

A Multilocus Technique for Risk Evaluation of Patients with Neuroblastoma

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Abstract

Purpose: Precise and comprehensive analysis of neuroblastoma genetics is essential for accurate risk evaluation and only pangenomic/multilocus approaches fulfill the present-day requirements. We present the establishment and validation of the PCR-based multiplex ligation-dependent probe amplification (MLPA) technique for neuroblastoma.

Experimental Design: A neuroblastoma-specific MLPA kit was designed by the SIOP Europe Neuroblastoma Biology Committee in cooperation with MRC-Holland. The contained target sequences cover 19 chromosomal arms and reference loci. Validation was performed by single locus and pangenomic techniques ($n = 174$). Dilution experiments for determination of minimal tumor cell percentage were performed and testing of reproducibility was checked by interlaboratory testing ($n = 15$). Further 156 neuroblastomas were used for establishing the amplification cutoff level.

Results: The MLPA technique was tested in 310 neuroblastomas and 8 neuroblastoma cell lines (including validation and amplification cutoff level testing). Intertechnique validation showed a high concordance rate (99.5%). Interlaboratory MLPA testing ($\kappa = 0.95$, $P < 0.01$) revealed 7 discrepant of 1,490 results (0.5%). Validation by pangenomic techniques showed a single discordance of 190 consensus results (0.5%). The test results led to formulation of interpretation standards and to a kit revision. The minimal tumor cell percentage was fixed at 60%.

Conclusions: The recently designed neuroblastoma-specific MLPA kit covers all chromosomal regions demanded by the International Neuroblastoma Risk Group for therapy stratification and includes all hitherto described genetic loci of prognostic interest for future studies and can be modified or extended at any time. Moreover, the technique is cost effective, reliable, and robust with a high interlaboratory and intertechnique concordance. *Clin Cancer Res*; 17(4); 792–804. ©2011 AACR.

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Translational Relevance

Consensus exists in the International Neuroblastoma Risk Group (INRG) to use a series of tumor genomic features for risk evaluation. Increasing attention is now focused on utilizing pangenomic or multilocus datasets to better assign patients to certain genetic risk groups and also to discover new regions of interest exploring their potential clinical impact. So far, molecular-genetic investigations for routine diagnosis have been almost exclusively performed by FISH, PCR, and flow cytometric analysis. The INRG Biology Committee suggests studying the prognostic impact of at least 10 genomic changes in neuroblastoma. Consensus also exists on the use of commercialized platforms. We present a neuroblastoma-specific MLPA kit fulfilling all these requirements including interlaboratory and intertechnique validation as well as interpretation guidelines.

Introduction

In the past 2 decades, a great deal of information on genetic aberrations in many distinct tumor entities has been gathered. Although new scientific results await implementation into clinical practice for many tumor types, this is already the case for neuroblastoma, the most common tumor in infants less than 1 year of age (7%–10% of all childhood tumors), where several genetic markers have already entered risk classification systems (INRG, classification schema, SIOPEX Studies; refs. 1, 2). Because of the unique biological features of neuroblastoma showing a wide range of clinical behavior (highest rate of spontaneous regression of any human malignancy, a potential for undergoing both induced and spontaneous maturation, and also aggressive clinical courses with poor survival), markers identifying risk are of vital importance. In particular *MYCN* amplification (MNA) signifies aggressiveness and a high metastatic potential, but occurs only in a subgroup of tumors (20%–23%; refs. 3, 4). Segmental chromosome aberrations (SCA; gains and losses of parts of chromosomes) and tumor cell ploidy have also been shown to influence the biologic tumor behavior (5–13). In the INRG schema for pretreatment risk evaluation, tumor cell ploidy and loss of genetic material at the long arm of chromosome 11 are incorporated in addition to the status of the *MYCN* oncogene (1). In 2009, international consensus was reached to study a series of loci at 10 different chromosomes besides typically amplified genes (2). In the forthcoming SIOPEX LINES (Low and Intermediate Neuroblastoma European Study) study, to be launched shortly, it is planned to take into account SCA at any of 7 specific chromosome arms (including losses at 1p, 3p, 4p, and 11q and gains at 1q, 2p, and 17q) known to be recurrently altered and of prognostic impact in neuroblastoma for risk stratification, if *MYCN* is not amplified. The identification of the described genetic aberrations has so far been facili-

tated by the use of fluorescence in situ hybridization (FISH) and loss of heterozygosity (LOH) studies in most laboratories dealing with neuroblastoma diagnosis all over the world. However, for the evaluation of 7 chromosomal regions, cost-effective standardized methods allowing a higher throughput are urgently needed, which is fulfilled by the multiplex ligation-dependent probe amplification (MLPA) technique as a "semihigh throughput" technique (14). The cost effectiveness of this technique is taking effect when a larger number of samples is analyzed in one experiment. Here, we report on the establishment of a neuroblastoma-specific MLPA kit by the SIOPEX Biology Committee together with MRC-Holland; the generation of a clear and comprehensible graphic representation together with the Austrian Institute of Technology (AIT); the validation of the kit by other techniques, including FISH, comparative genome hybridization (CGH)-array, and single nucleotide polymorphism (SNP)-array; and the interobserver reproducibility evaluated by an interlaboratory testing.

Materials and Methods

The MLPA technique

In the PCR-based MLPA (for details concerning the probes, primer sequences, and PCR conditions see www.mrc-holland.com, MRC-Holland, Amsterdam, The Netherlands), 2 DNA probes for every target sequence are hybridized to the tumor sample DNA (0.3–0.5 µg). After binding to the target sequence, the probes are ligated and amplified (14). The amplification of the added probes by PCR reaction depends on the presence of the target sequence (the tumor DNA). Quantification of losses, gains and amplifications can be determined by analyzing the ratio of the reaction product between the test DNA and the reference DNA from healthy individuals. The reaction product is proportional to the number of target sequences and thus the number of target recognition sites can be quantified. In case of nullisomy, no reaction product will be obtained. The identification of the target sequences is enabled by the lengths of the spacers (stuffer sequences), which are specific for the different probes. Thus, every individual PCR product can be identified and quantified after running the products on a capillary electrophoresis. For guidelines concerning the technical aspects of the MLPA method see ref. 15.

