Chapter 3

Multiplex Ligation-Dependent Probe Amplification for the Detection of 1p and 19q Chromosomal Loss in Oligodendroglial Tumors

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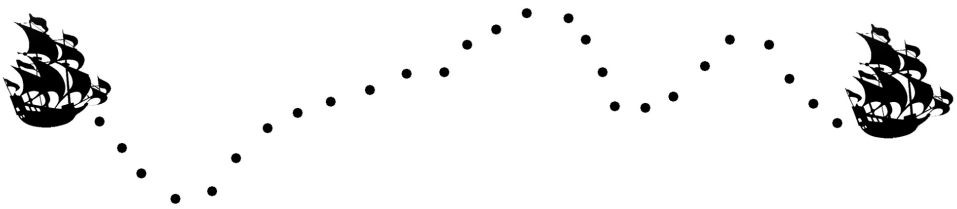


Abstract
Multiplex Ligation-Dependent Probe Amplification (MLPA) is a new assay for the detection of multiple chromosomal deletions in tumor tissue in a single experiment. Since genotyping of gliomas with oligodendroglial features by the detection of 1p/19q chromosomal deletions became essential for treatment decisions, we developed and validated an MLPA- based assay to determine these losses in formalin fixed and paraffin embedded oligodendroglial tumors (OG). Nineteen OG, and 10 control samples were analyzed by MLPA and the results were correlated with those obtained by fluorescent in situ hybridization (FISH). The MLPA results were reproducible in all samples in which repeated experiments were performed. In 18 of 19 OG MLPA and FISH were concordant for presence or absence of 1p deletion. In 3 OG, MLPA detected a 19q deletion not shown by FISH. For the other 15 OG MLPA and FISH were concordant. In one sample with 50-75% of tumor, MLPA failed to detect the 1p/19q deletions revealed by FISH (though with borderline values of significance). We conclude that MLPA is a valid and reproducible method for the detection of 1p/19q chromosomal deletions in OG stored on formalin fixed, paraffin embedded tissue.

Introduction
Gliomas are a group of primary brain tumors presumably originating from the astrocytic and/or oligodendroglial cell lineage. An oligodendroglial morphology in gliomas is associated with chemosensitivity and a relatively favorable prognosis, as well as deletions of the short arm of chromosome 1 and the long arm of chromosome 19 (1,2,8,10). More specific, chemosensitvity and 1p/19q deletions are most frequent in oligodendrogliomas, less frequent in gliomas showing both oligodendroglial and astrocytic phenotypes (oligo-astrocytomas) and least frequent in astrocytomas (8,10,13). The determination of 1p/19q deletions in gliomas is likely to result in a more reproducible and biologically and clinically more meaningful sub-classification of gliomas than the current classification, which is, to a large degree, based on subjective morphological criteria. Since the presence of 1p and/or 19q physical deletions in gliomas appears to be a better predictor for chemosensitivity and prognosis than morphology alone, there is growing need for a practical assay to determine these deletions in the routine clinical practice (3,11,13).
So far, a variety of methods for detection of 1p and 19q loss in gliomas have been used, like Fluorescent In Situ Hybridization (FISH) (10,12), detection of loss of heterozygosity (LOH) by microsatellite repeat analysis (5), and quantitative microsatellite analysis by real-time PCR (QUMA) (6).
Multiplex ligation dependent probe amplification (MLPA) is a recently developed technique for the relative quantification of DNA sequences that can detect chromosomal deletions or amplifications (4,7,9,14). The principle of MLPA is that two DNA oligonucleotides are directly adjacent hybridized to their complementary target sequences on the template DNA, followed by ligation of these two oligonucleotides. One oligonucleotide contains a target specific part with an M13 forward tail, the second probe contains a target specific part linked to a variable

length stuffer sequence and an M13 reversed tail. The two oligonucleotides can only be ligated together if both target specific parts are hybridized adjacently to their DNA template. PCR is then performed making use of the M13 primer sequences on the ligation product. PCR can only start after successful adjacent hybridization and ligation of both oligonucleotides, which ensures specificity of probe amplification. MLPA is thus characterized by PCR performed on the ligated two oligonucleotides (forming the probe) and not on the template DNA. The amount of ligated probes is related to the number of specific primer binding sites, making this method suitable for the detection of chromosomal deletions or amplifications.

