Rapid and sensitive detection of CpG-methylation using methyl-binding (MB)-PCR

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ABSTRACT

Methylation of CpG islands is associated with transcriptional repression and, in cancer, leads to the abnormal silencing of tumor suppressor genes. We have developed a novel technique for detecting CpG-methylated DNA termed methyl-binding (MB)-PCR. This technique utilizes a recombinant protein with high affinity for CpG-methylated DNA that is coated onto the walls of a PCR vessel and selectively captures methylated DNA fragments from a mixture of genomic DNA. The retention and, hence, the degree of methylation of a specific DNA fragment (e.g. a CpG island promoter of a specific gene) is detected in the same tube by gene-specific PCR. MB-PCR does not require bisulfite treatment or methylation-sensitive restriction and provides a quick, simple and extremely sensitive technique allowing the detection of methylated DNA, in particular in tumor tissue or tumor cells from limited samples. Using this novel approach, we determined the methylation status of several established and candidate tumor suppressor genes and identified the ICSBP gene, encoding the myeloid and B-cell-specific transcription factor interferon consensus sequence-binding protein, as a target for aberrant hypermethylation in acute myeloid leukemia.

INTRODUCTION

During the past few years it has become increasingly clear that the formation of tumors is supported not only by genetic lesions (e.g. mutations or translocations) but also by epigenetic changes, including alterations in the methylation status of DNA (1,2). In mammals, methylation of DNA occurs at specific cytosine residues which precede a guanosine residue (CpG-dinucleotides) and generally correlates with stable transcriptional repression (3–5). The aberrant gain of DNA methylation (hypermethylation) in neoplastic cells frequently affects DNA sequences with a relatively high content in CpG-dinucleotides, the so-called CpG islands. These regions often contain transcription initiation sites and promoters and with only few exceptions are generally not methylated in normal cells (3,4,6–8). It is particularly in tumors that CpG islands that are normally not methylated can be present in a hypermethylated form. In many cases, genes affected by the hypermethylation encode proteins that counteract the growth of a tumor such as tumor suppressor genes (1,2,9,10). Reasons for the tumor-specific hypermethylation are unknown. Interestingly, certain kinds of tumors seem to have their own hypermethylation profiles (11,12). Promoter-hypermethylation events may actually provide some of the most promising markers that can be used to improve cancer detection and the assessment of cancer risk. Hence, the detection of CpG-methylated DNA and thus the identification of dysregulated tumor suppressor genes and/or oncogenes is of utmost clinical interest (2,13,14).

Here, we present a novel, single tube, PCR-based technique that we termed methyl-binding (MB)-PCR, allowing the sensitive detection of CpG island-specific methylation. This novel method avoids the manipulation of DNA by methylation-sensitive restriction endonucleases or bisulfite treatment and relies on the ability of a recombinant, bivalent, methyl-CpG-binding polypeptide to specifically bind CpG-methylated DNA fragments. The single tube assay involves the fractionated binding of restricted genomic DNA by the recombinant methyl-CpG-binding polypeptide immobilized on the walls of a PCR vessel and the subsequent detection of bound DNA by PCR in the same tube. Given the enormous amplification capability and specificity of PCR, MB-PCR can reliably detect the methylation degree of a specific genomic DNA fragment from <30 cells.

Using the described technique, we determined the methylation status of several established (ESR1; CDKN2B) and candidate tumor suppressor genes (ICSBP, ETV3, DDX20) in leukemia cell lines, normal cells and blasts from 35 patients with newly diagnosed, untreated acute myeloid leukemia (AML). We confirm earlier studies showing the frequent methylation of the estrogen receptor (ESR1) as well as the CDKN2B (also known as p15\(^{INK4b}\)) CpG island promoter.
(15–17). We also identify ICSBP as a target for aberrant promoter methylation in a subset of AML patients.

