ORIGINAL PAPER

Clinical investigation of malignant mesothelioma in Japan

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Abstract

Purpose The asbestos-related problems caused much social concern; however, no large-scale study was conducted about clinical features of MM in Japan. Patients with MM who have a history of occupational asbestos exposure (AE) are provided worker's compensation in Japan. However, only about 10% of MM cases were actually claimed and compensated. So there is still controversy over the association between MM and AE. The aim of this study is to investigate the clinical features of MM. We also aimed to clarify the association between MM and occupational AE in Japan.

Methods We examined the clinical features of MM cases. Clinical information was obtained including gender, age, site of origin, pathological subtype, radiological findings, and treatment outcome. To investigate the association between MM and AE, investigators interviewed all patients regarding work and residential history.

Results Between January 2005 and December 2007, 105 cases (median age: 63 years, range 35–80, male/female: 88/17) were diagnosed with MM in the Rosai Hospital group and related facilities. Among them, 94(89.5%) cases originated in the pleura, 7(6.7%) in the peritoneum, 2(1.9%) in the pericardium, and 1(0.9%) in the tunica vaginalis testis. There were 69(65.7%) epithelioid, 19(18.1%) biphasic, 16(15.2%) sarcomatoid, and 1 unclassified pathological subtypes of MM. A favorable survival rate was indicated in the patient group of MPM that underwent surgery compared to others, though it was not statistically significant ($P = 0.1743$). The occupational AE was indicated in 89 cases (84.8%). Three patients had no history of occupational AE, but lived with someone who was in an occupation that handled asbestos. There were two patients in which AE was indicated in their life environment. Altogether, AE was indicated in 93(88.6%) patients.

Conclusions This study stresses the urgent need for physicians to acknowledge the association between MM and AE, and to inquire thoroughly regarding AE to the patients with MM.

Keywords Pleura · Peritoneum · Pericardium · Pemetrexed

Dr. K. Yamazaki passed away in 2008.

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Introduction

Malignant mesothelioma (MM) is an aggressive tumor that develops from the mesothelial cell of the pleura, peritoneum, pericardium, or testicular tunica vaginalis. A newspaper article published in June 2005 reported that 5

residents who lived near a now-closed asbestos cement pipe plant in Amagasaki, Japan, developed pleural mesothelioma (Ohshima 2005). The asbestos-related problems that this article raised caused much social concern; however, no large-scale study was conducted about clinical features of MM in Japan. One of the most important issues is the association of MM and asbestos exposure (AE). An association between MM and AE has been well-known worldwide since the 1950s (Magnani et al. 2000; Newhouse and Thompson 1965; Rees et al. 1999). There is so far one report that demonstrated association between AE and MM in western parts of Japan (Kishimoto et al. 2004). Patients who have a history of occupational AE and developed MM are provided worker's compensation in Japan. However, among 2,641 cases who died of MM between 2002 and 2004, only 287 cases claimed the compensation and 269(10.2%) was actually compensated (Ministry of Health, Labor, and Welfare of Japan, http://www.mhlw.go.jp/houdou/2006/05/h0530-1.html). As a result, there is still controversy over the association between MM and AE in Japan.

Based on these statistics, we hypothesized that there would be more MM cases in which patients and/or physicians were unaware of occupational AE. One of the reasons for the uncertainty might be the long latency of the disease after AE and that the work history of each patient has not been fully investigated. In such cases, retrospective investigation of medical records after the death is often unsuccessful in clarifying occupational AE.

The Rosai Hospital group, specialized facilities established to treat occupational illnesses, conducted this study to investigate the clinical features of MM in Japan. These features include the site of origin, pathological subtype, radiological findings, and treatment outcome. Especially, we aimed to clarify the association between MM and occupational AE in Japan. For this purpose, all the patients were interviewed regarding their entire work history and living environment since their youth at the diagnosis of MM.

Materials and methods

Enrolled patients were those who were diagnosed MM and in treatment between January 2005 and December 2007. The essential aim of this study was to make face-to-face interview to the patients to clarify the history of AE, so the patients diagnosed before 2004 and were in treatment in 2005 were also included. Clinical information was obtained from each facility by survey sheet including gender, age, site of origin, pathological subtype, and treatment outcome. The radiological images and pathological specimens were sent to Okayama Rosai Hospital for review of the diagnosis and analyses.

The clinical stage of malignant pleural mesothelioma (MPM) was determined according to International Mesothelioma Study Group (IMIG) criteria (Rusch 1996) based on the staging procedure including computed tomographic (CT) scans of the chest and abdomen, magnetic resonance images of the brain, and Technetium-99 m hydroxymethylene diphosphonate bone scans. Characteristic radiological findings that indicated AE were assessed concerning the presence of pleural fluid, asbestosis, rounded atelectasis, and pleural plaque based on chest X-ray and CT. 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 using SPSS 11.0 software (SPSS, Inc., Chicago, IL).

