

Promoter Methylation of Glutathione S-Transferase π 1 and Multidrug Resistance Gene 1 in Bronchioloalveolar Carcinoma and its Correlation With DNA Methyltransferase 1 Expression

Peng Gao, MD¹; Xi Yang, MD²; Yu-Wen Xue, MD²; Xiao-Fang Zhang, MD¹; Yan Wang, MD¹; Wen-Jun Liu, MD¹; and Xiao-Juan Wu, MD¹

BACKGROUND: The presence of glutathione S-transferase (GST) π 1 (GSTP1) or multidrug resistance gene 1 (MDR1) promoter methylation in lung cancer was studied for the first time to the authors' knowledge; and, to date, the clinical significance of methylation is not clear. The objective of the current study was to determine the promoter methylation status of GSTP1 and MDR1, which encode GST- π and P-glycoprotein (Pgp), respectively, in patients with bronchioloalveolar carcinoma (BAC) and to investigate whether methyltransferase 1 (DNMT1)-mediated GSTP1 or MDR1 methylation are responsible for disease progression and prognosis in patients with BAC. **METHODS:** Protein expression levels of DNMT1, GST- π , and Pgp were determined by immunohistochemistry in samples from 36 patients with BAC. Promoter methylation status of the GSTP1 and MDR1 genes was determined by using methylation-specific polymerase chain reaction analysis. **RESULTS:** The results demonstrated a significant correlation between the methylation of the GSTP1 or MDR1 promoters and negative expression of their respective proteins in BAC ($P < .05$). A significant correlation also was demonstrated between GSTP1 methylation and recurrence-free and overall survival of patients with BAC. DNMT1 protein expression levels were correlated with GSTP1 promoter methylation and patient prognosis ($P < .05$). However, no correlation was observed between DNMT1 expression and MDR1 methylation. **CONCLUSIONS:** GSTP1 promoter methylation mediated by DNMT1 may promote BAC progression and could serve as a poor prognostic indicator for patients with this disease. DNMT1 protein expression also may be considered as a prognostic indicator. Methylation of the MDR1 promoter may be mediated through pathways other than DNMT1 in BAC and does not appear to be associated with disease progression or patient prognosis. **Cancer 2009;115:3222-32.** © 2009 American Cancer Society.

KEY WORDS: glutathione S-transferase π 1, DNA methyltransferase 1, methylation, prognosis, bronchioloalveolar carcinoma.

Corresponding author: Peng Gao, MD, Department of Pathology, School of Medicine, Shandong University, Jinan Wen Hua Xi Road 44, 250012, P.R. China; Fax: (011) 86-531-88382052; gaopeng@sdu.edu.cn

¹Department of Pathology, School of Medicine, Shandong University, Jinan, People's Republic of China; ²Department of Pulmonary Medicine, Qilu Hospital, Shandong University, Jinan, People's Republic of China

The first two authors contributed equally to this article.

This article was conceived and designed by Peng Gao and Yu-Wen Xue. Data for the article were collected and assembled by Peng Gao, Xi Yang, Yu-Wen Xue, Xiao-Fang Zhang, Yan Wang, Wen-Jun Liu, and Xiao-Juan Wu. Data were analyzed and interpreted by Peng Gao and Xi Yang. The article was written by Peng Gao and Xi Yang. All authors gave their final approval of the article.

Received: October 3, 2008; **Revised:** December 20, 2008; **Accepted:** December 29, 2008

Published online: May 29, 2009 © 2009 American Cancer Society

DOI: 10.1002/cncr.24369, www.interscience.wiley.com

Lung cancer is 1 of the most common cancers in the world. In recent years, the incidence of adenocarcinoma gradually has risen and has surpassed squamous carcinoma as the most common histologic subtype of lung cancer in many countries.¹ Bronchioloalveolar carcinoma (BAC) is a subtype of lung adenocarcinoma that is characterized by the growth of neoplastic cells along pre-existing alveolar structures in the absence of stromal and vascular invasion.² BAC is almost always moderately differentiated or well differentiated, and patients with BAC typically have a favorable prognosis or are diagnosed at an early stage of lung cancer.²⁻⁴

The administration of chemotherapeutics after surgical procedures is 1 of the principal treatments for patients with lung cancer. However, some patients eventually develop drug resistance, which results in chemotherapy failure.⁵ Previous studies have demonstrated that the overexpression of glutathione S-transferase π (GST- π) and P-glycoprotein (Pgp) may result in drug resistance. It is believed that GST- π , which is encoded by GST- π 1 (GSTP1), helps catalyze the conjugation of hydrophobic and electrophilic compounds, such as cisplatin to glutathione (GSH).⁶ The GSH-cisplatin complex actively is displaced from cells, thus reducing the cytotoxic efficacy of drugs. The overexpression of Pgp, encoded by multidrug resistance gene 1 (MDR1), is 1 of the most important mechanisms of drug resistance. Pgp may function as a drug efflux pump to decrease the intracellular concentration of a variety of anticancer drugs with various structures and functions.⁷ The overexpression of GST- π and Pgp has been demonstrated in a variety of human cancers.⁸⁻¹⁰ However, the mechanisms of their regulation are not clear. The potential impact of MDR1 and GSTP1 promoter methylation on the regulation of GST- π and Pgp expression in patients with BAC is not known.

It was reported previously that Pgp expression is correlated inversely with methylation of CpG sites on the MDR1 promoter in patients with bladder cancer.¹¹ Hypermethylation of the GSTP1 promoter also was observed in prostate cancer¹² and in breast cancer cells¹³ versus benign disease from the same tissues. We hypothesize that methylation of the GSTP1 and MDR1 promoters is mediated by DNA methyltransferase 1 (DNMT1) and may contribute to regulation of protein expression.