MATERIALS AND METHODS

Cells

Peripheral blood mononuclear cells (MNC) were separated by leukapheresis of healthy donors, followed by density gradient centrifugation over Ficoll/Hypaque. Monocytes were isolated from MNC by countercurrent centrifugal elutriation in a J6M-E centrifuge (Beckman, München, Germany) as described previously (18). Drosophila S2 cells were obtained from ATCC and cultured in Insect-Xpress medium (Bio Whittaker) containing 10% fetal calf serum (FCS; PAA) in an incubator at 21°C. The human myeloid leukemia cell lines THP-1, NB-4, KG-1, K562, HL-60 and U937 were grown in RPMI 1640 medium supplemented with 10% FCS. The human myeloid leukemia cell line Mono Mac 6 was grown in RPMI 1640 medium plus 10% FCS and 1% OPl media supplement (Sigma). The human myeloid leukemia cell line MUTZ-3 was maintained in αMEM plus 20% FCS and 10 ng/ml stem cell factor. For DNA-demethylation, U937 cells were treated with the indicated amounts of Decitabine (2-deoxy-5′-azacytidine; Sigma) for several days.

Patient samples

Fresh peripheral blood samples and bone marrow specimens from 35 patients with newly diagnosed and untreated de novo or secondary AML were used for the study. All patients were treated according to the protocol AMLCG-2000 of the German AML Cooperative Group. The study was approved by the Institutional Ethics Committee, and written informed consent was obtained from each patient before entering the study.

Recombinant expression of the methyl-binding polypeptide MBD-Fc

A detailed description of the design and generation of the MBD-Fc protein will be given elsewhere (19). In brief, a cDNA corresponding to the methyl-CpG-binding domain of human MBD2 (amino acids 144–230) was PCR-amplified, fused to the Fc-tail of human IgG1, and subcloned into the inducible expression vector pMTBiiP/V5-His (Invitrogen). The resulting vector was stably transfected into Drosophila S2 cells using Effectene transfection reagent (Qiagen) and hygromycin selection. For large-scale protein production, 1–5 × 10⁸ cells were cultured in 100–200 ml Insect-Xpress medium without FCS in 2000 ml roller bottles for 2 days before the addition of 0.5 mM CuSO₄. Culture medium was harvested every 4–7 days and cells were replaced in medium plus CuSO₄ for continued protein production. Cell culture supernatants were pooled and purified using a protein A–Sepharose (Amersham) column. The purified protein (200–700 µg/ml) was dialyzed against TBS and 0.6% gelatine/0.02% NaN₃ was added as preservative.

DNA preparation

Genomic DNA from various cellular sources was prepared using the Blood and Cell Culture DNA Midi Kit from Qiagen. Quality of the genomic DNA preparation was controlled by agarose gel electrophoresis and DNA concentration was determined by UV spectrophotometry. Genomic DNA was digested with MseI (NEB) and quantified using PicoGreen dsDNA Quantification Reagent (Molecular Probes). Where indicated, DNA was in vitro methylated using SsI methylase (NEB).

Preparation of PCR tubes for MB-PCR

MBD-Fc-coated PCR tubes were prepared using heat stable TopYield™ Strips (Nunc). Recombinant MBD-Fc protein (50 µl, diluted at 15 µg/ml in 10 mM Tris–HCl, pH 7.5) were added to each tube and incubated overnight at 4°C. Tubes were washed three times with 200 µl TBS (20 mM Tris, pH 7.5, containing 170 mM NaCl) and blocked for 3 h with 100 µl blocking solution [10 mM Tris, pH 7.5, containing 170 mM NaCl, 5% skim milk powder, 5 mM EDTA and 1 µg/ml of each poly(dI–dC), poly(dA–dT) and poly(dC–dG)] (Amersham)]. Tubes were washed two times with 200 µl TBST (TBS containing 0.05% Tween-20) and once with binding buffer (20 mM Tris, pH 7.5, containing 400 mM NaCl, 2 mM MgCl₂, 0.5 mM EDTA and 0.05% Tween-20).

