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Promoter Methylation Analysis on Micro- dissected Paraffin- Embedded Tissues Using Bisulfite Treatment and PCR-SSCP

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ABSTRACT

Methylation-sensitive single-strand conformation analysis (MS-SSCA) is a new method of screening for DNA methylation changes. The combination of bisulfite modification and PCR results in the conversion of unmethylated cytosines to thymines, whereas methylated cytosines remain unchanged. This sequence conversion can

lead to methylation-dependent alterations of single-strand conformation, which can be detected by SSCA. An analysis of mixtures of methylated and unmethylated DNA at known ratios revealed that the relative intensities of the corresponding bands following MS-SSCA were maintained. MS-SSCA was applied for methylation analysis of human p16 promoter region using genomic DNA obtained from either frozen, fixed, or microdissected fixed tissue sections. MS-SSCA is a rapid, specific, and semiquantitative approach that allows the detection of methylation of the p16 gene promoter. In reconstruction experiments, the method permits the detection of 10% or less of cells harboring a methylated p16 promoter. We have been successful in analyzing by MS-SSCA almost all (96%) tumor samples microdissected from archival paraffin-embedded fixed tissue sections and obtaining reproducible results. In addition, when microdissection was performed, the clonality of this genetic alteration could be identified.

INTRODUCTION

Hypermethylation of the 5'CpG island in the promoter region is one of the mechanisms for inactivation of tumor suppressor genes in human neoplasia (8,9). Detection of DNA methylation has recently attracted considerable attention. Although the exact sequence location of methylated cytosines can be determined by bisulfite genomic sequencing (4,5), this approach is technically difficult and time consuming. Methylation-specific PCR (MSP) is an alternative qualitative method for methylation analysis that is easy to perform and highly sensitive (7). However, we obtained false-positive results using DNA from fixed tissues due to PCR overamplification. Southern blot analysis is a quantitative method to analyze DNA methylation levels at specific gene loci, but this requires much more DNA (> 5 µg) and cannot be performed

on DNA isolated from paraffin sections. Combined bisulfite restriction analysis (COBRA) provides an excellent methylation analysis with both high sensitivity and quantitative accuracy (11), but this method also potentially generates false-positive results when enzymatic digestion and/or bisulfite modification are incomplete (10). These limitations hinder the routine analysis of samples, especially from paraffin-embedded fixed tissues. Recently, a new method of screening for methylation changes has been developed based on bisulfite treatment of DNA followed by PCR and single-strand conformation analysis (SSCA) (2,3). In this study, we applied this procedure to the methylation analysis of p16 promoter region in human cancers. We specially investigated the use of this semiquantitative method on bisulfite-treated genomic DNA from frozen, fixed, and microdissected archival paraffin-embedded fixed tissue sections.

MATERIALS AND METHODS

Tissues, Cell Lines, and DNA Extraction

Frozen tumor samples from cases of bladder, colon, esophagus, and soft tissue tumors, as well as their associated normal tissues, were obtained from the Tissue Bank of the Institute of Pathology, University of Lausanne. Archival formalin-fixed and paraffin-embedded tissues of each case were also studied. All tissue samples, frozen and fixed, were histologically characterized. A colon cancer-derived cell line (SW620) and placenta tissue were also used. Genomic DNA from frozen and archival fixed tissues and cell lines were extracted by using the DNeasy™ Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer.

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Figure 1. Outline of the MS-SSCA procedure. This method is based on bisulfite treatment of genomic DNA followed by PCR using primers specific to the modified strand and without CpG sites (indicated by large bands) and then semiquantitatively analyzed by nonradioactive SSCP. According to the sequence differences created by bisulfite PCR, three patterns are distinguishable by SSCP: unmethylated, methylated, and a mixture the two alleles.

Figure 2. Determination of semiquantitative methylation by MS-SSCA. DNAs from a human colorectal cancer cell line (SW620) and from normal human tissue (placenta) were mixed at various ratios, as indicated above the gel, to determine the dynamic range of semiquantitative methylation by MS-SSCA. The p16 promoter was fully methylated for SW620 (100%) and unmethylated for placenta (0%). Linear regression analysis of the data shows that MS-SSCA yields reliable semiquantitative results across a wide range of DNA methylation levels and that the technique is sensitive to 10% methylated alleles of a given CpG island locus.

