Advanced genome-wide screening in human genomic disorders

Knijnenburg, J.

Chapter 4

Diagnosis of genetic abnormalities in developmentally delayed patients: A new strategy combining MLPA and array-CGH.

published in Am J Med Genet A (2007);143(6):610-4.

Diagnosis of Genetic Abnormalities in Developmentally Delayed Patients:
A New Strategy Combining MLPA and Array-CGH

Marjolein Kriek,[1*] Jeroen Knijnenburg,[2] Stefan J. White,[1] Carla Rosenberg,[2,3] Johan T. den Dunnen,[1] Gert-Jan B. van Ommen,[1] Hans J. Tanke,[2] Martijn H. Breuning,[1] and Károly Szuhai[2]

[1]Center for Human and Clinical Genetics, Leiden University Medical Center, RC Leiden, The Netherlands [2]Department Molecular Cell Biology, Leiden University Medical Center, RC Leiden, The Netherlands [3]Department of Genetics and Evolutionary Biology, Institute of Biosciences, University of Saõ Paulo, Saõ Paulo, Brazil

*Correspondence to: Marjolein Kriek, M.D., Center for Human and Clinical Genetics, Leiden University Medical Center, Albinusdreef 2, Leiden, The Netherlands. E-mail: M.Kriek@lumc.nl

Marjolein Kriek and Jeroen Knijnenburg contributed equally to this work.

To the Editor:

Developmental delay (DD) affects ~3% of the general population and the underlying cause remains unknown in about half of the cases. G-banded karyotyping is the most common approach for the detection of genomic alterations, however, despite its indisputable success, this tool has limited resolution, usually being unable to detect genomic changes ~3–5 Mb. It is known that micro alterations that escape detection by classical cytogenetics contribute substantially to the etiology of DD [Flint et al., 1995; Vissers et al., 2003]. This limitation has been partly overcome by fluorescence in situ hybridization (FISH) with a resolution of 5–500 kb, however, it has a limited possibility for multiplexing, for example, in most of the routine practice only 2–3 regions can be analyzed simultaneously. Therefore, candidate probes (especially for microdeletion syndromes) need to be selected a priori for FISH investigation, based on the patient's phenotype.

Recent technological developments, such as array-based comparative genomic hybridization (array-CGH) [Pinkel et al., 1998; Antonarakis, 2001; Snijders et al., 2001] and Multiplex Ligation-dependent Probe Amplification (MLPA) [Schouten et al., 2002], are efficient methods for screening for copy number imbalances in multiple genomic regions simultaneously. MLPA especially has already found its way into the diagnostic laboratories for several indications (e.g., BRCAI gene and NFI gene screening); however, the standard of practice for the assessment of

developmental delay does not currently include MLPA and array-CGH testing. In this article, it is argued that both techniques are extremely valuable tools for the diagnostic setting in DD patients, and the implementation of both techniques should be considered.

Data regarding the robustness of both techniques have been provided previously [Price et al., 2005; Rooms et al., 2005]. In the case of array- CGH, thousands of sites can be simultaneously investigated in one patient, allowing partial or total coverage of the genome. The number of targets that can be screened by MLPA is limited to <60 loci per assay, however, 96 samples can be simultaneously tested at a cost less than one array-CGH hybridization. As MLPA analysis requires relatively little hands-on time (Table I), it is more suitable for the initial screening of large patient numbers.

To assess their value in clinical diagnosis, we have independently tested 58 developmentally delayed (DD) patients using both array-CGH and MLPA. This study was reviewed and approved by the Institutional Review Board of the Leiden University Medical Center, conforming to Dutch law and the World Medical Association Declaration of Helsinki. The patients had, in addition to DD, either dysmorphic features or congenital malformations or both (DD ''plus'' patients). All patients had a normal karyotype and, where tested (the vast majority of the patients), had tested negative for Fragile X syndrome. The array-CGH results were partly reported elsewhere [Rosenberg et al., 2006] without the comparative analysis with MLPA.