It has been demonstrated that epigenetic modifications like DNA methylation are involved in the early stages of carcinogenesis.¹⁴ Overall DNA hypomethylation accompanied by regional DNA hypermethylation generally is observed in human cancers.^{14,15} Gene promoter methylation plays an important role in transcriptional regulation, such as silencing of gene transcription through the methylation of CpG islands.¹⁵ It has been confirmed that DNA methyltransferases (DNMTs), including DNMT1, DNMT3a, and DNMT3b, possess DNMT activity. DNMT1 is a major enzyme involved in the establishment of DNA methylation.^{16,17} Some reports have indicated that DNMT1 possesses both maintenance and de novo DNA methylation activity in vivo, regardless of its in vitro preference for hemimethylated rather than unmethylated substrates.^{18,19} Promoter methylation of tumor suppressor genes, such as *p16*, *p14*, the human mutL homolog 1 gene *hMLH1*, and the fragile histidine triad gene *FHIT*, may act to inactivate the expression of their respective proteins, thus promoting tumor development.²⁰⁻²² Currently, to our knowledge, there is no information in the literature regarding the role of MDR1 and GSTP1 promoter methylation in the progression and prognosis of patients with BAC.

In the current study, protein expression levels of DNMT1, Pgp, and GST- π were determined by immunohistochemistry in 36 samples of BAC. The promoter methylation status of MDR1 and GSTP1 was determined using methylation-specific polymerase chain reaction (MSP). The correlation between DNMT1 expression and promoter methylation status of MDR1 or GSTP1 was analyzed. In addition, we investigated whether these findings were correlated with patient's prognosis.

MATERIALS AND METHODS

Patients

Thirty-six patients with BAC who underwent pneumonoresection with lymph node dissection at Qi Lu Hospital of Shandong University from 2004 to 2006 were studied. The patients received clinical follow-up at a median of 38 months (range, 10-55 months). The study was approved by the Ethics Committee of Shandong University. The patients ranged in age from 35 years to 76 years, and their median age was 62 ± 9 years. All clinical specimens were

Table 1. Methylation of Multidrug Resistance Gene 1 and Glutathione S-Transferase π 1, Expression of DNA Methyltransferase 1, and Their Correlation With Clinical Pathologic Parameters in Bronchioloalveolar Carcinoma

Variable	No. of Patients								
	Methylation of MDR1			Methylation of GSTP1			DNMT1 Expression		P
	M	U	P	M	U	P	Positive	Negative	
Age, y									
35-61	10	5	.709	4	11	.694	7	8	.175
62-76	16	5		4	17		15	6	
Sex									
Men	8	4	.700	2	10	.691	8	4	.727
Women	18	6		6	18		14	10	
Smoker									
Yes	6	4	.413	2	8	1.00	7	3	.706
No	20	6		6	20		15	11	
Size, cm									
<3	13	5	1.00	3	15	.691	11	7	1.00
\geq 3	13	5		5	13		11	7	
Positive lymph nodes									
Yes	10	3	.716	2	11	.682	11	2	.039
No	16	7		6	17		11	12	
Clinical stage									
I	14	6	.305	3	12	.035	11	9	.625
II	5	0		2	3		3	2	
III	7	4		3	7		8	3	
Recurrence rate, %	43.7	44.4	.622	52.6	16.67	.029	58.82	12.50	.046
Survival rate, %	50.2	50	.262	31.2	100	.045	38.24	80	.040

MDR1 indicates multidrug resistance gene 1; GSTP1, glutathione S-transferase π 1; DNMT1, DNA methyltransferase 1; M, methylated; U, unmethylated.

collected and embedded in paraffin for histologic diagnosis and immunostudy. Designation of tumor stage and criteria for histologic classification were performed according to the World Health Organization classification.²³ Clinicopathologic parameters that were measured included patient age, sex, tumor size (>30 mm or <30 mm), pathologic tumor (pT) classification (pT1-pT3), smoking habit, and lymphatic metastases. These parameters are summarized in Table 1.

Methylation-specific Polymerase Chain Reaction Analysis

High-molecular-weight DNA was extracted from 36 BAC samples using phenol-chloroform extraction and dialysis. Bisulfite conversion was performed using 1 μ g of genomic DNA and a CpG genome DNA modification kit (Chemicon, Temecula, Calif). This process converts unmethylated cytosines to uracils, which ultimately are detected as thymidines after polymerase chain reaction

(PCR) amplification. In contrast, methylated cytosine residues will remain unchanged after amplification. MSP of bisulfite-treated genomic DNA was carried out according to the method reported previously.²⁴ The PCR products of methylated and unmethylated genomes differ after bisulfite conversion and may be distinguished using MSP. The primers that we used for analysis of GSTP1 and MDR1 are listed in Table 2. The PCR cycling conditions included 40 cycles at 95°C for 30 seconds, 58°C for 45 seconds, and 72°C for 60 seconds. Product sizes of the methylated and unmethylated reactions for GSTP1 and MDR1 also are listed in Table 2. The products were separated electrophoretically on a 3% agarose gel. MSP results were classified as either 1) unmethylation if unmethylated alleles were detected and no methylated alleles were observed or 2) methylation if methylated alleles were observed and no unmethylated alleles were detected. Positive results produced from preliminary experiments were used as positive controls for methylation analysis. Double-distilled water was used as a negative control by the

Table 2. The Sequence of Primers for Glutathione S-Transferase π 1 and Multidrug Resistance Gene 1

