Original Article

# Epigenetic Mechanisms for Silencing Glutathione *S*-Transferase M2 Expression by Hypermethylated Specificity Protein 1 Binding in Lung Cancer

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**BACKGROUND:** Glutathione *S*-transferases M2 (GST-M2) is a detoxifying enzyme. Low expression levels of GST-M2 have been detected in lung cancer cells. However, little is known about the regulation of GST-M2 in lung cancer cells. In this study, the authors investigated the epigenetic regulatory mechanisms of GST-M2 in lung cancer cells. **METHODS:** The authors evaluated the promoter methylation of GST-M2 in lung cancer cells after treatment with the DNA methyltransferase (DNMT) inhibitor 5′-aza-2′-deoxycytidine (5′-aza-dC). Reporter activity assays, chromatin immunoprecipitation (ChIP), electrophoretic mobility-shift assays, and small interfering RNA (siRNA) assays were used to determine whether the methylation of specificity protein 1 (Sp1) affected binding to the GST-M2 promoter or regulated GST-M2 transcription. Real-time polymerase chain reaction was used to determine GST-M2 and DNMT-3b messenger RNA levels in 73 nonsmall cell lung cancer (NSCLC) tissues. **RESULTS:** GST-M2 expression was restored after treatment with 5′-aza-dC in lung cancer cells. GST-M2 exhibited high frequency of promoter hypermethylation in lung cancer cells and NSCLC tumor tissues. CpG hypermethylation abated Sp1 binding to the GST-M2 promoter in lung cancer. Knockdown of Sp1 in normal lung cells reduced GST-M2 expression, and silencing of DNMT-3b increased GST-M2 expression in lung cancer cells. In addition, DNMT-3b expression was significantly higher in lung tumors with low levels of GST-M2 expression than in lung tumors with high levels of GST-M2 expression, especially among women and among patients who had stage I disease. **CONCLUSIONS:** Epigenetic silencing of GST-M2 was distinguished from Sp1-mediated GST-M2 transcriptional expression. The authors concluded that this represents a mechanism that leads to decreased expression of GST-M2 in lung cancer cells. ***Cancer* 2011;117:3209–21.** © *2011 American Cancer Society.*

**KEYWORDS:** glutathione S-transferases, lung cancer, specificity protein 1, DNA methylation, DNA methyltransferase.

**Glutathione** *S*-transferases (GSTs) are a family of inducible phase 2 detoxification enzymes.[1] Among them, GST-M2 is an isoenzyme of Mu-class GST and has unique clinical features compared with other members in the same class.[2] In lung cancer cells, we previously observed that GST-M2 was effective in reducing benzo(a)pyrene diolepoxide (BPDE)-induced DNA damage,[3] and its overexpression alleviated DNA double-strand breaks.[4] Those results suggested that GST-M2 activity is important for protecting against DNA damage in the lungs.

Epigenetic regulation is a key mechanism in either the activation or the silencing of gene transcription.[5] Gene silencing through methylation can occur in early stages of lung cancer.[6] Aberrant DNA methylation of CpG islands on the promoter of many genes, including p16, human MutL homolog 1 (hMLH1), and E-cadherin (CDH1), is associated with

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gene silencing.[7] DNA methylation catalyzed by specific DNA methyltransferase (DNMT) plays an important role in the epigenetic control of gene expression and the maintenance of genome integrity.[8] DNMT-3 enzymes are responsible for the establishment of new methylation patterns in genomic DNA maintenance and de novo pathways.[9] It has been reported that overexpression of the DNMT-3b variant Δ form is related to the promoter methylation of tumor suppressor genes (p16, Ras association domain family member 1 [RASSF1A]) in NSCLC.[10,11] Cigarette smoke condensate increases DNMT-3b expression in lung cancer cells.[12] Moreover, DNMT-3b is correlated with the hypermethylation of tumor suppressor genes in tobacco-induced lung cancers and diminishes the survival of patients who have these neoplasms.[13] Our previous study indicated that DNA hypermethylation reduces GST-M2 expression in lung cancer cells.[4] However, to date, the role of DNMT in the regulation of GST-M2 expression remains unknown.

Methylation that occurs at the CpG islands of the specific binding element may interfere sterically with the binding of transcription factors to DNA and, thus, inhibit transcription.[14] It is also known that CpG methylation represses transcription factor specificity protein 1 (Sp1) binding to the promoter of extracellular superoxide dismutase and regulates its cell-specific expression in the lung.[15] It is noteworthy that removal of the Sp1 motif exposes the CpG-rich regulator regions of several genes to DNA methylation, indicating that Sp1 participates in maintaining gene expression by preventing the silencing effects of DNA methylation.[16] Sequencing analysis of the methylation-specific polymerases chain reaction (PCR) (MSP) products from lung cells and tissues revealed 2 Sp1 binding sites located at positions −196 and −153 of cytosine. Therefore, it is possible that CpG methylation abates Sp1 binding ability to the promoter of GST-M2 and that Sp1 regulates expression of the GST-M2 gene.

The objective of the current study was to determine whether DNMT-3b plays a role in GST-M2 promoter methylation and blocks Sp1 binding to the GST-M2 promoter in lung cancer cells and in early stage NSCLC tumors. Our specific objectives were 1) to investigate in depth the relation between GST-M2 gene expression and hypermethylation in lung cells, 2) to clarify that Sp1 regulates GST-M2 expression in lung cells, 3) to demonstrate that CpG methylation abates Sp1 binding ability of the GST-M2 promoter in lung cells, and 4) to evaluate the possible correlation between DNMT-3b expression and GST-M2 expression in patients with early stage NSCLC.

## MATERIALS AND METHODS

### Study Participants and Tissue Collection

The protocol for this study was approved by the Institutional Review Board Committee of Taichung Veterans General Hospital. Confidentiality issues were explained to the participants, and informed consent was obtained. From January 2003 to August 2006, we collected lung tumor samples and adjacent normal tissue samples from 73 patients with stage I through II NSCLC who underwent surgical resection. There were 48 men and 25 women, and the median patient age was 68 years. The histologic types of NSCLC included 26 squamous cell carcinomas and 47 adenocarcinomas. The pathologic stage was stage I in 50 patients and stage II in 23 patients. There were 11 well differentiated tumors, 33 moderately differentiated tumors, and 29 poorly differentiated tumors. Tumor samples were acquired from the solid part of the mass, and gross necrotic areas were avoided. The tissues were stored at −70°C. None of the patients received preoperative chemotherapy or radiotherapy. Tumor staging was performed according to American Joint Committee on Cancer criteria.[17]

### Cell Culture and Drug Treatments

The cell lines for this study were obtained from the American Type Culture Collection and were grown as described previously.[4] Cell lines were maintained at 37°C in a 5% $CO_2$ humidified atmosphere in Dulbecco modified Eagle medium (Gibco, Rockville, Md) and Eagle basal medium (Sigma, St. Louis, Mo) that contained 10% fetal bovine serum (Life Technologies, Inc., Rockville, Md) and 100 ng/mL each of penicillin and streptomycin (Life Technologies, Inc.). Cells either were left untreated or were treated with demethylation reagent 5′-aza-2′-deoxycytidine (5-aza-dC) (A3656; Sigma-Aldrich, Inc., St. Louis, Mo) at final concentrations of 1 μM and 10 μM for 96 hours.

### Genomic DNA Isolation and Bisulfite Modification

Genomic DNA was isolated with the Genomic DNA Isolation Kit (Qiagen, Inc., Hilden, Germany). Bisulfite treatment was carried out as described previously.[18] Briefly, genomic DNA was sheared into 500 to 1000 base-pair (bp) fragments by sonication. Then, 2 μg of DNA were diluted to 25 μL and denatured for 20 minutes at 37°C with 25 μL of 0.4 M NaOH. This was followed by the addition of 35 μL of freshly prepared hydroxyquinone (10 mM; Sigma-Aldrich) and 520 μL of freshly prepared sodium bisulfite (3 M), pH 5.0 (Sigma-Aldrich), mixing, and incubation at 55°C for 16 hours.