The generation of a neuroblastoma-specific MLPA kit

For the neuroblastoma-specific MLPA kit (P251, P252, P253), it was decided to include all published chromosomal regions of possible prognostic impact (2). Interlaboratory testing, establishment of interpretation guidelines and validation by SNP-array led to a modification of the original A1 version of the kit by including additional probes at 1q, 3q, 4p, 4q, 7p, 9q, and 11p, exchanging probes on 2p and omitting probes for *MYCN*, and several loci of less impact. A further change was the unification of the reference probes for all 3 probe mixes. The revised kit B1

consists of 3 panels of probes. The SALSA P251-B1 contains 34 probes for chromosomes 1, 3, and 11; P252-B1 comprises 33 probes for chromosomes 2 and 17; and P253-B1 consists of 33 probes for chromosomes 4, 7, 9, 12, and 14. In each panel, control probes (10 each plus 9 control fragments) located in chromosomal regions rarely altered numerically in neuroblastomas are included: 5p, 6p, 15q, 16p, 16q, 19q, 20q, and 21q. In a single assay, 12 or 24 different tumor samples can be analyzed. The remaining slots can be left free or be filled with retrospective neuroblastoma samples and DNA from other tumors with similar genomic aberrations. One single kit allows the analysis of approximately 80 tumor samples (excluding the control samples) with the simultaneous evaluation of 106 (A1 version) and 100 (revised B1 version) genetic loci (corresponding to the 19 chromosomal regions/arms of interest plus reference loci). Moreover, adaptation of the kit by adding newly identified relevant loci is possible.

The graphic representation: the MLPAVizard

When this study began there was no software available to offer an easy-to-use and robust way to analyze and visualize MLPA data. Those shortcomings have been overcome by developing the MLPAVizard (MLPA Visualization Wizard), a client-based research prototype software created at the AIT (www.ait.ac.at) to perform the data analysis and to create visualizations for MLPA experiments. Special focus was set on usability and robustness, therefore a hybrid architecture was created by incorporating 2 different technologies: Java and GNU R. All user interactions and the data management of assays (e.g., saving and loading of an MLPA assay) are written in Java. The mathematical analysis and creation of graphs is carried out by GNU R. The calculations were created using the information provided by MRC-Holland in 2007 (and mirror those used in the software "Coffalyser 5.3"). MLPA data analysis is based on the comparison of relative amounts of probe amplification products between test DNA (tumor DNA from a neuroblastoma patient) and reference DNA (DNA from healthy humans). Prior to the ratio computation, the peaks in the electrophoresis signal reflecting copy number variation of the targeted DNA sequences have to be extracted. The extracted peak values are normalized in various steps to calculate the MLPA output ratio values. The MLPAVizard carries out additional quality checks to ensure a valid experiment (DNA quantity control, PCR control). During the final step of the process, the application creates a graphical visualization of the ratio values in the form of a bar chart, allowing investigators the immediate interpretation of the data. The 95% CI for each probe is given in the center of each bar.

Tumor DNA dilution experiments

To analyze the tumor cell percentages needed for the detection of amplifications and SCA, mixing experiments of tumor cell DNA (derived from a neuroblastoma tumor and a neuroblastoma cell line, STA-NB-9) with reference DNA from peripheral blood of the same patient have been

carried out. For STA-NB-9, MLPAs (kit A1) have been carried out undiluted (designated as 100%), in 70%, 50%, 30% dilution steps and without tumor cells (0%). In the case of the neuroblastoma tumor, the dilution steps and the tumor cell contents were as follows: 95% (undiluted), 66.5% (70%), 47.5% (50%), 28.5% (30%), 9.5 (10%), and without tumor cells (experiments were carried out with the revised B1 MLPA kit). The STA-NB-9 cell line shows *MYCN* amplification as well as 1p loss and 17q gain as known by cytogenetic and FISH analyses. The analyzed tumor exhibited gains of the chromosomal regions 2p, 4q, and 17q and loss of 11q, confirmed by SNP-array.

Validation by FISH, CGH-array, and SNP-array

MLPA results were validated by the following. (i) Interphase FISH (I-FISH; 149 primary neuroblastomas consecutively received at the Children's Cancer Research Institute (CCRI) resulting in evaluable profiles from 125 tumors) with the following probes: BAC clone containing the *MYCN* gene, RP11-355H10 (kindly provided by Pieter de Jong, BACPAC, Resource Center at Children's Hospital, Oakland Research Institute, Oakland, CA), D1Z1 (16), D1Z2 (kindly provided by J. Giannakudis, Germany), 2pter, 11p (PAC-908-H22), 11q (MLL: PAC-dJ 167 K 13), 17p (BAC-bA 22 G 12), and 17q (RP13-516 M14) were purchased from Dr. M. Rocchi (Resources for Molecular Cytogenetics, University of Bari, Italy), and D2Z was obtained from Qbiogene. (ii) Cytogenetic results and I-FISH (8 neuroblastoma cell lines established in the CCRI). (iii) SNP-array (Affymetrix GeneChip Human Mapping 250K Nsp Array; Software: CNAG 3.0, carried out by one of the coauthors, T.M.) and 5K BAC array-CGH (performed by the coauthors, G.S. and J.C.): 10 DNA samples (7 tumor DNA samples, 1 in duplicate, and 2 DNA samples from *MYCN* amplified neuroblastoma cell lines). (iv) Fourteen informative tumors were derived from the LNESG 1 (Localized Neuroblastoma European Study Group). (v) Fifteen informative tumors derived from the INES (Infant Neuroblastoma European Study) were compared with array-CGH data as well. The LNESG1 and INES tumors were validated with the 5K BAC array (G.S. and J.C.). All analyses were performed with the A1 kit version.

