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Methylation of *p16* and *Ras Association Domain Family Protein 1a* during Colorectal Malignant Transformation

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Abstract

Accurate assessment of gene methylation in formalin-fixed, paraffin-embedded archived tissue (FF-PEAT) by microdissection remains challenging because the tissue volume is small and DNA is damaged. In addition, methods for methylation assessment, such as methylation-specific PCR (MSP), require sodium bisulfite modification (SBM) on purified DNA, which causes major loss of DNA. On-slide SBM, in which DNA is modified *in situ* before isolation of tumor cells, eliminates DNA purification steps and allows histology-oriented assessment of gene methylation. This study describes a protocol and use of on-slide SBM using 20 FF-PEAT of colorectal cancers with intratumoral adenoma components to detect accumulation of gene methylation during colorectal malignant transformation. Deparaffinized tissue sections were incubated in sodium bisulfite solution for 8 hours at 60°C, stained with hematoxylin, and then microdissected. Proteinase K lysate was directly used as a template in subsequent PCR. Using on-slide SBM, 282-bp-long bisulfite direct sequencing was possible. Yield of modified DNA was 2.6-fold greater than standard SBM on average. The mean conversion rate was 97%, and false-positive or false-negative results were not observed in subsequent MSP. Intratumoral heterogeneity by accumulation of *p16* and *Ras association domain family protein 1a* methylation during malignant transformation were shown by MSP comparing cancer with adenoma parts within a single section. On-slide SBM is applicable in most methylation studies using FF-PEAT. It allows detailed, intratumoral analysis of methylation heterogeneity within solid tumors. On-slide SBM will significantly improve our approach and understanding of epigenetic events in minimal disease and the carcinogenic process.

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Introduction

In the mammalian genome, ~5% of cytosines are modified to 5-methylcytosine (1), and this modification has important regulatory effects on gene expression especially when it involves cytosine-guanine dinucleotide (CpG) rich areas (CpG islands) in promoter regions (2, 3). Epigenetic gene silencing by promoter hypermethylation of tumor suppressor genes is as significant as deletions or mutations (4-6), and it can play a significant role in malignant transformation and immortalization of cells. Therefore, assessment of promoter hypermethylation has become important for understanding the mechanisms of malignant transformation (7, 8). Sodium bisulfite modification (SBM) of genomic DNA is commonly used to detect methylation of CpG islands in promoter regions. Sodium bisulfite converts cytosines but not 5-methylcytosines to uracils (9). Therefore, the methylation status reflects the changes in the sequence of the DNA after modification. The modified DNA can be analyzed by sequencing (10), methylation-specific PCR (MSP; ref. 11), combined bisulfite restriction analysis (12), high-throughput quantitative methylation assay (MethyLight; ref. 13), quantitative analysis of methylated alleles (14), and other methods.

The most widely available starting material for assessment of solid tumors is formalin-fixed, paraffin-embedded archived tissue (FF-PEAT) used for pathologic diagnosis after surgical removal of the tumor or after tumor biopsy. Use of FF-PEAT for epigenetic analysis, such as MSP, would have significant use to evaluate patients' specimens. As described in a previous report, there are critical issues with SBM of FF-PEAT (15, 16), formalin fixation causes fragmentation and cross-linking of the DNA that is dependent on the acidity of the formalin and soaking time. In addition, 84% to 96% of the source DNA may be lost during conventional SBM (17). This significant loss of template DNA hampers assessment of methylation; a previous study reported that 50 cycles of thermal cycling in MSP were needed for SBM DNA extracted from a small area of FF-PEAT by microdissection (18). This high number of PCR cycles would detect only a single copy of DNA, which increases the risk of false-positive results due to incomplete SBM, and may not be reproducible. Extracting DNA from a larger tumor area or whole-tumor sections increase yields of DNA would result in more reproducible results, but it increases the risk of contamination with DNA from noncancer cells.

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On-slide SBM, in which DNA is modified in the tissues, was initially developed for MSP *in situ* hybridization (19), but its application has been very limited to date. However, on-slide SBM should minimize loss of DNA because it eliminates DNA purification steps. We have developed and optimized a protocol for on-slide SBM that can be easily adapted in laboratories to do histology-oriented methylation analyses. We validated the use of on-slide SBM using primary colorectal cancers with intratumoral adenoma component. Specificity was assessed by estrogen receptor 1 (*ESR1*) and thymine-DNA glycosylase (*TDG*) promoter methylation status. Intratumoral heterogeneity of *p16* and Ras association domain family protein 1a (*RASSF1a*) promoter methylation status was also assessed. The feasibility of direct sequencing of a relatively long PCR product was tested to show that DNA modified by the on-slide SBM technique is degraded to a lesser extent compared with conventional SBM.

