

원저

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Methylation Abnormality in Body Fluid Cytology: A Supplemental Molecular Marker for the Diagnosis of Malignant Mesothelioma

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Malignant mesothelioma (MM) is a highly lethal neoplasm arising in pleura and the peritoneum and a rapid and accurate diagnosis is crucial for treatment of the disease. However, the sensitivity of cytological analysis using pleural or ascitic fluid is relatively low, yielding an accurate diagnosis in only 32 ~ 79% of cases. We tested the diagnostic value of epigenetic alterations in body fluid cytology as a supplement to conventional methods. Paraffin-embedded tissue blocks from 21 MM patients and associated body fluid cytology slides considered no evidence of malignancy were used to test for epigenetic alteration. Using methylation-specific PCR, we detected methylation of *RASSF1A* and *p16* in 47.6% (10/21) of both surgically resected tumor samples, respectively. Body fluid samples of MM also showed abnormal methylation of *RASSF1A* and *p16INK4a* genes in 38.1% (8/21) and 33.3% (7/21) of cases. The concordance in the rates of *RASSF1A* and *p16INK4a* gene-methylation abnormalities determined from cytology samples and tissue samples were 61.9% (13/21) and 66.7% (14/21), respectively. Combining both genes increases the sensitivity of the test to 57.1% (12 of 21) of cases. Our results suggest that testing for methylation abnormalities in selected individual genes or gene combinations has diagnostic value as an alternative or adjunct method to conventional cytological diagnosis.

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Key Words : Mesothelioma, Methylation, *p16^{INK4a}*, *RASSF1A*,
Body Fluid

INTRODUCTION

Malignant mesothelioma (MM) is a highly lethal cancer with a strong causal relationship to asbestos exposure for which no effective treatment modality exists.¹ Although rapid and accurate diagnosis is crucial for

treatment, most patients are unfortunately diagnosed at an advanced stage. Currently, diagnosis mostly often relies solely on histopathological examination after invasive tissue sampling. A less invasive cytological examination is widely accepted; however, the accuracy of this method in diagnosing mesothelioma is generally

low and highly variable. Although a diagnostic sensitivity of up to 76% with cytology of serous effusions has been reported in a relatively large series by Di Bonito et al.,² another series reported a much lower diagnostic sensitivity ($\leq 32\%$).^{3,4} A review of reports on cytologic diagnosis of mesothelioma published over the past 30 years shows a wide range of diagnostic sensitivity (0~93%), with better results coming from laboratories located where there is a high local incidence of the disease - presumably those with much greater experience with the disease.^{5,6} Serous fluid in pleural and peritoneal cavities of mesothelioma patients potentially harbors malignant cells, degraded cells, and cellular components and metabolites. Therefore, efforts to increase the diagnostic accuracy of MM tests should include an analysis of molecular components of serous fluid in addition to a traditional examination of cellular morphology.

Aberrant methylation of promoter CpG islands is a well-known mechanism by which tumor suppressor genes are inactivated in various human malignancies, including lung cancers,⁷ bladder cancers⁸ and colon cancers.⁹ This epigenetic event is known to occur at an early step in the carcinogenic process and has been used to develop methods to improve early detection and prognosis of various human cancers.^{7,10-12} Although epigenetic abnormalities of MM are less well studied, several studies have demonstrated that the *p16INK4a(p16)*^{13,14} and *RASSF1A*^{15,16} tumor suppressor genes, which are frequent targets of epigenetic abnormalities in non-small cell lung cancer (NSCLC), are hypermethylated in MM. As in NSCLC, these changes are frequent early events in MM,¹⁷⁻²⁰ so the detection of methylation abnormalities in the *p16* and *RASSF1A* genes in cytologic specimens of serous fluid represents a potential ancillary approach to improve early detection of MM.