The interlaboratory MLPA testing

To further validate the test kit (version A1) and to learn about its robustness when applied in different laboratories, interlaboratory testing was performed. DNA from 7 tumors (1 tumor DNA was sent in duplicate) and DNA from 2 neuroblastoma cell lines were distributed to 8 different SIOOPEN reference laboratories, giving a total number of 9 investigators. All but one DNA (test sample 4) were derived from tumor specimens with tumor cell contents more than 60%. The laboratories had no information on the genetic makeup of the samples. The results were returned within 2 weeks. The interpretations were given by each laboratory after collaborative establishment of common rules and guidelines for evaluation of the graphs. Validations of the MLPA results were performed by CGH- and SNP-arrays

on all samples. The κ -statistic was used to describe the agreement between raters (17, 18). The statistical significance (P value) is calculated as the fraction of permutation values that are at least as extreme as the original sample. Another interlaboratory test involving 4 laboratories was carried out using 5 neuroblastoma samples with numerical aberrations only and a control DNA from peripheral blood.

Cutoff level for MYCN amplification

For determination of a reliable cutoff level for *MYCN* amplification, the values for *MYCN* in 156 neuroblastomas, 17 with and 139 without amplification (verified by FISH), have been compared using the revised B1 neuroblastoma MLPA kit.

Results

The neuroblastoma-specific MLPA kit and the graphic MLPA representation: the MLPAVizard

The current neuroblastoma-specific MLPA kit (B1 series) consists of 100 genetic loci corresponding to all chromosomal regions, which have been recommended to be analyzed by the INRG Biology Subcommittee. The chromosomal regions under investigation are located in the following chromosomal arms: 1p, 1q, 2p (including *MYCN*, *DDX1*, *NAG*, and *ALK*), 3p, 4p, 7q, 9p, 11q, 12q, 14q, 17p, and 17q. If additional loci are found to be prognostically relevant, probes can be promptly implemented into the neuroblastoma detection kit.

The MLPA data presented in this study have been analyzed with the currently available data analysis prototype MLPAVizard in a desktop application. The MLPAVizard is mainly focused on the direct visualization of the MLPA data according to chromosomal position, thus facilitating the interpretation of the data in the daily work. Figure 1 shows 3 characteristic graphs with numerical aberrations only, MNA and/or SCA, and gives further details concerning the graphic representation. Table 1 gives information on definition, nomenclature, and cutoff levels.

Determination of minimal tumor cell content needed for reliable MLPA results and interpretation

Tumor cell dilution experiments revealed that *MYCN* amplification was clearly detectable in the cell line, in every dilution step downwards to 30%. In case of the tumor sample, the 4 SCA were clearly visible with mean values more than 0.25 in the 70% dilution (66.5% tumor cells). 11q loss and 17q gain, in contrast to 2p and 4q gains, gave significant differences in mean values (>0.25) in the 50% dilution (corresponding to 47.5% tumor cells) and also with only 28.5% tumor cells in the sample. However, still visible SCA in higher dilutions most likely reflect great chromosome arm ratio differences, that is, greater than 2:1. In case of the neuroblastoma cell line STA-NB-9, SCA were clearly detectable with significant differences in mean values at every dilution step (>0.3 for 1p loss and 17q gain in all dilutions). As expected, 0% tumor cells resulted in a flat profile in both test series.

The MLPA interlaboratory testing

Table 2 gives the consensus results of the interlaboratory MLPA testing for each sample and also includes the observed discordances. Consensus was defined as agreement of at least 7 of 9 investigators (or $\geq 75\%$ agreement, including 7 of 8 or 6 of 7 agreements). In 6 of 10 test samples complete concordance was achieved. No discordances (all test samples, all investigators) occurred for MNA, 1p loss, 2p gain, and 17q gain. Discordances occurred in 4 test samples for the following chromosomal arms: 7p (1 \times false-positive according to MLPA consensus, but correct according to array data), 7q (2 \times false-negative), 11q (1 \times false-positive), 12p (2 \times false-positive), and 12q (1 \times false-negative). The total discordance rate was 0.5% (0.27% false-positive, 0.2% false-negative), that is, 7 discrepant results of 1,490 interpretable loci ($\kappa = 0.95$, $P < 0.001$). Table 3 shows the agreement rate for each chromosomal arm. Because gains or losses of single DNA probes (visible as more than 0.25 increase or decrease of a single bar) may be due to technical reasons, they have not been considered as SCA, but recorded as so-called "outlier." The highest number of outliers was found in the chromosomal arms 7q, 14q, and 4p. For chromosome 4p, the frequency of outliers was markedly diminished in the B1 kit by adding an additional 4p probe in the region of observed losses. Outliers in 14q frequently concerned the NFKBIA probe, which will be replaced in the next version of the kit.

The second interlaboratory test using neuroblastoma samples with numeric chromosome changes only, led to no discrepancies. All involved laboratories diagnosed numeric aberrations, with chromosomes 1, 2, 7, 12, and 17 most frequently involved. The control DNA was uniformly interpreted as disomic for all chromosomes.