Unmethylated cytosine residues were converted to uracil, whereas methylated cytosine residues remained unchanged. Modified DNA was purified using the DNA Clean-Up Kit according to the manufacturer's instructions (A7280; Promega, Madison, Wis) and was eluted into 100 μL of water. DNA modification was completed with NaOH (0.3 M) treatment for 20 minutes at room temperature followed by ethanol precipitation. DNA was resuspended in water and was either used immediately or stored at −20°C.

### *Isolation of RNA, Reverse Transcriptase-Polymerase Chain Reaction (PCR), and Quantitative Real-Time PCR Analysis*

Total RNA was isolated using Trizol reagent (Life Technologies, Inc.). Then, combinational DNA was reverse-transcribed from 2 μg total RNA using random hexamer primers and MMLV-RTase (Promega). The reverse transcriptase-PCR (RT-PCR) amplifications for GST-M2 and β-actin were preformed as described in our previous study.[4] For DNMT-1 primers, we used 5′-CCCCTG AGCCCTACCGAAT-3′ (forward) and5′-CTCGCTGG AGTGGACTTGTG-3′ (reverse); and, for DNMT-3b primers, we used 5′-TCGACCTCACAGACGACACA-3′ (forward) and 5′-CACGACGCACCTTCGACTTAT-3′ (reverse). The PCR protocol for DNMT-1 and DNMT-3b entailed 5 minutes at 95°C; then 32 cycles of 45 seconds at 95°C, 45 seconds at 55°C, and 45 seconds at 72°C; and a 10-minute final extension at 72°C.

By using the ABI PRISM 7000 real-time PCR System and a TaqMan gene expression probe (Applied Biosystems, Foster City, Calif), real-time PCR analyses of GST-M2, DNMT-1, and DNMT-3b gene expression levels were carried out as described previously (assays-on-gene expression products: DNMT-1 probe Hs00945899_m1 and DNMT-3b probe Hs00171876_m1; Applied Biosystems).[4] Concentrations of synthesized primers and probes were optimized for each assay; then, real-time PCR was performed in triplicate in a 25-μL reaction volume.

### *Methylation-Specific PCR*

The sequence databases of the GST-M2 promoter region and the CpG island were obtained from a previous study.[19] One-microliter aliquots of bisulfite-modified DNA were used as templates for the first-round PCR reactions. The PCR mixture contained 1 × PCR buffer (16.6 mM ammonium sulfate; 67 mM Tris, pH 8.8; 6.7 mM $MgCl_2$; and 10 mM 2-mercaptoethanol), 10 pmole of each primer, 0.2 μM of each deoxynucleoside triphosphate, and 2 U Pro-Taq polymerase (PROtech Technologies, Inc., Taipei, Taiwan). The amplification conditions were as follows: initial denaturation for 5 minutes at 95°C; 35 cycles for 45 seconds at 95°C, 45 seconds at 50°C, and 45 seconds at 72°C; and final 10-minute extension at 72°C. The primer sequences were 5′ AG AATTTTTTTGTTTTAGAGAGAG-3′ (forward) and 5′-CTAACTTTATAACAACTCTACACG-3′ (reverse) for GST-M2 promoter and the amplified region from −306 bp to +313 bp relative to the transcription start site. For the second round of PCR, the primer sequences were 5′-TTTTTTGTTTAATTTAGAGTGTAGTGGT GTG-3′ (forward) and 5′-CAAACCAATCTAAAAACC TAAAACACA-3′ (reverse) for GST-M2 promoter unmethylation and amplified region from −83 bp to −276 bp; 5′-TTGTTTAATTTAGAGTGTAGCGGCG(forward)-3′ and 5′-CGAACCAATCTAAAAACCTAAA ACG-3′(reverse) for GST-M2 promoter methylation and the amplified region from −83 bp to −272 bp relative to the transcription start site. Cytidine residues modified by bisulfite treatment are underlined. The PCR conditions were the same as those described above, and the annealing temperatures were 62°C and 64°C for the unmethylation and methylation reactions, respectively. These methylation-specific PCR (MSP) fragments were separated onto 3% agarose gels, stained with ethidium bromide, and observed under an ultraviolet illuminator. The known methylated DNA was used as a positive control for methylation,[20] and water was used as a negative control. The PCR products were purified and inserted into vector pGEM-T Easy Vector System (A3610; Promega) using the Fast DNA Ligation System (Promega). Four clones from each cell line or tissue were sequenced to assess the level of methylation at each CpG site as described previously.[21]

### *Site-Directed Mutagenesis, Transient Transfection, and Luciferase Reporter Assay*

The GST-M2 promoter construct (pGL3-GST-M2p-WT) was described in our previous report.[4] Deleted GST-M2 promoter constructs that carried deletions at the Sp1 sites within the GST-M2 promoter region (from −295 bp to +245 bp) were generated using the Quik Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, Calif). In addition, the introduction of a deletion into the Sp1 site (CCACGGGGC) to generate pGL3-GST-M2pdel$_{-156-148}$ was performed using the forward primer 5′-GATCTCTCTCCTGAAACTGGGAGGT

GGGAG-3′, which corresponded to the region from −173 bp to −135 bp. Another deletion that was introduced into the Sp1 site (CCCGCCC) to generate pGL3-GST-M2del$_{-198\text{-}-192}$ was performed using the forward primer 5′-CAACCAGTTCTCCTGGGCTTCTGGGC GTC-3′, which corresponded to the region from −211 bp to approximately −176 bp. The nucleotide sequences of the mutant constructs were confirmed by DNA sequencing.

Transient transfections were carried out using the jetPEI reagent (Polyplus, New York, NY) according to the manufacturer's instructions. Briefly, $1 \times 10^5$ cells were seeded onto each well of a 24-well tissue culture plate. Cells in each well were transfected with 0.8 μg of pGL-3 plasmid DNA by using 2 μL jetPEI reagent. The bacterial β-galactosidase gene was cotransfected in each experiment to serve as an internal control of transfection efficiency. After transfection for 24 hours, the cells were collected, and transcriptional activity was assayed with the Luciferase Assay System (Promega). Extracts from at least 3 independent transfection experiments were assayed in triplicate. The results are presented as mean ± standard deviation values.

### Chromatin Immunoprecipitation

A chromatin immunoprecipitation (ChIP) assay was conducted using the ChIP Assay Kit according to the manufacturer's instructions (Upstate-Millipore Corporation, Billerica, Mass). A549 cells were treated either with dimethyl sulfoxide or with 1 μM or 10 μM 5-aza-dC for 96 hours. Protein-DNA complexes were cross-linked using formaldehyde at a final concentration of 1%. The genomic DNA of lysed cells was sheared using a sonicator into a size range from 200 bp to 1000 bp. The final lysate was incubated with antibodies specific to Sp1 (07-645; Millipore Corporation), DNMT-3b (2161s; Cell Signaling Technology, Beverly, Mass), DNMT-1 (GTX116011; GeneTex Inc., Irvine, Calif), and total histone 3 (ab1791; Abcam, Boston, Mass) and was precipitated with Protein G-Plus Agarose (Zymed, South San Francisco, Calif). In addition, 10% of the solution that was collected before adding antibodies was used as an internal control for the amount of input DNA. After extensive washing, DNA-protein complexes were eluted and reverse cross-linked by incubation at 65°C overnight. DNA was purified followed by phenol/chloroform/isoamyl alcohol extraction (25:24:1); then, it was resuspended in water. The primers for GST-M2 promoter were 5′-TTTCTTGCCTAACCTAGAGTGCAGCG GCG-3′ (sense) and 5′-CGGGCCAATCTGGAGGCC TGGAGCGC-3′ (antisense) and amplified the region from −83 bp to −276 bp relative to the transcription start site. Products of PCR amplification were separated onto 2% agarose gel and were observed using ethidium bromide and ultraviolet light.

