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Advances in Brief

Aberrant Methylation and Simian Virus 40 Tag Sequences in Malignant Mesothelioma[1]

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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[3] 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 (*p16*), retinoic acid receptor-β (*RARβ*), adenomatosis polyposis coli (*APC*), H-cadherin (*CDH13*), and O^6-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° until analyzed. Cell lines were initiated by one of the authors (H. I. P.; Ref. 14) or A. F. G. 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% CO_2 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/proteinase K (Life Technologies, Inc., Rockville, MD) digestion, phenol-chloroform extraction, and ethanol precipitation.

MSP. Aberrant methylation of the *RASSF1A*, *GSTP1*, *p16*, *RARβ*, *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 Sss1 methyltransferase (New England BioLabs, Beverly, MA) 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. All of the results were confirmed by repeat assays performed on DNA samples independently treated with bisulfite.

Detection of SV40 Tag Sequences. PCR were performed to amplify the 105 bp (6). Primers SVFor3 and SVRev, which specifically amplify a 105 bp of the retinoblastoma protein binding domain of the large T antigen region of SV40 and not that of any other virus (16).

Expression of *RASSF1* Transcripts. Expression of *RASSF1* transcripts *A*, *C*, and *F* (GenBank nos. AF102770, AF040703, and AF286217, respectively) were analyzed by RT-PCR as described by us previously (12). Total RNA was

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[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; RASSF1, ras association domain family 1; Tag, large T-antigen.

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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 χ^2 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. 1*A* 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. 1*A*). The methylation index of MM was significantly lower than that of lung adenocarcinoma (Fig. 1*A*).

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. 1*B*). The methylation index of SV40 Tag-sequence positive MMs was significantly higher than that of negative samples (Fig. 1*B*). 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. 1*C*; Table 1).

SV40 Tag sequences were present in four of the six mesothelioma cell lines (NCI-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. 2*B*). 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. 2*B*). 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.

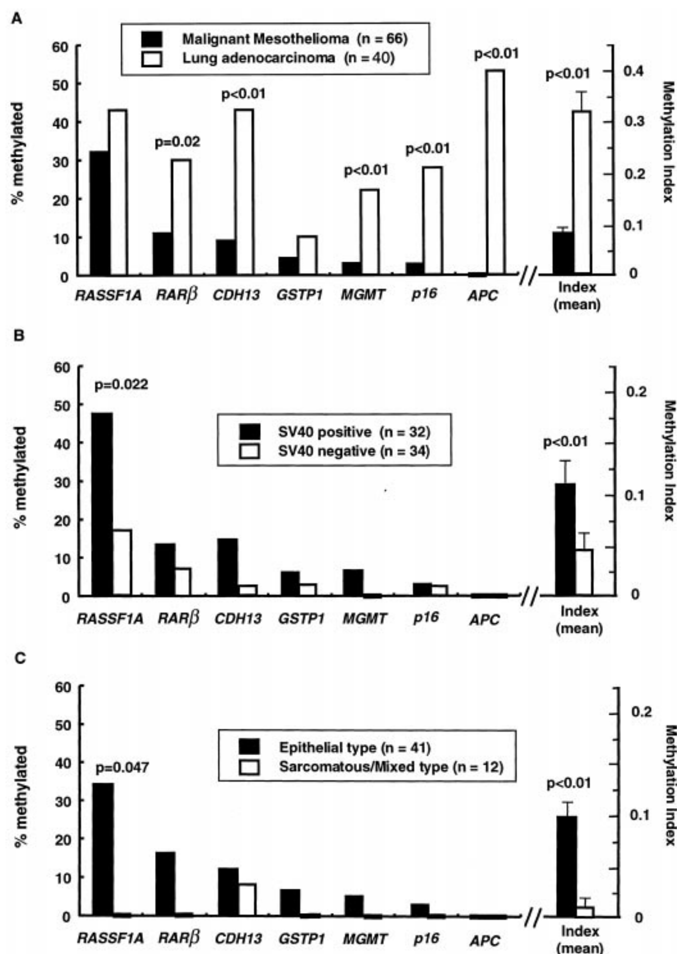

Fig. 1. *A*, comparison of frequency of aberrant methylation and methylation index between MM and lung adenocarcinoma. *B*, comparison of frequency of aberrant methylation and methylation index between positive and negative of SV40 Tag sequence. *C*, comparison of frequency and methylation index of aberrant methylation between epithelial and sarcomatous/mixed-type MMs. The frequencies of methylation in MMs and lung adenocarcinomas were compared using χ^2 tests. The methylation index of different groups were compared using the Mann-Whitney *U* nonparametric test. The *P* is shown when there is a significant difference between two groups. n = number of examined samples.

Table 1 *Clinicopathological features and methylation indices of MMs and lung adenocarcinomas*

Although 66 MM samples were examined, clinical records were available from only 53 of the cases.

