Multiplex ligation-dependent probe amplification (MLPA) genetic testing in the diagnostics of children with developmental delay/intellectual disabilities

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Summary

Developmental delay/intellectual disability (DD/ID) affects 1%-3% of the children population. Specific genetic factors are the most common cause of DD/ID. Results of the new molecular karyotyping techniques indicate a high incidence of submicroscopic rearrangements in patients with DD/ID. The implementation of Multiplex Ligation-Dependent Probe Amplification (MLPA) has enabled analysis of microdeletion/microduplication syndromes and subtelomeric chromosome aberrations. MLPA follow-up probe mix for specific subtelomeric regions and microdeletion syndromes has proven to be suitable for confirmation and better characterization of selected aberrations for better diagnostic and prognostic information on the patient.

Key words: intellectual disabilities, child, chromosome aberrations, genetic testing

INTRODUCTION

Developmental delay/intellectual disabilities (DD/ID) are present in 1%-3% of the population; while as many as 5%-10% of children suffer retardation in one or two developmental areas including gross/fine motor skills, speech development, cognitive development, social skill development, and abilities of inclusion in daily life and activities. The term 'developmental delay' is usually used for children younger than 5 years because intelligence cannot be objectively tested in this age, thus precluding reliable diagnosis of ID. There are numerous causes of developmental disorders; however, more than half of DD/ID are believed to be due to genetic factors (1). Therefore, genetic evaluation of these patients is of utmost importance, whereby the use techniques testing genetic aberrations play a major role. The development and availability of multiplex ligation-dependent probe amplification (MLPA) and array based comparative genomic hybridization (arrayCGH) techniques for the accurate assessment of copy number changes at multiple loci has provided a better approach for subtelomere and microdeletions testing in routine settings (2). These methods have been gradually replacing G-banded karyotyping and fluorescence *in situ* hybridization (FISH) studies using probes targeted to the subtelomeres or known microdeletion loci. As the choice of diagnostic work-flow depends on the availability of techniques at laboratories, the MLPA method appears to be more available to modest laboratories because of its low cost and simpler result processing.

The telomeric regions are rich in repeat sequences that are homologous among different chromosomes, thus increasing the risk of erroneous matching during meiotic division. As subtelomere regions are very rich in genes, their restructurings generally entail more severe phenotypic consequences. In 1992, Ledbetter was the first to point to the need of investigating the chromosomal telomeric ends in ID patients (3). A number of screening methods investigating restructurings in subtelomeric regions have been presented to date, e.g., fluorescent *in situ* hybridization (FISH), microsatellite analysis, high-resolution CGH, MLPA and array CGH (4-8). The prevalence of submicroscopic disorders of the subtelomeric regions detected by MLPA ranges from 1.8% to 15%, depending on the patient selection criteria. The latest studies indicate the need of testing of all patients with DD/ID irrespective of the presence of dysmorphic features and congenital anomalies (9). Microdeletion syndromes have been reported to occur in approximately 5% of patients with unexplained DD/ID (10). Since microdeletions are not visible by conventional karyotyping, FISH has been

used as a complementary cytogenetic method. FISH was for years the method of choice in the diagnosis of known microdeletion syndromes (11). The improving resolution of molecular cytogenetic techniques has enabled detection of a number of new microdeletion and microduplication syndromes in the same chromosomal regions (12).

In the present study, screening for subtelomeres and microdeletion/microduplication syndromes by MLPA was performed to determine the prevalence of submicroscopic chromosomal aberrations in DD/ID patients with or without dysmorphic features (DF) and/or congenital malformations (CA).

PATIENTS AND METHODS

This prospective study includes three hundred patients referred because of DD/ID. All patients were evaluated by clinical geneticist and had a normal routine karyotype (450-500 band level resolution). Patients were not additionally clinically selected/subdivided on the basis the presence of dysmorphic features, CA or severity of the DD/ID, thus enabling a broad screening regardless of the level of ID or the presence of associated abnormalities.

Two distinct MLPA kits (SALSA P036 and SALSA P070) designed for detection of deletions/duplications of subtelomeric regions of all chromosomes were used for subtelomere screening. Both MLPA kits contain 46 probes (target regions for acrocentric chromosomes are located in the proximal segment of the -q arm) with different binding sites in subtelomeric regions. The patients with normal MLPA findings of subtelomere screening underwent testing for microdeletions using the MLPA SALSA P245 kit. The MLPA was carried out according to the manufacturer's instructions (MRC Holland, Amsterdam, The Netherlands). Amplification products were identified and quantified by capillary electrophoresis on an ABI 310 genetic analyzer, using GenMapper, vs 4.0 (Applied Biosistems, Foster City, CA, USA).