### Electrophoretic Mobility-Shift Assay (EMSA)

Oligonucleotides were synthesized by MDBio Technologies, Inc. (Taipei, Taiwan). These included the wild-type consensus Sp1 element of the GST-M2 promoter (5′-$_{-201}$TCCCCCGCCCTGGGCTTC$_{-184}$-3′), the methylated Sp1 element (5′-$_{-201}$TCCCCC$^{m}$GCCCT GGGCTTC$_{-184}$-3′), and the methylated Sp1 element (5′-$_{-158}$ACCCAC$^{m}$GGGGCTGGGAGG$_{-141}$-3′. The consensus Sp1 binding site is underlined. The synthetic oligonucleotide probes were labeled with biotin. The end-labeled oligonucleotides were heated at 95°C for 5 minutes then cooled gradually to room temperature for annealing. Nuclear extracts of A549 cells were extracted as described previously.[22] DNA and protein binding reactions were performed at 25°C for 15 minutes in 20 μL reaction buffer (10 mM Tris-HCl, pH 7.5; 50 mM NaCl; 1 mM ethylenediamine tetracetic acid [EDTA]; 10% glycerol; 0.1 μg poly-L lysine; 1 μg of poly[dI-dC]; 1 mM dithiothreitol; and 10 μM biotin-labeled oligonucleotide probes for Sp1) with or without Sp1 antibody (07-645; Millipore Corporation) as competitor. To destroy binding of the complexes, nuclear extracts were preincubated with Sp1 antibodies (1 μg) at 37°C for 60 minutes before adding biotin-labeled oligonucleotide. DNA-protein complexes were separated from unbound DNA probe on native 6% polyacrylamide gels (100 volts in 0.5 times Tris-borate-EDTA buffer), and the gels were transferred to positively charged nylon membranes (Roche Molecular Biochemical, Mannheim, Germany). Electrophoretic mobility-shift assay (EMSA) detection experiments were performed according to the manufacturer's instruction (LightShift Chemiluminescent EMSA Kit; Thermo Scientific, Rockford, Ill).

### RNA Interference

Lentiviral infection of cell lines was used to stably integrate and express short hairpin RNA (shRNA) that targeted the Sp1 and DNMT-3b messenger RNA (mRNA) sequences. Cells that stably expressed control or target shRNAs were generated as described previously using a lentiviral system.[23] Individual clones were identified by their unique number assigned by the RNAi Consortium (TRCN) as follows: TRCN00000772246 was used for

vector control targeted to luciferase (responding sequence, CAAATCACAGAATCGTCGTAT), TRCN0000020 445 (responding sequence, GCAGCAACTTGCAGCA-GAATT) was used for shSp1-45 targeted to Sp1, TRCN0 000020448 (responding sequence, GCTGGTGGTGA TGGAATACAT) was used for shSp1-48 targeted to Sp1, TRCN0000035685 (responding sequence: GCCC GTGATAGCATCAAAGAA) was used for DNMT-3b-685, and TRCN0000035686 (responding sequence: CCATGCAACGATCTCTCAAAT) was used for DNMT-3b-686 targeted to DNMT-3b. Cells were subcultured at $5 \times 10^5$ cells per 6 cm on tissue culture plates. After 24 hours in culture, the cells were infected with recombinant lentivirus vectors at a multiplicity of infection of 1. The next day, medium was removed, and the cells were selected by puromycin for 2 days. For stable transfections, clones were passaged into selection media that contained puromycin (2 μg/mL).

### Statistical Analysis

Statistical analysis was performed using the SPSS statistical software program (version 15.0; SPSS Inc., Chicago, Ill). The relative expression level of target genes was calculated by using the $2^{-\Delta Ct}$ method. The median level of GST-M2 expression was used to stratify patients into high and low expression groups.

The correlation between DNMT-3b and GST-M2 expression levels was evaluated with the Spearman correlation test. The Wilcoxon rank-sum test was used for nonparametric comparisons in variables of interest.

## RESULTS

### Expression of GST-M2, DNMT-1, and DNMT-3b in Lung Cell Lines

We performed RT-PCR (Fig. 1, top) and real-time PCR (Fig. 1, bottom) analyses to detect the expression of GST-M2, DNMT-1, and DNMT-3b in 7 lung cancer cell lines. The results revealed that lung cancer cell lines expressed low levels of GST-M2 but high levels of DNMT-1 and DNMT-3b mRNA. GST-M2 mRNA was detectable at low levels in lung cancer cell lines, including A549, H1299, H1355, and CaLu-1. DNMT-1 and DNMT-3b expression was detectable at high levels in lung cancer cell lines.

### Induction of GST-M2 Expression by the Demethylation Agent 5-Aza-dC

To understand the molecular mechanism by which GST-M2 presents with low levels of expression in lung cancer cell lines, we examined the expression of GST-M2 after treatment with the demethylation reagent 5-aza-dC in the lung cancer cell lines A549, H1355, and H1299. Cells were treated with 1 μM and 10 μM of 5-aza-dC for 96 hours. In Figure 2, top reveals that GST-M2 mRNA expression was restored depending on the 5-aza-dC concentration. GST-M2 mRNA expression was verified further by real-time PCR experiments (Fig. 2, bottom). The results revealed increased GST-M2 mRNA expression in A549 cells (5-fold; $P < .01$), H1299 cells (8-fold; $P < .01$), and H1355 cells (5-fold; $P < .01$) relative to controls after 5-aza-dC treatment at 10 μM. Thus, the results indicated that GST-M2 expression probably is elevated by aberrant hypermethylation in lung cancer cells.

### GST-M2 Promoter Methylation in Lung Cancer Cells and Early Stage Lung Tumors

Next, we used MSP to investigate whether DNA hypermethylation occurred at the core promoter (between −276 bp and −83 bp relative to the transcription start site) of GST-M2 in lung cell lines and in early stage NSCLC tumors. We observed that methylation of the GST-M2 promoter occurred in lung cancer cells but not in normal lung cells (Fig. 3, top left). Methylation of the GST-M2 promoter also was observed in tumor tissues from patients with stage I and II NSCLC but not in lung normal tissues (Fig. 3, middle). We confirmed those results in 8 NSCLC tissue samples. Real-time PCR analysis revealed low levels of GST-M2 mRNA expression in tumor tissues that had a methylated allele (Fig. 3B, middle bottom). These results indicated that aberrant hypermethylation of CpG sites in GST-M2 promoter silences gene expression in lung cancer cells.

Then, we analyzed the methylation status of CpG islands at the GST-M2 promoter with MSP products in the cell lines, including 9 CpG sites within a 194-bp fragment from −276 bp to −83 bp. Densely methylated clones were detected in lung cancer cell lines and early stage NSCLC tumor tissues but not in normal lung cell lines or normal tissues (Fig. 3, bottom). These data suggested that aberrant hypermethylation of the GST-M2 promoter occurs in lung cancer cells and early stage NSCLC tumor tissues.

### CpG Methylation Abates Sp1 Binding to the GST-M2 Promoter in Lung Cancer Cells

To determine whether Sp1 is essential for the transcriptional activation of GST-M2, luciferase assays were performed using reporter plasmids that had 2 deletions of the core promoter in lung cancer cells. Deletions of the