In this study, we evaluated methylation abnormalities of *p16* and *RASSF1A* genes in MM tissue samples from 21 patients and associated body fluid cytology samples initially diagnosed as negative for malignancy, and

assessed the diagnostic value of these methylation abnormalities as a potential supplement to conventional diagnostic methods.

MATERIALS AND METHODS

Patients and sample collection

The study population comprised 21 MM patients who had undergone body fluid aspiration cytologic examination and surgical excision for diagnostic and therapeutic purposes at the Asan Medical Center from 2000 to 2006 (Table 1). Hematoxylin-eosin-stained slides were reviewed and appropriate tissue blocks were selected so as to include at least 50% tumor tissue. Conventional cytologic slides initially scored as negative for malignancy were reviewed, and slides confirmed to be cytologically negative for malignancy were selected. Cytologic samples from eight patients in which effusion was confirmed as resulting from infection or liver cirrhosis were included as non-malignant controls (Table 2). Pleural fluid samples from NSCLC patients who were confirmed as pleural metastasis-negative were used as tumor controls (Table 2).

The mean age of the study population was 53.8 years (range, 38-77) and there was a slight predominance of males (13/21, or 61.9%). Histologically, 15 cases were classified as epithelioid, and 3 each were classified as sarcomatoid and biphasic types; none was metastatic. Tissue and body fluid samples were obtained before adjuvant chemotherapy or radiation therapy with the consent of the patient.

DNA extraction

Genomic DNA was extracted as previously described.²¹ Briefly, 10-mm-thick sections obtained from a representative paraffin-embedded block were deparaffinized by xylene, and were incubated with 1 ml digestion buffer (100 mM NaCl, 10 mM Tris pH 8.0, 25 mM

Table 1. Clinicopathologic characteristics of patients with malignant mesothelioma and methylation patterns of p16 and RASSF1A genes

patient	Age	sex	Histologic subtype	Recur	Treatment	F/U period(d)	Specimen	p16-S	p16-C	RASSF1A-S	RASSF1A-C
patient1	50	M	Epithelioid	No	Surgery+CTX	67	Pleural fluid	Negative	Negative	Negative	Negative
patient2	65	F	Epithelioid	No	Surgery+CTX	270	Ascites	Positive	Negative	Negative	Negative
patient3	56	M	Epithelioid	No	Surgery only	85	Pleural fluid	Positive	Positive	Negative	Negative
patient4	58	M	Epithelioid	Yes	Surgery+RTX	1303	Pleural fluid	Negative	Negative	Negative	Negative
patient5	51	F	Epithelioid	Yes	Surgery+CTX	905	Ascites	Positive	Positive	Positive	Negative
patient6	38	F	Epithelioid	No	Surgery+CTX	1605	Ascites	Positive	Negative	Positive	Negative
patient7	54	M	Epithelioid	No	Surgery+CTX	58	Pleural fluid	Negative	Negative	Negative	Positive
patient8	53	M	Epithelioid	No	Surgery+CTX	456	Pleural fluid	Negative	Negative	Negative	Positive
patient9	38	F	Biphasic	No	Surgery+CTX	455	Pleural fluid	Negative	Negative	Negative	Positive
patient10	53	M	Epithelioid	Yes	Surgery+CRTX	1140	Pleural fluid	Positive	Positive	Positive	Positive
patient11	50	M	Epithelioid	No	Surgery only	21	Pleural fluid	Negative	Negative	Negative	Negative
patient12	51	F	Epithelioid	No	Surgery only	10	Ascites	Negative	Positive	Positive	Positive
patient13	62	F	Epithelioid	No	Surgery+CTX	960	Ascites	Positive	Negative	Positive	Negative
patient14	77	F	Sarcomatoid	No	Surgery only	23	Pleural fluid	Positive	Positive	Positive	Positive
patient15	63	M	Sarcomatoid	No	Surgery+CTX	849	Pleural fluid	Negative	Negative	Positive	Negative
patient16	48	M	Sarcomatoid	No	Surgery+CRTX	129	Pleural fluid	Positive	Negative	Negative	Negative
patient17	56	M	Epithelioid	No	Surgery+CRTX	201	Pleural fluid	Positive	Positive	Negative	Negative
patient18	50	F	Biphasic	No	Surgery only	59	Ascites	Positive	Negative	Positive	Positive
patient19	47	M	Epithelioid	No	Surgery+CTX	66	Pleural fluid	Negative	Negative	Positive	Positive
patient20	65	M	Epithelioid	No	Surgery+CTX	120	Pleural fluid	Negative	Negative	Negative	Negative
patient21	45	M	Biphasic	No	Surgery+CTX	455	Pleural fluid	Positive	Positive	Positive	Negative