Binding of methylated DNA (M-reaction)

Binding buffer (50 µl) (20 mM Tris, pH 7.5, containing 400 mM NaCl, 2 mM MgCl₂, 0.5 mM EDTA and 0.1% Tween-20) was added to each coated, blocked and washed tube and 2 µl digested DNA (5 ng/µl) was added to every second tube (M-reaction). The salt concentration in the binding buffer was initially determined in pilot experiments to allow the efficient binding of highly methylated but not unmethylated CpG island fragments. The salt concentration determines the ‘cut-off’ and may be adjusted for individual DNA fragments. Tubes were incubated on a shaker at room temperature for 40 min, washed two times with 200 µl binding buffer and once with 200 µl of 10 mM Tris–HCl, pH 8.0.

Detection of methylated DNA fragments

PCR was carried out directly in the treated and washed TopYield™ Strips. The PCR mixture (50 µl/well) included 1 pmol of each gene-specific primer (synthesized by Metabion). Primer sequences were P15 S (5′-GCG TCA GCT TCA TTA CCC TCC-3′), P15 AS (5′-AAA GCC CGG AGC TAA CGA C-3′), ESR1 S (5′-GAC TGC ACT TGC TCC CGT C-3′), ESR1 AS (5′-AGG ACA GCC CGA GGT TAG-3′), ICSBP S (5′-CCG AAT TCC TGG GAA AGC C-3′), ICSBP AS (5′-TTC CGA GAA ATC ACT TTC CCG-3′), METS S (5′-ATT TGC GTG TTA AGT CTG CGG-3′), METS AS (5′-TCC CAC ACA ACA GAG AGG CG-3′), DP103 S (5′-GCT GTT AGT CCA GTT CCA GGT TCC-3′), DP103 AS (5′-GTC TAA CCA CCA CAT TTA TCT CGG G-3′), Chr6-S (5′-GAA ACC CTC ACC CAG GAG ATA CAC-3′), Chr6-AS (5′-AGT TGG GTG ACT TTA TTC CAT AGA AGA G-3′), Chr9-S (5′-GTG TCC ACA TCT CTG GGT AAC TC-3′) and Chr6-AS (5′-AGT AAG CTC TTT CCT GGT ACA C-3′). After
adding the PCR mixture, 1 μl MseI-digested DNA (5 ng/μl) was added to every other well, that was not incubated previously with DNA fragments (\(P\)-reaction). PCR was performed on a MJResearch engine with the following cycling conditions: 95°C for 3 min (denaturation), 94°C for 20 s, 60°C for 20 s and 72°C for 70 s (36 cycles) and 72°C for 5 min (final extension). Initial pilot experiments showed that the reaction was still in the linear phase of amplification using the above conditions (Figure 2B). The MB-PCR products (20 μl) were analyzed using 3% agarose gel electrophoresis and the ethidium bromide stained gels were scanned using a Typhoon 9200 Imager (Amersham/Pharmacia).

**Sodium bisulfite sequencing**

Modification of DNA with sodium bisulfite (20) was performed as described previously (21). Bisulfite-treated DNA was amplified in a nested PCR using the primers icsbp-out S (5'-GGG GTA GTT AGT TTT TGG TTG-3') and icsbp-out AS (5'-ATA AAT AAT TCC ACC CCC AC-3') for the first and icsbp-in S (5'-TTG TGG ATT TTG ATT AAT GGG-3') and icsbp-in AS (5'-CCR CCC ACT ATA CCT ACC TAC C-3') for the second round of amplification. PCR products were cloned using TOPO-TA Cloning Kit (Invitrogen) and several independent clones were sequenced.

