tic accuracy of spindle-cell lesions in the pleura. In this study, 6 of 7 (87.5%) pulmonary sarcomatoid carcinomas tested positive for calretinin and 3 of 6 cases (50%) tested positive for D2-40. On the other hand, 33 of 43 (76.7%) sarcomatoid mesotheliomas tested positive for calretinin and 15 of 21 cases (71.4%), for D2-40. D2-40 positivity in the case sarcomatoid mesothelioma tended to be higher than that in the case of pulmonary sarcomatoid carcinoma; however, a definite conclusion could not be drawn from the limited number of pulmonary sarcomatoid carcinoma cases in this study.

The other problem is differential diagnosis between desmoplastic mesothelioma and fibrous pleuritis. In this study, 9 of 65 cases (13.8%) in the "definitely not/unlikely" category were considered to be fibrous pleuritis. Churg et al. [26] provided a summary of distinguishing desmoplastic mesothelioma from fibrous pleurisy (pleuritis) and/or organizing pleuritis in their review article. They showed that fibrous pleuritis typically exhibits "zonation" with high cellularity and cytologic atypia toward the pleural space and increasing fibrosis with decreasing cellularity and lesser atypia toward the chest wall. On the other hand, sarcomatous (desmoplastic) mesothelioma does not exhibit this type of zonation and is sometimes accompanied with bland necrosis and overtly sarcomatous foci. To differentiate between these 2 diseases, a relatively large amount of tissue and/or pleural whole layer tissues is necessary for a thoracoscopic biopsy. Churg documented that needle biopsy is generally inadequate, because "small" thoracoscopic biopsy may not be sufficient to evaluate "zonation" [16].

In this study, 10 carcinomas, i.e., serous papillary adenocarcinomas, adenocarcinoma, NOS, and carcinosarcomas of the genital tract or peritoneum were included in the case of peritoneal cases in females, and only 4 cases were categorized as "probable/definite" epithelioid mesothelioma. The ratio of peritoneal mesotheliomas in Japanese females among all mesothelioma cases is reportedly higher than that in Western countries [27]. In females, the most difficult differential diagnosis is that between peritoneal epithelioid mesothelioma and serous papillary adenocarcinoma of the ovary and/or peritoneum [28]. Baker et al. [29] described the morphological differences between peritoneal epithelioid mesothelioma (PEM) and serous papillary adenocarcinoma (SC). Peritoneal mesothelioma often invades the peritoneal cavity with multiple nodule formation, associated with ascites, or occasionally forms a localized abdominal mass, including mass formation in the ovary [30]. Therefore, SC of ovarian or peritoneal origin [28] is the most important and difficult malignant tumor for differentiation due to the clinical and histological similarities in the 2 cases [29]. Recently, reports on the application of immunohistochemistry for differential diagnosis have emerged. Attanoos et al. [31] described that calretinin and Ber-EP4 are useful discriminant markers for distinguishing PEM in women from SCs and peritoneal carcinoma. Ordonez [9,32] reported that combinations of best positive markers (D2-40 and calretinin) and negative markers (Ber-EP4, MOC-31 and ER) were useful for discriminating between the 2 tumors. The relatively high rate of misdiagnosed cases among clinically diagnosed female peritoneal mesothelioma clinically is a problematic issue. We described calretinin and thrombomodulin as positive markers and Ber-EP4, MOC-31, CA19-9, and ER as negative markers with relatively high sensitivity and specificity [11]. Chemotherapy and/or radiotherapy can significantly improve patient survival and decrease recurrence, especially in primary and secondary SC, as compared with PEM [33–35]. However, Sugarbaker et al. recently reported a remarkable prolongation in the median survival of peritoneal mesothelioma patients treated with an intensive local-regional treatment strategy that included cytoreductive surgery with peritonectomy and hyperthermic intraoperative intraperitoneal chemotherapy; the prolongation was also reported for some patients that received early postoperative intraperitoneal chemotherapy [36]. Therefore, appropriate antibody selection for immunohistochemistry and for sampling a relatively large amount of tumor tissue by laparotomy are necessary for early and precise diagnosis [37]. The application of immunohistochemistry for cytology materials (ascites) is also effective, especially for the epithelioid type mesothelioma [38].

There are various methods to obtain mesothelioma cells and tissues, such as cytology, needle biopsy, and biopsy under thoracoscopy or laparoscopy. Among these methods, pleural or peritoneal biopsy under thoracoscopy or laparoscopy is a reliable method to obtain sufficient tumor tissue from the pleura and peritoneum for accurate pathological diagnosis [39,40].

Cytology from body cavity fluids is also useful for cancer diagnosis; however, morphological observation only by Papanicolaou, Giemsa, and PAS staining would not be sufficient, even for experienced pathologists [41]. In particular, as small amounts of tumor cells from sarcomatoid or desmoplastic mesothelioma are shed into the body cavity, diagnosis by only cytology is impossible [7]. In this study, of the 46 cases for which only the cytological tests were performed, 18 cases (39.2%) (all of epithelioid subtype) belonged to the "probable/definite" category according to the cytological and radiological features, the level of hyaluronan in body fluids, and so on. The cytological criteria for epithelioid mesothelioma were based on classical morphological features such as high cellularity, uniform cell population, intercellular gap, central or paracentral nucleus, multinucleation with atypia, villosity, nuclear pleomorphism, and so on. [41]. On the other hand, 5 cases where cytology specimens were stained by immunohistochemistry, including calretinin, D2-40, CEA, TTF-1, and so on, were included in this study and among them 3 cases (60%) (all of epithelioid subtype) belonged to "probably/definite" category. Therefore, it is assumed that cytology with immunohistochemistry using an appropriate antibody panel may increase the accuracy of mesothelioma diagnosis, especially in the case of epithelioid type mesothelioma [38]. Lyons–Boudreaux recommended the use of D2-40 and MOC-31, which are sensitive and specific markers for mesothelial and epithelial cells, respectively, to improve the diagnostic accuracy with body cavity effusions [38]. Pu et al. reported the utility of WT1, p63, MOC31, and cytokeratin (K903 and CK5/6) immunostains in differentiating adenocarcinoma, squamous cell carcinoma, and malignant mesothelioma in pleural effusions [42]. However, no effective markers are available thus far with sensitivity and specificity high enough to differentiate between epithelioid mesothelioma and benign mesothelial lesions (reactive mesothelial and reactive hyperplasia cells), because the diagnosis of epithelioid mesothelioma is based on the "invasiveness of mesothelial cells" [16]. Therefore, we considered that the cases of the "probable/definite" category among the "cytology-only" category in this study should belong to the "probable" mesothelioma category and not to the "definite" category. We supposed that body fluid cytology is useful for mesothelioma diagnosis; however, adequate tissue sampling by biopsy and immunohistochemistry using an appropriate antibody panel are necessary for "definite" mesothelioma diagnosis. Recently, Hanley et al. reported the utility of anti-L523S antibody (antibody to K homolog domain containing protein overexpressed in cancer (KOC)) in combination with calretinin and CK5/6 for differentiating reactive mesothelial cells from malignant mesothelioma and metastatic carcinoma [43]. Further efforts to evaluate new markers useful for differentiating mesothelioma especially from benign mesothelial lesions are required.

In conclusion, it is ascertained that the diagnosis of mesothelioma in females and in the case of peritoneal and sarcomatoid subtype cases has relatively low diagnostic accuracy, and approximately 15% of the deaths by mesothelioma in Japan are diagnostically suspicious. Therefore, precise pathological procedures, including immunohistochemistry using an appropriate antibody panel selected based on histology and clinical information, are necessary for accurate mesothelioma diagnosis. Moreover, the nationwide mesothelioma registration system must also be

established for obtaining precise data on mesothelioma for epidemiological study. These efforts will help promote early detection and therapy of mesothelioma and facilitate significant improvements in patient prognoses.

Conflict of interest statement

None.

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Soluble mesothelin-related protein in pleural effusion from patients with malignant pleural mesothelioma

NOBUKAZU FUJIMOTO, KENICHI GEMBA, MICHIKO ASANO, SAE WADA, KATSUICHIRO ONO, SHINJI OZAKI and TAKUMI KISHIMOTO

Department of Respiratory Medicine, Okayama Rosai Hospital, Okayama 7028055, Japan

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Abstract. Malignant pleural mesothelioma (MPM) is a highly aggressive neoplasm primarily arising from surface serosal cells of the pleura and is strongly associated with asbestos exposure. Patients with MPM often develop pleural fluid as initial presentation. However, cytological diagnosis using pleural fluid is usually difficult and has limited utility. A useful molecular marker for differential diagnosis particularly with lung cancer (LC) is urgently needed. The aim of the present study was to investigate the diagnostic value of soluble mesothelin-related protein (SMRP) in pleural fluid. Pleural fluids were collected from 23 patients with MPM, 38 with LC, 26 with benign asbestos pleurisy (BAP), 5 with tuberculosis pleurisy (TP) and 4 with chronic heart failure (CHF), and the SMRP concentration was determined. All data were analyzed by using non-parametric two-sided statistical tests. The median concentration of SMRP in MPM, LC, BAP, TP and CHF were 11.5 (range 0.90-82.80), 5.20 (0.05-36.40), 6.65 (1.45-11.25), 3.20 (1.65-6.50) and 2.03 (1.35-2.80) nmol/l, respectively. The SMRP concentration was significantly higher in MPM than in the other diseases (P=0.001). The area under the ROC curve (AUC) values of the MPM diagnosis was 0.75 for the differential diagnosis from the other groups. Based on the cut-off value of 8 nmol/l, the sensitivity and specificity for diagnosis of MPM were 70.0 and 68.4%, respectively. These results indicate that the SMRP concentration in pleural fluid is a useful marker for the diagnosis of MPM.

Introduction

Malignant pleural mesothelioma (MPM) is a highly aggressive tumor with a poor survival rate that arises from the surface cells of the pleura. It is a rare tumor; however, MPM has become a very serious public health concern in Japan. A newspaper article, published in June 2005, reported that five residents who had lived near a now-closed asbestos cement pipe plant in Amagasaki, Japan, developed pleural mesothelioma (1). The industrial use of asbestos has been banned in Japan since 2006, but the incidence of MPM is expected to continue increasing for the next few decades due to the past usage of asbestos (2).

