Epigenetic Inactivation of *Betaig-h3* Gene in Human Cancer Cells

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Abstract

Gene silencing by CpG island methylation in the promoter region is one of the mechanisms by which tumor suppressor genes are inactivated in human cancers. It has been shown previously that *Betaig-h3* gene, which encodes an extracellular matrix protein involved in cell adhesion and tumorigenesis, is down-regulated or silenced in a variety of human cancer cell lines. To unravel the underlying molecular mechanism(s) for this phenomenon, DNA methylation patterns of *Betaig-h3* CpG island were examined in normal, immortalized, and cancer cell lines derived from lung, prostate, mammary, and kidney. A good correlation was observed between promoter hypermethylation and lost expression of *Betaig-h3* gene, which was supported by the data that demethylation of promoter by 5-aza-2′-deoxycytidine reactivated *Betaig-h3* and restored its expression in *Betaig-h3*-silenced tumor cell lines. This result was further substantiated by a luciferase reporter assay, showing the restoration of promoter activities and increased response to transforming growth factor-β treatment in *Betaig-h3*-negative 293T cells when transfected with unmethylated *Betaig-h3* promoter. In contrast, activity of *Betaig-h3* promoter was completely inactivated by *in vitro* methylation. Furthermore, CpG methylation of *Betaig-h3* promoter was also shown in primary lung tumors that expressed decreased level of Betaig-h3 protein. These results suggest that promoter methylation plays a critical role in promoter silencing of the *Betaig-h3* gene in human tumor cells. (Cancer Res 2006; 66(9): 4566-73)

Introduction

DNA methylation, one of the most commonly occurring epigenetic events in the mammalian genome, is an important regulator of gene transcription and plays an important role in cancer biology, genetic imprinting, developmental abnormalities, and X-chromosome inactivation (1–4). Alteration of DNA methylation is a common event in a variety of tumors, including breast, lung, esophageal, ovarian, prostate, renal, colon, gastric, and brain cancers, as well as in leukemia and lymphoma. Numerous genes have been found to undergo hypermethylation, such as those involved in cell cycle control (*p15INK4b, p16INK4a*, and *Rb*), DNA repair (*BRAC1* and *MGMT*), and apoptosis (*DAP* and *TMS1*; refs. 1, 5). Certain genes, such as *p16INK4a* and *RASSF1A*, are commonly methylated in a variety of cancers, whereas others are methylated only in specific cancer types (6).

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Betaig-h3 gene, also known as *transforming growth factor-β–induced* (*TGFBI*) gene, was first identified in a human lung adenocarcinoma cell line (A549) treated with TGF-β (7). This gene product is composed of 683 amino acids containing four homologous internal repeat domains (Fas-1) and has been reported to function as an extracellular matrix protein to mediate cell adhesion and migration through interacting with integrin ($\alpha_3\beta_1$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$), collagen (I, IV, and VI), laminin, and fibronectin (8, 9). In addition, *Betaig-h3* is involved in cell growth, cell differentiation, wound healing, apoptosis, and tumorigenesis (10–13). *Betaig-h3* gene mutations were identified in families affected with human autosomal dominant corneal dystrophies (14).

Epigenetic inactivation of tumor suppressor genes (TSG) or tumor-related genes by hypermethylation in promoter region is a common event in human tumor cell lines and human cancers. Several TSGs are inactivated by this mechanism (4, 5, 15). In addition, a novel class of TSGs has been identified where epigenetic inactivation plays the predominant role, whereas somatic mutations are a relatively rare event (16, 17). We have shown previously that *Betaig-h3* gene is ubiquitously expressed in normal human tissues, whereas its expression was either down-regulated or lost in a variety of human tumor cell lines (11–13). Down-regulation of this gene has been shown to correlate with the tumorigenic phenotype in human bronchial epithelial cells, suggesting an antitumor function of this gene (12, 13). Therefore, understanding the molecular mechanism(s) responsible for the inactivation of *Betaig-h3* gene in tumor cells is a high priority. In this study, we investigated the methylation patterns of *Betaig-h3* gene promoter in human tumor cell lines and primary lung tumors. The results showed that the promoter region of *Betaig-h3* gene is hypermethylated in five tumor cell lines compared with their normal controls, and demethylation of promoter in these cells resulted in reactivation of *Betaig-h3* gene. In addition, promoter methylation was also shown in primary lung tumors with decreased level of Betaig-h3 protein.

Materials and Methods

Cell lines. Normal human bronchial epithelial cells (NHBE), prostate epithelial cells (PHEC), and mammary epithelial cells (HMEC) were purchased from Clonetics/BioWhittaker (Walkersville, MD) and maintained in serum-free bronchial, prostate, and mammary epithelial basal media supplemented with growth factors, respectively. Non–small cell lung cancer (NSCLC) cell lines (A549, H522, H810, and H1417), prostate cancer cell lines (PC3, LNCaP, and Du145), breast tumor cell lines (MCF-7, MDA-MB231, and MDA-MB361), and 293T cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA) and grown in 10% fetal bovine serum (FBS)/DMEM or RPMI 1640. Immortalized normal adult human prostate cells (RWPE-1) and spontaneously immortalized mammary epithelial cells (MCF-10F) were purchased from ATCC. RWPE-1 cells were maintained in serum-free medium supplemented with 5 ng/mL epidermal growth factor (EGF) and 0.05 mg/mL bovine pituitary extract. MCF-10F

cells were cultured in DMEM/F-12 (1:1) supplemented with 10% FBS, 10 μg/mL insulin, 0.5 μg/mL hydrocortisone, and 0.02 μg/mL EGF. Immortalized human bronchial epithelial cells (BEP2D) were kindly provided by Dr. James C. Willey (Medical College of Ohio, Toledo, OH) and maintained in serum-free LHC-8 medium (Biofluids, Inc., Rockville, MD).