**RNA-preparation, real-time PCR**

Total RNA was isolated from different cell lines by the guanidine thiocyanate/acid phenol method (22). RNA (2 μg) was reverse transcribed using Superscript II MMLV-RT (Invitrogen). Real-time PCR was performed on a Light-Cycler (Roche) using the Quantitect kit (Qiagen) according to the manufacturer’s instructions. Primers used were ICSBP, sense 5'-CGT GTG GTG CAA AGG CAG-3', antisense 5'-CTG TTA TAG AAC TGC TGC AGC TCT C-3'; and ACTB, sense 5'-TGA CGG GGT TCA CCC ACA CTG TGC CCA TCT A-3', antisense 5'-CTA GAA GCA TTT GTG GTG GAC GAT GGA GGG-3'. Cycling parameters were denaturation 95°C, 15 min, amplification 95°C, 15 s, 57°C, 20 s, 72°C, 25 s, for 50 cycles. The product size was initially controlled by agarose gel electrophoresis and melting curves were analyzed to control for specificity of the PCRs. ICSBP data were normalized for expression of the ACTB gene. The relative units were calculated from a standard curve plotting three different concentrations of log dilutions against the PCR cycle number (CP) at which the measured fluorescence intensity reached a fixed value. The amplification efficiency \(E\) was calculated from the slope of the standard curve by the formula \(E = 10^{-1\text{/slope}}\). \(E_{\text{ICSBP}}\) ranged from 1.87 to 1.98 and \(E_{\text{ACTB}}\) ranged from 1.76 to 1.84. For each, sample data of three independent analyses were averaged.

**RESULTS**

**Outline of the MB-PCR technique**

We reasoned that a methyl-CpG DNA-binding polypeptide covalently coupled to the vessel walls of a PCR tube might allow the binding and detection of CpG-methylated DNA in a manner comparable to the standard ELISA technique.!
To enable the high-affinity binding of double-stranded, CpG-methylated DNA, we used a fusion protein comprising the methyl-CpG-binding domain (MBD) of human MBD2, a flexible linker polypeptide and the Fc portion of human IgG1. MBD2 was chosen, because previous binding studies suggested that its methyl-binding domain shows a high affinity to CpG-methylated DNA when compared to other mammalian MBD proteins. The bivalent structure of the antibody-like MBD-Fc protein likely further increases its affinity and avidity towards CpG-methylated DNA. Design, production and properties of the MBD-Fc protein will be described elsewhere (19). Briefly, its affinity to a given DNA fragment depends on the following variables: (i) the amount of salt in the reaction or washing buffer (low salt requires little CpG methylation for binding, high salt requires more CpG methylation for binding); (ii) the number of methylated CpG-dinucleotides; and (iii) the density of the MBD-Fc protein on the interaction surface.

The binding reaction and two washing steps are performed in a stringent high salt buffer (400 mM NaCl) assuring that fragments with little or no methylation are washed off. In contrast to most previous methods, detection of CpG methylation by MB-PCR therefore largely depends on the degree/number of methylated cytosine residues in a given DNA fragment.

We explored the MB-PCR method by analyzing the degree of CpG methylation of single CpG island promoters that were shown previously to be frequently methylated in leukemia cells, namely the human \(CDKN2B\) gene (also known as \(p15^\text{INK4b}\)) and the human estrogen receptor 1 (\(ESR1\)) gene. In addition to the well-established tumor markers we selected three additional genes with CpG island promoters that could potentially act as tumor suppressor genes: the human interferon consensus-binding protein (\(ICSBP\)) gene, the human Ets variant 3 gene (\(ETV3\)) and the human DEAD box polypeptide 20 gene (\(DDX20\)). ICSBP, a transcription factor of the interferon (IFN) regulatory factor family (IRF), is frequently down-regulated in human myeloid leukemia (23) and ICSBP-deficient mice display hematological alterations similar to chronic myelogenous leukemia (CML) in humans (24), suggesting a tumor suppressor function for ICSBP in hemopoietic cells. In mice, the Ets repressor ETV3 (also known as METS or PE1) and its co-repressor DDX20 (also known as DP103) were shown to link terminal monocytic differentiation to cell-cycle arrest (25), which may also indicate a possible tumor suppressor role.

