Hypermethylation of the GATA Genes in Lung Cancer

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ABSTRACT

Purpose: In lung cancer, DNA hypermethylation is known to be a common event.

Experimental Design: Gene expression and methylation status of GATA-4, GATA-5, and GATA-6 were analyzed with cell lines and primary human lung cancers. Methylation profiles of primary lung cancers were analyzed and correlated with clinical as well as histopathological data.

Results: Complete methylation was detected by methylation-specific PCR for both GATA-4 and GATA-5 in four cell lines (H358, DMS-53, A549, and H1299), all of which had no expression by reverse transcription-PCR analysis. Demethylation with 5-aza-2'-deoxycytidine restored expression in each case. GATA-6 was ubiquitously expressed in all of the six cell lines. GATA-4 bisulfite sequencing revealed complete methylation of the GATA-4 promoter in H358 cells, correlating well with its lack of expression at the mRNA level. Only a few CpG sites showed methylation by bisulfite sequencing within the GATA-4 promoter in a cell line that expressed the gene. In 63 cases of primary lung cancers, GATA-4 and GATA-5 promoter methylation was detected in (42 of 63) 67% and (26 of 63) 41%, respectively. GATA-6 remained unmethylated both in cell lines and primary tumors. Six autopsy specimens of normal lung tissue showed no aberrant promoter hypermethylation for the GATA genes. Correlation of concomitant GATA-4 and GATA-5 methylation with clinical and histopathological parameters only found a statistically significant increase in methylation frequency with increasing patient age (P < 0.001).

Conclusions: These epigenetic changes in the GATA genes in lung cancer are tumor-specific, relate to the loss of GATA gene expression, and occur increasingly in the elderly.

INTRODUCTION

As a clinical entity worldwide, lung cancer remains the most common cause of cancer death, accounting for more life lost than breast, prostate, colon, and rectal cancer combined (1). Due to a high case-fatality rate, ~90% of all lung cancer patients die from their disease—over 157,000 deaths annually in the United States alone (2). Tobacco smoking remains the most important etiological agent, making lung cancer the end stage of a multistep carcinogenesis process that extends from premalignant precursor lesion to neoplasm. Although genetic events are prominent, epigenetic silencing of wild-type tumor suppressor genes via aberrant promoter hypermethylation has also been shown to be frequent in lung cancer (3–6). There is growing evidence that individual tumors may epigenetically silence multiple genes by direct promoter methylation of the targeted genes or indirectly through aberrant promoter methylation of upstream transcription factors.

The possible regulatory significance of epigenetic silencing of upstream and downstream genes via the aberrant methylation of a single transcriptional factor has recently been shown to occur in the GATA gene family of transcription proteins (7). The GATA family contains two conserved zinc-finger DNA binding domains that recognize the sequence WGATAR and has been studied primarily for its role in gene regulation, epithelial differentiation (8), and organogenesis in many developing organ systems (9). Although GATA-1, -2, and -3 are expressed mainly in the hematopoietic system, GATA-4, GATA-5, and GATA-6 are found in many organs including the heart, lung, and gastrointestinal tract (6, 10–15).

There is growing evidence to link the loss of GATA-4 and GATA-5 gene functions with malignancy in various organs and histologic types including serous ovarian cancers (16), gastric cancer (17), colorectal cell lines, and in primary colon cancers (7). In addition, allelic imbalances in the chromosomal loci for GATA-4, 8p23.1-p22, and deletions of GATA-5, 20q13.2-q13.3, are frequent areas of chromosomal deletion in neoplasms (18). GATA-6 could be predicted to have an oncogenic effect because its expression is found in many proliferating progenitor cells (8, 19–21). Moreover, GATA-4 and GATA-5, but not GATA-6, are potent inducers of early endodermal marker genes, suggesting that GATA-4 and GATA-5 may drive endodermal-specific differentiation (22). We recently described that methylation and silencing of both GATA-4 and GATA-5 were frequent events in colon and gastric cancer (7). Because the endoderm gives rise to the lining of the lung and the GI tract, we hypothesized that GATA-4 and GATA-5 might be targets for loss of function in lung cancer as well and that promoter hypermethylation could be responsible for loss of gene expression in this tumor, as was seen for colon and gastric cancer. To answer this question, we analyzed promoter hypermethylation in...
GATA-4, GATA-5, and GATA-6 in lung cancer cell lines in primary lung carcinomas and in normal lung specimens procured from nonsmoking patients without lung cancer.