The array used in the study contained ~3,500 large genomic insert clones spaced at ~1Mb intervals over the genome, meaning that the resolution of the arrays used is 0.3–3 Mb. Array-CGH testing was performed as described by [Knijnenburg et al., 2005]. The clones were provided by the Wellcome Trust Sanger Institute (UK), and information regarding the full set is available at the Ensemble web site.

The MLPA probe design and assay was performed as described previously [White et al., 2004]. It included a set of synthetic probes designed for 71 regions known to be frequently altered in DD patients (probe sequences are available on request). This set targets 42 chromosome ends (except for the p-arms of the acrocentric chromosomes), five pericentromeric regions on the q-arm of acrocentric chromosomes (the regions tested included the first gene-specific unique sequence near the centromere on the q-arm) and 24 probes (Table II) containing microdeletion syndrome-related sequences. The size of the probes used was between 75 and 125 bp, and the number of sites investigated by MLPA corresponds to ~2% (71/3,500) of all regions tested by array-CGH.

Seventeen alterations were detected by array-CGH analysis, of which 14 were verified using either FISH or MLPA (14/58=24%). (The MLPA probes were specifically designed for confirming these alterations. They were not part of the screening set.) As far as was tested the remaining three changes could not be confirmed using FISH or MLPA.

MLPA analysis identified eight alterations, all of which were confirmed by FISH, MAPH or sequencing (8/58=14%). Table III provides an overview of the alterations found. The eight alterations found solely by array-CGH were all located in regions not covered by MLPA probes. In contrast, the two alterations detected by MLPA only were too small to be detected by array-CGH analysis. One of these alterations was a point mutation near the ligation site of the MLPA probe, which disturbed the ligation and appeared as a deletion. The point mutation (that was never reported before) has been proven by bi-directional sequencing. It is a silent mutation, and it was also present in one of the parents. Therefore, it was considered to be a single nucleotide polymorphism (SNP). Although all MLPA probes have been designed outside the sequences containing known SNPs, theoretically, a low frequency SNP could be present at or near the ligation site. Therefore, it is necessary to confirm copy number variations by a second MLPA probe covering an adjacent sequence or by sequencing.
Of the eight alterations detected by MLPA, we considered six to be probably causative as the phenotype of the patients agreed with the clinical features described in literature for those chromosome alterations. All these rearrangements were also detected by array-CGH. In two of these six cases, however, we could not confirm that the rearrangement was de novo. Two of the eight alterations detected by MLPA are likely to be polymorphic variants, as they are also present in unaffected family members.

TABLE I. A Comparison of the Man-Hours and Material Required for Both Karyotyping and MLPA Analysis

	Karyotyping	*MLPA*
Number of samples performed per week	12	5x96 wells plate
Total time before result per sample	32–40 hr	8 hr[a]
Materials needed	Cell culture, reagents	DNA reagents, probe set

This table shows that MLPA is suitable for the screening of copy number variations in a large number of patients within relatively short time. Compared to karyotyping, this technique is much faster and requires less hands-on time. As it is also possible to analyze a part of a fragment run or use a DNA sequencer with less throughput capacity, it is not necessary to wait for 96 patient samples requiring MLPA testing.
[a]Recently, it was shown that MLPA analysis can be performed within 8 hr (Kalf et al. in preparation).

Nine of the fourteen confirmed rearrangements detected by array-CGH are probably pathogenic, four alterations might be polymorphic variants as they are present in unaffected family members. The clinical consequences of the remaining alteration are currently unknown, because the patients' parents were unavailable for testing. This latest FISH confirmed array-CGH finding which was not detected by MLPA, was located near the chromosome end of the long arm of chromosome 10. The corresponding ''subtelomeric'' MLPA probe in our study mapped proximal to the altered BAC.