To evaluate the yield and conversion rate (i.e., percentage of modified DNA) of on-slide SBM, we have newly developed a highly sensitive quantitative real-time PCR (qPCR) assay for bisulfite-modified and unmodified DNA (i.e., genomic DNA). *Alu*, which is the most abundant repeat sequence in the human genome with a copy number of $\sim 1.4 \times 10^6$ in a cell (20), was used as a target of qPCR to maximize sensitivity. It allowed accurate quantification and precise evaluation for samples having very low amount of modified DNA. Bisulfite-modified DNA and the remaining unmodified DNA were separately quantified by *Alu*-qPCR with specifically designed primer sets.

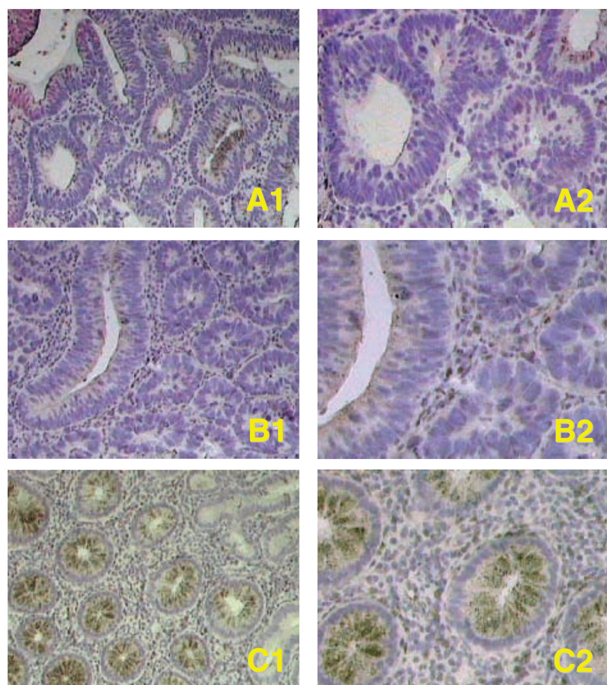

FIGURE 1. Microscopic photographs of three tissue sections after on-slide SBM treatment and hematoxylin staining. Magnification, ×100 (**A1, B1**, and **C1**) and ×200 (**A2, B2**, and **C2**).

Results

Effect of On-Slide SBM on Microscopic Histology of Tissue Sections

Because on-slide SBM uses alkaline pretreatment for denaturation of DNA, potent acid treatment for modification of cytosines at relatively high temperatures for long durations, and alkaline treatment for desulfonation of cytosines, we expected some deterioration of microscopic tissue architectures. However, as shown in Fig. 1, tissue architecture was maintained after on-slide SBM at optimal conditions of 60°C for 8 hours, and there was no impairment of pathologic assessment after hematoxylin staining. Consequently, microdissection could be precisely done without difficulty. H&E and methyl green staining was also done on post–on-slide SBM slides and showed similar quality (data not shown).

Bisulfite Direct Sequencing of p16 *Gene Promoter Region*

Bisulfite direct sequencing of a 282-bp amplicon in the *p16* gene promoter region was done in an initial evaluation set of three colorectal cancer specimens comparing two incubation temperatures of on-slide SBM. Sequencing of on-slide SBM samples incubated at 70°C for 4 or 8 hours was unsuccessful. This suggests that template DNA may be damaged and truncated during incubation at 70°C, thus not suitable for assessment. In contrast, on-slide SBM samples incubated at 60°C for 4 or 8 hours were successfully sequenced (Fig. 2). All cytosines in the sequencing results were correctly converted to uracil with incubation at 60°C for 8 hours. Therefore, incubation at 60°C was used for on-slide SBM in the subsequent assessments.

Direct sequencing of DNA after standard SBM or agarose-bead SBM was unsuccessful with the same sequencing protocol.

The Yield and Conversion Rate of Modification

The yield and conversion rate of modification was evaluated using eight tissue samples for on-slide, standard, and agarose-bead SBM by assessing the copy numbers of modified and unmodified *Alu* repeat sequences. Each sample was assessed in triplicate, and the median intersample coefficient of variance for modified and unmodified DNA was 6.8% and 11.2%, respectively.

The yield of modified DNA increased with the duration of incubation of on-slide SBM. When modified *Alu* copy numbers of on-slide and standard SBM were compared, the relative yield of modified DNA by on-slide SBM was higher by a mean factor of 1.3 ± 0.8 and 2.6 ± 1.0 (mean ± SD) at incubation settings of 60°C for 4 hours and 60°C for 8 hours, respectively (Fig. 3). The relative yield of modified DNA by agarose-bead SBM was only 0.9 ± 1.0 (mean ± SD) after 14 hours of incubation at 50°C.