To allow for a semi-quantitative analysis of the genomic PCR, we initially tested a series of DNA dilutions at various cycle numbers. As shown in Figure 2B using 36 cycles our PCR approach was able to detect a range of 5 ng down to at least 250 pg, suggesting that ethidium bromide stained PCR fragments could be semi-quantitatively analyzed under these conditions. As a validation of our MB-PCR approach, genomic DNA from normal cells was either left untreated or methylated \textit{in vitro} using SssI, digested with MseI and subjected to MB-PCR. Genomic DNA was digested with MseI because this enzyme is methylation insensitive and cuts DNA into small fragments while leaving CpG islands relatively intact (26). Locations of the detected MseI fragments relative to the first exon of their respective genes as well as positions of gene-specific primers used for

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**Figure 2.** MB-PCR detects methylation of CpG island promoters. (A) Schematic presentation of the detected MseI-fragments (indicated as gray boxes) of \(ESR1\), \(CDKN2B\) (\(p15^\text{INK4b}\)), \(ICSBP\), \(ETV3\) and \(DDX20\). The position of CpG-dinucleotides, MseI-restriction sites, transcription start site, first exon and relative position of primers are marked. (B) \(P\)-reaction for \(ICSBP\) using a serial dilution of MseI-digested genomic DNA. (C) Representative MB-PCR results of normal (unmethylated) and \textit{in vitro} methylated genomic DNA for the indicated promoters and CpG-free regions. The \(P\)-reaction directly amplifies the genomic DNA, whereas the \(M\)-reaction only amplifies CpG-methylated DNA fragments.
MB-PCR are shown in Figure 2A. All fragments in CpG-containing regions include the putative proximal promoter regions. As shown in Figure 2C, the M-reactions of all five gene loci were negative when normal DNA was used, indicating that these genomic regions are, as expected, free of methylation in normal blood cells. However, each locus was amplified in the corresponding M-reaction when the same DNA was in vitro methylated using SssI-methylase before it was subjected to MB-PCR. The CpG-free genomic regions were not amplified in untreated and SssI-treated DNA, suggesting that the amplification of a genomic DNA fragment requires the presence of DNA methylation and that no unmethylated DNA is retained after washing. Hence, MB-PCR is able to discriminate the methylated and unmethylated state at these loci.

**Methylation status of specific CpG island promoters in tumor cell lines analyzed by MB-PCR**

To test whether MB-PCR is able to detect the methylation status of the above loci in biological samples, we analyzed several leukemia cell lines. Routinely, a total of 10 ng of restricted DNA was used for the M-reaction and 5 ng of the same digested genomic DNA was used for the P-reaction. The result of a representative MB-PCR experiment from eight different leukemia cell lines is shown in Figure 3A. The ESR1 promoter was amplified to varying degrees in the M-reaction of all eight samples, which is in line with previous reports demonstrating its aberrant methylation in >80% of human hemopoietic tumors. The P-reaction for the CDKN2B promoter failed completely in three cell lines (THP-1, NB-4 and K562) suggesting mutations or deletions on both alleles, which has been demonstrated previously in the cases of NB-4 (16) and K562 (27). The two cell lines KG-1 and MUTZ-3 showed a positive M-reaction for the CDKN2B promoter, whereas three cell lines (U937, MonoMac6 and HL-60) were negative. The observed results were in good concordance with previously published methylation analyses of ESR1 (28) and CDKN2B promoters (15,16,27). In some cases, P-reactions were weaker in comparison with other cell types, suggesting the loss or mutation of one allele (e.g. ESR1 in U937 cells). Interestingly, the ICSBP promoter was also amplified in M-reactions of six cell lines, whereas no significant methylation was detected at the promoters of ETV3 and DDX20 genes. The two negative control fragments on chromosomes 6 and 9 were positive in the P-reaction but also negative in the M-reaction (data not shown).

