Multiplex ligation-dependent probe amplification as a screening test in children with autism spectrum disorders

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Abstract

Background. Autism spectrum disorders (ASDs) are a heterogeneous group of neurodevelopmental disorders, characterized by the presence of various symptoms related to deficits in communication and social interactions as well as stereotyped and repetitive behavior. Increasing evidence indicates the contribution of genetic factors in the etiology of ASDs. Genetic diagnosis in ASDs is based on identifying chromosome aberrations, microaberrations and point mutations in specific genes. One of the diagnostic tools is multiplex ligase-dependent probe amplification (MLPA) with a set of probes dedicated to ASDs (SALSA MLPA P343 Autism-1; MRC-Holland BV, Amsterdam, the Netherlands) targeting the genes located in the regions 15q11-q13, 16p11 and the *SHANK3* gene in the 22q13 region.

Objectives. Our study included 240 patients referred to the clinical genetics unit because of ASDs and/or developmental delay and/or an intellectual disability. Before genetic testing, the patients underwent a comprehensive medical work-up.

Material and methods. Multiplex ligase-dependent probe amplification was performed in 256 DNA samples from 240 probands and 16 family members using the SALSA MLPA P343 Autism-1 probe mix (MRC-Holland BV) according to the manufacturer's protocol.

Results. We obtained 234 normal results and 22 abnormal results (15 probands and 7 abnormal results for probands' parents or siblings). We diagnosed 1 16p11 microdeletion syndrome and 1 16p11 microduplication syndrome. We also found 3 deletions and 1 duplication in 15q13 region including 2 or 3 genes and 9 single probe alterations in the regions examined (1 duplication and 7 deletions).

Conclusions. Due to the low costs, MLPA test may be a good tool for the genetic screening of ASD patients.

Key words: diagnostics, autism, MLPA

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The prevalence of autism spectrum disorders (ASDs) has been estimated at 20–60/10,000 children, corresponding to 1–2% of the general population.[1–3] Importantly, ASDs are highly heterogeneous neurodevelopmental disorders, characterized by the presence of various symptoms, including deficits in communication and social interactions as well as stereotyped and repetitive behaviors. Moreover, patients with ASDs present high rates of comorbid intellectual disability, sensory abnormalities or sleep disturbances.[2] The role of genetic factors and metabolic conditions in the development of ASDs is strongly suggested on the basis of family studies, especially twin studies.[4] Although several genes have been identified and their association with ASD susceptibility is well-documented, a large group of genes is still suspected to be involved in this disease.[2] Additionally, diagnoses of several genetic syndromes need to be excluded before a final diagnosis of ASD is established.[5] Diagnostic considerations are even more complex due to multifactorial inheritance and a number of environmental risk factors potentially involved in the etiology of ASDs.[6]

Over the last few years, the laboratory methods used for ASD diagnosis have moved from classical cytogenetics (banding analysis) or fluorescent in situ hybridization (FISH) to array comparative genomic hybridization (aCGH) and next-generation sequencing (NGS). Several single nucleotide variants (SNV) have been identified in many genes and confirmed by family-sequencing using whole-exome sequencing (WES) and whole-genome sequencing (WGS). These studies have revealed that ASDs may be caused by numerous alterations in genes acting in different molecular pathways with incomplete penetrance and variable expressivity.[2,7]

The WES and WGS methods make it possible to screen large parts or the entire genome and to identify all potential genomic alterations, but their costs and laboratory equipment requirements are high. Taking into account these considerations, multiplex ligation-dependent probe amplification (MLPA) is an appealing alternative for initial screening in patients with ASD.[5,8] The dedicated MLPA probe mix for ASD contains probes for 3 chromosomal regions: 15q11-q13, 16p11 and 22q13. Therefore, the aim of our study was to evaluate the rate of positive results in a large sample of ASD patients, using the MLPA mix dedicated to this disease.