To investigate the association between the occurrence of MM and AE, all the patients were interviewed regarding their work history and that of the family members, and residential history, since their youth, which may suggest environmental exposure to asbestos.

Results

Patient characteristics

Between January 2005 and December 2007, 105 cases (median age: 63 years, range 35–80, male/female: 88/17) were diagnosed with MM in 31 Rosai Hospitals and related facilities. Among them, 94(89.5%) cases originated in the pleura, 7(6.7%) in the peritoneum, 2(1.9%) in the pericardium, and 1(0.9%) in the tunica vaginalis testis. There was one case in which the origin, whether the pleura or pericardium, was undetermined. There were 69(65.7%) epithelioid, 19(18.1%) biphasic, 16(15.2%) sarcomatoid, and 1 unclassified pathological subtypes of MM. According to the IMIG staging system, there were 19 Stage I, 8 Stage II, 34 Stage III, and 29 Stage IV patients with MPM. The characteristics of the patients are summarized in Table 1.

Diagnostic procedure

Fifty-five patients were diagnosed based on video-assisted thoracoscopic biopsy under either general or local anesthesia or laparoscopy. Twenty-eight patients were diagnosed based on open-chest biopsy. Percutaneous needle biopsy was performed in 18 patients for diagnosis. Three patients were diagnosed based on cytological examination of pleural fluid. A patient with mesothelioma in the tunica vaginalis testis was diagnosed after the tumor resection.

Table 1 Patient characteristics

Age	
Median (range)	63(35–80)
Gender	
M/F	88/17
Site of origin	
Pleura	94
Peritoneum	7
Pericardium	2
Tunica vaginalis testis	1
Undetermined	1
Subtypes	
Epithelioid	69
Biphasic	19
Sarcomatoid	16
Unclassified	1

Radiological findings

Radiological findings were available in 103 cases. Pleural effusion was documented in 74(71.8%) cases, pleural plaque in 42(40.8%) cases, and asbestosis was not found. In the case of MPM, radiological findings were available in 88 cases. Among them, pleural effusion was documented in 69(78.4%) cases, pleural plaque in 35(39.8%) cases.

Treatment outcome

Among 94 patients with MPM, 36 patients underwent surgery as the principal treatment modality. Adjuvant chemotherapy was delivered in 12 cases and radiotherapy was added in 7 of these 36 cases. Six patients had multimodality treatment comprising surgery, radiotherapy, and systemic chemotherapy. Systemic chemotherapy was delivered in 49 patients as the initial treatment. Major chemotherapy regimens are as follows; Cisplatin + pemetrexed were administered in 18 cases, vinorelbine + gemcitabine were administered in 12 cases, and cisplatin + gemcitabine were administered in 6 cases.

Survival analysis was performed with patients with MPM diagnosed between 2005 and 2007. The median overall survival time (MST) of the patients was 13.2 months (95% confidential interval: 11.23–15.17). Overall survival according to the clinical stage is shown in Fig. 1a. A favorable survival rate is indicated in the earlier stages (I and II) where the MST is 17.9 months (95% C.I. 9.07–26.66) rather than in the advanced stages (III and IV) where the MST is 12.8 months (95% C.I. 10.34–15.19) ($P = 0.0707$). A favorable survival rate is indicated in the patient group that underwent surgery with the MST of 15.1 months (95% C.I.

10.23–20.04) compared to the other groups with the MST of 12.7 months (95% C.I. 8.95–16.45) ($P = 0.1743$) (Fig. 1b), though these were not statistically significant.

Patients with malignant peritoneal mesothelioma were treated with platinum-based chemotherapy combined with pemetrexed (3 cases) or paclitaxel (one case). Two cases with malignant pericardial mesothelioma were treated with platinum-based chemotherapy combined with pemetrexed. Surgical resection was performed on the MM patient with tunica vaginalis testis.