Microdissection and DNA Extraction from Fixed Tissue Sections

After deparaffinization in xylene and rinsing in methanol, sections were stained with 0.01% toluidin blue, and selected areas were microdissected and extracted as previously described (1). In brief, nontumorous tissues were completely eliminated before tumor cell collection. By this procedure, microdissected areas were determined to contain at least 95% cancer cells. The microdissected cells were immediately resuspended in 27 μ L TE buffer, 3 μ L digestion buffer (500 mM KCl, 150 mM Tris-HCl, pH 8.0, 15 mM MgCl₂, and 1.5% Tween[®] 20), and 0.5 mg/mL proteinase K, incubated for at least 24 h at 55°C. An equal volume of a 10% Chelex[®]-100 resin solution (Bio-Rad Laboratories, Hercules, CA, USA) was added, and the mixture was incubated for 1 h at 55°C, then boiled for 10 min. After quick centrifugation, the supernatant containing the DNA was subsequently purified with phenol followed by phenol/chloroform and co-precipitated in ethanol with 10 μ g glycogen at -20°C. Extracted DNA was resuspended in 20 μ L 10 mM Tris-HCl, pH 8.0, solution and kept at -20°C until use.

Bisulfite Modification

Genomic DNA (1 μ g) from cell lines, frozen, or fixed tissue, and all the DNA obtained in the case of microdissected tissues, in a volume of 40 μ L water, were first heated at 100°C for 10 min and then denatured for 10 min at 37°C by addition of 2.7 μ L freshly prepared 3 M NaOH. Twenty-eight microliters of 10 mM hydroquinone and 500 μ L 4.8 M sodium bisulfite (both from Sigma, St. Louis, MO, USA) at pH 5.0, both freshly prepared, were added, and the reagents were well mixed. The mixture was incubated at 55°C for 4 h (7). After adding 80 μ L water and 365 μ L 100% EtOH, the modified DNA was purified by passing through a column from the DNeasy Tissue Kit. After washing with the wash buffer contained in the kit, DNA modification was completed on the column by addition of 500 μ L 0.2 M NaOH/90% EtOH. The reaction was performed during 10 min at room temperature, washing was done twice, and

DNA was eluted into 50 μ L 10 mM Tris-HCl solution and stored at -20°C .

PCR-SSCP

A 194-bp fragment of the p16 gene promoter containing 11 CG dinucleotides was amplified by PCR using the following primers specific to the upper modified strand: 5'-GGGGGA GATTTAATTTGG-3' (sense) and 5'-CAACCCCTCCTCTTTCTT-3' (anti-sense). After an initial denaturation for 5 min at 95°C , the PCR was carried out in the presence of 5% dimethyl sulfoxide (DMSO) for 35 (with DNA from frozen tissues) or 40 (with DNA from fixed tissues) cycles using *Taq* DNA polymerase and the following amplifi-

cation profile: denaturation at 94°C for 30 s, annealing at 54°C for 45 s, and extension at 72°C for 75 s. Amplified PCR products were analyzed by electrophoresis on a 2% agarose gel. Five microliters of PCR product were denatured with 5 μ L denaturing buffer (100 mM NaOH and 2 mM EDTA) at 50°C for 10 min. After the addition of 1.5 μ L formamide dye, the samples were immediately analyzed in a 30% MDE gel (AT Biochem, Malvern, PA, USA) in $0.5\times$ TBE (45 mM Tris-HCl, 1 mM EDTA, 40 mM boric acid) with a constant voltage of 20 V/cm for 5 h at 20°C . After electrophoresis, gels were released from plates, stained for 20 min in the dark with a SYBR[®] Gold gel stain (Molecular Probes, Eugene, OR,

Figure 3. MS-SSCA of human esophageal squamous cell carcinomas and their associated normal tissues. To test whether this method could be applied to the routine analysis of any kind of clinical material: frozen (lanes 4 and 5), fixed (lanes 6 and 7), and microdissected fixed (lanes 8 and 9) tissues were used. Mixtures of SW620 and placenta genomic DNAs in different ratios (0%, 80%, and 100%) were used as control (lanes 1–3). T, tumor tissue; N, normal tissue.

