Technical Advance

A New Dimethyl Sulfoxide-Based Method for Gene Promoter Methylation Detection

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The identification of gene promoter methylation is a useful tool for the molecular diagnosis of human diseases. We have developed a new PCR-based technique for detecting the methylation status of CpG islands of gene promoters. This new method, named methyl-sensitive dimethyl sulfoxide-PCR (Ms-DMSO-PCR), is based on the finding that methylated and unmethylated DNAs show a different sensitivity to the amount of DMSO used in the PCR reaction. For the amplification of methylated DNA, more DMSO is required in comparison to unmethylated DNA. This finding resulted in the development of a simple PCR screening of CpG islands with addition of DMSO in the range from 0 to 8% (v/v), and the same pair of primers is sufficient for distinguishing hyper- or hypomethylated gene promoters from normally methylated sequences. This new technique is a one-step procedure and does not require any modifications of DNA or expensive equipment. Therefore, Ms-DMSO-PCR has the potential to be widely used for clinical applications as well in basic research. (J Mol Diagn 2007, 9:574–581; DOI: 10.2353/jmoldx.2007.070025)

Changes in gene expression occur in many physiological and pathological conditions including carcinogenesis.^{1,2} The main epigenetic event responsible for these changes is the alteration of the DNA methylation pattern. Both local increases in DNA methylation and global decreases in genomic methylation are extremely common in human cancer.³ In particular, the hypermethylation of CG-rich promoter regions of tumor suppressor genes is a frequent event in carcinogenesis.^{4,5} Although the DNA hypermethylation causing gene silencing and tumor progression has been intensively studied,^{4,5} the role of demethylation is only beginning to be clarified. Cancer-associated aberrant demethylation has been detected in DNA sequences that are methylated in normal tissues and the hypomethylation of repeated sequences in ge-

nome has been shown to increase the homologous recombination and rearrangements of chromosomes.⁶ Demethylation also changes expression of imprinted genes, tissue-specific genes, proto-oncogenes, and genes associated with invasion and metastases.⁶ Evidence is therefore accumulating that not only local hypermethylation but also global demethylation are involved in the neoplastic process.

The increased interest for DNA methylation has led to the development of many sophisticated techniques. Currently, two main methods are used to study the degree of DNA methylation: nonbisulfite and bisulfite methods. The first relies on the digestion of DNA with methylation-sensitive restriction enzymes followed by Southern blot or PCR. The second technique is based on the bisulfite modification of tested DNA. Bisulfite converts unmethylated cytosine to uracil, whereas methylated cytosine remains unaltered. Subsequently, the modified DNA can be analyzed by different methods including methyl-sensitive PCR, combined bisulfite restriction analyses, methylation-sensitive single nucleotide primer extension, methylation-sensitive single-stranded conformational polymorphism, and others.⁷

This article describes a new technique, the methylation-sensitive dimethyl sulfoxide-PCR (Ms-DMSO-PCR), which allows the one-step methylation analysis of the entire CpG island of gene promoter region. The developed method is quick, inexpensive, and does not need any DNA manipulation before the test. It is based on the different sensitivity of methylated and unmethylated DNAs to the amount of DMSO used in PCR reaction. We developed and tested the new method using *DAPK*, *RASSF1A*, *TIMP3*, and *MGMT* gene promoters, which are known as methylation markers of cancer. *DAPK* is a positive mediator of apoptosis, and the methylation of its

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Table 1. Primer Sequences

Gene name	GenBank no.	Primers and annealing temperature (T _{ann})	Location*	Size [†] (bp)
Death-associated protein kinase 1 (<i>DAPK</i>)	NT_023935.17	F: 5'-CAGTACACATCTCGGGACGG-3' R: 5'-GTCCCCGGCGTTGGCTCG-3' T _{ann} = 58°C	-371+24	395
O-6-methylguanine-DNA methyltransferase (<i>MGMT</i>)	AL355531	F: 5'-GGTCGCTTGACGCCCCGC-3' R: 5'-AAGAGAGCGCGCGGGGC-3' T _{ann} = 70°C	-435-102	333
Ras association domain family 1, transcript variant A (<i>RASSF1A</i>)	NM_007182	F: 5'-CGCTGAAGTCGGGGCCCG-3' R: 5'-CAGGCCTTTGCGCACGACG-3' T _{ann} = 62°C	+45+371	326
Tissue inhibitor of metalloproteinase 3 (<i>TIMP3</i>)	NT_011520.10	F: 5'-CCCCAACAGCCCGAGGCG-3' R: 5'-TTGCCGCTGCTGCCGCCG-3' T _{ann} = 63°C	+825+1192 [‡]	367

*Location of CpG island to the start of transcription.

