대한세포병리학회지
ISSN 1017-0391
10.3338/kjc.2008.19.2.126

원 저

체액 세포 도말 검사에서 메틸화 이상이 악성 중피종 진단의 부가적인 분자 표지자로서의 기능

울산대학교의과대학 서울 아산병원 병리과

송 준 선 · 정 진 경 · 강 지 혜 · 황 일 선 · 장 세 진

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

Joon Seon Song, M.D, Jin Kyung Jung, M.S, Ji Hye Kang, M.S, Ilseon Hwang, M.D and Se Jin Jang, M.D, PhD

Department of Pathology, University of Ulsan College of Medicine, Asan Medical Center, Seoul, Korea

논문접수 : 2008년 7월 16일
논문수정 : 2008년 7월 31일
게재승인 : 2008년 8월 26일

책임저자 : 장 세 진
주 소 : (138-736) 서울 송파구 풍납동 388-1
울산대학교 의과대학 서울 아산병원 병리과
전 화 : 02-3010-5966
팩 스 : 02-472-7898
E-mail address : jangsejin@amc.seoul.kr

* This work was supported by KOSEF research grant R01-2004-000-10670-0.

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.

(Korean J Cytopathol 2008;19(2):126-135)

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 (≤ 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 cancers[8] 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 no definite evidence of 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. Clinocopathologic 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	Epitheliod	No	Surgery+CTx	67	Pleural fluid	Negative	Negative	Negative	Negative
patient2	65	F	Epitheliod	No	Surgery+CTx	270	Ascites	Positive	Negative	Negative	Negative
patient3	56	M	Epitheliod	No	Surgery only	85	Pleural fluid	Positive	Positive	Negative	Negative
patient4	58	M	Epitheliod	Yes	Surgery+RTx	1303	Pleural fluid	Negative	Negative	Negative	Negative
patient5	51	F	Epitheliod	Yes	Surgery+CTx	905	Ascites	Positive	Positive	Positive	Negative
patient6	38	F	Epitheliod	No	Surgery+CTx	1605	Ascites	Positive	Negative	Positive	Negative
patient7	54	M	Epitheliod	No	Surgery+CTx	58	Pleural fluid	Negative	Negative	Negative	Positive
patient8	53	M	Epitheliod	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	Epitheliod	Yes	Surgery+CRTx	1140	Pleural fluid	Positive	Positive	Positive	Positive
patient11	50	M	Epitheliod	No	Surgery only	21	Pleural fluid	Negative	Negative	Negative	Negative
patient12	51	F	Epitheliod	No	Surgery only	10	Ascites	Negative	Positive	Positive	Positive
patient13	62	F	Epitheliod	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	Epitheliod	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	Epitheliod	No	Surgery+CTx	66	Pleural fluid	Negative	Negative	Positive	Positive
patient20	65	M	Epitheliod	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. Clinocopathologic characteristics of patients with inflammatory/ non–cancer body fluid and Non–small cell lung cancer

Patient	Age	Sex	Category	Specimen	Disease	*p16*-C	*RASSF1A*-C
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℃ 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℃ for 10 min. For samples containing nanogram quantities of DNA, 1 ㎍ 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℃ 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 NH_4OAc and 20㎍ glycogen (Roche Molecular Biochemicals, Mannheim, Germany) were added at －20℃ and DNA was purified by precipitation with 70% ethanol. The precipitated DNA, ready for PCR, was resuspended 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℃ for 15 min; 40 cycles of 95℃ for 1 min, 54～68℃ for 1 min and 72℃ for 1 min; and a final extension at

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

Gene	Forward primer (5' →3')	Reverse primer (5' →3')	Size(bp)
p16-M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	150
p16-U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	151
RASSF1A-M	GGGTTTTGCGAGAGCGCG	GCTAACAAAAGCGAACCG	170
RASSF1A-U	GGTTTTGTGAGAGTGTGTTTAG	CACTAACAAACACAAACCAAAC	171

M represents methylated-specific primers
U represents unmethylated -specific primers

72℃ 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.