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USA), diluted 1:10 000 in 1× TBE buffer, and visualized with UV light using a charge-coupled device (CCD) camera. For each sample, the final results were confirmed by the simultaneous analysis of two PCR products.

RESULTS AND DISCUSSION

Semiquantitative MS-SSCA Analysis

The differences of methylation-dependent sequence are introduced into the genomic DNA by sodium bisulfite modification, and then the modified DNA is amplified by PCR using primers without CpG repeats and complementary to the deaminated DNA strand. This combination of bisulfite modification and PCR results in the conversion of unmethylated cytosine residues to thymine, whereas methylated cytosine residues, present at CpG sites, are retained as cytosine. This sequence conversion can lead to the methylation-dependent alteration of single-strand conformation, which can be detected by single-strand conformation polymorphism (SSCP). The outline of the MS-SSCA, bisulfite modification followed by PCR-SSCP analysis, is shown in Figure 1. Three patterns of band mobility were detected by MS-SSCA: no methylation (0%), full methylation (100%), and a mixture of both. The percentage of DNA methylation can therefore be directly reflected by the ratio of intensity between the methylated and unmethylated bands. To check the reliability of this approach, this analysis was performed on genomic DNAs for which the p16 promoter region was found to be either fully methylated (SW620 colorectal cell line) or fully unmethylated (placenta) at all CpG sites. To determine the dynamic range of semiquantitative methylation analysis by PCR-SSCP, we prepared mixtures of SW620 and placenta genomic DNAs in different ratios (0%, 10%, 20%, 50%, 80%, 90%, and 100%) before the bisulfite modification and processed as independent samples throughout a complete bisulfite modification followed by a PCR-SSCP analysis. The results of this experiment are shown in Figure 2. Linear regression analysis of the data shows that MS-SSCA yields reliable semi-

quantitative results across a wide range of DNA methylation levels. This technique allows the detection of at least 10% fully methylated p16 gene promoter in the presence of unmethylated one (Figure 2, lane 2).

Application of MS-SSCA in Frozen and Fixed Human Cancer Tissues

To test if this method could be applied to the routine analysis of clinical tumor specimens of frozen or archival fixed tissues, we analyzed human cancers (from bladder, colon, esophagus, and soft tissue) and their associated normal tissues for p16 methylation status. All the genomic DNAs tested were found to be amplifiable by PCR after bisulfite treatment. Only three patterns of mobility were detected by MS-SSCA: no methylation, full methylation, and a mixture of the two. These results suggested that partially methylated alleles (methylation only occurs in some of the CpG sites within the analyzed region) were not present in these tissues or cell lines. Analysis of p16 promoter methylation in three cases of esophageal squamous cell carcinoma by using frozen and fixed tissues are shown in Figure 3. In comparison with the pattern of unmethylated normal esophageal squamous epithelium, case 1 had no methylation (Figure 3, case 1, compare lane 5 and 7 to lanes 4 and 6), while a mixture between no and full methylation was observed for cases 2 and 3 (Figure 3, cases 2 and 3, compare lanes 5 and 7 to lanes 4 and 6). Since the tumor tissues are constituted of tumor and nontumor cells (e.g., inflammatory cells, stroma) in variable proportions, it remains unclear whether it is the tumor cells that harbor a methylated p16 promoter. In addition, it could be necessary to determine the level of tumor cells that contain one or both methylated p16 alleles. This crucial problem cannot be resolved by the analysis using pieces of tissues, fixed or frozen, and only microdissection can answer this question.