MLPA has several advantages over currently used techniques: 1) up to 40 genomic loci can be analyzed in one reaction (9), 2) paired non-neoplastic tissue from the same patient is not needed and there are no non-informative loci, 3) requires only small amounts of DNA, (20ng is sufficient for 1 reaction in which 40 loci can be tested) (9), 4) is probably less sensitive to DNA degradation because probe target sequences are small (50-70bp) (9).

To the best of our knowledge, there are no reports about the feasibility of MLPA for the detection of 1p/19q deletions in oligodendroglial tumors (OG). The objective of the present study was to develop an MLPA based assay for the detection of 1p/19q chromosomal deletions in formalin fixed, paraffin embedded OG and to test the validity and reproducibility of this assay.

Materials and methods

Patients

Nineteen OG from 19 patients were analyzed. From all tumors FISH data were available. The tissue was obtained by stereo tactic biopsy or surgical excision and routinely formalin fixed (around 16 hours) and paraffin embedded (4-11 years). All tumors received a histological diagnosis according to the WHO criteria (JMK) (Table 2). Tumor percentages in isolated tissue were above 75% in 15 tumors, 50-75% in 3 tumors and around 50% in one tumor. Control brain tissue was obtained from microscopically unaffected brain areas of 3 of the above patients with an OG, 6 autopsy brains and 1 lymph node (5 of these autopsy brains were without abnormalities and 1 brain was from a patient with hereditary cerebral hemorrhage with amyloidosis, Dutch type). Autopsy brains were fixed for 24 hours (3X), 3 days (1X) or unknown (2X).

DNA isolation

Guided by the HE section, tumor or non-neoplastic tissue was punched from the paraffin embedded tissue blocks with a tissue array needle (Beecher Instruments, Silver Spring, MD) and deparaffinized by 2x 15 minutes incubation in xylol, followed by 2x 15 minutes incubation in ethanol. Tissue cores were resuspended in 128 μL of PK-1 lysis buffer [50 mM KCl, 10 mM Tris (pH 8.3), 2.5 mM MgCl2, 0.45% NP40, 0.45% Tween 20, 0.1 mg/mL gelatine] containing 5% Chelex beads (Biorad, Herculaes, USA) and 100 μg of proteinase K. The suspension was

Table 1. MLPA and FISH probes included in the analysis.

Probeset	Cyto pos	Map-view 31	probe number	PCR product length
FLJ10782-D01-247-M	01p36.33	01-002.45	16	247
FISH-probe D1S32	01p36			
TP73-D01-256-M	01p36	01-003.6	17	256
TNFRSF1B-D01-166-M	01p36.3	01-012.1	7	166
MUTYH-D02-310-M	01p34.3-1p32.1	01-044.8	23	310
BCAR3-D01-418-M	01p13.2	01-093.4	35	418
F3-D01-139-M	01p22-p21	01-094.2	2	139
BCAS2-D01-400-M	01p13.3	01-114.0	33	400
FISH-probe PUC 1.77	01cen			
TANK-D01x-220-M	02q24	02-160.6	13	220
CHL1-D01-265-M	03p26	03-000.3	18	265
MLH1-D13-355-M	03p21.3	03-036.3	28	355
MLH1-D17-436-M	03p21.3	03-036.3	37	436
APC-D09B-283-M	05q21-q22	05-112.6	20	283
APC-D01-175-M	05q21-q22	05-112.6	8	175
IL4-D01-154-M	05q31.1	05-132.5	5	154
IL12B-D01-382-M	05q31.1-q33.1	05-159.3	31	382
RAD54B-D01-292-M	08q21.3-q22	08-095.3	21	292
RB1-D03-193-M	13q14.3	13-043.0	10	193
RB1-D17B-160-M	13q14.3	13-043.0	6	160
FISH-Probe BAC2310A1	19p13			
BAX-D01-301-M	19q13.3	19-049.8	22	301
BAX-D02-211-M	19q13.3	19-049.8	12	211
FISH-probe BAC127F23	19q13			
KLK3-D02-391-M	19q13	19-051.7	32	391
LOC125905-D01-450-M	19q13.43	19-059.4	38	450

incubated for 12 h at 56 °C, 10 min at 100 °C and after 10 minutes centrifugation at 13.000g, the DNA containing supernatant was collected.

