DNA Methylation Profiles of Lung Tumors

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Abstract

Aberrant methylation of CpG islands in promoter regions of tumor cells is one of the major mechanisms for silencing of tumor suppressor genes. We determined the frequency of aberrant promoter methylation of the p16, adenomatous polyposis coli (APC), H-cadherin (CDH13), glutathione S-transferase P1 (GSTP1), O6-methylguanine-DNA-methyltransferase (MGMT), retinoic acid receptor β-2 (RARß), E-cadherin (CDH1), and RAS association domain family 1A (RASSF1A) genes in 198 tumors consisting of small cell lung cancers [SCLCs (n = 43)], non-small cell lung cancers [NSCLCs (n = 115)], and bronchial carcinoids (n = 40). The profile of methylated genes in the two neuroendocrine tumors (SCLC and carcinoids) were very different from that of NSCLC. However, whereas the overall pattern of aberrant methylation of carcinoids was similar to that of SCLC, carcinoids had lower frequencies of methylation for some of the genes tested. There were also significant differences in the methylation profiles between the two major types of NSCLC, adenocarcinoma and squamous cell carcinoma. We performed cluster analysis and found that SCLCs clustered with other SCLCs and carcinoids but not with NSCLCs, whereas the NSCLCs tended to cluster together. Within NSCLCs, adenocarcinomas and squamous cell carcinomas clustered with their respective histological types. Finally, we compared the methylation profiles of SCLC and NSCLC tumors and their respective cell lines (n = 44). In general, methylation frequencies were higher in tumor cell lines, but these differences were seldom significant. Thus, tumor cell lines appear to be suitable models to study aberrant DNA methylation. We conclude that SCLC, carcinoids, squamous cell carcinomas, and adenocarcinomas of the lung have unique profiles of aberrant methylation. Our findings should help us understand differences in the pathogenetic mechanisms of lung cancers.

Introduction

Lung cancer is the leading cause of cancer mortality in the United States and in the world (1, 2). Lung cancer is divided into two major histological categories, SCLC and NSCLC. SCLC accounts for 20–25% of all bronchogenic malignancies and follows a highly aggressive clinical course. Metastases (clinically apparent or occult) are usually present at diagnosis of SCLC, and the treatment of choice is usually combination chemotherapy. Less than 5% of patients currently survive 5 years past the initial diagnosis (3), whereas the 5-year survival rate for patients diagnosed with NSCLC is 15% (4). SCLC is initially highly responsive to radiation and systemic chemotherapeutic regimes. NSCLC is less sensitive to chemotherapy, and curative intent surgical resection is the treatment of choice (5). Bronchial carcinoids are tumors of the lung of low-grade malignancy, although a subtype, the atypical carcinoid, follows a more aggressive course (6). However, the overall survival of patients with carcinoids is far better than that of patients with SCLC. Carcinoids and SCLC are neuroendocrine tumors, although their pathogenesis is believed to be very different. Carcinoids occur at an earlier age and are not smoking related. In contrast to SCLC, they are highly resistant to chemotherapy, and most carcinoids are cured by surgical resection. There are both similarities and differences in the molecular changes present in SCLC and carcinoid (7, 8). In particular, mutations of the menin gene, characteristic of many carcinoids, are absent in high-grade neuroendocrine tumors including SCLC (9).

Whereas most lung cancers are smoking related, there are major somatically acquired genetic differences between SCLC and NSCLC (5, 10–14) and, to a lesser extent, between squamous cell carcinomas and adenocarcinomas (5, 15, 16). Aberrant methylation of the promoter region of tumor suppressor genes and the resultant gene silencing play an important role in the pathogenesis of most if not all types of
human cancer (17, 18). Individual tumor types frequently have their characteristic pattern of acquired aberrant methylation (18, 19). We and others have identified several genes methylated frequently in NSCLC (19–25). Whereas several of these reports also describe gene promoter methylation in SCLC cell lines, there is very little published information about SCLC tumors (26) or the subtypes of NSCLC, and there is no published information about bronchial carcinoids. In this study, using eight genes previously studied for DNA methylation in lung cancers, we compare and contrast the aberrant methylation profiles of the neuroendocrine tumors SCLC and carcinoid and the squamous cell carcinomas and adenocarcinoma types of NSCLC. In addition, because tumor cell lines are frequently used as models to study DNA methylation, we compared the patterns of SCLC and NSCLC tumors with those of their respective cell lines.