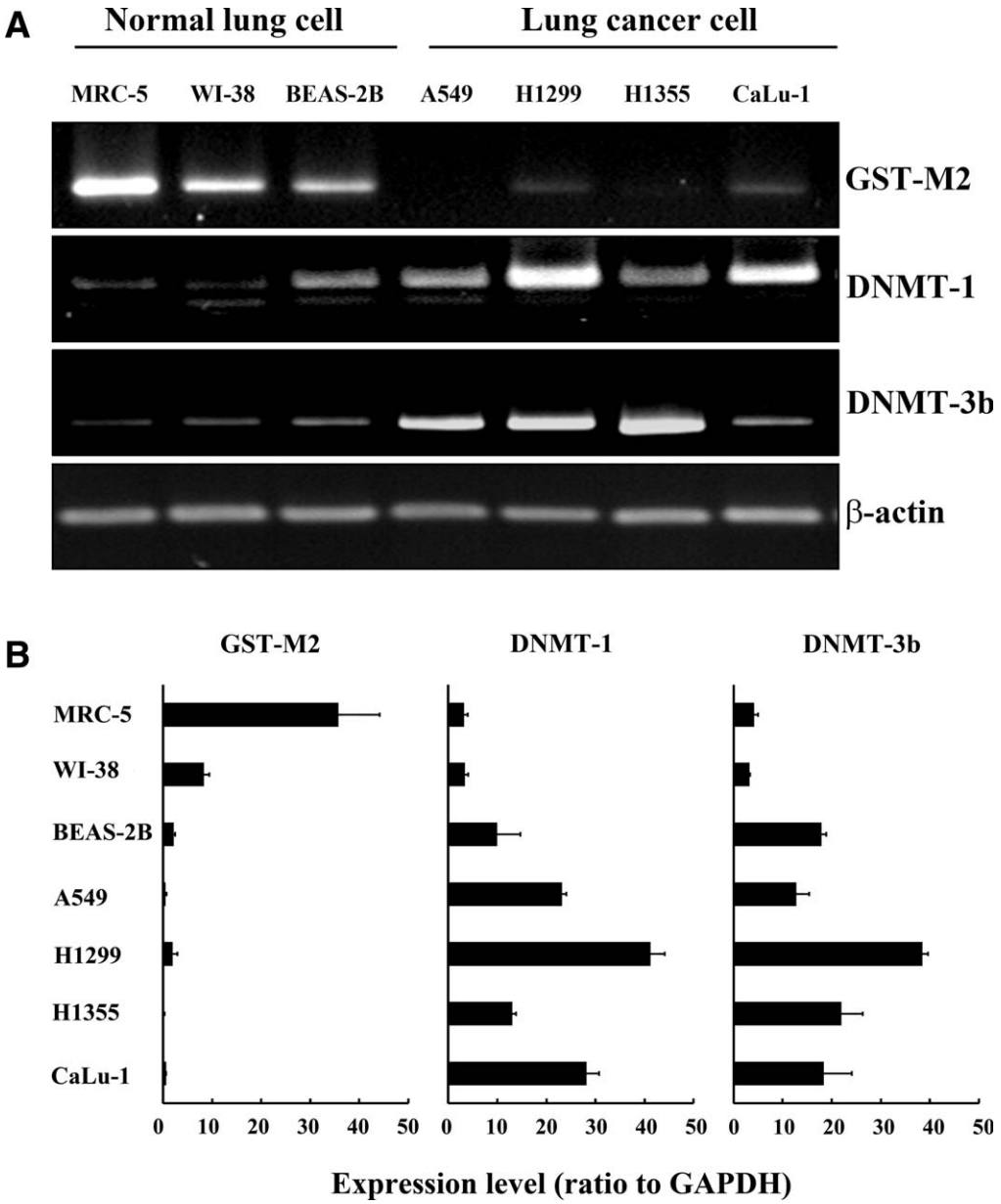


**Figure 1.** The expression of glutathione *S*-transferase M2 (GST-M2), DNA methyltransferase 1 (DNMT-1), and DNMT-3b messenger RNA is illustrated in normal lung cells and in lung cancer cell lines. (*Top*) GST-M2, DNMT-1, and DNMT-3b expression was analyzed in total RNA (2 μg) amplified by reverse transcriptase-polymerase chain reaction (PCR). Equal amounts of DNA were loaded, as confirmed by the intensity of β-actin after ethidium bromide staining. (*Bottom*) Real-time PCR quantification of GST-M2, DNMT-2, and DNMT-3b was carried out by using TaqMan analysis. All values are normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and are the averages of 3 independent readings.

promoter from −156 to −148 led to a slight reduction in transcriptional activity in H1355 cells. Further deletions of the region from −198 to −192 resulted in a significant 32% reduction ($P < .01$) in transcriptional activity, suggesting that the GST-M2 promoter, which contains a GC-box that binds to Sp1, is important for transactivation (Fig. 4, top left). We also investigated the effects of CpG methylation on transcription factor binding to the GST-M2 promoter. A ChIP assay was used to detect the binding of transcription factor Sp1 to the GST-M2 promoter in A549 cells that were either treated or untreated with 5-aza-dC. Figure 4 (bottom left) reveals that A549 cells that were treated with 10 μM 5-aza-dC for 96 hours had significantly increased Sp1 binding ability. The

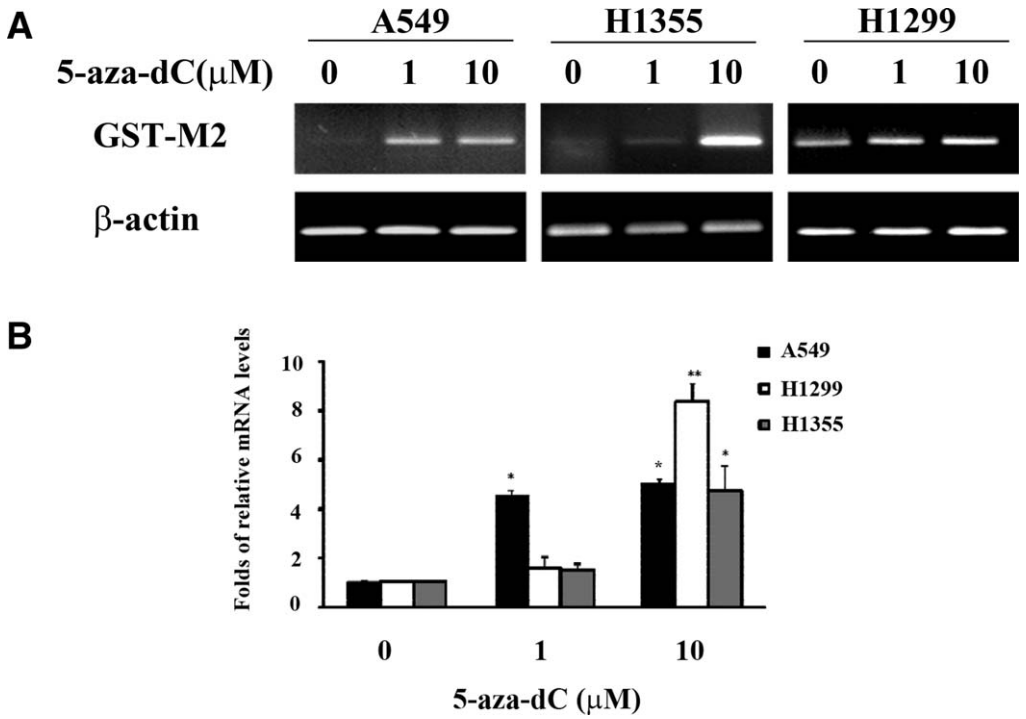


**Figure 2.** Transcription re-expression of methylated genes after 5′-aza-2′-deoxycytidine (5-aza-dC) treatment is illustrated. (*Top*) The A549, H13l55, and H1299 cell lines were treated with 1 μM or 10 μM 5-aza-dC for 96 hours. Reverse transcriptase-polymerase chain reaction (PCR) analysis of equal amounts of combinational DNA from the cells demonstrated the up-regulation of glutathione *S*-transferase M2 (GST-M2) messenger RNA (mRNA) in treated cells compared with the loading control (β-actin). (*Bottom*) Real-time PCR analysis confirmed the same model. mRNA levels were calculated as the relative expression compared with glyceraldehyde 3-phosphate dehydrogenase. Columns indicated the mean values from 3 determinations, and error bars indicate the standard deviation (single asterisk, $P < .05$; double asterisks, $P < .01$ vs nontreated control).

presence of DNMT-3b, but not of DNMT-1, in the GST-M2 promoter was diminished significantly after treatment with 1 &mgrM 5-aza-dC treatment. An EMSA also was used to test whether the Sp1 site (at positions −196 or −153 of cytosine) affected the binding of Sp1 to its recognition elements. We designed a 17-bp oligomer (positions −201 to −184 and positions −158 to −141) that spanned the −196 and −153 binding site in the GST-M2 promoter. The nucleic extracts from A549 cells were used to form a DNA-protein complex. The results revealed differences in formation and binding patterns at different Sp1 binding sites of these 2 complexes (Fig. 4, right). Compared with the binding of Sp1 to positions −158 through −141 of the recognition binding site (Fig. 4, right; lane 7), the binding to positions −201 through −184 of the recognition binding site (Fig. 4, right; lane 5) was stronger. It is noteworthy that CpG methylation within the Sp1 element caused a significant decrease in the binding of Sp1 (Fig. 4, right; lane 6). Antibody always produced a supershift of pre-existing DNA-protein complex. Sp1 antibody potentially interferes with Sp1 binding to DNA. Thus, when nuclear extracts were incubated with Sp1 antibodies, the binding of the complex was destroyed (Fig. 4, right; lane 9).

