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Methylation-Specific MLPA (MS-MLPA): simultaneous detection of CpG methylation and copy number changes of up to 40 sequences

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ABSTRACT

Copy number changes and CpG methylation of various genes are hallmarks of tumor development but are not yet widely used in diagnostic settings. The recently developed multiplex ligation-dependent probe amplification (MLPA) method has increased the possibilities for multiplex detection of gene copy number aberrations in a routine laboratory. Here we describe a novel robust method: the methylation-specific MLPA (MS-MLPA) that can detect changes in both CpG methylation as well as copy number of up to 40 chromosomal sequences in a simple reaction. In MS-MLPA, the ligation of MLPA probe oligonucleotides is combined with digestion of the genomic DNA–probe hybrid complexes with methylation-sensitive endonucleases. Digestion of the genomic DNA–probe complex, rather than double-stranded genomic DNA, allowed the use of DNA derived from the formalin treated paraffin-embedded tissue samples, enabling retrospective studies. To validate this novel method, we used MS-MLPA to detect aberrant methylation in DNA samples of patients with Prader–Willy syndrome, Angelman syndrome or acute myeloid leukemia.

INTRODUCTION

Alterations of DNA methylation patterns have been recognized as a common change in human cancers. Aberrant methylation of normally unmethylated CpG-rich areas, also known as CpG islands, which are located in or near the promoter region of many genes, have been associated with transcriptional inactivation of important tumor suppressor genes, DNA repair genes and metastasis inhibitor genes (1,2). Therefore, the detection of aberrant promoter methylation of cancer-related genes may be essential for diagnosis, prognosis and/or detection of metastatic potential of tumors. As the number of genes known to be hypermethylated in cancer is large and increasing, sensitive and robust multiplex methods for the detection of aberrant methylation of promoter regions are therefore, desirable. In addition, the amount of DNA available for large-scale studies is often limited and of poor quality since this DNA is isolated from the formalin treated, paraffin-embedded tissues that have been stored at room temperature for years.

Most current approaches for the detection of methylation are based on the conversion of unmethylated cytosine residues into uracil after sodium bisulphite treatment (3), which are converted to thymidine during subsequent PCR. Thus, after bisulphite treatment, alleles that were originally methylated have different DNA sequences as compared with their corresponding unmethylated alleles. These differences can be exploited by several techniques, such as methylation-specific PCR (MSP), restriction-digestion (COBRA), Methylight, direct sequencing, denaturing high-performance liquid chromatography (DHPLC), nucleotide extension assays (MS-SnuPE), methylation-specific oligonucleotide (MSO) microarray and HeavyMethyl (3–10). However, most of these methods are labor intensive and/or allow the study of the methylation status of only one gene at a time. In addition, most of these techniques are not suitable to study large numbers of paraffin-embedded tissue samples.

The recently developed multiplex ligation-dependent probe amplification (MLPA) technique (11) has been accepted as a simple and reliable method for multiplex detection of copy number changes of genomic DNA sequences using DNA samples derived from blood (12–16), amniotic fluid (17) or tumors (18). Here, we describe a rapid and easy method to apply MLPA based method, methylation-specific MLPA (MS-MLPA) for the detection of changes in methylation status as well as copy number changes of up to 40 selected sequences

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in a reaction using only 20 ng of DNA. The general outline of this method is shown in Figure 1. Similar to a conventional MLPA assay (11) genomic DNA is first denatured, followed by the addition of MS-MLPA probes and a 16 h hybridization step. Subsequently, this probe–DNA complex is simultaneously ligated and digested by methylation-specific enzymes. If the CpG site is methylated, a normal MLPA product will be detected. If the CpG site is not methylated, the DNA–probe complex will be digested by the methylation-sensitive enzyme and no amplification product is formed. The MS-MLPA method described here extends the MLPA method for multiplex copy number quantification to a method for simultaneous analysis of the copy number, as well as the methylation status of up to 40 sequences in a simple reaction.