The conversion rates of modified DNA by on-slide SBM were $75.4 \pm 15.2\%$ and $97.1 \pm 2.7\%$ (mean ± SD) at incubation settings of 60°C for 4 hours and 60°C for 8 hours, respectively (Fig. 4). Conversion rates increased with increasing duration of incubation. The conversion rate of on-slide SBM with 4 hours of incubation was obviously low for specific assessment of methylation status, but it was satisfactory and similar to the

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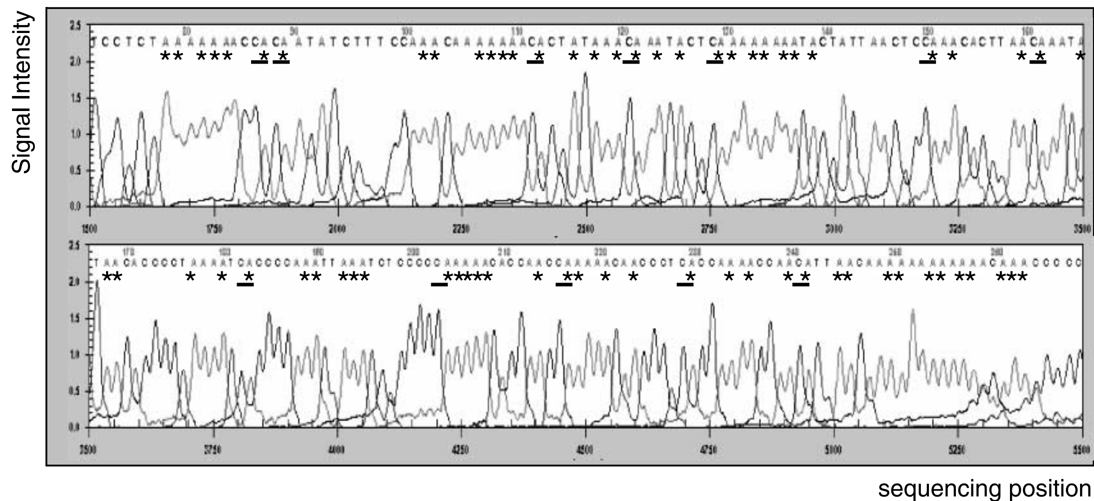

FIGURE 2. Representative standard sequencing diagram for bisulfite direct sequencing of *p16* gene promoter region after on-slide SBM DNA with incubation at 60°C for 8 hours depicting the high quality of modified DNA. Bisulfite direct sequencing of a 282-bp amplicon was made possible by on-slide SBM from 1-mm^2 microdissected tissue of 10-μm FF-PEAT section of a colorectal cancer. All cytosines in this sequence (*) were converted to uracil, shown as "A" in reverse strand. Signals of false conversion were not observed. CpGs are underlined. The *p16* gene promoter region of this colorectal cancer is not methylated.

standard SBM method with 8 hours of incubation. The conversion rate of standard SBM was 97.6±1.3% after 3 hours of incubation at 60°C. Assessment of cross-reaction of *Alu* primers on the salmon sperm DNA used as a carrier in the standard SBM revealed that it contributed only <0.1% of the total copy numbers. The agarose-bead SBM method had a conversion rate of 99.4 ± 0.5% after 14 hours of incubation at 50°C.

MSP for ESR1, TDG, p16, *and* RASSF1a

To evaluate the possibility of false-positive or false-negative MSP results after on-slide SBM, we assessed the methylation status of CpG islands in *ESR1* and *TDG* gene promoter of 16 pairs of cancer and adenoma tissues. All showed methylation of *ESR1* (Fig. 5A), and none showed methylation of *TDG* (Fig. 5B). These results indicate that on-slide SBM did not cause false-positive or false-negative results by MSP.

Methylation status of the CpG island in the *p16* and *RASSF1a* gene promoter region was assessed to show the use of on-slide SBM for assessment of intratumoral heterogeneity of epigenetic alterations. MSP results from cancer and adenoma parts within the same tissue section were compared. Among the 16 evaluated pairs, 4 showed methylation of the *p16* promoter in both the cancer and the adenoma parts, and 3 showed methylation only in cancer part (Fig. 5C). These results show that certain colorectal cancers arisen from adenoma acquire *p16* promoter hypermethylation during malignant transformation, and others acquire it at an earlier stage. Methylation of *RASSF1a* promoter was assessed for a total of 20 pairs of colorectal cancers and adenomas. One sample showed methylation of *RASSF1a* promoter in both the cancer and the adenoma parts, and two showed methylation only in cancer part among the 19 informative pairs. Specific involvement of *RASSF1a* in malignant transformation of certain colorectal cancers was shown. On-slide SBM was proved to be a powerful tool for precise assessments of gene methylation status, such as intratumoral heterogeneity.