TABLE II. Overview of the Microdeletion Syndrome Related Probes Used by MLPA Screening

Disorder	Chromosome band	Gene
Alagille syndrome	20p12.2	JAG1
Angelman syndrome	15q12	UBE3A
Cat eye syndrome	22q11.1	CECR2
DiGeorge syndrome	22q11.2	DGCR2
DiGeorge syndrome	22q11.2	HIRA
DiGeorge syndrome	22q11.2	TBX1
DiGeorge syndrome	22q11.2	UFD1L
DiGeorge syndrome like region	10p14	CUGBP2
Extostosis	8q24	EXT1
Jacobsen syndrome	11q25	HNT
Miller–Dieker syndrome	17p13.3	LIS 1
Mowat–Wilson syndrome	2q22	SIP1
Prader–Willi syndrome	15q12	SNRPN
RETT syndrome	Xq28	MECP2
Rubinstein–Taybi syndrome	16p13,3	CBP
Smith–Magenis syndrome	17p11.2	RAI1
Smith–Magenis syndrome	17p11.2	COPS3
Smith–Magenis syndrome	17p11.2	DRG2
Sotos syndrome	5q35	NSD1
Trichorhinophalangeal syndrome	8q23.3	TRPS1
William–Beuren syndrome	7q11.23	ELN
William–Beuren syndrome	7q11.23	FKBP6
Wolf–Hirschhorn syndrome	4p16.2	MSX1
X-linked hydrocephalus	Xq28	L1CAM

Based on the data on the human genome variation database, the region involved might be polymorphic. Moreover, the clinical features of the patient do not resemble those corresponding with previously described 10q chromosome end alterations [Waggoner et al., 1999]. The sizes of the reported alterations, however, are larger than the one obtained in this study.

The comparison between the screening results for detecting copy number variations using the different approaches shows the reliability and specific strengths of both techniques. In summary, using 2% of the loci tested by array-CGH, MLPA detected 50% (8/16) of all alterations. Three potentially pathogenic alterations were not detected using MLPA, as they were localized outside the regions tested.

Based on the outcome of this parallel screening and costs considerations, we suggest the following strategy for diagnostic purposes: when a patient presents with DD of unclear etiology and the G-banding karyotype is normal, the first screening will use MLPA for the commonly altered regions in DD patients (currently, chromosome ends and microdeletion syndrome-related regions). Subsequently, when MLPA is negative and the patient's phenotype is suggestive of a chromosome abnormality, array-CGH follows.

Alternatively, the order of testing could be reversed. MLPA using subtelomeric probes is capable of detecting trisomies as well as the vast majority of the unbalanced translocations, both of which comprise a substantial part of the alterations diagnosed using cytogenetic tools. Table I shows that MLPA requires

less manpower (hence is cheaper) and is considerably faster compared to karyotyping, and thus, it seems more effective to use MLPA as an initial screening tool. In addition to the time- and cost-effectiveness, MLPA has a much higher resolution for detecting copy number variations compared to karyotyping, and therefore, this technique is capable of detecting copy number variations that remain undiagnosed using this cytogenetic tool. Applying MLPA testing first will even be more effective when a MLPA probe set encompassing the most frequent microdeletion related regions is added. In a diagnostic setting, it is preferable to have at least two MLPA probes per regions of interest (instead of one as was used in this study) to limit false positive and false negative results as much as possible. Implementing microdeletion syndromerelated regions and two probes per region will increase the costs related to MLPA screening, however, this will also reduce the necessity of performing FISH for the detection of microdeletion syndromes, and the need for additional confirmation tests (with the exception of sequencing, see above).

It is obvious that balanced translocations and inversions will not be detected using this or other molecular techniques (unless they are specifically designed to detect breakpoints). Also, for a proportion of the samples with a positive outcome using the initial MLPA screening, subsequent karyotyping is essential for localization of these structural rearrangements. These include, for example, aneusomies for which Robertsonian translocations have to be excluded. Based on these arguments, karyotyping will maintain its essential role in a diagnostic process, however it will only be implemented for selected samples.

