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Multiplex Ligation-Dependent Probe Amplification Accurately Detects Turner Syndrome in Girls with Short Stature

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Keywords
Short stature · Multiplex ligation-dependent probe amplification · Karyotype · X chromosome aneuploidies · Screening

Abstract
Aims: We aimed at evaluating a standard multiplex ligation-dependent probe amplification (MLPA) probe set for the detection of aneuploidy to diagnose Turner syndrome (TS). We first fixed an MLPA ratio cutoff able to detect all cases of TS in a pilot TS group. We then tested this value on a second group of TS patients and a short-stature population to measure specificity and sensitivity. ***Methods:*** 15 TS patients with X mosaicism or X structural abnormalities (Pilot TS Group), 45 TS karyotype-assessed patients (TS Group), and 74 prepubertal female patients with apparent idiopathic short stature (Short-Stature Group) were enrolled. All subjects underwent MLPA and karyotype analysis. In the TS and Short-Stature Groups, MLPA testing was performed in blind. ***Results:*** The choice of an MLPA threshold ratio of 0.76 for at least 1 probe allowed us to detect all TS cases, including mosaicisms. Sensitivity and specificity were 100% (CI 95%, 0.92–1) and 88.89% (CI 95%, 0.79–0.94), respectively. The positive predictive value was 88.5%, and the negative predictive value was 100%. MLPA detected the presence of Y chromosome material in 2 patients. ***Conclusion:*** MLPA is an accurate and inexpensive tool to screen for TS in girls with short stature. A customized MLPA kit may be useful for the screening of an even larger population.

Introduction

Turner syndrome (TS) is a common genetic syndrome affecting the female sex, with an incidence of about 1 in 1,500–2,500 newborn girls worldwide [1]. TS is characterized by the absence of all or part of an X chromosome in all cell lines (45, X0) or in a subset of cells (TS

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mosaicism) [2, 3]. This pathology requires a multidisciplinary approach to address the combination of genetic, developmental, endocrine, cardiovascular, psychosocial, and reproductive symptoms [4]. TS patients present short stature, hypergonadotropic hypogonadism as well as renal and cardiac structural abnormalities [2–4]. Early detection of TS allows both timely growth hormone therapy with improvement in statural prognosis [5] and timely management of comorbidities [4]. In addition, treatment with low-dose ethinyl estradiol, if initiated in childhood, has been reported to subsequently normalize the onset and tempo of puberty [6]. Unfortunately, many girls with TS are only diagnosed at the age of 10 years or later [7, 8] and, in any case, not usually before the seventh year of age when short stature becomes clinically evident [9].

A standard 30-cell karyotype analysis [10] represents the gold standard for TS diagnosis. A diagnosis of TS can be made when the typical clinical features are accompanied by complete or partial absence of the second sex chromosome (with or without cell line mosaicism) [4]. However, karyotyping is labour-intensive and impractical for large-scale population or high-throughput testing. Therefore, geneticists do not advocate karyotyping as a first-line diagnostic technique in their guidelines for evaluating short stature [11]. In contrast, since 1 in 50–100 girls with short stature is affected by TS [1, 12], paediatric endocrinologists recommend that short girls (≤5th percentile) be tested for this condition [4]. Although PCR-based approaches have been proposed for TS detection [13–16], they do not effectively identify patients with mosaicism or partial X chromosome deletions, which account for over 40% of the karyotypes in TS [11].

Multiplex ligation-dependent probe amplification (MLPA) based on relative quantification of different DNA target sequences in a single reaction is a quantitative method both faster and cheaper than karyotyping. It was introduced in clinical practice to rapidly detect aneuploidies of chromosomes 13, 18, 21, X, and Y in prenatal screening [17–20]. Here, for the first time, we used MLPA to screen for putative X aneuploidies and X structural abnormalities in the postnatal period. We initially performed MLPA analysis in a population of karyotype-assessed TS patients with X mosaicism or X structural abnormalities to determine the ratio able to detect all cases of TS. We then tested this ratio by analyzing both a TS and a short-stature population using MLPA, thereby establishing its specificity and sensitivity.