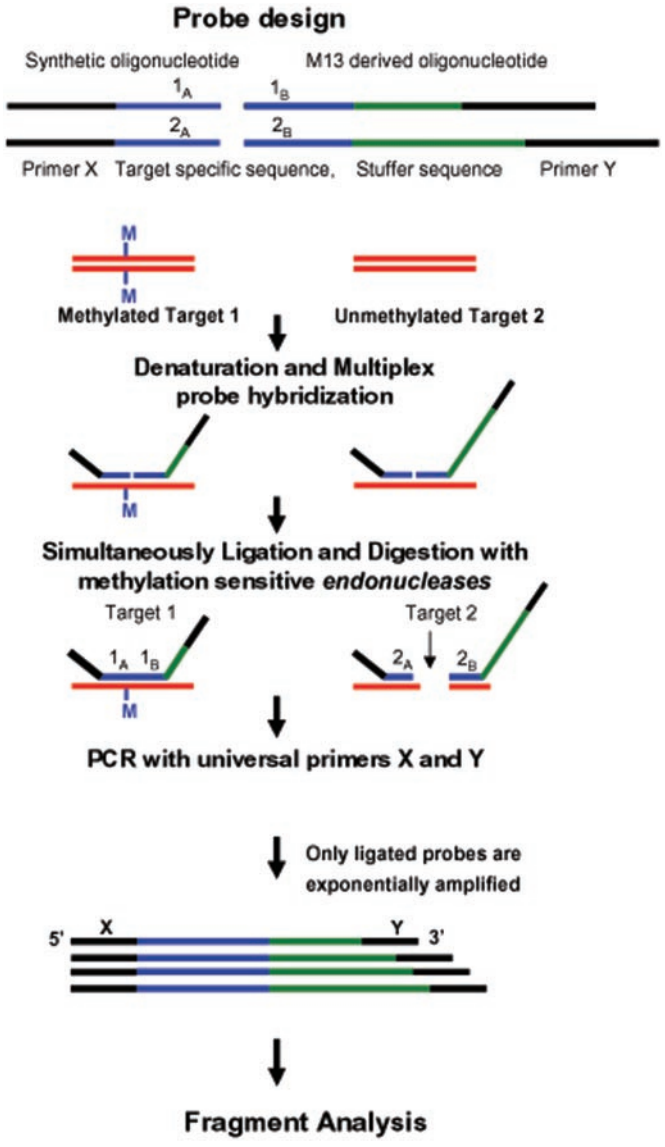

Figure 1. Outline of the MS-MLPA procedure. An ordinary MLPA probe harbors two oligonucleotides, one short synthetic and one long M13-derived oligonucleotide and up to 50 probes can be added to each MLPA reaction. Both oligonucleotides contain universal primers sites. For each MLPA probe, the M13 oligonucleotide is cloned in a M13-vector that contains stuffer sequence that varies in length between the different probes. Subsequently, these long M13-oligonucleotides are obtained by restriction-digestion from the M13 clones. For MS-MLPA, the probe design is similar to an ordinary MLPA probe except that the sequence detected by the MS-MLPA probe contains a recognition sequence for HpaII or HhaI. Upon digestion of the DNA/MS-MLPA probe complex with one of the methylation-sensitive enzymes, probes of which the recognition sequence is methylated will generate a signal. If the CpG site is unmethylated the genomic DNA–MS-MLPA probe complex will be digested and prevent exponential amplification and no signal will be detected after fragment analysis.

In this study, we demonstrate the use of the MS-MLPA assay on DNA samples from Prader–Willy syndrome (PWS) and Angelman syndrome (AS) patients and on DNA samples derived from acute myeloid leukemia (AML) cell lines. Furthermore, we show that this newly developed technique can also be applied successfully to DNA derived from paraffin-embedded tissues.

METHODS

DNA samples

DNA samples of 16 anonimized patients diagnosed with PWS or AS were kindly provided by Ans van den Ouweland (Erasmus MC, Rotterdam, The Netherlands).

Genomic DNA was isolated from 21 AML cell lines of patients that had high blast counts. Tumor DNA samples, either paraffin-embedded or fresh-frozen, were kindly provided by Petra Nederlof (Netherlands Cancer Institute, NKI-AvL, Amsterdam, The Netherlands).

Methylated DNA was obtained by treating human genomic DNA (Promega) with HhaI methylase (New England Biolabs) in the presence of *S*-adenosylmethionine according to the manufacturer's instructions.

Paraffin-embedded DNA extraction

Slides with a slice of paraffin-embedded tissue (5 mm × 5 mm, 10 μm of thickness) were heated for 15 min at 75°C to melt the paraffin. The hot slides were placed in Xylol for 5 min. This was repeated until the paraffin oil was completely dissolved. The slides were then incubated for a period of 30 s in 99, 96 and 75% ethanol, tap water and finally placed in 1 M NaSCN at 37°C overnight. The next day the slides were washed with TE buffer [10 mM Tris–HCl (pH 8.5) and 1 mM EDTA] and air dried. A few drops (20–40 μl) Proteinase K solution [2 mg/ml recombinant Proteinase K (Roche) in 25 mM Tris–HCl (pH 8.2)] were applied in the tissue. The tissue was transferred to a 1.5 ml tube containing 100 μl Proteinase K solution and incubated overnight at 37°C. After 20 min of incubation at 80°C to inactivate the Proteinase K, the tubes were centrifuged for 10 min at 13 000 rpm on an Eppendorf microcentrifuge. Finally, 2 μl of the supernatant was used for each MS-MLPA reaction.

MLPA probe design

The design of the MS-MLPA probes was performed as described by Schouten *et al.* (11). However, each probe used in this study for methylation quantification analysis contained one HhaI restriction site in the target recognition sequence. In this study three probe mixtures were developed, the P028 PWS/AS, the P041A and the P041B mixture. The P028 PWS/AS probe mixture contains 25 probes specific for most of the genes in the PWS/AS critical region of chromosome 15q11–q13 and two probes for genes that are located

outside this region. Among the probes in the critical region 10 probes contained a HhaI recognition site. Furthermore, 14 control MLPA and MS-MLPA probes are included that are not specific to genes on chromosome 15 (Supplementary Table 1).

The methylation mixture, P041A, contains a panel of 41 probes specific to 22 tumor suppressor genes (Supplementary Table 2). The mixture contained for 19 genes in a single probe that detects a HhaI sequence within the promoter region of these genes. For *VHL* and *CDKN2A*, two probes are included. For the promoter region of *MLH1*, three probes are included. The remaining 15 probes in this mixture lack a HhaI sites in their recognition sequence and serve as control probes. These probes are used for quantification of the methylation levels. The P041B mixture contains MS-MLPA probes for the same genes detected by the P041A mixture, except that these probes recognize a different CpG site in the corresponding promoter regions. Details on probe sequences, gene loci and chromosome locations can be found at www.mlpa.com.

MS-MLPA assay

MLPA reagents were obtained from MRC-Holland, Amsterdam, The Netherlands (EK1 kit; www.mlpa.com). Approximately 25 ng of genomic DNA in 5 µl of TE buffer [10 mM Tris–HCl (pH 8.5) and 1 mM EDTA] was denatured for 10 min at 98°C. SALSA MLPA buffer (1.5 µl) and MS-MLPA probes (1 fmol each and 1.5 µl vol) were then added and after incubation for 1 min at 95°C, were allowed to hybridize to their respective targets for ~16 h at 60°C. After hybridization, the mixture was diluted at room temperature with H_2O and 3 µl Ligase buffer A to a final volume of 20 µl and then equally divided in two tubes. While at 49°C a mixture of 0.25 µl Ligase-65 (MRC-Holland), 5 U HhaI (Promega) and 1.5 µl Ligase buffer B in a total volume of 10 µl was added to one tube. The second tube was treated identical except that the HhaI enzyme was replaced with H_2O. Simultaneous ligation and digestion was then performed by incubation for 30 min at 49°C, followed by 5 min heat inactivation of the enzymes at 98°C. The ligation products were PCR amplified by the addition of 5 µl of this ligation mixture to 20 µl PCR mixture containing PCR buffer, dNTPs, SALSA polymerase and PCR primers (one unlabeled and one D4-labeled) at 60°C as described by Schouten *et al.* (11).