Since aberrant CpG methylation at the ICSBP locus has not been described previously, the degree and effect of ICSBP promoter methylation was analyzed to further validate the experimental potential of MB-PCR. To determine how MB-PCR results correlate with the exact pattern of CpG methylation at the ICSBP promoter in individual cell lines, we analyzed ICSBP promoter methylation by bisulfite sequencing. The results shown in Figure 3B indicate that the degree of promoter methylation corresponds with results obtained by MB-PCR. Strong amplification signals (comparable with the corresponding P-reaction) as seen in KG-1, U937, MUTZ-3, HL-60 and K562 cell lines, appear to indicate a high degree, whereas weaker signals (as observed for NB-4 cells) indicate a lesser degree of methylation. In the absence of DNA methylation (THP-1 and MonoMac6 cells) the MB-PCR is negative. As shown in Figure 4A, mRNA expression levels analyzed by LightCycler real-time PCR inversely correlated with methylation degree as determined by MB-PCR and bisulfite sequencing. Treatment of U937 cells, which show a high degree of ICSBP promoter methylation with the demethylating agent Decitabine (5-Aza-2'-Deoxycytidine) led to a marked, dose- and time-dependent induction of ICSBP mRNA expression (Figure 4B), indicating that the methylation-induced repression of ICSBP transcription is reversible in these cells.

**Figure 3. Detecting CpG methylation in leukemia cell lines by MB-PCR.** (A) Shown are representative MB-PCR results of eight different leukemia cell lines for the indicated promoters. (B) Genomic DNA from the same cell lines was analyzed by bisulfite sequencing. The indicated region of the ICSBP gene was amplified and cloned. Several independent inserts were sequenced and results are presented schematically. Squares mark the position of CpG-dinucleotides (open, unmethylated; closed, methylated).
Linearity and sensitivity

Samples derived from tumors may contain significant numbers of normal cells, that would be expected to be unmethylated at most CpG islands. To test how linear the detection of CpG methylation is with respect to cell purity, MB-PCR was performed using mixtures of DNA from normal blood cells (freshly isolated blood monocytes) and a leukemia cell line KG-1 showing high levels of CpG island methylation at the promoters investigated in this study. As shown in Figure 5A, the ICSBP promoter fragment was only detected in samples containing KG-1 DNA and the signal gradually increased with the proportion of methylated DNA in the sample. Similar results were obtained for ESR1 and CDKN2B loci (data not shown).

To test the sensitivity of our approach, we performed MB-PCR experiments using decreasing amounts of DNA. Dilutions of genomic DNA from three different cell lines (U937, NB-4 and THP-1) were subjected to MB-PCR. To account for the reduced amount of template DNA in the PCRs, the cycle number was increased proportionally. As shown in Figure 5B, comparable results were obtained using either 10 ng, 2.5 ng, 625 pg or even 160 pg of digested genomic DNA, suggesting that MB-PCR is able to detect the methylation status of a single promoter in DNA derived from as few as 30 cells.

Detecting methylation of CpG island promoters in primary tumor cells

DNA was prepared from blood monocytes of several healthy persons (n = 4) and leukemic blasts of patients with previously untreated AML (n = 35), digested with MseI, and subjected to MB-PCR. Figure 6 shows representative ICSBP MB-PCR and corresponding bisulfite sequencing results for nine AML patients and one normal individual. In general, the intensity of the band observed in the M-reaction (as compared with the corresponding P-reaction) showed good correlation with the mean density of methylation in the sample.

Out of 35 AML patients tested, 7 patients (20%) showed positive MB-PCR results for ICSBP, 21 patients (60%) for ESR1 and 25 patients (71%) for CDKN2B (data not shown). The frequencies for ESR1 and CDKN2B methylation observed concur with those described in previous studies (16,28). ICSBP methylation apparently only affects a subgroup of patients. Twelve patients were tested for methylation of ETV3 and DDX20 genes and, as observed for the leukemia cell lines, no significant methylation was detected in any of the samples.
DISCUSSION

We have developed a novel one tube assay, termed MB-PCR, to detect genomic DNA fragments according to their level of CpG methylation. The novel technique requires little amounts of DNA and allows the rapid screening of multiple loci. MB-PCR is particularly useful to screen for methylation levels of candidate genes in tumor tissue or tumor cells as exemplified for acute myeloid leukemia in this report. It may, however, also be useful to detect changes in DNA methylation in other situations, including normal cellular differentiation and aging.