MPM has therapeutic and diagnostic challenges. Surgical resection, often combined with radiotherapy or adjuvant chemotherapy, is indicated for the treatment of MPM in the earlier stage. There is a small population of patients who achieve prolonged disease-free survival. Yet the majority of cases are already progressive at the time of diagnosis, and these patients exhibit an extremely poor prognosis (3). Systemic chemotherapy or radiotherapy to date has not had an impact on patient survival for advanced cases. Thus, it is quite important to diagnosis MPM at an early stage. Most MPM cases demonstrate pleural effusion at the time of diagnosis, but cytological diagnosis with pleural effusion is usually difficult and has limited utility. To obtain a definite diagnosis, a thoracoscopic or percutaneous biopsy should be performed to obtain adequate specimens for pathological and immunohistochemical analyses. Yet, even with these procedures, it is sometimes difficult to differentiate MPM from other pleural diseases including benign asbestos pleurisy (BAP), tuberculosis pleurisy (TP), or pleural metastasis of lung cancer (LC). Several investigators have sought to improve the differential diagnosis of pleural effusion by measuring tumor markers. Shi *et al* reported the usefulness of measuring the pleural carcinoembryonic antigen for the diagnosis of malignant pleural effusion (4). Similar findings were reported regarding cytokeratin 19 fragment 21-1 and carbohydrate antigen (CA) 125, CA15-3 and CA19-9 (5). Aoe *et al* previously reported that the concentration of receptor-binding cancer antigen expressed on Siso cells (RCAS1) was higher in malignant pleural effusion than in non-malignant effusion (6), but the usefulness of these markers has not yet been fully established in clinical practice. A useful molecular marker for the differential diagnosis of these diseases is therefore urgently needed.

Mesothelin is a 40-kDa cell surface glycosylated phosphatidylinositol (GPI)-anchored glycoprotein which has putative functions in cell-to-cell adhesion (7). Mesothelin

Correspondence to: Dr Nobukazu Fujimoto, Department of Respiratory Medicine, Okayama Rosai Hospital, 1-10-25 Chikkomidorimachi, Minamiku, Okayama 7028055, Japan
E-mail: nfuji@okayamaH.rofuku.go.jp

Key words: mesothelin, mesothelioma, asbestos

Table I. Patient characteristics.

	MPM	PMLC	BAP	TP	CHF
No.	23	38	26	5	4
Age (years)					
Median (range)	64 (47-89)	70 (48-90)	75.5 (58-88)	82 (68-88)	74 (68-82)
Gender					
Male/Female	21/2	28/10	26/0	5/0	3/1
Asbestos exposure period (years)					
Median (range)	33 (5-51)	-	30 (3-46)	-	
Histology					
Epithelioid	15	-	-	-	
Biphasic	2	-	-	-	
Sarcomatoid	4	-	-	-	
Unknown	2	-	-	-	
Adenocarcinoma		24			
Squamous cell carcinoma		3			
Small-cell carcinoma		4			
Not determined		7			
Stage					
I	3	-	-	-	
II	2	-	-	-	
III	9	-	-	-	
IV	6	-	-	-	
Unknown	3	-	-	-	

MPM, malignant pleural mesothelioma; PMLC, pleural metastasis of lung cancer, BAP, benign asbestos pleurisy; TP, tuberculosis pleurisy; CHF, chronic heart failure.

is expressed on normal mesothelial cells (8); however, it is highly overexpressed in cancers such as MPM (9,10), pulmonary carcinomas (11-14) and other neoplasms (15,16). Soluble mesothelin-related protein (SMRP) is recognized as a cleaved fragment of membrane-bound mesothelin (17). Robinson and colleagues reported that serum SMRP levels were elevated in MPM when compared with healthy asbestos-exposed and non-exposed subjects, and with other pulmonary diseases including LC (18). Similar results were reported by Cristaudo *et al* (19) and Schneider *et al* (20) who demonstrated that SMRP blood concentrations were significantly higher in MPM than in LC cases. These findings suggest the usefulness of serum SMRP as a diagnostic or screening marker of MPM.

The SMRP value in pleural fluid was evaluated by Scherpereel *et al* (21) and Pass *et al* (22). Both research groups reported that the pleural SMRP value was higher than that in serum, and the level was higher in MPM than in other pulmonary diseases. Therefore, the aim of the present study was to investigate the SMRP level in pleural fluid in Japanese patients with MPM. For this purpose, SMRP concentrations in pleural fluid from Japanese patients with MPM were examined and compared with those of patients with BAP, TP or LC. Correlations between SMRP and asbestos exposure were also examined.

Materials and methods

Materials. Pleural fluid was collected from patients with MPM. For these cases, pathological diagnosis of MPM was confirmed based on standard H&E staining and positive immunohistochemical reactivity to mesothelial markers such as calretinin, Wilms' tumor 1, or thrombomodulin, and negative reactivity to carcinoembryonic antigen. The clinical stage of MPM was determined according to the International Mesothelioma Interest Group (IMIG) criteria (23) and was based on staging procedures including computed tomographic (CT) scans of the chest and abdomen, magnetic resonance images of the brain and Technetium-99m hydroxymethylene diphosphonate bone scans. Survival data of the patients with MPM were determined from the day of diagnosis to the day of death or last follow-up. Pleural fluid was also collected from patients with LC, BAP, TP and with chronic heart failure (CHF) as controls. LC was diagnosed in cases where lung cancer cells were detected in the pleural effuion. Histological subtypes of LC were based on the World Health Organization (WHO) classification (24). The clinical stage of the disease was assessed using the International Staging System (25). TP was diagnosed in cases in which *Mycobacterium tuberculosis* was detected in the pleural fluid. TP was also diagnosed in cases with higher concentrations of adenosine deaminase

(>50 IU/l) and when lymphocyte dominancy was shown in the fluid. CHF was diagnosed in cases which demonstrated transudate fluid with known cardiac diseases. The diagnosis of BAP was determined by exclusion of other specific causes in patients with past asbestos exposure, in which malignant diseases were ruled out with thoracoscopy. Informed consent was provided by all patients, and the study was conducted with approval of the appropriate institutional review boards.

SMRP measurement. SMRP was measured using the chemiluminescent enzyme immunoassay (CLEIA) (Fujirebio Diagnostics, Malvern, PA, USA) based on the 2-step sandwich method. In brief, 20 µl of sample was mixed with 180 µl of sample diluents, then 20 µl of the diluted sample was incubated with 250 µl of anti-SMRP antibody-coated ferrite particles at 37˚C for 10 min. After washing, 250 µl of anti-SMRP antibodies coupled with alkaline phosphate was added and incubated at 37˚C for 10 min. After a washing step, 200 µl of substrate [3-(2'-spiroadamantane)-4-methoxy-4-(3"-phosphoryloxy) phenyl-1,2-dioxetane disodium salt; AMPPD] solution was added, followed by incubation at 37˚C for 5 min. Luminescence at a wavelength of 477 nm was measured, and the SMRP concentration of each sample was calculated with the standard curve method.

Asbestos body burden. Quantification of asbestos bodies was performed using the protocol modified by Kohyama and Suzuki (26). In brief, portions of paraffin-embedded normal lung tissue (1-2 g) obtained from surgery or autopsy were deparaffinized with xylene, then microcut. These were digested with solution containing 5-20% sodium hypochlorite and KOH for 6 h at 60˚C. Following digestion, samples were pelleted and resuspended in distilled water. Samples were then mixed well and filtered through a cellulose ester membranous filter which was dehydrated and cut in half. Pieces of the filter were mounted on microscope slides and dried with acetone vapor. Asbestos bodies were then counted, and the asbestos bodies per (wet weight) gram of lung were calculated.

Statistical analyses. Comparisons between groups were performed using the Kruskal-Wallis test and non-parametric analysis using the Mann-Whitney U test. Areas under receiver operating curves (ROC) were calculated using standard techniques. Survival data were determined from the day of diagnosis to the day of death or last follow-up and analyzed based on the Kaplan-Meyer method. Correlations between pleural SMRP values and asbestos body or patient survival were calculated based on Pearson's correlation coefficient (PCI). Statistical calculations were performed with SPSS Statistical Package version 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics. Between January 2004 and July 2007, pleural fluids were collected from 23 patients with MPM, 38 with LC, 26 with BAP, 5 with TP and 4 with CHF at the Okayama Rosai Hospital. Of the 23 cases (median age 64 years; range 47-89; male/female 21/2) diagnosed with MPM, there were 15 epithelioid, 2 biphasic, 4 sarcomatoid and 2 unknown pathological subtypes. According to the IMIG staging system, there were 3 cases in stage I, 2 in stage II, 9 in stage III, 6 in stage IV and 3 unknown. Of the 38 cases (median age 69.5 years; range 46-91; male/female 29/9) diagnosed with LC, there were 24 patients with adenocarcinoma, 4 with small-cell carcinoma, 3 with squamous cell carcinoma and 7 undetermined pathological subtypes. The characteristics of the patients are summarized in Table I.

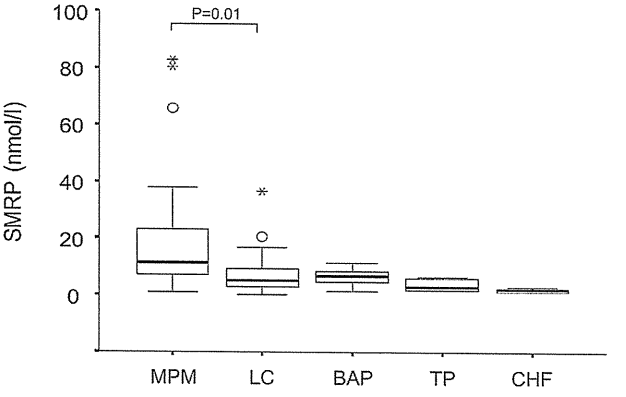

Figure 1. SMRP concentrations in pleural fluid. MPM, malignant pleural mesothelioma; LC, lung cancer; BAP, benign asbestos pleurisy; TP, tuberculosis pleurisy; CHF, chronic heart failure.

SMRP value in MPM. According to the clinical stage and pathological subtypes of MPM, a trend was noted in which the SMRP value was higher in advanced stages (III and IV, n=16; median 13.8, range 2.85-82.8 nmol/l) compared with the value in early stages (I and II, n=5; median 7.9, range 2.5-33.9 nmol/l), and higher in epithelioid type (n=13; median 15.4, range 2.2-82.8 nmol/l) than in sarcomatoid (n=4; median 13.8, range 2.85-10.45 nmol/l), though there were no significant differences (P=0.158 and 0.389, respectively).