Primers	Sequence	Product Size, bp
GSTP1		
M (upstream)	5'-TTCGGGGTGTAGCGGTCGTC-3'	92
M (downstream)	5'-GCCCAATACTAAATCACGACG-3'	
U (upstream)	5'-GATGTTTGGGGTGTAGTGGTTGTT-3'	99
U (downstream)	5'-CCACCCCAATACTAAATCACAACA-3'	
MDR1		
M (upstream)	5'-CGTTGTTAGATTTTTAATTTTGTTC-3'	100
M (downstream)	5'-CCA ACTACTCTA ACCGCG AT-3'	
U (upstream)	5'-TGTTGTTAGATTTTTAATTTTGTTC-3'	100
U (downstream)	5'-TACCCC AAC TAC TCTAACCCAC AAT-3'	

bp Indicates base pairs; GSTP1, glutathione S-transferase π 1; M, methylated; U, unmethylated; MDR1, multidrug resistance gene 1.

replacement of genomic DNA. All PCR reactions were performed with positive controls for both unmethylated and methylated alleles and for a negative "no DNA" control. In addition, 10 samples of normal lung tissue from patients with inflammatory pseudotumors were analyzed in the MSP assay for CpG island methylation of the MDR1 and GSTP1 promoters.

Immunohistochemical Staining

Paraffin-embedded BAC sections (4 μ m thick) and corresponding, noncancerous samples obtained from within 3 cm of the tumor border were dewaxed and subjected to antigen retrieval by microwaving in 0.01 M citric buffer, pH 6.0, twice for 15 minutes each at 100°C; this was followed by incubation in 3% H₂O₂ for 10 minutes to quench endogenous peroxidase. Nonspecific binding was prevented by incubation in 5% normal horse serum for 20 minutes in a humid chamber. Next, slides were incubated with antibodies against DNMT1 (goat polyclonal antibody; 1:200 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif), Pgp (mouse monoclonal antibody; 1:100 dilution; NeoMarkers, Fremont, Calif), and GST- π (mouse monoclonal antibody; 1:200 dilution; DAKO, Glostrup, Denmark) overnight at 4°C. After washing, the primary antibody was detected with an appropriate secondary antibody for 30 minutes at 37°C. After washes, slides were incubated with a streptavidin biotin complex (Zymed, South San Francisco, Calif) for 30 minutes at

37°C, washed 3 times, observed using diaminobenzidine, rinsed in distilled water, and counterstained with hematoxylin.

Assessment of Immunohistochemistry

Immunoreactivity for DNMT1 was detected in the nucleus, and expression of Pgp was observed in the cellular membrane. Positive expression of GST- π was observed in both the cytoplasm and the nucleus. For each sample, at least 500 cells were counted randomly. To distinguish definitively positive cells from weak background signals, such as proliferating zones of normal lung tissues or lymphocytes, only samples that had >30% of tumor cells with nuclear staining were considered positive for DNMT1, as reported previously.^{22,25} For the analysis of Pgp expression, the cutoff point is 10% of tumor cells, as reported previously.²⁶ For the analysis of GST- π , the cutoff point has varied from 10% to 50% of tumors cells in different reports.²⁷⁻²⁹ In the current study, the cutoff point of 30% was established by using receiver operating characteristic (ROC) curve analysis in SPSS software (version 10.0; SPSS Inc., Chicago, Ill).

Statistical Analysis

Potential correlations between the methylation status of the GST- π promoter, the MDR1 promoter, or DNMT1 expression and clinicopathologic parameters were

analyzed using the Fisher exact test. Correlations between protein expression of DNMT1, GST- π , or Pgp and GSTP1 or MDR1 promoter methylation also were analyzed using the Fisher exact test. Survival curves were plotted using the Kaplan-Meier method and were compared using the log-rank test. A Cox proportional hazards model was used to calculate the hazard ratio of each factor in univariate and multivariate analyses. Analyses were performed using the statistical package SPSS (version 10.0; SPSS Inc.). Differences were considered statistically significant for P values $< .05$.

RESULTS

Methylation Status of the GSTP1 and MDR1 Promoters in Bronchioalveolar Carcinoma and Their Correlation With Clinical Parameters

Eight of 36 BAC samples (22.2%) revealed methylated alleles in MSP analysis for the GSTP1 promoter, whereas none of the 10 samples of normal lung tissue revealed GSTP1 promoter methylation (Fig. 1 Top). No correlation was observed between GSTP1 promoter methylation and patient age, sex, smoking habit, lymph node metastases, or tumor size. However, there was a statistically significant correlation between GSTP1 methylation and the recurrence rate or the survival rate ($P < .05$) (Table 1). GST- π protein expression was observed in 30 of 36 samples (Fig. 2 Top). In this study, 25% (2 of 8) methylated samples and 14.3% (4 of 28) unmethylated samples had negative expression of GST- π . There was a tendency for more frequent negative expression of GST- π in the methylated samples ($P = .046$) (Table 3). However, given our small sample size, further study that includes a larger number of patients will be needed to confirm this correlation.