Application of MS-SSCA in Microdissected Formalin-Fixed and Paraffin-Embedded Tissues

Methylation analysis has not been extensively applied to microdissected

archival fixed tissues. Gonzalzo et al. (6) developed a rapid quantitative method (Ms-SNuPE) for assessing methylation differences at specific CpG sites based on bisulfite treatment of DNA, followed by single-nucleotide extension, and claimed that this technique could be applied to DNA obtained from microdissected pathological sections. However, this approach is more laborious than other techniques and also uses radioactive tracer. We tested two other alternative methods, including MSP (7) and COBRA (11), and found that both resulted in a relatively high number of false-positive cases, probably due to an incomplete bisulfite modification of the genomic DNA (10), and false negative cases, essentially due to poor PCR amplification when the MSP approach was used (data not shown).

There are some points that are important for the successful analysis of methylation status by MS-SSCA using microdissected formalin-fixed and paraffin-embedded tissue sections. Since a significant amount of DNA is lost during bisulfite modification, three- to fivefold more tissue is necessary compared with PCR-SSCP analysis of unmodified genomic DNA, for which 1 mm² of tissue generally suffices (1). In addition, DNA extraction from microdissected sections needs to be optimal. Addition of Chelex (a product known to be a chelator of metals, which are potential inhibitors of *Taq* DNA polymerase) during DNA extraction after the proteinase K digestion step but before phenol/chloroform and ethanol precipitation steps allowed most of samples to be amplified (1). Bisulfite modification is then performed essentially as described for genomic DNA obtained from frozen or fixed tissues. Part of this modification, mainly the washing steps and the final alkali treatment, was done on the DNeasy Tissue Kit column. This procedure is easy to perform, limits the risk of contamination, and yields a large amount of relatively pure modified DNA, which is essential for the next PCR amplification step. Nevertheless, to avoid false results due to the poor quality of extracted DNA in some microdissected samples, two PCRs need to be performed. The same mobility of the PCR products analyzed by SSCP assures a correct result.

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In only a few cases (about 4%), different mobility was observed. In these cases, the whole procedure has to be repeated from the beginning.

This approach has been applied to the analysis of clinical tumor specimens. Figure 3 shows, as an example, the analysis of p16 promoter methylation in esophageal squamous carcinoma. Normal esophageal mucosal cells were also microdissected, but MS-SSCA never showed p16 promoter methylation. For the tumor tissues, similar results were obtained from frozen, fixed, microdissected formalin-fixed, and paraffin-embedded tissues (Figure 3). In case 2, even after microdissection, MS-SSCA showed a mixture of fully methylated and unmethylated p16 promoter, suggesting that half of the DNA molecules was fully methylated and the remainder was unmethylated. (Figure 3, case 2, compare lane 9 to lanes 5 and 7). Conceivably, either the p16 promoter was methylated at all CpG sites in one allele with the other allele totally unmethylat-

ed or a part of the tumor cells was fully methylated. In case 3, the p16 promoter region was fully methylated at all the CpG sites and in all microdissected tumor cells (Figure 3, case 3, compare lane 9 to lanes 5 and 7). As shown in cases 2 and 3, microdissection was necessary to confirm that the p16 promoter was methylated in tumor cells. Analysis after microdissection also revealed that the methylation status of the p16 promoter is complex. Indeed, during our experiments, together with loss of heterozygosity analysis of chromosome 9p21, where p16 is located (data not shown), we found that (i) complete methylation represents either methylation of one allele and deletion of the other allele or methylation of both alleles; (ii) a mixture of complete methylation and no methylation at a ratio of 1:1 represents methylation of only one allele; and (iii) a mixture with other ratios is probably due to intratumor heterogeneity (only part of tumor cell population harbors a methylated p16 promoter). From this point of view, microdissection is a very useful tool to determine the methylation status of the p16 promoter within tumor cells in a tissue.

In conclusion, our improved MS-SSCA has several advantages over existing methods. It is rapid, specific, semiquantitative, and works very well with DNA extracted from microdissected fixed tissues. Indeed, MS-SSCA can detect methylation changes in as little as 1 ng DNA. This technique is also sensitive and allows the detection of 10% or even less of fully methylated p16 gene promoter in the presence of the unmethylated one. The advantage of this method not only allows the analysis of methylation status within a lesion but also gives, when microdissection is performed, further information about the clonality of this genetic alteration.

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