DNA quality

DNA concentration was measured with PICO green (Molecular Probes Europe BV, Leiden, the Netherlands) according to the manufacturers protocol. DNA concentration ranged from 5.2 – 154ng/ul, median 9.9 ng/ul. For testing tumor DNA quality, a PCR reaction was performed on P53 exon 8 generating a 239-basepair product in controls. The primer sequences were: ex8-forward, GTA GGA CCT GAT TTC CTT ACT GCC TCT TGC, ex8-reversed ATA ACT GCA CCC TTG GTC TCC TCC ACC GC. Each reaction was performed in 25 μL using AmpliTaq Gold ™ at 94 °C for 10 minutes initial denaturation followed by 35 cycles of 94 °C for 45 s, 57°C for 30 s and 72°C for 1 min. Products were analyzed on a 2% agarose gel to confirm size and quantity (data not shown).

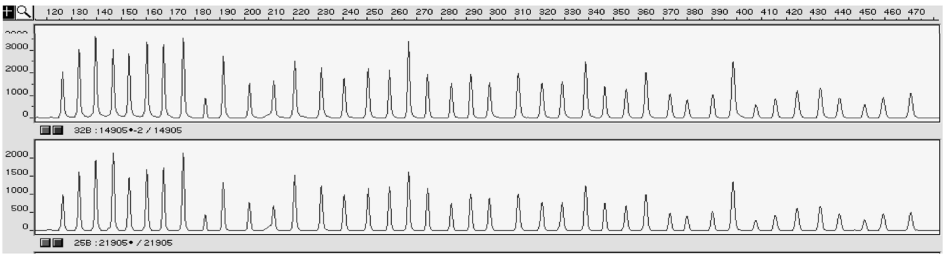
MLPA detection

MLPA has previously been described (9). In brief, MLPA is based on the ligation of two DNA oligonucleotides that hybridize adjacently to DNA target sequence. The first oligonucleotide was synthesized with an on average 26bp (min: 21bp, max: 39bp) target specific part and a universal M13-forward tail. The second oligonucleotide was an M13-derived single stranded DNA containing an, on average, 42bp (min: 31bp, max: 50bp) target specific part, a stuffer sequence of variable length (130-480 base pairs) and an M13-reversed tail. Thus, a probe consists of two oligonucleotides of which the target specific parts hybridize adjacently and ligate. The M13 forward and reversed tails attached to all probes and the different length of each probe made it possible to perform a single primer multiplex PCR.

Twenty-two probes of the 40 probes in the kit were included for analysis and selected on the basis of localization on 1p or 19q (focus probes), likelihood to be unaltered in OG (1) (reference probes) and performance (Table 1). The MLPA kit was assembled by MRC-Holland (Amsterdam, the Netherlands). Details of MLPA and probes can be found at http://www.mlpa.com.

After denaturing 15-250ng DNA for 5 minutes at 95°C, the probe mix, containing all probe sets, was added. After overnight hybridization at 60°C the hybridized probes were ligated for 15 minutes at 54°C with a DNA-ligase. An aliquot was taken out of the ligation mix and the ligated products were amplified in a multiplex PCR with forward and reverse M13 primers for 20 sec at 95°C, 30 seconds at 60°C and 60 seconds at 72°C for 33 cycles in an Applied Biosystems® 9700 PCR machine. After PCR 3 μL of the PCR products were mixed with 1 μL 500 TAMRA (Applied Biosystems ®) internal size marker and 20μL deionised formamide and injected for 5 seconds in an ABI310® capillary filled with POP5 polymer. After a 30 minutes run the data were collected and analyzed with Genescan analysis and Genotyper software (Applied Biosystems®) (Figure 1). A Genotyper output file was generated combining probe set number, size and peak heights. This table was exported to a database where probe annotation is added to the data table. Subsequently normalization and diagnosis of the profiles were performed.