Twenty-six of 36 BAC samples (72.2%) revealed methylated alleles in MSP analysis for the MDR1 promoter, 10 samples revealed unmethylation of the MDR1 promoter, and all 10 samples of normal lung tissue revealed promoter methylation of MDR1 (Fig. 1 Bottom). There was no correlation between promoter methylation of MDR1 and patient age, sex, smoking habit, lymph node metastasis, tumor size, tumor stage, recurrence rate, or survival rate (Table 1). Pgp-positive expres-

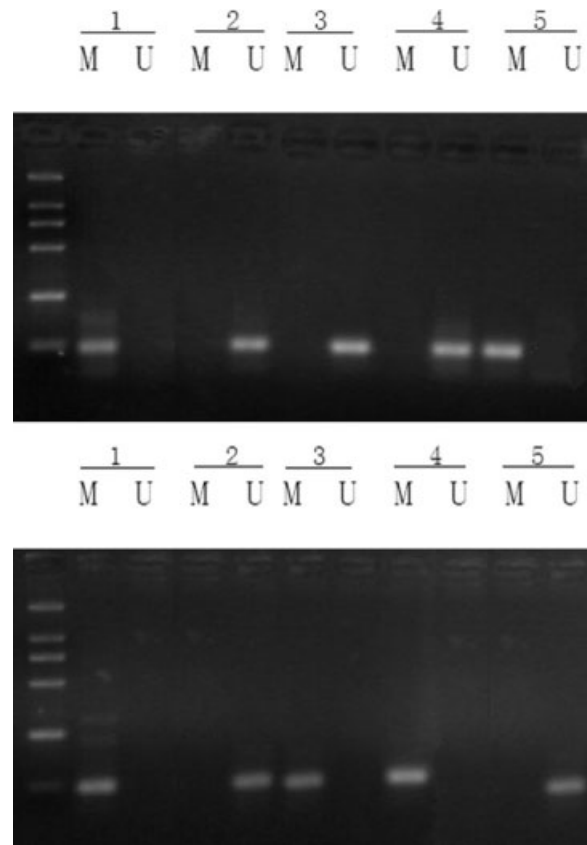


FIGURE 1. These are examples of methylation-specific polymerase chain reaction (MSP) products from a methylation analysis of (top) the glutathione S-transferase π 1 gene (GSTP1) promoter and (bottom) the multidrug resistance gene 1 (MDR1) promoter in patients with bronchioalveolar carcinoma (BAC). MSP products marked M and U reflect the presence of methylated and unmethylated genes, respectively. Lanes 1 and 2 are positive controls for the M and U alleles. (Top) In normal lung tissue (lane 3), the GSTP1 promoter is unmethylated (U). However, 8 of 36 BAC samples had methylated (M) alleles (a representative example is shown in lane 5), and the other samples had unmethylated (U) alleles (a representative example is shown in lane 4). (Bottom) In normal lung tissue (lane 3), the MDR1 promoter is methylated (M). However, 26 of 36 BAC samples had MDR1 promoter methylation (a representative example is shown in lane 4), and the other 10 samples were unmethylated (a representative example is shown in lane 5).

sion was observed in 15 of 36 samples (Fig. 2 Middle), and the other 21 samples had negative expression. A significant correlation between MDR1 promoter methylation and negative expression of Pgp was demonstrated in the BAC samples (Table 3). Samples that had MDR1 promoter methylation tended to have negative Pgp expression.

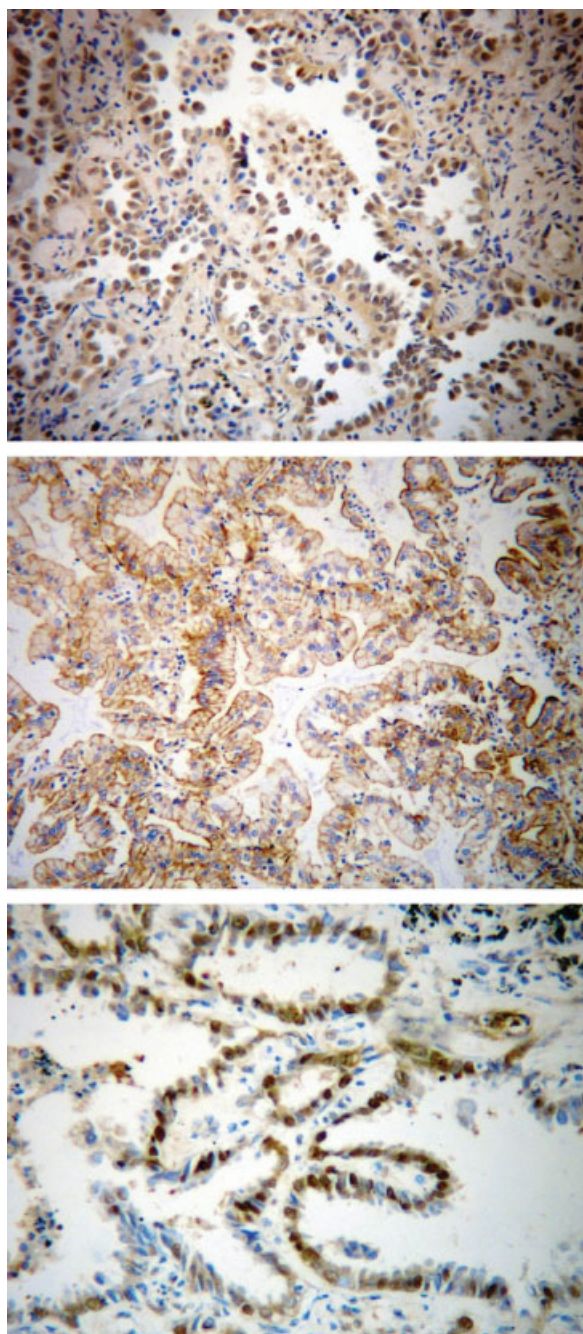


FIGURE 2. These are photomicrographs from immunohistochemical studies of glutathione S-transferase π (GST- π), P-glycoprotein (Pgp), and DNA methyltransferase 1 (DNMT1) expression in bronchioalveolar carcinoma. GST- π protein was expressed in both cytoplasm and nucleus. (Top) Samples in which >30% of tumor cells demonstrated nuclear staining were considered positive for GST- π (original magnification, $\times 200$). (Middle) Pgp protein was expressed in cell membranes. Samples that had >10% tumor cells with membrane staining were considered positive (original magnification, $\times 200$). (Bottom) DNMT1 protein was expressed in the nucleus. Only samples in which >30% of tumor cells had nuclear staining were considered positive for DNMT1 (original magnification, $\times 400$).