Recur represents recurrence; F/U represent follow-up; p16-S represents methylation of p16 gene in surgically resected tissue sample; p16-C represents methylation in body fluid cytology samples; RASSF1A-S represents methylation of RASSF1A gene in surgically resected tissue sample; RASSF1A-C represents methylation in body fluid cytology samples

Table 2. Clinicopathologic characteristics of patients with inflammatory/ non-cancer body fluid and Non-small cell lung cancer

Patient	Age	Sex	Category	Specimen	Disease	<i>p16-C</i>	<i>RASSF1A-C</i>
patient1	31	M	Non-neoplastic	Pleural fluid	Tuberculosis	Negative	Negative
patient2	32	M	Non-neoplastic	Pleural fluid	Tuberculosis	Negative	Negative
patient3	56	M	Non-neoplastic	Pleural fluid	Tuberculosis	Negative	Negative
patient4	35	F	Non-neoplastic	Pleural fluid	Pneumonia	Negative	Negative
patient5	42	M	Non-neoplastic	Pleural fluid	Pneumonia	Negative	Negative
patient6	67	M	Non-neoplastic	Pleural fluid	Pancreatitis	Negative	Negative
patient7	46	M	Non-neoplastic	Ascites	Liver cirrhosis	Negative	Negative
patient8	58	M	Non-neoplastic	Ascites	Liver cirrhosis	Negative	Negative
patient1	48	F	NSCLC	Pleural fluid	Adenocarcinoma	Negative	Negative
patient2	62	M	NSCLC	Pleural fluid	Adenocarcinoma	Negative	Negative
patient3	68	F	NSCLC	Pleural fluid	Adenocarcinoma	Negative	Negative
patient4	68	M	NSCLC	Pleural fluid	Adenocarcinoma	Negative	Negative
patient5	59	M	NSCLC	Pleural fluid	Squamous cell ca.	Negative	Negative
patient6	75	M	NSCLC	Pleural fluid	Squamous cell ca.	Negative	Negative
patient7	50	F	NSCLC	Pleural fluid	Adenocarcinoma	Negative	Positive
patient8	65	F	NSCLC	Pleural fluid	Adenocarcinoma	Negative	Negative
patient9	66	M	NSCLC	Pleural fluid	Squamous cell ca.	Negative	Negative
patient10	58	F	NSCLC	Pleural fluid	Adenocarcinoma	Positive	Negative

NSCLC represents non-small cell lung cancer; ca. represents carcinoma; *p16-C* represents methylation in body fluid cytology samples; *RASSF1A-C* represents methylation in body fluid cytology samples.

EDTA pH 8.0) and proteinase K (0.1 mg/ml) at 50 °C for 12 ~ 18 h. Cytology slides were treated with xylene to remove the cover glass, rinsed, scraped into proteinase K-containing digestion buffer and incubated as described above. Genomic DNA was purified using the phenol/chloroform method.

