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Diagnostics of *SHOX* gene rearrangement in 46,XX women with idiopathic short stature

Diagnostyka rearanżacji genu SHOX u kobiet 46,XX z idiopatyczną niskorosłością

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Abstract

Introduction: The *SHOX* gene has been mapped at the pseudoautosomal region 1 (PAR1) of chromosomes X (Xp22.33) and Y (Yp11.32). The loss of *SHOX* gene functionality is assumed to be responsible for the Leri-Weill syndrome formation and the disproportionate short stature (DSS). The *SHOX* gene rearrangements constitute the majority of cases of gene functionality loss. Therefore, a practical application of the method, which allows for the diagnostics of the gene rearrangements, becomes a primary issue. With such an assumption, the MLPA technique (multiplex ligation — dependent probe amplification) becomes the method of choice.

Material and methods: DNA samples were evaluated in the study by means of the MLPA method. The DNA was isolated from peripheral blood of sixty-three (63) 46,XX patients with short stature.

Results: Out of the examined patients, deletions within the *SHOX* gene were found in five (5) patients, and duplication at the PAR1 regulatory region of the *SHOX* gene in one (1) case.

Conclusions: The obtained results confirm the opinion that the MLPA method, while enabling the diagnostics of the etiopathogenetic factor of short stature, identified in approximately 9.5% of cases, is a useful tool in the diagnostics of *SHOX* gene deletion and duplication. **(Endokrynol Pol 2016; 67 (4): 397–402)**

Key words: *short stature; MLPA; SHOX*

Streszczenie

Wstęp: Gen *SHOX* został zmapowany w regionie pseudoautosomalnym 1 (PAR1) chromosomów X (Xp22.33) i Y (Yp11.32). Utratę funkcji genu *SHOX* czyni się odpowiedzialną za powstanie zespołu Leri-Weill i nieproporcjonalnie niski wzrost (DSS, *disproportionate short stature*). Delecje genu *SHOX* stanowią większość przypadków utraty funkcji genu. Z tego tytułu sprawą pierwszoplanową staje się aplikacja do praktyki diagnostycznej metody, która umożliwia diagnostykę rearanżacji tego genu. Przy takim założeniu technika MLPA (*multiplex ligation — dependent probe amplification*) staje się metodą z wyboru.

Materiały i metody: W badaniach analizowano z użyciem metody MLPA próbki DNA wyizolowanego z krwi obwodowej 63 pacjentek 46,XX z niskorosłością.

Wyniki: Spośród przebadanych pacjentek u 5 wykryto delecje w obrębie genu *SHOX*, u jednej pacjentki wykryto duplikację w regionie regulatorowym PAR1 genu *SHOX*.

Wnioski: Uzyskane wyniki potwierdzają opinię, że metoda MLPA, umożliwiając diagnostykę czynnika etiopatogenetycznego niskorosłości w około 9,5 % przypadków, jest użytecznym narzędziem w diagnostyce delecji i duplikacji genu *SHOX*. **(Endokrynol Pol 2016; 67 (4): 397–402)**

Słowa kluczowe: *niskorosłość; MLPA; SHOX*

Introduction

The height of a short patient is below the third centile or < –2SD for a given age, sex, and race. Short stature is a multi-causal symptom which may be genetically determined. The frequent causes of short stature are rearrangements of the *SHOX* (*short stature homeobox*) gene on chromosome X or Y at PAR1 region (Xp22.33 and Yp11.32). The presence of abnormalities within the *SHOX* gene was demonstrated in patients with the Leri-Weill syndrome or Turner's syndrome, as well as in patients with idiopathic short stature (ISS). Rearrangements in a non-coding sequence, localised in a certain distance from the *SHOX* gene, at the regulatory region in size of up to 346 kbp (locus Xp22.32), may also disturb normal gene expression [1].