Tumors	*n* (%)	Methylation index (mean ± SD)
Lung adenocarcinoma	40 (100)	0.32 ± 0.25
Mesothelioma	66 (100)	0.086 ± 0.11
Epithelial	41 (77)	0.10 ± 0.12
Sarcomatous/mixed	12 (23)	0.012 ± 0.040
SV40(+)[a]	32 (48)	0.12 ± 0.13
SV40(−)[b]	34 (52)	0.050 ± 0.085
Survival ≤36 mo	49 (92)	0.090 ± 0.11
Survival >36 mo	4 (8)	0

[a] SV40(+), SV40 Tag sequence positive cases.
[b] SV40(−), SV40 Tag sequence negative cases.

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

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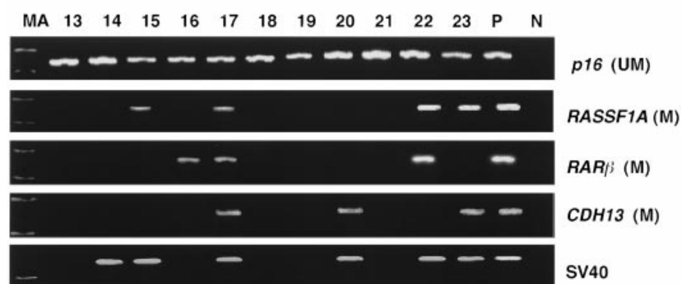

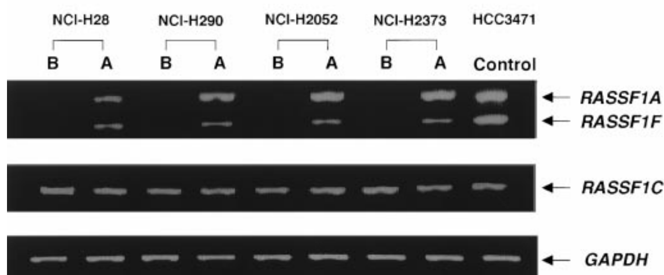

Fig. 2. *A*, representative examples of MSP assay and SV40 Tag sequence amplification. Data from 11 MMs are illustrated. The unmethylated form of *p16* was run as a control for DNA integrity. Results of testing for the methylated forms of *RASSF1A*, *RARβ*, and *CDH13* and for the presence of SV40 Tag DNA sequences are illustrated. *M*, size ladder; *P*, positive control; *n*, negative control (water blank). *B*, expression of *RASSF1* transcripts was examined by RT-PCR in MM cell lines before and after treatment with Aza-CdR. In four MM cell lines (all of which were methylated for *RASSF1A*), expression of *RASSF1A* and *RASSF1F* were absent, but were restored after treatment with Aza-CdR. All of the cell lines express the *RASSF1C*. In contrast, the nonmalignant mesothelial cell culture (HCC3471) expresses all three of the transcripts. All cultures express the housekeeping gene *GAPDH*, which was run as a control for RNA integrity. Before (*B*) and after (*A*) treatment with Aza-CdR.

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 MMs 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 and lung adenocarcinoma may be difficult (3). Our data indicate that a profile of aberrant methylation may help to distinguish between MMs and lung adenocarcinomas. Of particular interest, *APC* promoter 1A methylation was completely absent in MMs, although it was the gene most frequently methylated in adenocarcinomas (52%). The *p16* gene is frequently inactivated in both tumor types. In lung cancers, a variety of inactivating mechanisms occur, including homozygous deletions, aberrant methylation, and mutations (17, 18). However, in MM, it appears that the major mechanism in tumors and cell lines may be homozygous deletion (4).

The highest frequency of methylation in MMs was of the *RASSF1A* gene, located at 3p21.3, a chromosomal region frequently deleted in MMs and in lung carcinomas of all histologies (4, 19, 20). The *RASSF1* gene encodes two major transcripts that are produced by alternative promoter selection and alternative mRNA splicing: RASSF1A (340 amino acids, encoding a M_r 39,000 peptide) containing a predicted NH_2-terminal diacylglycerol (DAG)-binding domain and a predicted COOH-terminal RAS-associated domain; and RASSF1C (270 amino acids, encoding a M_r 32,000 peptide) with a different NH_2 terminus lacking the DAG domain but exhibiting a similar COOH terminus containing the RAS-association domain (11, 12). *RASSF1A* has been demonstrated to function as a tumor suppressor gene in lung cancer (11, 12). Three transcripts are derived from two known promoters, and promoter 1A controls expression of transcripts 1A and 1F, whereas promoter 1B controls expression of transcript 1C. As in lung and breast cancers, all of the MM cell lines tested expressed transcript 1C, whereas there was selective loss of transcripts 1A and 1F associated with methylation of their promoter 1A. Treatment with Aza-CdR restored expression of *RASSF1A* and *F* in methylated MM cell lines, which confirmed that methylation was responsible for loss of gene expression in these lines. As 3p21.3 loss is frequent in MMs (21), these results suggest that relatively frequent inactivation of products of the *RASSF1A* promoter occurs in MMs, by a combination of aberrant methylation and allelic loss. Of interest, differential methylation of specific promoters and inactivation of their transcripts also occurs for the *RARβ* P2 and *APC* 1A promoter (10, 15).

In lung cancer, we reported that aberrant methylation of *RASSF1A* was associated with poor prognosis (12). Although we did not demonstrate a significant relationship between prognosis of MMs 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 MMs, 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 *RASSF1* 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 MMs.

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