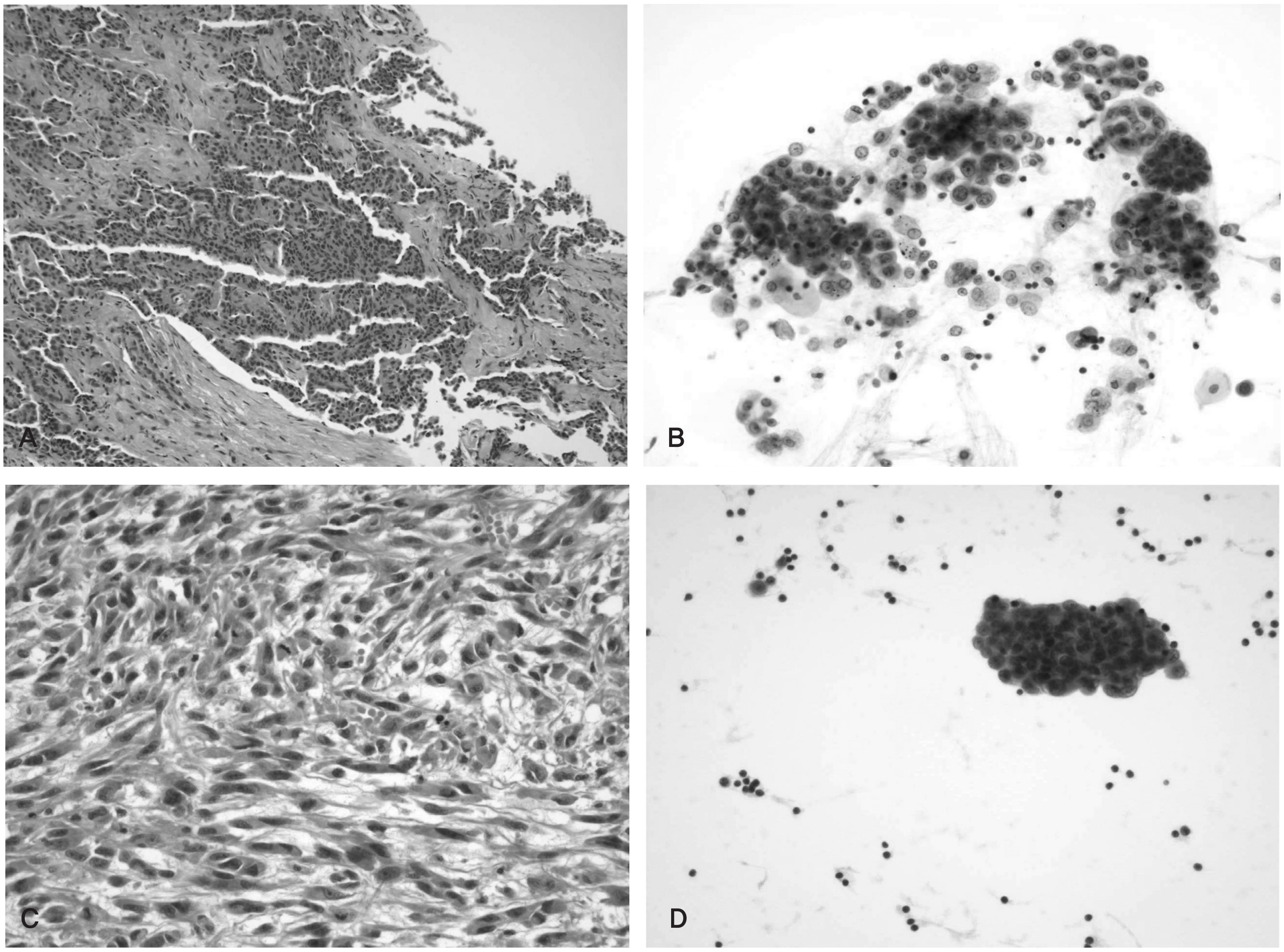

Fig. 1. A. Epithelioid pleural malignant mesothelioma (MM) exhibits tubulopapillary profiles of polygonal cells (patient 8). B. Cytologic specimen of epithelioid pleural MM scored as negative for malignancy and reactive mesothelial hyperplasia. C. An example of sarcomatoid mesothelioma (patient 13) showing 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.