Asbestos exposure

The occupational history was obtained from all the patients and occupational AE was indicated in 89 cases (84.8%), including 23 cases in the shipbuilding industry, 16 in the construction industry, 9 in plumbing, 8 in
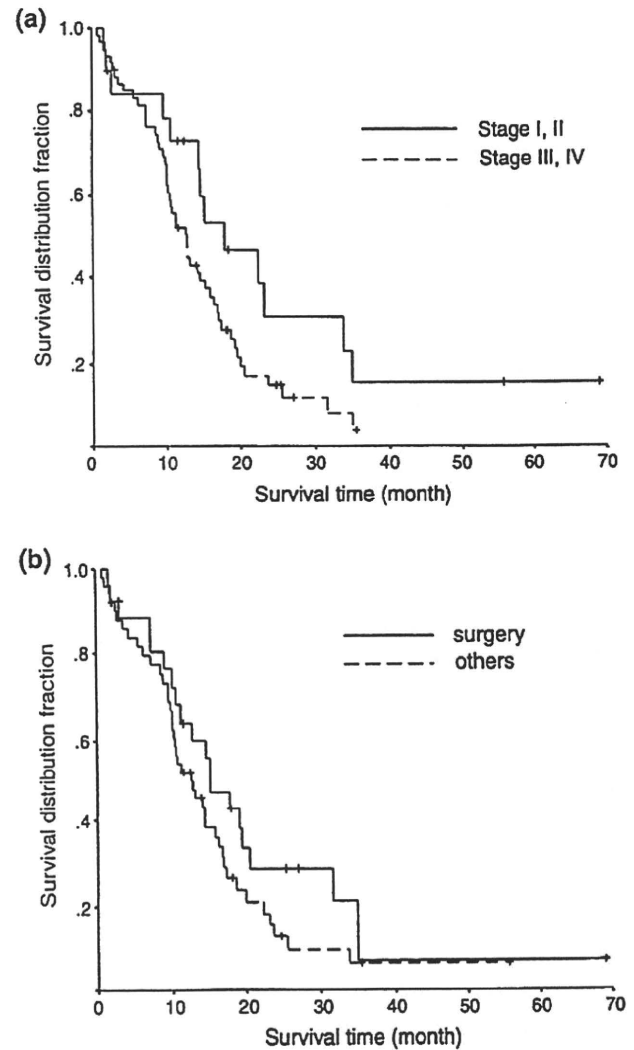

Fig. 1 Overall survival curve of patients with malignant pleural mesothelioma according to clinical stage (a) and treatment (b)

Table 2 Occupational history related to asbestos exposure

Shipbuilding	23
Construction	16
Plumbing	9
Electric work	8
Manufacturing	7
Asbestos products industry	5
Automobile manufacture	5
Steel production	3
Pottery and porcelain	3
Fiber product	2
Coating industry	2
Warehouse management	2
Chemical works	1
Cement manufacture	1
Metallic manufacture	1
Other asbestos-handling work	1
Total	89

Table 3 Patients characteristics of patients without asbestos exposure

Age	Gender	Site of origin	Subtype	Occupation
40	F[#]	Pleura	Sarcomatoid	Designer
43	M[§]	Pleura	Sarcomatoid	Bank staff
44	M	Pleura	Biphasic	Medical doctor
47	F	Pericardium	Biphasic	Office worker
48	F	Peritneum	Epithelioid	Homemaker
50	F	Pleura	Epithelioid	Homemaker
56	F	Pleura	Epithelioid	Homemaker
61	F	Pericardium	Epithelioid	Office worker
62	M	Pleura	Epithelioid	Utility worker
63	M	Pleura	Epithelioid	Cook
63	M	Pleura	Sarcomatoid	School teacher
64	F	Pleura	Epithelioid	Office worker

[#] female, [§] male

electrical work, and 7 in the manufacturing industry as shown in Table 2. Three patients had no history of occupational AE, but lived with someone who was in an occupation that handled asbestos. There were two patients in which AE was indicated in their life environment, i.e., living in a neighborhood near an asbestos products factory. The remaining 12 of the 105 patients had no history of occupational or environmental AE. The characteristics of these 12 patients were summarized in Table 3. As a result, AE was indicated in 93(88.6%) patients. The median time of AE was 29(1–60) years and the median time between the first AE and development of MM was 41(4–60) years.

Discussion

The clinical features of the 108 MM cases were investigated. This is the first nationwide study in Japan to clarify the characteristics and treatment outcome of MM cases.

MM is diagnostically challenging. Significant numbers of patients with MM demonstrate pleural effusion at the initial presentation, but there are many other diseases or conditions that demonstrate pleural effusion. In our cohort, pleural effusion was documented in 70(77.8%) of the cases with MPM. However, Aleman et al. reported that MPM accounted for only 6.7% of cases with malignant pleural effusion (Aleman et al. 2007). In patients demonstrating pleural fluid and/or diffuse pleural thickening, MPM could be one of the causes, especially if the patient has a history of AE, or some characteristic radiological findings indicating AE such as pleural plaque. However, the frequency of radiological findings indicating AE was very low. In our cohort, pleural plaque was found in 41% of the cases with MPM, and asbestosis was not found. MM should be kept in mind in the case of pleural effusion, even when characteristic radiological findings that suggest AE are not found.

Definite pathological diagnosis of MM should be based on immunohistochemical reactivity to some markers such as calretinin and thrombomodulin, in addition to the usual hematoxylin-eosin staining (Kushitani et al. 2008). Recently, Takeshima et al. reported that the diagnosis of MM was suspicious in approximately 15% cases who died of "MM" in Japan (Takeshima et al. 2009). In our cohort, 101 (96.2%) cases were diagnosed based on the materials obtained through procedures such as thoracoscopy or percutaneous biopsy. We are convinced that the diagnoses in our cases based on central review of pathological examination containing immunohistological analysis.

