Original Article

Investigation of Microdeletions in Syndromic Intellectual Disability by MLPA in Iranian Population

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Abstract

Background: Intellectual Disabilities (ID), defined as a state of developmental deficit, result in significant limitation of intellect and poor adaptation behavior. A number of genetic factors can result in ID, such as chromosomal abnormalities, copy number variation, and single gene defect. Karyotyping is the routine method for detecting chromosomal abnormalities in patients with ID. More recently, the Multiplex Ligation-dependent Probe Amplification (MLPA) method has been applied for detecting microdeletion/duplication in patients with dysmorphism and ID.

Methods: A total of 100 patients with dysmorphism and ID have been referred to us since 2011. All patients were first evaluated clinically and a number of these individuals had normal karyotypes. We investigated duplications and deletions for 21 different microdeletion syndromes using MLPA kit (MRC-Holland).

Results: We were able to identify aberrations in 12 (12%) patients clinically ascertained as follows: 5 Williams syndromes, 3 Miller- Dieker syndromes, 1 Sotos syndrome, 1 Angelman Syndrome, 1 Di-George syndrome and one patient with an abnormal 4p chromosomal region.

Conclusion: Our MLPA results indicate a high degree of concordance between the clinical data and the genotype. We suggest MLPA as the first screening method for children suffering from MR with normal karyotypes. In those cases where clinical findings were not compatible with the microdeletion syndrome identified by MLPA investigation, further studies such as FISH and aCGH were performed.

Key words: Intellectual disability, Iranian population, microdeletion syndromes, Multiplex Ligation-dependent Probe Amplification assay.

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Introduction

Chromosomal rearrangements which result in deletions or duplications of a part of a chromosome, usually spanning several genes, are a major cause of congenital anomalies. A mixture of numerous abnormalities and important clinical conditions, including intellectual disabilities (IDs), structural dysmorphisms and congenital malformations can indicate a specific syndrome, which are normally caused by microdeletion and microduplication syndromes. The extent of the anomaly is usually too small to be detected under the microscope using conventional cytogenetic methods (less than 5 – 10 megabases in size). Approximately 5% of ID cases are due to a microdeletion or microduplication. They are termed as microdeletion in case of genetic material loss, or microduplication if genetic material is gained. Normally, a group of common phenotypic features are observed in a specific microdeletion or duplication, which leads us to a clinical diagnosis of a microdeletion/duplication syndrome.[1]

Regardless of the specific symptoms, some cases of microdeletions/duplications still remain undiagnosed due to the range of variety and severity of the symptoms. Recently, clinical diagnosis of these syndromes has became more successful with the application of high-resolution G-banded karyotyping, fluorescence *in situ* hybridization (FISH) and high-resolution metaphase comparative genomic hybridization (HR-CGH) analysis or BAC array-CGH. Whole-genome array screening (WGAS) is suggested as the first diagnostic tests for detection of chromosomal abnormalities.[2] On the other hand, these techniques are relatively labor-intensive and expensive.[3,4]

The introduction of a new technique, Multiplex Ligation-dependent Probe Amplification (MLPA) offered by MRC-Holland, provided a considerably easier, cost-effective and faster alternative which has since been used in several experiments (MRS-MLPA, MRC-Holland, Amsterdam, The Netherlands). MLPA is a method detecting abnormal copy numbers of up to 50 different genomic DNA or RNA sequences in a single multiplex PCR, which is able to distinguish sequences differing in only one nucleotide.[5]

In this study, we investigated the presence of a microdeletions or microduplications in patients referring to our laboratory with ID and/or dysmorphic features. Many of these patients had been previously karyotyped with no aberration detected. We used the MLPA probe mix p245-microdeletion syndromes-1, which analyzes the following microdeletions and microduplications:

1p36 deletion syndrome, 2p16 microdeletion, 3q29 microdeletion, 9q22.3 microdeletion, 15q24 deletion syndrome, 17q21 mi-

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crodeletion, 22q13 / Phelan-McDermid, Cri-du-chat syndrome, 5p15, DiGeorge syndrome 22q11, DiGeorge region 2, 10p15, Langer-Giedion syndrome, 8q, Miller-Dieker syndrome, 17p, NF1 microdeletion syndrome, Prader-Willi / Angelman, MECP2 / Xq28 duplication, Rubinstein-Taybi syndrome, Smith-Magenis syndrome, Sotos syndrome 5q35.3, WAGR syndrome, Williams syndrome, Wolf-Hirschhorn 4p16.3.