Material and methods

Materials

The analyses were performed in 240 individuals with ASDs and 16 healthy family members. The patients' group included 54 women and 202 men, with a mean age of 8.7 ±8.06 years. The patients had been referred to the clinical genetics unit with ASDs and/or developmental delay and/or intellectual disability, with or without dysmorphic features or additional congenital abnormalities. The diagnosis of ASD was established according to ICD-10 criteria.[9] All the probands and/or their legal guardians signed a consent form. The MLPA test was performed after excluding genetic disorders that had been diagnosed based on clinical examinations or specific genetic tests (cytogenetics and fragile-X testing). The study was accepted by the Ethics Committee of Wroclaw Medical University (approval No. 320/2018).

DNA isolation

Genomic DNA was isolated from 200 µL of peripheral blood lymphocytes, using the Prepito DNA Blood kit and the chemagic Prepito-D isolator (both from PerkinElmer Inc., Waltham, USA) according to the manufacturer's protocol. DNA was diluted to 20 ng/µL and 5 µL (100 ng) was used in the MLPA reaction.

The MLPA analysis

The SALSA MLPA P343 Autism-1 probe mix (MRC-Holland BV, Amsterdam, the Netherlands) containing MLPA probes for 3 chromosomal regions 15q11-q13 (including, among others, *UBE3A*, *GABRB3* and in 15q13 *CHRNA7*), 16p11 (including, among others, *LAT, MAZ, MAPK3*, and *HIRIP3*) and 22q13 (including *SHANK3*) was used to assess the deletions and duplications in these regions. The MLPA reaction was prepared using a TC512 thermocycler (Techne Inc., Burlington, USA). The producer's protocol for MLPA was followed precisely.

The MLPA products were separated using the ABI 310 Genetic Analyzer with GeneScan Analysis v. 3.1.2 software, POP-4 Polymer and GeneScan™ 500 LIZ™ dye Size Standard (all from Thermo Fisher Scientific, Waltham, USA). The analysis of the results was prepared using GeneMarker v. 1.85 software (SoftGenetics LLC, State College, USA).

In the analysis of copy number variants (CNVs), a change in the peak values of over +0.3 was considered a duplication and –0.3 a deletion.

Microarray comparative genomic hybridization analysis

In aCGH analysis, 300 ng of each DNA sample was labeled with Cy-5 fluorescent dye using the CGH Labelling Kit for Oligo Arrays (Enzo Life Sciences Inc., Farmingdale, USA) according to the manufacturer's protocol. As a reference genotype for hybridization, 300 ng of gender-matched human reference DNA (Agilent Technologies, Santa Clara, USA) was simultaneously labeled with the Cy-3 dye. The hybridization of the samples and the processing of the microarrays were performed according to a standard oligonucleotide array-based CGH protocol

(Agilent Technologies). The microarray design used for the analysis was SurePrint G3 CGH ISCA v. 2, 8 × 60K (Agilent Technologies). The design focuses on 498 regions in the human genome of high clinical significance, defined on the basis of the International Standards Cytogenomic Arrays (ISCA) Consortium.[10] The approximate coverage of the genome is about 60 kb, with median practical resolution for accurate detection of imbalances estimated at 300 kb (lower in ISCA regions). The data was analyzed using CytoGenomics software (Agilent Technologies) with the default CGH analysis method, and was interpreted in reference to available databases (DGV [last update: 2016-05-15], ClinVar [2018-06-01 03:26], DECIPHER v. 9.23 [2018-05-23] May, 2018]).

Results

The MLPA analysis was performed on 256 DNA samples from patients and their families. We obtained 234 normal results and 22 abnormal results (in 15 patients and 7 parents or siblings of patients) (Table 1).

In the patients' group, we diagnosed 1 16p11 microdeletion syndrome and 1 16p11 duplication syndrome. We also found 1 duplication in the 15q13 region, 3 deletions in the 15q13 region, including *KLF1* and *CHRNA7* or *ABPA2*, *NDNL1* and *TJP1* genes, as well as 9 single-probe alterations in all the regions examined (2 duplication and 7 deletions) (Table 1).