The penetration of *SHOX* deficit is high, but its clinical manifestation is variable. Growth disorders are initiated in the first years of life. The body length of a newborn after birth is characterised by a slight deficit which, however, increases when the child is at kindergarten age. In female patients affected by the above-

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mentioned rearrangement, no normal pubertal spurt is observed. The presence of certain small morphological abnormalities plus subtle radiological symptoms are significant markers, indicating the necessity of genetic studies [2–5].

The SHOX gene

The *SHOX* gene has been mapped during studies on deletions in the short arm of chromosome X, accompanied by short stature. The studies were performed by Rao [6] and, independently, by Ellison et al. [7], who provided the studied gene with the name of *PHOG* (*pseudoautosomal homeobox-containing osteogenic gene).* The gene is localised at the terminal part of the short arms of chromosomes X and Y at the telomere sub-region of the PAR1 pseudoautosomal region, and comprises 2.6 Mbp. The PAR1 region comprised inactivation-avoiding genes [8, 9]. The *SHOX* gene is then active on both copies of chromosome X in women and on chromosomes X and Y in men. It causes the occurrence of disease phenotypes in cases of a modified number of pseudoautosomal gene copies, i.e. in the case of occurrence of aneuploidy or polysomy of the sex chromosomes (e.g. 45,X, 47,XXX, 47,XXY, 47,XYY). The PAR1 region undergoes an obligatory *crossing over* process between chromosomes X and Y, which may induce abnormalities, promoting an emergence of mutations in the *SHOX* region in the course of male meiosis [10].

The *SHOX* gene is built of two motifs: the first one encodes a homeodomain, which enables the specific DNA binding with the protein and ensures the transactivation of genes. The other motif encodes the C-terminal OAR domain, which is important for the *SHOX* gene activity. The mRNA of the *SHOX* gene is encoded by seven exons (1-5, 6a and 6b), corresponding to approximately 49 kbp of genomic DNA. An alternative *splicing* provides the following two products: the OAR domain containing *SHOXa* and *SHOXb*, its shorter version, which, while missing the OAR domain, does not activate the transcription process. The functionality of *SHOXb* has not yet received sufficient attention of researchers and thus remains rather obscure [7, 11].

The *SHOX* gene functions are impaired in almost 100% of Turner's syndrome cases, 50–90% of Leri-Weill syndrome cases, and in 2–15% of patients with idiopathic short stature (ISS) [4, 12, 13]. The *SHOX* gene plays an important role in the development of the bone system [7]. The homeodomain protein of the *SHOX* gene plays the function of transcription activator, which controls the expression of genes responsible for the growth process. A loss of gene copy or mutation of one gene allele causes shortening of metacarpal bone IV, abnormalities in facial skeleton structure, gothic palate, short, broad neck, and scoliosis (however, often in cases of Leri-Weill syndrome, as well as in Turner syndrome).

The most frequent rearrangements of the *SHOX* gene include deletions of various sizes, encompassing the *SHOX* gene or the regulatory region [14]. Deletions stand for approximately 80% of all rearrangements [15]. The loss of both *SHOX* alleles brings about a complete deficit of *SHOX* protein and an extreme phenotype of osteodysplasia, described as Langer syndrome [16]. An additional copy of the *SHOX* gene, which occurs in this case of polysomy, may result in higher patient's stature [17, 18].

Short stature may also be caused by partial duplication of the *SHOX* gene and of the regulatory region [18, 19]. The rate of patients with identified duplications amounts to 7.3% of those with Lerie-Weill dyschondrosteosis (LWD) and 1% of patients with idiopathic short stature (ISS). The clinical symptoms associated with partial *SHOX* duplication depend on the physical localisation of the duplicated sequence, which may cause gene expression decrease. Additional copies of the gene or its fragments in the neighbourhood of the normal gene may change the distance between the gene, the *SHOX* promoter, and the regulatory region, which may in turn result in gene expression reduction [19].