A



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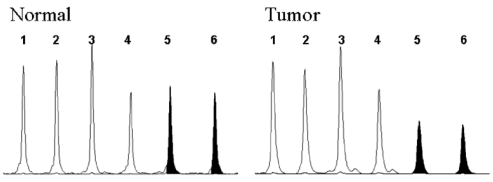

Probeset	Gene Name	Chr pos
1	CHL1	03p26
2	CDKN2A	09p21
3	APC	05q21-q22
4	RAD54B	08q21.3-q22
5	BAX	19q13.3
6	MUTYH	1p32.1

Figure 1. Examples of ABI electrophoresis signals in MLPA. A: Two control DNA samples showing reproducibility of MLPA patterns of all 40 probes in the kit, of which 22 were selected for analysis. Peakheight differences for the different loci are due to probe length (smaller probes give higher peaks/signals due to more efficient PCR) and differences in binding kinetics for each oligonucleotide. Differences in absolute peakheights between samples are probably due to differences in DNA concentration. B: Fragment of an MLPA pattern showing decreased peak heights of probes on 1p and 19q in an oligodendroglioma, indicating chromosomal loss.

Data analysis for MLPA

Normalization

Because MLPA traces analyzed with Genescan and Genotyper are not calibrated, raw data files had to be normalized. Peak heights were dependent on sample quality, DNA concentration, hybridisation parameters and instrument settings. Also peaks from different probe sets differed in magnitude in a systematic way (Figure 1). To calibrate the data, we used the following algorithm:

1. Distinguish focus probes (seven and four loci on chromosome 1p and 19q respectively) and reference probes (11 loci usually unaltered in OG).
2. Select the reference probes from the control (non-tumour) samples. Perform steps 3 to 5 with this subset of data.
3. Within each sample divide all peak heights by the median peak height of the concerning sample. This is to correct for the sample-to-sample variation.
4. Within each probe, divide all peak heights by the median peak height of the concerning probe. This is to correct for systematic differences between probes. The result of 3 and 4 we call normalized peak heights.
5. Determine which (reference) probes are most stable. Subtract 1 from each normalized peak height and take the absolute value. Compute the median of these numbers for each probe. This is the median of the absolute deviations: MAD.
6. Select the 5 reference probes with the lowest MAD. These 5 reference probes are named calibration probes and are used to normalize the complete experiment as described in step 7 and 8.
7. Within each sample (OG and normal control samples) divide all peak heights (focus and all reference probes) by the median peak height of the five calibration probes of the concerning sample. This is to correct for the sample-to-sample variation.
8. Within each probe (focus and reference probes), take the median peak height of the control samples. Then, within each probe (focus and reference probes) divide all peak heights (OG and normal control samples) by the median peak height of the concerning probe. This is to correct for systematic differences between probes.

Computing was initially performed in Matlab (The Mathworks inc., Natick, MA, USA). A Windows analysis interface was constructed using Delphi. The application is available on request (J.Oosting@lumc.nl).

Data visualization and interpretation.

Each experiment was normalized and analyzed separately. Heatmaps were generated for each experiment using Matlab (Figure 2) and scatter plots for each individual Tumor and Normal were generated in Matlab (Figure 3) and anonymized. Two authors (SvD an RN) independently decided if a scatter plot showed a deletion or not. The principle decision rule for a deletion was that for 1p at least 4 probes and for 19q at least 2 focus probes had normalized peak heights at least 0.25 below the median normalized peak height of the reference probes.