Concerning the treatment strategy, surgical resection such as extrapleural pneumonectomy (EPP) was performed in cases at an earlier stage (Stage I or II) with good performance status. The overall survival rate was relatively favorable in the group that underwent surgery with the MST of 20.0 months. A few patients received postoperative adjuvant chemotherapy and/or radiotherapy. Trimodality therapy, consisting of EPP, systemic chemotherapy, and adjuvant hemithoracic radiotherapy, has been reported to offer long-term survival in selected patients with MPM (Sugarbaker et al. 1999). The comparison of the survivals after treatment needs to be evaluated carefully, because these results are containing patient selection bias. An extrapleural pneumonectomy has been indicated in selected patients with earlier stage and better performance status. Further studies as prospective clinical trials are warranted to evaluate the feasibility and effectiveness of these combined modalities. For patients with advanced disease,

systemic chemotherapy was administered. As a chemotherapy regimen, a combination of cisplatin and pemetrexed, gemcitabine, or vinorelbine was mainly administered. Recently, pemetrexed, a multi-targeted antifolate, has demonstrated modest activity against MPM in combination with cisplatin (Vogelzang et al. 2003) or carboplatin (Castagneto et al. 2008). Since the approval of pemetrexed by the Ministry of Health, Labour and Welfare in Japan in 2006, the combination of cisplatin and pemetrexed has been considered as the standard regimen against MM. However, the treatment outcome is still unsatisfactory with an MST of only about one year. In addition, there are many aged patients with MM with some concomitant medical problems. Novel approaches are needed that incorporate new chemotherapeutic or molecular-targeted therapies.

Another principal objective of this study was to clarify the association between MM and AE. For this purpose, the patients were interviewed concerning their work and residential histories. As a result, occupational AE was revealed in more than 80% of the cases. This was reported in other countries (Wagner et al. 1960), but this is the first report to describe the detailed proportion of AE in MM in Japan. These include cases in which occupational AE was not described in the clinical record, but was revealed based on the interviews. The median duration of AE was 29 years and the median time of latency between AE and development of MM was about 40 years. The industrial use of asbestos was banned in Japan in 2006, but the number of incidences of MM is anticipated to continue to .increase for the next few decades due to past usage of asbestos (Robinson and Lake 2005). This study stresses the urgent need for physicians to acknowledge the association between MM and AE, and to inquire thoroughly regarding AE in their work history and living environment since their youth.

In conclusion, the clinical features of Japanese MM cases were investigated. A strong association with occupational AE was demonstrated.

References

Aleman C, Sanchez L, Alegre J, Ruiz E, Vazquez A, Soriano T, Sarrapio J, Teixidor J, Andreu J, Felip E, Armadans L, Fernandez De Sevilla T (2007) Differentiating between malignant and idiopathic pleural effusions: the value of diagnostic procedures. Qjm 100:351–359

Castagneto B, Botta M, Aitini E, Spigno F, Degiovanni D, Alabiso O, Serra M, Muzio A, Carbone R, Buosi R, Galbusera V, Piccolini E, Giaretto L, Rebella L, Mencoboni M (2008) Phase II study of pemetrexed in combination with carboplatin in patients with malignant pleural mesothelioma (MPM). Ann Oncol 19:370–373

Kishimoto T, Ozaki S, Kato K, Nishi H, Genba K (2004) Malignant pleural mesothelioma in parts of Japan in relationship to asbestos exposure. Ind Health 42:435–439

Kushitani K, Takeshima Y, Amatya VJ, Furonaka O, Sakatani A, Inai K (2008) Differential diagnosis of sarcomatoid mesothelioma from true sarcoma and sarcomatoid carcinoma using immunohistochemistry. Pathol Int 58:75–83

Magnani C, Agudo A, Gonzalez CA, Andrion A, Calleja A, Chellini E, Dalmasso P, Escolar A, Hernandez S, Ivaldi C, Mirabelli D, Ramirez J, Turuguet D, Usel M, Terracini B (2000) Multicentric study on malignant pleural mesothelioma and non-occupational exposure to asbestos. Br J Cancer 83:104–111

Newhouse ML, Thompson H (1965) Mesothelioma of pleura and peritoneum following exposure to asbestos in the London area. Br J Ind Med 22:261–269

Ohshima H (2005) Five cases with mesothelioma living near a now-defunct asbestos cement plant in Amagasaki city (in Japanese). In Mainichi newspaper pp 1(June 29)

Rees D, Myers JE, Goodman K, Fourie E, Blignaut C, Chapman R, Bachmann MO (1999) Case-control study of mesothelioma in South Africa. Am J Ind Med 35:213–222

Robinson BW, Lake RA (2005) Advances in malignant mesothelioma. N Engl J Med 353:1591–1603

Rusch VW (1996) A proposed new international TNM staging system for malignant pleural mesothelioma from the International Mesothelioma Interest Group. Lung Cancer 14:1–12