[†]Size of PCR product.

[‡]Start of translation is at 1193 bp from start of transcription.

gene promoter has been associated with early recurrence of tumors.⁸ The hypermethylation of *MGMT*, a gene involved in repair of O6-methylguanine, has been shown to be a useful predictor of the responsiveness of tumors to alkylating agents,⁹ whereas the methylation of *RASSF1A* in serum DNA is associated with poor cancer prognosis.¹⁰

Materials and Methods

Cell Culture and Tissues

Cervical cancer-derived cell lines C33 [human papillomavirus (HPV)-negative], SiHa, and CaSki (containing 1 copy and 500 copies of HPV16 genome, respectively) were grown under standard conditions.¹¹ Normal human keratinocytes were isolated from biopsies of uterine cervix and cultivated as described before.¹¹ Frozen biopsy specimens, cell scrapings, and formalin-fixed, paraffin-embedded tissue (FFPE) corresponding to cervical squamous cell carcinoma (SCC) and normal tissues were retrieved from the Tumor Bank of the University Hospital of Liege. The histological and cytological diagnoses were confirmed after hematoxylin and eosin and Papanicolaou staining.

Treatment with 5-Aza-2'-Deoxycytidine

SiHa cells (1×10^5) were seeded in 6-cm tissue dishes. One hundred $\mu\text{mol/L}$ 5-aza-dC (Sigma-Aldrich, Schnelldorf, Germany) in DMSO (Sigma-Aldrich) was added the next day. For control, cells were mock-treated with the identical volume of DMSO. The cells were cultured for 6 days, and the media with 5-aza-dC or DMSO were changed every second day. At the end, cells were collected and chromosomal DNA was isolated.

DNA Isolation

Chromosomal DNA from cell lines, cervical scrapes, frozen biopsies, and FFPE blocks were isolated using QIAmp DNA mini kit (Qiagen, Venlo, The Netherlands) ac-

cording to the manufacturer's recommendations. After purification DNA was extracted with phenol-chloroform, precipitated with ethanol, and dissolved in water.

Methylation in Vitro

Methylation *in vitro* was performed with site-specific SssI (CG) methylase and S-adenosylmethionine (New England Biolabs, Leusden, The Netherlands). Two μg of DNA were methylated in 50 μl of reaction mixture containing 6 U of SssI and 640 $\mu\text{mol/L}$ S-adenosylmethionine at 37°C for 30, 60, or 120 minutes. Methylation efficiency was monitored by *Hpa*II digestion and gel electrophoresis. The DNA was purified by phenol extraction followed by ethanol precipitation and dissolved in water.

Restriction of DNA with *Msp*I/*Hpa*II Endonucleases

Two hundred μg of genomic DNA were digested with restriction endonucleases in 20 μl of reaction mixture containing 10 U of *Msp*I or *Hpa*II (both from Fermentas, St. Leon-Rot, Germany) at 37°C for 16 hours. Restriction digestion was checked by gel electrophoresis. One μl of reaction mixture was taken for PCR. PCR was performed in 25 μl containing 50 pmol of each primer, 6% DMSO, and 0.5 U of *Taq* polymerase (Fermentas). Thermocycling included 30 cycles at annealing temperature of 58°C for 30 seconds and extension at 72°C for 45 seconds. Five μl of PCR mixtures were analyzed by 2% agarose gel electrophoresis. The sequences of primers for PCR are shown in Table 1.

Ms-DMSO-PCR

The location of CpG islands in gene promoters, the sequences of primers, and their annealing temperatures for PCR are shown in Tables 1 and 2. The DMSO-PCR was performed in 25 μl containing 1 $\times$ *Taq* buffer with $(\text{NH}_4)_2\text{SO}_4$, 0.2 mmol/L of each dNTP, 1.5 mmol/L MgCl_2 (for *DAPK*, *TIMP3*, and *RASSF1*) or 2 mmol/L MgCl_2 (for