Materials and Methods

Patient Enrolment

Written, informed consent was obtained from all enrolled children and/or their parents after the nature of the investigation had been explained. The Institutional Review Board of the Second University of Naples approved the study protocol.

This was a multicentre study. 15 TS patients with known mosaicism or X structural abnormalities (karyotype assessed) attending the Paediatric Endocrinology Department of the Second University of Naples (Pilot TS Group) were enrolled with the aim to identify the MLPA cutoff able to detect all cytogenetic abnormalities (Fig. 1). Moreover, 45 known TS karyotype-assessed patients (TS Group) from 4 different Italian paediatric endocrinology centres (Second University of Naples, University of Naples "Federico II", University of Catania, and University of Messina) were enrolled to test the identified MLPA cutoff (Fig. 1). A karyotype analysis of these patients had previously been performed at the cytogenetic laboratory of each paediatric endocrinology centre. All centres sent peripheral blood samples to the Laboratory of Medical Genetics at the Second University of Naples, where the MLPA analyses were performed.

We also enrolled 74 prepubertal female patients (mean age 6.5 ± 2.9 years; mean height standard deviation score [SDS] -2.8 ± 0.7) with apparent idiopathic short stature (Short-Stature Group; Fig. 1) attending the Paediatric Endocrinology Department of the Second University of Naples to evaluate the MLPA cutoff in a general population with short stature. Pubertal stage according to Tanner criteria was clinically assessed.

The Short-Stature Group inclusion criteria were stature ≤2 SDS and growth velocity <10th percentile with no evidence of diseases following complete evaluation (full blood cell count; hepatic, renal, thyroidal, and adrenal function; sexual hormones; transglutaminase antibodies; total immunoglobulin A; C-reactive protein; erythrocyte sedimentation rate, and insulin growth factor-1) including stimulated growth hormone levels but excluding karyotype analysis. This population underwent both karyotyping and MLPA analysis. Height was measured to the nearest 1.0 mm with a Harpenden wall-mounted stadiometer.

DNA Samples

DNA was extracted from peripheral blood using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA), and was then quantified using a NanoDrop (Thermo Fisher Scientific, Wilmington, MA, USA) spectrophotometer according to the manufacturer's instructions. DNA quality was assessed by agarose gel electrophoresis.

Multiplex Ligation-Dependent Probe Amplification

MLPA is a multiplex PCR method, which can detect abnormal copy numbers of up to 50 different genomic DNA or RNA sequences and which is able to distinguish sequences differing in only 1 nucleotide.

For each genomic target, a set of 2 probes is designed to hybridize immediately adjacent to target sequences in order to be ligated into a single probe. Each probe is made up of the target sequence and a nonhybridizing "stuffer sequence" of variable length and a universal PCR primer-binding site. After hybridization, the probes are ligated, and PCR is performed with a universal fluorescent-labelled primer pair. Each ligated fragment has a unique amplicon