Optimization on Glass Slides

Because tissue sections did not adhere consistently enough to noncoated glass slides during on-slide SBM treatment, we initially tested the adhesive-coated, positive-charged slides (Superfrost Plus microscope slides, Fisher Scientific, Pittsburgh, PA) that are commonly used for immunohistochemistry. However, long incubation periods at increased temperatures in acidic or alkaline solutions sometimes caused tumor tissue sections to detach, and experiments had to be repeated. Therefore, we tested silane coated slides (Tekdon, Inc., Myakka City, FL) to improve the robustness of the protocol on 20 colorectal cancer tissue samples. Consequently, none of the tissue sections detached during the on-slide SBM process. The yield of modified DNA and conversion rate was equivalent to

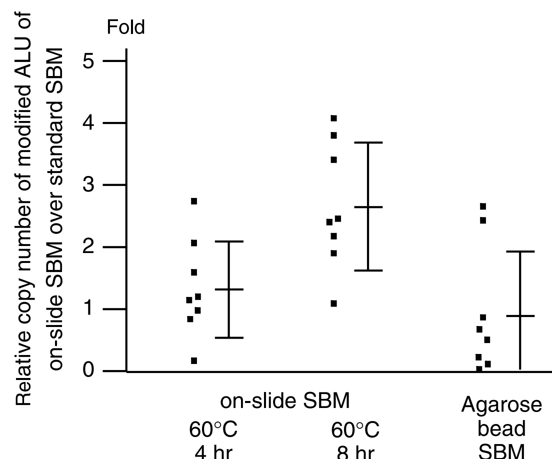

FIGURE 3. Relative yield of modified DNA of on-slide and agarose-bead SBM over standard SBM, calculated as the ratio of absolute copy numbers of modified *Alu*. On-slide SBM at incubation settings of 60°C for 8 hours had the highest relative yield, which was 2.6 ± 1.0-fold (mean ± SD) greater than the yield of standard SBM.

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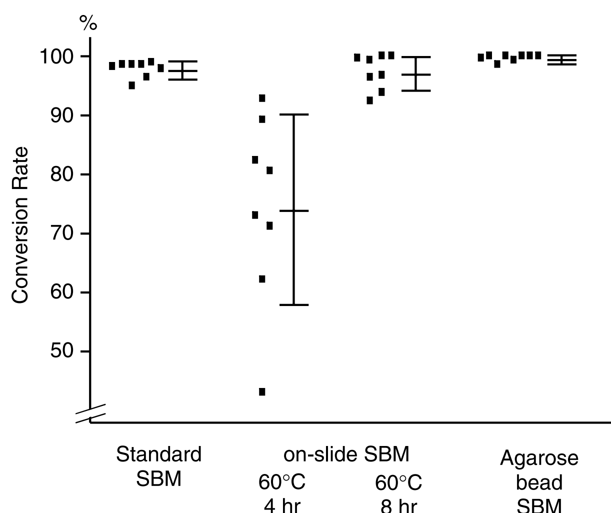

FIGURE 4. Conversion rates of standard, on-slide, and agarose-bead SBM, calculated as the ratio of absolute copy numbers of bisulfite-modified *Alu* over total absolute copy numbers of modified and unmodified *Alu*. On-slide SBM at incubation settings of 60°C for 8 hours had similar conversion rate as standard SBM.

the other kinds of glass slides (data not shown). Because of the improvement of robustness of the protocol, we advocate use of silane coated slides for on-slide SBM.

Discussion

As shown by our results, we were able to study malignant tumor progression by assessing intratumoral heterogeneity in silencing by hypermethylation using a single section of FF-PEAT. On-slide SBM technique is widely applicable because FF-PEAT is the most abundant tissue source for cancer-related studies or any other disease-related studies. The use of on-slide SBM could greatly enhance our knowledge of the relation between histopathologic observations and epigenetic events. To date, to study such phenomena *in vivo* has been difficult because of technical limitations of conventional SBM methods. By simply applying the SBM step *in situ* previous to DNA isolation from the cells of interest, we were able to obtain sufficient amounts of DNA to do PCR analyses. Consequently, we could provide evidence that epigenetic changes of *p16* and *RASSF1a* might be involved in a multistep manner in the progression of the adenomas to colorectal cancers similar to the structural DNA changes (21).