DNMT1 Protein Expression in Bronchioalveolar Carcinoma and Its Correlation With Clinicopathologic Parameters and Methylation Status of the GSTP1 and MDR1 Promoters

Twenty-two of 36 BAC samples (61.1%) demonstrated positive expression of the DNMT1 protein (Fig. 2 Bottom), whereas none of the corresponding noncancerous tissues or normal lung tissues were positive for DNMT1. DNMT1 protein expression was correlated significantly with lymph node metastasis, recurrence rate, and survival rate ($P < .05$) (Table 1). No significant correlations were observed between DNMT1 expression and patient age, sex, smoking habit, tumor size, or tumor stage ($P > .05$).

DNMT1 protein expression was correlated with GSTP1 promoter methylation in BAC samples ($P < .05$) (Table 3). However, no correlation was observed between DNMT1 expression and MDR1 methylation ($P > .05$).

Prognostic Significance of MDR1 and GSTP1 Promoter Methylation and DNMT1 Expression in Bronchioalveolar Carcinoma

During follow-up, 8 of 36 patients (22.2%) patients died of their disease, and 11 of 36 patients (30.6%) developed a recurrence. No correlation was observed between MDR1 promoter methylation and recurrence-free survival (Fig. 3 Top Left) or overall survival (Fig. 3 Top Right). However, both GSTP1 promoter methylation and DNMT1 protein expression were correlated with the recurrence and survival rates ($P < .05$) (Table 1). In survival analyses using Kaplan-Meier curves and log-rank tests, GSTP1 promoter methylation was associated with poor recurrence-free survival ($P < .01$) (Fig. 3 Middle Left) and poor overall survival ($P < .01$) (Fig. 3 Middle Right). The survival rate for patients who had GSTP1 promoter methylation was significantly lower compared with the rate among patients without methylation. DNMT1 protein expression also was associated with poor recurrence-free survival ($P < .01$) (Fig. 3 Bottom Left) and poor overall survival ($P < .01$) (Fig. 3 Bottom Right). Patients who had positive DNMT1 expression had a poorer prognosis than those who had negative expression. Univariate and multivariate analyses demonstrated that

Table 3. Correlation Between the Expression of DNA Methyltransferase 1, P-glycoprotein, and Glutathione S-Transferase π Protein and the Methylation Status of Multidrug Resistance Gene 1 and Glutathione S-Transferase π 1

Methylation Status	DNMT1			Pgp			GST- π		
	Positive	Negative	P	Positive	Negative	P	Positive	Negative	P
MDR1									
M	18	8	.140	10	16	.026			
U	4	6		7	3				
GSTP1									
M	6	2	.036				6	2	.046
U	16	12					24	4	

DNMT1 indicates DNA methyltransferase 1; Pgp, p-glycoprotein; GST- π , glutathione S-transferase π ; MDR1, multidrug resistance gene 1; M, methylated; U, unmethylated; GSTP1, glutathione S-transferase π 1.

lymph node metastasis, GSTP1 promoter methylation, and DNMT1 expression could serve as prognostic factors independent of other factors (Table 4).

DISCUSSION

Intensive investigations have been performed regarding correlations between protein expression of GST- π and patient responses to chemotherapy.^{8,9,29} However, the regulation of protein expression by promoter methylation of GSTP1 is not understood well. In addition, the clinical significance of promoter methylation is not clear. CpG island methylation of GSTP1 reportedly was detected in 93% of patients with prostate cancer.¹² Methylation at a single gene locus encompassing GSTP1 did not correlate with any clinicopathologic variables in that study. In contrast, methylation at 2 gene sites (GSTP1/adenomatous polyposis coli [APC] and GSTP1/prostaglandin G/H synthase and cyclooxygenase [PTGS2]) was correlated significantly with clinical stage and Gleason score.¹² Methylation of GSTP1 also was detected in the serum from 27.8% of patients with metastatic prostate cancer and has potential as a useful biomarker for patients with hormone-refractory prostatic cancer.³⁰ In the current study, none of the normal lung tissue samples contained promoter methylation of GSTP1. However, 8 of 36 BAC samples (22.2%) had GSTP1 methylation. GSTP1 methylation was correlated significantly with poor overall survival and recurrence-free survival. Multivariate analysis suggested that GSTP1 methylation may serve as a prognostic indicator independent of other factors for patients with BAC. A significant correlation was observed between DNMT1 protein expression and methylation status of the

GSTP1 promoter, and there was a tendency for samples with GSTP1 methylation to have more frequent negative GST- π expression. Given the function of DNMT1 to silence gene transcription through methylation of promoter CpG islands, our results suggest that the DNMT1 protein may mediate promoter methylation of GSTP1 and that GSTP1 methylation, in turn, may result in decreased expression of the GST- π protein in BAC.

The precise molecular mechanisms responsible for Pgp protein expression have been controversial in the literature. Some reports indicated that amplification of the MDR1 gene may result in its overexpression and may be implicated in acquired chemoresistance.^{31,32} However, other reports have demonstrated that chemotherapeutic drugs may induce specific epigenetic modifications (hypomethylation) at the MDR1 locus and may up-regulate MDR1 by transcriptional activation.^{33,34} In the current study, there was a significant correlation between MDR1 methylation and negative Pgp expression. Our results support the conclusion that methylation of the MDR1 promoter negatively regulates Pgp protein expression in BAC. Furthermore, no correlation was observed between the methylation status of the MDR1 promoter and DNMT1 protein expression in this study, suggesting that MDR1 methylation may be mediated by some other pathway or by DNA methyltransferases, such as DNMT3a or DNMT3b, rather than DNMT1 in BAC.