15q11-13

In our study, alterations of region 15q11-13 were found in 11 out of 15 patients with a positive result: 4 out of 15 patients had abnormal results for more than 1 probe in this region (3 deletions and 1 duplication) while 7/15 had an alteration of 1 exon in this region (5 deletions and 2 duplications). In 1 case, the deletion of 2 probes in the 15q13 region (*KLF13*ex2 and *CHRNA7*ex4) was confirmed using aCGH; it occurred as part of a 1.1 Mb deletion (arr 15q33.3[31516639_32635959]x1). In the group of individuals with alterations in the 15q11-13 region, we observed ASDs with other clinical symptoms (6 cases) or without (4 cases); only 1 patient had developmental delay with epilepsy (Table 1).

In 5 patients we observed a deletion of 1 exon in the *GABRB3* gene (15q12), 4 in exon 9 (exon 6 according to new numbering) and 1 in exon 4. Three of these deletions were also present in 1 healthy parent (2 families were not examined). In this group of patients, we observed the following clinical picture: 1) ASDs without other clinical problems in 3 cases (1 with ASD family history); 2) ASD with developmental delay and epilepsy in 1 case; 3) other problems in 1 case; and 4) developmental delay with epilepsy in 1 case (Table 1).

We found 2 duplications and 2 deletions in the *CHRNA7* gene (the probe for exon 4), 1 duplication and 3 alterations being parts of a more complex rearrangement. In this group of patients, we observed ASDs in all cases. The patients with deletions presented with other comorbidities, such as intellectual disability in 1 case as well as developmental delay, speech delay and short stature in another 1 (Table 1). A single duplication of *CHRNA7* exon 4 was also present in a healthy sister of the proband.

16p11.2

Out of 15 patients, 3 were carriers of 16p11.2 alterations. We found 2 deletions: a small deletion (represented by 1 probe for *LAT4*), confirmed with aCGH as a 187 kb deletion (arr 16p11.2[28843773_29031059]x1]; 1 deletion exposed by 9 out of 11 probes for this region in the MLPA analysis; and 1 duplication, also for 9 out of 11 probes. In this group of patients, we observed that patients with deletions presented with more clinical symptoms than patients with duplications (Table 1).

22q13

In 1 patient, 1 probe found a deletion of exon 15 of the *SHANK3* gene.

Discussion

Genetic testing is an important part of the diagnostic process in children with ASDs and/or developmental delay and/or intellectual disability. Identification of a genetic cause of a disorder may elucidate the issue of etiology, enable assessment of the prognosis, facilitate care and management planning, and permit an estimation of the risk of recurrence. Using currently available standard laboratory methods (other than WES or WGS), it is possible to find a genetic cause in about 10% of ASD cases.[11] According to the literature, the aCGH analysis is recommended as a first-tier diagnostic method for ASD patients.[11] Additionally, single-gene tests, such as fragile-X syndrome testing, should be applied.[11] Given that aCGH and WES are expensive procedures, it is reasonable to use more cost-effective methods for screening and selecting patients for further analysis. One of these methods is MLPA – a method mainly used for the detection of small deletions and duplications. The SALSA MLPA P343 Autism-1 probe mix (MRC-Holland BV) is dedicated for ASD patients and allows deletions and duplications to be found in the 15q11-13, 16p11.2 and 22q13 chromosomal regions. Alterations identified in our study include deletions and duplications in regions 15q11-13, 16p11.2, and 22q13, as well as deletions and duplications in the *GABRB3* and *CHRNA7* exons.