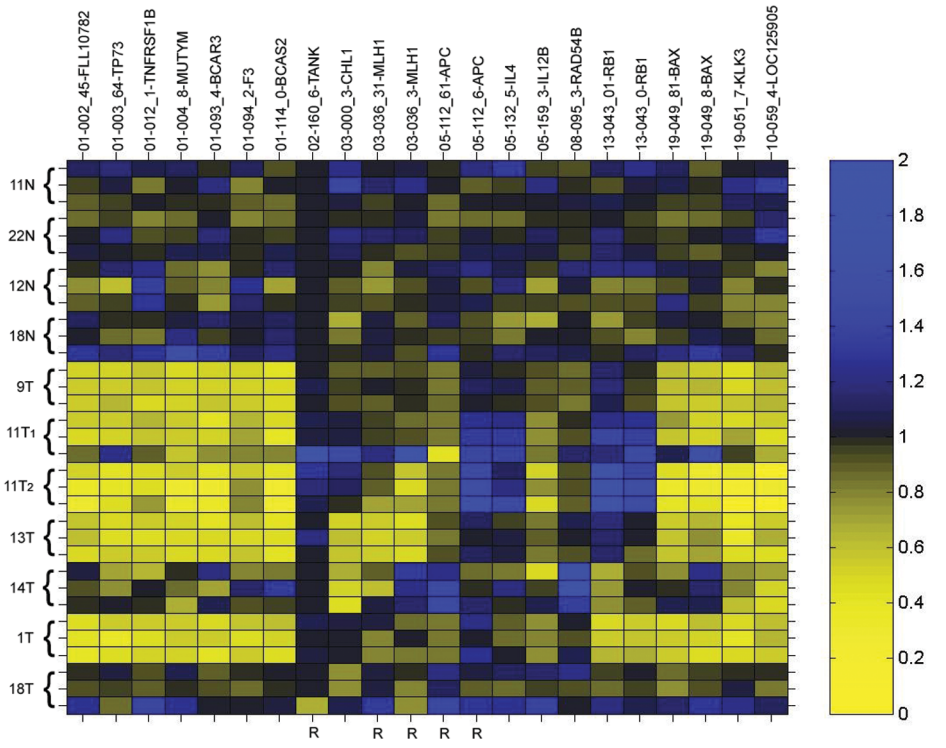

Figure 2. Heatmap representing an overview of a complete experiment. On the Y-axis samples are shown. The X-axis shows the different probes in chromosomal order. R indicates the 5 probes with least variance the Normal samples, and these were used for normalization. Chromosomal loss (yellow) or gain (blue) is shown.

FISH

The 1p and 19q status of all 19 OG was determined with fluorescence in situ hybridization on paraffin embedded, archival material using locus specific probes as earlier described (13). The cut off value to decide whether a 1p or 19q deletion was present or absent was 0.8.

Results

DNA quality was checked by a standard 239bp PCR, which was negative in 3 tumor and 2 control samples and weak in 4 tumor and 3 control samples. However, MLPA showed reproducible peak heights in these samples (Table 2). Electrophoresis was repeated once or twice in each experiment and gave highly reproducible results (Figure 2 and 3).

In 15 OG and 5 controls 2 to 4 experiments were performed. For all samples the different experiments showed consistent results, with little variation in the amount of focus probes with a normalized peak height of at least 0.25 below the median of the reference probes (Table 2).

In 18/19 OG, results of MLPA and FISH were concordant for presence or absence of a 1p deletion. In 15/19 OG, results of MLPA and FISH were concordant for a 19q deletion. In 3 OG, MLPA detected a 19q deletion, which was not shown by

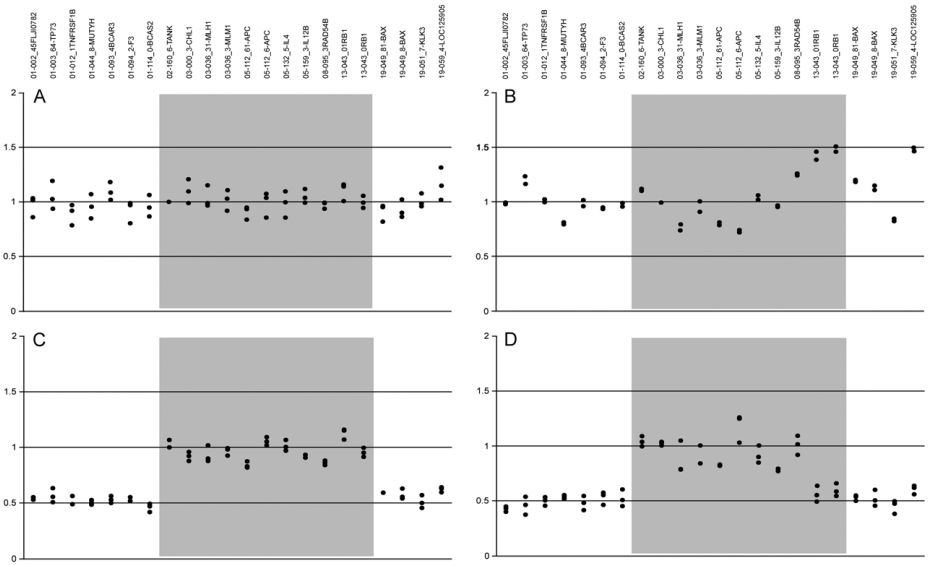

Figure 3. Representative scatterplots of normalized peakheights. Each plot represents one sample from one experiment with electroforesis in duplicate or triplicate. On the left in white 1p, on the right in white 19q, in grey the reference probes. A: Non-neoplastic brain tissue. B: Oligodendroglioma with FISH 0.99 for 1p and FISH 0.95 for 19q. C: Oligodendroglioma with FISH 0.59 for 1p and FISH 0.57 for 19q. D: Oligodendroglioma with FISH 0.54 for 1p and FISH 0.58 for 19q.