MDR1 methylation was not correlated with tumor stage or patient prognosis in this study, suggesting that MDR1 methylation would not be a good prognostic indicator for BAC. This result differs from the results from studies performed on prostate cancer³⁵ and neuroblastoma samples,³⁶ which suggested that MDR1

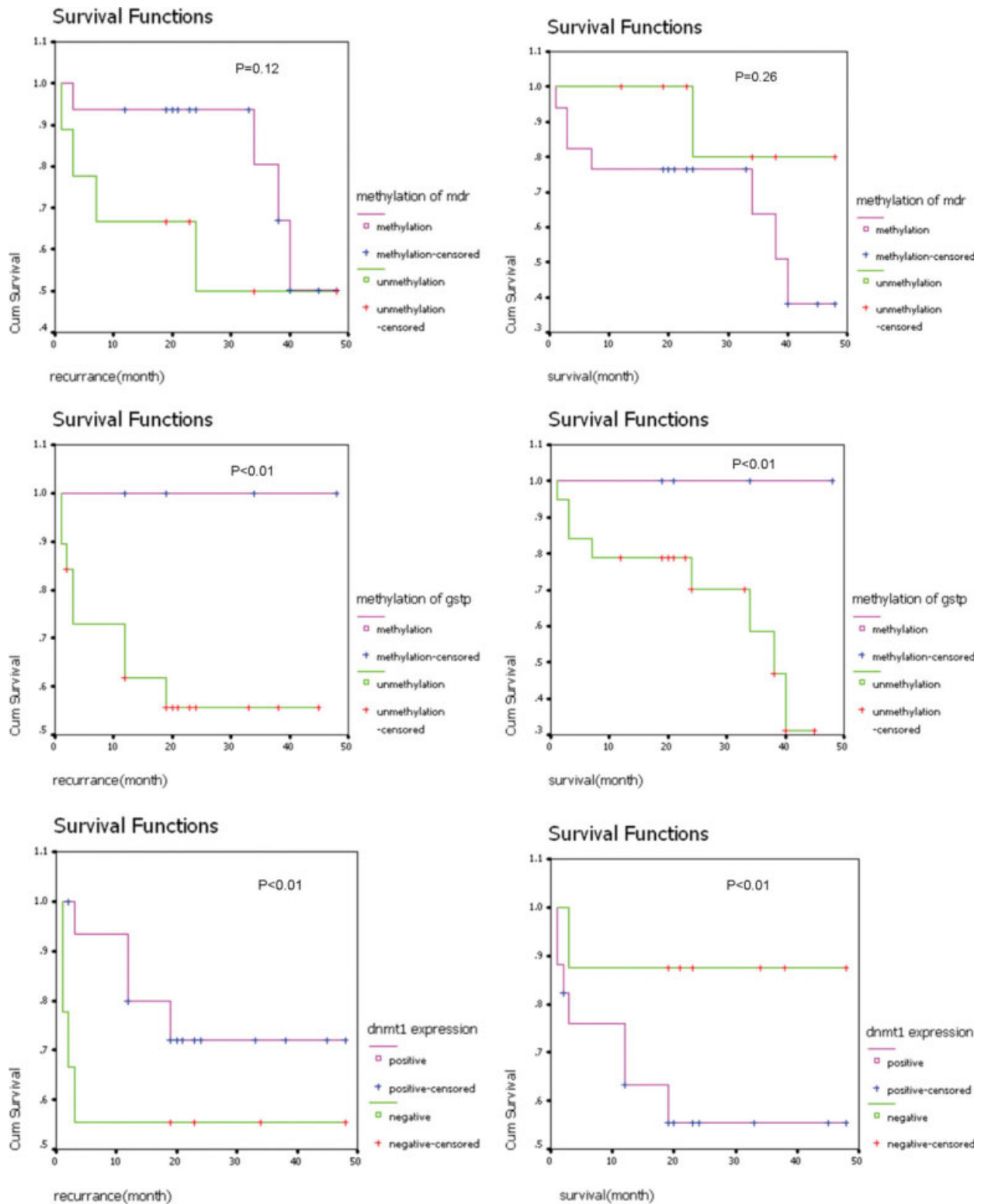


FIGURE 3. These Kaplan-Meier survival curves for patients with bronchioloalveolar carcinoma were calculated according to methylation status of the glutathione S-transferase $\pi 1$ (GSTP1) promoter or the multidrug resistance (mdr) gene 1 (MDR1) promoter and DNA methyltransferase 1 (DNMT1) expression. No correlation was observed between MDR1 promoter methylation and (Top Left) recurrence-free survival or (Top Right) overall survival. GSTP1 promoter methylation was associated with (Middle Left) poor recurrence-free survival ($P < .01$) and (Middle Right) poor overall survival ($P < .01$). The survival rate of patients who had GSTP1 promoter methylation was significantly lower than the survival rate of patients without GSTP1 promoter methylation. DNMT1 protein expression also was associated with (Bottom Left) poor recurrence-free survival ($P < .01$) and (Bottom Right) poor overall survival ($P < .01$). Patients who had DNMT1-positive expression had a poorer prognosis than patients who had DNMT1-negative expression. Cum survival indicates cumulative survival.

