications by comparing relative signal strengths of amplified probes between a sample taken from the patient and a normal DNA sample.

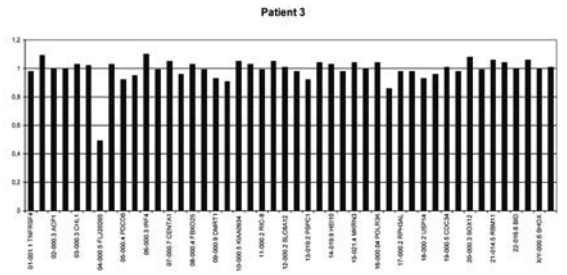


Fig. 3. 4p deletion detected in Patient 3

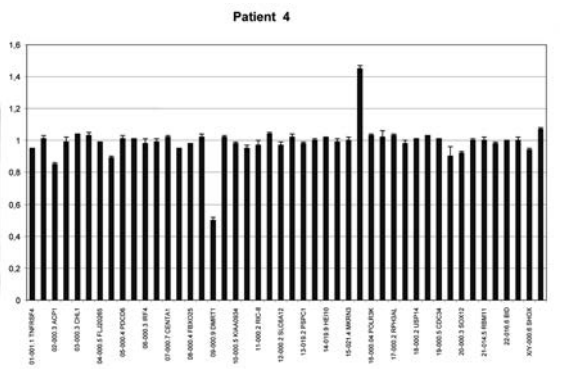
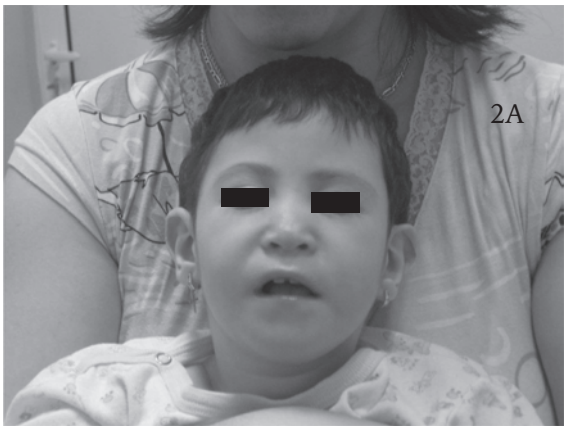
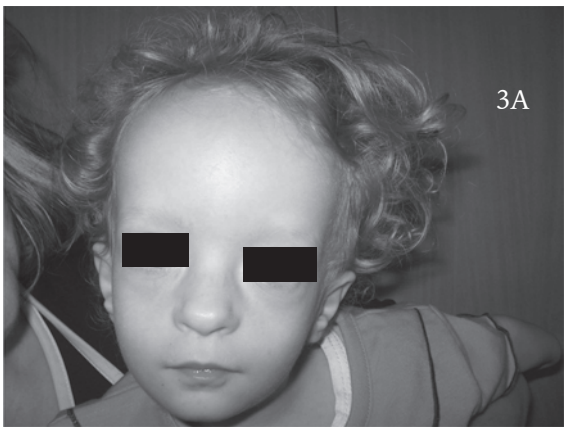


Fig. 4. 9p deletion and 15q duplication detected in Patient 4





MLPA is a very useful tool in detecting the main causes of DD/ID, such as aneuploidy or submicroscopic deletions or duplications in human chromosomes or in a specific gene. Moreover, this technique can be used in the molecular diagnosis of various genetic diseases where pathogenesis is related to specific deletions or duplications, and in diagnosing genetic diseases characterized by the presence of abnormal DNA methylation.

Our study is the first to analyze submicroscopic, subtelomeric aberrations in Serbian patients with DD/ID using the MLPA method. Clinically relevant submicroscopic aberrations were identified in four patients (4/80, 5.00%). We found that the aberrations detected (three deletions and two duplications) were not present in the patients' parents; however, when using this technique, it is not possible to exclude balanced rearrangements in one of the parents.

A commonly used tool for preselection of patients to be tested for subtelomeric rearrangements is a checklist developed by de Vries et al.[12], which includes certain clinical characteristics such as facial dysmorphism, congenital malformations and family history. In our study, submicroscopic chromosomal aberrations were identified only in patients with a clinical score ≥3, pointing to an increased rearrangement rate in this group of patients (4⁄57, 7.02%). The rate of detection of chromosomal imbalances was higher in children with facial dysmorphism (4/39, 10.25%) and epilepsy (2/10, 20.0%). Our results are in agreement with those of Mandal et al.[16], and our study supports the conclusions of previous reports suggesting that the de Vries criteria are a useful tool for preselecting patients for MLPA analysis[17].