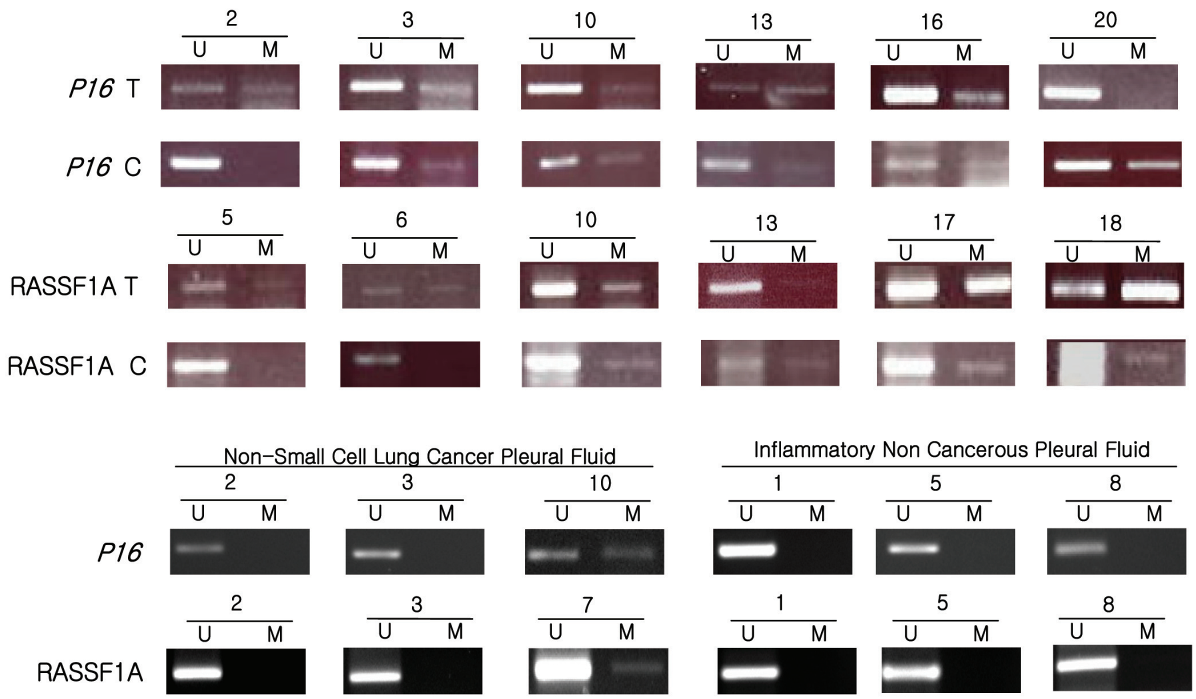

Fig. 2. Examples of representative MSP analyses of malignant mesothelioma patients, NSCLC controls and inflammatory non-neoplastic controls. Products were amplified with primers that recognize methylated and unmethylated sequences, respectively

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 serum[29-32] 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.