Fragment and data analysis

Automated fragment and data analysis was performed by exporting the peak areas to an excel based analysis program. In brief, for copy number quantification, every sample peak area was divided by the nearest control peak areas. Relative copy number was obtained by comparing this ratio with the same ratio obtained from a control sample. Quantification of the methylation status of a particular CpG site was done by dividing the peak area with the combined areas of the control probes lacking a HhaI site. Finally, the relative peak area of each target probe from the digested sample was compared with those obtained from the undigested sample. Aberrant methylation was scored when the calculated methylation percentage was >10%. Any methylation percentages below this level were regarded as background. All MS-MLPA reactions were performed at least three times.

MSP and bisulphite sequencing

The DNA samples were converted by sodium bisulphite using the EZ DNA Methylation Kit (Baseclear, The Netherlands) following the manufacturer's instructions. The modified DNA was amplified using methylated and unmethylated specific primers to amplify the same fragment within the promoters of *SNRPN* and *p15* as the respective MS-MLPA probes. The PCR conditions were for reactions: a denaturation step at 95°C for 5 min; 32 cycles at 95°C for 40 s, 65°C for 30 s, 72°C for 60 s. Finally, the PCR products were visualized on a 2.5% agarose gel.

For SNRPN the following methylated primers used were 5′-CGCGGTCGTAGAGGTAGGTTGGCGC and 5′-GACACAACTAACCTTACCCGCTCCATCGCG resulting in a 167 bp product.

For the unmethylated reaction the following primers were 5′-GTATGTTTGTGTGGTTGTAGAGGTAGGTTGGTGT and 5′-CACCAACACAACTAACCTTACCCACTCCATCACA resulting in a 180 bp product.

For MSP of p15 the following methylated primers used were 5′-GAAGGTGCGATAGTTTTTGGAAGTCGGCGC and 5′-GACGATCTAAATTCCAACCCCGATCCGCCG resulting in a 160 bp product. For the unmethylated reaction the following primers were 5′-GTGGAGAAGGTGTGATAGTTTTTGGAAGTTGGTGT and 5′-CATCAACAATCTAAATTCCAACCCCAATCCACCA resulting in a 169 bp product.

For bisulphite sequencing of the *p15* gene the following primers were used to amplify a 291 bp of the promoter region including the target sequences recognized by the p15 MS-MLPA probe: p15-forward 5′-TAGGTTTTTTAGGAAGGAGAGAGTG-3′ and p15-reverse 5′-CCTAAAACCCCAACTACCTAAATC. Subsequently, the nested forward primer 5′-AGGAGAATAAGGGTATGTTTAGTGG-3′ was used for sequencing.

RESULTS

MS-MLPA with PWS and AS

To validate MS-MLPA, we used the P028 PWS/AS probe mixture to analyze DNA samples of patients with PWS and AS. These syndromes are distinct neurogenetic disorders, which are characterized by deletions or uniparental disomy resulting in aberrant expression of genes located in the imprinted region on chromosome 15q11–q13. Absence of a paternal contribution of chromosome 15q11–q13, due to hemizygous deletion or uniparental disomy, results in PWS. The absence of the corresponding maternal copy of the same region causes AS (19). Among the probes in the P028 PWS/AS mixture seven probes are specific to the *SNRPN* gene, which is located in the imprinting center. Five are MS-MLPA probes containing a HhaI restriction site. If the site is not methylated, HhaI digestion will prevent exponential amplification of the MS-MLPA probe (Figure 1). Patient with AS due to uniparental disomy harbor two unmethylated alleles and accordingly no MS-MLPA signal is observed (Figure 2a). DNA from a patient with the PWS syndrome due to uniparental disomy shows no differences in peak areas of the *SNRPN* specific MS-MLPA probes between the digested and undigested sample DNA (Figure 2b), indicating that all