Sugarbaker DJ, Flores RM, Jaklitsch MT, Richards WG, Strauss GM, Corson JM, DeCamp MM Jr, Swanson SJ, Bueno R, Lukanich JM, Baldini EH, Mentzer SJ (1999) Resection margins, extrapleural nodal status, and cell type determine postoperative long-term survival in trimodality therapy of malignant pleural mesothelioma: results in 183 patients. J Thorac Cardiovasc Surg 117:54–63 (Discussion 63–5)

Takeshima Y, Inai K, Amatya VJ, Gemba K, Aoe K, Fujimoto N, Kato K, Kishimoto T (2009) Accuracy of pathological diagnosis of mesothelioma cases in Japan: Clinicopathological analysis of 382 cases. Lung Cancer 66:191–197

Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kaukel E, Ruffie P, Gatzemeier U, Boyer M, Emri S, Manegold C, Niyikiza C, Paoletti P (2003) Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. J Clin Oncol 21:2636–2644

Wagner JC, Sleggs CA, Marchand P (1960) Diffuse pleural mesothelioma and asbestos exposure in the North Western Cape Province. Br J Ind Med 17:260–271

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

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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 *SFRPs* 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 *β-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

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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 1×10^5 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	X/Y	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 *SFRPs* in mesothelioma and NPLVP.

	Mesothelioma (%)				NPLVP[a] (%)
	Epithelioid	Sarcomatoid	Biphasic	Total	
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 491 genes 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
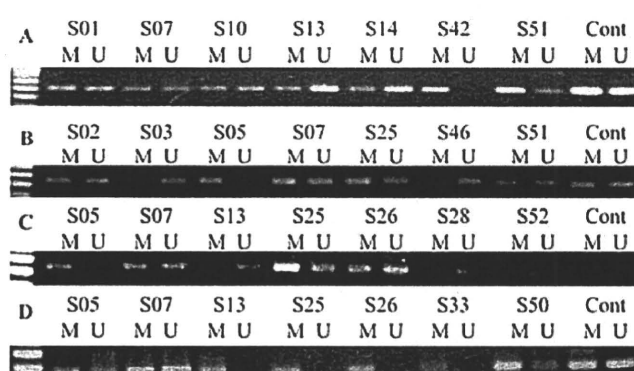

Figure 1. Methylation-specific PCR. Mesothelioma tissues show *WIF-1* (A), *SFRP1* (B), *SFRP2* (C) and *SFRP4* (D) promoter methylation as methylated DNA products (lane M) amplified using methylation-specific primers and unmethylated DNA products (lane U) amplified using unmethylation-specific primers.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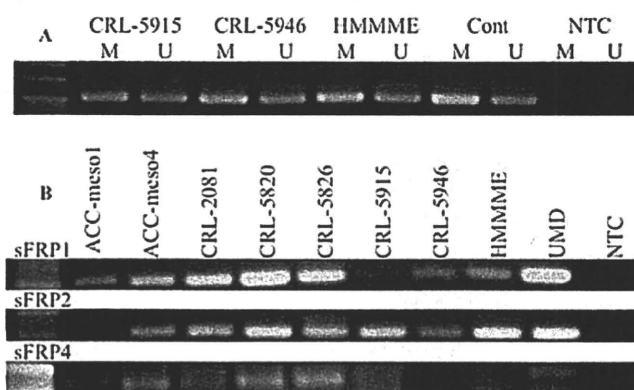

Figure 2. Methylation-specific PCR analysis in mesothelioma cell lines. Mesothelioma cell lines show *WIF-1* (A) and *SFRP1, 2* and *4* promoter methylation [(B), only methylated bands are shown]. M, methylated DNA product amplified with methylation-specific primers; U, unmethylated DNA product amplified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

were also found in 7, 7 and 3 of 8 mesothelioma cell lines, respectively (Fig. 2B).