When an aberration was found, confirmatory testing was performed with follow-up MLPA kits with more probes in specific chromosomal region, which are able to confirm and specify the abnormalities in more detail. In addition, high-resolution G-banded karyotyping studies at a >700-band level were performed in all patients with newly discovered chromosomal imbalances.

RESULTS

Screening for subtelomere aberrations and microdeletion syndromes using MLPA technique was performed in 300 patients with unexplained DD/ID with or without dysmorphic features or congenital anomalies. Forty-three aberrations were detected (43/300, 14.3%), including 23 (23/300, 7.6%) subtelomeric aberrations (Table 1) and 20 (20/300, 6.6%) microdeletion/microduplication syndromes (Table 2). Subtelomere imbalances were detected in 20 patients by both probe sets, while in two patients (T7, T11) the imbalances were detected only by P036 set and in patient T21 the imbalance dup9p was detected only by P070 set. In this patient SALSA P036 showed del22qsubtel and P070 analysis detected dup9psubtel and del22qsubtel. MLPA follow-up set for 9p (P230) showed terminal duplication that included only probe from SALSA P070 set. In patient T7, MLPA P036 revealed a *de novo* 15q subtelomere deletion. Subsequent analysis with P070 showed normal result. The patient presented with severe short stature which is in agreement with the haploinsufficiency of *IGF1R* gene located in the 15q subtelomere region. The ambiguous result was resolved by further analysis of healthy parents with P070. This revealed maternal polymorphism (dup 15qsubtel, duplicated signal for *TM2D3* gene) resulting in the false normal MLPA SALSA P070 signal in the patient. Follow-up MLPA with SALSA P291 additionally confirmed the presence of del 15qsubtel. In patient T11 MLPA SALSA P036 detected dup 9psubtel, but P070 analysis was normal. MLPA follow-up set for 9p (P230) showed interstitial duplication that included only probe from SALSA P036 set. In one patient (T23) with 9p subtelomere deletion follow-up set P249 revealed proximal duplication. In three patients (T12, T13, T14), the same abnormality was detected in the respective affected parent. Using P245 kit we have additionally found 20 (6.6%) microdeletions/microduplications: eight 22q11.2 deletions and one duplication 22q11.2, two del5q35.3, del7q11.2, del8q24.22, del15q24.22 and 17q21.31 microdeletions, and one del17q11.2 (NF1).

Table 1. Overview of the detected abnormalities found in patients with subtelomeric MLPA

Patient	Age	Gender	DF	CA	P036	P070	Follow-up MLPA
T1	12y	F	+	-	del1p	del1p	P147
T2	2y	F	+	+	del4p	del4p	P096
T3	6y7m	M	+	-	del8p	del8p	P249
T4	1y7m	M	+	+	del9q	del9q	P286
T5	7y 3m	F	+	+	del14q	del14q	P291
T6	3y5m	F	+	+	del15q	del15q	P291
T7	2y1m	F	+	+	del15q	x2	P291
T8	2y2m	F	+	+	del20p	del20p	P249
T9	3y	F	+	+	del22q	del22q	P188
T10	8y	F	+	-	delXp	delXp	P018
T11	4y2m	M	+	-	dup9p	x2	P230*
T12	3y11m	F	+	-	dupX/Yp	dupX/Yp mat	P018*
T13	1y4m	M	+	-	dupX/Yp	dupX/Yp mat	P018*
T14	2y4m	M	+	-	dupX/Yp	dupX/Yp pat	P018
T15	1y6m	M	+	+	dupXq/del11q	dupX/del11q	P015/P286
T16	11y9m	F	+	+	dup3p/del18q	dup3p/del18q	P208/P320
T17	9y4m	F	+	-	dup4p/del8p	dup4p/del8p	P358/P249
T18	4y2m	M	+	-	dup4p/del8p	dup4p/del8p	P358/P249
T19	6y9m	M	+	+	del8p/dup12p	del8p/dup12p	P249/P230
T20	2y4m	M	+	+	dup8p/del18q	dup8p/del18q	P208/P320
T21	6y5m	M	+	+	del22q	dup9p/del22q	P230/P356
T22	7y11m	F	+	+	del12p/dup22q	del12p/dup22q	P230/P188
T23	13y4m	F	+	+	del19p	del19p	P249**

DF -dysmorphic features, CA -congenital anomalies, M -male, F -female.

* Intrachromosomal duplication

** Terminal deletion and intrachromosomal duplication

Table 2. Overview of the detected abnormalities found in patients with microdeletion/microduplication.