SMRP and asbestos exposure. Occupational asbestos exposure was revealed in 21 patients with MPM. We examined the duration of asbestos exposure and the SMRP value in the pleural fluid, but no correlation was shown (PCI, -0.069). Quantification of asbestos bodies was performed in 17 cases of MPM. The median number of bodies was 2,180 (239-526,000) per gram of dried lung. We examined the correlation between the SMRP value in pleural fluid and the number of asbestos bodies, but no correlation was found (PCI, -0.156). Survival data was available in 22 cases. No correlation was found between the SMRP value and survival (PCI, -0.179). We compared the survival of two groups, those with a lower concentration of SMRP (≤8.0 nmol/l) and those with a higher concentration, but no statistical difference was demonstrated (data not shown).

SMRP value for differential diagnosis. The median concentration of SMRP in MPM, LC, BAP, TP and CHF were 11.5 (range 0.9-82.8), 5.2 (0.05-36.4), 6.65 (1.45-11.25), 3.20 (1.65-6.5) and 2.03 (1.35-2.8) nmol/l, respectively. The SMRP concentration was significantly higher in MPM than in the other diseases (P=0.001, Kruskal-Wallis test, Fig. 1). The area under the ROC curve (AUC) values of the MPM diagnosis

was 0.75 [95% confidence interval (CI), 0.615-0.884] for the differential diagnosis from the other groups. Based on the cut-off value of 8 nmol/l, the sensitivity and specificity for diagnosis of MPM were 70.0 and 68.4%, respectively. The SMRP concentration in MPM was significantly higher than that in LC (P=0.004, Mann-Whitney U test). The AUC for the differential diagnosis of MPM and LC was 0.724 (95% CI, 0.583-0.866). Based on the cut-off value of 8 nmol/l, the sensitivity and specificity for diagnosis of MPM were 69.6 and 68.4%, respectively. The SMRP concentration in MPM was significantly higher than in BAP (P=0.004, Mann-Whitney U test). The AUC value for the differential diagnosis of MPM and BAP was 0.74 (95% CI, 0.586-0.894). Based on the cut-off value of 8 nmol/l, the sensitivity and specificity for diagnosis of MPM were 69.6 and 69.2%, respectively.

Discussion

In this study, we first examined the SMRP value in pleural fluid from patients with MPM. SMRP was higher in the epithelioid subtype than in the sarcomatoid, and higher in advanced stages (III and IV) than in early stages (I and II), though the differences were not statistically significant. These findings collaborate a previous study by Scherpereel *et al* (21). They examined the SMRP values, both in serum and pleural fluid, and reported that SMRP both in serum and pleural fluid was higher in the epithelioid subtype and in advanced diseases of MPM. The differences in our study were not statistically significant, probably due to the small number of samples, but our results reflect a similar trend in MPM in Japan. In addition, we examined the correlation between pleural SMRP and overall survival of patients with MPM, but no correlation was found. The role of serum SMRP as a prognostic marker was examined by Cristaudo *et al*. In their study, a high SMRP level in serum was an independent negative prognostic factor in patients with MPM (19). The present study is the first report to examine the role of pleural SMRP as a prognostic factor, but these results should be interpreted carefully because of the small number of cases. Further studies are warranted to clarify the role of pleural SMRP as a prognosis predictive marker.

We next examined the usefulness of pleural SMRP as a diagnostic marker of MPM. We compared the SMRP value in the pleural fluid of MPM to that of LC, BAP, TP and CHF. The SMRP value in MPM was significantly higher than in the other diseases. Similar findings were also reported by Scherpereel *et al* (21). They reported that the serum or pleural fluid SMRP level was significantly higher in patients with MPM than in subjects with benign pleural lesions related to asbestos exposure (BPLAE) or in LC. In their report, BPLAE was defined based on the definition by the American Thoracic Society (27), which corresponds with BPE in our study. In our study, subjects with TP and CHF were also included as controls. TP is the single most frequent cause of death by an infectious agent and is also a major cause of pleural effusion (28). Several molecular markers in pleural effusion have been examined as diagnostic markers of TP (29), but the differential diagnosis is still often problematic in clinical practice. Our results revealed, for the first time, the usefulness of pleural SMRP to distinguish MPM and TP.

We also analyzed the correlations between the SMRP concentration and asbestos exposure. We determined the number of asbestos bodies in the lungs of patients with MPM. The duration of occupational asbestos exposure was determined through patient interview. As a result, no correlation was revealed between SMRP values and the duration of asbestos exposure or asbestos bodies in the lung. These findings indicate that elevation of SMRP in the pleural effusion of MPM is not influenced by asbestos, but is one of the cancer-specific events. The mechanisms of accumulation of SMRP in pleural fluid have not as yet been established. SMRP is reported as a proteolytically cleaved fragment of membrane-bound mesothelin (17). The release of SMRP could also be due to a frameshift mutation of the protein (21). Further studies are warranted to examine the mechanisms involved in the elevation of SMRP in MPM.

In conclusion, we examined the SMRP concentration in pleural fluid from patients with MPM, LC, BAP, TP and CHF and demonstrated that the SMRP value in MPM was significantly higher than that in the other diseases. These results indicate the usefulness of pleural SMRP as a diagnostic marker of MPM.

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Aberrant promoter methylation of *WIF-1* and *SFRP1, 2, 4* genes in mesothelioma

HIDEKAZU KOHNO[1], VISHWA JEET AMATYA[1], YUKIO TAKESHIMA[1], KEI KUSHITANI[1], NOBORU HATTORI[2], NOBUOKI KOHNO[2] and KOUKI INAI[1]

[1]Department of Pathology, [2]Molecular and Internal Medicine, Hiroshima University, Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

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Abstract. *WIF-1* is a negative regulator of the Wnt-signaling pathway that may have important implications for tumorigenesis. Microarray analysis of whole genome expression in mesothelioma tissue revealed down-regulation of 491 genes and up-regulation of 167 genes involved mainly in Jak-STAT signaling (8 genes), MAPK signaling (16 genes) and Wnt signaling (13 genes) pathways. Of these, *WIF-1* gene was down-regulated in mesothelioma 72-fold compared to normal tissue. We also analyzed *WIF-1* and *SFRP*s promoter methylations in 46 mesothelioma tissues, 8 mesothelioma cell lines by methylation-specific polymerase chain reaction (MSP). *WIF-1* promoter methylation was observed in 34 of 46 mesothelioma tissues (73.9%) and in all 8 mesothelioma cell lines. *SFRP1, 2* and *4* promoter methylation was observed in 21 of 37 (56.8%), 26 of 42 (61.9%) and 17 of 36 (47.2%) mesothelioma tissues, respectively. Promoter methylation of any *WIF-1* and/or *SFRP* genes was observed in 44 of 46 (95.6%) mesothelioma tissues. The treatment of mesothelioma cell lines with 5-aza-2'-deoxycytidine (5-aza-2dC) showed *WIF-1* promoter methylation recovery followed by restoration of WIF-1 expression in 6 of 8 mesothelioma cell lines. The cytoplasmic expression of ß-catenin was observed in 38 of 43 cases of mesothelioma without any nuclear reactivity. The eight mesothelioma cell lines and 27 cases of mesothelioma examined showed no mutation in exon 3 of *ß-catenin* suggesting no alteration of canonical Wnt signaling pathway. Our data suggest that *WIF-1* promoter methylation is a common event in mesothelioma.

Introduction

Mesothelioma derived from pleura or other mesothelial surfaces is an aggressive tumor with a poor prognosis (1). Exposure to asbestos fibers is considered to be the main cause of mesothelioma, although the mechanism of tumorigenesis from mesothelial cells to mesothelioma is still unknown. Although mutations and deletions of *p53* or *RB* tumor suppressor genes occur frequently in many cancers, those changes are extremely rare in mesothelioma (2,3). Cytogenetic analyses have shown frequent deletions of various chromosome loci in mesothelioma (4,5). The loss and/or inactivation of tumor suppressor genes, *CDKN2A/ARF* at 9p21 or *NF2* at 22q12, are primarily reported in mesothelioma (1). Recent studies on tumorigenesis have focused on epigenetic alterations including promoter methylation or histone deacetylation for playing roles in gene silencing without altering DNA sequence (6,7). *RASSF1A*, *ESR1*, *IGFBP3*, *APC*, *CCND2*, *HPPBP1*, *BMP3b* and *BMP6* have been reported to be down-regulated by promoter methylation in mesothelioma. It has been suggested that these types of epigenetic changes contribute to tumorigenesis in mesothelioma (8-11).

Down-regulation of *WIF-1* gene due to hypermethylation of its promoter has also been observed in other human cancers, including prostate, breast, lung, urinary bladder and gastrointestinal malignancies (12-14). Furthermore, *WIF-1* promoter methylation was found in 69.4% of malignant pleural effusions in NSCLC patients (13), in 81.4% of early colorectal tumors (15) and in 92% of frozen mesothelioma tissue samples (16). In the present study, we analyzed the whole gene expression of mesothelioma using genechip microarray and examined *WIF-1* promoter methylation in mesothelioma tissue and in mesothelioma cell lines as well as the effects of a demethylating agent. Our aim was to ascertain the role of *WIF-1* promoter methylation in tumorigenesis of mesothelioma.

Materials and methods

Gene expression analysis. Frozen tissues from four mesothelioma were crushed in liquid nitrogen and total RNA was isolated using the TRIzol reagent (Invitrogen Corp., Carlsbad, CA, USA), following by DNase treatment for 30 min at 37°C (Turbo DNase, Ambion, TX, USA) and tested with RNA StdSens Analysis kit using Experion automated electrophoresis system (Bio-Rad Laboratories, CA, USA). Human genome focus array (Affymetrix Inc., CA, USA), containing 8500 gene probes was used to analyze gene expression profiles. cDNA was synthesized from 5 μg total RNA using

Correspondence to: Professor Kouki Inai, Department of Pathology, Hiroshima University, Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
E-mail: koinai@hiroshima-u.ac.jp

Key words: mesothelioma, WIF-1, methylation, demethylation, SFRP

Table I. Primer sequence and PCR conditions.