Materials and Methods

Patients

A total of 100 patients referring to the Kariminejad-Najmabadi Pathology & Genetics Center, Tehran, Iran, were selected for this study. All patients had ID and/or dysmorphic features. After genetic counseling, clinical data collection, and taking a letter of consent, 10 mL of blood was drawn from each patient and sent for genomic DNA extraction.

DNA extraction

DNA was extracted from 10 mL of patients' fresh blood using the salting out method according to the standard protocol.[6]

MLPA (Multiplex Ligation-dependent Probe Amplification)

The MLPA procedure was performed using the SALSA P245-A2 Microdeletion syndromes probe set, according to the manufacturer's protocol (MRS-MLPA, P245 probe set, MRC-Holland, Amsterdam, the Netherlands). PCR was performed using an Eppendorf AG theromcycler (Eppendorf, Hamburg, Germany), and then one microliter of the PCR product was sent for capillary electrophoresis with an Applied Biosystems 3130 analyzer (Applied Biosystems). This probe set contains several probes for 21 different microdeletion syndromes (Table 1). The results were normalized and analyzed using the Coffalayser software (available for free downloadathttp://old.mlpa.com/coffalyser/).

Results

Among the 100 patients studied, we detected 12 aberrations (12%) presented in Table 1. The clinical findings were completely compatible with the microdeletion syndrome identified by MLPA.

Sotos syndrome

In the case of Sotos syndrome, the patient was a two and a half year-old girl with unusual phenotype including macrocephaly, prominent and wide forehead, coarse facies, hypertelorism, wide mouth, prominent jaw, premature eruption of teeth, low-set ears, hypotonia, hallux valgus, wrinkled skin, hearing loss, developmental delay and growth retardation (height and weight below the 3rd percentile). Based on the clinical findings, chromosomal study and MLPA were requested.

MLPA detected a deletion in the two probes pertaining to Sotos syndrome. The deletion was in the 5q35 region, in the *NSD1* gene exons 17 and 22.

Karyotyping confirmed terminal deletion of the segment distal to 5q35.2. Our patient had additional findings, namely coarse features, wrinkled skin, hyperextensibility of joints, mainly in digits, and was different from Sotos syndrome in having growth retardation, while Sotos patients have excessive growth (> 97th centile). Array comparative genomic hybridization (aCGH) was performed showing a 6.4 Mb terminal deletion in 5q35.2 → ter with maximum 174400000 and minimum174800000 breakpoint boundaries. The additional findings are most probably related to deletion of genes adjacent to NSD.

Williams syndrome

We detected 5 patients with Williams syndrome, all manifesting the deletions in all the three probes in the 7q11.23 chromosomal region specific to this disorder. The probes include the *ELN* gene, exons 1 and 20 (2 probes) and the *LIMK1* gene (1 probe).

One of the patients is the first child born to first-cousin Iranian parents. After an uneventful pregnancy, he was born at term by Caesarean section. Birth weight was 2400 grams (< 3rd centile), but length and head circumference (HC) were not documented. The milestones were delayed; he sat at 7 months and walked at 18 months. He was referred to the lab with a suspicion of Williams syndrome. Examination at 4 years revealed periorbital fullness, epicanthal fold, anteverted nares, long philtrum, prominent lips, open mouth and dental caries. He had a friendly but anxious personality. The voice was hoarse. He had mild ID. Weight, length and HC were 11 Kg (< 3rd centile), 92 cm (< 3rd centile) and 47 cm (3rd centile), respectively. Echocardiography had been performed and supravalvular aortic stenosis was detected. He had a herniography operation at 3 months. MLPA confirmed the suspicion of Williams syndrome and since clinical features were compatible with the diagnosis, no further workup was performed.

Miller- Dieker syndrome

We detected 3 patients with Miller-Dieker syndrome, all of whom manifested deletions in the two probes indicating deletions in the 17p13.3 chromosomal region which carries the *PAFAH1B1* gene (probes available for exons 3 and 7).

One of the patients was a 6-year old girl born to unrelated par-

Table 1. The table illustrates the diagnosed patients using the MLPA P245 probemix: among the 100 patients whom we studied, ten aberrations (12%) were found, with the following probe characteristics.