**MATERIALS AND METHODS**

**Lung Cancer Cell Lines.** Six well-established human lung cancer cells of different histologies were included. All of the cell lines used, H157, H358, H727, DMS-53, A549, and H1299, were purchased through the American Type Culture Collection (Manassas, VA) and are widely studied. H157 is a squamous cancer cell, H727 is a well-differentiated bronchial carcinoid cell line, H358 is derived from bronchoalveolar carcinoma, and A549 is adenocarcinoma. DMS-53 is small cell carcinoma of the lung, whereas H1299 is a human non–small-cell cancer cell line. All of the cell lines were cultivated and maintained in 10% fetal bovine serum and 90% RPMI 1640 (Invitrogen, Carlsbad, CA) or 45% RPMI 1640 + 45% Ham’s F12 (Invitrogen) + 10% fetal bovine serum. Cells were passaged 1:3 once total confluence was reached on a 75 cm² culture flask (Sarstedt, Newton, NC).

**Human Lung Cancer Samples.** Sixty-three cases of primary lung cancer were procured after surgical resection as snap-frozen fresh tissue and stored at −70°C at the Johns Hopkins Hospital (Baltimore, MD) under the approved guidelines of the university’s institutional review board. All of the malignancies were examined, and tumor staging was codified according to the tumor-node–metastasis classification of the American Board of Cancer. For each patient, histologically confirmed malignant sections were available for H&E staining and hypermethylation studies. In addition, six lung specimens were obtained at autopsy from patients without associated lung carcinoma as normal comparisons.

**Clinical and Pathological Features of the Study Population.** The mean age of the study population was 66 years (+/− 10.4 years (median age 68 years, range 40 to 84)), and there was a slight predominance of males (54%). Approximately 90% of the cohort had a history of tobacco smoking, although most patients (40 of 63, or 64%) were former smokers. The mean pack-year history was 52 pack years. Because all of the patients were selected preoperatively to undergo resections with curative intent, the pathological staging of disease in the cohort consists predominantly of stage I (61.9%) and stage II (15.9%) disease. Pathological subtypes consisted of 28 (44.4%) patients with adenocarcinoma, and 23 (36.5%) with squamous and 8 (12.7%) with large cell cancers. Four patients (6.4%) had lung cancer of another histologic subtype. There were 53 (84.1%) lobectomies, 6 (9.5%) pneumonectomies, and 4 (6.4%) other surgeries. All of the patients had bronchial margins negative for malignancy by histopathologic analysis.

**DNA Preparation.** Genomic DNA from the human lung cancer tissue was extracted by standard methods with a simplified protease K digestion method. The lung cancer cell cultures were trypsin-EDTA digested before collection, and cell line DNA was then prepared with the standard protease K method. DNA was dissolved in low TE buffer and stored at −20°C.