Materials and Methods

Sample Collection. A total of 198 tumors consisting of 43 SCLCs (mean age, 64.4 years; age range, 43–78 years; male:female ratio = 4.0), 115 NSCLCs (77 adenocarcinomas and 38 squamous cell carcinomas; mean age, 60.3 years; age range, 32–87 years; male:female ratio = 2.0), and 40 bronchial carcinoids (33 typical and 7 atypical carcinoids; mean age, 54.2 years; age range, 31–73 years; male:female ratio = 1.4) were obtained from multiple centers. All NSCLC cases were from the United States, most carcinoid cases were from France, and most SCLC tumors were from Japan. With the exceptions noted later, the tumors were from previously untreated cases receiving curative intent surgical resections. Eleven of the SCLC tumors were recurrences occurring after prior chemotherapy. We also studied 20 SCLC and 24 NSCLC cell lines initiated from United States cases in the laboratory of A. F. G. and J. D. M. (27).

DNA Extraction. Genomic DNA was isolated from frozen tissues or cell pellets by homogenization, SDS/proteinase K (Life Technologies, Inc., Rockville, MD) digestion, phenolchloroform extraction, and ethanol precipitation. (28).

MSP. Aberrant methylation of p16, APC, CDH13, GSTP1, MGMT, RARβ, CDH1, and RASSF1A was determined by MSP using primers specific for the methylated and unmethylated alleles of each gene after treatment of the DNA with sodium bisulfite (22–24). Briefly, 1 μg of genomic DNA was denatured by NaOH and modified by sodium bisulfite, which converts all unmethylated cytosines to uracils, whereas methylated cytosines remain unchanged. The modified DNA was purified using the Wizard DNA purification kit (Promega, Madison, WI), treated again with NaOH to desulfonate it, precipitated with ethanol, and resuspended in water. PCR amplification was done with treated DNA as template as described previously using specific primer sequences for the methylated and unmethylated forms of each gene. DNA from peripheral blood lymphocytes and scraped buccal mucosa (from 10 healthy subjects each) was used as negative control for the methylated genes along with water blanks. Aberrant methylation of nonmalignant lung tissue was, at most, 14%, as reported previously (20). DNA from lymphocytes of healthy volunteers treated with Sss1 methyltransferase (New England Biolabs) and then subjected to bisulfite treatment was used as a positive control for methylated alleles. PCR products were visualized on 2% agarose gels stained with ethidium bromide.

Cluster Analysis. Clustering analysis was done as described previously (13) using a Visual Basic program (available on request).

Data Analysis. Frequencies of methylation of two groups were compared using Fisher exact tests. The MI, a reflection of the methylation status of all of the genes tested, is defined as the total number of genes methylated divided by the total number of genes analyzed. The MIs for each case were determined, and the median MI for each tumor group was calculated. The MIs of different groups were compared using the Mann-Whitney nonparametric U test. For all tests, probability values of P < 0.05 were regarded as statistically significant. All statistical tests were two-sided.

Results

Methylation Profiles of Lung Tumor Types. The methylation profiles of the different lung cancer groups were determined by testing the methylation status of the eight genes. Examples of the bands obtained by MSP are illustrated in Fig. 1. Fig. 2 shows complete data for 242 tumor and tumor cell line DNAs sorted using a clustering algorithm, and the frequency of aberrant methylation is summarized in Fig. 3. The mean MIs are tabulated in Table 1. Tumor tissues consist of mixtures of tumor and nonmalignant cells, and the unmethylated forms of all genes were present in all tumor samples. The presence of the unmethylated bands confirmed the integrity of DNA in all tumor samples. Whereas the unmethylated bands could come from the tumor, noninvolved tissue, or both, in our prior studies of tumor cell lines, we usually found only methylated or unmethylated alleles (21–24). We first compared the methylation profile of neuroendocrine tumors (SCLCs and carcinoids) with that of NSCLC tumors (squamous cell carcinomas and adenocarcinomas; Fig. 3A). There was no significant difference in the
Fig. 2. Clustering analysis of DNA methylation profiles for 242 thoracic malignancies and tumor cell lines. Identical methylation patterns are clustered and separated by horizontal lines. Generally, neighboring clusters have similar methylation patterns. Sky blue, methylated; yellow, unmethylated. SCLC, red; SCLC cell lines, pink; carcinoid, cherry; adenocarcinoma, dark blue; squamous cell carcinoma, orange; NSCLC cell lines, purple. The right panel is a continuation of the left panel.
Methylation Profiles of Lung Tumors

The frequencies of methylation of several genes were higher in the tumor cell lines, these differences were significant in only two cases (MGMT in SCLC and RASSF1A in NSCLC).

Clustering analysis was performed to confirm these results and to identify other potential similarities between the methylation pattern of different tumor samples (Fig. 2). Consistent with the above-mentioned analysis, carcinoids (cherry), SCLC (red), and SCLC cell lines (pink) are often seen clustered together (or belong to neighboring clusters). Adenocarcinomas (dark blue) cluster together, as do squamous cell carcinomas (orange), which also tend to cluster with adenocarcinoma. Similarly, tumors and/or cell lines of the same histological type are usually clustered.