Analysis of CpG island in *Betaig-h3* promoter. A CpG island 620 bp long, spanning the proximal promoter and the first exon, was identified by screening the entire *Betaig-h3* genomic sequence using CpG plot prediction analysis. The putative transcription start site (TSS) is located at −65 relative to the translation start codon ATG (18). The CpG analysis program can define a CpG island as a region where there has a rich CpG 0.2 to 1 kb long. CpG islands are frequently found in the promoter regions of genes. The detailed information about this program can be found at the Web site: http://www.ebi.ac.uk/emboss/cpgplot.

Bisulfite sequencing. Genomic DNAs were isolated from the culturing cells or lung tissues by using Easy-DNA kit (Invitrogen, Carlsbad, CA). DNA (1 μg) was treated with sodium bisulfite as described (19), purified using EZ DNA Methylation kit (Zymo Research, Orange, CA), and dissolved in 10 μL distilled water. Bisulfite-modified DNA (2 μL) was used as template in the first round of PCR (primer set M1/M2) to amplify the promoter region and the first exon of *Betaig-h3* gene. The PCR products were then diluted from 1:1 to 1:100 based on the amplification efficiency, and diluted products (1 μL) were used as templates for the second round of PCR using primer set M3/M4. For lung tumors and the matched noncancerous tissues, methylation-specific primer set (MSP1/MSP2) was used in the second round of PCR in addition to use of the M3/M4 primer set as described above. Primers M1, M2, M3, and M4 were designed to avoid potential methylation sites (CpGs) so that both methylated and unmethylated DNA would be amplified equally. Primers MSP1 and MSP2 were designed mainly based on the bisulfite sequencing data of primary tumors generated from PCR products using M3/M4 primers. Two to four methylated CpG sites that were found to be methylated in both primary tumors and tumor cell lines were designed into the primers; meanwhile, the resulted PCR product covers most of CpG sites of promoter region. The MSP1 and MSP2 primers were then used to do the second round of PCR using the first round of PCR products as templates in tumor samples. Methylated DNA templates will selectively be enriched, and, therefore, in a limited number of clones sequenced, methylated DNAs could be shown easily. FailSafe PCR System (2× PreMix J buffer, Epicentre, Madison, WI) was used for all of the PCRs, and the PCR conditions were as follows: a hot start at 95°C for 5 minutes followed by 35 cycles of PCR (94°C for 30 seconds, 53°C for 30 seconds, and 72°C for 60 seconds) with a final extension for 5 minutes at 72°C. For tumor samples, a higher annealing temperature (67°C) and 2× PreMix D buffer were used in the second round of PCR. The resulted PCR products were subcloned into the pGEM-Teasy vector (Promega, Madison, WI). Five to eight individual clones were isolated from each PCR and sequenced by DNA Sequencing Facility at Columbia University (New York, NY). Primer pairs used were as follows: 5′-AGTTGGGGAG-GGTGGTTAGTT-3′ (M1; −548 to −529), 5′-ACCCCAACTACCTAACCTT-CC-3′ (M2; +164 to +184), 5′-TTTGTAGTGTTTTGTAGTTTTAAGATT-3′ (M3; −458 to −432), 5′-TAACCTTCCACAACCCCTAACCAA-3′ (M4; +149 to +172), 5′-GTAGGAT*C*GAAGTTTT*C*GAGAT-3′ (MSP1; −403 to −381), and 5′-CCC*G*CCAAAATC*G*C*G*AC*G*AA-3′ (MSP2; +53 to +72). Nucleotide positions were numbered relative to translation start site (+1), and methylated nucleotides were italicized in the methylation-specific primers.

5-Aza-2′-deoxycytidine treatment. The NSCLC cells (H522, H810, and H1417), prostate cancer cells (Du145 and LNCaP), and human fetal kidney 293T cells (30-40% confluent) were incubated in culture medium containing 20 to 40 μmol/L 5-aza-2′-deoxycytidine (5-Aza-CdR; dissolved in DMSO; Sigma, St. Louis, MO) for 4 days with medium change each day. The final concentration of 5-Aza-CdR used for 293T, H522, H810, H1417, Du145, and LNCaP cells were 40, 30, 30, 20, 40, and 20 μmol/L, respectively. Total RNA and genomic DNA were isolated from the treated cells using Trizol reagent and Easy-DNA kit.

Luciferase reporter assay. To generate *Betaig-h3* promoter-luciferase reporter constructs, two fragments (1.53 and 0.79 kb) of the human *Betaig-h3* gene promoter were amplified using the following primers: 5′-CCG*CTCGAG*CTCATCTACCTGGCAAGCCTGC-3′ (P1), 5′-CCG*CTCGAG*-GCAGGACTCTTGCCTTTGCAGA-3′ (P7; *Xho*I restriction site), and 5′-GCG-*AAGCTT*GGAGCGGGACGACGCGCACC-3′ (P2; *Hin*dIII restriction site). The PCR products were digested with *Xho*I and *Hin*d III and cloned into the reporter vector *pGL3-Basic* (Promega) to generate *pGL3-BP1* or *pGL3-BP7*. To analyze the effects of methylation on promoter activity, *pGL3-BP7* was methylated *in vitro* with CpG methylase (M. *Sss*I, New England Biolabs, Beverly, MA) in the presence of 0.016 mmol/L *S*-adenosylmethionine at 37°C for 4 hours (New England Biolabs). 293T Cells (2×10^5) were seeded in 12-well plates and, in the 2nd day, transfected with 0.5 μg control vector or *pGL3-BP1* or *BP7* vectors using LipofectAMINE Plus (Invitrogen). Twenty-four hours after transfection, cells were incubated in fresh serum-free medium containing TGF-β1 (10 ng/mL; R&D Systems, Minneapolis, MN) or control vehicle (4 mmol/L HCl containing 1 mg/mL bovine serum albumin). Luciferase activities were determined 48 hours after transfection using Luciferase Reporter Assay System (Promega).