Table 2. Primers and Characterization of PCR Products of *DAPK* Promoter

Size of PCR product (bp)	Location of PCR product to the start of transcription	Primer name and annealing temperature (T_{ann})	Number of CG in primer	Number of CG in PCR product	C + G content in PCR product (%)
161	-137 +24	Up 3 Down 2 $T_{ann}=60^{\circ}\text{C}$	1 3	16	74.5
395	-371 +24	Up 2 Down 2 $T_{ann}=58^{\circ}\text{C}$	2 3	45	72.4
448	-424 +24	Up 1 Down 2 $T_{ann}=58^{\circ}\text{C}$	0 3	47	70.3
649	-137 +512	Up 3 Down 1 $T_{ann}=64^{\circ}\text{C}$	1 0	65	73.2
883	-371 +512	Up 2 Down 1 $T_{ann}=60^{\circ}\text{C}$	2 0	95	72.6
936	-424 +512	Up 1 Down 1 $T_{ann}=60^{\circ}\text{C}$	0 0	97	71.6

MGMT1), 0 to 8% DMSO, 5 to 15 ng of DNA template, 50 pmol of each primer, and 0.5 U *Taq* polymerase (Fermentas). PCR cycling conditions for all of the amplified fragments and the temperatures used for primer hybridization (Table 1) were the same: 95°C for 9 minutes, then 30 cycles with denaturation at 95°C for 30 seconds, annealing for 30 seconds, and polymerization at 72°C for 30 to 60 seconds. All the PCR products were analyzed by a 2% agarose gel electrophoresis.

Results

Direct Detection of DNA Methylation by Ms-DMSO-PCR

The DNA extracted from normal human keratinocytes was split in two parts. One remained untreated whereas the other one was methylated *in vitro* by *SssI* methylase as described in the Materials and Methods. In an attempt to find optimal PCR conditions for the amplification of *DAPK* promoter, we observed that methylated and untreated DNAs showed a different sensitivity to the amount of DMSO used in the PCR mixture (Figure 1A). For untreated DNA, PCR products appeared at 3% DMSO whereas at least 5% DMSO was necessary for the methylated DNA. The same results were observed with DNAs from C33 and CaSki cell lines (data not shown). To confirm that this different sensitivity to DMSO was related to methylation, we used an enzymatic method for detecting DNA methylation. After digestion of untreated and *in vitro* methylated DNAs with the methylation-sensitive enzyme *HpaII* and subsequent PCR, amplified products were observed only in the case of methylated DNA (Figure 1B).

Methylation Analysis of *TIMP3*, *RASSF1A*, and *MGMT* Gene Promoters

To verify whether this different sensitivity to DMSO is a general feature of methylated DNA, we performed *Ms-DMSO-PCR* of *TIMP3*, *RASSF1A*, and *MGMT* gene promoters. Chromosomal DNA was isolated from CaSki cells and methylated *in vitro* by *SssI* methylase. Again, we found that DNA methylation changed the sensitivity to DMSO for all tested promoters (Figure 2), although the amplified gene products appeared at different DMSO concentrations. Indeed, the PCR signal for the *TIMP3* promoter started to be seen at 2% DMSO (Figure 2A), whereas those of *RASSF1A* (Figure 2B) and *MGMT* (Figure 2C) were detected at 1% and 4% DMSO, respec-

Figure 1. Analysis of *DAPK* promoter methylation in human cervical keratinocytes. Isolated DNA was methylated *in vitro* by *SssI* methylase and compared with the same untreated DNA preparation. **A:** *Ms-DMSO-PCR*: 15 ng of each DNA template were taken for each PCR reaction with the concentration of DMSO ranging from 0 to 8%. **B:** PCR of *MspI*- and *HpaII*-digested DNA. N, noncut DNA; M, *MspI* digest; H, *HpaII* digest; L, 100-bp DNA ladder (Fermentas).

Figure 2. Ms-DMSO-PCR of *TIMP3*, *RASSF1A*, and *MGMT* gene promoters. CaSki cell DNA was methylated *in vitro* by *SssI* methylase and compared with the same untreated DNA preparation. Ten ng of each DNA template were taken for each PCR reaction with the concentration of DMSO ranging from 1 to 7%. L, 100-bp DNA ladder.

tively. Interestingly in the case of methylated and untreated DNA for *DAPK*, *TIMP3*, and *RASSF1A* gene promoters, the difference in the sensitivity to DMSO was the same (~2%). A lower difference in the sensitivity to DMSO of methylated and untreated DNA from CaSki cells was observed in the case of *MGMT* (Figure 2C). However, the promoter of *MGMT* gene was found to be already methylated in cancer cells,¹² and additional methylation *in vitro* did not change significantly the promoter configuration.