Syndrome	Williams syndrome	Miller-Dieker syndrome	Sotos syndrome	DiGeorge syndrome	Angelman syndrome	4p dup
Chromosomal region	7q11.23 / del	17p13.3 / del	5q35.3 / del	22q11.21 / del	15q12/ del	4p16.3 / dup
No. of probes	3	2	2	3	4	2
No. of patients detected	5	3	1	1	1	1
Geographical distribution	All of them from Tehran	One of them from Qom*	-*	Gilan	Ardebil	Alborz

* No information was available on geographical distribution of the patients diagnosed with Sotos syndrome and two patients diagnosed with Miller-Dieker syndrome.

ents. The pregnancy was complicated by polyhydramnios. Delivery was by Caesarean section at 35 weeks because of fetal distress. Birth weight, length and HC were 2200 grams (< 3rd centile), 47 cm (< 3rd centile) and 31.5 cm, respectively. She was hospitalized for 2 weeks at birth due to respiratory distress which was followed by jaundice that led to seizure. She had hypotonia and severe developmental delay. She had the next seizure at 11 months which was refractory to treatment. Brain CT scan showed lissencephaly. Karyotyping was performed which was normal 46,XX. She was referred to our center for MLPA for microdeletion syndromes. At examination, she was noted for microcephaly, bitemporal narrowing, prominent forehead, upslanting palpebral fissures, protruberant upper lip and micrognathia, small nose and anteverted nostrils, and arched eyebrows. She had bilateral single palmar crease and tapering fingers. Weight, length, and HC were 12 Kg (< 3rd centile), 85 cm (< 3rd centile), and 46 cm (< 3rd centile), respectively.

Chromosome 4 abnormality

The case is a two and a half year-old girl with multiple congenital anomalies, seizure disorder, and severe ID. The child is the second child of first cousin parents once removed. The parents and her elder brother are all apparently healthy. The pregnancy was complicated by gestational diabetes. Delivery was by obligatory repeat Caesarean section at 37 weeks. Birth weight, length, and HC were 1900 grams, 45 cm, and 27 cm, respectively. She developed seizure at birth and was hospitalized for 19 days. The child has had repeated tonic-clonic seizures refractory to treatment. On examination, her weight, height, and HC were 9400 grams (3rd centile), 80 cm (10th centile), and 45 cm (< 10th centile). She had micrognathia, deep-set eyes, mild hypotelorism, arched eyebrows, bulbous nose, high nasal bridge, anteverted nares, long philtrum, short nasal septum, and low-set ears. She had proximal insertion of thumb and tapering fingers. Ultrasonography of kidneys was normal. Echocardiography revealed ventricular septal defect.

First tested by MLPA, a duplication in the 4p16.3 region (using the microdeletion kit) and a subtelomeric deletion in 4p (using the subtelomeric PO36 and PO70 kits) were detected. Array-CGH was performed to determine the exact size of duplication and deletion of chromosome 4. Genomic deletion of 4p16.3 from 995760→1169503, a 173 kb deletion and genomic duplication of 4p16.3 to 4p13 extending from 1689962→41433598, 39 Mb duplication were detected. Karyotype detected a large additional chromosomal segment on 4p region confirming the duplication and terminal deletion.

Discussion

MLPA is a rapid and sensitive technique with the ability of relative quantification of several target DNA sequence aberrations in a single reaction by using specific probes for each targeted sequence.[7,8]

In our study, we found three cases of Miller-Dieker syndrome (MDS) in 100 patients (3%). MDS is a specific rare human malformation syndrome that exhibits classical lissencephaly, literally "smooth brain" as well as characteristic facial features. This condition occurs by a deletion of genetic material from the short arm of chromosome 17 at 17p13.3.

Children with MDS present with severe developmental delay, epilepsy, and feeding problems. In a Dutch study in 1991, a prevalence of approximately 11.7 cases of classical lissencephaly per million live births was reported; the author suggested a higher prevalence due to the wide use of MRI and estimated that about 25% – 30% of classical lissencephaly cases suffer from MDS.[9,10]

Due to the rare occurrence of this disease it is nearly impossible to archive a sufficiently large number of these patients to make a definite assessment of the incidence of the disease. In a retrospective study of two hundred and fifty-eight patients, Kirchhoff, et al. (2007) detected 15 imbalances (5.8%) by MLPA in all chromosomal regions represented in their probe set, except for 17p13 (Miller-Dieker syndrome).[3] In our study, we found 3 MDS cases (3%) which can reflect the appropriate selection criteria or a higher incidence of MDS in Iranian patients.

