Abstract

Aberrant promoter methylation and resultant silencing of several genes plays an important role in the pathogenesis of many tumor types. We compared the methylation profile of 66 malignant mesotheliomas (MMs) and 40 lung adenocarcinomas using methylation-specific PCR for seven genes frequently methylated in lung cancer. We also compared the methylation frequencies of these genes as well as the methylation index, a reflection of all of the gene frequencies, with the presence of SV40 large T-antigen (Tag) sequences, histological subtype, and patient survival. Our major findings are: (a) with the exception of the RASSF1A promoter of the RASSF1 gene, frequencies of aberrant methylation were significantly lower in MMs than in adenocarcinomas; (b) the frequency of RASSF1A aberrant methylation and the value of the methylation index were significantly higher in SV40 sequence positive MM than in negative MM; and (c) the methylation index was higher in epithelial MM than in sarcomatous/mixed MM. Our results demonstrate a relationship between SV40 and aberrant methylation in MMs.

Introduction

Aberrant methylation of CpG islands in the promoter region of tumor suppressor genes is a frequent mechanism of gene silencing (1). MM is an aggressive tumor that develops from the pleura or other mesothelial surfaces and is strongly associated with exposure to asbestos (2). Most MMs have epithelial histological features, and the pathological distinction from metastatic carcinomas, especially adenocarcinoma of the lung, may be difficult (3). However, sarcomatous or mixed patterns also occur. Compared with lung carcinomas, the molecular biology of mesotheliomas is less well studied. However, several genomic regions of frequent chromosomal loss or gain have been identified, as well as inactivation of the p16 and NF2 genes (4). Mutations of the p53 and ras genes, which are frequently mutated in lung carcinomas, are rare in MMs. In contrast, SV40 Tag sequences are frequently present in MMs, but are absent in adjacent lung tissues and in lung carcinomas (5, 6). The biological and clinical significance of this finding is not fully understood.

Aberrant promoter methylation in MMs has received scant attention. Expression of GPC3, an X-linked recessive overgrowth gene, is silenced in MM tumors and cell lines (7). We and others have documented that several genes are frequently methylated in lung and other carcinomas. These genes include the RASSF1A promoter of the newly described RASSF1 gene, glutathione S-transferase (GSTP1), CDKN2A/p16, retinoic acid receptor-β (RARβ), adenomatosis polyposis coli (APC), H-cadherin (CDH13), and O6-methylguanine-DNA methyltransferase (MGMT) (8–13). We determined the methylation status of these genes in MMs and correlated them with patient survival, histological pattern, and the presence of SV40 Tag sequences. We also compared the methylation profiles of MMs with those of lung adenocarcinomas.

Materials and Methods

Tumors and Cell Lines. Tumor tissues were obtained from 66 patients with MM resected by one of the authors (H.I.P.) and from 40 patients with primary lung adenocarcinomas resected at M.D. Anderson Cancer Center, Houston TX. These tumor samples are different from those that we examined previously (6). Tissues and clinical data were collected after obtaining appropriate Institutional Review Board approval and signed patient informed consent. Clinical records of 53 MM patients were reviewed to determine clinical features including histological type, stage, and survival of the MM cases. Forty-one patients were male (mean age, 54 years old; range, 30–78 years) and 12 patients were female (mean age, 47 years old; range, 34–68 years). Tissues were stored at −80°C until analyzed. Cell lines were initiated by one of the authors (H. I. P.; Ref. 14) or A. G. F. Six MM cell lines (NCI-H28, NCI-H290, NCI-H2052, NCI-H2373, NCI-H2452, HP1), were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) supplemented with 5% fetal bovine serum and were incubated in 5% CO2 at 37°C. Four nonmalignant mesothelial primary cell cultures (HCC3466, HCC3468, HCC3469, HCC3471) were established by A. F. G. from pleural effusions that arose in patients free of cancer and that demonstrated normal or reactive cytological changes.

DNA Extraction. Genomic DNA was isolated from frozen tissue by homogenization, SDS/protease K (Life Technologies, Inc., Rockville, MD) digestion, phenol-chloroform extraction, and ethanol precipitation.

MSP. Aberrant methylation of the RASSF1A, GSTP1, p16, RARB, APC, CDH13, and MGMT genes was determined using gene-specific primers by the method of MSP (8) as described previously (9, 10, 13, 15). DNA from 10 peripheral blood lymphocytes and 10 buccal mucosa scrapings of healthy subjects and water blanks were used as negative controls for the methylated genes. DNA from lymphocytes of healthy volunteers treated with 5-aza-2′-deoxycytidine; Aza-CdR, 5 aza 2′-deoxycytidine; RASSF1a, ras association domain family 1; Tag, large T-antigen.

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2 To whom requests for reprints should be addressed, at Hamon Center for Therapeutic Oncology Research, University of Texas Southwestern Medical Center, 6000 Harry Hines Boulevard, Dallas, Texas 75390. Phone: (214) 648-4921; Fax: (214) 648-4940; E-mail: Adi.GAZDAR@UTSouthwestern.edu

3 The abbreviations used are: MM, malignant mesothelioma; MSP, methylation-specific PCR; RT, reverse transcription; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Aza-CdR, 5 aza 2′-deoxycytidine; RASSF1a, ras association domain family 1; Tag, large T-antigen.

4 GenBank nos. AF102770, AF40703, and AF266217, respectively.

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extracted from the cell lines with Trizol (Life Technologies, Inc.) following the manufacturer’s instructions. RT reaction was performed on 2 μg of total RNA with the SuperScript II First-Strand Synthesis using oligo(dT) primer System (Life Technologies, Inc.). The house keeping gene GAPDH was used as an internal control to confirm the success of the RT reaction. The primers for RT-PCR were confirmed not to amplify genomic DNA, and PCR products were analyzed on 2% agarose gels.