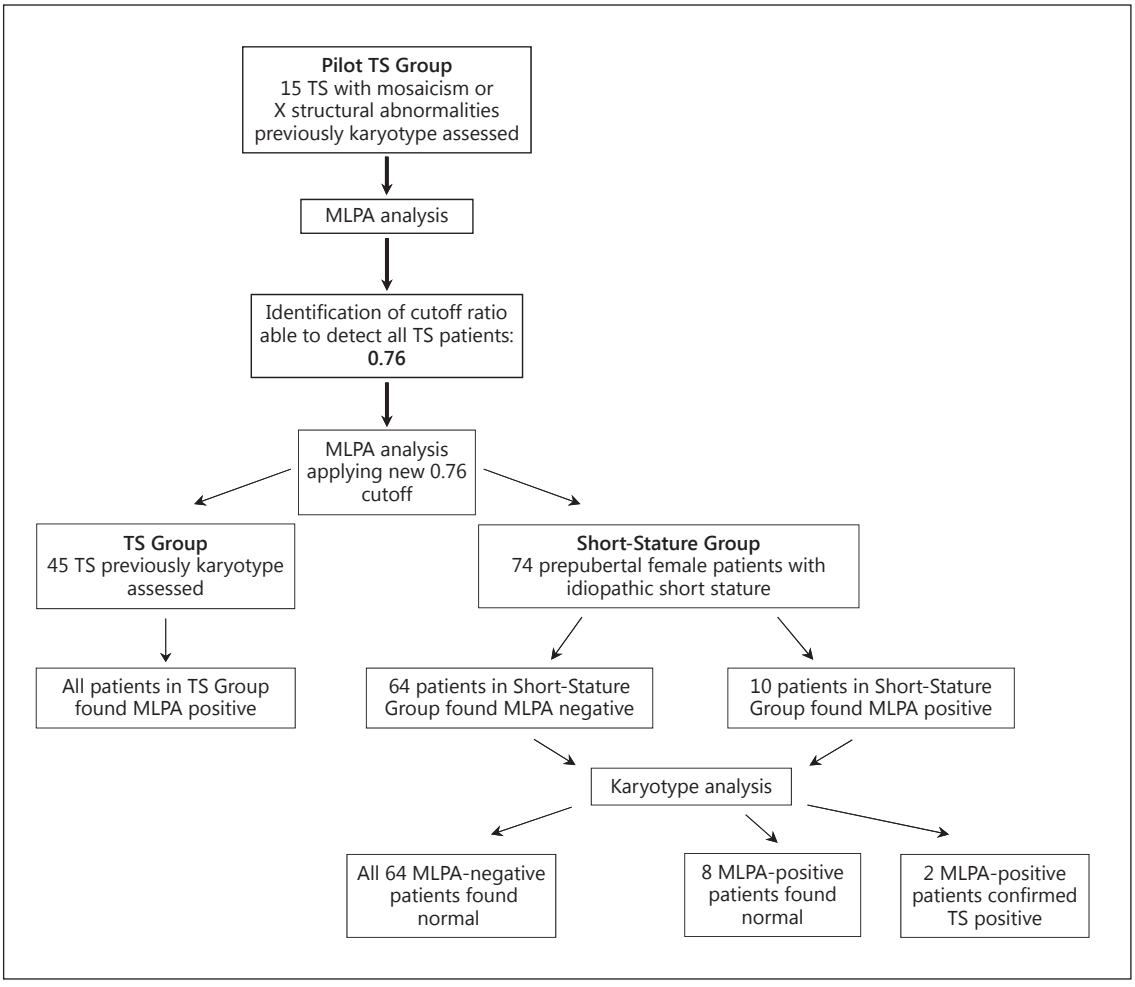

Fig. 1. Study design. MLPA, multiplex ligation-dependent probe amplification; TS, Turner syndrome.

length, ranging between 130 and 500 nt, and is separated by capillary electrophoresis. Sequences showing aberrant copy numbers are identified by comparing the peak pattern obtained to that of reference samples. MLPA analyses were performed using the SALSA MLPA kit P095 Aneuploidy (MRC-Holland, Amsterdam, The Netherlands), containing probes designed to detect aneuploidy of chromosomes 13, 18, 21, X, and Y [20]. Information on the localization, sequences, and lengths of the probes is available on the MRC-Holland website (https://www.mlpa.com/WebForms/WebFormProductDetails.aspx?Tag = _tz2fAPIAupKyMjaDF-E-t9bmuxqlhe_Lgqfk8Hkjuss.&ProductOID = _b4E4JKHdVds).

The P095 probe mix contains 36 MLPA probes with amplification products between 130 and 454 nt: 8 probes for chromosomes 13, 18, 21, and X, and 4 probes for the Y chromosome. In addition, it contains 9 control fragments generating an amplification product smaller than 120 nt: 4 DNA quantity fragments (Q-fragments) at 64, 70, 76, and 82 nt; 3 DNA denaturation control fragments (D-fragments) at 88, 92, and 96 nt; 1 X-fragment at 100 nt, and 1 Y-fragment at 105 nt.