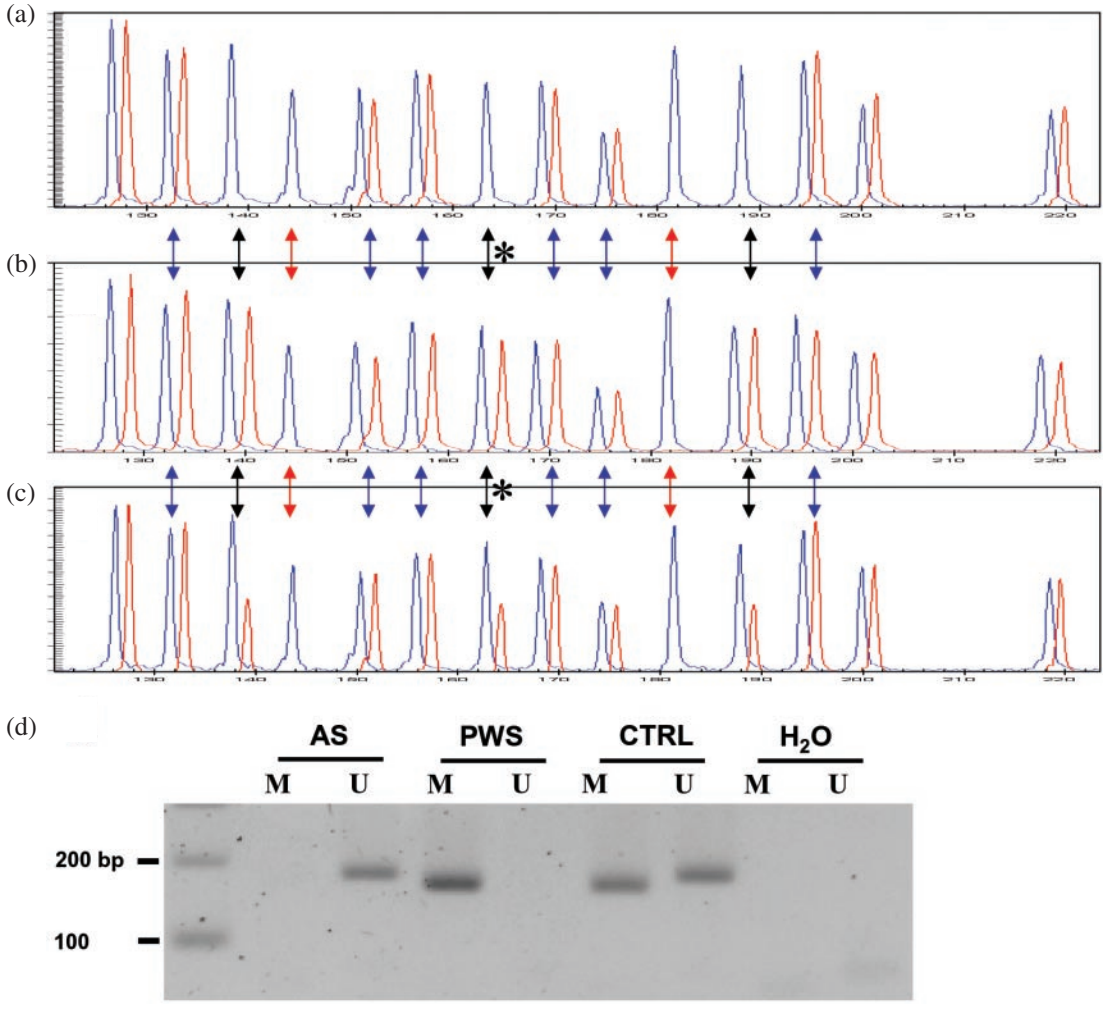

Figure 2. Detection of the methylation status of the imprinting center in chromosome 15 by MS-MLPA. Approximately 100 ng of DNA from patients diagnosed with either PWS or AS and control DNA from healthy persons was subjected to MS-MLPA using the P028 PWS/AS probe mixture. Only a part of the capillary electrophoresis (CE) pattern is shown. Blue signals correspond with undigested DNA. Red signals correspond with the same samples but digested with HhaI. Black arrows indicate three different HhaI sites in the promoter region of the *SNRPN* gene. The asterisks indicate the probe for which MSP was performed. Red arrows indicate two HhaI sites; one within the chromosome 15q11 imprinted center and one outside that are not methylated and serve as controls for proper digestion. Blue arrows correspond with other probes located in the chromosome 15q11 region without a HhaI site that serves as a control for the copy number quantification. (**a**) CE-pattern from an AS patient which has both alleles unmethylated therefore, after MS-MLPA no signal is generated from the MS-MLPA probes. (**b**) CE-pattern generated from a PWS patient. Patients diagnosed with PWS due to uniparental disomy inherit only the maternally methylated allele in the promoter of the *SNRPN* gene, thus both alleles are methylated and therefore will generate a normal signal. (**c**) CE-pattern from control DNA. Normal individuals have one methylated and one unmethylated allele, thus a 50% reduction of the signal is seen. (**d**) MSP results on the three samples from above confirming the MS-MLPA results for the region recognized by the 166 bp SNRPN promoter specific MS-MLPA probe.

CpG sites are methylated. DNA of control individuals shows a 50% reduction of the MS-MLPA signal, corresponding to the presence of one methylated allele (Figure 2c).

Aberrant methylation in AML cell lines

AML is a heterogeneous disorder with respect to morphology and chromosome aberrations detected in the leukemic cells. Various genes known to be silenced by promoter methylation have been analyzed in AML. Frequent aberrant promoter methylation of the tumor suppressor genes $p15^{INK4b}$, $p16^{INK4a}$ and *p73*, has been described by different groups (20–22). To evaluate MS-MLPA, we analyzed DNA samples derived from 21 AML cell lines for promoter methylation using probe mixture P041A that contains MS-MLPA probes for 22 different genes (Supplementary Table 2). Of the 21 AML samples, frequent aberrant methylation of the genes $p15^{INK4b}$ and *p73* occurred in nine (42.9%) and in ten (47.6%) samples, respectively. Aberrant methylation was also found in the following genes: *IGSF4* (28.6%), *TIMP-3* (23.8%), *ESR1* (19.1%), *FHIT* (9.5%) and *CDH13* (9.5%). Two examples of a MS-MLPA profile are shown in Figure 3. In one sample of an AML cell line aberrant methylation of the *p15* (211 bp amplification product) and the *p73* (238 bp) gene was detected (Figure 3a). In addition, a decrease in copy number of the *MEN1* and *HIC1* promoter is seen as depicted by the black arrows in this sample (Figure 3b, 193 and 355 bp fragments). The other AML sample shows aberrant methylation of several genes including *TIMP-3* (142 bp), *KLK10* (184 bp), *p*15 (211 bp), *p*73 (238 bp), *CDH13*