Table 4. Univariate and Multivariate Analysis of Prognostic Factors in Bronchioloalveolar Carcinoma

Variable	Univariate Analysis		Multivariate Analysis	
	HR (95% CI)	P	HR (95% CI)	P
Tumor size	2.63 (1.12-5.93)	<.05	2.46 (0.92-4.11)	NS
Smoking status	1.26 (0.43-3.59)	.72		
Lymph node status	3.89 (1.76-6.92)	<.01	3.36 (1.53-6.26)	<.01
GSTP1 methylation	4.03 (1.81-7.86)	<.01	3.85 (1.63-7.05)	<.01
MDR1 methylation	1.75 (0.86-3.75)	.11		
DNMT1 expression	3.37 (1.58-6.07)	<.01	3.15 (1.43-5.65)	<.01

HR indicates hazard ratio; CI, confidence interval; NS, not significant; GSTP1, glutathione S-transferase π 1; MDR1 indicates multidrug resistance gene 1; DNMT1, DNA methyltransferase 1.

methylation may contribute to tumor progression and may serve as a poor prognostic factor. One explanation for this discrepancy is that the MDR1 promoter is not methylated in the majority of benign prostate disease¹²; whereas, in the current study, normal lung tissue demonstrated MDR methylation.

Because it deregulates the expression of tumor suppressor genes, promoter methylation is a crucial event in cancer initiation and progression.¹³⁻¹⁵ Overexpression of DNMT1 messenger RNA has been reported in several human tumors, including gastric cancer, prostate cancer, renal cancer, and lung cancer.^{19,25,37} In the current study, all samples of normal lung tissue and noncancerous tissues near the primary cancer demonstrated negative expression of DNMT1, whereas 22 of 36 BAC samples (61.1%) were positive for DNMT1 expression. The results indicate that DNMT1 protein expression is correlated with positive lymph node metastasis, disease recurrence, and poor overall survival. These data suggest that DNMT1 protein expression has the potential to become a predictor of patient prognosis. This conclusion is coincident with other investigations. In patients with nonsmall cell lung cancer, increased DNMT1 messenger RNA expression³⁷ and protein expression²² reportedly were associated with a poor prognosis.

No correlation was uncovered between DNMT1 expression and other clinicopathologic parameters, including smoking habits. However, previous reports have indicated that exposure to tobacco smoke may induce DNMT1 expression in nonsmall lung cancer (specifically, in squamous cell carcinoma).^{22,38} We believe that this discrepancy lies in the method with which the

samples were collected from patients with BAC, a subtype of early adenocarcinoma with well differentiated histology. Because the tumorigenesis of lung adenocarcinoma is not associated as closely with smoking as squamous carcinoma, tobacco exposure may not play an important role in mediating DNMT1 expression in BAC.

In summary, the current results suggest that methylation of the GSTP1 promoter by DNMT1 may promote disease progression and could serve as a poor prognostic indicator for patients with BAC. DNMT1 protein expression also could be a prognostic indicator. Methylation of the MDR1 promoter may be mediated by pathways other than DNMT1 in BAC and is not associated with disease progression or patient prognosis. The contribution of GSTP1 promoter methylation to progression and prognosis in BAC deserves a further study in larger groups of patients. Demethylation experiments, such as using 5-azadeoxycytidine in cultures from lung adenocarcinoma cells and nude mouse xenografts, also are needed to supply further evidence of the correlations between DNMT1 expression and GSTP1 methylation or GSTP1 methylation and GST- π expression.

Conflict of Interest Disclosures

The authors made no disclosures.

References

- Colby TV, Koss M, Travis MD. Tumors of the Lower Respiratory Tract. 3rd ed. Washington, DC: Armed Forces Institute of Pathology; 1995.
- Travis WD, Colby TV, Corrin B, Shinmosato Y, Brambilla E; In collaboration with Sobin LH and pathologists from 14 countries. Histologic Typing of Lung and Pleural Tumours. WHO International Histological Classification of Tumors. 3rd ed. Berlin, Germany: Springer; 1999.
- Terasaki H, Niki T, Matsuno Y, et al. Lung adenocarcinoma with mixed bronchioloalveolar and invasive components: clinicopathological features, subclassification by extent of invasive foci, and immunohistochemical characterization. *Am J Surg Pathol.* 2003;27:937-951.
- Noguchi M, Morikawa A, Kawasaki M, et al. Small adenocarcinoma of the lung. Histologic characteristics and prognosis. *Cancer.* 1995;75:2844-2852.
- Huang Y, Sadee W. Membrane transporters and channels in chemoresistance and -sensitivity of tumor cells. *Cancer Lett.* 2006;239:168-182.
- Jakoby WB. The glutathione S-transferases: a group of multifunctional detoxification proteins. *Adv Enzymol Relat Areas Mol Biol.* 1978;46:383-414.