Discussion

Our results demonstrate important differences in the methylation profiles of the major types of lung tumors. In a comparison of neuroendocrine and NSCLC tumors, the frequency of RASSF1A methylation was significantly higher in the neuroendocrine tumors, and the frequencies of p16,
 APC, and CDH13 methylation were significantly higher in NSCLC tumors. These findings are consistent with our previous studies using SCLC cell lines (21, 23, 24). They also confirm the findings of Dammann et al. (26) and Burbee et al. (24), who both found a high frequency of RASSF1A methylation in a few uncultured SCLC tumors. Cell cycle perturbations in lung tumors usually involve abnormalities of the p16 gene in NSCLC and the RB gene in SCLC (11). The p16 gene can be inactivated by multiple mechanisms in NSCLC, including methylation (34), but inactivation of p16 in SCLC is rare (11, 35). Our findings of frequent p16 methylation in NSCLC tumors combined with infrequent methylation in neuroendocrine tumors are consistent with these reports.

SCLC and bronchial carcinoids share expression of neuroendocrine features but have different pathogenetic mechanisms and clinical courses. Carcinoids are not associated with smoking, have a limited metastatic potential, and are chemoresistant. However, the patterns of methylation of these two very different neuroendocrine tumors of the lung were more similar to each other than to NSCLCs, although the frequencies of methylation of RARβ, CDH1, and RASSF1A were higher in SCLC. Although conclusions must be tempered by the small number of samples available for study, there were no significant differences between previously treated and untreated SCLC tumors. In addition, whereas atypical carcinoids had a higher frequency of methylation of RASSF1A, similar to that of SCLC, there were no significant differences in the methylation frequencies of any individual gene or of the mean MIs between typical and atypical carcinoids. In a comparison of the methylation profiles of squamous cell carcinomas and adenocarcinomas, p16 was methylated more frequently in squamous cell carcinomas, as has been reported by others (36), whereas APC and CDH13 methylation was more frequent in adenocarcinomas. With rare exceptions, the profiles of NSCLC and SCLC tumors and their respective cell lines for the individual genes and the mean MIs were not significantly different. These findings suggest that cell lines are useful models for studying methylation. Of interest, the pattern of aberrant methylation in bladder cell lines is similar to that in the tumors from which they originated (37).

SCLC tumors are seldom resected in the United States, and carcinoids are relatively rare tumors. For these reasons, the methylation profiles of uncultured specimens from these tumors have not been determined. To obtain enough tumors for our studies, we had to obtain SCLC samples from Japan, where resection of localized SCLC tumors is more common than in the United States and Europe. In addition, the numbers were supplemented with tumors that had recurred after combination chemotherapy. There are no squamous cells in the normal bronchus, and squamous cell carcinomas are believed to arise from metaplastic cells frequently present in the bronchus of smokers (38). By contrast, most adenocarcinomas are peripherally located and may express features of Clara cells or type II pneumocytes. In addition, adenocarcinomas are the most frequent histological type of lung cancer arising in women and never smokers (39, 40). These findings demonstrate the very different origins of the two major forms of NSCLC. Whereas both squamous cell carcinomas and SCLCs are strongly related to smoking and usually arise from the bronchial tree, the molecular changes present in the bronchial epithelium of SCLC patients are much more extensive than those in the mucosa adjacent to squamous cell carcinomas (16). Thus, we have suggested that SCLC may arise directly from bronchial mucosa that demonstrates extensive molecular changes but few histological changes, whereas squamous cell carcinomas arise after a defined sequence of molecular and histological changes (16).

Our findings are consistent with these differences in pathogenesis, and clustering analysis further confirmed these results by showing tumors of the same histological type to have similar methylation patterns. Together, they demonstrate important differences in the methylation profile of the four types of lung cancer studied, as well as similarities within each subtype, and indicate that lung cancer cell lines are appropriate models for studying methylation.

Understanding the methylation profiles of tumors may impact on several clinical parameters. Methylation of some genes, such as Deach-associated protein kinase in lung, APC in breast tumors, and several genes as well as the mean MI in bladder cancer, correlates with survival or other parameters of poor prognosis cancers (23, 41). Treatment with demethylating agents results in temporary restoration of gene expression and may be a potential method of tumor
therapy or chemoprevention (42, 43). We have suggested that loss of expression of the RARβ gene by methylation in smoking-damaged epithelium may be the cause of failure of chemoprevention by its ligands (44). Thus, our findings may help stratify lung cancer patients into clinically important subsets and lead to improved chemoprevention strategies.

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References


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