In our study, subtelomeric rearrangements were detected predominantly in girls with DD. Given the age of the patients, it was not possible to establish the degree of ID in three of the four cases with detected chromosomal aberrations. Nevertheless, clinically recognizable DD in newborns usually results in moderate or severe ID, and we may assume that patients with detected submicroscopic chromosomal abnormalities will develop moderate or severe ID. In different studies, subtelomeric rearrangements were detected, depending on the method and cohort, in 0.5% to 16.5% of patients with unexplained ID[2,18]. Some studies described a high frequency of detected subtelomere rearrangements (17%), but most of these imbalances were inherited and not causative for the phenotype[19]. Other publications described a low frequency of detected imbalances because of specific clinical preselection criteria and constraints of the methodology[8,20]. The frequency of abnormalities observed in our study is comparable to that indicated by most studies, identifying subtelomeric defects in approximately 5% of patients[21-24].

The most common subtelomeric chromosomal aberrations detected in patients with DD/ID are 1p, 4p and 9p deletions. For each of these deletions, there are at least 50 reported cases with a relatively consistent phenotype[25]. In almost half of the published cases, the telomeric deletions appear to be *de novo*[26]. We identified four submicroscopic subtelomeric deletions in this study: del 1p (patients 1 and 2), del 4p (patient 3) and del 9p (patient 4).

Deletion 1p36 is one of the most common cryptic subtelomeric deletions in patients with severe mental retardation (2-3.75%)[25]. The majority of patients have hypotonia, growth abnormalities such as short stature and microcephaly, and facial dysmorphism with a large anterior fontanelle, a prominent forehead, deep-set eyes, a depressed nasal bridge and midface hypoplasia with a flat appearance[23,26-28]. Additionally, various cardiac malformations and orofacial clefts may be observed in those patients. In some cases sensorineural hearing loss, visual problems and seizures have been reported[29], usually indicating more severe intellectual disabilities. In this study, Patients 1 and 2 had dysmorphic facial features, hypotonia and microcephaly. Additionally, Patient 1 had ventricular septal defect and Patient 2 had seizures. All of these features are part of the 1p deletion syndrome spectrum.

Terminal deletions of 4p are associated with, in particular, severe growth retardation and hypotonia, profound intellectual disabilities, microcephaly, seizures and a distinctive facial appearance (e.g., hypertelorism, broad forehead and nasal bridge, "Greek helmet" appearance)[4,30]. This phenotype is well known, as Wolf-Hirschhorn syndrome. In Patient 3, the most prominent clinical features were severe, prenatal-onset growth retardation, intellectual disability and seizures, while facial dysmorphia was mild[13]. Such nonspecific facial dysmorphia and an absence of major congenital anomalies meant that there was slight chance for clinical diagnosis, which in this case was made possible by subtelomeric MLPA screening. .

Clinical features of 9p deletion syndrome include dysmorphic facial features (trigonocephaly, upward-slanting palpebral fissures, hypoplastic supraorbital ridges and a long philtrum), intellectual disabilities and, usually, normal growth[31]. Patient 4 in our study presented with a relatively typical phenotype, including moderate developmental delay and craniostenosis.

We found duplications (12q and 15q) in two patients. Both duplications were associated with deletions and are registered in the Toronto database of copy number variations. Ruiter et al.[32] described a patient with moderate intellectual disability, microcephaly, epilepsy, dysmorphism and 12q duplication, and a case with moderate intellectual disability, mild dysmorphism, autism and 15q duplication. Both reported duplications appear to be inherited from an unaffected parent, and are not causative for the phenotype. Although we did not detect 12q and 15q duplications in patients' unaffected parents, the observed deletions rather than the duplications may be responsible for our patients' phenotypes (Patients 2 and 4).

Published data have shown concordance in rearrangements detected in patients where FISH, cytogenetic microarray and MLPA[33] were performed. In the present study, 80 DD/ID patients were analyzed using two MLPA probe sets. The MLPA technique has the advantage that one kit is used for analyzing all subtelomeric regions, and the other for confirming detected abnormalities. We have shown that MLPA is a reliable method for detecting subtelomeric rearrangements. Although MLPA screening of subtelomeric regions may be conducted for all patients with intellectual disabilities, clinical preselection increases the possibility of detection of submicroscopic rearrangements. For this reason, MLPA screening of all subtelomeres in selected patients is a valuable diagnostic tool for DD/ID.

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