Table 1. Results of the MLPA analysis

Patient number	Sex	Age [years]	Clinical diagnosis	MLPA result	Chromosomal region	MLPA family results	aCGH result
1	M	10	Fragile X syndrome suspected (excluded)	del *SHANK3ex15*	22q13	nd	nd
2	M	5	ID, ASD	del *KLF13ex2*, *CHRNA7ex4*, *TRPM1ex27*	15q13	nd	nd
3	M	9	DD, epilepsy, epilepsy in family	del *GABR3ex9 (6)*	15q12	nd	nd
4	F	15	DD, ASD	dup *CHRNA7ex4*	15q13	mother – normal result, healthy one sister – duplication, healthy	nd
5	M	7	ASD	del *GABR3ex9(6)*	15q12	mother – deletion, healthy father – normal results, healthy	nd
6	F	5	ASD	del *GABR3ex4*	15q12	mother – deletion, healthy	nd
7	M	8	ASD	dup *HIRIP3ex3*, *SEZ6L2ex1*, *DOC2Aex4*, *MAZex5*, *MVPex5*, *SPNex3*, *MAZex6*, *HIRIP3ex4*, *MAPK3ex5*	16p11	mother – deletion, healthy father – normal results, healthy	nd
8	F	7	ASD, DD, ID	del *HIRIP3ex3*, *SEZ6L2ex1*, *DOC2Aex4*, *MAZex5*, *MVPex5*, *SPNex3*, *MAZex6*, *HIRIP3ex4*, *MAPK3ex5*	16p11	nd	nd
9	M	2	DD, dysmorphy	del *LATex4*	16p11	mother – deletion, healthy father – normal results, healthy	arr 16p11.2(28843773_29031059)x1
10	M	11	DD, ADS, ID, speech delay, short stature, short stature in family	del *KLF13ex2*, *CHRNA7ex4*	15q13	3 siblings – healthy, normal result	arr 15q33.3(31516639_32635959)x1
11	M	13	ASD, epilepsy, gynecomastia	del *ABPA2ex14*, *NDNL2ex1*, *TJP1in1*	15q13	mother – normal result, healthy father – deletion, healthy	nd
12	F	11	DD, speech delay, epilepsy, ASD, short stature, autoagression	del *GABR3ex9 (6)*	15q12	mother – deletion, healthy father – normal results, healthy	nd
13	M	4	ASD, ID, ASD in family	del *GABR3ex9 (6)*	15q12	nd	nd
14	F	4	ASD	dup *HIRIPex3*	15q13	nd	nd
15	F	6	ASD	dup *HIRIPex3*, *CHRNA7ex4*, *TRPM1ex27*	15q13	nd	nd

MLPA – multiplex ligase-dependent probe amplification; aCGH – array comparative genomic hybridization; ID – intellectual disability; ASD – autism spectrum disorder; DD – development delay; nd – no data.

Deletions and duplications in region 15q11-13

Copy number variants in the 15q11-13 region are among the most common autosomal alterations in ASD patients.[5] A substantially higher rate of ASDs has been found in individuals with Prader–Willi syndrome, especially in those with the uniparental disomy (UPD) subtype.[12]

The proximal part of chromosome 15 is one of the most unstable regions in the human genome, because it contains 6 low copy repeat (LCR) elements that are grouped in 6 breakpoints involved in non-homologous recombination. Microdeletions within this region are observed in patients with Prader–Willi and Angelman syndromes, while microduplications are associated with ASDs, learning disabilities and seizures.[13] Similarly, CNVs in the 15q13.3 region, located downstream to the 15q11-13 locus, are reported in patients with neuropsychiatric phenotypes, attention deficit hyperactivity disorder and ASDs.[13]

In 1 of our patients (No. 10), we found a 15q13 deletion not observed in 3 healthy siblings; using the aCGH method, it was characterized as a 1.1 Mb deletion. Moreover, a smaller deletion in this region, covering genes *ABPA2* (exon 14), *NDNL2* (exon 1) and *TJP1* (intron 1) was diagnosed in patient No. 11. Although this alteration was also observed in the presumably healthy father, it may still be the cause of ASD in the proband if the penetrance of the alteration is incomplete. To verify this hypothesis, further genetic testing in numerous family members is necessary.

Deletions and duplications of *GABRB3* exons

Alterations in the *GABRB3* gene reported in ASD patients mainly involve variations of single or several nucleotides, and most of them are familial.[14] Due to high incidence of this deletion (5 patients and 3 familial cases, with no data for 2 families) it is likely that this alteration is not causative of clinical features in these patients.