Williams syndrome (WS) is a rare neurodevelopmental disorder, with an approximate prevalence of 1 in 7,500 to 1 in 20,000.[11] Although the etiology of this syndrome is well known, its pathogenesis is still unclear. The syndrome is caused by a deletion in the long arm of chromosome 7 (7q11.23) in more than 96% of patients,[12,13] which causes a haploinsufficiency in the elastin gene. The protein product of this gene is responsible for the elastic properties of several tissues, such as the skin, lungs and large blood vessels.[14]

Until the 1990s, Williams syndrome was diagnosed based on clinical characteristics of the patients and results of assays of calcium metabolism function, which plays an important role in muscle structure. The emergence of FISH and detection of the 7q11.23 microdeletion locus provided a more accurate technique whereby precise diagnosis has become available.[15]

Using the MRS-MLPA, Kirchhoff, et al. found one patient showing enhanced ratios for seven probes targeting 7q11; they also confirmed the duplication by Real-time PCR. In their case, the *FKBP6* and *GTF2I* genes were duplicated.[3]

In our study, we found five cases of WS in 100 patients (5%), manifesting a relatively high percentage in our panel in comparison with the study by Kirchhoff, et al. Furthermore, there were only three probes for diagnosis of WS; however, it seems that these probes can be sufficient for detection of WS in comparison to the six probes used by Kirchhoff, et al. and EunHae Cho, et al.[3,7]

EunHae Cho, et al. concluded that MLPA is a reliable technique for screening microdeletion syndromes with a relatively low cost compared to FISH.[7] The percentage of diagnosis in our study may confirm this suggestion.

Sotos syndrome (SoS) is a severe physical growth condition characterized by mild ID, macrocephaly and distinctive facial features. The disease is caused by mutations in the *NSD1* gene (Nuclear receptor binding Su-var, enhancer of zeste, and trithorax domain protein 1) located at the 5q35 chromosomal region. Generally, this syndrome occurs in patients with no previous family history and is *de novo*, although there have been reports of autosomal dominant inheritance, as well. MLPA can provide an appropriate technique for detection of SoS deletions regardless of the family background.[16]

Kirchhoff, et al. (2007) found only one case of Sotos syndrome, presenting a duplication in the *NSD1* gene in their two hundred and fifty-eight patients.[3] In our study, we found one case of SoS which was also confirmed by array-CGH; the patient showed deletion in both of the two probes that were set for SoS detection.

In this article, we also report a two and a half year-old girl who presented with multiple anomalies consistent with trisomy for 4p. The chromosomal abnormality was first identified by microdeletion syndromes P245- MLPA kit, showing duplication in the 4p

region and then also manifesting a deletion in the subtelomeric region by Subtelomeric PO36 and PO70 MLPA kit. The *de novo* terminal inversion duplication and subtelomeric deletion was also confirmed by karyotyping, array CGH and FISH.

To date, this is the fifth case with the *de novo* terminal inversion duplication of the short arm of chromosome 4 accompanied by a subtelomeric deletion[17,18,19] and the first to be identified by the MLPA technique. This case is still under analysis and will be reported soon in an individual article.

We have also found a DiGeorge syndrome and an Angelman syndrome case. The clinical data of these two patients strongly correlated with their diagnosis.

To sum up, we suggest the MLPA technique as an easy and appropriate detection method for several diseases, especially such multi-factorial disorders which can be caused by several mechanisms and mutations. Although we can never overstate the benefits and accuracy of FISH and array-CGH, we strongly recommend MLPA as a reliable, routine diagnostic test as it is much more cost-effective and less labor-intensive.

Nevertheless, there are some drawbacks to this technique; for example, in some cases MLPA will not be sufficient for understanding the whole underlying cause of the disorder. MLPA probes are specific for genome targets, but they are limited in size and thus the rearrangements could be sometimes much more complicated. For example, in our Sotos case, some of the patients' symptoms were not compatible with the main Sotos symptoms and further analysis revealed the deletion to be much bigger than could be detected by P245-MLPA kit since there is two probes for this chromosomal region and MLPA is not able to identify the specific breakpoints of the deletion and its power to detect the boundaries depends on the number of probes used for each region.

Furthermore, it should be noted that the MRC-MLPA probe mixes are not FDA approved yet and they are not certified for diagnostic tests. For this validation to occur, further research must be done to analyze its accuracy further. In this regard, the results of MLPA P245-A2 kit could be confirmed by other MLPA kits that have more probes for each specific chromosomal region or other techniques such as FISH or aCGH. Since some of our results have been confirmed by array-CGH and FISH, and the MLPA results correlate strongly with the clinical characteristics of the patients, we recommend this technique as a future routine diagnostic test for an initial understanding of rearrangements followed by an additional complimentary technique in some cases. However, in view of the fact that we have not confirmed the MLPA results for all of our samples with the second method, further conclusions depend on advanced studies.

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