REFERENCES

1. Antman KH, Schiff PB and Pass HI Cancer: Principles and Practice of Oncology. 5th edition ed. Philadelphia: Lippincott, 1997; 1853-78.
2. DiBonito L, Falconieri G, Colautti I, Bonifacio Gori D, Dudine S, Giarelli L. Cytopathology of malignant mesothelioma: a study of its patterns and histological bases. *Diagn Cytopathol* 1993;9:25-31.
3. Renshaw AA, Dean BR, Antman KH, Sugarbaker DJ, Cibas ES. The role of cytologic evaluation of pleural fluid in the diagnosis of malignant mesothelioma. *Chest* 1997;111:106-9.
4. Teirstein AS. Diagnosing malignant pleural mesothelioma. *Chest* 1998;114:666-7.
5. Whitaker D, Shilkin KB. Mesotheliomas. Gray W 2nd ed. New York: Churchill Livingstone, 1995; 195-224.
6. Whitaker D, Shilkin KB. Early diagnosis of malignant mesothelioma: the contribution of effusion and fine needle aspiration cytology and ancillary techniques. New York: Garland Law Publishing, 1989; 71-115.
7. Tsou JA, Hagen JA, Carpenter CL, Laird-Offringa IA. DNA methylation analysis: a powerful new tool for lung cancer diagnosis. *Oncogene* 2002;21:5450-61.
8. Marsit CJ, Houseman EA, Christensen BC, et al. Examination of a CpG island methylator phenotype and implications of methylation profiles in solid tumors. *Cancer* Res 2006;66:10621-9.
9. Jair KW, Bachman KE, Suzuki H, et al. De novo CpG island methylation in human cancer cells. *Cancer Res* 2006;66:682-92.
10. Fujiwara K, Fujimoto N, Tabata M, et al. Identification of epigenetic aberrant promoter methylation in serum DNA is useful for early detection of lung cancer. *Clin Cancer Res* 2005;11:1219-25.
11. Wang J, Lee JJ, Wang L, et al. Value of *p16INK4a* and *RASSF1A* promoter hypermethylation in prognosis of patients with resectable non-small cell lung cancer. *Clin Cancer Res* 2004;10:6119-25.
12. Toyooka S, Suzuki M, Maruyama R, et al. The relationship between aberrant methylation and survival in non-small-cell lung cancers. *Br J Cancer* 2004;91:771-4.
13. Hirao T, Bueno R, Chen CJ, Gordon GJ, Heilig E, Kelsey KT. Alterations of the *p16*(INK4) locus in human malignant mesothelial tumors. *Carcinogenesis* 2002;23:1127-30.
14. Wong L, Zhou J, Anderson D, Kratzke RA. Inactivation of *p16INK4a* expression in malignant mesothelioma by methylation. *Lung Cancer* 2002;38:131-6.
15. Toyooka S, Carbone M, Toyooka KO, et al. Progressive aberrant methylation of the *RASSF1A* gene in simian virus 40 infected human mesothelial cells. *Oncogene* 2002;21:4340-4.
16. Toyooka S, Pass HI, Shivapurkar N, et al. Aberrant methylation and simian virus 40 tag sequences in malignant mesothelioma. *Cancer Res* 2001;61:5727-30.
17. Chan EC, Lam SY, Tsang KW, et al. Aberrant promoter methylation in Chinese patients with non-small cell lung cancer: patterns in primary tumors and potential diagnostic application in bronchoalevolar lavage. *Clin Cancer Res* 2002;8:3741-6.
18. Toyooka S, Toyooka KO, Maruyama R, et al. DNA methylation profiles of lung tumors. *Mol Cancer Ther* 2001;1:61-7.
19. Yanagawa N, Tamura G, Oizumi H, Takahashi N, Shimazaki Y, Motoyama T. Promoter hypermethylation of tumor suppressor and tumor-related genes in non-small cell lung cancers. *Cancer Sci* 2003;94:589-92.
20. Harden SV, Tokumaru Y, Westra WH, et al. Gene promoter hypermethylation in tumors and lymph nodes of stage I lung cancer patients. *Clin Cancer Res* 2003;9:1370-5.
21. Zhou JX, Niehans GA, Shar A, Rubins JB, Frizelle SP, Kratzke RA. Mechanisms of G1 checkpoint loss in resected early stage non-small cell lung cancer. *Lung Cancer* 2001;32:27-38.
22. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996;93:9821-6.
23. Fackler MJ, McVeigh M, Evron E, et al. DNA methylation of *RASSF1A*, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. *Int J Cancer* 2003;107:970-5.
24. Pu RT, Laitala LE, Alli PM, Fackler MJ, Sukumar S, Clark DP. Methylation profiling of benign and malignant breast lesions and its application to cytopathology. *Mod Pathol* 2003;16:1095-101.
25. Pu RT, Sheng ZM, Michael CW, Rhode MG, Clark DP, O'Leary TJ. Methylation profiling of mesothelioma using real-time methylation-specific PCR: a pilot study. *Diagn Cytopathol* 2007;35:498-502.
26. Kobayashi N, Toyooka S, Yanai H, et al. Frequent *p16* inactivation by homozygous deletion or methylation is associated with a poor prognosis in Japanese patients with pleural mesothelioma. *Lung Cancer* 2008 [EPub ahead of print].
27. Kim H, Kwon YM, Kim JS, et al. Tumor-specific methylation in bronchial lavage for the early detection of non-small-cell lung cancer. *J Clin Oncol* 2004;22:2363-70.
28. Topaloglu O, Hoque MO, Tokumaru Y, et al. Detection of promoter hypermethylation of multiple genes in the tumor and bronchoalveolar lavage of patients with lung cancer. *Clin Cancer Res* 2004;10:2284-8.
29. Benlloch S, Galbis-Caravajal JM, Martin C, et al. Potential diagnostic value of methylation profile in pleural fluid and serum from cancer patients with pleural effusion. *Cancer* 2006;107:1859-65.
30. Fischer JR, Ohnmacht U, Rieger N, et al. Promoter methylation of *RASSF1A*, RARbeta and DAPK predict poor prognosis of patients with malignant mesothelioma. *Lung Cancer* 2006;54:109-16.
31. Ramirez JL, Rosell R, Taron M, et al. 14-3-3sigma methylation in pretreatment serum circulating DNA of cisplatin-plus-gemcitabine-treated advanced non-small-cell lung cancer patients predicts survival: The Spanish Lung Cancer

Group. *J Clin Oncol* 2005;23:9105-12.
32. Ramirez JL, Sarries C, de Castro PL, et al. Methylation patterns and K-ras mutations in tumor and paired serum of resected non-small-cell lung cancer patients. *Cancer Lett* 2003;193:207-16.
33. Esteller M, Sanchez-Cespedes M, Rosell R, Sidransky D, Baylin SB, Herman JG. Detection of aberrant promoter hypermethylation of tumor suppressor genes in serum DNA from non-small cell lung cancer patients. *Cancer Res* 1999;59:67-70.