Bisulfite treatment and methylation-specific PCR

Methylation-specific PCR (MSP) was performed for *p16INK4a* and *RASSF1A* as described by Herman et al.²² The genomic DNA was modified by bisulfite treatment for MSP as follows: Genomic DNA (2 ml) was dissolved in 50 ml water in a microfuge tube and denatured in 0.2 M NaOH at 37 °C for 10 min. For samples containing nanogram quantities of DNA, 1 µg salmon sperm DNA (Sigma Chemical company, St Luis, MO) was added as carrier. Freshly made 10 mM hydroquinone (30 ml) and 3 M sodium bisulfite pH 5 (520 ml) were added and the tubes were incubated at 50 °C

for 16 h. After purification using the Wizard DNA Clean-Up system (Promega, Madison, WI) and elution in 50 ml water, the modified DNA was denatured at room temperature for 5 min in 3 M NaOH, followed by precipitation with 100% ethanol. A 50% volume of 7.5 M NH₄OAc and 20 µg glycogen (Roche Molecular Biochemicals, Mannheim, Germany) were added at -20 °C and DNA was purified by precipitation with 70% ethanol. The precipitated DNA, ready for PCR, was re-suspended in 50 ml DNase/RNase-free water. Primer pairs are described in Table 3.

Five microliters of bisulfite-modified DNA (100 ng) was amplified using primers selective for methylated or unmethylated alleles. The PCR mixture contained 10x PCR buffer, dNTPs (each at 1.25 mM), primers (300 ng each per reaction), bisulfite-modified DNA (50 ng) and 0.5 U Hotstar Taq polymerase (Qiagen, Germany) in a final volume of 20 ml. PCR conditions were as follows: 95 °C for 15 min; 40 cycles of 95 °C for 1 min, 54 ~ 68 °C for 1 min and 72 °C for 1 min; and a final extension at

Table 3. PCR primer sequences and PCR product sizes used for MSP

Gene	Forward primer (5' →')	Reverse primer (5' →')	Size(bp)
<i>p16</i> -M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150
<i>p16</i> -U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151
<i>RASSF1A</i> -M	GGGTTTTGCGAGAGCGCG	GCTAACAAAAGCGAACCG	170
<i>RASSF1A</i> -U	GGTTTTGTGAGAGTGTGTTTAG	CACTAACAAACACAAACCAAAC	171

M represents methylated-specific primers

U represents unmethylated -specific primers

72 °C for 5 min. The PCR products were analyzed by electrophoresis in 2.5% agarose gels and visualized by ethidium-bromide staining.

RESULTS

Frequent hypermethylation of *p16* and *RASSF1A* genes in MM tissue.

Methylated DNA was detected in surgically resected tumor samples from 47.6% (10/21) of patients for *p16* and *RASSF1A*, respectively. Overall, 14 of 21 patients (66.7%) were methylation-positive for at least one of the two genes tested.

The surgically resected tumor samples of 7 of 13 male (53.8%) and 3 of 8 (37.5%) female patients were positive for *p16* methylation, while 8 of 15 patients with the epithelioid subtype and 2 of 3 patients with sarcomatoid histologic features showed hypermethylation for *p16*. There was no significant relationship between *p16* methylation status and sex ($p = 0.47$, $r = 0.21$), histologic subtype ($p = 0.19$, $r = 0.17$) or age ($p = 0.40$, $r = 0.19$) by Pearson χ^2 test.

The surgically resected tumor samples of 4 of 13 male (30.8%) and 6 of 8 (75%) female patients were positive for *RASSF1A* methylation, while 8 of 15 patients with the epithelioid subtype, 1 of 3 patients with sarcomatoid subtype, and 1 of 3 patients with biphasic histologic features showed hypermethylation for *RASSF1A*. There was no significant relationship between *RASSF1A* methylation status and histologic subtype ($p = 0.71$, $r = 0.20$) or age ($p = 0.84$, $r = 0.74$)

by Pearson χ^2 test; however, *RASSF1A* methylation was more frequent among females than among males ($p = 0.049$, $r = 0.19$).