Reverse transcription and quantitative PCR. The expression of *Betaig-h3* gene was analyzed by quantitative real-time reverse transcription-PCR (RT-PCR; Applied Biosystems 7300, Foster City, CA) using RT^2 Real-time SYBR Green/ROX Gene Expression Assay kit (SuperArray Bioscience Corp., Frederick, MD). The first strand of cDNA was synthesized from 4 μg total RNA using SuperScript II First-Strand Synthesis System (Invitrogen). Quantitative real-time RT-PCR was then done in a reaction volume of 25 μL, including 1 μL RT^2 PCR Primer Set [human TGFBI or glyceraldehyde-3-phosphate dehydrogenase (GAPDH); SuperArray Bioscience] and 1 μL cDNA. Three PCRs were done for each sample. PCR conditions were as follows: 95°C for 15 minutes followed by 40 cycles at 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds. Each plate included multiple water blanks and a negative template control. *GAPDH* was used as an internal reference gene to normalize the expression of *Betaig-h3*. Relative quantification of *Betaig-h3* gene was analyzed by comparative threshold cycle (C_T) method as described at http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf (User Bulletin 2: Relative Quantification of Gene Expression). In brief, a comparative threshold cycle (C_T) was used to determine the expression level normalized to the expression in PC3 or 5-Aza-CdR-treated LNCaP cells. Thus, expression levels were expressed as an n-fold difference relative to the calibrator. For each sample, the *Betaig-h3* C_T value was normalized using the formula: ΔC_T = C_T *Betaig-h3* − C_T *GAPDH*. To determine relative expression levels, the following formula was used: $\Delta\Delta C_T$ = ΔC_T sample − ΔC_T calibrator. The value was used to plot the *Betaig-h3* expression using the expression $2^{-\Delta\Delta CT}$.

Lung tumor samples. Five frozen human lung tumor samples that have decreased level of Betaig-h3 protein and four matched noncancerous lung

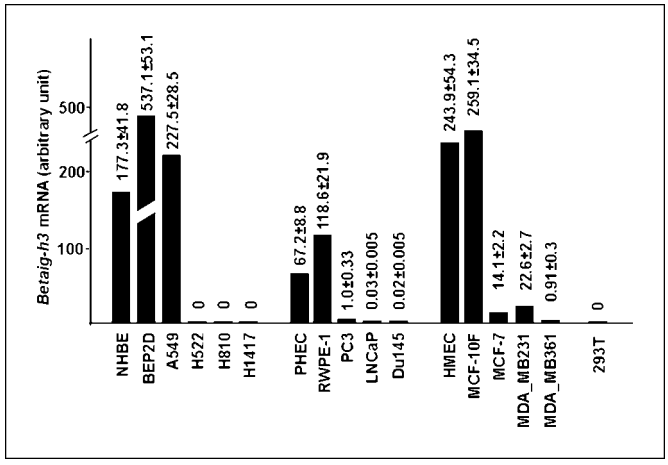

Figure 1. Expression of *Betaig-h3* in normal, immortalized, and cancer cell lines of lung, prostate, and mammary as well as in 293T cell lines. Relative quantification of *Betaig-h3* expression was done by using real-time PCR, calibrated and normalized to its expression in PC3 cells.