The success of methylation analysis depends on yield of modified DNA and completeness of conversion. When the yield of modified DNA is low, it becomes difficult to detect the methylation status of small amounts of tissue. If the conversion rate is low, false-positive results for methylation can be observed in unmethylated DNA by PCR-based nonquantitative methods because of misgenerated DNA coincidentally having the similar sequence of methylated DNA. Our results showed that *TDG*, which was unmethylated by standard SBM in a pilot study using 26 colorectal cancer tissues and six colorectal cancer cell lines, was unmethylated by on-slide SBM in all 16 pairs of cancer and adenoma tissues. In addition, *ESR1*, which is reportedly methylated in all colorectal cancers (22), was methylated by on-slide SBM in all 16 pairs. Therefore, on-slide SBM is accurate enough for highly-sensitive methods, such as MSP, and does not cause false-negative or false-positive results. After incubation at 60°C for 8 hours, the conversion rate of on-slide SBM was similar to that of standard SBM. Furthermore, the yield was greater than that of agarose-bead SBM, which is commonly used for specimens containing minimal DNA, such as microdissected FF-PEAT tissue or blood serum.

We did not pretreat the tissue samples with a digestive enzyme, such as pepsin or proteinase K, that elevates permeability of reaction solution routinely done in a previous MSP *in situ* hybridization report (19), because predigestion can damage the microscopic tissue structure. Moreover, in our preliminary assessment, predigestion did not significantly increase the conversion rate or the yield of modified DNA (data not shown). Eliminating the predigestion step maintains the microscopic morphology of the tissue during SBM and allows subsequent hematoxylin staining and microdissection.

We initially planned to use a SBM direct sequencing technique to evaluate the conversion rates between three SBM methods. Assessment of 282 bp of the *p16* promoter region of samples treated with the on-slide SBM protocol showed a complete, 100% conversion. Therefore, doing sequencing on modified DNA of a limited number of samples to evaluate the conversion rate was considered not fit to assess subtle differences among the three SBM methods. In addition, our preliminary assessment of SBM DNA revealed that unmodified cytosines are not randomly scattered but exist as clusters in the genome (data not shown) probably because of regional protection from bisulfite, such as fixed double helix. Therefore, we aimed to develop an assay to quantify bisulfite-modified and unmodified DNA using *Alu*-qPCR for evaluation of yield and conversion rate of SBM DNA. This assay can be widely used as a tool for preevaluation of quality of SBM DNA. In addition, it can be widely used to standardize the amount of template SBM DNA in each reaction of methylation assessment. Using this technique, we showed that on-slide SBM yielded 2.6 times more modified DNA than standard SBM. This is likely caused by loss of DNA in purification steps before and during the standard SBM. The high yield of on-slide SBM is a great advantage when the target tissue is small. In addition, on-slide SBM allowed 282-bp bisulfite direct sequencing from very small amount of FF-PEAT, reflecting the high quality of modified DNA.

Total reagent cost and labor cost for on-slide SBM were relatively low because it does not require DNA purification. This is particularly advantageous when multiple areas of a tissue section are assessed simultaneously because DNA modification is completed before microdissection. Another advantage of on-slide SBM is consistency of modification conditions, such as incubation temperature, incubation duration, and concentration of bisulfite within a slide. Therefore, on-slide SBM should be especially suitable for assessments of intratumoral heterogeneity.

In conclusion, 8-hour incubation at 60°C in 3 mol/L sodium bisulfite solution without predigestion maintained tissue architecture during on-slide SBM. Yield of modified DNA was 2.6-fold greater than that of standard SBM. Bisulfite direct

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sequencing of a 282-bp amplicon was possible. Conversion rate was 97.1%, and no false-positive or false-negative results in the subsequent MSP for *ESR1* and *TDG* were observed. Intratumoral heterogeneity of *p16* and *RASSF1a* gene methylation representing pathologic malignant transformation was shown. On-slide SBM should be applicable in most methylation studies using FF-PEAT especially in assessments of intratumoral heterogeneity in solid tumors. The approach provides a unique opportunity to assess retrospective archived procured tissues from clinical trials in which there is limited tissue sections availability.