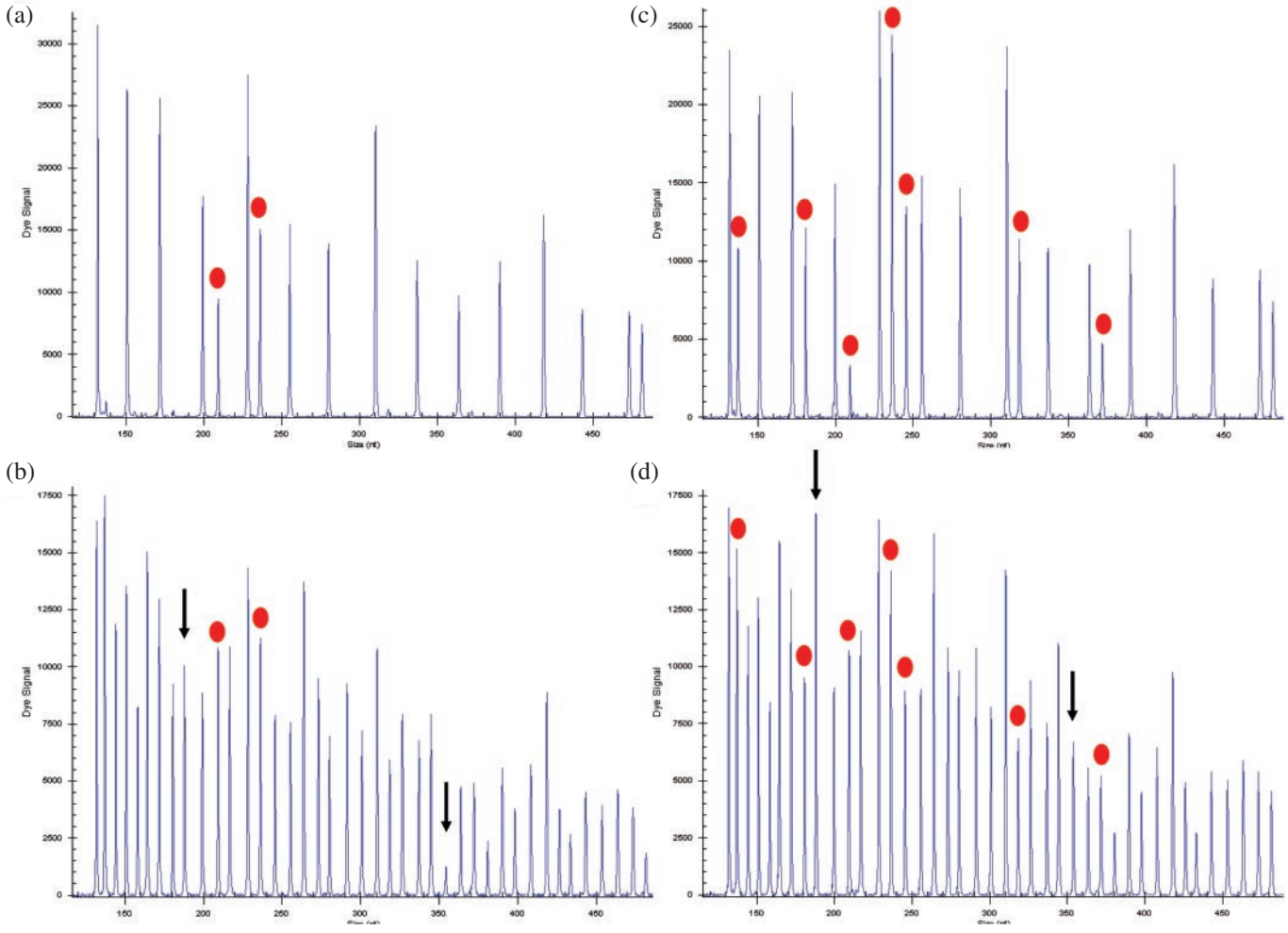

Figure 3. Detection of aberrant methylation patterns in AML cell lines by MLPA using the P041A probe mixture. (**a**) CE-pattern from an AML cell line showing 50% methylation of *p15* (211 bp) and *p73* (238 bp) genes (red dots). Total absence of all other MS-MLPA probes indicates 100% efficiency in the digestion reaction. (**b**) CE-pattern from the same cell line [as shown in (a)] but without HhaI treatment, showing the undigested peak heights that were used for quantification of the methylation levels. When compared to control DNA samples, a reduced probe signal specific for the *MEN1* and *HIC1* promoters is seen as shown by the black arrows, indicating a decrease in copy number of these genes [(b), 193 and 355 bp fragments]. The expected normal probe signals specific for the *MEN1* and *HIC1* promoters is depicted by black arrows in (d). (**c**) CE-pattern from an AML cell line showing methylation of several genes including *TIMP-3* (142 bp), *KLK10* (184 bp), *p*15 (211 bp), *p*73 (238 bp), *CDH13* (247 bp), *IGSF4* (319 bp) and *ESR1* (373 bp) (red dots). (**d**) CE-pattern from the same cell line [as shown in (c)] but without HhaI treatment.

(247 bp), *IGSF4* (319 bp) and *ESR1* (373 bp) (Figure 3c). Also shown are the undigested MS-MLPA profiles that were used for quantification of the methylation levels (Figure 3b and d). A summary of the MS-MLPA test results including SD is shown in Supplementary Table 3. MS-MLPA experiments have been performed at least three times.

The $p15^{INK4b}$ gene is commonly inactivated in association with promoter region hypermethylation involving multiple sites in its 5′-CpG island (23). In some gliomas and all of the primary leukemias, this event occurs without epigenetic alteration of the adjacent gene, $p16^{INK4a}$. In other tumors, including lung, head, neck, breast, prostate and colon cancer, inactivation of $p15^{INK4b}$ occurs rarely and only with concomitant inactivation of $p16^{(INK4a)}$ (21,24,25). Indeed, we did not observe hypermethylation of the $p16^{INK4a}$ gene in any of the 21 AML samples.

To ensure that the disappearance of the MS-MLPA signals was not caused by any other event than the HhaI endonuclease treatment, we treated human genomic DNA (Promega) with HhaI methylase. In this way the internal cytosine residue in de HhaI recognition sequence (GCGC) becomes methylated. As expected, MS-MLPA with 20 ng of HhaI methylase treated DNA showed the presence of all MS-MLPA signals (data not shown), confirming that methylation of the sample DNA CpG sites prevents HhaI endonuclease digestion of the sample DNA–probe hybrids.