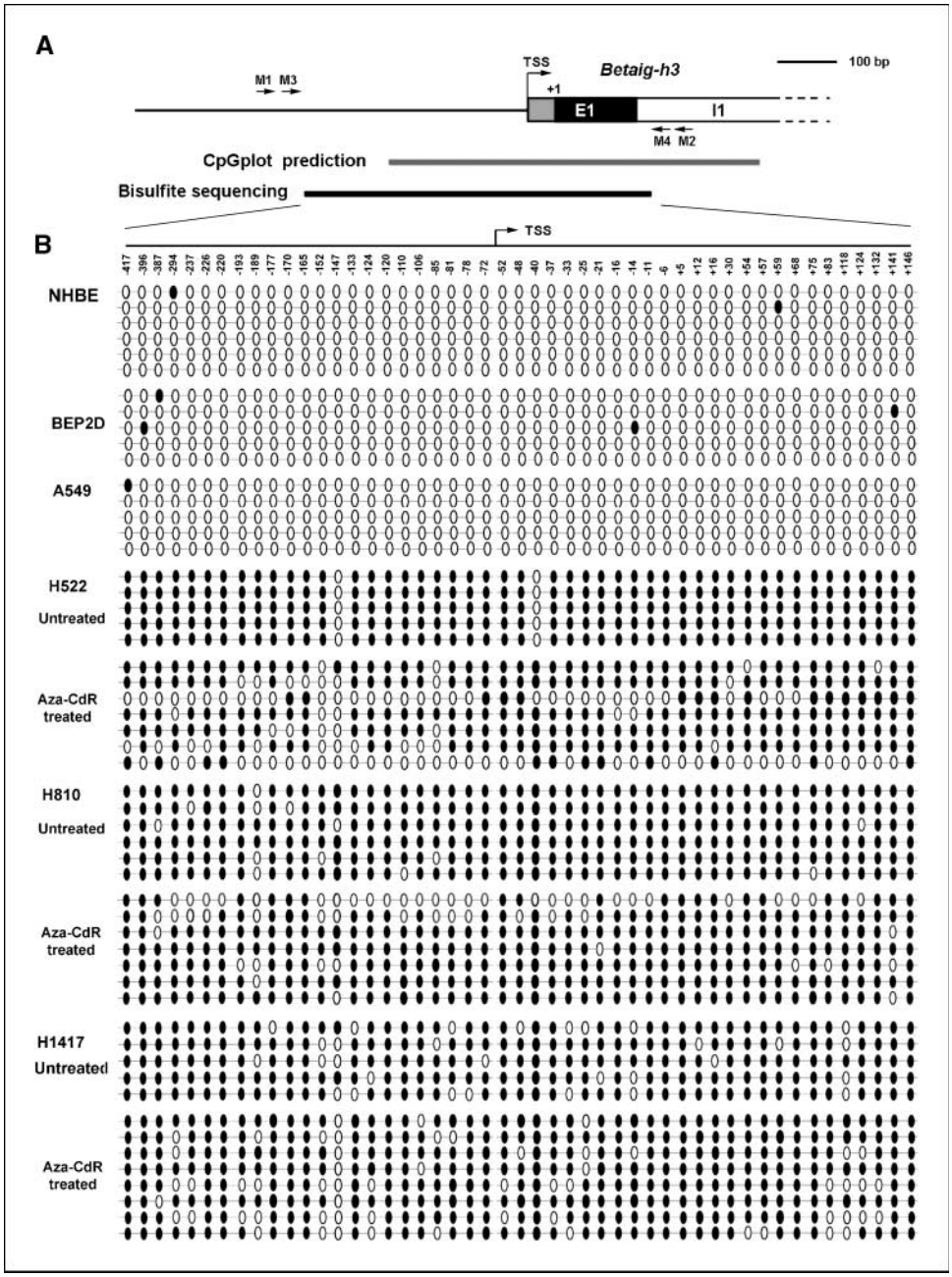

Figure 2. *A,* schematic diagram indicating CpG island on *Betaig-h3* promoter region. TSS is located at −65 relative to the ATG start codon (+1). *Black column,* bisulfite sequencing fragment containing the promoter region and the first exon. *B,* fine methylation mapping of 49 CpG sites of *Betaig-h3* promoter region obtained from bisulfite sequencing in lung cell lines. *White symbols,* unmethylated CpG sites; *black symbols,* methylated CpGs.

tissues were provided by Department of Pathology at Columbia University. Noncancerous tissues were acquired from the same lobe but were distant from the tumor. Sections of noncancerous tissues were found to be free of tumor cells examined by pathologist. Genomic DNAs were isolated from these tissues, treated with sodium bisulfate, PCR amplified, and sequenced to determine the methylation status of CpG island.

Results

***Betaig-h3* expression in normal, immortalized, and tumor cell lines.** Our previous studies showed that expression of *Betaig-h3* gene was down-regulated or lost in a variety of tumor cell lines (12, 13). In this study, real-time RT-PCR was used to quantify the expression of *Betaig-h3* gene in a series of normal, immortalized, and tumor cell lines derived from lung, prostate, mammary, and kidney. The results showed that *Betaig-h3* gene was expressed at a relatively high level in normal and immortalized cell lines, whereas it was down-regulated in most of the tumor cell lines. Interestingly, *Betaig-h3* expression was undetectable in three lung cancer cell lines (H522, H810, and H1417) and in one kidney cell line 293T (Fig. 1).

Analysis of *Betaig-h3* promoter hypermethylation. To determine whether loss of *Betaig-h3* expression resulted from promoter hypermethylation, methylation status of a total of 49 CpGs across 0.6 kb of the *Betaig-h3* locus in these cell lines was characterized by bisulfite genomic sequencing. Most of the CpG island of *Betaig-h3* gene was selected for DNA methylation analysis (Fig. 2*A*). The detailed CpG methylation status of lung cell lines was shown in Fig. 2*B*. Most of the CpG sites of *Betaig-h3* promoter were not

methylated in normal NHBE, immortalized BEP2D, and A549 tumor cells that expressed a relatively high level of *Betaig-h3* gene. Although several methylated CpG sites were identified, they were randomly distributed and limited to one methylated site per clone. In contrast, *Betaig-h3*-negative cell lines, including H522, H810, and H1417, showed dense methylation in the *Betaig-h3* promoter. Among all of the CpG sites examined, only two sites (−147 and −40) were found to be unmethylated in the clones obtained from H522 tumor cells. Similarly, H810 cells showed one to three sites, and H1417 cells showed five to eight sites that were not methylated and randomly distributed in each clone. These results clearly show that the *Betaig-h3* promoter was hypermethylated in these three tumor cell lines that lack *Betaig-h3* expression.

Similar results were also obtained from prostate cell lines (Fig. 3). Most of CpG sites of *Betaig-h3* promoter was unmethylated in normal PHEC and immortalized RWPE-1 cells that expressed a high level of *Betaig-h3* gene. Three prostate tumor cell lines that expressed a low level of the *Betaig-h3* gene displayed a largely different pattern of CpG methylation. Du145 cells showed a dense methylation of the promoter region. Compared with Du145 cells, a lower degree of methylation was found in LNCaP cells with sparse or partial methylation in the promoter region. The difference in the number of methylated CpG sites among individual clones may be due to internal heterogeneity of LNCaP cell line. PC3 cells showed a sparse methylation pattern, indicating that, in addition to promoter methylation, other mechanisms probably exist to regulate *Betaig-h3* expression in PC3 cells.

Methylation of *Betaig-h3* promoter was not observed in normal, immortalized, and mammary tumor cell lines with exception of MDA-MB361 tumor cells, which showed sparsely methylated sites in the promoter region (data not shown). Human fetal kidney cell line 293T, a *Betaig-h3*-negative cell line, was densely methylated

