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USEFULNESS OF THE MULTIPLE LIGATION-DEPENDENT PROBE AMPLIFICATION IN THE DETECTION OF SHOX GENE DELETIONS

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Abstract: *The multiple ligation-dependent probe amplification (MLPA) is currently the first recommended molecular biological method used for the detection of deletions that occur in the SHOX gene and associates short stature. We summarize the results of the MLPA assay for identification and characterization of SHOX deletions in 109 short stature patients examined at the Endocrine Genetics Laboratory of the Semmelweis University, Budapest, Hungary. This is one of the first studies on Hungarian and Romanian population in which deletions of the SHOX gene were tested with MLPA in short stature patients.*

Key words: *SHOX gene, MLPA, short stature, PAR1.*

1. Introduction

The process of growth is determined by various environmental and genetic factors [9]. To date, many different etiologies of short stature are known and more than two hundred genes underlying growth control have been identified [15].

The isolated haploinsufficiency of the short stature homeobox-containing gene (SHOX, MIM 312865) is one of the most common reasons for undergrowth caused by the monogenic mutations [9].

The gene was described almost at the same time in 1997 by two different research groups, the Rappold-led German working group and the US based Ellison and co-workers [4], [11], as a gene responsible for the short stature in Turner syndrome patients [16]. Further studies have shown that it is localized in the pseudoautosomal region 1 of the sex chromosomes and encodes a transcription factor implicated in skeletal development and human growth [2].

Heterozygous mutations in its coding or regulatory regions have been identified in up to 2-15% of patients diagnosed with idiopathic short stature (ISS, MIM 300582), in 50-90% of patients with Leri-Weill Dyschondrosteosis (LWD, MIM 127300) carrying different size of deletions and almost 100% in patients with Turner syndrome, while homozygous loss of the *SHOX* gene underlies the severe short stature and

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dysmorphic features in Langer mesomelic dysplasia (LMD, MIM 249700) [15].

Genetic alterations, as copy number variations (CNVs) in non-coding regions with conserved non-coding elements (CNEs) outside the transcription unit of their target gene can cause genetic diseases [18]. Previous studies have identified highly conserved non-coding DNA elements (CNEs) with cis-regulatory activity in the upstream and downstream regions in human cells, chicken limb buds and zebra fish [3] which can produce the same clinical findings [8].

SHOX gene deletions can be detected by fluorescence in situ hybridization (FISH) or microsatellite analysis. However, neither technique is able to provide a clear-cut identification of the deletion breakpoints or to disclose the involvement of other genes in the rearrangement.

In recent years, the multiple ligation-dependent probe amplification (MLPA) methodology has been demonstrated as a useful tool in the detection of abnormal copy numbers of exons, genes, or chromosome regions [9], [10]. MLPA technique uses probes that permit the analysis of different regions in a single reaction [9], [17].

In this study, the MLPA method was used to determine the frequency of *SHOX* gene mutations in Hungarian and Romanian patients presented with short stature.

2. Objectives

Our objective was to perform molecular genetic analysis of the *SHOX* gene with MLPA technique in patients with suspicion of Turner syndrome, based on phenotypic features, but with normal chromosome karyotype**,** in children with signs of Leri-Weill Dyschondrosteosis syndrome, in idiopathic short stature (ISS) and in children with Langer mesomelic dysplasia.

3. Material and Methods

All participants provided informed consent for the performed studies, and ethical approval was obtained from the responsible institutions.

A total of 109 patients were included in the study between 2011 and 2016. Patients were referred from different Hungarian and Romanian medical centers.

Short stature is statistically defined as a height that is less than 2 standard deviations (SD) or one that is below the third percentile for the mean population height corrected for age, gender and ethnicity [19]. The diagnosis of idiopathic short stature (ISS) was made by the mentioned parameters, without any apparent clinical signs of systemic, endocrine, nutritional and chromosomal abnormalities [15], [12].

A suspected LWD diagnosis included two or more of the following features: 1) short stature less than -2 SD; 2) Madelung deformity; 3) mesomelic shortening of the arms [12].

We received the completed data sheet from the patients and the declaration of consent from their parents, and then 6 ml of blood was taken from the patients in EDTA tubes.