Table 2. MLPA and FISH results. PCR= standard polymerase chain reaction to test DNA quality, see “Methods”. Exp=number of MLPA experiments. N=non-neoplastic brain tissue. O=oligodendroglioma, AO=anaplastic oligodendroglioma. OA=oligoastrocytoma. AOA=anaplastic oligoastrocytoma. ret/del= retention/deletion of chromosome1p/19q. The principle decision rule for a deletion was that for 1p at least 4 (out of 7) focus probes and for 19q at least 2 (out of 4) probes had normalized peak heights at least 0.25 below the median normalized peak height of the reference probes. Numbers between brackets state for each MLPA experiment the number of focus probes with a normalized peak height of at least 0.25 below the median normalized peak height of the reference probes.

								MLPA/FISH concordant	
DNA-ID	Tissue	PCR	exp	MLPA 1p	MLPA 19q	FISH 1p	FISH 19q	1p	19q
109N	N	pos	1	ret (0)	ret (0)				
111N	N	pos	2	ret (0-0)	ret (0-0)				
113N	N	pos	1	ret (0)	ret (0)				
114N	N	pos	1	ret (0)	ret (0)				
115N	N	neg	1	ret (0)	ret (0)				
116N	N	weak	1	ret (0)	ret (0)				
122N	N	pos	2	ret (0-0)	ret (0-0)				
13N	N	weak	4	ret (1-0-0-0)	ret (0-0-0-0)				
15N	N	neg	3	ret (0-0-2)	ret (0-1-0)				
18N	N	weak	4	ret (1-0-0-0)	ret (0-0-0-0)				
1T	AO	pos	3	del (7-7-7)	del (4-4-4)	0.54	0.58	+	+
2T	OA	neg	1	ret (0)	ret (0)	1	1.05	+	+
3T	AO	pos	2	del (7-7)	del (4-4)	0.66	0.78	+	+
4T	AO	pos	2	del (7-7)	del (3-3)	0.67	0.75	+	+
5T	AO	neg	2	del (4-5)	del (2-2)	0.46	0.85	+	-
6T	AO	pos	2	del (7-7)	del (4-3)	0,68	0,60	+	+
7T	AO	pos	2	ret (0-0)	ret (0-0)	0.99	0.95	+	+
8T	AO	weak	1	ret (1)	ret (0)	1.06	0.89	+	+
9T	AO	pos	3	del (7-7-7)	del (4-4-4)	0.59	0.57	+	+
10T	AO	pos	2	ret (0-0)	ret (0-1)	1.06	1.04	+	+
11T	AO	pos	4	del (5-7-7-7)	del (4-4-4-4)	0.73	0.68	+	+
12T	AO	pos	2	del (7-7)	del (4-4)	0.67	0.64	+	+
13T	AO	weak	3	del (6-7-6)	del (3-4-4)	0.74	0.85	+	-
14T	OA	neg	3	ret (0-0-3)	ret (0-2-0)	1.02	1.06	+	+
15T	AOA	weak	1	ret (1)	ret (0)	0.95	0.97	+	+
16T	AO	pos	1	del (4)	del (3)	0.42	1.13	+	-
17T	AO	pos	2	del (4-7)	del (2-3)	0.66	0.59	+	+
18T	O	pos	3	ret (0-0-0)	ret (0-0-0)	0.73	0.79	-	-
19T	AO	pos	2	del (7-7)	del (4-4)	0.66	0.57	+	+

FISH. These 3 OG carried a 1p deletion according to both FISH and MLPA. In two of these samples, FISH results were close to the cut off value of 0.8. Only one sample showed inconsistent MLPA- and FISH results for both 1p and 19q status. This sample contained 50-75% tumor cells and the FISH results for 19q were close to the cut off value (Table 2, tumor 18T). None of the controls showed 1p/19q deletions. The amount of focus probes with a normalized peak height of at least 0.25 below the median of the reference probes is shown for each DNA sample and each experiment in Table 2. If not all focus probes in tumor samples with a deletion showed a normalized peak height of at least 0.25 below the median of the reference probes there was often a trend towards this value. The study was not designed for, and the data did not permit, the identification of breaking points.
The 3 tumor DNA's with negative control PCR all showed reproducible MLPA results consistent with FISH (Table 2).