Interlaboratory testing: validation of MLPA results by CGH-array and SNP-array

MLPA, CGH-array, and SNP-array results are all summarized in Table 4. SNP array analyses depicted the highest number of SCA and amplicons, followed by array-CGH. These additional aberrations are only infrequently seen in neuroblastic tumors with yet unknown prognostic relevance. In test samples 3, 6, 7, 8, and 9, in comparison with MLPA, additional aberrations have been found in the following chromosomal regions (not covered by MLPA): 1p (1p21.2–3), 1q (1q43–44), 2p, 4p, 4q, 5p, 5q, 6p, 6q, 8p, 8q, 10p, 10q, 12q, 16p, 16q, 19q, and 22q. In 2 tumors (test samples 6 and 7), multigenomic techniques revealed loss of 4p (4pter to 4p15.31 and 4p16.1 to 4p12, respectively). The original MLPA kit version (A1) contained only 1 probe in the concerned area. All MLPA profiles showed a loss of this probe. However, because 2 adjacent probes are required for the diagnosis of a SCA, an additional probe was included located at 4p16.3 in the modified kit version, B1. The new MLPA kit clearly showed 2 lost probes for these tumors. Therefore, this finding was not considered as divergent from the multigenomic results. One genuine discrepancy has been found for one chromosomal region concerning: 7p12.1 to 7qter gain in sample 6. Only 1

observer found a gain as seen by multigenomic techniques, 2 MLPAs showed only 1 increased value, 1 was suggestive of a gain but in the borderline range, 3 showed a balanced ratio, and 2 were not interpretable. Because 19 chromosomal "arms" have been analyzed in 10 test samples, the rate of discrepancies between MLPA and BAC array-CGH and SNP array is 0.5% (1 of 190).

Intertechnique testing: MLPA, I-FISH, and array-CGH

From 149 neuroblastomas, 125 tumors (154 tumor pieces) have been informative, whereas 24 gave unclear results either due to insufficient tumor cell content or degraded DNA. The 125 informative tumors genetically belonged to the following subgroups: 51 neuroblastoma with numeric chromosome aberrations only, 41 tumors with SCA, but without MNA, and 33 neuroblastomas with MNA, mostly with additional SCA and with co-amplifications of *NAG* and *DDX1* and rarely *ALK*. Among neuroblastomas with numeric aberrations only, no discrepancies with FISH have been observed (198 markers analyzed). Neuroblastoma with SCA but without *MYCN* amplification (116 markers analyzed) revealed a single discrepancy, those with *MYCN* amplification (62 markers analyzed) also showed a single discrepancy, which, however, did not concern the *MYCN* status itself (no discordances out of 154 investigated tumor areas in 125 tumors). The latter was only "discrepant" if *MYCN* amplification was heterogeneous and the number of MNA cells was too small to be detected by MLPA (i.e., under 10%-15%). In total, 376 markers have been analyzed by MLPA and FISH and 2 discrepancies concerning SCA have been encountered (0.5%). Both concerned chromosome 1p and were observed in 2 tumors, 1 with and 1 without *MYCN* amplification. In both cases, chromosome 1p loss was detected by MLPA but not by FISH.

Fourteen informative tumors derived from LNESG 1 patients were investigated by MLPA and array-CGH. Among 260 regions evaluable by MLPA, 6 imbalances have been diagnosed by both techniques without any discordance. For another 15 tumors from the INES 99 (Infant Neuroblastoma European Study), 279 regions were evaluable by MLPA, 5 aberrations have been found by MLPA, 4 by array-CGH. The one discrepancy concerned 17q gain and could also be explained by tumor heterogeneity.

Cutoff level for *MYCN* amplification

MYCN amplified tumors showed a signal ratio range between 4.3 and 38 (see Fig. 2). The fluctuation of signals along the baseline is due to variation of copy number of chromosome 2 in the individual tumors, which can be markedly overrepresented. Nevertheless, the highest value in this series of tumors was 2.6 and the values for all other genes of the same chromosome were increased as well. Therefore, a signal excess of at least 12×0.25 , that is, 3, was decided as cutoff level for definite gene amplification (see also Table 1). In case of clear signal excesses for *MYCN* (with or without *DDX1*, *NAG*, or *ALK*), which are, however, between 1.0 and 3, FISH investigations should be performed to exclude heterogeneous (focal or diffuse) MNA. Flat MLPA profiles with "low excess" *MYCN* (with or without adjacent genes) values hint at MNA but low tumor cell content, whereas clearly visible numeric and/or SCA and "low excess" *MYCN* values rather point to intratumoral *MYCN* heterogeneity.

Discussion

The SIOPEN Biology Committee has always considered it a priority to work toward a single diagnostic test system that will reliably and accurately detect allelic deletions and gains, as well as MNA and estimate overall DNA content (ploidy) on a single platform. This has been achieved using in-house arrays (13). However, a commercially available platform was needed to implement this technology uniformly in clinical laboratories across various groups and countries. To fulfill not only the contemporary but also the future need of genetic diagnosis in neuroblastoma, a "semi-high throughput" technique enabling the investigation of all currently known aberrations of possible prognostic impact in one single experiment was established. The robustness of the PCR-based technique relies on making use of a single primer pair in the amplification reaction for all included chromosomal probes. Moreover, the flexibility of this technique allows adaptation of future requirements with the inclusion of additional markers. Although chip-based techniques cannot be considered as routine techniques (they are time consuming, costly, and not standardized across Europe), they allow a complete view of all gains and losses in a given tumor and will therefore continue to play an important position in molecular diagnosis

Figure 1. The graphs show the signal intensity ratios reached for each of the multiple DNA probes translated to heights of the bars. The single bars representing the DNA probes are arranged according to the chromosomal regions starting from the telomeric regions of the short arms followed by probes for the long arms. Each chromosome is represented by a specific color; q arms are shown in a darker color than p arms. The shaded area represents the baseline (between -0.75 and $+1.25$), which usually indicates a disomic pattern. Bars within this area are given in yellow; bars under the baseline, representing losses, are given in red; bars above the baseline area, representing gains or amplifications, are indicated in green. On the x-axis the different target genes are listed. A, graphic MLPA (kit 251-, 252-, 253-A1) representation of a neuroblastoma with numeric but no segmental chromosome aberrations. The conclusion reads as follows: a0 s0/10. B, the graph shows amplification of the genes *NAG*, *DDX1*, and *MYCN* (values between 11.04 and 15.86), besides loss at the short arm of chromosome 1, loss at the long arm of chromosome 14 (2 adjacent probes, mean difference 0.25), and gain of chromosomal material at the long arm of chromosome 17. The elevated value of TP53 probes is regarded as so-called outlier. The conclusion for this tumor is as follows: a3 (*MYCN*, *DDX1*, *NAG*) s3/10: 1p-, 14q-, +17q. C, MLPA results on a tumor sample identifying gains of genetic material at the long arms of chromosomes 1 and 17 and at the short arm of chromosome 2 and losses of genetic material at the short arms of chromosomes 3 and 9 and at the long arm of chromosome 11. According to the recommended nomenclature this tumor would be diagnosed as follows: a0 s7/10: +1q, +2p, 3p-, 9p-, 11q-, +17q; ++17q. Reference probes are not represented in the graphs.