Both *p16* and *RASSF1A* were methylated in surgical samples from 6 of 21 patients (28.6%) and both were unmethylated in 7 of 21 patients (33.3%). Four of 21 patients were positive for *p16* methylation and negative for *RASSF1A* methylation, and an identical number of patients were positive for *RASSF1A* methylation and negative for *p16* methylation. There was no correlation between methylation of the two genes ($p = 0.28$, $r = 0.21$; Pearson correlation coefficient analysis).

Methylation abnormalities in body fluid cytology samples.

Cytologically negative body fluid samples from MM patients showed a higher rate of abnormal methylation of *p16* and *RASSF1A* genes: seven (33.3%) and eight (38.1%) of 21 cases showed methylation of *p16* and *RASSF1A* genes, respectively. Twelve of 21 patients (57.1%) were positive for methylation of at least 1 of these 2 genes by MSP.

The body fluid cytology samples from 4 of 13 male (30.8%) and 3 of 8 (37.5%) female patients were positive for *p16* methylation. Six of 15 patients with the epithelioid subtype and 1 of 3 patients with sarcomatoid histologic features showed hypermethylation for *p16* in cytologic specimens. There was no significant relationship between *p16* methylation status in body fluid and sex ($p = 0.75$, $r = 0.76$), histologic subtype ($p = 0.41$, $r = 0.22$) or age ($p = 0.41$, $r = 0.98$) by Pearson χ^2 test.