	Sense primer	Antisense primer	PCR product (bp)	Cycle no.	Annealing temperature (°C)
WIF-1 promoter					
Methylated	GGGCGTTTTATTGGGCGTAT	ACGAAACCAACAATCAACGAAAC	201	40-45	60
Unmethylated	GGGTGTTTTATTGGGTGTATTGTA	AAAACCAACAATCAACAAAACAAAT	199	40-45	55
SFRP1 promoter					
Methylated	GGGGATTGCGTTTTTTGTTTTC	CATACCGACTCTACGCCCTA	109	40-45	62
Unmethylated	GTTTTTTGTTTGTTGGGGTT	ATAAAAATACACACCACCTC	109	40-45	62
SFRP2 promoter					
Methylated	GGGTTTGTAGCGTTTCGTTC	ACCCGCTCTCTTCGCTAAAT	113	40-45	60
Unmethylated	GGGTTTGTAGTGTTTTGTT	ACCCACTCTCTTCACTAAAT	113	40-45	56
SFRP4 promoter					
Methylated	GTTTTTTGTTTGTCGGGGTC	ATAAAAATACGCACCGCCTC	133	40-45	58
Unmethylated	GTTTTTTGTTTGTTGGGGTT	ATAAAAATACACACCACCTC	133	40-45	54
ß-catenin exon3					
ß-cat1	AAAGTAACATTTCCAATCTACTAATGC	CTGTGGTAGTGGCACCAGAA	163	40	60
ß-cat2	GAATCCATTCTGGTGCCACT	TGACTTTCAGTAAGGCAATGAAAA	178	40	60
WIF-1 mRNA					
RT-PCR	CCGAAATGGAGGCTTTTGTA	TGGTTGAGCAGTTTGCTTTG	188	30	62
GAPDH mRNA					
RT-PCR	CGGAGTCAACGGATTTGG	GGCAACAATATCCACTTTACC	79	30	62

the SuperScript II Reverse Transcriptase (Invitrogen Corp.) and purified using Genechip Sample Cleanup Module (Affymetrix Inc.) according to the manufacturer's instructions. Biotin-labeled cRNA was synthesized using the Genechip IVT Labeling kit (Affymetrix Inc.) and purified using Genechip Sample Cleanup Module (Affymetrix Inc.). Yield and size distribution of the labeled transcripts were determined with NanoVue spectrophotometer (GE Healthcare Bio-Sciences, Uppsala, Sweden) and Experion automated electrophoresis system (Bio-Rad Laboratories). After fragmentation using the fragmentation buffer from Genechip Sample Cleanup Module, cRNA were hybridized to the Human Genome Focus Array. Genechip were automatically stained with streptavidin-phycoerythrin by using a fluidic station (Affymetrix Inc.) and scanned by Genechip Scanner 3000 (Affymetrix Inc.). The resulting images were processed by the accompanying software (Microarray Suite 5.0; Affymetrix Inc.). A global scaling approach was used to normalize signal intensities (TGT value = 500). The generated .CEL files after normalized data, with signal intensities >1.5, were analyzed with web-based GeneSifter analysis (Geospiza Inc., WA, USA). This program also produced gene ontology and z-score reports. The ontology were organized according to the principles of the Gene Ontology Consortium and included biological processes, molecular functions and cellular components. Statistical analysis of individual gene expression was performed with Student's t-test and corrected with Benjamini and Hochberg.

Formalin-fixed paraffin-embedded tissue samples. Forty-six mesothelioma tissue samples were obtained from the Department of Pathology at Hiroshima University. These samples included tissues from 38 male and 8 female patients with a mean age of 62.3 years, ranging from 26 to 83. The microscopic slides were reviewed in order to confirm the diagnosis, and they were reclassified using the current WHO histological classification (17). Histologically, the samples comprised 24 epithelioid, 9 biphasic and 13 sarcomatoid mesotheliomas. Twenty-four non-neoplastic peripheral lung tissues containing visceral pleura (NPLVP) were also examined as a control in the study, 12 of the cases were obtained from lung cancer patients. The anonymized (unlinkable) tissue samples are provided by the Department of Pathology to investigators for molecular analyses. This is in accordance with the Ethics Guidelines For Human Genome/Gene Research enacted by the Japanese Government as tissue specimens are collected and used, based on the approval of the Ethics Review Committee of the Hiroshima University.

DNA extraction and methylation-specific PCR. Genomic DNA was extracted from mesothelioma tissues and cell lines using QIAamp DNA Mini kit (Qiagen, Hilden, Germany). Bisulfite modification of ~2 μg of genomic DNA was carried out with Epitect Bisulfite kit (Qiagen) according to the manufacturer's protocol. Universal methylated DNA (Chemicon International, Temecula, CA, USA) and unmethylated DNA were also included. Methylation-specific PCR was conducted on approximately 200 ng of bisulfite-modified DNA with methylation-specific and unmethylation-specific primers in 20 μl PCR volume using Epitect MSP kit (Qiagen) according to the manufacturer's protocol. The PCR products were resolved by electrophoresis on a 2% agarose gel. The gel was stained with ethidium bromide and photographed under UV

Table II. Highly altered gene expression in mesothelioma.

Gene ID	Mean expression (in log2 value)		Expression ratio	Regulation	Adjusted P-value[a]
	Normal tissue	Mesothelioma			
SFTPA2B	14.7453	2.879625	3731.85	Down	0.0005
SFTPC	13.9464	2.66835	2483.31	Down	0.0005
SCGB1A1	11.1261	2.49493	396.5	Down	0.00006
SFTPB	12.7158	4.393872	320	Down	0.0002
LAMP3	11.3303	3.27922	265.23	Down	0.0009
AGER	10.6757	2.716178	248.92	Down	0.0004
SFTPD	12.0554	4.18174	234.54	Down	0.0184
CYP2B6	9.99538	2.147642	230.36	Down	0.0004
FOLR1	9.99264	2.667368	160.37	Down	0.0016
C4BPA	9.68517	2.846748	114.44	Down	0.00006
CYP4B1	10.6188	3.85538	108.64	Down	0.0071
LMO3	9.75603	3.025715	106.18	Down	0.0017
PGC	8.95029	2.748875	73.59	Down	0.0004
WIF1	10.2253	4.06439	71.55	Down	0.0066
CPA3	8.01564	1.8815	70.24	Down	0.000005
CACNA2D2	9.10564	3.428962	51.15	Down	0.0004
SPP1	2.73033	8.330343	48.5	Up	0.181
CA4	8.34102	2.902375	43.37	Down	0.0005
VIPR1	8.38322	3.087003	39.29	Down	0.0289
EMP2	12.4312	7.18791	37.88	Down	0.0873

[a]Statistical analysis of log tranformed data by t-test with Benjamini and Hochberg correction.

transillumination. The primers and their annealing temperatures for the methylated and unmethylated sequences are summarized in Table I.

ß-catenin mutation analysis. The 10-μm tissue sections, after brief staining with hematoxylin were subjected to DNA extraction from tumor cell nests using sterilized curette. The DNA from dissected samples was extracted using QIAamp DNA Mini kit (Qiagen). For mutational analysis, the samples were subjected to PCR with two pair of primers (Table I) encompassing exon 3 of the ß-catenin gene, which contains the consensus GSK-3ß phosphorylation sites, using Primestar HS (Takara Bio, Tokyo, Japan). The electrophoresis of PCR products was done in a 2.5% (w/v) agarose gel, visualized under UV light with ethidium bromide staining, the specific PCR product was recovered using a QIAquick Gel Extraction kit (Qiagen). Isolated PCR products were sequenced on an Applied Biosystems 3130x Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) using BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems Inc.).

Mesothelioma cell lines and treatment with 5-aza-2-deoxycytidine (5-aza-2dC). Eight mesothelioma cell lines, ACC-MESO1, ACC-MESO4 and HMMME purchased from the RIKEN BRC Cell Bank, Japan and MSTO-211H, NCI-H28, NCI-H226, NCI-H2052, NCI-H2452 purchased from American Type Culture Collection, Manassas, VA, USA. All cell lines were cultured in RPMI-1640 Glutamax media supplemented with 10% fetal bovine serum and 1% kanamycin, 1% fungizone at 37°C in a humid incubator with 5% CO_2 (all purchased from Invitrogen Corp.). The cells were seeded at 10^5 cells in a 60-mm culture dish, allowed to attach for 24 h and then treated with 10 and 30 μM of 5-aza-2dC (Wako Pure Chemical Industries, Osaka, Japan) for 6 days. Culture media and 5-aza-2dC were changed every 48 h.

Real-time reverse transcription polymerase chain reaction (real-time RT-PCR). Total RNA from $1x10^5$ harvested cell lines treated with and without the demethylating agent was extracted, and amplification of *WIF-1* mRNA was performed using the Power SYBR Green Cells-to-CT kit (Ambion, Austin, TX, USA) with Mx3000P real-time PCR system (Stratagene, Madison, WI, USA). RT-PCR primer sequences for WIF-1 and GAPDH are listed in Table I.

Western blot analysis. Total proteins from the mesothelioma cell line cultured with and without 5-aza-2dC treatments were prepared using cell lysis protein extraction reagent (Cell-LyEX1 kit, ToyoB-net, Tokyo, Japan). Equal amounts of proteins were subjected to electrophoresis on a 10% SDS-polyacrylamide ReadyGel J using Mini-protean tetra cell (Bio-Rad Laboratories). The proteins were transferred to Hybond-P PVDF membrane (GE Healthcare Life Sciences, Buckinghamshire, UK) using semi-dry blot (Bio-Rad Laboratories). Proteins were detected using the WesternDot Western blotting kit (Invitrogen Corp.) using Western Q (Scithrobe, Tokyo, Japan). The primary polyclonal antibodies were anti-WIF-1 (1:1000; Cell Signaling Technologies, Danvers, MA, USA) and ß-actin (N21, 1:1000; Santa Cruz Biotechnology, CA, USA) as a normalizing reference.

Table III. JAK-STAT signaling, MAPK signaling and Wnt signaling pathway gene expression in mesothelioma.