MLPA reactions were performed in a thermocycler (Applied Biosystems, Foster City, CA, USA). The reaction products were electrophoresed in an ABI Prism 3130 XL Genetic Analyzer (Applied Biosystems).

The size and peak areas for each probe were quantified and analyzed by Coffalyser.Net (MRC-Holland), a free software program designed specifically for MLPA data analysis. The analysis is performed in 2 steps: DNA fragment analysis and comparative analysis of samples. Relative probe signals are calculated and compared with samples of normal male and female sex. In chromosomally normal samples, the relative probe signal is expected to be 1 for all probes.

By performing MLPA analysis in the Pilot TS Group, we determined the ratio able to detect all X aneuploidies and X structural abnormalities. This ratio was then tested in both the TS Group and the Short-Stature Group.

Karyotyping

The same operator performed all karyotyping assays in the Short-Stature Group following MLPA analysis. Chromosomal

Table 1. Karyotype results and MLPA X probe ratios of Pilot-TS-Group patients

Patient	Karyotypes	MLPA probes									
		[Xp [ARX-4]	[Xp RPS6KA3-3]	[Xp [DMD-69]	[Xp [TSPAN7-5]	[Xq [AR-1]	[Xq [ACSL4-17]	[Xq [AIFM1-15]	[Xq [L1CAM-4]	[Yp [SRY-1]	[Yp [ZFY-4]
1	mos 45,X[92]/46,X,+r(y)[8]	0.56	0.53	0.59	0.54	0.56	0.6	0.52	0.57	0.09	0.08
2	mos 45,X[80]/47,XXX[20]	0.12	0.53	0.58	0.61	0.55	0.52	0.5	0.68		
3	mos 45,X[36]/46,X,del(X)(p11.2)[14]. ish del(X)(wcpX+)	0.59	0.46	0.53	0.58	0.64	0.72	0.74	0.68		
4	46,X,i(X)(q10)	0.54	0.51	0.59	0.61	1.46	1.33	1.43	1.56		
5	mos 45,X [24]/46,X X [76]	0.87	0.76	0.91	0.86	0.82	0.88	0.92	0.85		
6	mos 45,X[25]/46,X,add (X)(p?). ish dup (X)(q?)(wcpX+)[25]	0.52	0.7	0.74	0.63	0.74	0.73	0.62	0.71		
7	mos 46,X,+r[24]/45,X[21].ish r(X)(wcpX+)	0.46	0.45	0.53	0.54	0.74	0.49	0.52	0.68		
8	mos 45,X[45]/46,X,+r .ish r(X)(wcp X +)[17]	0.52	0.74	0.63	0.54	0.59	0.59	0.47	0.49		
9	mos 45,X [45]/47,XXX[45]	0.65	0.64	0.72	0.67	0.69	0.63	0.66	0.59		
10	mos 45,X[80]/46,X,i(X)(q10)[20]	0.53	0.63	0.59	0.61	0.61	0.64	0.7	0.62		
11	mos 46Xr(X) [29]/ 45,X [71]	0.61	0.63	0.65	0.59	0.62	0.53	0.54	0.53		
12	46,X,i(X)(q10)	0.57	0.53	0.56	0.54	1.25	1.25	1.23	1.29		
13	46,X,i(X)(q10)	0.64	0.45	0.56	0.49	1.94	2.68	1.23	1.82		
14	mos 45X[90]/46X+mar[10]. ish der (X) (DXZ1+, SRY–), Yp11.3 (SRY–, DXZ1+)	0.56	0.58	0.53	0.62	0.57	0.55	0.59	0.64	0.04	
15	mos 45,X[70]/46,X,i(X)(q10)[30]	0.53	0.5	0.51	0.52	0.62	0.66	0.58	0.63		

MLPA, multiplex ligation-dependent probe amplification; TS, Turner syndrome.

analyses were carried out on 2 whole blood cultures according to standard methods. GBG banding (G bands by BrdU using Giemsa stain) was performed as described elsewhere [21].