**RNA Isolation and Semiquantitative Reverse Transcription (RT)-PCR.** Total RNA was isolated with the TRIzol reagent (Life Technologies, Inc., Gaithersburg, MD). Agarose gel electrophoresis at 1% and spectrophotometric analysis (A260:280 nm ratio) were used to assess RNA quality. RNA was stored at −70°C before use. First-strand cDNA was synthesized by using oligo-(dT)12-18 primer included with the Superscript II-reverse transcriptase kit (Invitrogen). Five micrograms of total RNA was used for each reaction. After first-strand synthesis, the reaction mixture was diluted to 100 μL with water. Subsequently, 2.5 μL of the diluted cDNA mixture was used for PCR amplification in a final 25 μL reaction volume. PCR amplification was carried out with primer sets derived from the published GATA-4, GATA-5, and GATA-6 gene sequences (7): GATA-4–sense, 5′-CTGGCCCTGTCATC-TCACTAGC-3′; GATA-4–antisense, 5′-GGTCCGTGCCAGG-AATTGGAGG-3′; GATA-5–sense, 5′-TCGCCAGACTGAC-AGCTCAG-3′; GATA-5–antisense, 5′-TGGTGTGTTCCAGG-CTGTCC-3′; and GATA-6–sense, 5′-TTCATACTCAGTGGATTGACG-3′; GATA-6–antisense, 5′-GCTGCACAAAGAGCAGCACG-3′. The primer sets for GATA genes were designed to span intronic sequences between adjacent exons to control for genomic DNA contamination and are located as described previously (7). A total of 35 cycles of amplification was done for each of the RT-PCR experiments. As an internal control, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was amplified with 25 cycles to ensure cDNA quality and quantity for each RT-PCR. Amplified products were analyzed on 1% agarose gels.

**Methylation-Specific PCR.** Genomic DNA from primary lung cancer tissues and cell lines was bisulfite modified as described previously (23). Methylation-specific PCR primers were designed according to genomic sequences flanking the presumed transcription start sites for GATA-4, GATA-5, and GATA-6 and are located as described previously (7). Primer sequences were oligo-synthesized (IDT) to allow methylation-specific PCR to detect bisulfite-induced changes affecting unmethylated (U) and methylated (M) alleles. Methylation-specific PCR primers for GATA-4, GATA-5, and GATA-6 are as follows: GATA-4-M-sense, 5′-GTATAGTTCTGGTAGTTT-GCGTTTAGC-3′; GATA-4-M-antisense, 5′-AACCTCGCCAC-TGAATCCCCG-3′; GATA-4-U-sense, 5′-TTTGATATGTTTGTAGTTTGTATGG-3′; GATA-4-U-antisense, 5′-CCTCCTCACAACCTCAAATCCCCC-3′; GATA-5-M-sense, 5′-AGTTGCTTTTAGGTTAGTTTTCGCG-3′; GATA-5-M-antisense, 5′-CCTTACATAACCAAACTGAAAACC-3′; GATA-5-U-sense, 5′-TGGAGTTTGTGTTTTAGGTTTTTTCGCG-3′; GATA-5-U-antisense, 5′-CAACAGCAAAACTGAAAACC-3′. Methylation-specific PCR reaction incorporated 100 ng of bisulfite-treated DNA as template, 10 pmol/L of each primer, 100 pmol/L deoxynucleoside triphosphate, 10× PCR buffer, and 1 unit of JumpStart Red Taq Polymerase (Sigma-Aldrich, St. Louis, MO) in a final reaction volume of 25 μL. Cycle conditions were as follows: 95°C×5 minutes; 35 cycles × (95°C×30 seconds,
60°C × 30 seconds, and 72°C × 30 seconds); and 72°C × 5 minutes. Methylation-specific PCR products were analyzed with non-denaturing 6% polyacrylamide gel electrophoresis and stained with ethidium bromide.

**Bisulfite Sequencing.** Bisulfite-treated DNA was subjected to PCR with primers flanking the targeted methylation-specific PCR regions above. Sequencing primers were as follows: GATA-4-forward, 5'-TAATAAAGTTGATTTTGGGG-TATTAG-3'; and GATA-4-reverse, 5'-CCCTACCTACTAACCTAAAATTC-3'. Conditions for PCR were as follows: 95°C × 5 minutes; 35 cycles × (95°C × 30 seconds, 56°C × 50 seconds, and 72°C × 50 seconds); and 72°C × 5 minutes. PCR products were gel-purified and cloned into the vector pCR2.1-TOPO according to the manufacturer’s protocol (Invitrogen). Colonies were grown on agar plates and randomly selected. Plasmids were then isolated and purified with Wizard mini-prep kits (Promega Corp., Madison, WI) following the manufacturer’s recommendations, and integrated PCR fragments were confirmed with EcoRI digestion (New England Biolabs, Beverly, MA). The cloned PCR fragments were sequenced with the M13 reverse primer via automated sequencing (Johns Hopkins University School of Medicine Biosynthesis and Sequencing Facility, Department of Biological Chemistry, Baltimore, MD).