Materials and Methods

Tissue Specimens

FF-PEAT of colorectal cancers were obtained from 20 patients who underwent colectomy or proctectomy between 1995 and 1998 at Saint John's Health Center (Santa Monica, CA) and consented for research use of their tissue specimens according to the guidelines of Saint John's Health Center/John Wayne Cancer Institute's (Santa Monica, CA) review board. Cases were selected in which the pathologic report described presence of intratumoral adenoma component. All tissue specimens had been fixed in 10% buffered formalin for 24 hours and paraffin embedded. A set of eight colorectal cancer specimens was used to evaluate yield, conversion rate, and a set of three colorectal cancer specimens was used to show feasibility of relatively long sequencing of on-slide SBM DNA. Sections (10 μm) were cut with a microtome from each FF-PEAT block.

On-Slide SBM and Subsequent Microdissection

To evaluate the yield and conversion rate of on-slide SBM, deparaffinized and rehydrated tissue sections were incubated in 0.2 mol/L NaOH at room temperature for 15 minutes and then incubated in 3 mol/L sodium bisulfite solution with 0.5 mmol/L hydroquinone (pH 5) in the dark. We tested four incubation settings (60°C for 4 hours, 60°C for 8 hours, 70°C for 4 hours, and 70°C for 8 hours) for three colorectal cancer specimens in the initial evaluation set. Eight colorectal cancer specimens were used with two incubation settings (60°C for 4 hours and 60°C for 8 hours) to compare conversion rate and yield of modified DNA between on-slide SBM and conventional SBM methods. After incubation, sections were rinsed with distilled water, soaked in 0.3 mol/L NaOH for 15 minutes to desulfonate the modified cytosines, and then desalted in distilled water at 60°C for 2 hours. After light staining with hematoxylin, 25-mm^2 cancer tissue was microdissected under a microscope from each section. The microdissected areas of each section were identical for each incubation setting. Microdissected tissues were digested in 50 μL lysis buffer containing 4 μg proteinase K, 2.5% Tween 20, 50 mmol/L Tris, and 1 mmol/L EDTA at 50°C for 5 hours followed by heat deactivation of proteinase K at 95°C for 10 minutes. In each subsequent qPCR, 2 μL lysate was used as a template without DNA purification.

To validate the use of on-slide SBM to study intratumoral heterogeneity in methylation status, paired cancer and adenoma tissues were microdissected from each on-slide SBM section treated at the conditions that were found to be optimal. From each specimen, ~1-mm^2 tissue was microdissected and

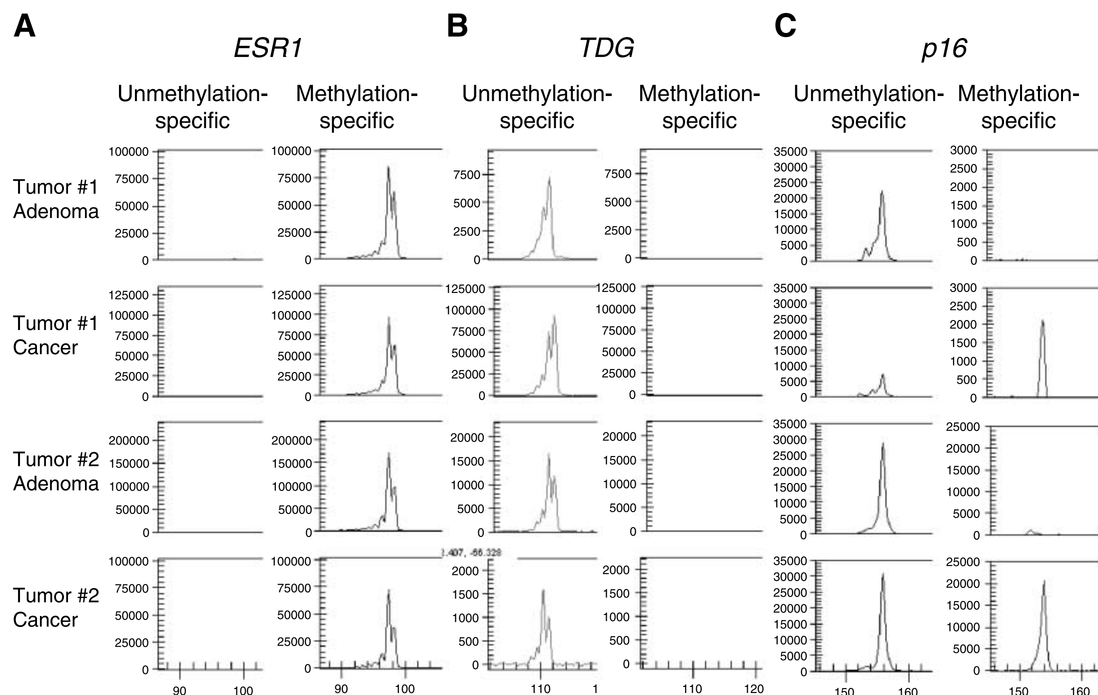

FIGURE 5. Representative MSP results for promoter regions of *ESR1* (**A**), *TDG* (**B**), and *p16* (**C**) genes after on-slide SBM of 16 pairs of cancer and adenoma tissues. For each MSP, 1-mm^2 tissue from 10-μm FF-PEAT section was microdissected and 1 of 10 tissue lysate was used as a template. X axis, amplicon size; Y axis, relative amounts of the PCR amplicons.