Poseidon whole-chromosome probes specific for chromosomes X and Y (Kreatech, Amsterdam, The Netherlands) were used according to the manufacturer's protocols.

Routine G-band karyotyping included counting of at least 30 metaphases, 5 of which were fully analyzed.

Mosaicism was defined as the presence of at least 5 cells showing the same chromosomal aberration (numerical chromosome abnormalities or a structural rearrangement) in 2 separate cultures.

Karyotypes and fluorescence in situ hybridizations (FISH) were analyzed using a Nikon Eclipse-1000 epifluorescence microscope (Nikon Instruments, Tokyo, Japan), and images captured and elaborated using the Genikon system v. 3.8.5 (Nikon Instruments S.p.a., Calenzano, Italy).

Statistical Analysis

Statistical analysis was performed using the Fisher exact test in a 2 × 2 crossover design and *t* test calculator.

Results

To assay whether MLPA is able to accurately detect TS cases, we first analyzed a cohort of known mosaic TS patients (Pilot TS Group). Using this group, the ratio able to detect all mosaicisms and X structural abnormalities was 0.76 in at least 1 probe (Table 1). This ratio also allowed for the identification of the lowest grade of mosaicism (15%; mos 45,X[15]/46,XX[85]) among patients in the Pilot TS Group. Interestingly, in this population, MLPA detected the presence of Y chromosome material in 2 patients (Table 1). Applying this 0.76 cutoff in the TS Group, MLPA detected all X aneuploidies and X structural abnormalities including X monosomy, 45X0/46XX and 45X0/47XXX mosaicisms, isochromosome X, and deletion of X chromosome. In the TS Group, the prevalence of mosaicism, X structural abnormalities, and X monosomy was 31.0% (14/45), 15.5% (7/45), and 53.3% (24/45), respectively. The means of MLPA X probe ratios grouped by karyotype results are shown in Table 2. Noteworthy, X monosomy showed mean ratios <0.6 for all probes, X structural abnormalities showed mean ratios <0.6 on Xp probes and >1.2 on Xq probes, and mosaicisms presented high variability in the means of X probe ratios, but with means always <0.76 (Table 2). We then used this ratio cutoff in the short-stature population. Among these patients, MLPA results were suggestive of X aneuploidies or X structural abnormalities in 10 cases. Karyotype analysis confirmed the diagnosis of TS in 2 pa-

Table 2. Karyotype results and mean MLPA X probe ratios of TS-Group-patients

Karyotype results	Patient	MLPA probes	Mean (SD)	Range
X monosomy	24	[Xp [ARX-4]	0.52 (0.037)	0.12–0.58
		[Xp [RPS6KA3-3]	0.58 (0.057)	0.49–0.71
		[Xp [DMD-69]	0.53 (0.060)	0.37–0.59
		[Xp [TSPAN7-5]	0.52 (0.094)	0.15–0.62
		[Xq [AR-1]	0.56 (0.032)	0.52–0.65
		[Xq [ACSL4-17]	0.55 (0.055)	0.39–0.63
		[Xq [AIFM1-15]	0.52 (0.030)	0.46–0.58
		[Xq [L1CAM-4]	0.55 (0.030)	0.48–0.62
X structural abnormalities	7	[Xp [ARX-4]	0.53 (0.066)	0.43–0.64
		[Xp [RPS6KA3-3]	0.56 (0.097)	0.45–0.75
		[Xp [DMD-69]	0.51 (0.054)	0.39–0.56
		[Xp [TSPAN7-5]	0.59 (0.230)	0.42–1.15
		[Xq [AR-1]	1.34 (0.316)	0.96–1.94
		[Xq [ACSL4-17]	1.47 (0.520)	1.05–2.68
		[Xq [AIFM1-15]	1.27 (0.105)	1.15–1.49
		[Xq [L1CAM-4]	1.37 (0.208)	1.13–1.82
X mosaicisms	14	[Xp [ARX-4]	0.58 (0.15)	0.12–0.88
		[Xp [RPS6KA3-3]	0.64 (0.14)	0.45–0.99
		[Xp [DMD-69]	0.62 (0.12)	0.44–0.92
		[Xp [TSPAN7-5]	0.62 (0.10)	0.50–0.86
		[Xq [AR-1]	0.70 (0.19)	0.55–1.46
		[Xq [ACSL4-17]	0.69 (0.21)	0.49–1.33
		[Xq [AIFM1-15]	0.70 (0.26)	0.47–1.49
		[Xq [L1CAM-4]	0.72 (0.26)	0.49–1.56