**The 5-Aza-2’dexyycytidine Treatment.** For de-methylation studies, cells were daily treated with 2 μmol/L 5-aza-2’dexyycytidine (Sigma-Aldrich, St. Louis, MO) for 48 hours as described previously (24).

**Statistics.** Disease-specific survival was the primary endpoint of the analysis. Survival was calculated from the date of surgery to the time of death or censor. Correlation between variables was estimated with the Fisher’s exact test, or the Student t test when appropriate, all of the P values were two-sided, and all of the significant associations were considered when P ≤ 0.05 (Stata Statistical Software, College Station, TX).

**RESULTS**

**GATA Gene Silencing by Hypermethylation.** Using RT-PCR, we initially analyzed the expression patterns of GATA-4, GATA-5, and GATA-6 among 6 established lung cancer cell lines (Fig. 1A). GATA-6 was ubiquitously expressed in each of the cell lines, whereas GATA-4 and -5 expression varied. A colorectal cancer cell line, (RKO), treated with 5-aza-dc, served as a positive control. A 1-kb molecular weight marker indicated an appropriate size for the amplified products. Strong GATA-6 expression was present in each of the cell lines, whereas GATA-4 and -5 expression varied. A. GATA gene family methylation-specific PCR in lung cancer cell lines. U represents amplification of unmethylated alleles, and M represents methylated alleles. In vitro methylated DNA (IVD) and normal human peripheral lymphocytes (NL) served as the positive and negative methylation controls, respectively. The presence of methylation of GATA-4, -5, and -6 correspond directly to the loss of expression of the respective genes in each of the cell lines, shown in A. (MW, molecular weight; MSP, methylation-specific PCR)
H1299, whereas both genes were expressed in H157 and H727. In all of these cases in which these genes were not expressed, treatment with the demethylating agent 5-aza-2’-deoxycytidine for 48 hours resulted in a robust restoration of expression of both GATA-4 and GATA-5 (Fig. 1A).

Methylation-specific PCR was then done to determine whether the silencing of these GATA genes was related to promoter region methylation, as suggested by the demethylating experiments (Fig. 1B). GATA-6 methylation was not present in any of the cell lines studied, which was expected because all of the six cell lines expressed GATA-6 by RT-PCR. Complete methylation was detected for both GATA-4 and GATA-5 in four cell lines (H358, DMS-53, A549, and H1299), all of which had no expression by RT-PCR analysis, whereas both H157 and H727, which express GATA-4 and GATA-5, were unmethylated in this promoter region. Both the RT-PCR and methylation-specific PCR results are summarized in Table 1. These results are consistent with previous studies (7) and confirm that promoter methylation in this region silences gene expression (7).

To determine a more detailed map of the methylation changes detected by methylation-specific PCR, we did bisulfite sequencing on the targeted promoter region of the GATA-4 gene in some of the cancer cell lines studied above. Representative GATA-4 bisulfite sequencing results of lung cancer cell lines H358 and H727 are shown (Fig. 2). Bisulfite sequencing of 10 individual clones of PCR products from H358 revealed densely methylated CpGs within the promoter regions of all of the clones. Accordingly, these bisulfite sequencing data not only confirm the complete methylation of the GATA-4 promoters in H358 cells previously determined by methylation-specific PCR but also explain the lack of GATA-4 expression of H358 cells results obtained by RT-PCR analysis. In contrast, only one of 10 clones from the H727 cell lines showed methylated CpGs within the GATA-4 promoter by bisulfite sequencing of PCR products, and this was present at only a few CpG sites. This correlated well with our previous results, where H727 was unmethylated for GATA-4 by methylation-specific PCR analysis and expressed the gene at the mRNA level.