Diagnostics

Cytogenetic study is a commonly used diagnostic method to identify chromosome X monosomy or structural aberration. This method is characterised by low cost and the possibility of simultaneous evaluation of the entire genome organisation in the studied subject. Tests that enable the identification of selected chromosome aberrations by other methods are increasingly common: FISH (fluorescence in situ hybridization), QF-PCR (quantitative fluorescence polymerase chain reaction), and MLPA (multiplex ligation-dependent probe amplification) [20–32]. These methods, having comparable efficacy and diagnostic reliability, making it possible to diagnose *SHOX* gene rearrangements [33]. Studies employing comparative genomic hybridisation (CGH) are expensive but also are more often used, allowing for simultaneous evaluation of tens of thousands of sequences. In our conditions, with the results of cytogenetic study, excluding the presence of structural aberrations on the Xp arm, the diagnosis of which could be possible at the level of a single chromosome band, we decided to apply the MLPA technique [20–22].

Material and methods

The presented research project was approved by the Bioethical Committee of the Medical University in Lodz, No. RWS-1173/11. Patients or legal guardians

expressed their conscious consent to the tests. Sixty-three (63) female patients with normal 46,*XX* karyotype and short stature were qualified to the study, all of them inhabitants of the Lodz Province. Some of the patients demonstrated such features as: compromised physique proportions, asymmetric bodies, and changed facial skeleton proportions, as well as features characteristic for patients with chromosome X monosomy, i.e. primary amenorrhoea, dysmorphic facial features, shortened metacarpal bone IV, short and broad neck, and low posterior hairline.

Cytogenetic studies of chromosomes employed the classical "*band after band*" analysis with a minimal resolution of 550. DNA was isolated from 6 ml of whole blood, collected under vacuum on EDTA. Genomic DNA from the patients was collected by the column isolation method, using a Qiamp DNA Blood Midi Kit (Qiagen, Germany). The obtained DNA concentrations amounted to 50–500 ng in 1 μl of TE buffer. The samples were analysed by the MLPA method, using *SALSA MLPA probe mix* (P018-F1 SHOX probes of MRC-Holland), following the manufacturer's instructions. In the first step, DNA was denatured following by an overnight incubation period with a mixture of MLPA probes. MLPA probes consist of two oligonucleotides, each containing a single pair of PCR primers used for amplification. The two probe oligonucleotides hybridise to immediately adjacent target sequences. Only when both oligonucleotides hybridised to their adjacent targets can they be ligated during the ligation reaction, and they can be exponentially amplified during the subsequent PCR reaction. The resulting amplification products of *SALSA MLPA kits* range between 130 and 480 bp in length. The PCR product was separated by the capillary electrophoresis method. The obtained results were analysed by means of *GeneMarker V1.70* software (SoftGenetics, LLC, State College, USA). The probe signal level in the analysed sample was comparable to the probe signal level in the synthetic control. The values of relative probe fluorescence (the relative peak values) of normal samples were within the range 0.7–1.3. The values above 1.3 indicated duplication, while those below 0.7 were informative of deletion (Fig. 1).

Results

Among DNA samples, obtained from 63 patients, in 57 there were no *SHOX* gene rearrangements that wereidentifiable by means of the MLPA method and commercially available probes. Abnormalities in the *SHOX* gene were identified in six patients, including five cases of deletion and one case of duplication. Deletions in exon 5 were found in two patients. One