MSP and bisulphite sequencing

To validate the MS-MLPA findings in the DNA samples from PWS and AS patients and DNA from AML samples, MSP was carried out. The MSP primers were designed to amplify CpG regions in the *SNRPN* and *p15* genes. Each primer pair was designed in order to contain at least two CpG sites including the ones that are recognized by MS-MLPA. As shown in Figures 2d and 4, all samples that showed methylation of either *SNRPN* or *p*15 by MS-MLPA were also shown to be methylated by MSP. For the *p15* gene we also performed

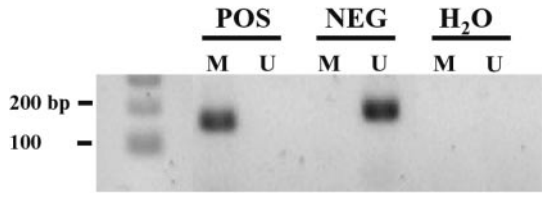

Figure 4. MSP using specific primers to amplify the *p15* promoter region of two AML samples; one AML sample that showed positive methylation of the *p15* gene with MS-MLPA (POS) and one AML sample that was negative with MS-MLPA (NEG). Also a MSP is included only with H_2O as a control. As expected a PCR product of 160 bp was detected with primers designed to amplify methylated sequences (M) in the AML cell line positive for p15 methylation. In the AML cell line that was negative for p15 methylation with MS-MLPA only a 169 bp PCR product was detected indicating that this cell line was indeed not methylated for the *p15* gene.

bisulphite sequencing of the promoter region that is detected by the p15 MS-MLPA probe. Bisulphite sequencing was performed on three DNA samples: one control DNA sample treated with HhaI methylase (Figure 5a), one DNA sample of an AML cell line that was negative for p15 methylation (Figure 5b) and one AML cell line that was positive for p15 methylation after MS-MLPA (Figure 5c).

The DNA sample that is treated with HhaI methylase only the internal cytosine residue of the GCGC sequence becomes methylated and thus is protected from bisulphite conversion which is clearly seen in Figure 5a. All the other CpG sites are converted (underlined). In the DNA sample that is negative for p15 methylation all the CpG site are converted (Figure 5b), whereas in the sample with positive p15 methylation all the six CpG sites are protected including the CpG

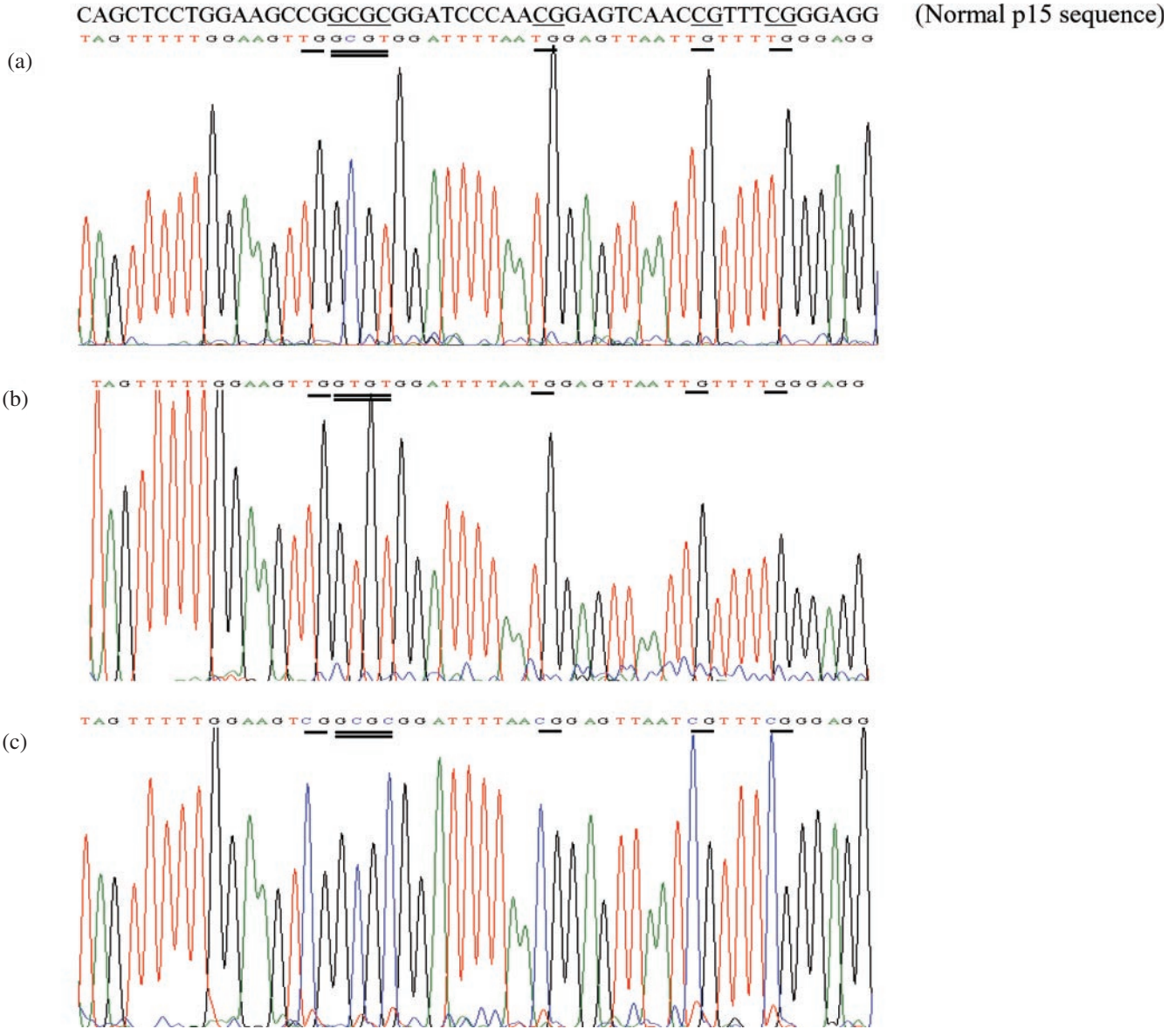

Figure 5. Bisulphite sequencing. Methylation status of p15 promoter region recognized by MS-MLPA analyzed by bisulphite DNA sequencing. Three samples were sequenced (**a**) One control DNA sample treated with HhaI methylase, (**b**) One DNA sample of an AML cell line that was negative for p15 methylation after MS-MLPA and (**c**) One AML cell line that was positive for p15 methylation after MS-MLPA. The HhaI site recognized by the MS-MLPA probe is double underlined. All the CpG sites in this region are indicated (underlined). On top, part of the DNA sequence of the normal p15 sequence (without bisulphite treatment) is shown.