Gene ID	Chromosome	Expression ratio	Direction	P-value[a]
Wnt signaling pathway				
MMP7	11	7.59	Down	1.20E-05
CAMK2G	10	5.8	Up	8.53E-06
PPP2R5A	1	4.47	Down	0.048016839
FZD4	11	3.76	Down	0.000287084
FRAT1	10	3.46	Down	0.04216696
DKK2	4	2.73	Down	0.000369127
MAPK10	4	2.65	Down	0.002119702
PLCB4	20	2.59	Down	0.011296314
PPP2R5C	14	2.2	Down	0.041980397
LRP5	11	1.91	Down	0.02905675
PPP2CB	8	1.83	Down	0.047037039
GSK3B	3	1.67	Up	0.016049389
MAPK signaling pathway				
CACNA2D2	3	51.15	Down	4.20E-06
PLA2G1B	12	11.35	Down	2.87E-05
FGFR4	5	9.68	Down	7.81E-06
HSPB1	7	4.4	Up	0.022335849
RAPGEF2	4	3.4	Down	0.020796777
MAP4K4	2	3.3	Up	0.024964528
PLA2G10	16	2.75	Down	0.038125675
MAPK10	4	2.65	Down	0.002119702
CACNA1D	3	2.38	Down	0.017128842
JUND	19	2.23	Down	0.04987878
FGFR2	10	2.05	Down	0.041253873
PDGFB	22	2	Down	0.040237895
MAP2K2	19	1.97	Up	0.031953726
BDNF	11	1.95	Down	0.00173388
SRF	6	1.72	Down	0.000149666
MAP4K1	19	1.66	Down	0.00933087
Jak-STAT signaling pathway				
SOCS2	12	6.21	Down	0.004980347
PIM1	6	5.87	Up	0.0185666
CSF2RB	22	5.52	Down	0.039279884
LEPR	1	4.36	Down	0.010789499
IL3RA	XIY	2.57	Down	1.57E-05
STAT4	2	2.28	Down	0.00699128
IL2RG	X	2.13	Down	0.040775092
IL11RA	9	2	Down	0.041638272

[a]Statistical analysis of log tranformed data by t-test with Benjamini and Hochberg correction.

Immunohistochemistry. Immunohistochemistry for the detection of WIF-1 expression was performed using 3-μm tissue sections from formalin-fixed paraffin-embedded tissue blocks and cell line blocks on APS coated slides. The tissue sections were deparaffinized by four changes of xylene and rehydrated through a graded series of ethanol. The antigens were retrieved by autoclaving the tissue sections at 121˚C for 10 min, immersed in citrate buffer (0.01 M, pH 6.0). Endogenous peroxidase was inactivated by treatment with 0.3% H_2O_2 in PBS for 30 min, and then the tissue sections were incubated with anti-WIF-1 antibody (1:1000; Cell Signaling Technologies) and anti-ß-catenin antibody (1:50; Dako, Glostrup, Denmark) at 4˚C overnight in humidified chamber. The reaction was visualized using Simple Stain MAX PO kit and diaminobenzidine (Nichirei Biosciences Inc., Tokyo, Japan). The tissue sections were weakly counterstained for nuclei with Mayer's hematoxylin. The bronchial epithelial cells in and around each tumor were considered to be internal positive controls and immunohistochemical staining with the omission of the primary antibody was performed as a negative control. The immunohistochemical scoring was based on the cytoplasmic staining in tumor cells. Weak immunoreactivity

Table IV. Frequency of promoter methylation of *WIF-1* and *SFRP*s in mesothelioma and NPLVP.

	Mesothelioma (%)				
	Epithelioid	Sarcomatoid	Biphasic	Total	NPLVP[a] (%)
WIF-1	19/24 (79.2)	9/13 (69.2)	6/9 (66.7)	34/46 (73.9)	4/24 (16.7)
SFRP1	10/20 (50.0)	7/10 (70.0)	4/7 (57.1)	21/37 (56.8)	9/24 (37.5)
SFRP2	12/23 (52.2)	7/11 (63.6)	7/8 (87.5)	26/42 (61.9)	12/22 (54.5)
SFRP4	7/21 (33.3)	4/8 (50.0)	6/7 (85.7)	17/36 (47.2)	4/15 (26.7)

[a]Positive methylation in NPLVP was observed in the normal tissue obtained from lung cancer patients.

in <5% of the tumor cells was considered negative and scored as 0. Immunoreactivity in 5-50% of the tumor cells was scored as 1+ and immunoreactivity in >50% of the tumor cells was scored as 2+. Cases were considered positive when >5% of the tumor cells showed WIF-1 expression.

Results

Gene expression analysis of malignant mesothelioma. Analysis of whole genome microarray expression in mesothelioma revealed down-regulation of 491genes and up-regulation of 167 genes with highly expressed genes as mentioned in Table II. Of these, 8, 16 and 13 genes were involved with JAK-STAT signaling, MAPK signaling and Wnt signaling pathways are indicated in Table III. *WIF-1* gene was down-regulated in mesothelioma 72-fold compared to normal tissue. This finding led us to analyze the promoter methylation of *WIF-1* and its similar *SFRP* (secreted frizzled-related protein) genes. Both of these genes inhibit activation of the Wnt-signaling pathway by preventing the binding of Wnt ligands to the frizzled transmembrane receptors.

WIF-1 and SFRP promoter methylation in mesothelioma and non-neoplastic pleural tissue. *WIF-1* promoter methylation was found in 34 of 46 (73.9%) of mesothelioma tissue samples and in 4 of 24 (16.7%) NPLVP. All four of the methylated NPLVP samples were obtained from lung cancer cases. The frequency of *WIF-1* promoter methylation in mesothelioma was significantly higher than that in non-neoplastic pleural tissue ($P<0.01$) and did not differ among the histologic types, including epithelioid, sarcomatoid and biphasic types. *SFRP1*, *2* and *4* promoter methylation was found in 56.8, 61.9 and 47.2% of mesothelioma tissues and in 37.5, 54.5 and 26.7% of NPLVP, respectively. The frequencies of SFRP promoter methylation were higher in mesothelioma than in non-neoplastic pleural tissue, although the differences were not statistically significant. The number of cases with methylation of the promoter for any one of *WIF-1* or *SFRP1*, *2* and *4* was significantly higher in mesothelioma tissues (44 of 46, 95.6%) than in NPLVP (12 of 24, 50%) ($P<0.01$) (Table IV). Electropherograms showing *WIF-1* and *SFRP* promoter methylation of representative cases are shown in Fig. 1.

WIF-1 and SFRP promoter methylation in mesothelioma cell lines. Methylation-specific PCR revealed *WIF-1* promoter methylation in the 8 mesothelioma cell lines (Fig. 2A) (Table IV). Promoter methylation of *SFRP1*, *2* and *4* genes were also found in 7, 7 and 3 of 8 mesothelioma cell lines, respectively (Fig. 2B).

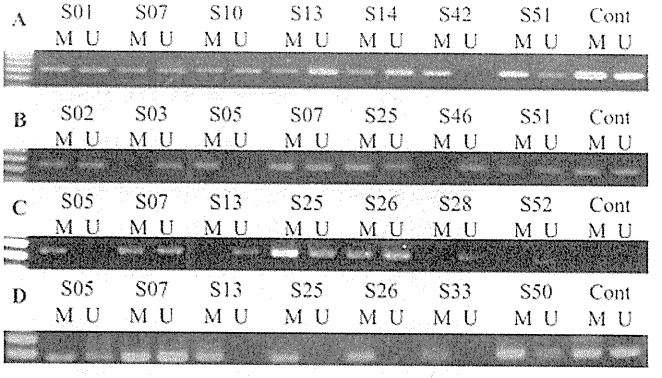

Figure 1. Methylation-specific PCR. Mesothelioma tissues show *WIF-1* (A), *SFRP1* (B), *SFRP2* (C) and *SFRP4* (D) promoter methylation as methylated DNA products (lane M) amplified using methylation-specific primers and unmethylated DNA products (lane U) amplified using unmethylation-specific primers. Methylated (universal methylated DNA) and unmethylated control DNA samples were amplified as controls.

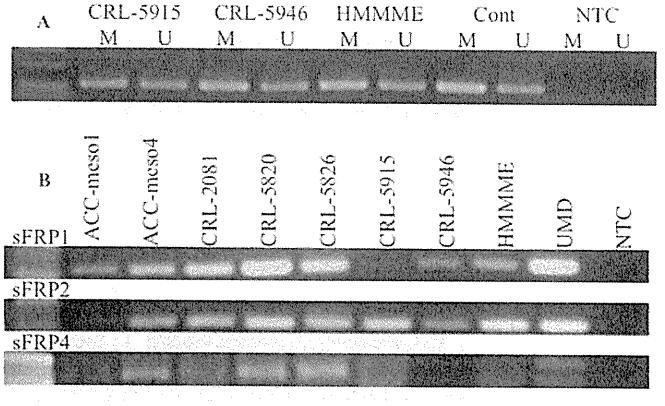

Figure 2. Methylation-specific PCR analysis in mesothelioma cell lines. Mesothelioma cell lines show *WIF-1* (A) and *SFRP1*, *2* and *4* promoter methylation [(B), only methylated bands are shown]. M, methylated DNA product amplified with methylation-specific primers; U, unmethylated DNA product amplified with unmethylation-specific primers; Cont, control methylated or unmethylated DNA; NTC, non-template control; UMD, universal methylated DNA.

WIF-1 promoter methylation status and WIF-1 expression after 5-aza-2dC treatment. *WIF-1* mRNA was detected in 6 of 8 mesothelioma cell lines (ACC-MESO1, ACC-MESO4,

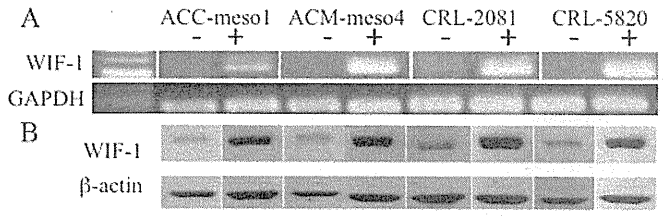

Figure 3. Real-time RT PCR shows amplification of WIF-1 mRNA in a 5-aza-2dC treated mesothelioma cell line compared to amplification of GAPDH mRNA in cell lines treated either with or without 5-aza-2dC. The PCR product from real-time RT-PCR was electrophoresed in a 2% agarose gel followed by ethidium bromide staining (A). Western blot analysis revealed re-expression of WIF-1 protein in cell lines treated with 5-aza-2dC (B). -, cell line without 5-aza-2dC treatment; +, cell line with 5-aza-2dC treatment.

HMMME, MSTO-211H, NCI-H28, NCI-H226) after 5-aza-2dC treatment (Fig. 3A). Western blot analysis (Fig. 3B) showed increased expression of WIF-1 protein in all of these 6 mosothelioma cell lines after 5-aza-2dC treatment.