Discussion

We showed that MLPA is a valid, reproducible, fast and simple method for the detection of physical 1p/19q deletions in DNA isolated from formalin fixed, paraffin embedded brain tissue. To the best of our knowledge, the use of MLPA for the detection of 1p/19q chromosomal loss in OG has not been described before.
MLPA is less limited by poor DNA integrity than conventional PCR because the probe target sequences (the combined binding specific domains of the 2 ligated oligonucleotides) are only 50-70bp (9). In the present study the use of formalin fixed, paraffin embedded material, even when stored for over 10 years (2 samples 11 years, 1 sample 10 years) and with only little amounts of DNA (as little as 15ng in the present series) yielded satisfactory results. Moreover, MLPA appeared to be more sensitive than conventional PCR. MLPA gave reproducible peak heights in 2 control- and 3 tumor DNA's with negative conventional PCR, normally generating a 239bp product.
MLPA is probably less sensitive to variations in DNA quality between samples than other techniques because, from the first round of amplification, ligated probes are amplified and not template DNA. Furthermore, the variable length of stuffer sequences in one of the probes permits the performance of multiplex PCR (40, and potentially more, reactions in one well), enabling the analysis of a large number of loci in a highly efficient way.
As compared to FISH, MLPA is performed in shorter time, is less elaborate and less dependent on individual interpretation. In addition, only one marker per experiment can be assessed using FISH. Tracing of chromosomal losses in individual tumor cells, for instance in infiltration margins of gliomas, is possible using FISH, but in the majority of studies FISH is used for detection of chromosomal aberrations in fields of tumor cells, not individual cells. For MLPA probably the majority of tissue needs to consist of tumor cells. In the present study we analyzed one tumor (tumor 18, table 2) with 50-75% of preexistent cells and were not able to detect a 1p or 19q deletion shown by FISH, although with borderline values of significance.

MLPA certainly has advantages over QUMA or LOH detection by microsatellite analysis. From the first round of amplification, PCR is performed on ligated probes instead of template DNA making PCR conditions more comparable between samples. Furthermore, MLPA is likely to work better on paraffin embedded material because probe target sequence (50-70bp) is smaller than the product lengths used in QUMA (in which expected product lengths length varied between 81-93bp and 145-157bp among the different probes) (6) or LOH (in which expected product lengths length varied between 69-110bp and 130-185bp among the different probes) (5). In addition, extended multiplexing with 40, and potentially more, loci in on reaction is possible, which is difficult with LOH detection by standard microsatellite markers or QUMA. To test the same amount of loci in conventional LOH or QUMA analysis is more elaborate and takes much more DNA. For QUMA it was reported that at least 15ng DNA per locus was required (6). In the present study, only 15ng of DNA was enough to determine the relative amount of DNA at all 22, and potentially more, loci. An additional major advantage of MPLA over LOH detection with microsatellite markers is that patient-matched normal DNA is not necessary. Any normal DNA can be used as a control. This is especially relevant for brain tumor biopsies and excisions in which 'normal' surrounding tissue is either not available or often contains variable amounts of infiltrating tumor cells.
We tested and validated a MLPA based assay for the detection of 1p/19q deletions in formalin fixed, paraffin embedded brain tissue. MLPA enables the introduction of many more probes in one reaction than presented in the present paper, potentially enabling the routine analysis of all clinically relevant or scientifically interesting chromosomal deletions and amplifications of oligodendrogliomas, oligo-astrocytomas and astrocytomas in one assay. It is likely that this information will improve classification of these tumors, enable a more reliable estimation on prognosis and guide therapeutic decisions. The present results suggest that MLPA on paraffin embedded tissue may also be used for clinically or scientifically relevant chromosomal deletions or amplifications in other malignancies like HER-2/neu amplification in breast cancer.

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