**Aza-CdR Treatment.** Five tumor cell lines with RASSF1A promoter methylation and absent gene expression were incubated in culture medium with Aza-CdR at a concentration of 2 μg/ml for 6 days, with medium changes on days 1, 3, and 5 (10).

**Data Analysis.** The frequencies of methylation in MMs and lung adenocarcinomas were compared using χ² tests. To compare the overall degree of methylation for the panel of genes examined, we calculated the methylation index. The methylation index is defined as the total number of genes methylated divided by the total number of genes analyzed. The methylation index for each case was determined and the median methylation index for each tumor group calculated. The methylation index of different groups was compared using the Mann-Whitney U nonparametric test. Survival curves were calculated by use of the Kaplan-Meier method, and survival curves were compared with the log-rank statistic. For all of the tests, probability values of $P < 0.05$ were regarded as statistically significant. All of the statistical tests were two-sided.

### Results

We studied 66 MMs and 40 lung adenocarcinoma samples (Table 1; Figs. 1A and 2). Aberrant methylation was present less frequently in MMs than in lung adenocarcinoma for all of the genes studied, although the differences for RASSF1A and GSTP1 were not significant (Fig. 1A). The methylation index of MM was significantly lower than that of lung adenocarcinoma (Fig. 1A).

SV40 Tag sequences were present in 48% (32 of 66) of MMs but were not detected in any of the 40 pulmonary adenocarcinomas (Table 1). The frequency of RASSF1A aberrant methylation was significantly higher in SV40 Tag-sequence positive MMs than in negative samples (Fig. 1B). The methylation index of SV40 Tag-sequence positive MMs was significantly higher than that of negative samples (Fig. 1B).

There was no significant relationship between aberrant methylation of any gene and patient survival by Kaplan-Meier analysis. However, the MMs of the four patients who lived longer than 36 months lacked methylation of any of the genes tested, and three of the four lacked SV40 sequences. Aberrant methylation was predominantly limited to the epithelial MMs and was rare in the sarcomatous or mixed types. The methylation index was significantly higher in epithelial MMs than in those with sarcomatous/mixed patterns (Fig. 1C; Table 1).

SV40 Tag sequences were present in four of the six mesothelioma cell lines (NCl-H28, NCI-H290, NCI-H2052, and HP1). RASSF1 transcript expression was examined by RT-PCR in six MM cell lines. Although RASSF1C was expressed in all six of the cell lines, RASSF1A and RASSF1F were absent in four cell lines (NCI-H28, NCI-H290, NCI-H2052, and NCI-H2373; Fig. 2B). Two cell lines (NCI-H2452 and HP1) expressed all three of the transcripts. The four cell lines lacking expression of the A and F transcripts showed aberrant methylation of the RASSF1A promoter and three of the four lines lacked the unmethylated allele by MSP analysis, whereas cell line NCI-H2373 had both methylated and unmethylated alleles. In all of these four cell lines, RASSF1A and RASSF1F expression were restored by 5Aza-CdR treatment (Fig. 2B). We also examined four short-term cultures derived from nonmalignant reactive mesothelial cells. All four of the cultures lacked aberrant methylation, expressed all three of the transcripts of RASSF1, and were negative for SV40 TAG sequences.

### Discussion

Because very little information is available regarding the aberrant methylation profile of MM, we selected seven genes previously studied in lung cancers. We compared the methylation profiles of MMs and lung adenocarcinomas. Although asbestos exposure predisposes to both tumor types and they share certain histological features, they arise in different tissues, and MMs are not associated with smoking exposure (2).

The methylation frequencies in lung adenocarcinomas for all of the
genes studied varied from 10 to 52%. However, for MM, only two of the genes, RASSF1A (32%) and RARβ (11%), were methylated at frequencies above 10%. In all instances, the methylation frequencies for MM were lower than those of adenocarcinomas, and, except for RASSF1A and GSTP1, these differences were significant. In addition, the methylation index, a reflection of overall methylation frequency, was significantly lower in MMs. The differential diagnosis of MM was notable absent in the four cases with survival >36 months. Furthermore, although Procopio et al. (22) reported that SV40 sequences are a negative prognostic cofactor for MM, we did not detect a statistically significant difference in survival between SV40 sequence positive and negative MMs.

RASSF1A was associated with poor prognosis (12). Although we did not demonstrate a significant relationship between prognosis of MM and aberrant methylation of any gene in this study, aberrant methylation was notably absent in the four cases with survival >36 months. Furthermore, although Procopio et al. (22) reported that SV40 sequences are a negative prognostic cofactor for MM, we did not detect a statistically significant difference in survival between SV40 sequence positive and negative MMs.

MM has two major histological types, epithelial and sarcomatous, although mixed forms may also exist. Aberrant methylation was present more frequently in epithelial than in sarcomatous/mixed types, which suggests differences in the pathogenesis of these two forms of MM. Of interest, we have reported previously that SV40 Tag sequences (which are associated with a higher methylation index) are more frequent in epithelial MMs (6).

Our results indicate that the pattern of aberrant methylation in MMs is very different from that in pulmonary adenocarcinomas. Of the genes tested, only methylation and silencing of the 1A promoter of the RASSF1A gene was frequent in MMs. Methylation was significantly more frequent in epithelial tumors and in those with SV40 Tag sequences. Our results demonstrate a relationship between SV40 and aberrant methylation in MM.

References


Aberrant Methylation and Simian Virus 40 Tag Sequences in Malignant Mesothelioma


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