### Table 1: RT-PCR and MSP for lung cancer cell lines

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**Fig. 2** Sodium bisulfite sequencing of GATA-4 in lung cancer cell lines that were found to have only unmethylated alleles by methylation-specific PCR (H157) or only methylated alleles (H358). The region of the CpG island studied by methylation-specific PCR (double-headed arrow) spanned 142 bp. • methylated CpG sites within the GATA-4 CpG island, ○ unmethylated CpG sites. Bisulfite sequencing focused on a 385 bp (+47 bp to +432 bp) CpG island just downstream of the GATA-4 transcription start site. (MSP, methylation-specific PCR)
GATA-4 and GATA-5 Hypermethylation in Primary Lung Cancer. To determine whether GATA-4 and GATA-5 promoter methylation was limited to cultured lung cancer cell lines or whether this event occurred in noncultured lung carcinomas, we examined resected non–small-cell lung carcinomas for methylation of GATA-4 and GATA-5 (Fig. 3). In 63 cases of primary lung cancers, GATA-4 and GATA-5 promoter region methylation was detected in (42 of 63) 67% and (26 of 63) 41% of the tumors examined (Table 2). We also observed that like the lung cancer cell lines, methylation of both GATA-4 and/or GATA-5 for many of the primary lung cancers (LCJH; see Table 2). The presence of unmethylated alleles (U Lanes) among cancer cases with GATA methylation likely results from sample inclusion of adjacent normal mucosa from the same patient. Methylation of GATA-4 was not found among lung tissue taken from patients without cancer (NL). (MSP, methylation-specific PCR)

**Table 2** MSP results for primary human lung samples

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Abbreviations: MSP, methylation-specific PCR.
Epigenetics of Lung Cancer

...genes often occurred in the same tumors, 26 of 63 (41%) cases had methylation of both GATA-4 and GATA-5. An additional 16 tumors (25%) had methylation of GATA-4 only, but in no tumor was GATA-5 methylation detected without methylation of GATA-4. Methylation of GATA-4 or GATA-5 was not detected in six cases of normal lung autopsy specimens collected from nonsmoking patients without lung cancer, showing that this process was part of the transformed phenotype. We did not examine GATA-6 methylation by methylation-specific PCR in any primary lung tumor because there was no evidence of transcriptional silencing or methylation of the GATA-6 promoter region in any of the lung cancer cell lines analyzed nor had methylation of this gene been observed in colon or gastric cancer (7).

We correlated DNA hypermethylation of GATA-4 and GATA-5 with clinical and histopathological variables to determine whether these alterations were associated with any particular phenotype. There was no significant correlation between methylation of GATA-4 or GATA-5 and tumor stage, consistent with the known importance of GATA-4 and GATA-5 function in the early steps of lung morphogenesis (14, 25). There was also no association of methylation of these targets with tumor histology. There was, however, a statistically significant association between GATA-5 methylation and concomitant GATA-4, which can be easily seen in both cell lines and primary tumor data presented above. In addition, there was an association of GATA-5 methylation and the chronological age at which lung cancer was diagnosed in the patients studied (*P* < 0.01; Table 3). GATA-5 methylation was also significantly associated with the length of time from the age a patient started smoking a cigarette to the time of lung cancer diagnosis. There was no association, however, between GATA family methylation and either the smoking status or the total pack-year history of a smoker. There was, however, a trend toward statistical significance of more frequent GATA-4 and GATA-5 methylation in primary lung cancer specimens from patients who had quit smoking cigarettes longer (*P* = 0.08).

**DISCUSSION**

We have found aberrant methylation involving the promoter regions of GATA-4 and GATA-5 in different histologies of lung cancer. In cancer cell lines, DNA promoter hypermethylation correlated with loss of gene expression and could be efficiently restored with the de-methylation agent, 5-aza-2-deoxycytidine alone. In addition, our findings of a lack of GATA-4 and GATA-5 methylation in normal adult lung tissue support the fact that epigenetic silencing of these genes is a tumor-specific process. This fits the paradigm that has now been widely documented in many malignancies of a reciprocal relationship between the density of methylated cytosine residues in the 5' region of some gene promoters and the transcriptional activity of that gene.