TABLE III. Copy Number Variations Detected by Two Techniques Independently

	Only by a-CGH			Only by MLPA			By a-CGH and MLPA			Total
Altered	11			2			6			19
Confirmed	8[a]			2[b]			6			16
	De novo	Present in parents	Unknown	De novo	Present in parents	Unknown	De novo	Present in parents	Unknown	
Deletion	2	3	1[c]	0	1[d]	0	2[c]	0	1[c,e]	10
Duplication	1	1	0	0	1[d]	0	1[c]	0	0	4
del./dup.	0	0	0	0	0	0	0	0	1[d,e]	1
UT	0	0	0	0	0	0	1[c]	0	0	1
Confirmed total	3	4	1	0	2	0	4	0	2	16

An overview of the results obtained by screening of 58 DD patients using array-CGH and MLPA. All rearrangements were not detected by routine karyotyping.

UT, unbalanced translocation.

[a]These regions were not covered by MLPA analysis.

[b]These alterations were too small to be detected by array-CGH.

[c]Alterations localized at the chromosome ends.

[d]Alterations present in regions related to micro-deletion syndromes.

[e](One of) the patient's parents were (was) unavailable for testing. The phenotype of the patient, however, resembles that described in literature. Therefore, this alteration is thought to be pathogenic.

After MLPA testing, additional array-CGH can be performed for patients with a clinical phenotype suggestive for chromosomal alterations. Although this will increase the cost, it will also increase the number of copy number variations detected.

Array-based techniques are evolving rapidly. Several reports have described the results of testing developmentally delayed patients tested using a 3,000-clone array [Vissers et al., 2003; Tyson et al., 2005; Menten et al., 2006; Rosenberg et al., 2006; Shaw-Smith et al., 2006]. In addition, de Vries et al. used an array with 32,000 clones for the detection of copy number variations. Recently, SNPbased arrays have successfully been used to detect genome-wide copy number variations [Friedman et al., 2006]. These type of arrays have an even higher resolution than the array used in de Vries et al. Future comparative studies will help to determine which array platform is the most appropriate to implement.