Table 1. Nomenclature and threshold levels for MLPA results, partly published in Ambros and colleagues (2)

Terms	Description	Comments
Normal status	Balanced ratio between the majority of signals ^a of both chromosomal arms	In the case of a balanced ratio, a uniparental isodisomy ^b with a complete or partial LOH of loci located on the investigated chromosome cannot be excluded Caveat: flat profiles most likely reflect a too small tumor cell content and are designated as " no result "
Segmental chromosome aberration Loss	Unbalanced ratio between the signals of the chromosomal region of interest (decrease of the mean value of at least 2 adjacent probes of at least 0.25) and the reference signals (at least 2 adjacent probes in direct vicinity)	This result could correspond to a FISH deletion, which reflects an LOH, or a FISH imbalance, which does not necessarily indicate an LOH
Gain	Unbalanced ratio (low signal excess, between at least 0.25 and 1.0) between the signals of the chromosomal region of interest (at least 2 adjacent probes) and the reference signals (at least 2 adjacent probes in direct vicinity) of the chromosomal region of interest	In case of chromosome 2p gain , a type 1 gain is discriminated from a type 2 gain (see also Discussion); the latter is suspicious for either <i>MYCN</i> amplification in a minority of tumor cells (intratumoral heterogeneity) or <i>MYCN</i> amplification in samples with very low tumor cell numbers (e.g., after therapy), resulting in flat curves except for <i>MYCN</i> (and possibly co-amplified genes); clarification by I-FISH is highly recommended Signal excess ratios between 1.0 and 3 are suspicious for the presence of small numbers of <i>MYCN</i> amplified tumor cells—either because of low tumor cell content (flat MLPA profile) or due to heterogeneous <i>MYCN</i> amplification and have to be clarified by I-FISH
Amplification	Unbalanced ratio (high signal excess, at least 12 × 0.25, i.e., a ratio of 3) between the signals of one or more genes (at least 2 adjacent probes) and all other probes located on the same chromosome	
Borderline mean values	Differences of mean values between 0.2 and 0.24 are regarded as borderline. MLPA should be repeated or other techniques be used for clarification. A homogenous increase or decrease of several adjacent probes not reaching the borderline value of 0.2 to 0.24 can be regarded as suspicious for a SCA and should be clarified by another technique.	
Outlier probe	Only 1 probe shows a more than 0.25-fold increased or decreased value—not taken into account in the calculation of mean values of other probes located on the concerned chromosome. FISH, aCGH, and/or SNP array can be used for clarification. In case a chromosomal arm is only covered by 3 probes, it cannot be evaluated (in the current kit, the 3q, 7p, 12p and 12q arms are concerned).	
No result	Should be specified: unclear or not interpretable (e.g., flat profile or too much fluctuation, mostly due to inadequate tumor cell content or degraded DNA, respectively) No tumor; not done	

^aSignal intensity is visualized in the graphic representation of the MLPA results as height of the bars.

^bA uniparental isodisomy means that both chromosomes or parts thereof are derived from one parent. Thus, the 2 chromosomes are identical. However, uniparental isodisomies are rare in NB tumors (they are found in 1%; G.S. and J.C., unpublished).

Table 2. Consensus MLPA data and discrepancies for the 10 test samples of the interlaboratory testing

Sample	MYCN	1p	1q	2p	2q	3p	3q	4p	4q	7p	7q	9p	9q	11p	11q	12p	12q	14q	17p	17q
1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	loss	-	-	-	-	gain
2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	loss	-	-	-	-	gain
3	-	-	-	gain	-	-	-	-	-	-	-	-	-	-	loss	-	-	-	-	gain
4	amp^a	itcc	itcc	itcc	itcc	itcc	itcc	itcc	itcc	itcc	itcc	itcc	itcc	itcc	itcc	itcc	itcc	itcc	itcc	itcc
5	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1dis	-	-	-	-
6	-	-	-	gain	-	loss	-	-	-	1dis	-	-	-	-	-	-	gain	1dis	-	gain
7	-	-	-	-	-	-	-	-	-	-	-	-	-	-	loss	1dis	-	-	-	gain
8	amp^a	loss	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
9	amp^b	loss	-	gain	-	-	-	-	-	-	gain	-	-	-	-	-	-	-	-	-
10	amp^a	loss	-	-	-	-	-	-	-	-	2 dis	-	-	-	1dis	-	-	-	-	gain

NOTE: Consensus is indicated in case at least 7 of 9 investigators (or similar proportion) came to the same conclusion. In sample 4, the MLPA result shows no segmental aberrations; however, when considering tumor cell content (test sample 4 was a post-therapy specimen with a tumor cell content of 30%), the result has to be scored as inconclusive. Concordant results are given in bold. Abbreviations: amp, amplification; dis, discrepancy; itcc, inadequate tumor cell content.

^aMYCN, DDX1, NAG

^bMYCN

of tumor samples (10, 12, 19–25). For MYCN analysis, I-FISH (although exceeding the costs of MLPA) will continue to represent the frontline technique in the diagnostic work up of neuroblastomas (INRG), because it allows a cell to cell analysis (if necessary, also under preservation of the architecture of the tumor), simple discrimination of tumor cells from nontumor cells in case of aneusomies and identification of heterogeneous MNA, which occurs in 8% to 21% of all MNA cases (unpublished results and refs. 26, 27). This relatively high percentage observed in our laboratory (21%) is most likely due to investigations of different tumor areas (up to 4 samples) and inclusion of tumors detected by the Austrian neuroblastoma mass screening, which was performed at approximately 10 months of age (28). Heterogeneous MNA includes tumors with only a few amplified cells in a background of nonamplified tumor cells up to a large proportion of amplified tumor cells and a minority of nonamplified tumor cells. The genetic background of these tumors including ploidy is diverse and under current investigation.