Influence of the Length or C+G Content of Amplified Fragments and Primer Design on Ms-DMSO-PCR

To understand the effect of DMSO in the amplification of methylated versus unmethylated DNA templates, we tested the method for *DAPK* promoter fragments ranging from 161 to 940 bp (Figure 3, Table 2). Although the amplified DNA fragments contained a high percentage of C+G because of their locations in CpG islands, they had a different number of CG nucleotides, the substrate for methylation in eukaryotes. In addition, the primers used for PCR contained or not CG sequences (Table 2). We found that DNA methylation changed the sensitivity to DMSO for all the tested fragments (Figure 4). The best results were observed for the fragments of 395, 448, 883, and 940 bp. For these PCR products, the amplification of

Figure 3. Sequence region of the *DAPK* promoter. All potentially methylated CG sites are labeled with an asterisk. The primer sequences used for the amplification of different parts of the promoter and the starting point of transcription are indicated.

untreated DNA started at 2% DMSO, and the intensity of the bands did not change with increased DMSO concentration. In contrast, the PCR signals for the methylated DNA started to be weakly seen at 3% DMSO and reached the maximal intensity at 4% DMSO (Figure 4). Interestingly, strong amplification signals of untreated DNA for the fragments of 161 bp (which is a part of the 395- and 448-bp fragments, Table 2) and 649 bp (part of 883- and 936-bp fragments) were detected at 1% DMSO. The PCR products of *in vitro* methylated DNA for the 161- and 648-bp fragments were weak at 1% DMSO, but the maximum intensity was detected at 4% DMSO as for the other tested methylated DNA fragments. In these experiments, we found that the differential sensitivity to DMSO was not linearly dependent on the C+G content of amplified fragments. As shown in Table 2 and Figure 3, the highest C+G content was in the short 161-bp fragment (74.5%), but the PCR signal started to be detected earlier than for the fragment of 448 bp which has only 70.3% C+G. In addition, we demonstrated that primers designed for the test do not need to contain CG dinucleotides. Accordingly, the primers used for amplification of the 936-bp fragment do not contain any CGs, whereas the primers allowing the amplification of the 395-bp fragment have two or three CGs (Figure 3, Table 2). Because the sensitivity to DMSO was the same for both fragments (Figure 4), DNA internal secondary structures of methylated and unmethylated DNA fragments are likely to be more critical for the DMSO effect than the presence of CG dinucleotides in primer sequences.

Detection of Naturally Occurring DNA Methylation by Ms-DMSO-PCR

To demonstrate that this method can be used for detecting DNA methylated *in vivo*, Ms-DMSO-PCR of the

Figure 4. Methylation analysis of different fragments of *DAPK* promoter. CaSki cell DNA was methylated *in vitro* by *SssI* methylase and compared with the same untreated DNA preparation. Ms-DMSO-PCR: 5 to 10 ng of each DNA template were taken for each PCR reaction with the concentration of DMSO ranging from 1 to 5%. L, 100-bp DNA ladder. Primers and their location in promoter region are shown in Figure 3 and Table 2.

395-bp fragment of the *DAPK* promoter was performed in native DNA extracted from SiHa cells. The methylation of *DAPK* promoter in these cells was confirmed by the enzymatic method (Figure 5A). Agarose gel analysis of Ms-DMSO-PCR showed that amplification signal starts to be clearly seen at 4% and reached maximal intensity at 5% DMSO (Figure 5B), whereas the PCR products of the same fragment were detected at 3% DMSO for DNA isolated from normal keratinocytes (Figure 1A) and 2% DMSO for CaSki cells (Figure 4) and C33 cells (data not shown). There was no methylation of *DAPK* gene promoter detected by the enzymatic method in normal keratinocytes (Figure 1B), CaSki, and C33 cells (data not shown). To better characterize the level of *DAPK* promoter methylation in SiHa cells, we induced additional methylation of isolated DNA by *SssI* methylase. Interestingly, the Ms-DMSO-PCR technique showed a further shift of detected amplification signal toward a higher percentage of DMSO (Figure 5B), suggesting that the *DAPK* promoter is only partially or heterogeneously methylated in living SiHa cells. We also treated SiHa cells with an inhibitor of DNA methyltransferases (5-aza-dC) for 6 days. Because 5-aza dC was dissolved in DMSO, the cells were mock-treated with the same amount of DMSO as control. After treatment with 5-aza-dC, a PCR product of *DAPK* promoter appeared at 2% DMSO (Figure 5D), whereas after treatment with DMSO alone the appearance of the PCR