Normal lung cells were transfected with shRNA against Sp1 to evaluate the effects of Sp1 knockdown on normal lung cells. Figure 5 (top) reveals that silencing of the Sp1 gene resulted in the loss of GST-M2 transcriptional activity in normal lung cells (MRC-5 and BEAS-2B cells). We used shRNA to suppress DNMT-3b expression and observed that the robust knockdown of total DNMT-3b-686 cells increased GST-M2 mRNA expression in A549 cells (Fig. 5, middle). We also analyzed promoter region methylation in A549 cells using shRNA by MSP (Fig. 5, bottom). The knockdown of total DNMT-3b increased GST-M2 promoter methylation status. Therefore, we concluded that DNMT-3b is involved in silencing of the GST-M2 gene in lung cancer cells. Taken together, our data suggest that the methylation of CpG sites prevents the binding of Sp1 to the GST-M2

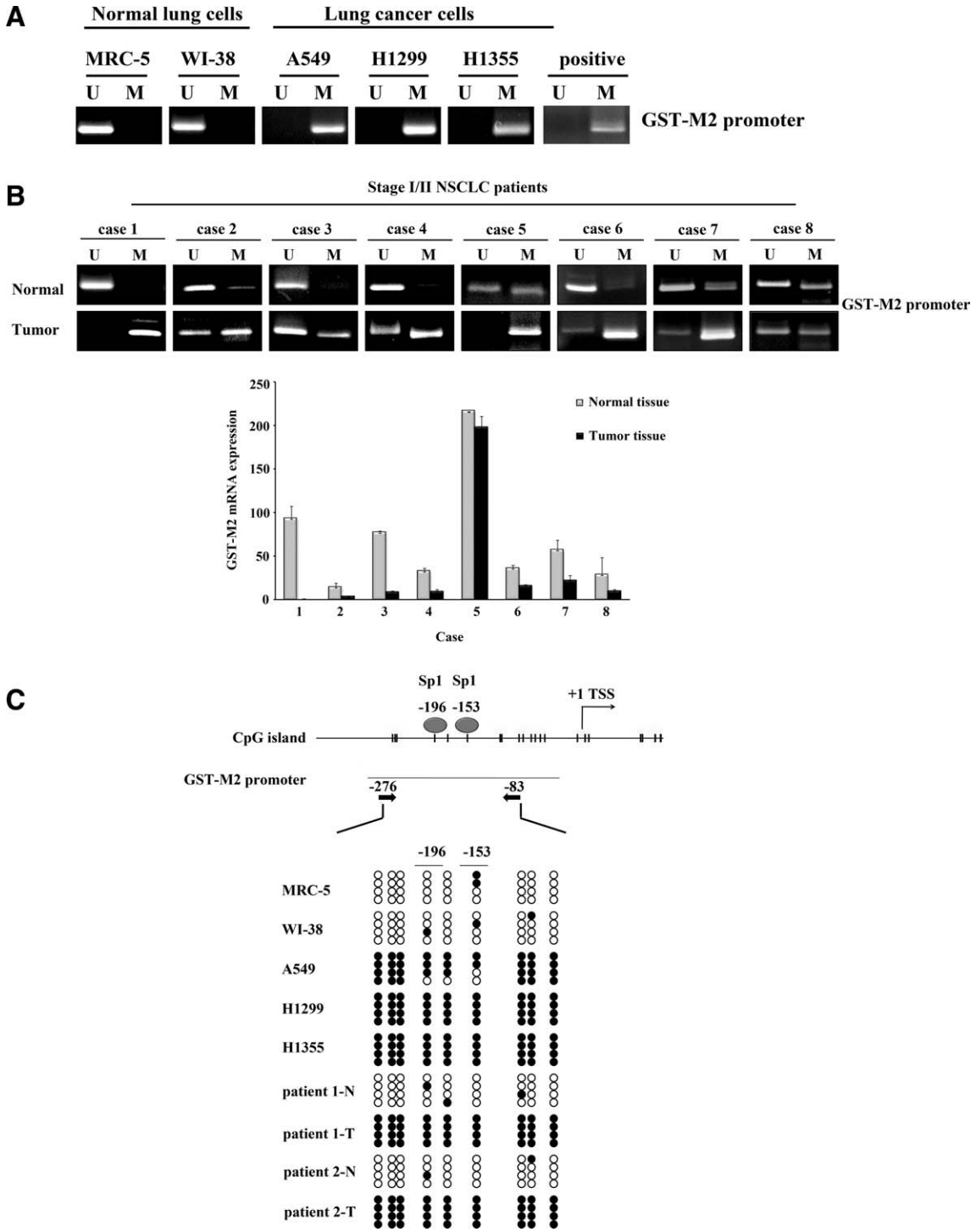


**Figure 3.** These are representative examples of methylation-specific polymerase chain reaction (PCR) (MSP) assays for glutathione *S*-transferase M2 (GST-M2) in lung cell lines and in nonsmall cell lung carcinoma (NSCLC) tissues. (*Top*) Representative results from MSP in 2 normal lung cell lines and 3 lung cancer lines are shown. Normal human lymphocyte DNA that was treated with SssI DNA methyltransferase (M0226S; New England Biolabs, Ipswitch, Mass) and subjected to bisulfate modification was used as positive methylated (M) control DNA for PCR analyses, and water was used as a negative control. U indicates unmethylated. (*Middle*) Representative results from MSP assays and real-time PCR analysis are shown from 8 paired samples of normal and tumor tissues from patients with NSCLC. U indicates amplification with specific primers for unmethylated GST-M2 DNA, M, amplification with primers specific for methylated GST-M2 DNA; mRNA, messenger RNA. (*Right*) A CpG plot of the GST-M2 promoter region is shown with the transcription start site (TSS) indicated by as +1 TSS. Sp1 indicates specificity protein 1. (*Bottom*) Below the plot, direct sequencing of the MSP products is illustrated for the cell lines and tissues (N indicates normal tissues; T, tumor tissues). Each circle denotes a CpG site across PCR fragments amplified from the MSP product. Solid circles indicate methylated; open circles, unmethylated.