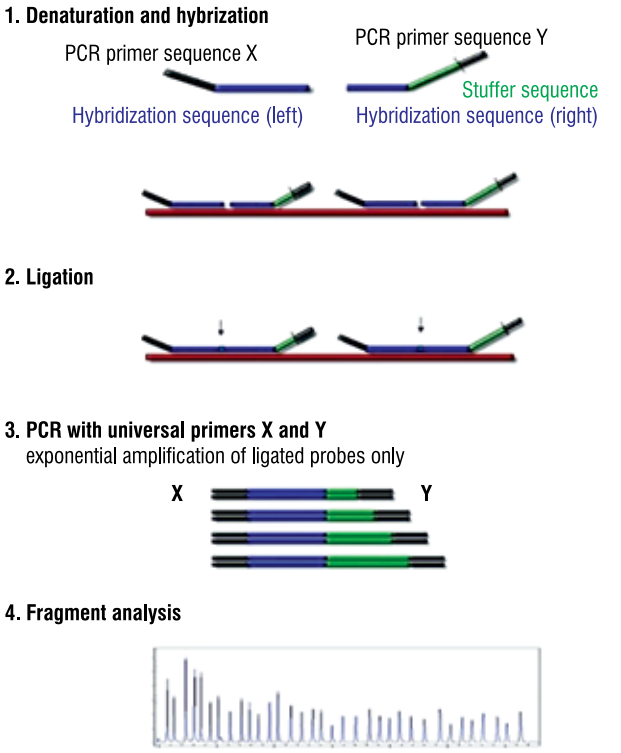

Figure 1. *Schematic MLPA reaction. Sources: Company Website MRC Holland, http://www.mlpa.com/WebForms/WebFormMain.aspx?Tag=_wl2zCji-rCGANQgZPuTixsEyIW1MscfzuKj2NDFYc-g. Access: 05.05.2016*

Rycina 1. *Schemat reakcji MLPA. Źródło: strona internetowa firmy MRC Holland, http://www.mlpa.com/WebForms/WebFormMain.aspx?Tag=_wl2zCji-rCGANQgZPuTixsEyIW1MscfzuKj2NDFYc-g; data pobrania: 05.05.2016*

of the results indicated deletions in exons 5 and 6a. The fourth patient demonstrated deletion in exon 4 and another one in exon 6. In one patient, duplication was observed, including the Xp22.33-PAR1 regulatory region and *CRLF2* and *CSF2RA* genes, located next to the region (Fig. 2, Table I).

Discussion

The MLPA enables a simultaneous analysis of a big number of samples, with a relatively little effort and small equipment needs. This method ensures study results to be obtained within 24 hours [20].

Among chromosomal aberrations of the PAR1 region, 80% are deletions. The incidence of heterozygotic deletions of the *SHOX* gene are estimated at 2–15% of idiopathic short stature cases [9]. This has been confirmed by the presented studies, where the obtained result (*i.e.* the proportion of heterozygotic deletions of the *SHOX* gene) amounted to 9.5% of cases.

The analysis of the *SHOX* gene was completed by an analysis of the Xp22.33-PAR1 region, localised beyond