GATA-4, GATA-5, and GATA-6 transcription factors may influence not only the genesis but also the later maintenance of murine pulmonary endothelial and epithelial cell differentiation (26–28). Because GATA-4 and GATA-5 have been shown also to be critical factors driving endoderm-specific differentiation (22), we hypothesized that these transcription factors may play a role in cell fate determinations of various histologies of lung cancer. There were no significant correlations, however, between DNA methylation of GATA-4 or GATA-5 and tumor stage or tumor histology.

Immunohistochemical analysis indicates that GATA-4 and GATA-6 proteins are present in human bronchial epithelial cells of adult normal and asthmatic subjects (29). Because GATA-6 is predominantly expressed in proliferating progenitor cells (30), it might be predicted to have an oncogenic effect. This is consistent with our data, which failed to show any promoter methylation in six lung cancer cell lines. In contrast, our data suggest that loss of transcriptional activation of GATA-4 and GATA-5 in human lung cancer is frequent and because of epigenetic silencing. GATA-4 and GATA-5 seem to behave more like putative tumor suppressor genes, especially in the intestine where increased expression levels correlate with terminal differentiation (7, 8). Loss of GATA-4 and GATA-5 gene expression, therefore, with the retention of the proliferative stimulus of GATA-6 would theoretically impede differentiation and expedite cancer progression.

The finding that the methylation of GATA-5, and combined methylation of GATA-4 and GATA-5, correlates with the increasing chronological age of the lung cancer study patients is intriguing. Increasing chronological age is a risk factor for the rising incidence in later life of almost all of the epithelial cancers, especially cancers of the lung, breast, prostate, and...
colorectal (31). Because younger patients in our dataset (<60 years old), who had fewer methylation events for GATA-4 and GATA-5, still developed lung cancer, we examined whether other cofactors may account for the accelerated kinetics of lung carcinogenesis in these patients. Indeed, patients <60 years old were significantly more likely to be current smokers than those >60 years old (P = 0.03).

Recent findings of Kim et al. (32), who observed that hypermethylation of the RASSF1A promoter was associated with starting cigarette smoking at an early age, suggest that epigenetic changes may cause progressive increase over time because of tobacco carcinogens. This is in agreement with our findings. Although GATA-4 and GATA-5 methylation were significantly associated with the length of time from the age a patient first started smoking to the age of lung cancer diagnosis, there was no correlation between methylation of the GATA genes and pack-year tobacco history of patients. Moreover, several patients had GATA-4 methylation alone, but we never observed GATA-5 methylation without methylation of GATA-4. This additionally suggests a progressive model of methylation of GATA-4 and GATA-5 that may be dependent on time. Our interpretation of these data are that the methylation of GATA-4 and GATA-5 genes is cumulative and ongoing over time in these heavy smokers who have a mean pack-year tobacco history of >50 years.

GATA-4 and GATA-5 have multiple downstream effects, with targets potentially affected by GATA gene inactivation including the Trefoil factor family (TFF) genes and inhibin-α. The expression of these candidate downstream genes, found in mammalian lung tissue (33, 34), has been reported to be up-regulated by GATA transcription factors (35). Akiyama et al. (7), using demethylation agents, re-expressed not only GATA-4 and GATA-5 in neoplastic cell lines but also their downstream targets including TFF1, TFF2, TFF3, and inhibin-α. This provides a basis for a model in which loss of transcriptional activation secondary to epigenetic silencing of upstream transcription factors disrupts established cell regulatory networks during carcinogenesis (7). Furthermore, McCluggage and Maxwell (36) reported weak or absent immunohistochemical staining for inhibin-α in 4 of 5 human lung adenocarcinomas, additionally supporting this model in lung neoplasms.

In summary, we report the frequent loss of GATA-4 and GATA-5 gene expression as a result of promoter hypermethylation in various lung cancer cell lines. These aberrant promoter methylation changes are also observed in various histologies of primary lung cancer. In addition, these methylation events become more frequent with increasing patient age, and we surmise that these methylation events are cumulative over time, perhaps initiated and promoted by tobacco carcinogens. The exact timing and sequence of the multiple promoter hypermethylation changes involved during lung carcinogenesis remain to be elucidated.

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