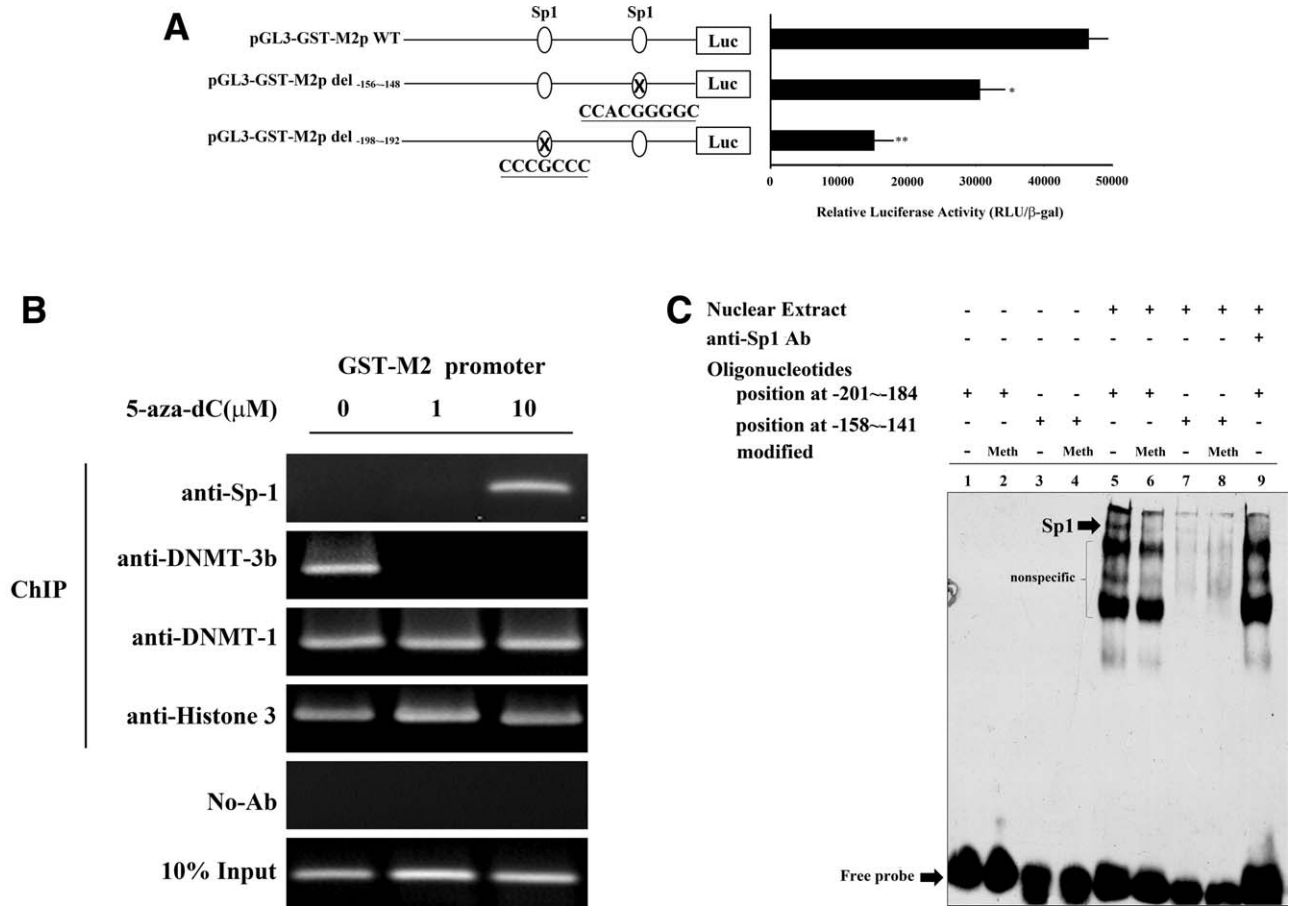


**Figure 4.** An analysis of glutathione *S*-transferase M2 (GST-M2) promoter occupancy by specificity protein 1 (Sp1), DNA methyltransferase 1 (DNMT-1), and DNMT-3b is shown. (*Top Left*) For deletion analyses of the GST-M2 core promoter, luciferase (Luc) reporter plasmids with 2 deletions of the core promoter in the Sp1 binding sites were prepared. Crossed-out circles indicate the deleted sites for Sp1 binding. These reporter plasmids were transfected into H1355 cells, and luciferase assays were performed. Solid horizontal bars indicate the mean values from 3 determinations, and error bars indicate the standard deviation (single asterisk, $P < .05$; double asterisks, $P < .01$. pGL3-GST-M2p-WT, GST-M2 promoter construct; del, deletion; RLU, relative light units; β-gal, β-galactoside. (*Bottom Left*) Chromatin immunoprecipitation (ChIP) assays using anti-Sp1, anti-DNMT-3b, and anti DNMT-1 were performed using the cell lysate from cells that were treated with or without 5′-aza-2′-deoxycytidine (5-aza-dC) for 96 hours. The amplified DNA fragments were observed by 1.5% agarose gel electrophoresis. The samples were immunoprecipitated with total histone 3 as a positive control. For a negative, lysate without the antibody (No-Ab) was used. (*Right*) The binding of Sp1 to the recognition Sp1 binding sights was analyzed by using an electrophoretic mobility-shift assay. Lanes 1 through 4 show biotin-labeled probe in the absence of nuclear extract; lanes 5 and 7 show binding reactions with labeled Sp1 at different positions as a probe, lanes 6 and 8 show binding reactions with labeled methylated (Meth) Sp1 as a probe, and lane 9 shows the destruction of binding activity when Sp1 antibody was added to the incubation mixture.

promoter and that Sp1 predominantly regulates transcription of the GST-M2 gene.

### *DNMT-3b Expression Levels Were Higher in Tumors With Low GST-M2 Expression*

We carried out real-time PCR for 73 pairs of matched lung tumor tissues and adjacent normal tissues from patients with stage I and II NSCLC to detect the expression of GST-M2 and DNMT-3b. The median of GST-M2 mRNA expression level was significantly higher in normal tissues than in tumor tissues (median level of $2^{-\Delta Ct}$ expression, 22.7 vs 14.0; $P = .019$; Wilcoxon rank-sum test). However, the correlation between DNMT-3b and GST-M2 expression levels was not significant in tumor tissues ($P = .121$; Spearman correlation). Furthermore, GST-M2 mRNA expression in tumor tissues was used to classify patients into those with high and low GST-M2 high expression levels. It is noteworthy that the expression of DNMT-3b was higher in patients who had low GST-M2 expression than in patients who had high

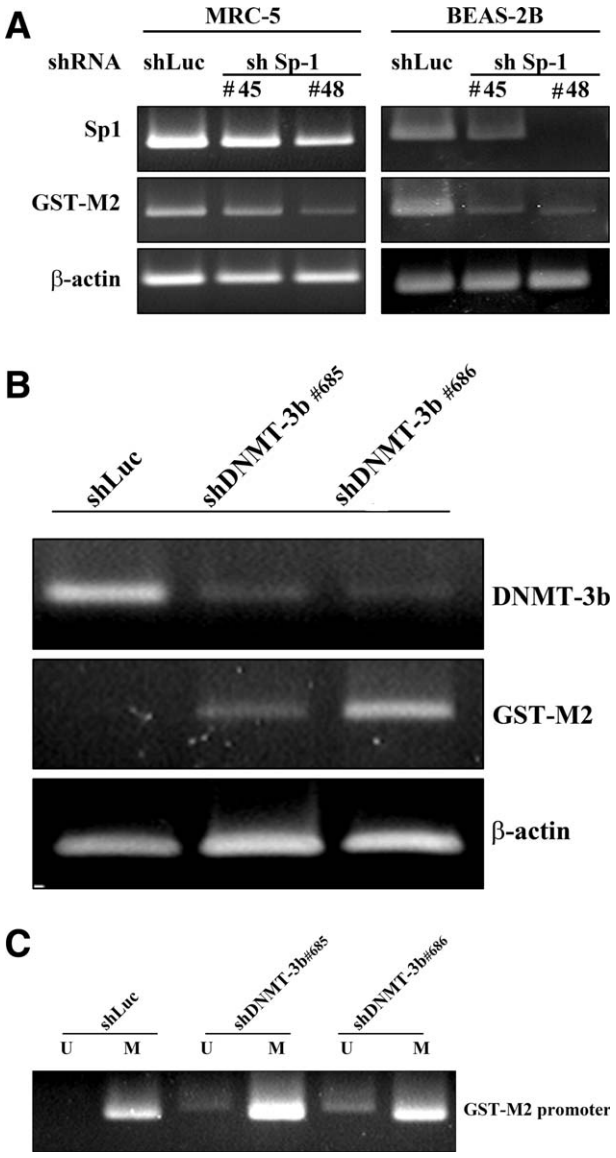


**Figure 5.** DNA methyltransferase 3b (DNMT-3b) affects specificity protein 1 (Sp1) binding to the glutathione *S*-transferase M2 (GST-M2) promoter and regulates its cell-specific expression. (*Top*) By using a lentiviral delivery system, short hairpin Sp1 (shSp1) (including cell lines [according to the RNAi Consortium number] TRCN0000020445 and TRCN0000020448) was stably expressed in MRC-5 and BEAS-2B cells. shLuc indicates short hairpin luciferase. (*Middle*) Short hairpin DNMT-3B (shDNMT-3b) (including the cell lines TRCN0000035685 and TRCN0000035686) was stably expressed in A549 cells. For cell harvest, cells ($5 \times 10^5$) with knockdown of short hairpin RNA (shRNA) or shLuc (mock) were seeded onto 6-cm dishes separately for 48 hours. Reverse transcriptase-polymerase chain reaction was used to detect messenger RNA expression and the expression of Sp1, GST-M2, and DNMT-3b in cell lines. Equal amounts of DNA were loaded, as confirmed by the intensity of β-actin after ethidium bromide staining. (*Bottom*) A methylation-specific assay was used to analyze the knockdown of DNMT-3b in A549 cells. U indicates unmethylated; M, methylated.