Figure 5. Analysis of *DAPK* promoter methylation in the SiHa cell line. **A:** PCR of *MspI*- and *HpaII*-digested DNA of methylated *in vitro* by *SssI* methylase and the same untreated preparation. N, Noncut DNA; M, *MspI* digest; H, *HpaII* digest; L, 100-bp DNA ladder. **B:** Ms-DMSO-PCR of the same samples: 10 ng of each DNA template were taken for each PCR reaction with the concentration of DMSO ranging from 1 to 6%. **C:** PCR of *MspI*- and *HpaII*-digested DNA from 5-aza-dC or mock-treated SiHa cells. N, Noncut DNA; M, *MspI* digest; H, *HpaII* digest; L, 100-bp DNA ladder. **D:** Ms-DMSO-PCR of the same samples: 10 ng of each DNA template were taken for each PCR reaction with the concentration of DMSO ranging from 1 to 5%.

product was not changed in comparison to untreated cells (Figure 5, B and D). The methylation analysis of *DAPK* promoter from treated SiHa cells by the enzymatic method also confirmed the demethylation of DNA (Figure 5C). In this experiment we still observed some level of methylation by both used methods, but it should be mentioned that complete demethylation is difficult to obtain *in vivo*. These data therefore suggest that the same Ms-DMSO-PCR is able to detect not only the methylation of DNA induced by DNA methyltransferases in living cells but also the demethylation of specific gene promoters.

Influence of Methylation Heterogeneity in DNA Samples on Ms-DMSO-PCR

To understand the sensitivity of Ms-DMSO-PCR to methylation heterogeneity in DNA samples, we performed two sets of experiments (Figure 6). We first performed a time course *in vitro* methylation of DNA isolated from CaSki

Figure 6. Sensitivity of Ms-DMSO-PCR to time course methylation and detection of methylation in CaSki and SiHa DNA mixtures. **A:** Ms-DMSO-PCR of *DAPK* promoter of DNA from CaSki cells methylated *in vitro* for 0, 30, 60, and 120 minutes: 10 ng of each DNA template were taken for each PCR reaction with the concentration of DMSO ranging from 1 to 5%. **B:** PCR of *MspI*- and *HpaII*-digested DNA of the same samples. N, Noncut DNA; M, *MspI* digest; H, *HpaII* digest. **C:** Ms-DMSO-PCR of *DAPK* promoter from CaSki and SiHa DNA mixtures: 10 ng of each DNA template or mixture were taken for each PCR reaction with the concentration of DMSO ranging from 1 to 4%.

cells and analyzed *DAPK* promoter methylation by Ms-DMSO-PCR. After 30 minutes of methylation the shift in sensitivity to DMSO was not observed, but the intensity of PCR signal at 2% DMSO for methylated DNA was significantly lower compared to the unmethylated one (Figure 6A). DNA methylation was not detected by the enzymatic method in the same preparation (Figure 6B). A clear shift from 2 to 3% DMSO appeared after 1 hour of methylation, and after 2 hours this shift increased to 4% DMSO (Figure 6A). The enzymatic method showed that *DAPK* promoter was methylated in both samples, but no differences in methylation level were observed (Figure 6B).

In a second set of experiments, we tested the ability of Ms-DMSO-PCR to detect the methylated gene against a background of the same, but unmethylated, gene. We mixed, in different proportions, the DNA isolated from CaSki cells (unmethylated *DAPK* promoter) with DNA isolated from SiHa cells (naturally partially methylated gene). As shown in Figure 6C, the PCR product was detected at 3% DMSO when the methylated gene was present in the DNA mixture at a percentage higher than 50%. When the proportion of methylated DNA was less than 50%, the strong PCR signal was detected also at 3% DMSO, but the weak signal corresponding to unmethylated gene was also detected at 2% DMSO.