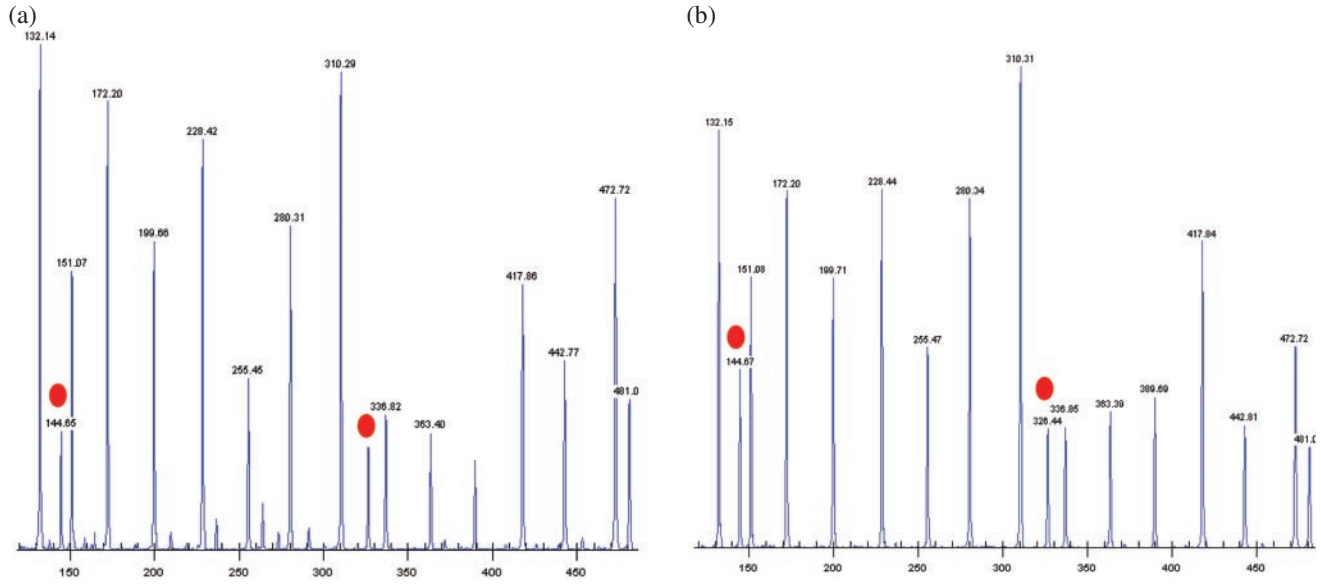

Figure 6. Comparison of MS-MLPA reactions performed on DNA obtained from the same breast tumors that were either paraffin-embedded or fresh-frozen. Samples were analyzed using the P041A probe mixture. Indistinguishable MS-MLPA results were obtained with DNA from paraffin-embedded or fresh-frozen tumor tissues. (**a**) CE-pattern from a MS-MLPA performed on DNA extracted from paraffin-embedded tissue. Red dots indicate methylation of one of the alleles of the *APC* promoter (148 bp) and the *ESR1* promoter (373 bp). (**b**) CE-pattern from a MS-MLPA performed on DNA from the same sample but derived from fresh-frozen material, showing the same methylation pattern.

site (double underlined) recognized by the MS-MLPA probe (Figure 5c).

MS-MLPA on paraffin-embedded tissue

We next tested whether MS-MLPA could be used on DNA derived from the formalin treated paraffin-embedded tissues. DNA extracted from paraffin material is usually of poor quality and is notoriously difficult to digest with restriction endonucleases. Storage of tissues in formaldehyde solution results in extensive crosslinking of proteins to other proteins and to nucleic acids and in nucleic acid fragmentation (26,27). Paraffin-embedding is commonly used and results in partial denaturation of the DNA, making digestion of the sample DNA very difficult. In MS-MLPA, fragmentation of sample DNA is not a problem, since the probes only require 50–60 bp for hybridization and ligation. Besides, the sample DNA does not need to be double-stranded as the digestion is performed on the MS-MLPA DNA–probe complex. Indeed, identical MS-MLPA results are obtained when using DNA derived from paraffin-embedded tissue as compared with fresh-frozen material prepared from the same tumors (Figure 6).

DISCUSSION

Here we describe a novel method, the MS-MLPA assay, for the detection of aberrant methylation patterns of CpG islands as well as copy number changes of a large number of genes in a simple reaction. To validate this method and to show the linearity of response, we analyzed genomic DNA samples of PWS and AS patients caused by uniparental disomy of chromosome 15. The respective methylation status (0, 50 and 100% methylation) and copy number of the genes in the 2 Mb 15q11–q13 PWS/AS region could simply be identified by MS-MLPA. In addition, MS-MLPA was applied to DNA samples from AML cell lines. In line with previous reports, we detected frequent aberrant promoter methylation of the tumor suppressor genes *p15* and *p73* (20–22). For two genes, *SNRPN* and *p15*, the methylation status of the CpG sites recognized by the MS-MLPA probes were independently confirmed by MSP. To provide further evidence that the MLPA results are in agreement with the observed methylation status of this gene, we applied bisulphite sequencing of the *p15* promoter region.

The principle of MS-MLPA is almost similar to the previously described MLPA (11), except that the target sequences detected by MS-MLPA probes contain a restriction site recognized by endonucleases such as HhaI or HpaII that are sensitive to cytosine methylation of one CpG site in their recognition sequence. Upon digestion with one of these enzymes, a probe amplification product will only be obtained if the CpG site is methylated. The level of methylation was determined by calculating the ratio of the relative peak area of each target probe from the digested sample and from the undigested sample.

Initially, in the process of developing MS-MLPA, the genomic DNA was first digested by CpG methylation-sensitive restriction endonucleases and was then denatured and hybridized with the MS-MLPA probes. Unmethylated recognition sites for the restriction endonuclease are digested, preventing the generation of probe amplification products as the two MLPA probe oligonucleotides bind to separate DNA fragments. Although this alternative procedure yielded excellent results, it has several drawbacks compared with the MS-MLPA method presented in this paper. First, the location of the restriction endonuclease site is restricted to the vicinity of the ligation site, whereas in MS-MLPA site can be anywhere in the probe recognition sequence. Second, digestion had to be performed in very small volumes, as the hybridization reaction in MLPA is limited to a maximum sample volume of 5 µl.