GST-M2 expression, especially among women ($P = .04$) and patients who had stage I disease ($P < .01$), a tumor classification of T1 or T2 ($P = .02$), and N0 lymph node status ($P = .03$). These results suggested that patients who have early stage tumors with increased levels of DNMT-3b expression have decreased levels of GST-M2 expression.

## DISCUSSION

Our current findings provide evidence to support the hypothesis that the hypermethylation of CpG islands within the GST-M2 proximal promoter is a major mechanism that mediates the silencing of GST-M2 gene expression in lung cancer cell lines and in stage I/II NSCLC tumor tissues. First, the transcriptional activities of the GST-M2 promoter were restored by treatment with the demethylator 5′-aza-dC in several lung cancer cell lines (Fig. 2, top). Second, the GST-M2 proximal promoter revealed a dense cluster of CpG islands in the region from −276 bp to −83 bp relative to the transcription start site. In addition, we observed hypermethylation of a CpG island that spanned the transcription factor Sp1 binding element, which determined GST-M2 gene expression in lung cells (Figs. 3, 4). Third, we demonstrated that the binding of Sp1 to the GST-M2 promoter is abolished by methylation of the Sp1 motif (Fig. 4). Fourth, we established that DNMT-3b is involved in methylation-based gene repression in lung cancer cell lines (Figs. 1, 4). Furthermore, we observed significant associations between clinical characteristics and DNMT-3b mRNA expression, such as being a women, T1 and T2 tumor classification, and N0 lymph node status, in patients with stage I/II NSCLC (Table 1).

Epigenetic information flows from histone to DNA through histone modification and DNA methylation. A self-reinforcing epigenetic cycle model has been proposed.[24] DNA methylation may exert positive feedback to histone modification. However, our previous study demonstrated that the histone deacetylase inhibitor trichostatin A at 0.5 μM is not effective in restoring the mRNA expression of GST-M2 in lung cancer cell lines.[4] Conversely, all 3 DNMTs that we studied participated in maintenance of the DNA methylation pattern, because there was a deficiency of DNMT-1 with loss of only 16% to 48% methylation of CG sites.[25] We also assessed the expression of DNMT-1 by using real-time PCR in 73 patients; however, we observed no significant correlation with GST-M2 gene loss (data not shown). There was relative DNMT-3b expression, especially in tumor tissues.

**Table 1.** Characteristics with DNA Methyltransferase-3b Gene Expression in 73 Patients With Nonsmall Cell Lung Carcinoma

| | Tumor Tissues (Median Separate=14) | | | | |
|---|---|---|---|---|---|
| | High GST-M2, n=37 | | Low GST-M2, n=36 | | |
| Characteristic | No. | Mean±SE | No. | Mean±SE | *P* |
| **Age, y**[a] | | | | | |
| <68 | 17 | 15.11±7.44 | 17 | 16.18±4.77 | .13 |
| ≥68 | 20 | 4.95±1.53 | 19 | 8.18±1.88 | .06 |
| **Sex** | | | | | |
| Men | 28 | 10.27±4.42 | 20 | 7.04±1.92 | .49 |
| Women | 9 | 7.58±5.37 | 16 | 18.11±4.79 | .04[b] |
| **Histology** | | | | | |
| AD | 23 | 11.88±5.58 | 24 | 11.80±3.51 | .17 |
| SQ | 14 | 5.9 ±2.08 | 12 | 12.23±3.00 | .06 |
| **Stage** | | | | | |
| I | 23 | 10.26±5.4 | 27 | 13.22±3.25 | .01[c] |
| II | 14 | 8.55±3.38 | 9 | 8.18±2.28 | .61 |
| **Tumor classification** | | | | | |
| T1,T2 | 30 | 10.68±4.36 | 31 | 13.08±2.87 | .02[b] |
| T3 | 7 | 5.08±1.99 | 5 | 5.04±1.73 | .87 |
| **Lymph node status** | | | | | |
| N0 | 30 | 9.05±4.16 | 32 | 11.94±2.80 | .03[b] |
| N1 | 7 | 12.07±6.44 | 4 | 12.10±4.11 | .63 |
| **Cell differentiation** | | | | | |
| Well/moderate | 22 | 10.99±5.58 | 22 | 14.37±3.87 | .08 |
| Poor | 15 | 7.60±3.38 | 14 | 8.17±2.01 | .13 |

GST-M2 indicates glutathione *S*-transferase M2; SE, standard error; AD, adenocarcinoma; SCC, squamous cell carcinoma.

[a] The median age of the 73 patients was 68 years.

[b] $P < .05$ (Mann-Whitney *U* test).

[c] $P < .01$. Mann-Whitney *U* test).

Furthermore, DNMT-3b knockdown restored the GST-M2 gene expression in lung cancer cells (Fig. 5, middle). These results suggest that, in tumors or lung cancer cells that have increased expression of DNMT-3b, there is promoter region methylation of GST-M2 that leads to decreased expression. Kassis et al reported that the depletion of DNMT-3b arrests lung cancer cell growth and reactivates the tumor suppressor genes, including RASSF1A, p16, p21 and tissue factor pathway inhibitor 2 (TFPI-2); and they claimed that DNMT-3b is essential for lung tumorigenesis and progression.[26] This indicates that DNMT-3b is more important in CpG islands of the GST-M2 promoter than DNMT-1. Conversely, loss of the nuclear factor (erythroid-derived 2)-like 2 (Nrf2) transcription factor causes a remarkable reduction in constitutive and inducible expression of the GST-alpha and GST-mu genes in the livers of mice.[27] Our results indicated that Sp1 regulates the gene expression of GST-M2 in lung cells and that methylation within the Sp1 binding site inhibits Sp1 binding and suppresses Sp1-regulated GST-M2 transcription in lung cancer cells.

We demonstrated that GST-M2 mRNA expression undergoes demethylation and is restored after exposure to 5′-aza-dC in lung cancer cells. These results are similar to previous findings for GST-P1 in human prostate cancer cells and clinical specimens.[28] The 5′-aza-dC nucleotide of DNA forms a covalent bond with the DNMTs and inactivates these enzymes, resulting in inhibition of maintenance and de novo methylation of genomic DNA.[29] 5′-aza-dC (decitabine) has been applied in clinical trials for such treatment.[30] By using different dose schedules, decitabine produces antineoplastic activity in human leukemia, myelodysplastic syndrome, and NSCLC[30-32] and is a potent antineoplastic agent against leukemia and tumors in animal models.[33] On the basis of the literature and the results from our current study, silencing of the GST-M2 gene is an indicator for 5-aza-dC treatment in patients with early stage NSCLC (especially those who have stage I

disease). It is vital to prevent NSCLC by interfering with silencing of the GST-M2 gene. Conversely, studies have demonstrated shown that the estrogen receptor (ER) promoter is hypermethylated and that the mechanisms of DNMT-3b are involved in breast cancer cells and lung cancer cells.[34] ER hypermethylation in lung tumors was associated with exposure to specific carcinogens in an animal study.[35] In addition, significant methylation at the ER locus was detected in 24% of human NSCLC tumors.[36] In the current study, women with stage I disease were affected by GST-M2 with DNMT-3b.

Recent studies have established that DNMT-induced epigenetic alterations occur mostly in the precancerous stage.[26] Furthermore, reduced GST-M2 expression results in susceptibility to benzo(a)pyrene-induced DNA damage.[4] GST-M2 methylation appears to be an early genetic alteration in lung tumorigenesis. The current study provides new insight into the molecular mechanism of GST-M2 expression in lung cancer cells and stage I/II NSCLC tissues. The results support our hypothesis that aberrant hypermethylation at the transcription factor Sp1 response element leads to the silencing of GST-M2 expression in lung cancer cells.