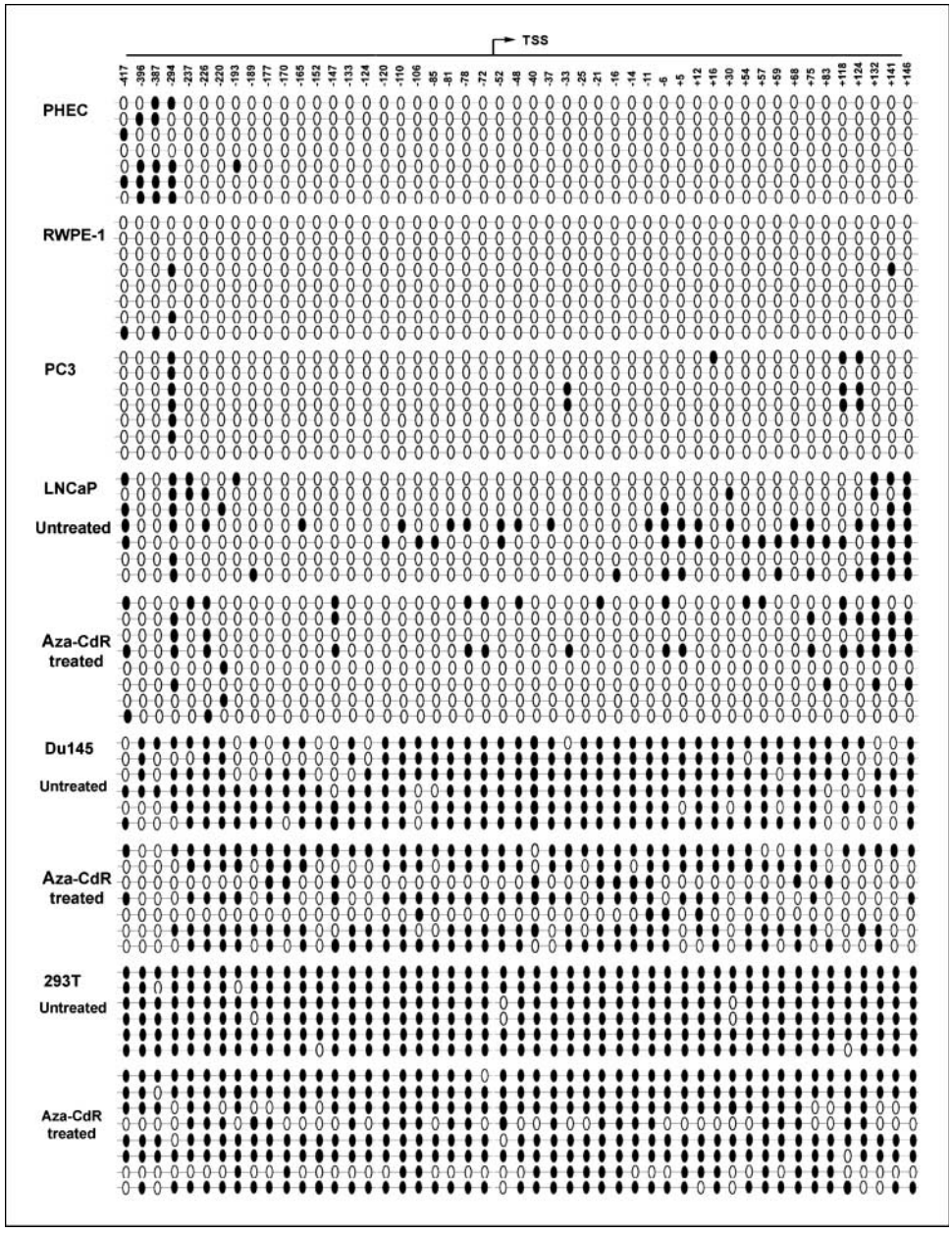

Figure 3. Bisulfite sequencing analysis of 49 CpG sites in the promoter region and the first exon of the *Betaig-h3* promoter in prostate and 293T cell lines. *White symbols,* unmethylated CpG sites; *black symbols,* methylated CpGs.

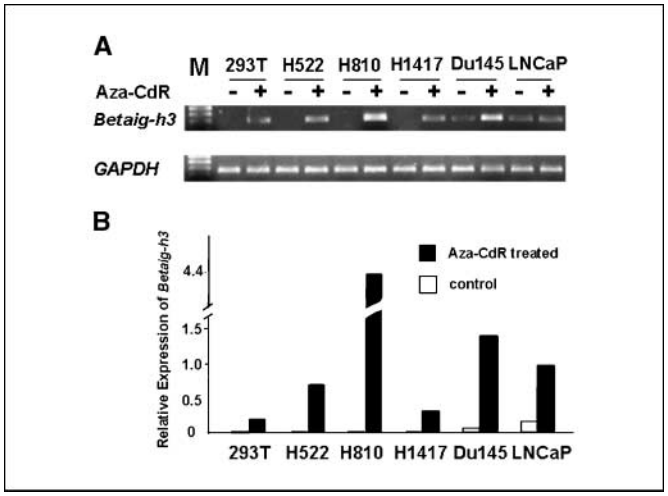

Figure 4. Reexpression of *Betaig-h3* following treatment with 5-Aza-CdR. *A,* levels of *Betaig-h3* expression in tumor cell lines after 5-Aza-CdR treatment analyzed by agarose electrophoresis. *B,* levels of *Betaig-h3* expression were quantified by real-time PCR and normalized to its expression in 5-Aza-CdR-treated LNCaP tumor cells.

throughout the CpG island (Fig. 3). Taken together, the overall patterns of DNA methylation correlated well with the levels of *Betaig-h3* expression determined by real-time PCR.

5-Aza-CdR treatment and reexpression of *Betaig-h3*. To determine the correlation between DNA methylation and *Betaig-h3* silencing, the tumor cell lines that showed hypermethylation of *Betaig-h3* promoter were treated with demethylation agent, 5-Aza-CdR. The expression levels of *Betaig-h3* gene were then quantified by real-time PCR. As shown in Fig. 4*A* and *B*, 5-Aza-CdR treatment resulted in the reexpression of *Betaig-h3* in all of the *Betaig-h3*-negative cell lines, including H522, H810, H1417, and 293T. A significantly increased expression of *Betaig-h3* was also detected in two *Betaig-h3*-positive cell lines, including LNCaP and Du145. To confirm the demethylation effect of 5-Aza-CdR, bisulfite sequencing was done on treated cell lines, and the results clearly showed that the promoter was truly demethylated (Figs. 2*B* and 3). These data strongly suggest that hypermethylation was responsible for *Betaig-h3* gene silencing.