Intertechnique and interlaboratory testing

The intertechnique and interlaboratory testing of the current neuroblastoma-specific MLPA kit revealed high concordance rates between MLPA and other techniques as well as a high interobserver concordance and reproducibility. The observed discrepancies between FISH and MLPA could be assigned either to the presence of intratumoral heterogeneity (and thus to "sampling error" making the analysis of more than 1 tumor area crucial) or, in 1 case, to misinterpretation of small paracentromeric signals by

FISH as cross-hybridization signals. In fact, the frequency of intratumoral heterogeneity of SCA is possibly higher than expected when more than 1 tumor area is investigated by MLPA (7 of 23 in our series). Additional reasons for divergent results could be a low tumor cell content especially in post-therapy specimens where amplification may still be visible but SCA are not and also the presence of a Schwann cell stroma (see Difficulties and pitfalls). In the interlaboratory testing, the 1 genuine discrepancy concerning chromosome 7 is possibly due to not ideally chosen chromosome 7 probes, which have been included as controls due to frequent overrepresentation of chromosome 7 in neuroblastomas. However, this chromosome is so far not intended to be used for prognostification. Concerning sample 4 of the interlaboratory testing, which is a typical post-therapy specimen of a MNA tumor, it is important to consider only MNA as interpretable (values still exceed a ratio >3); however, no conclusion about SCA can be given. Two members of the SIOPEX Biology group have already presented or published MLPA studies in limited numbers of tumor samples showing high intertechnique concordances (29, 30).

The role of tumor cell content

The reliability of molecular-genetic techniques based on DNA extraction like MLPA depends on the appropriate tumor cell content and on whether amplification or SCA need to be detected. Detection of amplifications also depends on the amplification grade and homo- or heterogeneity of amplification. In case of homogeneous and high amplification, the tumor cell content can be as low as 10%

Table 3. Observer agreement for the MLPA interlaboratory testing per chromosome

Chromosomal arm	Agreement	κ	P
1p	100% (82/82)	1	<0.001
1q	100% (83/83)	–	NA
2p	100% (80/80)	1	<0.001
2q	100% (79/79)	–	NA
3p	100% (84/84)	1	<0.001
3q	100% (83/83)	–	NA
4p	100% (63/63)	–	NA
4q	100% (76/76)	–	NA
7p	98.5% (66/67)	0.10506	0,12
7q	96.7% (59/61)	1	<0.001
9p	100% (84/84)	–	NA
9q	100% (80/80)	–	NA
11p	100% (85/85)	–	NA
11q	98.8% (84/85)	0.95033	<0.001
12p	97.7% (84/86)	–0.03765	0,5
12q	98.8% (83/84)	0.84435	<0.001
14q	100% (83/83)	–	NA
17p	100% (71/71)	–	NA
17q	100% (72/72)	1	<0.001
all	99.5% (1483/1490)	0.95371	<0.001

NOTE: The maximum of 90 agreements per chromosomal arms (10 test samples, 9 investigators) has not been achieved either due to fluctuation of probe values of the whole profile or of single arms, or due to single outliers concerning chromosomal arms for which only up to 3 probes were used. NA means not analyzable. Discordant results are given in bold.

(e.g., post-therapy specimen, sample 4 in the interlaboratory test and verified by the mixing experiments). The detection of heterogeneously (focally or diffusely) amplified tumors is possible in principle, but depends on the percentage of amplified tumor cells contained in the specimen and their amplification grade. For reliable detection of SCA, the tumor cell content has to be much higher (post-therapy specimens are usually inappropriate for SCA detection). On the basis of dilution experiments, 60% tumor cell content in the specimen can be recommended as threshold level for reliable detection of SCA, as previously recommended for PCR and Southern blot studies (31), although single SCA were detectable at lower tumor cell percentages. The latter most likely depends on the number of gained or lost copies and on whether the whole or only a subpopulation of tumor cells bear the aberration.

Interpretation of the MLPAVizard graph and guidelines for evaluation and terminology

Although the MLPAVizard software enables a clear graphic representation of the results, the SIOPEN Biology Committee had to develop exact guidelines and threshold levels for evaluation besides a useful terminology with involvement of the INRG Committee (2). In the MLPA graph, a lack of amplified genes and SCA is reflected by a balanced ratio between the majority of signals of both chromosomal arms, and are designated as "normal status." In case of disomies, the expected ratio is 1.0, the range between 0.75 and 1.25. In case of tri- and tetrasomies (and

penta-, hexasomies), the height of most or all bars of an individual chromosome will be more than 1.25 (see also Difficulties and pitfalls). Table 1 gives an overview of the terms used for MLPA interpretation, their definitions and comments. Figure 1A shows a profile without SCA, Figure 1B with MNA and SCA, and Figure 1C with SCA only. Two SCA on the same chromosome can be diagnosed only if 2 breakpoints on the same chromosome can be deduced from the profile and verified by calculation (Fig. 1C).

So far published thresholds for losses and gains relied on adjacently reduced gene dosages with a ratios of <0.7 and >1.3, respectively, without taking into account the ratios of gene probes located on the same chromosomal arm and/or on the opposite arm (32). Thus, in case 2 adjacent probes exceed values more than 1.3 whereas all other probes of this chromosome have a mean value of 1.2, for example, this situation could lead to a misinterpretation of a gain of the 2 probes. As aneusomies and thus probe ratios >1 of all probes along different chromosomes are frequently observed in neuroblastomas, the ratios of all probes along the whole chromosome or chromosome arm have to be taken into consideration when interpreting gains or losses of chromosomal segments.