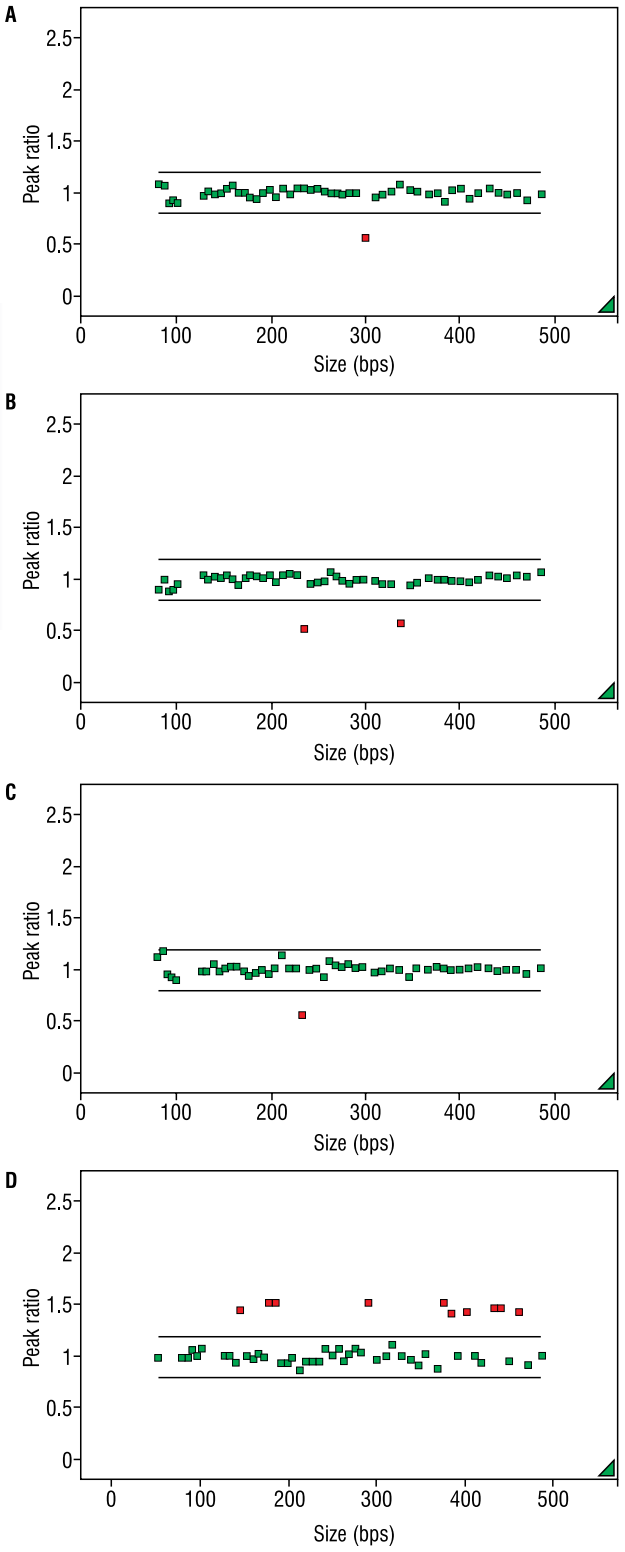

Figure 2. *Selected results of MLPA reactions:* **A.** *Deletion in exon 4;* **B.** *Deletion in exons 5, 6a;* **C.** *Deletion in exon 6;* **D.** *Duplication in the Xp22.33-PAR1 regulatory region and in the CRLF2 and CSF2RA genes*

Rycina 2. *Wybrane wyniki reakcji MLPA.* **A.** *Delecja w eksonie 4;* **B.** *Delecja w eksonach: 5, 6a;* **C.** *Delecja w eksonie 6;* **D.** *Duplikacja w regionie regulatorowym Xp22.33-PAR1 oraz genach CRLF2 oraz CSF2RA*

the *SHOX* gene; it has been suggested that this is the regulatory region of transcription. Deletions may occur in that region with the incidence rate of cases at 4–16% [13, 14, 34–36]. It means that 16% of all deletions may remain unidentified by the diagnostic method that does not encompass this particular region.

The possibility to identify *SHOX* gene duplications or its fragments, as well as duplications of the regulatory region, seems equally justified. Benito-Sanz et al. carried out studies employing the MLPA method in a group of 735 patients, identifying total duplications of the *SHOX* gene in 0.5% of cases, partial duplications encompassing several exons in 1.4% of patients, and duplications of the regulatory region in 2.0% of cases [19]. The presented study seems to confirm their results. In the presented study, one of the patients with idiopathic short stature demonstrated duplication, encompassing the Xp22.33-PAR1 regulatory region and *CRLF2* and *CSF2RA* genes, which comprises 1.8% of the studies cases. As expected, mutations within the regulatory region provide a similar clinical picture as those within the *SHOX* gene. This is probably associated with impaired gene transcription and reduced volume of normal *SHOX* protein.

In order to evaluate the diagnostic usefulness of the MLPA method, the following four aspects should be considered: the sensitivity of the method, its clinical limitations, the associated labour intensity, and the economic aspect. In many publications, the sensitivity and specificity of the MLPA method, regarding the identification of the most frequent rearrangements, amounted to 100% [31,32,40]. The frequency of failures resulting, among others, from poor DNA quality varied from 0.8% to 4.4% [30–32]. Many authors emphasise the fact that the MLPA method is sensitive to DNA quality. The rate of failures, when carrying out the test, was comparable with the rate of studies, in which neither the QF-PCR method nor the FISH method gave informative results, which were 0.1–3.7% and 0.0–4.9%, respectively [23–29, 40]. Taking into account the above observations, and ensuring DNA of good quality, the MLPA technique may be recommended as diagnostic standard.