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,
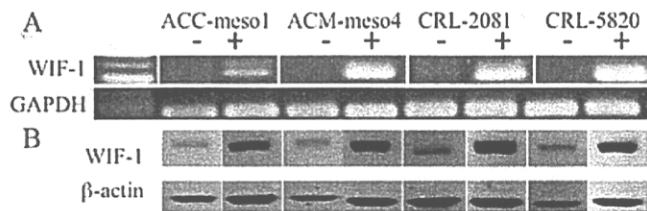
Figure 3. Real-time RT PCR shows amplification of WIF-1 mRNA in a 5-aza-2dC treated mesothelioma cell line compared to amplification of GAPDH mRNA in cell lines treated either with or without 5-aza-2dC. The PCR product from real-time RT-PCR was electrophoresed in a 2% agarose gel followed by ethidium 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-
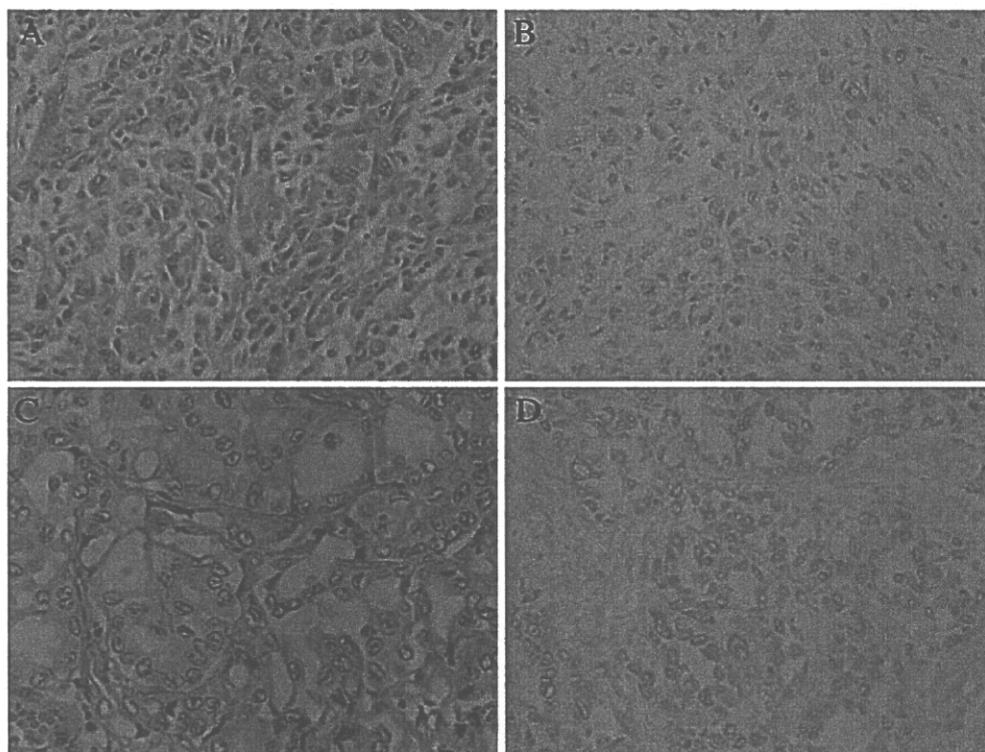
Figure 4. Immunohistochemistry. Sarcomatoid mesothelioma (A) with *WIF-1* promoter methylation shows no WIF-1 immunoreactivity (B). Epithelioid mesothelioma (C) without *WIF-1* promoter methylation shows WIF-1 immunoreactivity in the cytoplasm of tumor cells (D).
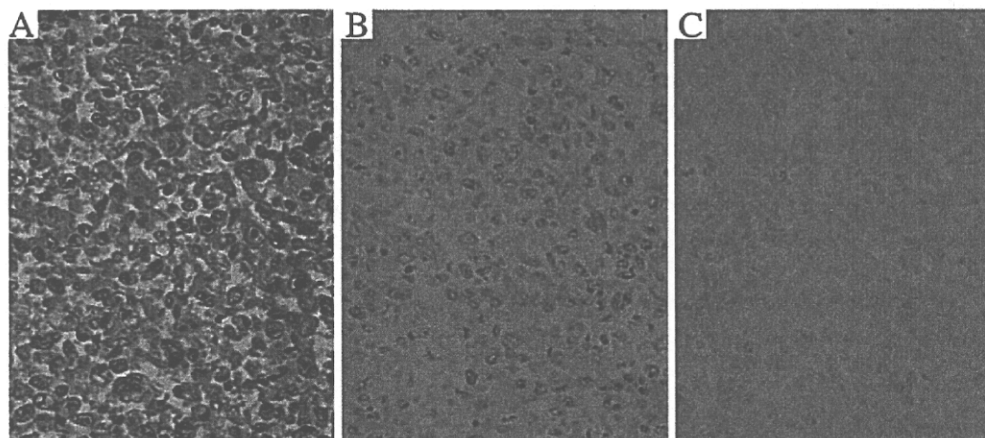

Figure 5. Immunohistochemistry. Mesothelioma cells, ACC-Meso1 (A) that had no expression of WIF-1 (B) prior to treatment with demethylating agents show membranous and cytoplasmic expression of WIF-1 after 5-aza-2dC treatment (C).

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.