Patient	Age	Gender	DF	CA	P036	P070	P245	Follow-up MLPA
M1	4y	F	+	+	x2	x2	del22q11	P250 del 14 probes at 22q11.23 [a]
M2	1y1m	F	+	+	x2	x2	del22q11	P250 del 14 probes at 22q11.23 [a]
M3	1y10m	F	+	+	x2	x2	del22q11	P250 del 14 probes at 22q11.23 [a]
M4	5y5m	F	+	+	x2	x2	del22q11	P250 del 14 probes at 22q11.23 [a]
M5	11m	M	+	+	x2	x2	del22q11	P250 del 14 probes at 22q11.23 [a]
M6	6y10m	M	+	+	x2	x2	del22q11	P250 del 14 probes at 22q11.23 [a]
M7	6y11m	M	+	+	x2	x2	del22q11	P250 del 14 probes at 22q11.23 [a]
M8	2y6m	M	+	+	x2	x2	del22q11	P250 del 5 probes at 22q11.23 [aa]
M9	2y2m	F	+	+	x2	x2	dup22q11	P250 dup 14 probes at 22q11.23 [a]
M10	15y6m	F	+	+	x2	x2	del17q21.31	P371 del 8 probes at 17q21.31 [b]
M11	3m	M	+	+	x2	x2	del17q21.31	P371 del 8 probes at 17q21.31 [b]
M12	5y	M	+	+	x2	x2	del17q11.2	P372 del 10 probes at 17q11.2 [c]
M13	17y2m	F	+	-	del15q11	del15q11	del15q11	P374 del 10 probes at 15q11.2 [d]
M14	3y7m	F	+	-	del15q11	del15q11	del15q11	P374 del 10 probes at 15q11.2 [d]
M15	11y2m	M	+	-	x2	x2	del8q24.1	P371 del 10 probes at 8q24.1 [e]
M16	16y2m	M	+	-	x2	x2	del8q24.1	P371 del 5 probes at 8q24.1 [ee]
M17	2y2m	F	+	+	x2	x2	del7q11.23	P374 del 10 probes at 7q11.23 [f]
M18	1y3m	F	+	+	x2	x2	del7q11.23	P374 del 10 probes at 7q11.23 [f]
M19	3y11m	M	+	+	x2	x2	del5q35.3	P372 del 8 probes at 5q35.3 [g]
M20	3y9m	F	+	+	x2	x2	del5q35.3	P372 del 8 probes at 5q35.3 [g]

DF -dysmorphic features, CA -congenital anomalies, M -male, F –female, LCR –low copy repeat

[a] *Breakpoint between LCR-A i LCR-D, commonly deleted DiGeorge region.*

[aa] *Breakpoint between LCR-B i LCR-D.*

[b] *Deletion of the CRHR1 and MAPT genes.*

[c] *Deletion of the NF1 gene*

[d] *Deletion of the SNRPN and UBE3A genes.*

[e] *Deletion of the TRPS1, EIF3H and EXT1 genes.*

[ee] *Deletion of the EIF3H and EXT1 genes.*

[f] *Deletion of the FZD9, STX1A, ELN, LIMK1, RFC2 AND CLIP2 genes.*

[g] *Deletion of FGFR4 and NSD1 genes.*

All imbalances were verified by follow-up MLPA kits. In 18 (18/23, 78.2%) subtelomere cases and in all nine patients with 22q11.2 aberrations, the follow-up MLPA kits determined approximate size of aberration. In five (21%) subtelomere cases all probes indicated an abnormality (deletion or duplication). In three patients with subtelomere imbalances high-resolution G-banding analysis (>850 band level resolution) confirmed subtelomeric aberration. Balanced translocation was detected in two patients (T16 and T20) and in one of their parents.