Comparison with existing methods

At present, mainly two technical approaches are used to detect the level of CpG methylation of known candidate gene loci: methylation-sensitive restriction or bisulfite treatment of DNA (29).

Isoschizomers of bacterial restriction endonucleases with different sensitivities for 5-methylcytosine can be used to determine the methylation status of specific CpG-dinucleotides (29). The use of methylation-sensitive restriction enzymes, however, has several limitations. Apart from the fact that incomplete restriction digests may complicate the analysis, the greatest disadvantage is that methylation-sensitive restriction merely informs on the methylation status of the cytosine residues which are recognized by the methylation-sensitive restriction enzymes used.

A global picture of the methylation pattern in a candidate gene locus may be obtained by bisulfite sequencing as originally described by Frommer et al. (20). The treatment of double-stranded genomic DNA with sodium bisulfite leads to the deamination of unmethylated cytosine residues (but not 5-methyl cytosine) into uracil residues. DNA treated with bisulfite can be used directly in PCR in which uracil residues (previously unmethylated cytosine) and thymidine residues are amplified as thymidine and only 5-methylcytosine residues are amplified as cytosine residues (20). Depending on the application, the primers used for the PCR differentiate between methylated and unmethylated sequences or amplify fragments independently of the methylation status (29). PCR fragments which have been amplified using non-discriminating primers can, for instance, be sequenced directly to determine the position of methylated and unmethylated CpGs. Other methodical approaches that allow high-throughput analyses utilize the differences in sequence for the specific amplification of methylated and unmethylated sequences by discriminating primers or probes (e.g. methylation-specific PCR, MethyLight) (29). In contrast to the methylation-sensitive restriction enzymes, the DNA treated with bisulfite can potentially provide information on the methylation status of several CpG residues in an amplified genomic fragment. The detection of CpG methylation by using discriminating primers or probes, however, is limited to the methylation status of single (or few) cytosine residues. Hence, the information provided by all currently known assays that are suitable for high-throughput methylation analysis of single gene loci is limited to one or only a few CpG residues within the gene of interest.

Rather than analyzing single CpG residues, MB-PCR analyses target DNA fragments according to their methylation degree. The information provided by MB-PCR will be at least as relevant as that obtained with other existing PCR techniques—the methylation density of a proximal promoter may actually correlate better with the transcriptional status of a gene than the methylation status of a single CpG residue within the region. We believe that the high methyl-CpG affinity of MBD2 combined with the bivalent, antibody-like structure of the recombinant MBD-Fc protein greatly increases its binding capacity, enabling the efficient retention of a DNA fragment on the basis of its degree of methylation. A comparable approach discriminating DNA fragments on the basis of their methylation density was developed in the laboratory of A. Bird already 10 years ago (26). A recombinant MeCP2 protein bound to a matrix was used in this and a number of recent studies for binding and enriching highly methylated DNA through affinity chromatography (26). Although we have not tested recombinant MeCP2, it is possible that it may also work as methyl-binding polypeptide in MB-PCR.

The company Panomics introduced recently a commercially available kit that differentiates promoters with methylated groups from unmethylated promoters. In principle, the company’s method consists of a spin column affinity purification using MeCP2. This method also appears to be rapid,
however, it requires a relatively large amount of starting material. It is not clear, to which extent the information can be quantified or whether the amount of isolated fragments correlates with the degree of promoter methylation. A recent report by Klose et al. (30) clearly demonstrated that MeCP2 requires an A/T run adjacent to the methylated CpG dinucleotide for efficient DNA binding, suggesting that all methods based on MeCP2-affinity chromatography, including the Panomics kit, will be biased towards certain CpG motifs. Therefore, it is not clear, whether MeCP2 will be able to detect every methylated CpG island fragment. No binding requirements or preferences of MBD2 were detected in this and previous studies (30). Owing to its binding properties, MeCP2 may be better suited to detect non-CpG island promoters with a lower CpG-density, e.g. CD14, IFNγ or IL-4 Promoters (as demonstrated in the user manual of the commercially available kit).