Third, a separate undigested sample had to be analyzed in order to be able to detect any copy number changes and to quantify the methylation. Fourth, the salt conditions required for restriction endonuclease digestion, prevented complete denaturation of the genomic CpG islands by a simple heating step. Finally, this alternative procedure did not allow analysis of most DNA samples derived from paraffin-embedded tissue, as the DNA could not be completely digested. This is probably caused by partial denaturation of DNA that is extracted from most paraffin-embedded tissues.

The MS-MLPA technique described here shows to be a robust method and is even suitable for large-scale analysis of DNA extracted from formaldehyde treated paraffin-embedded tissue. In MS-MLPA, the ligation of the probes while hybridized to their target sequence is combined with simultaneous digestion of these complexes with methylation-sensitive restriction endonucleases such as HhaI or HpaII. In this study the HhaI digestion was more effective than HpaII. Conditions of hybridization and ligation are nearly identical to conventional MLPA reactions, only the temperature in the ligation step had to be decreased from 54 to 49°C. HhaI activity decreases at temperatures >50°C (data not shown). Therefore, to ensure complete digestion of the DNA–MS-MLPA probe complex, the ligation and digestion time had to be extended from 15 to 30 min as compared with a convential MLPA reaction. In addition, complete digestion was also apparent by the disappearance of all MS-MLPA probes in a MS-MLPA reaction whereas, incomplete digestion results in general background peak signals of all MS-MLPA probes. In MS-MLPA, genomic DNA is first fully denatured, followed by the formation of a hemimethylated DNA complex with the MS-MLPA probes. Only methylation of the sample DNA strand of this complex showed to be sufficient to inhibit HhaI methylation-sensitive digestion. This is in line with earlier reports, which demonstrated that methylation of one strand is sufficient to block digestion by most methylation-sensitive restriction endonucleases (28,29) (www.rebase.neb.com). Similar to several other restriction endonucleases with a 4 nt recognition site, HhaI also digests single-stranded DNA although at a much lower rate. Several MS-MLPA probes used in initial experiments contained an additional HhaI recognition sequence in the stuffer sequence. This stuffer sequence is included in the M13-derived part of the MS-MLPA probes in order to generate size differences between different probe amplification products. Digestion of single-stranded DNA by HhaI is presumably dependent on the formation of secondary structures that render the HhaI site temporarily double-stranded. Although the digestion is performed at 49°C, which limits the formation of secondary structures, several probes were shown to be slowly digested at this additional HhaI site (data not shown). In order to avoid false negative results, these MS-MLPA probes were replaced by probes that only harbor HhaI recognition sites within the hybridizing sequences. In addition, a mutation or SNP very close or within the recognition site of the restriction enzyme could influence the digestion and might yield false positive results. Finally, not all CpG's within a promoter region are analyzed by MS-MLPA, but only those CpG's that block digestion of methylation-sensitive endonucleases. When designing the MS-MLPA probes, only one methylation-sensitive restriction site should be present within the recognition sequence, because not all CpG sites in a CpG island need to be methylated to silence the transcription of a particular gene (30,31). Thus, if a signal is generated from one MS-MLPA probe but not from a second probe located elsewhere in the same promoter, this indicates that the particular gene is methylated and additional tests should be performed.

The sensitivity of the MS-MLPA probes for the methylation status of sample DNA was demonstrated by the use of human sample DNA that was methylated *in vitro* by HhaI methylase. This resulted in amplification products for all probes, as the HhaI endonuclease is unable to cut methylated CpG sites. Further, specificity of MS-MLPA was demonstrated by the observation that all MS-MLPA probes that recognize a HhaI site within a CpG island resulted in the absence of amplification products after HhaI digestion of DNA samples from healthy individuals (data not shown). In contrast, MS-MLPA probes that recognize a HhaI site outside a CpG island showed the presence of an amplification product upon HhaI digestion of the sample DNA–probe hybrids. This is in agreement with the observation that CpG sites within CpG islands are unmethylated whereas, the great majority of isolated CpG sites are methylated in human DNA (32).

Several aspects contribute to the benefit of MS-MLPA: (i) a large number of genes can be studied using a minimum amount of only 20 ng sample DNA; (ii) owing to its simple procedure, large number of samples can be analyzed simultaneously; (iii) MLPA is quantitative and can discriminate between methylation of one, both or none of the alleles; and (iv) the simultaneous ligation and digestion reaction enables MS-MLPA to be used on paraffin-embedded tissue samples, because DNA degradation and partial DNA denaturation during embedding of the tissues or longtime storage do not influence the results.

In recent years, the identification of gene specific markers for cancer diagnosis has received much attention. Although the attention is primarily focused on mRNA and protein levels in tumor cells, the variation in expression level of many genes could be caused by changes in copy number and/or methylation status of these genes or their regulators. In neuroblastoma, e.g. certain genomic imbalances such as gain of 2p24 and 17q and loss of heterozygosity at 1p36 have been associated with a more aggressive phenotype (33,34). A recent study describes the use of microarray chip technology for DNA based clinical diagnostics in B cell chronic lymphocytic leukemia (B-CLL) (35). In CLL, trisomy of chromosomes 12 and 19 and loss of the 13q14 region, the *p53*, *ATM* and *PTEN* genes provide important markers for tumor diagnosis (34). In addition to genomic imbalances, epigenetic alterations might serve as an important prognostic marker. In this regard it is of note that recent studies imply that hypermethylation of the *p16* gene in ovarian cancer and myeloma is associated with poorer prognosis (36,37).

Due to its simplicity, the MS-MLPA method described here could serve as a powerful screening tool in tumor classification where often only limited amounts of DNA are available from tissue slices that have been characterized by histological examination. MS-MLPA can be used for the analysis of both methylation as well as copy number changes in DNA derived from blood samples of patients with various disorders such as PWS, AS, Beckwith-Wiedemann syndrome and FRAXE/FRAXA-mediated mental retardation.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at NAR Online.

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