Codification of amplifications and segmental chromosome aberrations

Lack of amplification is indicated by the letter and number code a0. In case of amplifications, the code indicates the number of amplified genes, for example: a1 means

Table 4. Segmental aberrations found by intertechnique testing: MLPA, BAC array-CGH, and SNP-array

tu sample	Chromosome																									
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	MLPA sum	aCGH sum	SNP sum	
T1 and T2	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	q-/	-/-	-/-	-/-	-/-	-/-	+q/+q/	-/-	-/-	-/-	-/-	-/-	-/-	a0 s2/10	a0 s2	a0 s2
T3	-/-	-/+p	-/-p-	-/-p-	-/+p	-/+p	-/-	-/-	-/-	-/-	q-/	-/-	-/-	-/-	-/-	-/+p	+q/+q/	-/-	-/-	-/-	-/-	-/-	-/-	a0 s3/10	a0 s8	a0 s13
T4	ni/ins/ni	MNA/ins/	ni/ins/ni	ni/ins/ni	ins/ni	ins/	ni/ins/	ins/ni	ni/ins/ni	ins/ni	ni/ins/ni	ni/ins/ni	ni/ins/ni	ni/ins/ni	ins/ni	ins/ni	ni/ins/ni	ins/ni	ins/ni	ins/ni	ins/ni	ins/ni	ins/ni	a3, sx	ni ^a	a5 sx
T5	-/-	-/-	-/-	-/-	-/-	ni	ni	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	a0 s0/10	a0 s0	a0 s0
T6	-/+p	+p/+p/	p-/p-	p-/p-	-q-	-/-	-/+p,	+p/	-/-	-p-	-/-	+q/+q/	-/-	-/-	-/-	-/-	+q/+q/	-/-	-/-	-/-	-/-	-/-	-/-	a0 s4/10	a0 s12	a0 s14
T7	-/-	-/-	-/-	-/-	-p-p-	-/-	q-/	-/-	-q-	-/-	q-/	-/-	-/-	-/-	-/-	-/-	+q/+q/	-/-	-/-	-/-	-/-	-/-	-/-	a0 s2/10	a0 s4	a0 s6
T8	p-/pa,	MNA/	-/-	-/-	-/-	q-	-/-	-/-	-/-	-/-	q-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	qa,	-/-	-/-	-/-	a3 s1/10	a ^b s3	a127 s6	
T9	p-p-/	MNA,	-/-	-/-	-/-	-/-	+q/+q/	-/-	-p-	-q-	-/-	-/-	-/-	-/-	-/-	-qa,	-/-	-/-	-/-	-/-	-/-	-/-	a1 s3/10	a ^c s4	a4 s7	
T10	p-	+p/MNA,	-/-	-/-	-/-	-/-	+q	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	q-,	-/-	-/-	-/-	-/-	-/-	-/-	-/-	a3 s2/10	a ^d s2	a4 s3
		+p/MNA,	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	+q	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
		+p	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
		MNA/	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-
		+p	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-	-/-

NOTE: Gray columns indicate chromosomes included in the MLPA neuroblastoma kit, white columns indicate those chromosomes (DNA probes for chromosomes 5, 6, 8, 10, 13, 15, 16, 18-22) only analyzed by pangenomic techniques. The only discrepancy between MLPA consensus results and aCGH and SNP array was observed in test sample 6 on chromosome 7p. Summary conclusion is indicated without further description of amplifications and segmental aberrations because of lack of space. "p" and "q" designate the short and the long arms of individual chromosomes.

Abbreviations: a, amplicon or amplification; ins, insufficient DNA; ni, not interpretable; nr, no result; pa, amplification on the p arm; qa, amplification on the q arm; s, segmental aberration.

^aInsufficient DNA quantity
^bAmplicons at 1p21.2, 2p24.3, and 19q13.33-19q13.42
^cAmplicons at 2p24.3 and 16q22.3