DISCUSSION

In this study, MLPA was used to search for subtelomere and microdeletion syndromes in patients with DD/ID and with or without dysmorphic features and/or congenital malformations and with normal routine karyotype at 450-500 band level resolution. Forty-three (43/300, 14.3%) aberrations were detected, including aberrations of the chromosomal subtelomeric regions in 23 (23/300, 7.6%) and microdeletions in 20 (20/300, 6.6%) patients. Studies of unselected patients with DD/ID and normal karyotype using different strategy of MLPA subtelomere screening have identified constitutional imbalances in 2.9-5.3% of patients. These studies have used only one MLPA set (13-15), or a combination of two sets (6,16,17). Our study yielded a higher proportion of positive results, suggesting good efficiency of the protocol employed. There is no generally accepted diagnostic protocol, but the simultaneous use of two distinct kits of subtelomeric primer sets as a mean of independent confirmation is recommended by the manufacturer and now commonly accepted as a standard procedure. In our study subtelomeric imbalances were found in 23 (7.6%) patients. The higher incidence of subtelomere aberrations illustrates the efficiency of using two subtelomere MLPA kits in all patients and evaluating further aberrant results with follow-up MLPA kits. Previous studies used FISH as the confirmation method. The shortcoming of this technique is that small duplications cannot be detected and, in addition, the target site of the commercial probes may be located more distally or proximally to the site of aberration, as suggested previously (6,16). In the present study, follow-up kits with more probes in specific chromosomal region were used for confirmation and further delineation of chromosome imbalances. The 23 subtelomeric aberrations detected included 10 terminal deletions (10/23, 43.3%), three interstitial duplications (3/23, 13%), eight unbalanced translocations (8/23, 34.7%), one terminal duplication (1/23, 4.3%) and one deletion and duplication (1/23, 4.3%). These results show an even proportion of deletions and unbalanced translocations, along with a considerable number of duplications detected by MLPA, whereas the studies of subtelomere screening by the FISH technique report on a much lower number of duplications detected (18). In addition, in one patient (T23) with distal subtelomere deletion at 19p, chromosome specific MLPA SALSA P249 revealed proximal duplication. This approach was quite effective as we were able to confirm (T11-T14) or detect (T23) duplications that would probably be missed by FISH.

In our study, we were able to determine the approximate size of aberration in 14 (14/23, 60%) patients, whereas in nine patients the aberration exceeded the area tested by the kit used. In four (4/23, 17.4%) patients, the lesion size was more precisely determined by high-resolution karyotyping at >800 band level. These results indicate that chromosome abnormalities up to 3 Mb in size can be detected by high-resolution karyotyping, but genome imbalance of 5-10 Mb can frequently be missed on routine karyotyping, depending on the area involved and/or resolution quality. Other studies also point to the fact that even greater structural changes may be missed on routine chromosome analysis (19,20). In the study by Stegmann *et al*., deletions of 3 Mb to even 14 Mb were confirmed by subsequent cytogenetic analysis in three (3/18, 16.6%) patients (17). Our results confirm the observation by Jehe et al. that, although confirmatory tests using other technologies such as FISH or array-CGH are welcomed, testing with MLPA kits in combination with high resolution karyotyping and/or revision of clinical findings is in most cases sufficient for establishing the diagnosis (21).

To the best of our knowledge, there are only two literature reports on subtelomere and microdeletion screening by the MLPA method (10,21). Kirchhoff *et al*. tested 258 ID patients with dysmorphism and normal karyotype. They detected 10.1% of aberrations, in 13 (13/258, 5%) patients by subtelomere screening and in 15 (15/258, 5.8%) patients by microdeletion screening. There were 5 microduplications and 10 microdeletions (10). Jeheej *et al*. tested 261 patients with multiple congenital malformations with or without ID and found 16% (43/261) of aberrations. Subtelomeric aberrations were detected in 7.3% (19/261) of patients, whereas screening for microdeletions revealed aberrations in 24 of 261 (9.2%) patients, including three microduplications and 21 microdeletions (21). Total, 10.1%-16% of aberrations have been detected by MLPA screening for subtelomere and microdeletion syndromes. Variation in the results reported could be explained by different structure of the study populations. The larger proportion of microdeletions were detected in the study where all patients had congenital malformations with or without ID, and the least part in the study of patients with ID and dysmorphic features (10,21). The incidence of common microdeletion/microduplication syndromes in our study was 6.6%. All microdeletions were confirmed by the appropriate follow-up MLPA kits. In the present study del 22q11.21 was the most frequent abnormality, found in 2.6% of patients and representing

one fourth of all detected aberrations. This is in agreement with other studies where del 22q11.21 is also the most frequent aberration found in 1.5% to 7% of patients (10,21).

In conclusion, we have presented the results of the investigation of patients with DD/ID obtained by using a combination of the MLPA sets for subtelomere aberrations and microdeletion syndromes, followed by the confirmation of the aberrant results by the region-specific MLPA kits. With the use of MLPA as the only molecular method and with the relatively simple strategy, we were able to detect clinically relevant aberrations in 14% of unselected patients with DD/ID. The lower cost, simplicity and reliability of MLPA makes her an effective first-tier cytogenetic diagnostic test for screening large cohorts of DD/ID patients and a good alternative option for diagnostic cytogenetic laboratories where arrayCGH is not readily available.