MLPA, multiplex ligation-dependent probe amplification; SD, standard deviation; TS, Turner syndrome.

Table 3. Mean MLPA X probe ratio of true-negative and false-positive Short-Stature-Group patients

	Patient	MLPA probes	Mean (SD)	Range
True negative	66	[Xp [ARX-4]	0.99 (0.039)	0.95–1.12
		[Xp [RPS6KA3-3]	1.03 (0.064)	0.92–1.17
		[Xp [DMD-69]	1.01 (0.054)	0.91–1.11
		[Xp [TSPAN7-5]	1.02 (0.064)	0.90–1.19
		[Xq [AR-1]	1.00 (0.053)	0.89–1.10
		[Xq [ACSL4-17]	1.00 (0.078)	0.78–1.24
		[Xq [AIFM1-15]	1.01 (0.046)	0.92–1.10
		[Xq [L1CAM-4]	1.00 (0.061)	0.91–1.13
False positive	8	[Xp [ARX-4]	1.05 (0.10)	0.93–1.23
		[Xp [RPS6KA3-3]	0.97 (0.19)	0.67–1.22
		[Xp [DMD-69]	1.05 (0.11)	0.86–1.23
		[Xp [TSPAN7-5]	1.09 (0.18)	0.77–1.34
		[Xq [AR-1]	0.77 (0.31)	0.10–1.08
		[Xq [ACSL4-17]	0.88 (0.28)	0.42–1.36
		[Xq [AIFM1-15]	0.99 (0.17)	0.58–1.17
		[Xq [L1CAM-4]	0.85 (0.20)	0.35–1.04

MLPA, multiplex ligation-dependent probe amplification; SD, standard deviation; TS, Turner syndrome.

tients, and then 8 patients showed a false-positive MLPA analysis. The mean of the MLPA X probe ratios of patients found true negative using MLPA analysis was about 1.0 for all probes (Table 3). MPLA did not miss any TS diagnoses, and therefore the detection rate was 100%. New aneuploidies identified by MLPA included the following karyotypes: 45,X[11]/46,XX[49] and mos46,X,i(X)(q10)[35]/45,X[15]. Evaluating together the TS and Short-Stature Groups, the ratio of 0.76 in at least 1 MLPA probe gave a sensitivity and specificity of 100% (CI 95%, 0.92–1) and 88.89% (CI 95%, 0.79–0.94), respectively. The positive predictive value was 88.5%, and the negative predictive value was 100%.

Discussion

Short stature is one of the most common reasons for referral to a paediatric endocrinologist. In the United States, about 2.2 million children under the age of 18 have heights below the 3rd percentile [22]. Although the majority will be healthy, a minority may be affected by a wide range of pathologies ranging from celiac disease to genetic disorders, requiring costly diagnostic tests. The low incidence of pathology detection coupled with the high cost of testing therefore makes correct screening of asymptomatic short children a matter of debate [23]. In addition to typical TS dysmorphisms, pubertal delay or ovarian failure, the presence of unexplained growth failure, short stature with growth velocity <10th percentile for age, and the distance between height SDS and target height SDS of 1.6–2.0 SDS in female patients are indications for karyotype analysis [4, 24, 25]. Since the prevalence of TS among female children with short stature is 1 out of 50–100, for every new TS diagnosis about 49–99 negative karyotype analyses are performed, with a significant impact in terms of economic costs.