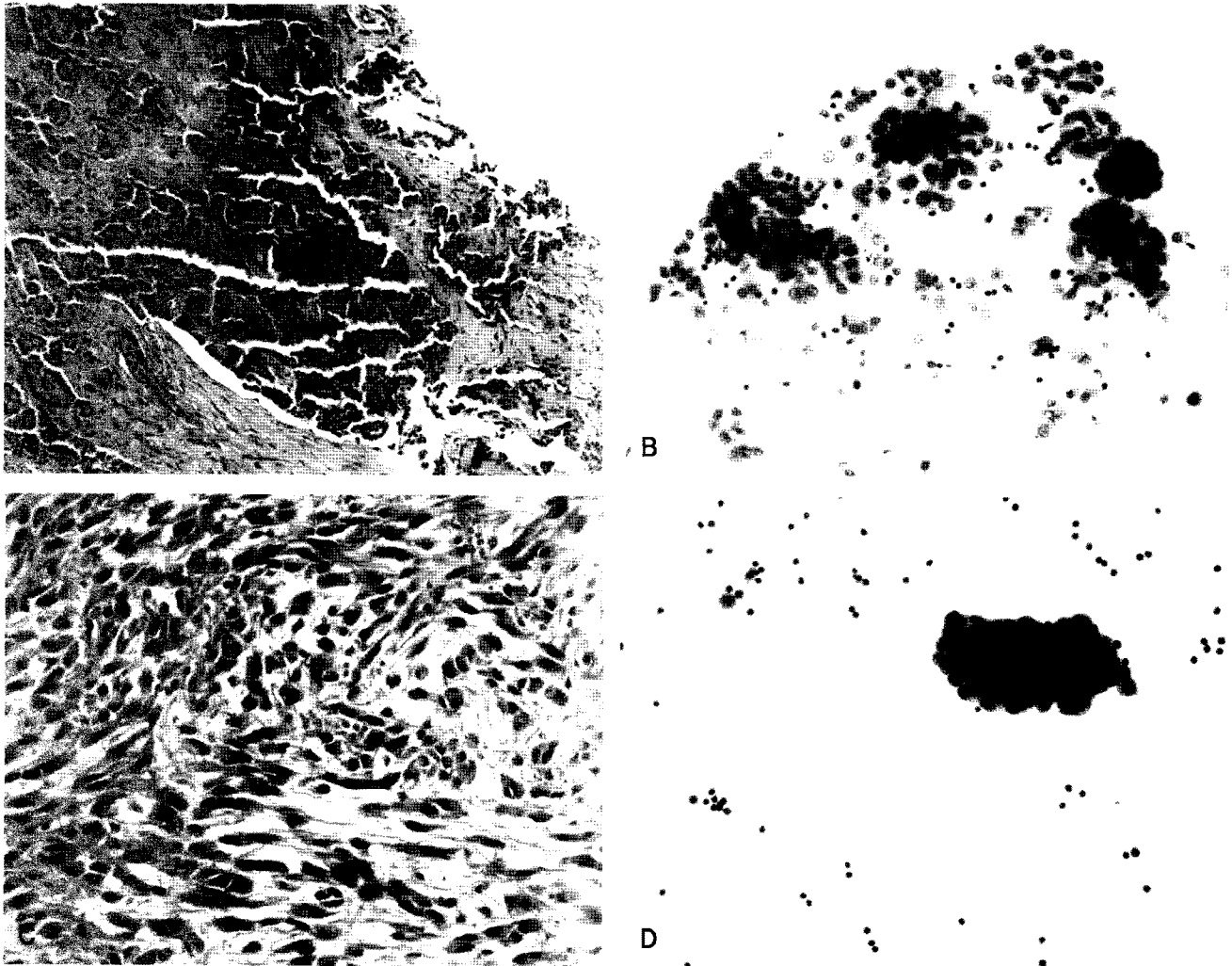


Fig. 1. A. Epithelioid pleural malignant mesothelioma (MM) exhibits tubulopapillary profiles of polygonal cells (patient 8). B. Cytologic specimen of epithelioid pleural MM scored as negative for malignancy and reactive mesothelial hyperplasia. C. An example of sarcomatoid mesothelioma (patient 13) showing atypical spindle cells arranged in fascicles. D. Cytologic specimens from a sarcomatoid pleural MM patient show reactive mesothelial cells. (A,C : H&E, B,D : Papanicolaou stain).

Body fluid cytology samples from 4 of 13 male (30.8%) and 4 of 8 (50%) female patients were positive for *RASSF1A* methylation. Six of 15 patients with the epithelioid subtype and 2 of 3 patients with biphasic histologic features showed hypermethylation for *RASSF1A* in cytologic specimens. There was no significant relationship between *RASSF1A* methylation status in body fluid and sex ($p = 0.38$, $r = 0.40$), histologic subtype ($p = 0.23$, $r = 0.74$) or age ($p = 0.26$, $r = 0.97$) by Pearson χ^2 test.

Both *p16* and *RASSF1A* were methylated in 3 of 21 patients and both were unmethylated in 9 of 21 patients. Four of 21 patients were positive for *p16*

methylation and negative for *RASSF1A* methylation, while 5 were negative for *p16* methylation and positive for *RASSF1A* methylation. There was no correlation between methylation of the two genes in body fluid ($p = 0.76$, $r = 0.069$; Pearson correlation coefficient analysis).

None of the eight body fluid cytology samples from patients with non-neoplastic diseases, which included tuberculosis, bacterial pneumonia and cardiac failure, showed methylation of *p16* or *RASSF1A* genes. Two of ten pleural fluid cytology samples, previously scored as malignancy negative, from surgically confirmed NSCLC patients showed methylation of *p16* and *RASSF1A* genes.