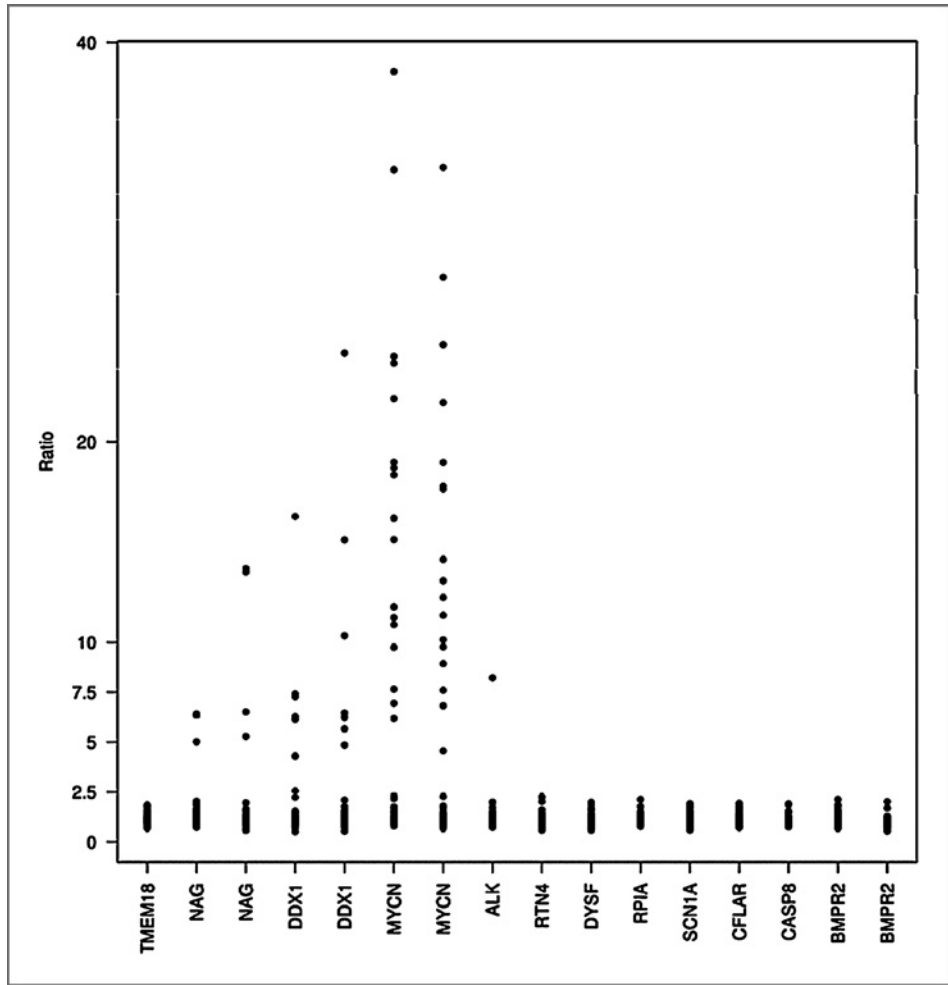


Figure 2. Summary of MLPA results for chromosome 2 of amplified and nonamplified neuroblastomas. The signal ratios range from 0.52 to 38. Note that the signal ratios of the nonamplified probes had a maximum value of 2.6 due to pentasomy/hexasomy of the given chromosome. However, the lowest value for an amplified probe was 4.3, and the highest value in this series of amplified tumor samples was 38. Note that co-amplification of the *ALK* gene occurred only in 1 tumor. In 2 cases, not the whole *NAG* gene was co-amplified, but only the proximal part of the gene. The graph is based on the modified B1 neuroblastoma MLPA kit containing 16 instead of 20 probes (used in A1) by reduction of *NAG*, *DDX1*, and *ALK* genes and inclusion of 2 genes on the short arm, that is, *DYSF* and *SCN1A* for better discrimination of chromosome 2p gain.

amplification of the *MYCN* gene without co-amplified genes; a3 means amplification of *MYCN*, *DDX1*, and *NAG* or amplification of *MYCN*, *DDX1*, and *MDM2*. Amplified genes or chromosomal regions should always be indicated after the code, for example, a2 (*MYCN*, 16q22.3).

No SCA are indicated by s0, whereby "s" stands for "segmental." The number of aberrations and the number of interpretable chromosomes (10 at maximum) is indicated and not interpretable chromosomes should be listed as not evaluable by n.e. (e.g., s0/10; or s2/9: 1p-, 2 n.e., +17q). To conform to the International System for Human Cytogenetic Nomenclature (33), it is recommended to put the minus behind and the plus before the concerned chromosomal region. If intratumoral heterogeneity is indicated by MLPA analyses of different tumor areas or by MLPA and FISH, "het" for heterogeneous together with the chromosomal region for which the heterogeneity has been found, should be added, for example, s3/8: 1p-, het +2p, 3 n.e., 4 n.e., 11q- (8 chromosomes have been interpretable).

In the case of 2p gain, 2 types are discriminated because of their different impact. Type 1, comprising either all presently included probes of the short arm of chromosome

2 or at least probes from *TMEM18* to *ALK*, reflects an SCA. Type 2, however, shows only *NAG* to *ALK* or even fewer probes affected and most likely indicates *MYCN* amplification (with co-amplification of adjacent genes) in a minority of cells, either due to genetic tumor heterogeneity or due to low tumor cell content (e.g., post-therapy specimen). Clarification by rechecking the tumor cell content and by I-FISH analysis is necessary.

A summary conclusion should be given taking into account the results of all techniques used for an individual tumor and for amplifications and SCA. Thus, the overall number of aberrations found by MLPA and FISH, for example, and the type of 2p gain, if possible, should be indicated, for example, MLPA results: a0 s2/10: +2p (type 1), +17q; FISH: no MNA, 1p-, +2p, +17q; summary: a0 s3/10: het1p-, +2p (type 1), +17q.

Difficulties and pitfalls

Difficulties in the interpretation of the numeric chromosome status are encountered if the reference chromosomes of the respective probe set are not disomic but tri- or tetrasomic. Under such circumstances the chromosome concerned seems to be underrepresented. Other difficulties

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may concern a pronounced fluctuation of the bar heights. In such cases, it is recommended to recheck the tumor cell content, to check the DNA quality, and to repeat the investigation, possibly using DNA from another tumor piece. A so-called flat profile can be caused by tumor cell content less than 60% or, possibly by a diploid tumor lacking SCA (whether such neuroblastomas indeed exist, is still unclear). Importantly, flat profiles can also result from DNAs from neuroblastomas with a higher amount of Schwann cell stroma (more than 50%), as is the case in ganglioneuroblastomas and ganglioneuromas, but occasionally also in differentiating neuroblastomas depending on the area used for DNA extraction. In these tumors, the reason for the flat profile are the Schwann cells, which are normal cells in differentiating/maturing neuroblastomas with a normal, diploid DNA content and lack of aberrations (6). Rarely, a 1p loss detected by I-FISH can show up as 1q gain in the MLPA profile. By definition, the baseline ploidy level of the tumor cell will be taken to decide whether a loss of 1p or a gain of 1q is present.

Conclusion

The robustness of the MLPA is based on the PCR technique itself and was achieved by validation by other molecular-genetic techniques leading to compelling interpretation guidelines resulting in a high interobserver and intertechnique concordance. Moreover, the relatively low costs and the use of standard PCR equipment make this technique attractive for routine neuroblastoma analysis. Single cell analysis by *MYCN* I-FISH remains valid and

indispensable in the diagnostic work up for the detection of intratumoral heterogeneity. Altogether, MLPA satisfies not only the current demands for INRG risk classification, covering all chromosomal regions of interest, showing a high reliability and reproducibility, but will hopefully also enable the development of a further refined reliable risk score to assess the tumor's aggressiveness at diagnosis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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