For DNA isolation we used peripheral blood and Qiagen isolation kits according to the supplier's protocol, then the quality of the DNA was assessed with agarose gel electrophoresis.

The MLPA is currently the first recommended molecular method for the detection of the deletions that occur in the *SHOX* gene with a rate of 70-75% success. It is a simple method that permits the analysis of a various number samples at the same time. Beside the *SHOX* gene partial or complete deletions and duplications of the *PAR1* region and the downstream enhancer region [1] can also be studied [9]. One positive and two negative control subjects should always be included into the analysis as a reference. In

our case the positive control subject was a previously diagnosed patient with Turner syndrome while the negative controls were two individuals (one man and one woman) with average height.

The genetic analysis was performed in the Laboratory of Endocrine Genetics Semmelweis University, Budapest, Hungary, on 109 patients using the SALSA P018 MLPA kit (MRC-Holland, Amsterdam, The Netherlands), in accordance with the manufacturer's protocol. About 100 ng of genomic DNA was used for the test. Data normalization and data analysis were performed according to the manufacturer's recommendation.

This SALSA® MLPA® probemix P018 SHOX contains 48 MLPA probes with amplification products between 124 and 503 nt: 26 probes located in the *SHOX* + Xp22 areas (including *SHOX* and its regulatory regions); 13 probes elsewhere on the X-chromosome and 9 reference probes detecting autosomal chromosomes [20].

The MLPA method includes 5 main phases: 1) DNA denaturation and hybridisation of MLPA probes; 2) ligation reaction; 3) PCR reaction; 4) separation of amplification products by capillary electrophoresis; and 5) data analysis (Figure 1). During the first step, DNA is denatureted following by an overnight incubation period with a mixture of MLPA probes. MLPA probes consist of two separate oligonucleotides, each containing a single pair of PCR primers used for amplification. The 2 probe oligonucleotides hybridise to immediately adjacent target sequences. Only when both oligonucleotides hybridised, can they be ligated through ligation reaction, and they can be exponentially amplified during the subsequent PCR reaction. The PCR product was separated by capillary electrophoresis [3], [13], [21].

All received data were aggregated in Excel spreadsheets on which we carried out the block normalization and we calculated the hypothetical patients and control subjects quotient gene-specific intensities measurements in each sample.

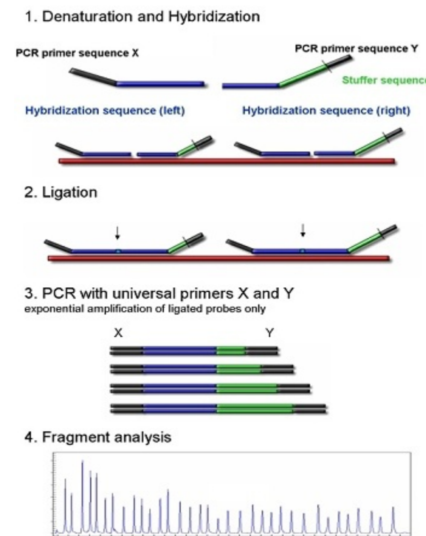

Fig.1. *Steps of the MLPA reaction* [21]

4. Results and Discussions

The presumed diagnosis from de 109 patients included in the study was: Turner-like syndrome (phenotypically Turner's syndrome, but normal karyotype) in 41 (37.6%) cases, idiopathic short stature in 64 (58.7%) cases and Leri-Weill Dyschondrosteosis in 4 cases. From the 109 examined samples 8 (7%) proved to be positive with MLPA for *SHOX* deletions. This result corresponds to the data in the literature. The details of these patients were summarized in the *Table 1.*

From the 8 positive cases 2 (25%) were boys. The deletions of this gene is characterized by feminine domination, which can be explained by the fact that the deletion of the short arm of the X chromosome is more frequent than the rupture of the Y chromosome's short arm.

Females are more severely affected than males, and this is explained by the presence of higher estrogen levels in females. The skeletal defects tend to worsen with puberty [6].

The age of the positive patients ranged from 5.5 years to 17 years, the mean age was 10.3 years. The presumed diagnosis in SHOX deletion carriers were Turner-like syndrome in 3 (37.5%) cases, idiopathic short stature in 4 (50%) cases and Leri-Weill Dyschondrosteosis in 2 (25%) cases.