Detection of DNA Hyper- and Hypomethylation in Clinical Samples

To check whether this method is also efficient in the analysis of clinical samples, we extracted chromosomal DNA from cervical scrapes and frozen biopsies and performed DMSO-PCR of *DAPK* and *MGMT* gene promoters. As shown in Figure 7, the *DAPK* promoter was found to be hypomethylated in cancer samples compared with normal tissues (Figure 7A), whereas *MGMT1* promoter was hypermethylated at the same time (Figure 7B), demonstrating that cancer cells may have concomitant hyper- and hypomethylation of promoter regions of specific genes. The technique for *DAPK*, *MGMT*, *RASSF1A*, and *TIMP3* gene promoters was also tested on DNA isolated from other tissues including thyroid and nervous system cancers and gave similar results (data not shown).

To test the possible application of the new method for the methylation analysis of DNA isolated from FFPE tissues, we extracted the DNA from cervical FFPE blocks and performed the Ms-DMSO-PCR of the *DAPK* promoter. Because extracted DNA was significantly de-

Figure 7. Ms-DMSO-PCR of *DAPK* and *MGMT* promoters in clinical samples of the uterine cervix: 10 ng of each DNA template were taken for each PCR reaction with the concentration of DMSO ranging from 2 to 5% for frozen biopsies and 0 to 2% for FFPE tissue. NI cells, HPV⁻, normal cells, HPV-negative; SCC HPV⁺, squamous cell carcinoma, HPV-positive. **A:** Demethylation of *DAPK* promoter in frozen biopsies of SCC. **B:** Methylation of *MGMT* promoter in frozen biopsies of SCC. **C:** Demethylation of *DAPK* promoter in FFPE tissues of SCC.

graded because of tissue fixation in formalin and the average size of DNA fragments was ~200 bp, we amplified the 161-bp fragment of the *DAPK* promoter (Table 2). Ms-DMSO-PCR showed that this fragment of the *DAPK* promoter was hypomethylated in cancer samples compared with normal tissues (Figure 7C). These results were in agreement with the data obtained with DNA isolated from frozen biopsies (Figure 7A).

Discussion

In the last decade, many researchers reported that the methylation status of individual or multiple genes can be associated with clinically useful information, suggesting that the discovery of DNA methylation markers and development of new techniques for promoter methylation studies are of potential interest for the clinical diagnosis, prognosis, and therapeutics of cancer and other diseases.¹³

DMSO helps the melting of DNA template and the proper primer annealing. Therefore, it is widely used in PCR to improve the specificity and efficiency of the amplification.¹⁴ For the first time, we showed that DMSO can act differently depending on the secondary structure of DNA caused by methylation of CpG islands. The fact that cytosine methylation affects the melting of DNA structure is shown by the possibility to separate methylated and unmethylated DNA fragments by denaturing gradient gel electrophoresis.¹⁵ These differences of DNA structure caused by methylation can be easily monitored by performing PCR with different amounts of DMSO (from 0 to 8% v/v) and subsequent agarose gel electrophoresis of PCR products. This new method, named methyl-sensitive DMSO-PCR (Ms-DMSO-PCR), allows the fast methylation analysis of CpG islands of gene promoters.

To better characterize the chemical basis of the DMSO effect in the amplification efficiency of methylated DNA templates, we tested the influence of C+G content or length of amplicons and primer design on Ms-DMSO-PCR. All the analyzed DNA fragments contained a high percentage of C+G (70 to 74%) because they were included in CpG island of promoter area. However, the fragments with the highest C+G content (more than 73%) were efficiently amplified with lower percentages of DMSO and methylated DNA showed a slightly lower sensitivity to DMSO in comparison to fragments of the same gene promoter with 70 to 73% C+G. When this technique was tested for detecting methylation in promoters of different genes (*DAPK*, *TIMP3*, and *RASSF1A*), the difference in sensitivity to DMSO was the same (~2%), although the PCR signals started to be seen at different DMSO concentrations. This different start of amplification probably reflects the level of promoter methylation in the cells and does not depend on the C+G-content of DNA. Accordingly, *DAPK* and *TIMP3* gene promoters contain 72 and 77% GCs, but PCR signals started to be seen at 3 and 2% DMSO, respectively. In contrast, *RASSF1A* and *MGMT* gene promoters have the same CG content (75% CGs), but amplification started to be observed at 1 and 4% DMSO, respectively. These data suggest that the

DNA secondary structure is more crucial for PCR efficiency than the simple calculation of C+G content and/or number of potentially methylated bases.