## CONFLICT OF INTEREST DISCLOSURES

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## REFERENCES

1. Tatemichi M, Iwasaki M, Sasazuki S, Tsugane S. Association between polymorphisms in glutathione S-transferase Mu3 and IgG titer levels in serum against Helicobacter pylori. *J Hum Genet*. 2009;54:557-563.
2. Ni H, Yu XJ, Liu HJ, et al. Progesterone regulation of glutathione S-transferase Mu2 expression in mouse uterine luminal epithelium during preimplantation period. *Fertil Steril*. 2009;91:2123-2130.
3. Weng MW, Hsiao YM, Chiou HL, et al. Alleviation of benzo[a]pyrene-diolepoxide-DNA damage in human lung carcinoma by glutathione S-transferase M2. *DNA Repair (Amst)*. 2005;4:493-502.
4. Tang SC, Sheu GT, Wong RH, et al. Expression of glutathione S-transferase M2 in stage I/II non-small cell lung cancer and alleviation of DNA damage exposure to benzopyrene. *Toxicol Lett*. 2010;192:316-323.
5. Issa JP. CpG island methylator phenotype in cancer. *Nat Rev Cancer*. 2004;4:988-993.
6. Cui JY, Choudhuri S, Knight TR, Klaassen CD. Genetic and epigenetic regulation and expression signatures of glutathione S-transferases in developing mouse liver. *Toxicol Sci*. 2010;116:32-43.
7. Chan AO, Rashid A. CpG island methylation in precursors of gastrointestinal malignancies. *Curr Mol Med*. 2006;6:401-408.
8. Tang M, Xu W, Wang Q, Xiao W, Xu R. Potential of DNMT and its epigenetic regulation for lung cancer therapy. *Curr Genomics*. 2009;10:336-352.
9. Luczak MW, Jagodzinski PP. The role of DNA methylation in cancer development. *Folia Histochem Cytobiol*. 2006;44:143-154.
10. Wang L, Wang J, Sun S, et al. A novel DNMT3B subfamily, DeltaDNMT3B, is the predominant form of DNMT3B in non-small cell lung cancer. *Int J Oncol*. 2006;29:201-207.
11. Wang J, Walsh G, Liu DD, Lee JJ, Mao L. Expression of Delta DNMT3B variants and its association with promoter methylation of p16 and RASSF1A in primary non-small cell lung cancer. *Cancer Res*. 2006;66:8361-8366.
12. Liu F, Killian JK, Yang M, et al. Epigenomic alterations and gene expression profiles in respiratory epithelia exposed to cigarette smoke condensate. *Oncogene*. 2010;29:3650-3664.
13. Xing J, Stewart DJ, Gu J, Lu C, Spitz MR, Wu X. Expression of methylation-related genes is associated with overall survival in patients with non-small cell lung cancer. *Br J Cancer*. 2008;98:1716-1722.
14. Zhu WG, Srinivasan K, Dai Z, et al. Methylation of adjacent CpG sites affects Sp1/Sp3 binding and activity in the p21(Cip1) promoter. *Mol Cell Biol*. 2003;23:4056-4065.
15. Zelko IN, Mueller MR, Folz RJ. CpG methylation attenuates Sp1 and Sp3 binding to the human extracellular superoxide dismutase promoter and regulates its cell-specific expression. *Free Radic Biol Med*. 2010;48:895-904.
16. Macleod D, Charlton J, Mullins J, Bird AP. Sp1 sites in the mouse aprt gene promoter are required to prevent methylation of the CpG island. *Genes Dev*. 1994;8:2282-2292.
17. Greene FL, Page DL, Fleming ID, et al, eds. AJCC Cancer Staging Manual. 6th ed. New York: Springer; 2002.
18. Law AY, Lai KP, Ip CK, Wong AS, Wagner GF, Wong CK. Epigenetic and HIF-1 regulation of stanniocalcin-2 expression in human cancer cells. *Exp Cell Res*. 2008;314:1823-1830.
19. Peng DF, Razvi M, Chen H, et al. DNA hypermethylation regulates the expression of members of the Mu-class glutathione S-transferases and glutathione peroxidases in Barrett's adenocarcinoma. *Gut*. 2009;58:5-15.
20. Chan AO, Peng JZ, Lam SK, et al. Eradication of Helicobacter pylori infection reverses E-cadherin promoter hypermethylation. *Gut*. 2006;55:463-468.
21. Milicic A, Harrison LA, Goodlad RA, et al. Ectopic expression of P-cadherin correlates with promoter hypomethylation early in colorectal carcinogenesis and enhanced intestinal crypt fission in vivo. *Cancer Res*. 2008;68:7760-7768.
22. Weng MW, Hsiao YM, Chen CJ, Wang JP, Chen WC, Ko JL. Benzo[a]pyrene diol epoxide up-regulates COX-2 expression through NF-kappaB in rat astrocytes. *Toxicol Lett*. 2004;151:345-355.

23. Nishitsuji H, Ikeda T, Miyoshi H, Ohashi T, Kannagi M, Masuda T. Expression of small hairpin RNA by lentivirus-based vector confers efficient and stable gene-suppression of HIV-1 on human cells including primary non-dividing cells. *Microbes Infect*. 2004;6:76-85.
24. Sarraf SA, Stancheva I. Methyl-CpG binding protein MBD1 couples histone H3 methylation at lysine 9 by SETDB1 to DNA replication and chromatin assembly. *Mol Cell*. 2004;15:595-605.
25. Hattori N, Abe T, Suzuki M, et al. Preference of DNA methyltransferases for CpG islands in mouse embryonic stem cells. *Genome Res*. 2004;14:1733-1740.
26. Kassis ES, Zhao M, Hong JA, Chen GA, Nguyen DM, Schrump DS. Depletion of DNA methyltransferase 1 and/or DNA methyltransferase 3b mediates growth arrest and apoptosis in lung and esophageal cancer and malignant pleural mesothelioma cells. *J Thorac Cardiovasc Surg*. 2006;131:298-306.
27. Chanas SA, Jiang Q, McMahon M, et al. Loss of the Nrf2 transcription factor causes a marked reduction in constitutive and inducible expression of the glutathione S-transferase Gsta1, Gsta2, Gstm1, Gstm2, Gstm3 and Gstm4 genes in the livers of male and female mice. *Biochem J*. 2002;365:405-416.
28. Jeronimo C, Usadel H, Henrique R, et al. Quantitation of GSTP1 methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma. *J Natl Cancer Inst*. 2001;93:1747-1752.
29. Miyamoto K, Ushijima T. Diagnostic and therapeutic applications of epigenetics. *Jpn J Clin Oncol*. 2005;35:293-301.
30. Momparler RL, Ayoub J. Potential of 5-aza-2′-deoxycytidine (Decitabine) a potent inhibitor of DNA methylation for therapy of advanced non-small cell lung cancer. *Lung Cancer*. 2001;34(suppl 4):S111-S115.
31. Momparler RL, Bouffard DY, Momparler LF, Dionne J, Belanger K, Ayoub J. Pilot phase I-II study on 5-aza-2′-deoxycytidine (Decitabine) in patients with metastatic lung cancer. *Anticancer Drugs*. 1997;8:358-368.
32. Momparler RL. Epigenetic therapy of cancer with 5-aza-2′-deoxycytidine (decitabine). *Semin Oncol*. 2005;32:443-451.
33. Lemaire M, Momparler LF, Bernstein ML, Marquez VE, Momparler RL. Enhancement of antineoplastic action of 5-aza-2′-deoxycytidine by zebularine on L1210 leukemia. *Anticancer Drugs*. 2005;16:301-308.
34. Sharma D, Blum J, Yang X, Beaulieu N, Macleod AR, Davidson NE. Release of methyl CpG binding proteins and histone deacetylase 1 from the Estrogen receptor alpha (ER) promoter upon reactivation in ER-negative human breast cancer cells. *Mol Endocrinol*. 2005;19:1740-1751.
35. Lai JC, Cheng YW, Chiou HL, Wu MF, Chen CY, Lee H. Gender difference in estrogen receptor alpha promoter hypermethylation and its prognostic value in non-small cell lung cancer. *Int J Cancer*. 2005;117:974-980.
36. Issa JP, Baylin SB, Belinsky SA. Methylation of the estrogen receptor CpG island in lung tumors is related to the specific type of carcinogen exposure. *Cancer Res*. 1996;56:3655-3658.