WIF-1 expression in mesothelioma tissue and cell lines. Eight of 37 cases (21.6%) of mesothelioma showed cytoplasmic staining of WIF-1 in tumor cells (Fig. 4) (Table IV). Furthermore, 3 of 28 (10.7%) mesothelioma cases with *WIF-1* promoter methylation and 5 of 9 (55.6%) mesothelioma cases without *WIF-1* promoter methylation showed WIF-1 expression. *WIF-1* promoter methylation was statistically correlated to WIF-1 expression (P=0.011). In addition, all 20 cases of non-

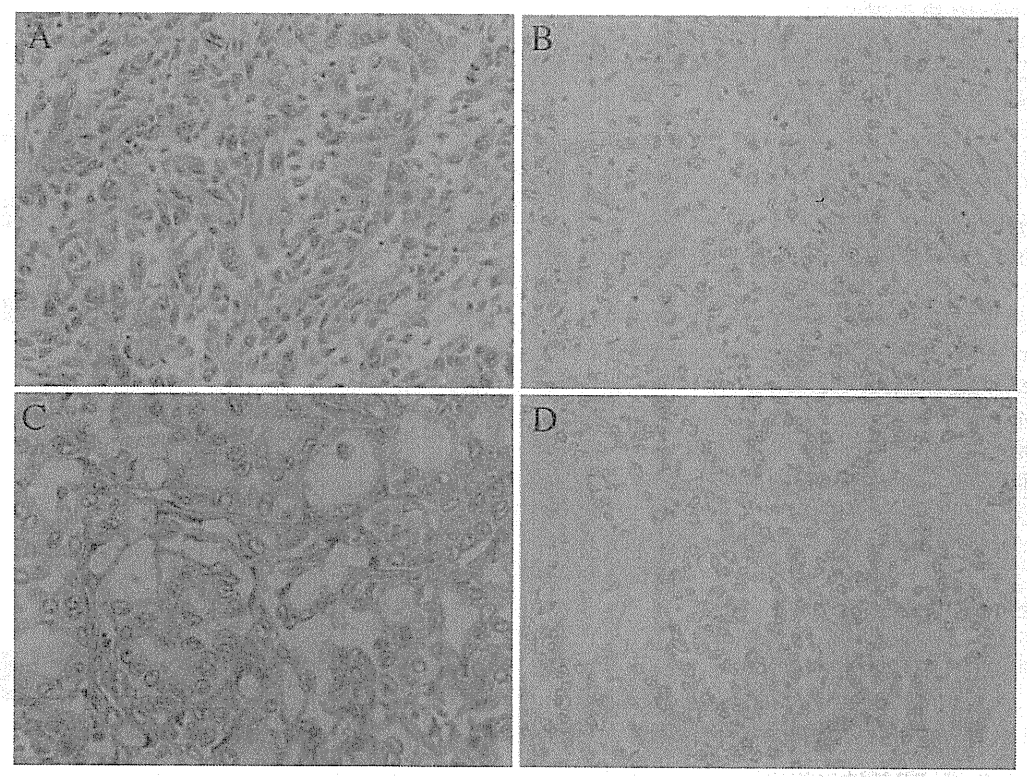

Figure 4. Immunohistochemistry. Sarcomatoid mesothelioma (A) with *WIF-1* promoter methylation shows no WIF-1 immunoreactivity (B). Epithelioid mesothelioma (C) without *WIF-1* promoter methylation shows WIF-1 immunoreactivity in the cytoplasm of tumor cells (D).

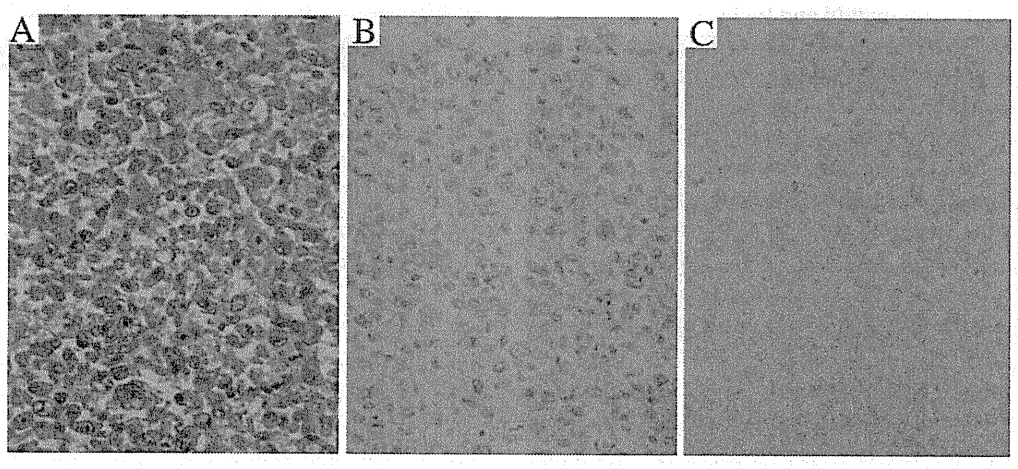

Figure 5. Immunohistochemistry. Mesothelioma cells, ACC-Meso1 (A) that had no expression of WIF-1 (B) prior to treatment with demethylating agents show membranous and cytoplasmic expression of WIF-1 after 5-aza-2dC treatment (C).

Table V. WIF-1 and SFRP promoter methylation, ß-catenin mutation, WIF-1 and ß-catenin expression in mesothelioma tissue and cell lines.

Sample ID	Age	Gender	Histology	Site	Methylation				ß-catenin mutation	Immunoreactivity score	
					WIF-1	*SFRP1*	*SFRP2*	*SFRP4*		WIF-1	ß-catenin
Mesothelioma tissue											
MM-A02	64	M	Sarcomatoid	Pleura	M	NI	NI	M	ND	0	2
MM-A03	50	M	Sarcomatoid	Peritoneum	MU	MU	MU	MU	ND	0	1
MM-A08	50	F	Biphasic	Peritoneum	U	U	NI	NI	ND	2	2
MM-A09	75	M	Sarcomatoid	Pleura	M	U	MU	NI	ND	0	1
MM-A10	76	F	Epithelioid	Pleura	U	U	MU	U	ND	1	1
MM-A11	86	M	Sarcomatoid	Pleura	MU	MU	U	NI	ND	0	0
MM-A13	70	M	Epithelioid	Pleura	MU	U	MU	M	ND	1	1
MM-A14	47	M	Epithelioid	Pleura	M	U	U	M	ND	1	1
MM-A16	83	M	Biphasic	Pleura	MU	NI	M	M	ND	1	1
MM-A17	78	M	Biphasic	Peritoneum	MU	U	U	NI	ND	2	1
MM-A18	65	M	Sarcomatoid	Pleura	MU	MU	M	NI	ND	0	0
MM-A21	49	M	Sarcomatoid	Pleura	U	NI	NI	MU	ND	1	1
MM-A22	56	M	Biphasic	Pleura	U	MU	MU	U	ND	0	2
MM-A24	65	M	Epithelioid	Pleura	MU	U	MU	NI	ND	1	2
MM-A25	70	M	Sarcomatoid	Pleura	U	MU	MU	U	ND	1	1
MM-A28	58	M	Biphasic	Pleura	U	U	MU	M	ND	2	NI
MM-A30	73	M	Epithelioid	Pleura	MU	MU	MU	MU	(-)	0	2
MM-A37	62	M	Epithelioid	Peritoneum	M	NI	NI	NI	ND	1	1
MM-A38	79	M	Sarcomatoid	Pleura	MU	U	U	NI	ND	0	0
MM-A39	66	F	Biphasic	Pleura	MU	NI	MU	MU	(-)	0	0
MM-A40	75	M	Epithelioid	Pleura	U	MU	MU	U	ND	1	2
MM-A41	42	F	Epithelioid	Pleura	U	U	U	NI	(-)	0	0
MM-S01	26	M	Epithelioid	Pericardium	MU	NI	U	U	(-)	2	2
MM-S02	68	M	Sarcomatoid	Pleura	U	MU	MU	U	(-)	0	1
MM-S03	53	M	Epithelioid	Tunica vaginalis	MU	U	U	U	(-)	0	2
MM-S05	69	M	Biphasic	Pleura	M	M	M	MU	(-)	0	2
MM-S07	73	M	Sarcomatoid	Pleura	MU	MU	MU	MU	(-)	0	2
MM-S10	56	M	Sarcomatoid	Pleura	MU	NI	U	NI	(-)	0	2
MM-S13	75	F	Epithelioid	Peritoneum	MU	U	U	U	(-)	0	2
MM-S14	69	M	Biphasic	Pleura	MU	M	MU	M	(-)	0	2
MM-S15	54	M	Sarcomatoid	Pleura	U	MU	U	U	(-)	0	2
MM-S16	61	M	Epithelioid	Pleura	M	NI	U	M	(-)	0	2
MM-S17	77	M	Epithelioid	Pleura	M	NI	M	U	(-)	2	2
MM-S20	67	M	Epithelioid	Pleura	M	M	U	M	(-)	0	1
MM-S25	66	F	Epithelioid	Pleura	MU	MU	MU	U	(-)	0	1
MM-S26	61	M	Sarcomatoid	Pleura	M	U	MU	U	(-)	0	1
MM-S31	72	M	Epithelioid	Pleura	U	MU	MU	U	(-)	1	1
MM-S33	51	M	Biphasic	Pleura	MU	MU	MU	M	(-)	0	2
MM-S35	46	M	Epithelioid	Pleura	U	MU	U	U	(-)	0	2
MM-S36	46	M	Epithelioid	Pleura	MU	MU	MU	U	(-)	0	1
MM-S42	56	M	Epithelioid	Pleura	M	U	U	U	(-)	2	2
MM-S46	48	F	Epithelioid	Peritoneum	MU	U	MU	U	(-)	0	NI
MM-S48	69	M	Epithelioid	Pleura	MU	MU	U	U	(-)	0	NI
MM-S50	48	F	Epithelioid	Pericardium	MU	U	U	MU	(-)	2	2
MM-S51	58	M	Epithelioid	Pleura	MU	MU	MU	U	(-)	1	2
MM-S52	60	M	Epithelioid	Pleura	MU	MU	MU	M	(-)	1	1

Table V. Continued.