Karyotyping is considered the gold standard to detect a range of numerical and structural chromosomal abnormalities with high accuracy (99.4–99.9%). It is, however, time-consuming, labour-intensive, and therefore very costly. A standard 30-cell karyotype analysis is recommended to rule out sex chromosome mosaicisms [26]. Although this test can identify 10% mosaicism with a confidence level of 95%, achieving a more sensitive detection level requires analysis of many metaphase cells, with a further increase in costs [26]. In recent years, DNA analysis techniques, such as quantitative fluorescence PCR or array comparative genomic hybridization, have become available for aneuploidy detection of the most common

chromosomal abnormalities (aneuploidies of chromosomes 13, 18, 21, X, and Y). MLPA is a rapid high-throughput technique shown to be robust, cost-effective, and flexible for DNA deletion and duplication but also for the detection of aneuploidies [27]. Specifically, the P095 MLPA probe set was designed to detect aneuploidies of chromosomes 13, 18, 21, X, and Y. We used this probe set in the diagnosis of TS as it provides comparable accuracy at lower costs [11, 16]. Compared to quantitative fluorescence PCR, which is able to find only copy number variations, MLPA has the advantage of also detecting structural changes [28]. This is crucial in TS diagnosis as partial X chromosome imbalances and mosaicisms may go undetected using molecular tests.

Compared to karyotyping and FISH, MLPA is less labour-intensive as it requires neither cell cultures nor skilled operators for microscopic analysis and metaphase counting [29]. The entire analysis can be performed in as little as 30 h, and can also be better automated. In addition, Y chromosome segments can be detected by MLPA, while karyotyping fails to attribute fragments to chromosomes. Furthermore, MLPA is reproducible, easy to perform, and large numbers of samples can be tested simultaneously [30, 31].

A molecular test able to exclude X aneuploidies or X structural abnormalities could be of particular interest to all paediatric endocrinologists evaluating short stature in younger females. As previously mentioned, early diagnosis has important practical implications in terms of improving outcomes in TS patients [4]. For the first time, we evaluated MLPA as a screening test to detect X aneuploidies and X structural abnormalities. Our results indicate that this technique provides excellent sensitivity (100%) and good specificity (88.89%). Since the main purpose of MLPA is to avoid missing TS diagnoses by giving false-negative test results and to reduce costs by requiring only MLPA-positive patients to undergo karyotype analysis, its relatively low specificity does not represent a limitation. However, false-positive results can raise anxiety in patients and parents. Clinicians should warn patients of the high rate of false-positive MLPA results to minimize apprehension. Interestingly, this technique was also able to identify mosaicism as low as 15% and 45X0/47XXX mosaicisms, and to detect Y chromosome material. Early detection of aberrant Y chromosome by means of MLPA could be useful in directing patients promptly to undergo Y-specific FISH at the same time as karyotype analysis.

We detected 2 new cases of TS in the Short-Stature Group, translating to a prevalence of 2.7%. This finding is in line with data available in the literature, which describes the prevalence of TS as about 1 case out of 50–100 short-stature female patients [1, 12].

From an economic perspective, a recent study reported that the short-term cost of MLPA testing was slightly under half that of karyotype analysis [28]. We estimate that evaluating a short stature female population by performing first-line MLPA with second-line karyotype testing instead of first-line karyotyping may achieve a saving of about 60%.

A further advantage of MLPA analysis is that it can be tested on saliva or other easily collectable specimens without recourse to invasive procedures.

Conclusions

This study is only a preliminary evaluation of the use of a standard MLPA protocol in detecting X aneuploidies and X structural abnormalities in females with short stature. Our encouraging findings could be improved by customizing the probe set for TS alone. Additional investigations will be necessary to confirm our results before MLPA can be used in routine clinical practice. Furthermore, in cases where the negative results of MLPA appear to be discordant with the clinical picture, karyotype analysis is advisable. Karyotyping still remains the diagnostic gold standard of TS and is required to confirm and refine a diagnosis.

In conclusion, MLPA may become a valid diagnostic tool for the screening of X aneuploidies and X structural abnormalities in girls of short stature.

Disclosure Statement

The authors have no conflicts of interest to disclose.

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