The application of modern methylation-sensitive techniques is restricted to the usage of short (up to 200 bp) DNA fragments and analysis of very few methylated cytosines, raising the question about the biological meaning of methylation of individual DNA bases and their role in epigenetic regulation of gene transcription. In our experiments we found that the new technique is applicable for fragments with lengths ranging from 161 to 936 bp, suggesting that short fragments as well as entire CpG islands may be analyzed with this method. There is accumulating evidence that methylation leads to global changes of promoter structures by recruiting complexes of methylation-binding proteins and histone deacetylases, which cause the blocking of transcription.^{16–18} However, it is likely that not only methyl-binding proteins change the promoter structure but also that DNA methylation itself causes formation of different structures of entire CpG islands. Most probably, the differences in sensitivity to DMSO reflect structural changes of promoter regions in relation to their methylation status, rather than methylation of separate bases within CpG islands. The method described here is the first technique allowing detection of methylation-induced structural DNA changes that may have more biological significance than methylated individual cytosines. Interestingly, the DMSO sensitivity of methylated versus unmethylated DNA was strongly correlated with changes of gene transcription. Accordingly, after treatment of SiHa cells with demethylation agent, we observed a notable shift from 4 to 2% DMSO when we analyzed *DAPK* promoter methylation by Ms-DMSO-PCR, and we also detected the appearance of significant amounts of *DAPK* mRNA in the treated cells (unpublished data).

We also showed that Ms-DMSO-PCR can be successfully used for detecting partial and/or heterogeneously methylated gene promoters. The methylation analysis of *DAPK* gene promoter in CaSki and C33 cells showed a strong PCR signal at 2% DMSO, suggesting that in these cells the promoter was not or only slightly methylated. After *in vitro* methylation, the amplification signal of *DAPK* promoter was clearly detected only at 5% DMSO. In SiHa cells, we observed the PCR signal of the analyzed fragment at 3% (very weak band) and 4% (strong band) of DMSO. These results, which are in agreement with the data obtained using the methylation-sensitive enzymatic method, suggest that *DAPK* promoter in SiHa cells is more methylated than in CaSki and C33 cells. However, *in vivo*-methylated DNA did not reach the maximal possible shift in sensitivity to DMSO, probably because of different DNA methylation pattern in individual cells. Time course *in vitro* methylation experiments also confirmed that this technique can be used for the detection of partial and/or heterogeneous methylation of gene promoters. However, it should be noted that the differences in DMSO sensitivity during the PCR amplification probably reflect the structural differences of methylated versus unmethylated DNA. Therefore, we believe that the DMSO depen-

dence for *in vitro*-methylated DNA might not be the same as for *in vivo*-methylated genes.

Experiments with mixtures of methylated and unmethylated DNA showed that Ms-DMSO-PCR allows the detection of methylated gene in a background of the same, but unmethylated, genes. We were able to detect methylation in samples containing only 30% methylated DNA. This issue is crucial for clinical application because biopsies normally represent mixtures of tumor and normal cells. Unfortunately, we could not determine the correspondence of DMSO sensitivity to the number of methylated bases in DNA because of the absence of suitable methods for methylation analysis of long fragments. Bisulfite conversion causes denaturation and significant degradation (up to 95%) of DNA making impossible the analysis of DNA fragments longer than 200 bp.¹⁹ In contrast to bisulfite-based methods, Ms-DMSO-PCR is a quick and qualitative test able to detect methylation of entire (up to 1 kb) CpG island of gene promoters.

This method also has several other advantages over existing methylation-detection techniques. In contrast to widely used protocols, Ms-DMSO-PCR does not require any additional DNA modification before methylation analysis. This is an important point because a limited amount of DNA is often available from biopsies and other medical materials. The technique described here can be performed with nanograms (25 to 50 ng) of DNA directly after isolation from analyzed tissues. In addition, this one-step procedure does not need expensive reagents and equipment and can be used widely in medical and research laboratories. Accordingly, the shift in DMSO sensitivity in clinical samples from cervical scrapes and frozen biopsies, corresponding to a mixture of normal and cancer cells, suggests that this new technique can be applied for the molecular diagnosis of cancer and other methylation-related diseases. Another interesting feature of Ms-DMSO-PCR is that it allows the concomitant detection of hyper- and hypomethylation. Only one reaction is indeed necessary to find any methylation changes occurring in the DNA. No additional steps and sets of primers are required, in contrast to other methods based on bisulfite conversion of DNA bases.

In conclusion, the new method described in this report should help to find and to re-evaluate new and existing markers for different diseases based on both DNA hypermethylation and hypomethylation events in the cells.

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