Five cases had isolated or combined deletions of exons 4, 5 and 2 of the *SHOX* gene, and in 3 cases the complete deletion was detected.

Examination of family members (mother, father, brothers or sisters) of SHOX deletion carriers no *SHOX* gene deficiency was observed suggesting that the deletions occurred *de novo.* The prevalence of the de novo pathogenic variants even in large populations is unknown [5].

There is no data about previous study of *SHOX* gene investigation with MLPA technique in Hungarian patients. In Romania Miclea at al. analyzed 79 patients with short stature with FISH technique using probes for *SHOX* and centromeric regions. They found one (2.3%) case with *SHOX* deletion in a patient presenting with short stature and normal karyotype. They suggested the FISH technique as initial analysis of the cases with short stature providing the explanation of the short stature by sex chromosomal abnormalities with a higher rate of mosaicism detection [14].

The genetical findings in our positive patients Table 1

	Gender	Age (years)	Diagnosis	Karyotype	Height (SDS)	Molecular genetic result
1	Girl	5.5	Turner syndrome – normal karyotype	46,XX	-2.35	Heterozygous deletion of exon 4
2	Girl	17	Turner syndrome – normal karyotype	46,XX	-4.1	Heterozygous deletion from the promoter region to exon 4
3	Boy	7	ISS	Not done	-3.7	Complete heterozygous gene deletion
4	Girl	7.5	ISS	46,XX 9qh+	-2.63	Complete heterozygous gene deletion
5	Girl	15	LWD	46,XX	-2.38	Homozygous deletion of exons 4 and 5
6	Girl	9	Turner syndrome – normal karyotype	46,XX	-2.19	Heterozygous deletion of exon 2
7	Girl	9	ISS	46,XX	-2.35	Heterozygous deletion of exons 4 and 5
8	Boy	13	LWD	Not done	-3	Complete heterozygous gene deletion

ISS: idiopathic short stature; LWD: Leri-Weill Dyschondrosteosis syndrome

In a study by Funari et al. three methods (the FISH technique, the microsatellite analysis and the MLPA technique.) used for the detection of the *SHOX* gene deletions in patients with disproportionate short stature (DSS) and LWD were compared. Considering sensitivity, cost and execution time the MLPA method proved to be more sensitive, less expensive and less time consuming, for detection of *SHOX* gene deletions [9], [16].

FISH analysis was the most expensive and has laborious methodology, needing approx. 8 days to obtain the results. Microsatellite analysis and MLPA were equivalent in execution time. MLPA was an effortless method analyzing a more extensive gene region and also had a lower cost.

The authors proposed a strategy containing in a start with MLPA analysis, which enables the screening of all *SHOX* exons and the 3' UTR of *SHOX* region. Small deletions should be confirmed using microsatellite analysis and in the absence of deletions, direct sequencing of *SHOX* gene exons is indicated [9].

In a recent study, searching for the new genes causing short stature, genom-wide analysis for copy number variants (CNVs, using single-nucleotide polymorphism arrays) was performed in 162 patients with short stature, and besides 6 CNVs known to be causative for short stature, 40 CNVs with possible pathogenicity were identified (HA an Duyvenvoorde et al.). Further studies are needed to support the role of these regions in longitudinal growth regulation and we would like to extend our researches in this direction in the future [7].

5. Conclusions

Molecular genetic examination is justified for short stature patients who present typical clinical symptoms of SHOX phenotype, or suffer of idiopathic short stature. Similarly, it is indicated in patients with Turner syndrome-like phenotype, in Leri-Weill Dyschondrosteosis syndrome, as well as in Langer mesomelic dysplasia.

The MLPA is currently the first recommended molecular method for the detection of deletions of *SHOX* gene, having a rate of 70-75% success.

It is a simple method that permits the concomitant analysis of wide number of samples and can be applied for detection of PAR1 deletions and duplications including *SHOX* and the downstream enhancer region. It also permits the evaluation of the extension of the deletion. MLPA is more sensitive, less expensive and less time consuming than other techniques (eg. FISH technique, or microsatellite analysis).

Based on the accurate genetic diagnosis, the physician can give proper genetic counseling for the patient and his/her family, informing them about the evolution of disease and the potential therapeutic strategy.

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