Conclusions

A reliable and early identification of the *SHOX* gene rearrangement allows for optimisation of the growth hormone therapy onset and duration, which helps achieve the highest relative indices of body height increase while preventing stature deficits to be revealed in the pre- and early school period [41]. It enables the hormonal replacement therapy to be initiated at close to the optimal time point. It also allows for an optimal introduction of psychological and educational sup-

Table I. *The obtained results: above 1.3 — duplication; below 0.7 — deletion*
Tabela I. *Uzyskane wyniki; powyżej 1,3 — duplikacja; poniżej 0,7 — delecja*

Locus	Patient					
	1	2	3	4	5	6
Xp22.33-PAR1	1.011	1.051	0.951	1.025	1.110	1.022
4 kb before SHOX-PAR1	1.162	1.037	0.978	1.023	1.026	1.063
Exon 1 PAR1	0.889	0.879	1.045	0.904	1.021	0.978
Exon 2 PAR1	0.878	0.985	1.150	1.105	1.111	0.948
Exon 3 PAR1	1.014	0.965	1.001	0.901	1.024	1.154
Exon 4 PAR1	1.121	0.542	1.012	1.128	1.078	1.134
Exon 5 PAR1	0.932	0.925	0.658	0.513	0.528	1.113
Exon 6 (6a) PAR1	0.984	0.965	0.913	0.504	1.054	0.520
Intron 6 PAR1	0.952	0.845	1.002	1.154	1.199	1.158
Intron 6 PAR1	0.987	0.963	0.956	0.945	0.875	0.846
Xp22.33-PAR1	0.874	0.982	1.015	1.198	1.201	0.987
Xp22.33-PAR1	0.932	0.952	0.994	0.965	1.025	1.032
Xp22.33-PAR1	0.923	0.987	1.023	0.885	1.021	0.954
Xp22.33-PAR1	1.025	1.147	0.964	0.948	1.085	1.112
Xp22.33-PAR1 — the regulatory region	1.087	1.096	0.998	0.985	1.010	0.012
Xp22.33-PAR1 — the regulatory region	1.456	1.013	1.102	1.098	1.002	0.982
Xp22.33-PAR1 — the regulatory region	1.438	1.154	1.069	0.958	0.948	0.892
Xp22.33-PAR1 — the regulatory region	1.514	1.002	1.051	1.145	1.005	1.009
Xp22.33-PAR1 — the regulatory region	1.531	0.951	0.956	1.023	1.023	1.056
Xp22.33-PAR1	1.442	1.059	1.007	1.195	0.948	1.085
Xp22.33-PAR1	1.522	1.002	1.021	1.048	1.063	1.145
Xp22.33-PAR1	1.467	0.879	1.045	1.065	1.023	1.095
Xp22.33-PAR1	1.522	1.004	0.948	1.201	0.987	1.144
CRLF2	1.435	1.011	0.956	1.025	1.032	1.165
CSF2RA	1.423	1.063	0.942	1.010	0.892	0.888
IL3RA	0.965	1.023	0.894	1.056	0.951	1.154
ASMT	0.854	1.062	1.145	1.111	1.117	0.945
ZBED1	0.869	1.021	1.146	1.025	0.932	0.874
ARSF	0.962	0.987	1.063	1.135	0.984	0.896
PRKX	0.935	0.978	1.185	1.165	0.846	0.945
NLGN4X	1.151	0.948	1.094	1.085	0.951	0.013
KAL1	1.025	1.154	1.023	0.985	0.965	0.966
FANCB	1.063	0.925	0.196	0.845	1.051	0.962
AIFM1	1.096	0.935	1.125	0.961	1.062	0.947
Xq28-PAR2	0.965	0.962	1.006	1.012	1.145	1.052

port to prevent differences in educational and social status opportunities. The obtained results confirm the view that the MLPA method, while enabling the diagnostics of the aetiopathogenetic factor of short stature, identified in approximately 10% of cases, is a useful tool in the diagnostics of *SHOX* gene deletion and duplication.

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