Promoter luciferase assay. To further verify whether promoter methylation plays a pivotal role in the regulation of *Betaig-h3* expression, we cloned the promoter region of *Betaig-h3* gene and analyzed its activity under methylated or unmethylated conditions in 293T cells using the promoter luciferase assay. The 293T tumor cells were chosen because the *Betaig-h3* promoter in these cells is hypermethylated and completely silenced. Additionally, 293T cells have high transfection efficiency. Two different fragments of the promoter sequence were constructed into luciferase reporter vector and transfected into 293T cells. Luciferase activity was measured as an indicator of *Betaig-h3* promoter activity. As shown in Fig. 5*A*, high luciferase activities were detected in 293T cells when transfected with *Betaig-h3* fragments *pGL3-BP1* or *pGL3-BP7*. Furthermore, significant increase in luciferase activities was found in cells treated with TGF-β1 (10 ng/mL) compared with untreated cells ($P < 0.01$). *In vitro* methylation of *pGL3-BP7* with M. *Sss*I methylase resulted in a complete abrogation of luciferase activity when transfected into 293T cells (Fig. 5*B*). To further validate the degree of methylation by methylase treatment, three independent clones from methylase-treated *pGL3-BP7* vector DNA were sequenced. The results confirmed that all of the CpG sites were indeed methylated (data not shown). These results indicate that promoter methylation is sufficient for the silencing of *Betaig-h3* promoter.

Methylation status of *Betaig-h3* promoter in primary lung tumors. To elucidate the methylation profile of *Betaig-h3* promoter in primary tumors, a total of 35 primary lung tumor samples with their matched normal lungs were used to prepare tissue arrays. Five sections from each sample were included in the array. Expression level of Betaig-h3 protein was then screened using polyclonal rabbit anti-human Betaig-h3 antibody and immunohistochemical method as described previously (20). After screening, we found that 13 tumors have significantly decreased level of Betaig-h3 protein, from which five frozen tumor samples with their matched noncancerous tissues were used in our methylation study. As shown in Fig. 6, Betaig-h3 staining was found in both lung stromal and nuclei of epithelial cells in normal lung tissues, which are consistent with the previous report (20). However, in tumor tissues, the staining was still positive in stromal but significantly decreased in tumor cells. The methylation profile of *Betaig-h3* promoter in lung tumors was summarized in Fig. 7. By using M3/M4 primers (Fig. 7*A*), 21 to 23 CpG sites in one of six clones from tumors T22 and T24 and 24 to 25 CpG sites in two of six clones from tumor T41 were found to be methylated. Only one to six methylated sites were observed in other clones due to the internal heterogeneity of lung tumor tissues. In contrast, CpG sites of *Betaig-h3* promoter were either unmethylated or sparsely methylated in matched noncancerous tissues and in two other tumors (T28 and T51; data not shown). However, if we chose the methylation-specific primers in the second round of PCR, most of the clones sequenced from tumors T22 and T41 showed a higher number (>21) of methylated CpG sites (Fig. 7*B*). A range of 10 to 22 CpG sites were observed in the clones generated from tumor

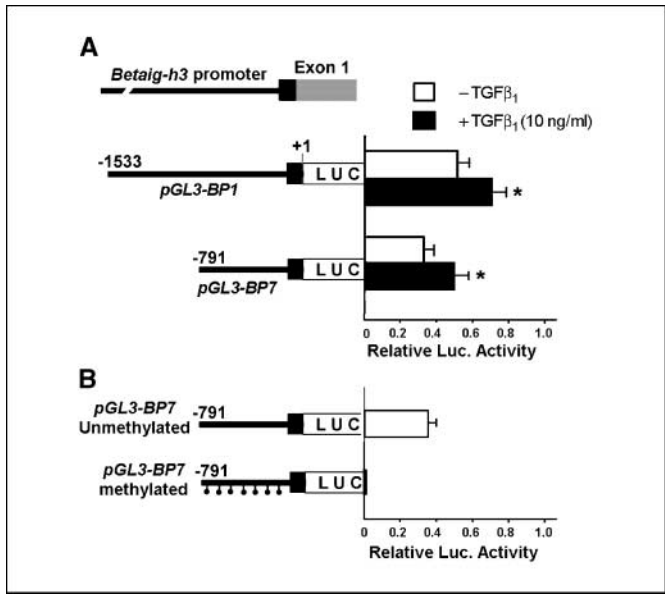

Figure 5. Effect of methylation on activity of the *Betaig-h3* promoter. *A,* relative luciferase (*LUC*) activity of 293T cells after transfection of *Betaig-h3* promoter-luciferase construct and treatment with 10 ng/mL TGF-β1. Transfection efficiency was normalized based on the luciferase activity after transfection of *pGL3-promoter* vector. *B,* methylation-dependent repression of *Betaig-h3* promoter. *Columns,* mean of triplicate experiments; *bars,* SD. *, $P < 0.01$, compared with *Betaig-h3* promoter-luciferase construct transfected cells without TGF-β1 treatment.