Sample ID	Age	Gender	Histology	Site	Methylation				ß-catenin mutation	Immunoreactivity score	
					WIF-1	*SFRP1*	*SFRP2*	*SFRP4*		WIF-1	ß-catenin
Mesothelioma cell lines											
ACC-MESO-1					MU	MU	U	U	(-)	0	2
ACC-MESO-4					MU	MU	MU	MU	(-)	0	2
MSTO-211H					MU	MU	MU	U	(-)	0	2
NCI-H28					MU	MU	MU	MU	(-)	0	2
NCI-H226					MU	MU	MU	MU	(-)	0	2
NCI-H2052					MU	U	MU	U	(-)	0	2
NCI-H2452					MU	MU	MU	U	(-)	0	2
HMMME					MU	MU	MU	U	(-)	0	2

M, methylated; U, unmethylated; NI, not informative; ND, not done.

neoplastic pleural tissue without *WIF-1* promoter methylation showed WIF-1 expression in the cytoplasm of mesothelial cells or bronchial epithelial cells. Immunohistochemical staining of mesothelioma cell lines showed no expression of WIF-1 (Fig. 5B), however, the restoration of WIF-1 expression was observed in all of these 6 mesothelioma cell lines after 5-aza-2dC treatment (Fig. 5C). Two other cell lines showed no expression of *WIF-1* mRNA or protein regardless of 5-aza-2dC treatment.

ß-catenin expression and mutation. ß-catenin nuclear immunoexpression was not observed in any of 43 cases. However, the cytoplasmic expression, crowded near to nucleus, was found 38 cases, 17 cases showing cytoplasmic expression in <10% of the tumor cells (immunoscore 1) and 21 cases showing cytoplasmic expression in >10% of the tumor cells (immunoscore 2). The 8 mesothelioma cell lines also showed cytoplasmic expression in >10% of the tumor cells (immunoscore 2). Mutation analysis of 27 cases of mesothelioma tissue and 8 mesothelioma cell lines did not show any mutation in GSK-3ß phosphorylation sites of exon 3 of *ß-catenin* gene (Table V).

Discussion

In the present study, microarray analysis of whole genome expression in mesothelioma revealed down-regulation of 491 genes and up-regulation of 167 genes. Thirteen genes were involved with Wnt signaling pathways. Wnt-signaling pathway has been shown to play a critical role in human carcinogenesis. Overexpression of Wnt has been reported in many cancer types and suggested to play an important role in Wnt signaling in both lung cancer and mesothelioma (18,19). The role of WIF-1, a wnt inhibitory protein, in carcinogenesis is becoming more clearly understood. We found WIF-1 expression was down-regulated in mesothelioma 72-fold compared to normal tissue. We tried to explain the down-regulation of *WIF-1* gene by MSP analysis of the promoter methylation of *WIF-1*. We also analyzed the promoter methylation of *SFRP1, 2, 4* (secreted frizzled-related protein) genes, other Wnt inhibitory proteins, although microarray analysis did not show alteration of SFRP expression. Both of these genes inhibit activation of the Wnt-signaling pathway by preventing the binding of Wnt ligands to the frizzled transmembrane receptors. The frequency of *WIF-1* methylation in mesothelioma tissues was similar to that reported in previous studies (13,15,29) and its frequency is higher in mesothelioma compared to that of other genes, such as *p16* (11.4%), *RASSF1A* (20.2%) and *IGFBP-3* (32%) (8,9,20). This result suggests that WIF-1 methylation might play an important role in mesothelioma. The three different Wnt-signaling pathways, Wnt-ß-catenin (canonical), planar cell polarity and Wnt-Ca^{2+} pathways (non-canonical) are known; canonical pathway being studied in details. We analyzed the expression of ß-catenin in mesothelioma tissue and cell lines. We found the cytoplasmic expression of ß-catenin in most of the tumor cells without its accumulation in nucleus. In addition, we could not detect any mutation in GSK-3ß phosphorylation sites of exon 3 of *ß-catenin* gene, suggesting that mesothelioma did not involve the canonical pathway. Microarray analysis of this study did not reveal *ß-catenin* alteration. Instead, we found alteration of PLC and CaMKII, genes involved in Wnt-Ca^{2+} pathway. Therefore, it may be presumed that Wnt-signaling pathway alteration in mesothelioma is not the canonical pathway, but the possibility of alteration in Wnt-Ca^{2+} pathway. Further detailed study is needed to confirm such hypothesis.

Treatment with 5-aza-2dC has been reported to result in re-expression of *WIF-1* in bladder cancer cell lines (21) and gastrointestinal cancer cell lines (14). In the present study, we treated 6 mesothelioma cell lines with the demethylating agent 5-aza-2dC and observed re-expression of *WIF-1* mRNA and WIF-1 protein. We also found that WIF-1 protein expression was down-regulated in 82.1% of the methylated mesothelioma tissues examined. These results suggest that the *WIF-1* gene is down-regulated by methylation of its promoter in

both mesothelioma cell lines and mesothelioma tissues. In the other two cell lines, however, no expression of *WIF-1* mRNA or protein was detected after 5-aza-2dC treatment. These results may be due to other epigenetic or genetic alterations. In conclusion, our data suggest that hypermethylation of *WIF-1* promoter is a common event in mesothelioma and play an important role in the regulation of WIF-1 expression.

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Longnan JIN, Vishwa Jeet AMATYA, Yukio TAKESHIMA,
Looniva SHRESTHA, Kei KUSHITANI and Kouki INAI*)

Department of Pathology, Graduate School of Biomedical Sciences, Hiroshima University, Japan

ABSTRACT

We evaluated apoptosis and the expression of apoptosis-related proteins in 3 mesothelioma cell lines and 34 paraffin-embedded tissue specimens. Apoptosis was evaluated by the TUNEL method, while expression of the apoptosis-related proteins, bax, bcl-2, survivin, caspase-3 and cleaved caspase-3 was evaluated by immunohistochemical staining. The mean apoptotic index of mesothelioma tissue was 17.6 (ranging from 0 to 41.9), which was significantly lower than that of other carcinomas. Thirty-one of 34 cases showed caspase-3 expression. However, the cleaved caspase-3 index in mesothelioma was only 14.7 (ranging from 0 to 36.5). There was a direct correlation between apoptotic index and cleaved caspase-3 index (p value = 0.03). All cases of mesothelioma tissue showed bax expression, while only 2 cases showed bcl-2 expression. Thirty of 31 mesothelioma cases showed cytoplasmic expression of survivin, and 16 cases out of 30 cases showed diffuse staining while 11 cases showed strong staining. Three mesothelioma cell lines also showed high cytoplasmic expression of bax, caspase-3 and survivin, while there was no expression of bcl-2, and apoptosis and cytoplasmic expression of cleaved caspase-3 were limited. mRNA expression of survivin was confirmed by RT-PCR and its protein was confirmed by western blotting. In conclusion, apoptosis is an uncommon event in mesothelioma and low mean cleaved caspase-3 index, suggesting the role of low activation of caspase-3 for inhibition of apoptosis. High expression of survivin in mesothelioma may play a role in inhibition of apoptosis.

Key words: *Mesothelioma, Apoptosis, Survivin, Caspase-3*

Mesothelioma, arising from the mesothelial cell linings of the pleural, peritoneal and pericardial cavities and tunica vaginalis, is mainly induced by exposure to asbestos[1]. Although the latent period between the initial exposure to asbestos and the development of mesothelioma ranges from 15 to 40 years of exposure to asbestos[14], mesothelioma is characterized by rapid growth and a grim prognosis.

Cell homeostasis is maintained by the balance between proliferation, growth-arrest, and apoptosis. It has been proposed that neoplastic cells acquire resistance to apoptosis by overexpression of the inhibitor of apoptosis proteins (IAPs)[5].

Apoptosis is primarily implemented by a family of cysteine proteases called caspases. Caspases are normally present in the cell in an inactive proenzyme form and require limited proteolysis for enzymatic activity[13] Activated caspase-3 cleaves DNA Fragmentation Factor-45 (DFF-45), leading to DNA fragmentation and apoptosis[10]. Two important groups of proteins that regulate apoptosis are Bcl-2 family and the inhibitor of apoptosis proteins (IAPs). The Bcl-2 family consists of pro-apoptotic protein, bax, which down-regulates anti-apoptotic protein, bcl-2. Bcl-2 inhibits the release of cytochrome c, leading to caspase-9 activation, and Smac, inhibiting function of IAPs[20]. To date, eight human IAPs have been identified: c-IAP1, c-IAP2, neuronal apoptosis inhibitory protein, survivin, XIAP, apollon, testis-specific IAP, and livin[2]. In general, IAPs inhibit the apoptotic action of caspases by preventing proteolytic cleavage of caspase proforms and/or directly inhibiting activated caspases[4]. Among these IAPs, the strongest evidence for IAP involvement of survivin in cancer has been report-

*Correspondence address: Kouki INAI, M.D., Ph.D.
Department of Pathology, Graduate School of Biomedical Sciences, Hiroshima University,
1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan
TEL: +81-82-257-5150, FAX: +81-82-257-5154 E-mail: koinai@hiroshima-u.ac.jp

ed[8]. Overexpression of survivin has been reported in human cancers, including mesothelioma[7, 21].

The aim of this study was to examine the level of apoptosis in mesothelioma and the expression of apoptosis-related proteins: survivin, bcl-2, bax, caspase-3 and cleaved caspase-3.

MATERIALS AND METHODS

Cell lines and tissue samples

Three mesothelioma cell lines were obtained from the following sources: ACC-MESO-1[19] and ACC-MESO-4[19] from the RIKEN BioResource Center (Tokyo, Japan) and NCI-H2452 from the American Type Culture Collection (Manassas, VA, USA). All of the mesothelioma cells were cultured in RPMI-1640 Glutamax supplemented with 10% fetal bovine serum at 37°C in a humidified incubator with 5% CO_2.

Thirty-four cases of mesothelioma, including 23 of the epithelioid type, 9 of the sarcomatoid type and 2 of the biphasic type, were obtained from the surgical and autopsy archives of the Department of Pathology, Hiroshima University. The male to female ratio was 26:8, and the average age of the mesothelioma patients was 59.2 years (range, 23-81 years). The microscopic slides were reviewed and reclassified by three pathologists (V.J.A., Y.T. and K.I.) using the current histological classification of lung and pleural tumors (WHO), 2004[1].