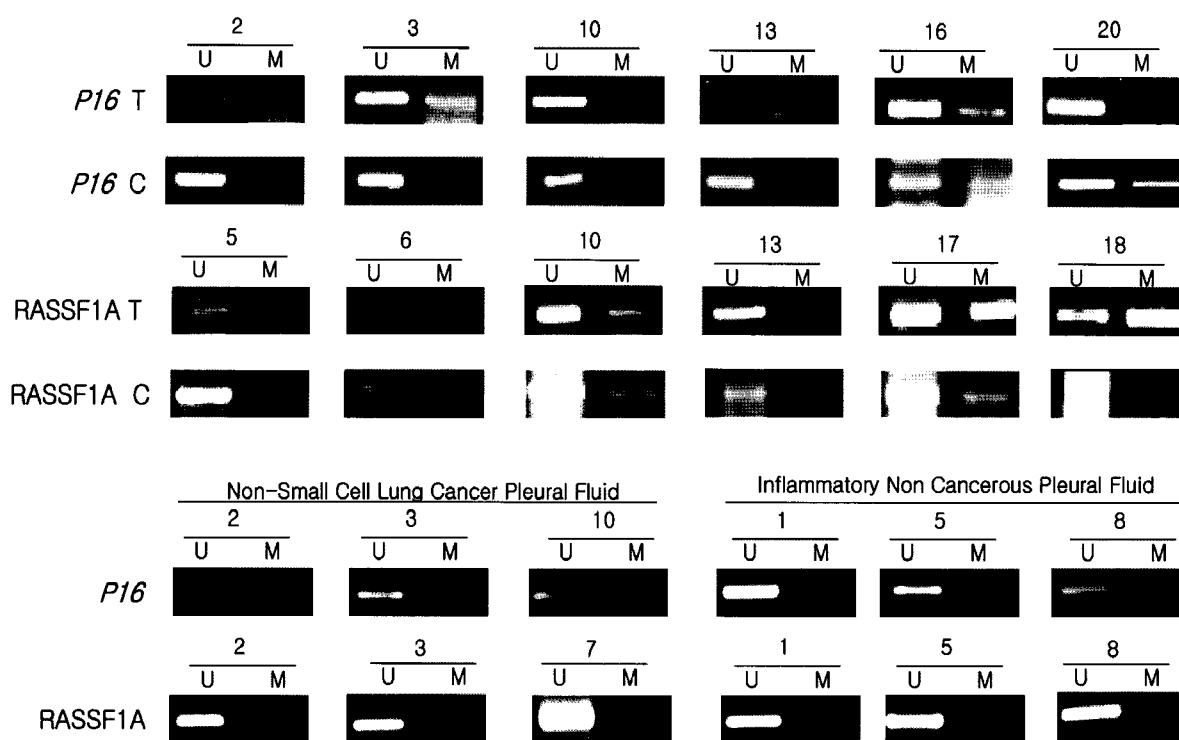


Fig. 2. Examples of representative MSP analyses of malignant mesothelioma patients, NSCLC controls and inflammatory non-neoplastic controls. Products were amplified with primers that recognize methylated and unmethylated sequences, respectively

Correlation of methylation abnormalities between tissues and body fluid cytology samples.

Five of ten patients positive for *p16* methylation in tissue samples and 2 of 11 patients negative for *p16* methylation in tissue samples showed methylation in body fluid samples. The *p16* methylation status concordance rate between cytology samples and tissue samples was 61.9% (13/21). Four of ten patients positive for *RASSF1A* methylation in tissue samples and 4 of 11 patients negative for *RASSF1A* methylation in tissue samples showed methylation in body fluid. The *RASSF1A* methylation status concordance rate between cytology samples and tissue samples was 66.7% (14/21).

DISCUSSION

In this study, we tested the methylation status of *p16* and *RASSF1A* in MM tissues and body fluid cytology

samples from MM, non-neoplastic diseases and NSCLC patients. DNA hypermethylation has been identified as a common mechanism by which tumor suppressor genes with CpG islands in their promoters are inactivated in various human cancers. This epigenetic abnormality is known to occur at an early step in carcinogenesis. Therefore, efforts have been made to use methylation abnormalities as a molecular method to detect cancers at an early stage.^{23,24} As in other malignancies, multiple tumor suppressor genes, including *p16* and *RASSF1A*, are known to be inactivated in mesothelioma.^{13,16} Inactivation of the *p16* gene is relatively common in MM; it occurs most often by homozygous deletion and less frequently as a result of a point mutation.¹³ Hypermethylation is another common *p16* gene-inactivating mechanism in MM, occurring at a frequency that ranges from 4%²⁵ to 80%.²⁶ The 47.6% frequency demonstrated in our study is within this range. *RASSF1A* is also frequently inactivated in MM by hypermethylation, with hypermethylated forms reported in up to 32% of MM patients.¹⁶ The frequency of *RASSF1A*

hypermethylation observed in our study is consistent with this previous report.