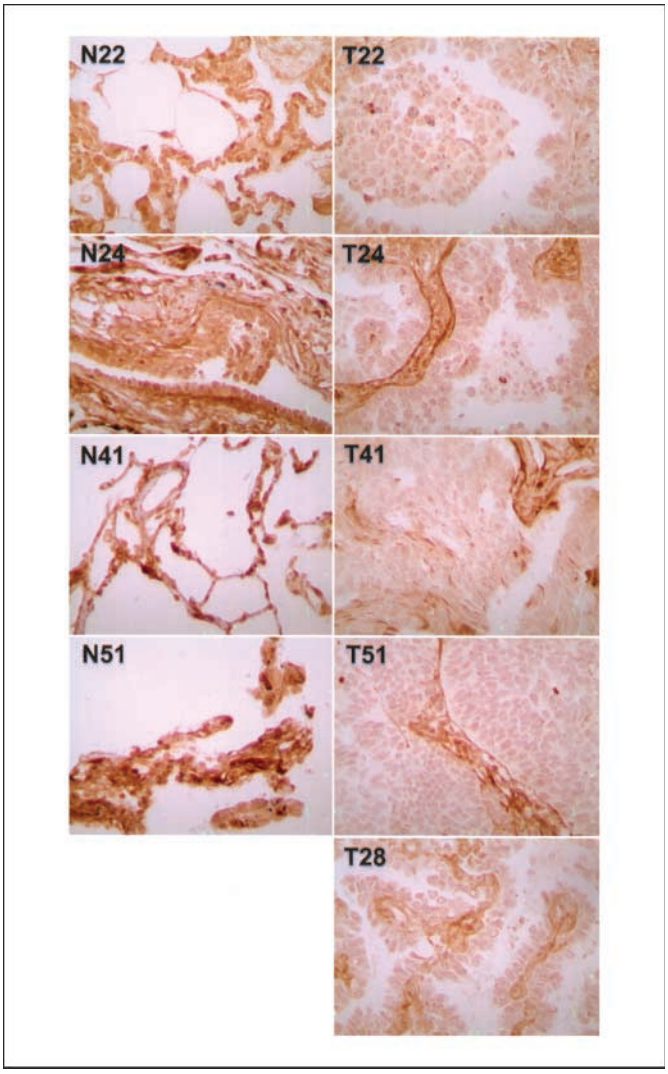

Figure 6. Immunohistochemical staining of Betaig-h3 protein in primary lung carcinomas (*T22, T24, T28, T41*, and *T51*) and matched noncancerous lung tissues (*N22, N24, N41*, and *N51*). Magnification, ×400.

T24 (Fig. 7*B*). PCR products were not found in tumors T28 and T51 and in matched noncancerous tissues using methylation-specific primers, which are consistent with the bisulfite sequencing results of PCR products amplified by M3/M4 primer set, suggesting that methylated DNAs could selectively be amplified by methylation-specific primers.

Discussion

CpG islands located in the promoter regions of certain TSGs undergo aberrant hypermethylation in cancer cells, which is an important mechanism responsible for gene silencing (1). It has been well documented that some known TSGs, such as cell cycle inhibitor (*p16INK4a*) and DNA repair genes (*hMLH1* and *BRCA1*), are modified and transcriptionally silenced by promoter hypermethylation (21). We have shown previously that *Betaig-h3* possesses an antitumor function in malignantly transformed human bronchial epithelial cells (12, 13). It is expressed ubiquitously in a variety of normal human tissues but is down-regulated or inactivated in many human tumor cell lines (12, 13). As DNA methylation of CpG island plays an important role in regulation of gene expression in human cancers (22, 23), methylation pattern of the promoter region and the first exon of *Betaig-h3* gene was investigated in 17 cell lines derived from lung, prostate, mammary, and kidney. All of the cell lines that expressed a high level of *Betaig-h3* gene, including normal, immortalized, and some tumor cell lines, did not show methylation in the promoter region. In contrast, tumor cell lines with low or undetectable level of *Betaig-h3* expression showed a dramatically increased pattern of methylation. In addition, treatment with the demethylation agent (5-Aza-CdR) resulted in the reexpression or up-regulation of *Betaig-h3* gene in these cell lines. The data provide strong evidence that CpG island methylation in the promoter region correlates with *Betaig-h3* silencing in some human tumor cell lines. It should be noted that in 5-Aza-CdR-treated H810 cells, the number of methylated CpG sites ranged from 21 to 48 among seven clones sequenced. This difference represents the varied demethylation effect in the cell population and might result from differences in cellular proliferation rates that can affect the intake of demethylation agent. In addition, minimal reduction of methylated CpG sites by 5-Aza-CdR treatment in H810 cells resulted in a dramatic increase of *Betaig-h3* expression. This may be due to the limited number of clones that were sequenced. Alternatively, demethylation of CpG island might activate the upstream factors of *Betaig-h3* gene that in turn exert their effects on *Betaig-h3* promoter and induce its expression in 5-Aza-CdR-treated H810 cells.

The methylation profile of individual genes seems to be different in various tumor types. Certain genes, such as *RASSF1A* and *p16INK4a*, are commonly methylated in multiple human cancers (24), whereas others show high frequencies of methylation only in specific tumors. One example is *GSTP1* gene that is highly methylated in breast and prostate cancers but is largely unmethylated in other types of tumors (1, 5). In this study, frequency of *Betaig-h3* promoter methylation was found to be different in various cell lines. For example, human lung tumor cells, including H522, H810, and H1417 with an undetectable level of *Betaig-h3* expression, showed dense methylation in the promoter region, whereas A549 lung cancer cells expressed a high level of *Betaig-h3* gene and, meanwhile, displayed substantially reduced methylation. These results suggest that hypermethylation correlates with the silencing of *Betaig-h3* promoter in lung cancer cell lines. However, in three prostate cancer cell lines examined, partial or dense methylation of the promoter region was only found in LNCaP and Du145 cells but not in PC3 cells, although all of the cell lines had a significantly decreased level of *Betaig-h3* expression. The findings are consistent with our data obtained from demethylation treatment that *Betaig-h3* can be reactivated significantly in Du145 and LNCaP cells but not in PC3 cells, suggesting that promoter methylation plays an important role in the down-regulation of *Betaig-h3* in Du145 and LNCaP cells. In mammary cancer cell lines, promoter methylation of the *Betaig-h3* gene was not shown in three fourths of the cell lines examined, except MDA-MB361 that showed a very low level of methylation, suggesting that DNA methylation is not a main mechanism for the down-regulation of the *Betaig-h3* gene among these cancer cell lines.