References

1. Barišić I, Marušić Della Marina B. Genetičke osnove razvojnog zaostajanja/mentalne retardacije. Paediatr Croat. 2007;51:191-200.
2. Miller DT, Adam MP, Aradhya S et al. Consensus statement: chromosomal microarray is a first-tier clinical diagnostic test for individuals with developmental disabilities or congenital anomalies. Am J Hum Genet. 2010;86:749-64.
3. Ledbetter DH, Minireview. Cryptic translocations and telomere integrity. Am J Hum Genet. 1992;51:451-6.
4. Petković I, Barišić I. Probir subtelomernih regija metodom fluorescentne in situ hibridizacije (FISH) u 31-og bolesnika sa smetnjama u razvoju. Paediatr Croat. 2004;48:187-9.
5. Rosenberg MJ, Vaske D, Killoran CE et al. Detection of chromosomal aberrations by a whole-genome microsatellite screen. Am J Hum Genet. 2000;66:419-27.
6. Kirchhoff M, Gerdes T, Brunebjerg S et al. Investigation of patients with mental retardation and dysmorphic features using comparative genomic hybridization and subtelomeric multiplex ligation dependent probe amplification. Am J Med Genet A. 2005;139:231-3.
7. Morozin Pohovski L, Dumic KK, Odak Lj, Barisic I. Muliplex ligation-dependent probe amplification workflow for the detection of submicroscopic chromosomal abnormalities in patients with developmental delay/intellectual disability. Mol Cytogenet. 2013;6(1):7
8. Shao L, Shaw CA, Lu XY et al. Identification of chromosome abnormalities in subtelomeric regions by microarray analysis: a study of 5,380 cases. Am J Med Genet A. 2008;146A:2242-51.
9. van Karnebeek CD, Jansweijer MC, Leenders AG et al. Diagnostic investigations in individuals with mental retardation: a systematic literature review of their usefulness. Eur J Hum Genet. 2005;13:6-25.
10. Kirchhoff M, Bisgaard AM, Bryndorf T et al. MLPA analysis for a panel of syndromes with mental retardation reveals imbalances in 5.8% of patients with mental retardation and dysmorphic features, including duplications of the Sotos syndrome and Williams-Beuren syndrome regions. Eur J Med Genet. 2007;50:33-42.
11. Trask BJ. Fluorescence in situ hybridization: applications in cytogenetics and gene mapping. Trends Genet. 1991;7:149-54.
12. Shaffer LG, Bejjani BA, Torchia B et al. The identification of microdeletion syndromes and other chromosome abnormalities: cytogenetic methods of the past, new technologies for the future. Am J Med Genet C. 2007;145:335–45.
13. Koolen DA, Nillesen WM, Versteeg MH et al. Screening for subtelomeric rearrangements in 210 patients with unexplained mental retardation using multiplex ligation dependent probe amplification (MLPA). J Med Genet. 2004;41:892-9.
14. Rooms L, Reyniers E, van Luijk R et al. Subtelomeric deletions detected in patients with idiopathic mental retardation using multiplex ligation-dependent probe amplification (MLPA). Hum Mutat. 2004;23:17-21.
15. Mandal K, Mandal K, Boggula VR et al: Use of Multiplex Ligation-Dependent Probe Amplification (MLPA) in screening of subtelomeric regions in children with idiopathic mental retardation. Indian J Pediatr. 2009, 76(10):1027-1031.
16. Rooms L, Reyniers E, Wuyts W et al. Multiplex ligation-dependent probe amplification to detect subtelomeric rearrangements in routine diagnostics. Clin Genet. 2006; 69:58-64.
17. Stegmann AP, Jonker LM, Engelen JJ. Prospective screening of patients with unexplained mental retardation using subtelomeric MLPA strongly increases the detection rate of cryptic unbalanced chromosomal rearrangements. Eur J Med Genet. 2008;51:93-105.
18. Ravnan JB, Tepperberg JH, Papenhausen P et al. Subtelomere FISH analysis of 11 688 cases: an evaluation of the frequency and pattern of subtelomere rearrangements in individuals with developmental disabilities. J Med Genet. 2006;43:478-89.
19. Bocian E, Hélias-Rodzewicz Z, Suchenek K et al. Subtelomeric rearrangements: results from FISH studies in 84 families with idiopathic mental retardation. Med Sci Monit. 2004;10:143-51.
20. Kriek M, White SJ, Bouma MC et al. Genomic imbalances in mental retardation. J Med Genet. 2004;41:249-55.
21. Jehee FS, Takamori JT, Medeiros PF et al. Using a combination of MLPA kits to detect chromosomal imbalances in patients with multiple congenital anomalies and mental retardation is a valuable choice for developing countries. Eur J Med Genet. 2011;54:425-32.