Interestingly, we found frequent hypermethylation of *p16* and *RASSF1A* genes, not only in tumor tissues but also in cytologically negative body fluid samples from MM and NSCLC patients; in contrast, no instances of *p16* or *RASSF1A* gene hypermethylation were detected in non-neoplastic diseases. These results suggest that hypermethylation of *p16* and *RASSF1A* is a cancer-specific phenomenon that can be readily detected in cancer tissues and body fluids. However, the methylation status of these genes in tumor tissues and cytologic samples was not 100% concordant. Theoretically, genetic or epigenetic changes in tumor tissue should also be present in cytologic samples if these samples contain tumor cells. On the other hand, if cytologic samples do not contain malignant cells, they should not show the genetic or epigenetic change characteristic of the tumor tissue. In our study, five of ten cases (50%) with hypermethylation of *p16* or *RASSF1A* genes in tumor tissue showed positive results for hypermethylation in cytology samples. These results suggest that even cytologically no definite evidence of malignancy fluid samples may contain degraded tumor cells or tumor-derived DNA that might potentially be useful for molecular diagnostic purposes. The presence of promoter methylation of various genes in bronchoalveolar lavage^{27,28} and serum²⁹⁻³² samples has been shown to serve as a surrogate for methylation of the same genes in tumor tissue. Moreover, circulating DNA found in serum harbors the same genetic characteristics that are observed in paired tumor DNA, as reported by several authors.³¹⁻³³ We agree with other researchers that the prevalence of methylation helps to increase the sensitivity of conventional cytology.

Unexpectedly, among 11 cases negative for hypermethylation in tumor tissues, 1 (patient 12) was hypermethylation-positive for *p16* and 3 (patients 7, 8 and 9) were hypermethylation-positive for *RASSF1A* in cytology samples. These results might be accounted for by the presence of proliferated precancerous mesothelial

cells with methylation abnormalities distinct from MM and concurrent degradation of tumor DNA in surgical samples. Recently, Pu et al.,²⁵ tested whether methylation profiles generated by real-time MSP are useful in differentiating benign, reactive mesothelial cell proliferation (RM) from MM. Using the five gene markers RARb2, GPC3, CDKN2A(*p16*), TERT and cyclinD2, they found DNA hypermethylation not only in MM but also in RM, although the frequency was much lower in RM (33%) than in MM (63%). There were distinct differences between RM and MM, however. For example, CDKN2A methylation was detected in MM but not in RM cases; similarly, *p16* methylation was detected in MM (4% of cases) but not in RM. These differences may be due, in part, to differences in body fluid samples. In our cohort, negative cytologic samples were also obtained from MM patients; these contained mesothelial cells, which are possibly generated during the course of neoplastic transformation. Our data on *p16* methylation status in MM and in non-neoplastic inflammatory body fluid supports this interpretation.

In our experiment, the amount of DNA obtained from cytologic slides of body fluid was limiting and prevented us from analyzing more than two molecular markers. Although analyzing multiple markers might have enhanced our ability to interpret the clinical utility of this approach, our results suggest that testing for methylation abnormalities in a selected single gene or a combination of multiple genes has diagnostic value as a supplementary method to conventional cytological diagnosis, especially in cases where cytological analyses yield ambiguous or unsatisfactory results.

CONCLUSION

Tests to detect methylated DNA in body fluid samples can be used as an less invasive, ancillary diagnostic method for MM. Larger studies are warranted to validate these findings.

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