Apoprosis assays by TUNEL immunostaining

Apoptosis was determined by the TUNEL method (terminal deoxynucleotidyl transferase end labeling) using the ApopTag Peroxides *In Situ* Apoptosis Detection Kit (Millipore, Billerica, MA, USA), following the manufacturer's instructions with a slight modification. In brief, after deparaffinization and rehydration, the sections in aminopropyltriethoxysilane-coated slides were incubated with Proteinase K (20 μg/ml, TaKaRa, Shiga, Japan) at room temperature for 15 min. Endogenous peroxidase activity was quenched in 3.0% hydrogen peroxide in PBS (pH 7.2). The free 3'-OH end of DNA termini *in situ* were labeled with digoxigenin-labeled nucleotides by terminal deoxynucleotidyl transferase for 75 min, followed by incubation with antidigoxigenin conjugate. The color was developed by peroxidase substrate containing diaminobenzidine. The sections were lightly counterstained with 2% methyl green. For the control, tissue sections from tonsils showing abundant apoptotic B cells within germinal centers were used. The nuclei of apoptotic cells were indicated by brown coloration.

Determination of the apoptotic index

Apoptotic bodies were defined as small, positively labeled globular bodies in the cytoplasm that could be found either singly or in groups. Apoptotic cells and bodies were counted from several areas of each case. The apoptotic index (AI) was estimated as the number of apoptotic cells and/or bodies per 1000 tumor cells.

Immunohistochemical staining

Immunohistochemical staining was performed on sections from formalin-fixed, paraffin-embedded tissue using Histofine Simple Stain MAX PO (MULTI) kit (Nichirei, Tokyo, Japan). The primary antibodies used in the present study were as follows; survivin (prediluted, Spring BioScience, Pleasanton, CA, USA), bax (1:50, Dako, Glostrup, Denmark), bcl-2 (prediluted, Nichirei BioScience, Tokyo, Japan), caspase-3 (1:50, Cell Signaling Tech., Beverly, MA, USA) and cleaved caspase-3 (1:100, Cell Signaling Tech., Beverly, MA, USA). Antigen retrieval was done by autoclaving the tissue section at 121°C for 20 min, except for survivin which was carried out by microwave for 10 min. The expression of survivin, bax, bcl-2 and caspase-3 was evaluated as follows: -, no immunoreactivity; +, <25% of tumor cells showing cytoplasmic positivity; ++, ≧25% of tumor cells showing cytoplasmic positivity. In addition, the expression of survivin was also evaluated as weak or strong reactivity. The cleaved caspase-3 index was estimated as the number of tumor cells with cleaved caspase-3 expression per 1000 tumor cells.

Survivin expression in mesothelioma cell lines

RNA isolation from 1×10^5 cultured mesothelioma cells followed by reverse transcription to cDNA was performed using the Power SYBR Green Cells-to-CT Kit (Applied Biosystems, Tokyo, Japan) according to the manufacturer's instructions. PCR amplification for survivin (BIRC5bF, TCCGGTTGCGCTTTCCT and BIRC5bR, TCTTC TTATTGTTGGTTTCCTTTGC, 121 bp) and a housekeeping gene (beta-actin sense GCCAACCG CGAGAAGATGA and anti-sense CATCACGATGC CAGTGGTA, 120 bp) was performed by using the KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Woburn, MA, USA) according to the manufacturer's instructions in an Mx3000P real-time PCR system (Agilent Technologies, CA, USA). The cyclic conditions for both the products were as follows: initial denaturation at 95°C for 10 min, 30 cycles of 95°C for 15 sec, and 62°C for 1 min, followed by dissociation analysis from 55°C to 95°C.

Protein was extracted from 1×10^6 mesothelioma cells using the Cell-LyEX1 protein extraction kit (TOYO B-Net, Tokyo, Japan) according to the manufacturer's protocol, and its concentration was determined by a NanoVue spectrophotometer (GE Healthcare Bio-Sciences, Tokyo, Japan). The extracted protein was separated on 10% SDS-PAGE and transferred onto PVDF membrane (HybondTM-LFP, GE Healthcare Bio-Sciences,

Tokyo, Japan). Immuno-labeling with anti-survivin antibody (NB500-237, clone 32.1, 1:500, Novus Biologicals, Littleton, CO, USA) and anti-actin antibody (SC-1616-R, 1:1000, Santa Cruz, CA, USA) using Western Dot 625 Kits (Invitrogen, Eugene, OR, USA) were performed in Western Q (SciTrove Inc, Tokyo, Japan) according to the manufacturer's protocols.

Statistical analysis

Descriptive statistics, Pearson's correlation and Unpaired Student's t-tests were used to determine statistical significance. Statistical significance was attributed to p-values lower than 0.05.

RESULTS

Apoptosis and cleaved caspase-3 expression in mesothelioma tissue and cell lines

The AI in mesothelioma tissue ranged from 0 to 41.9 (17.6 ± 11.3) (Table 1, Fig. 1. C). We also examined mean AI in other cancers including lung, stomach and colon cancers (Table 2). The mean AI of epithelioid mesothelioma (15.2) was lower than that of sarcomatoid mesothelioma (23.5), but it was not statistically significant (p=0.063). The mean AI in mesothelioma cell lines was 63.9 (Table 1, Fig. 1. D).

The cleaved caspase-3 index in mesothelioma ranged from 0 to 36.5 (14.7 ± 10.6) (Table 1, Fig. 1. K). Statistical analysis showed positive correlation between AI and cleaved caspase-3 index (p=0.036), but Pearson's correlation coefficient was not very high (r=0.373) (Table 3). The mean cleaved caspase-3 index in mesothelioma cell lines was 92.2 (Table 1, Fig. L).

Bax, caspase-3, bcl-2 expression in mesothelioma tissue and cell lines

Cytoplasmic expression of bax was found in all (100%) and caspase-3 was found in 31 (91.2%) of 34 of the mesothelioma cases (Table 1, Fig. 1. E, I). Expression of bcl-2 was detected in the cytoplasm of only 2 (5.9%) cases of mesothelioma (Table 1, Fig. 1. G). All three mesothelioma cell lines showed cytoplasmic expression of bax and caspase-3 (Table 1, Fig. 1. F, J), and non-expression of bcl-2 (Table 1, Fig. 1. H).

Survivin expression in mesothelioma tissue and cell lines

The cytoplasmic expression of survivin was found in 30 (96.8%) of 31 mesothelioma cases (Table 1, Fig. 1. M). Among them, 16 (51.6%) cases showed survivin expression in more than 25% of tumor cells and 11 (35.5%) cases showed strong reactivity. All three mesothelioma cell lines also showed cytoplasmic expression of survivin (Fig. 1. N). Survivin expression was also confirmed by expression of mRNA by real time RT-PCR and expression of protein by western blot (Fig. 2).

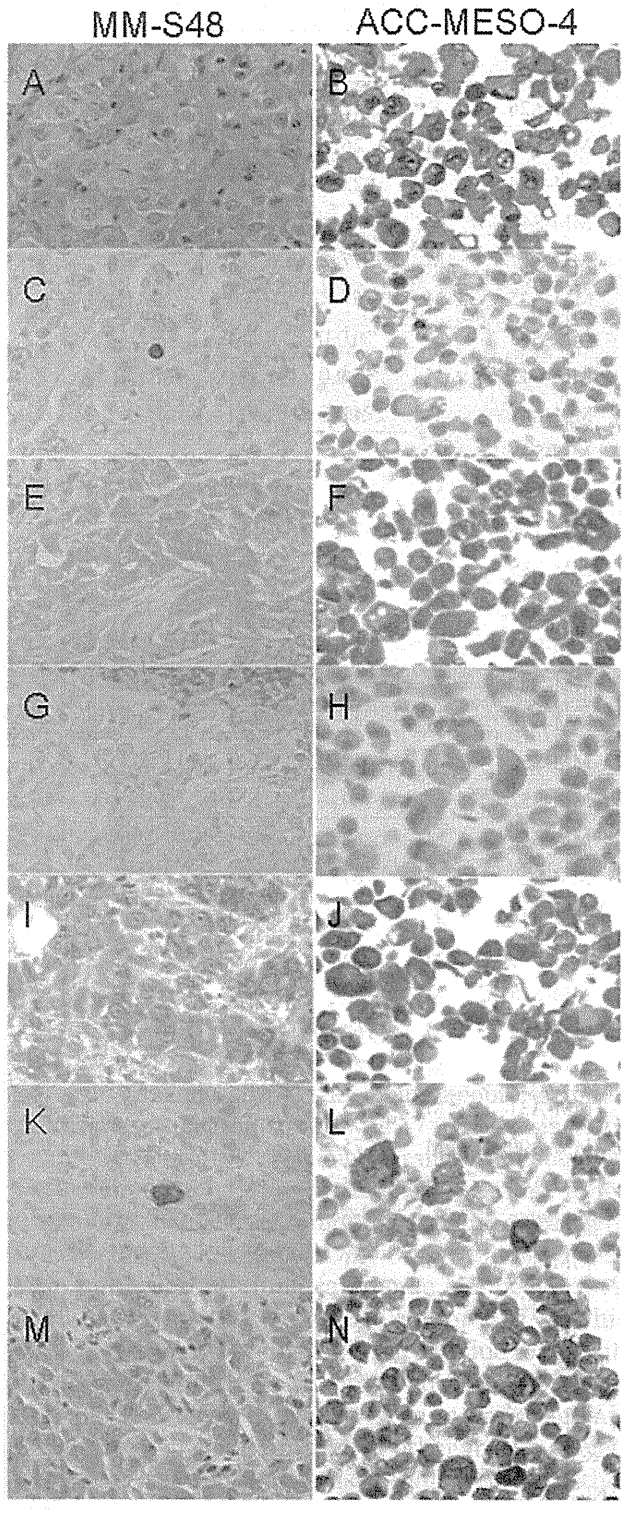

Fig. 1. MM-S48 is epithelioid mesothelioma showing solid growth (A) and ACC-MESO-4 is mesothelioma cell line obtained from epithelioid mesothelioma (B). Very few apoptotic bodies were detected by the TUNEL method (C and D). Immunohistochemically, both of them showed diffuse cytoplasmic expression of bax (E and F), no expression of bcl-2 (G and H), and diffuse cytoplasmic expression of caspase-3 (I and J). Very few tumor cells showed cytoplasmic expression of cleaved caspase-3 (K and L). Survivin expression was detected in the cytoplasm (M and N).