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consisted of ~2 to 10 ducts of cancer or adenoma tissue. Each specimen was digested in 10 μL lysis buffer; 1 μL lysate was used as a template in subsequent MSP or direct sequencing without DNA purification.

Standard SBM on Purified DNA from Microdissected Tissue

For comparison of standard SBM with on-slide SBM DNA, purified DNA from microdissected FF-PEAT sections was bisulfite modified with standard SBM method as described previously (23). DNA was extracted from the same area (25 mm^2) of each tissue section as on-slide SBM. DNA was purified with phenol-chloroform-isoamylalcohol from proteinase K–digested tissues microdissected from deparaffinized sections and modified by sodium bisulfite 60°C for 3 hours with 1 μg salmon sperm DNA as a carrier using the same bisulfite solution as described above. Modified DNA was recovered using the Wizard DNA Clean-Up System (Promega, Madison, WI), desulfonated with 0.3 mol/L NaOH, and resuspended in 50 μL Tris-EDTA buffer; 2 μL of the solution were used as a template in subsequent qPCR.

Agarose-Bead SBM on Microdissected Tissue

Bisulfite modification of DNA in digested tissues trapped in agarose beads (24) does not require the DNA purification steps and therefore allows a relatively high recovery rate of DNA from FF-PEAT samples. Same area (25 mm^2) of each tissue section was microdissected and treated. For bisulfite modification, digested tissues trapped in 2% agarose beads containing 0.2 mol/L NaOH were incubated in bisulfite solution, same as for on-slide and standard SBM, at 50°C for 14 hours. After modification, beads were desalted as described previously (24) and heat diluted up to 50 μL with Tris-EDTA buffer; 2 μL of the diluted agarose gel were directly used as a template in subsequent qPCR without DNA purification.

Quantification of Bisulfite-Modified and Unmodified DNA

To determine the yield and conversion rate of SBM DNA, bisulfite-modified and unmodified DNA was separately quantified by *Alu*-qPCR. Primers specific for bisulfite-modified *Alu* sequence independent to the methylation status were 5′-TTGTAATTTTAGTATTTTGGGAGGT-3′ (forward) and 5′-TCACCATATTAACCAAACTAATCTC-3′ (reverse); their amplicon size was 83 bp. Primers specific for unmodified *Alu* sequence were 5′-CCTGAGGTCAGGAGTTCGAG-3′ (forward) and 5′-CCCGAGTAGCTGGGATTACA-3′ (reverse); their amplicon size was 115 bp. The reaction mixture for each *Alu*-qPCR consisted of SBM DNA template, 0.2 μmol/L forward and reverse primers, 1 unit iTaq DNA polymerase (Bio-Rad Laboratories, Hercules, CA), 0.02 μL fluorescein calibration dye (Bio-Rad Laboratories), and 1× concentration of SYBR Gold (Molecular Probes, Eugene, OR) in a total reaction volume of 20 μL with 5 mmol/L Mg^{2+}. Real-time PCR amplification was done with a precycling heat activation of DNA polymerase at 95°C for 10 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 59°C (primer set for modified *Alu*) or 64°C (primer set for unmodified *Alu*) for 30 seconds, and extension at 72°C for 30 seconds using iCycler iQ Real-time PCR Detection System (Bio-Rad Laboratories). The absolute copy numbers of modified and unmodified *Alu* sequence in each sample were determined from the threshold cycle numbers with a standard curve done by serial dilutions of bisulfite-modified or unmodified *Alu* fragment, which was purified from PCR product and quantified by UV absorption. Triplicate reactions of qPCR were done, and each reaction plate included a negative control (no template). PCR products were electrophoresed on 2% agarose gels to confirm product size and specificity of the PCR. The relative yield was calculated as Q_{os} / Q_{std} for on-slide SBM and Q_{ag} / Q_{std} for agarose-bead SBM, where Q_{std}, Q_{os}, and Q_{ag} are the absolute copy numbers of modified *Alu* of standard, on-slide, and agarose-bead SBM, respectively. The conversion ratio was calculated as $Q_{mod} / (Q_{mod} + Q_{unmod})$, where Q_{mod} and Q_{unmod} are the absolute copy numbers of modified and unmodified *Alu*, respectively. Cross-reaction of *Alu* primers on the salmon sperm DNA used as a carrier in the standard SBM was assessed using a blank sample prepared without tissue DNA.