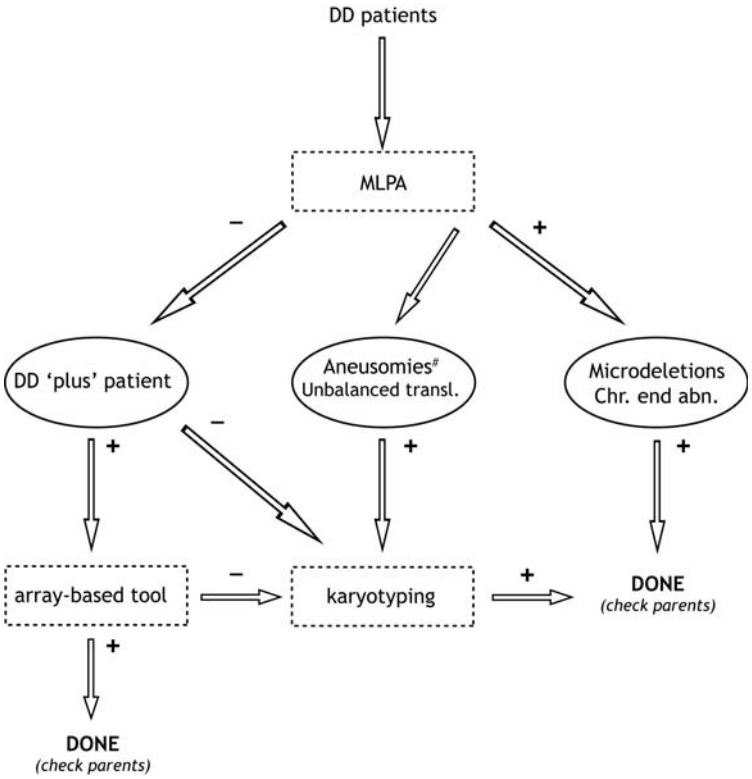

FIG. 1. This flow chart summarizes the alternative diagnostic approach for screening developmentally delayed patient samples. In this approach, karyotyping will only be requested for a selected group of samples: (1) Samples that had tested negative for MLPA (and array-based tool in the case of DD ''plus'' patients). (2) Samples for which information about the location of the structural rearrangement is essential for clinical practice. These include aneusomies for which a Robertsonian translocation should be excluded (acrocentric chromosomes (#)), unbalanced translocations and some of the alterations detected by array-CGH. Chr. end abn.: chromosome end abnormality, DD ''plus'' patients are patients with dysmorphic features and/or congenital malformations in addition to DD. These patients are suggestive for chromosomal imbalances.

In short, the alternative diagnostic approach would include MLPA for DD samples, with subsequent array-based testing (for DD ''plus'' patients that had tested negative for MLPA). Karyotyping could then be used to locate structural rearrangements for selected cases and for samples that showed no alteration using MLPA (and array-CGH) (Fig. 1). In this way, the screening of DD samples will be more effective in relation to the probability of finding a disease-causing rearrangement, which will improve the basis for counseling.

ACKNOWLEDGMENTS

M.K. is funded by Zon-Mw (AGIKO fellowship 940-37-032), S.W. is funded by ZonMw (nr 912-04- 047). C.R. is funded by CNPq. This work was partially supported by the ''Doelmatigheid'' grant from the Leiden University Medical Center (2002/2003). We thank Claudia Ruivenkamp for critically reading the manuscript and implementing the cytogenetics information and we thank the physicians for providing clinical information and patient material.

REFERENCES

Antonarakis SE. 2001. BACking up the promises. Nat Genet 27:230–232.

De Vries BB, Pfundt R, Leisink M, Koolen DA, Vissers LE, Janssen IM, Reijmersdal S, Nillesen WM, Huys EH, Leeuw N, Smeets D, Sistermans EA, Feuth T, Ravenswaaij-Arts CM, van Kessel AG, Schoenmakers EF, Brunner HG, Veltman JA. 2005. Diagnostic genome profiling in mental retardation. Am J Hum Genet 77:606–616.

Flint J, Wilkie AO, Buckle VJ, Winter RM, Holland AJ, McDermid HE. 1995. The detection of subtelomeric chromosomal rearrangements in idiopathic mental retardation. Nat Genet 9:132–140.

Friedman JM, Baross A, Delaney AD, Ally A, Arbour L, Asano J, Bailey DK, Barber S, Birch P, Brown-John M, Cao M, Chan S, Charest DL, Farnoud N, Fernandes N, Flibotte S, Go A, Gibson WT, Holt RA, Jones SJ, Kennedy GC, Krzywinski M, Langlois S, Li HI, McGillivray BC, Nayar T, Pugh TJ, Rajcan-Separovic E, Schein JE, Schnerch A, Siddiqui A, Van Allen MI, Wilson G, Yong SL, Zahir F, Eydoux P, Marra MA. 2006. Oligonucleotide microarray analysis of genomic imbalance in children with mental retardation. Am J Hum Genet 79:500–513.

Knijnenburg J, Szuhai K, Giltay J, Molenaar L, Sloos W, Poot M, Tanke HJ, Rosenberg C. 2005. Insights from genomic microarrays into structural chromosome rearrangements. Am J Med Genet Part A 132A:36–40.

Menten B, Maas N, Thienpont B, Buysse K, Vandesompele J, Melotte C, de Ravel T, Van Vooren S, Balikova I, Backx L, Janssens S, De Paepe A, De Moor B, Moreau Y, Marynen P, Fryns JP, Mortier G, Devriendt K, Speleman F, Vermeesch JR. 2006. Emerging patterns of cryptic chromosomal imbalance in patients with idiopathic mental retardation and multiple congenital anomalies: A new series of 140 patients and review of published reports. J Med Genet 43:625–633.