Acknowledgements

References

1. Robinson BW and Lake RA: Advances in malignant mesothelioma. N Engl J Med 353: 1591-1603, 2005.
2. Robinson B, Musk A and Lake R: Malignant mesothelioma. Lancet 366: 397-408, 2005.
3. Papp T, Schipper H, Pemsel H, et al: Mutational analysis of N-ras, p53, p16INK4a, p14ARF and cdk4 genes in primary human malignant mesotheliomas. Int J Oncol 18: 425-433, 2001.
4. Carbone M, Kratzke R and Testa J: The pathogenesis of mesothelioma. Semin Oncol 29: 2-17, 2002.
5. Murthy S and Testa J: Asbestos, chromosomal deletions and tumor suppressor gene alterations in human malignant mesothelioma. J Cell Physiol 180: 150-157, 1999.
6. Bird A and Wolffe A: Methylation-induced repression - belts, braces and chromatin. Cell 99: 451-454, 1999.
7. Merlo A, Herman J, Mao L, et al: 5' cpg island methylation is associated with transcriptional silencing of the tumour suppressor p16/cdkn2/MTS1 in human cancers. Nat Med 1: 686-692, 1995.
8. Destro A, Ceresoli G, Baryshnikova E, et al: Gene methylation in pleural mesothelioma: correlations with clinicopathological features and patient's follow-up. Lung Cancer 59: 369-376, 2008.
9. Tomii K, Tsukuda K, Toyooka S, et al: Aberrant promoter methylation of insulin-like growth factor binding protein-3 gene in human cancers. Int J Cancer 120: 566-573, 2007.
10. Tsou J, Galler J, Wali A, et al: DNA methylation profile of 28 potential marker loci in malignant mesothelioma. Lung Cancer 58: 220-230, 2007.
11. Kimura K, Toyooka S, Tsukuda K, et al: The aberrant promoter methylation of BMP3b and BMP6 in malignant pleural mesotheliomas. Oncol Rep 20: 1265-1268, 2008.
12. Wissmann C, Wild P, Kaiser S, et al: WIF1, a component of the wnt pathway, is down-regulated in prostate, breast, lung and bladder cancer. J Pathol 201: 204-212, 2003.
13. Mazieres J, He B, You L, et al: Wnt inhibitory factor-1 is silenced by promoter hypermethylation in human lung cancer. Cancer Res 64: 4717-4720, 2004.
14. Taniguchi H, Yamamoto H, Hirata T, et al: Frequent epigenetic inactivation of wnt inhibitory factor-1 in human gastrointestinal cancers. Oncogene 24: 7946-7952, 2005.
15. Nosho K, Yamamoto H, Takahashi T, et al: Genetic and epigenetic profiling in early colorectal tumors and prediction of invasive potential in PT1 (early invasive) colorectal cancers. Carcinogenesis 28: 1364-1370, 2007.
16. Batra S, Shi Y, Kuchenbecker K, et al: Wnt inhibitory factor-1, a wnt antagonist, is silenced by promoter hypermethylation in malignant pleural mesothelioma. Biochem Biophys Res Commun 342: 1228-1232, 2006.
17. Travis WD, Brambilla E, Müller-Hermelink HK and Harris CC (eds): Pathology and Genetics. Tumours of the lung, pleura, thymus and heart. World Health Organization Classification of Tumours. IARC Press, Lyon, 2004.
18. Uematsu K, He B, You L, Xu Z, McCormick F and Jablons D: Activation of the wnt pathway in non-small cell lung cancer: evidence of dishevelled overexpression. Oncogene 22: 7218-7221, 2003.
19. Uematsu K, Kanazawa S, You L, et al: Wnt pathway activation in mesothelioma: evidence of dishevelled overexpression and transcriptional activity of beta-catenin. Cancer Res 63: 4547-4551, 2003.
20. Pu R, Sheng Z, Michael C, Rhode M, Clark D and O'Leary T: Methylation profiling of mesothelioma using real-time methylation-specific PCR: a pilot study. Diagn Cytopathol 35: 498-502, 2007.
21. Urakami S, Shiina H, Enokida H, et al: Epigenetic inactivation of wnt inhibitory factor-1 plays an important role in bladder cancer through aberrant canonical wnt/beta-catenin signaling pathway. Clin Cancer Res 12: 383-391, 2006.

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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 downregulates 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-