Bisulfite Direct Sequencing for p16 *Gene Promoter Region*

To show the feasibility of on-slide SBM for bisulfite direct sequencing of relatively long target, we sequenced 282 bp of the modified *p16* gene promoter, which is usually difficult for DNA from FF-PEAT modified by standard SBM. Bisulfite direct sequencing was done as described previously (25). The primers were 5′-GGGTAGGTGGGGAGGAGTTTAGTTT-3′ (forward) and 5′-AATAACCAACCAACCCCTCCTCTTT-3′ (reverse). PCR amplification was done in a 50-μL reaction volume using 1-μL template with a precycling heat activation of DNA polymerase at 95°C for 10 minutes followed by 36 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds followed by final extension at 72°C for 7 minutes with 2.5 mmol/L concentration of Mg^{2+} and 0.2 μmol/L of each primer concentration. After PCR products were run on agarose gels and purified, they were directly sequenced using CEQ Dye Terminator Cycle Sequencing kit (Beckman Coulter, Inc., Fullerton, CA) according to the manufacturer's instructions. Cycling program includes 30 cycles of denaturation at 95°C for 20 seconds, annealing at 55°C for 40 seconds, and extension at 60°C for 4 minutes. Sequences were read by CEQ 8000XL CAE System (Beckman Coulter).

MSP for ESR1, TDG, p16, and RASSF1a

Methylation status of CpG islands in *ESR1, TDG, p16*, and *RASSF1a* gene promoter regions of paired cancer and adenoma tissues was assessed by MSP. Forward primers of MSP were labeled with D2, D3, or D4 WellRED dye-labeled phosphoramidites (Beckman Coulter). The methylation-specific primers for *ESR1* were 5′-D4-TAAATAGAGATATATCGGAGTTTGG-TACG-3′ (forward) and 5′-AACTTAAAATAAACGCGAA-AAACGA-3′ (reverse); the unmethylation-specific primers for *ESR1* were 5′-D3-TAAATAGAGATATATTGGAGTTT-GGTATGG-3′ (forward) and 5′-AACTTAAAATAAACA-CAAAAAACAAA-3′ (reverse). The methylation-specific

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primers for *TDG* were 5′-D4-TGAGCGTGATTGGGTTGTTT-TATATTAC-3′ (forward) and 5′-TTCTACCGTCCCCCACGA-3′ (reverse); the unmethylation-specific primers for *TDG* were 5′-D3-AGTGTGATTGGGTTGTTTTATATTATGT-3′ (forward) and 5′-CTTCTACCATCCCCCACAAA-3′ (reverse). The methylation-specific primers for *p16* were 5′-D4-TTATTA-GAGGGTGGGGCGGATCGC-3′ (forward) and 5′-CGATTTCGGGTCGCGGTCGTGG-3′ (reverse); the unmethylation-specific primers for *p16* were 5′-D2-TATTA-GAGGGTGGGGTGGATTGT-3′ (forward) and 5′-TGATTTTGGGTTGTGGTTGTGG-3′ (reverse). PCR conditions were optimized to achieve highest sensitivity without false-positive results using universal methylated control DNA and universal unmethylated control DNA as described previously (25). The optimized annealing temperatures for methylated-specific primers for *ESR1*, unmethylated-specific primers for *ESR1*, methylated-specific primers for *TDG*, and unmethylated-specific primers for *TDG*, methylated-specific primers for *p16*, and unmethylated-specific primers for *p16* were 61°C, 58°C, 62°C, 61°C, 62°C, and 61°C, respectively. Primers and PCR settings used for amplification of the *RASSF1a* promoter region were published previously (23). PCR amplification was done in a 10-μL reaction volume using 1-μL template with a precycling heat activation of DNA polymerase at 95°C for 10 minutes followed by 36 cycles of denaturation at 95°C for 30 seconds, annealing at optimized annealing temperature for 30 seconds, and extension at 72°C for 30 seconds followed by final extension at 72°C for 7 minutes with 2.5 mmol/L concentration of Mg^{2+} and 0.2 μmol/L of each primer concentration. PCR products were detected and analyzed by CEQ 8000XL CAE System as described previously (26).

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Molecular Cancer Research

Methylation of *p16* and *Ras Association Domain Family Protein 1a* during Colorectal Malignant Transformation

Naoyuki Umetani, Michiel F.G. de Maat, Eiji Sunami, et al.

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