Technical considerations

An important aspect of MB-PCR is the fragmentation of the genomic DNA. We have used the restriction enzyme MseI (T/TAA) in our study; however, other methylation-insensitive restriction enzymes such as Csp6I (G/TAC) or Tsp509I (AATT) may also be used (either alone or in combination) to achieve an appropriate fragmentation of the target gene. Most informative (with respect to the effects on transcription) and clearest results (in terms of noise and background) are obtained when a target gene fragment contains only the proximal promoter within the CpG island. In addition to enzyme restriction, DNA fragmentation may also be achieved by mechanical means, e.g. ultra-sonication.

As demonstrated, our current approach allowed the distinction between strong (>30–40% methylation), intermediate methylation levels (>10%) or no methylation. Owing to the limitations of standard PCR, a more detailed grading is technically difficult. In most cases, however, standard MB-PCR will be sufficiently informative to detect aberrant methylation in a tumor sample. A standardization of individual experiments may be achieved by using a series of mixtures of methylated and unmethylated DNA as a standard curve for each experiment. As a control for the completeness of restriction digestion as well as the washing procedure, a DNA fragment is amplified that contains no CpG residues and therefore should not be retained and amplified. Although we have not yet tested, it is conceivable that MB-PCR may also be run as a real-time PCR application, which may allow the quantification of amplified products and a better correlation with methylation levels in a sample.

Since the surface area of the PCR tube and hence its binding capacity for the recombinant methyl-DNA-binding polypeptide is limited, it is important to avoid the use of an excess amount of genomic DNA for the assay. We found that MB-PCR produces consistent results using 160 pg to 10 ng of restricted genomic DNA. The little amount of DNA required for MB-PCR is actually a great advantage of this technique, allowing the methylation analysis of candidate genes from very limited cell numbers which may include biopsy samples or cells collected by laser-mediated microdissection.

Screening for aberrant CpG methylation

To test the usefulness of MB-PCR for methylation analysis, we initially analyzed CpG island promoters that were known to us or described in the literature to be methylated or unmethylated in particular cell lines, including the promoters of CDKN2B (p15INK4B) and ESR1 genes. Since initial results obtained by MB-PCR from several leukemia cell lines corresponded with previously published observations, we selected a number of novel putative candidate genes (ICSBP, ETV3 and DDX20) for further analyses that are involved in cell-cycle arrest or cellular differentiation and represented good candidates as tumor suppressor genes. Using MB-PCR we show that the CpG island promoter of ICSBP is methylated in many leukemia cell lines and a subset of patients with AML. MB-PCR results were independently confirmed by bisulfite sequencing and methylation of the promoter correlated with the absence or down-regulation of ICSBP transcription in the leukemia cell lines. Our data suggest that epigenetic silencing may contribute significantly to the observed down-regulation of ICSBP in human myeloid leukemia. The CpG island promoters of ETV3 and DDX20 were not methylated in any of the samples tested so far. It will be interesting to analyze the methylation status of these genes, especially ICSBP, in other malignancies, e.g. CML or non-myeloid malignancies.

In summary, our report describes a rapid and sensitive procedure for detecting methylated DNA target sequences from limited sample material. MB-PCR will be particularly useful in screening methylation levels of candidate genes not only in tumor tissue but also in tumor cells. Our study also suggests that the promoter of ICSBP is hypermethylated in a subgroup of patients with myeloid leukemia, which may serve as a molecular marker for disease.

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