Pinkel D, Segraves R, Sudar D, Clark S, Poole I, Kowbel D, Collins C, Kuo WL, Chen C, Zhai Y, Dairkee SH, Ljung BM, Gray JW, Albertson DG. 1998. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. Nat Genet 20:207–211.

Price TS, Regan R, Mott R, Hedman A, Honey B, Daniels RJ, Smith L, Greenfield A, Tiganescu A, Buckle V, Ventress N, Ayyub H, Salhan A, Pedraza-Diaz S, Broxholme J, Ragoussis J, Higgs DR, Flint J, Knight SJ. 2005. SW-ARRAY: A dynamic programming solution for the identification of copy-number changes in genomic DNA using array comparative genome hybridization data. Nucleic Acids Res 33:3455–3464.

Rooms L, Reyniers E, Kooy RF. 2005. Subtelomeric rearrangements in the mentally retarded: A comparison of detection methods. Hum Mutat 25:513–524.

Rosenberg C, Knijnenburg J, Bakker E, Vianna-Morgante AM, Sloos W, Otto PA, Kriek M, Hansson K, Krepischi-Santos ACV, Fiegler H, Carter NP, Bijlsma EK, Van Haeringen A, Szuhai K, Tanke HJ. 2006. Array-CGH detection of micro rearrangements in mentally retarded individuals: Clinical significance of imbalances present both in affected children and normal parents. J Med Genet 43:180–186.

Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G. 2002. Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30:e57.

Shaw-Smith C, Pittman AM, Willatt L, Martin H, Rickman L, Gribble S, Curley R, Cumming S, Dunn C, Kalaitzopoulos D, Porter K, Prigmore E, Krepischi-Santos AC, Varela MC, Koiffmann CP, Lees AJ, Rosenberg C, Firth HV, de Silva R, Carter NP. 2006. Microdeletion encompassing MAPT at chromosome 17q21.3 is associated with developmental delay and learning disability. Nat Genet 38:1032–1037.

Snijders AM, Nowak N, Segraves R, Blackwood S, Brown N, Conroy J, Hamilton G, Hindle AK, Huey B, Kimura K, Law S, Myambo K, Palmer J, Ylstra B, Yue JP, Gray JW, Jain AN, Pinkel D, Albertson DG. 2001. Assembly of microarrays for genomewide measurement of DNA copy number. Nat Genet 29:263– 264.

Tyson C, Harvard C, Locker R, Friedman JM, Langlois S, Lewis ME, Van Allen M, Somerville M, Arbour L, Clarke L, McGilivray B, Yong SL, Siegel-Bartel J, Rajcan-Separovic E. 2005. Submicroscopic deletions and duplications in individuals with intellectual disability detected by array-CGH. Am J Med Genet Part A 139A:173–185.

Vissers LE, De Vries BB, Osoegawa K, Janssen IM, Feuth T, Choy CO, Straatman H, van der Vliet W, Huys EH, Van Rijk A, Smeets D, Ravenswaaij-Arts CM, Knoers NV, Van der Burgt I, De Jong PJ, Brunner HG, van Kessel AG, Schoenmakers EF, Veltman JA. 2003. Array-based comparative genomic hybridization for the genomewide detection of submicroscopic chromosomal abnormalities. Am J Hum Genet 73:1261–1270.

Waggoner DJ, Chow CK, Dowton SB, Watson MS. 1999. Partial monosomy of distal 10q: Three new cases and a review. Am J Med Genet 86:1–5.

White SJ, Vink GR, Kriek M, Wuyts W, Schouten J, Bakker B, Breuning MH, Dunnen JT. 2004. Two-color multiplex ligationdependent probe amplification: Detecting genomic rearrangements in hereditary multiple exostoses. Hum Mutat 24: 86–92.