7. Marchetti S, Mazzanti R, Beijnen JH, Schellens JH. Clinical relevance of drug-drug and herb-drug interactions mediated by the ABC transporter ABCB1 (MDR1, P-glycoprotein). *Oncologist*. 2007;12:927-941.
8. Shi H, Lu D, Shu Y, Shi W, Lu S, Wang K. Expression of multidrug-resistance-related proteins P-glycoprotein, glutathione-S-transferases, topoisomerase-II and lung resistance protein in primary gastric cardiac adenocarcinoma. *Cancer Invest*. 2008;26:344-351.
9. Hayes JD, Pulford DJ. The glutathione S-transferase supergene family: regulation of GST and the contribution of the isoenzymes to cancer chemoprotection and drug resistance. *Crit Rev Biochem Mol Biol*. 1995;30:445-600.
10. Marie JP, Legrand O. MDR1/P-GP expression as a prognostic factor in acute leukemias. *Adv Exp Med Biol*. 1999;457:1-9.
11. Tada Y, Wada M, Kuroiwa K, et al. MDR1 gene overexpression and altered degree of methylation at the promoter region in bladder cancer during chemotherapeutic treatment. *Clin Cancer Res*. 2000;6:4618-4627.
12. Ellinger J, Bastian PJ, Jurgan T, et al. CpG island hypermethylation at multiple gene sites in diagnosis and prognosis of prostate cancer. *Urology*. 2008;71:161-167.
13. Pasquali L, Bedeir A, Ringquist S, Styche A, Bhargava R, Trucco G. Quantification of CpG island methylation in progressive breast lesions from normal to invasive carcinoma. *Cancer Lett*. 2007;257:136-144.
14. Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet*. 2002;3:415-428.
15. Garinis GA, Patrinos GP, Spanakis NE, Menounos PG. DNA hypermethylation: when tumour suppressor genes go silent. *Hum Genet*. 2002;111:115-127.
16. Arora P, Kim EO, Jung JK, Jang KL. Hepatitis C virus core protein down-regulates E-cadherin expression via activation of DNA methyltransferase 1 and 3b. *Cancer Lett*. 2008;261:244-252.
17. Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. *Nat Genet*. 1998;19:219-220.
18. Bestor TH. Activation of mammalian DNA methyltransferase by cleavage of a Zn binding regulatory domain. *EMBO J*. 1992;11:2611-2617.
19. Rhee I, Bachman KE, Park BH, et al. DNMT1 and DNMT3b cooperate to silence genes in human cancer cells. *Nature*. 2002;416:552-556.
20. Kim DH, Nelson HH, Wiencke JK, et al. p16(INK4a) and histology-specific methylation of CpG islands by exposure to tobacco smoke in non-small cell lung cancer. *Cancer Res*. 2001;61:3419-3424.
21. Hsu HS, Wang YC, Tseng RC, et al. 5'-Cytosine-phosphoguanine island methylation is responsible for p14ARF inactivation and inversely correlates with p53 over-expression in resected non-small cell lung cancer. *Clin Cancer Res*. 2004;10:4734-4741.
22. Lin RK, Hsu HS, Chang JW, Chen CY, Chen JT, Wang YC. Alteration of DNA methyltransferases contributes to 5'CpG methylation and poor prognosis in lung cancer. *Lung Cancer*. 2007;55:205-213.
23. Jaffe ES, Harris NL, Stein H, Vardiman JV, eds. World Health Organization Classification of Tumours. Lyon, France: IARC Press; 2004.
24. Corn PG, Heath EI, Heitmiller R, et al. Frequent hypermethylation of the 5 CpG island of E-cadherin in esophageal adenocarcinoma. *Clin Cancer Res*. 2001;7:2765-2769.
25. Etoh T, Kanai Y, Ushijima S, et al. Increased DNA methyltransferase 1 (DNMT1) protein expression correlates significantly with poorer tumor differentiation and frequent DNA hypermethylation of multiple CpG islands in gastric cancers. *Am J Pathol*. 2004;164:689-699.
26. Kuo TH, Liu FY, Chuang CY, Wu HS, Wang JJ, Kao A. To predict response chemotherapy using technetium-99m tetrofosmin chest images in patients with untreated small cell lung cancer and compare with P-glycoprotein, multidrug resistance related protein-1, and lung resistance-related protein expression. *Nucl Med Biol*. 2003;30:627-632.
27. Arai T, Miyoshi Y, Kim SJ, Taguchi T, Tamaki Y, Noguchi S. Association of GSTP1 CpG islands hypermethylation with poor prognosis in human breast cancers. *Breast Cancer Res Treat*. 2006;100:169-176.
28. Lee JS. GSTP1 promoter hypermethylation is an early event in breast carcinogenesis. *Virchows Arch*. 2007;450:637-642.
29. Chandra RK, Bentz BG, Haines GK 3rd, Robinson AM, Radosovich JA. Expression of glutathione S-transferase pi in benign mucosa, Barrett's metaplasia, and adenocarcinoma of the esophagus. *Head Neck*. 2002;24:575-581.
30. Bastian PJ, Palapattu GS, Yegnasubramanian S, et al. CpG island hypermethylation profile in the serum of men with clinically localized and hormone refractory metastatic prostate cancer. *J Urol*. 2008;179:529-534.
31. Yasui K, Mihara S, Zhao C, et al. Alteration in copy numbers of genes as a mechanism for acquired drug resistance. *Cancer Res*. 2004;64:1403-1410.
32. Kuwano M, Uchiumi T, Hayakawa H, et al. The basic and clinical implications of ABC transporters, Y-box-binding protein-1 (YB-1) and angiogenesis-related factors in human malignancies. *Cancer Sci*. 2003;94:9-14.
33. Baker EK, Johnstone RW, Zalberg JR, El-Osta A. Epigenetic changes to the MDR1 locus in response to chemotherapeutic drugs. *Oncogene*. 2005;24:8061-8075.

34. Tsang WP, Kwok TT. Riboregulator H19 induction of MDR1-associated drug resistance in human hepatocellular carcinoma cells. *Oncogene*. 2007;26:4877-4881.
35. Enokida H, Shiina H, Igawa M, et al. CpG hypermethylation of MDR1 gene contributes to the pathogenesis and progression of human prostate cancer. *Cancer Res*. 2004;64:5956-5962.
36. Qiu YY, Mirkin BL, Dwivedi RS. MDR1 hypermethylation contributes to the progression of neuroblastoma. *Mol Cell Biochem*. 2007;301:131-135.
37. Kim H, Kwon YM, Kim JS, et al. Elevated mRNA levels of DNA methyltransferase-1 as an independent prognostic factor in primary nonsmall cell lung cancer. *Cancer*. 2006;107:1042-1049.
38. Kwon YM, Park JH, Kim H, et al. Different susceptibility of increased DNMT1 expression by exposure to tobacco smoke according to histology in primary non-small cell lung cancer. *J Cancer Res Clin Oncol*. 2007;133:219-226.