Deletions and duplications of *CHRNA7* exons

The α7 subunit of the nicotinic acetylcholine receptor is encoded by *CHRNA7* (15q13). This ion channel is reported to be expressed in the brain. A homopentameric form of the α7 subunit is involved in mediating signal transduction at synapses, the regulation of neurotransmitter release, synaptic plasticity, learning, and memory. The *CHRNA7* gene is located in the 15q13.3 region and has been associated with neurodevelopmental and neuropsychiatric disorders.[13] Deletion of the whole *CHRNA7* gene is observed in 1% of patients with idiopathic generalized epilepsies, while duplication of the gene occurs in patients with ASDs and cognitive impairment.[13,15–17] For patients with CNVs in the 15q13.3 regions, incomplete penetrance (about 80%) and variable expressivity have been reported.[13] It is difficult to conduct a sequence analysis of the *CHRNA7* gene because of the presence of a large identical sequence in the *CHRFAM7A* gene, which is therefore sequenced together with *CHRNA7*, but no rare damaging variants were reported in the *CHRNA7* gene in a group of 135 patients with ASD.[17] It should be noted that because of the limitations of MLPA analysis, this alteration may also be caused by a single nucleotide polymorphism or mutation. The same change was also observed in a healthy sister, which may suggest that this alteration was not responsible for ASD in the proband, or (as in previous reports) the penetrance of this mutation is incomplete.[13]

Deletion and duplication in the 16p11.2 region

Deletion and duplication in the 16p11.2 region are among the most frequent CNVs in patients with ASDs and neurodevelopmental disorders.[7] Several clinical characteristics, including speech articulation abnormalities, limb and trunk hypotonia with hyporeflexia, abnormalities of agility, sacral dimples, macrocephaly, and epilepsy, have been observed in patients with 16p11.2 deletions. Additionally, the 16p11.2 duplication syndrome has been associated with speech articulation abnormalities, hypotonia, abnormalities of agility, sacral dimples, and epilepsy along with action tremor and microcephaly.[7] In both deletion cases in our study, examination of the family members revealed a carrier status in mothers without any clinical features. As in cases of 15q11-13 microaberrations, incomplete penetrance and variable expressivity have been reported in 16p11.2 microdeletions and especially in microduplications.[7]

Deletion and duplication in the 22q13 region

Copy number variants in the 22q13 region are the cause of various neuropsychiatric disorders, including Phelan–McDermid syndrome (PMS), which is the result of a deletion of a critical region with the *SHANK3* gene. A high rate of ASDs (as high as 20–50%) has been reported in children with 22q13 aberrations. Other highly characteristic features include speech and developmental delays.[5]

Microduplications in the 22q13 region, including the *SHANK3* gene, are reported in patients with attention-deficit hyperactivity disorder (ADHD) and schizophrenia, while microdeletions and point mutations of the *SHANK3* gene are present in patients with mild autism without intellectual disability. Incomplete penetrance is also reported in cases of *SHANK3* gene alterations.[5,18] In 1 patient in our study, we found a deletion of 1 probe for exon 15 of the *SHANK3* gene. In this patient, intellectual disability and speech delay suggested fragile X syndrome (Table 1). Because of the severity of symptoms observed in our patient, it is highly unlikely that the microdeletion detected in the 22q13 region is responsible for his clinical features.

Conclusions

The MPLA analysis is an effective and low-cost technique for screening genetic causes in ASD patients. It allows patients to be selected for further diagnostic consideration with more expensive methods. Because deletions and amplifications in the 15q11-13, 16p11.2 and 22q13 regions have also been described in healthy subjects, confirmation of the results of the MLPA analysis is recommended using another MLPA probe mix or another diagnostic method, e.g., FISH or aCGH. The importance of any detected changes should always be interpreted in relation to clinical data. Abnormal results of an MLPA analysis can also arise from the presence of a point mutation or a single nucleotide polymorphism within the sequence analyzed. Therefore, if sequencing is not applied, the parents of the patient should be examined in order to determine the origin of the change (inherited/de novo).[18] Our analysis revealed that although MLPA can be an initial test in ASD patients, the majority of them will need further investigations, such as aCGH or WES.

Unraveling the genetic underpinnings of ASDs is an imperative not only for the patients but also for the whole family. It provides a basis for genetic counseling and an explanation of observed neurodevelopmental and behavioral characteristics. Finally, it facilitates comprehensive medical care for various comorbidities that might appear due to specific genetic alterations.

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