Previous studies using clinical samples have shown that methylation status in multiple genes is different at various stages of the carcinogenic process. *p16INK4a* methylation seems to be

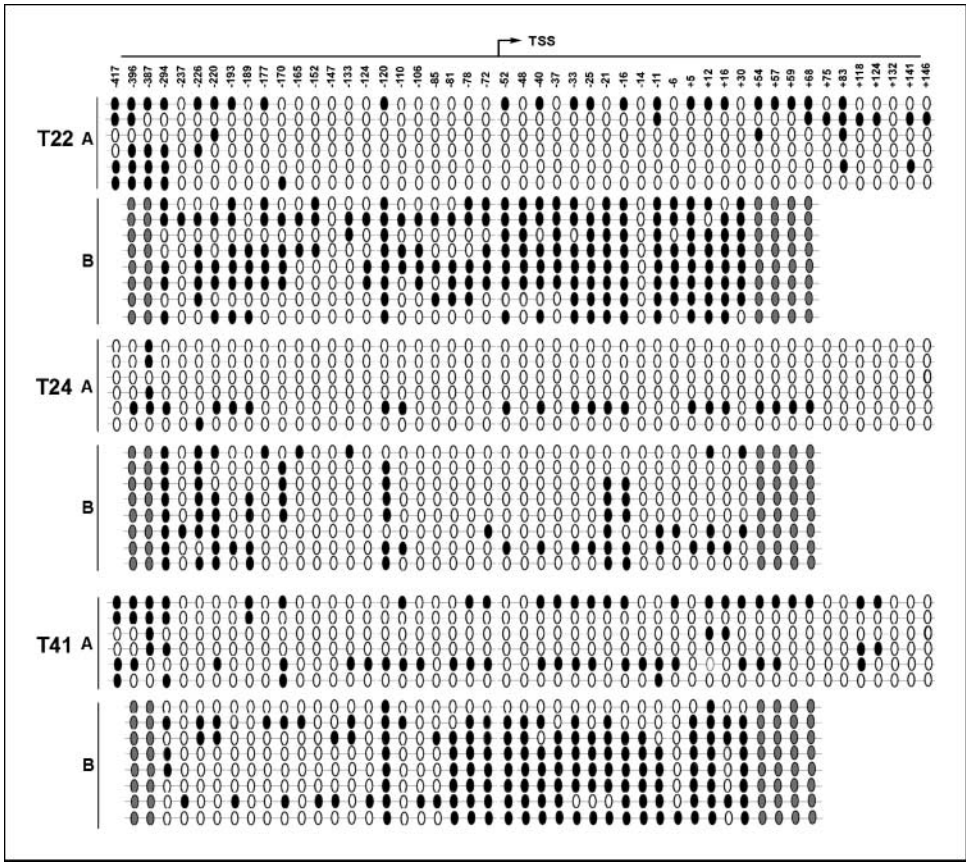

Figure 7. Bisulfite sequencing analysis of 49 CpG sites in the promoter region and the first exon of the *Betaig-h3* gene in primary lung tumors. *White symbols,* unmethylated CpG sites; *black symbols,* methylated CpGs; *gray symbols,* CpG sites used for designing of methylation-specific primers (MSP1/MSP2). *A* and *B,* clones generated from PCR products using M3/M4 primers and MSP1/MSP2 methylation-specific primers.

an early event in lung cancer with progressively increased frequency of methylation from 17% in basal cell hyperplasia to 60% in squamous cell carcinomas (25). In contrast, methylation of *MGMT* seems to be a late event because *MGMT* methylation was infrequent in normal and noncancerous tissues but increased during tumor progression (26). A methylation survey in a gastric tumor model provided evidence that the pattern of gene methylation could be age related (*APC* and *E-cadherin*), cancer specific (*GSTP1* and *RASSF1A*), low in noncancerous tissue but high in cancerous tissue (*hMLH1*), or increased with tumor stage (*DAP, p14ARF*, and *p15INK4b*; ref. 27). In the present study, methylation of *Betaig-h3* promoter was shown in primary lung cancer samples that have decreased level of Betaig-h3 protein. This observation suggests that promoter methylation of *Betaig-h3* gene occurs in primary tumors.

Betaig-h3 gene is one of the downstream effectors of TGF-β signaling pathway (7), which involves several of signaling molecules, including TGF-β receptor type I/II (TGF-βRI/II) and Smads 2, 3, and 4 (28). Any blockade or inactivation of this pathway caused by loss of the TGF-β receptors and mutations of Smads would lead to lack of response to TGF-β, which in turn would affect *Betaig-h3* expression. Recent data have shown that repression of TGF-βRII is a frequent event involved in progression of lung tumors (NSCLC; ref. 29). Of the cell lines examined in this study, LNCaP and MCF-7 cells have been shown previously to contain a defective *TGF-βRI* gene (30, 31). TGF-βRII was significantly decreased in MCF-7 and MDA-MB231 cell lines compared with nontumorigenic MCF-10F cells (32). Because promoter of *Betaig-h3* gene was sparsely or partially methylated in these cells, defects of TGF-β pathway might play an important role in the down-regulation of *Betaig-h3* expression. In the cell lines with a high degree of promoter methylation, including H522, H810, H1417, 293T, and Du145, it is currently unclear whether any defects in TGF-β pathway exist in these cells and whether DNA methylation is the main mechanism responsible for the gene down-regulation. However, the data obtained from *Betaig-h3*-negative 293T cells have shown the high levels of promoter activity of *Betaig-h3* in 293T cells after transfection with unmethylated *Betaig-h3* promoter. This suggests a functionally intact TGF-β pathway in 293T cells and further points to a critical role of promoter hypermethylation in *Betaig-h3* silencing.

Taken together, the present study shows for the first time that hypermethylation of *Betaig-h3* gene correlates with silencing of *Betaig-h3* promoter in lung, prostate, and 293T tumor cell lines and occurs in primary lung tumors. Further studies of methylation profiles of a large number of primary tumor samples will provide important insight in the role of CpG island hypermethylation of *Betaig-h3* promoter in tumor progression

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