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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).
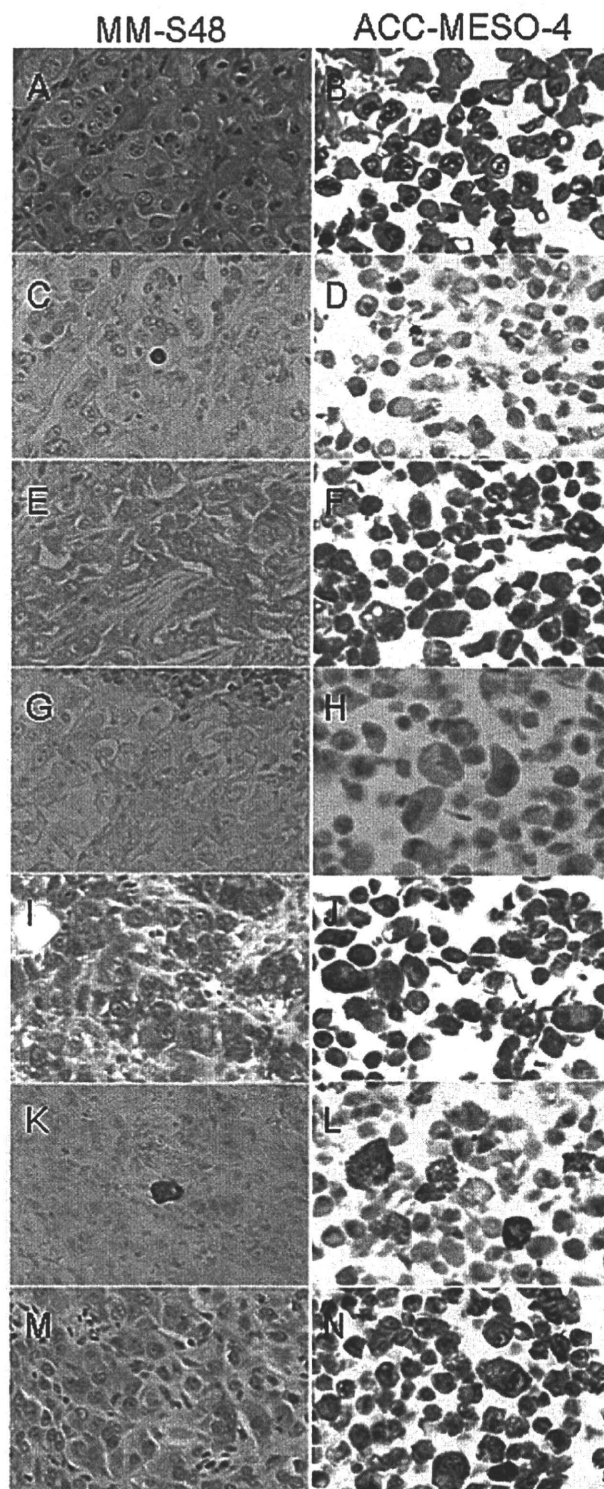
Fig. 1. MM-S48 is epithelioid mesothelioma showing solid growth (A) and ACC-MESO-4 is mesothelioma cell line obtained from epithelioid mesothelioma (B). Very few apoptotic bodies were detected by the TUNEL method (C and D). Immunohistochemically, both of them showed diffuse cytoplasmic expression of 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).

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Table 1. Expression of apoptosis related proteins and apoptotic index in mesothelioma tissues and cell lines

Cases	Sex	Age	Histology	AI	Bax[a]	Bcl-2[a]	Survivin[a]	Caspase-3[a]	Cleaved caspase-3 index[a]
MM-A26	F	52	EM	0	++	-	+/W	+	10.6
MM-S01	M	26	EM	2.0	++	-	++/S	++	15.7
MM-A04	M	23	EM	5.0	++	+	+/S	-	35.6
MM-S46	F	48	EM	6.9	++	-	++/W	++	4.0
MM-S17	M	77	EM	7.0	++	-	++/S	++	14.8
MM-A35	M	57	EM	8.0	++	-	++/S	++	17.4
MM-S16	M	61	EM	9.2	++	-	+/W	++	21.0
MM-S42	M	56	EM	9.8	++	-	++/S	++	2.0
MM-A41	F	42	EM	12.5	++	-	+/S	-	12.3
MM-S39	F	39	EM	12.7	++	-	++/W	++	8.8
MM-S31	M	72	EM	13.9	++	-	++/S	++	19.4
MM-A06	F	74	EM	15.0	++	+	NI	++	2.0
MM-S35	M	46	EM	17.4	++	-	++/S	++	1.0
MM-S41	M	81	EM	17.7	++	-	+/W	++	1.9
MM-S36	M	46	EM	19.8	++	-	+/W	++	14.4
MM-A40	M	75	EM	21.7	++	-	++/W	-	25.8
MM-A19	M	49	EM	22.1	++	-	++/W	+	18.8
MM-S50	F	48	EM	23.1	++	-	++/W	++	0
MM-S52	M	60	EM	32.5	++	-	+/S	++	8.0
MM-S48	M	69	EM	32.9	++	-	+/S	++	18.4
MM-S20	M	67	EM	34.9	++	-	++/W	++	24.4
MM-A10	F	76	EM	NI	++	-	++/W	+	35.1
MM-A30	M	73	EM	NI	++	-	+/W	++	2.0
MM-S15	M	54	SM	5.0	++	-	NI	++	16.0
MM-A02	M	64	SM	7.0	++	-	-	+	1.0
MM-A21	M	49	SM	15.0	++	-	++/W	+	2.0
MM-A31	M	65	SM	22.0	++	-	+/W	+	25.0
MM-A09	M	75	SM	23.0	++	-	+/W	+	3.0
MM-A18	M	65	SM	32.7	++	-	+/W	+	12.3
MM-A38	M	79	SM	32.7	++	-	+/W	+	21.7
MM-S26	M	61	SM	34.5	++	-	NI	++	22.9
MM-S02	M	68	SM	41.9	++	-	++/W	++	36.5
MM-S33	M	51	BM	11.1	++	-	++/S	++	12.8
MM-A39	F	66	BM	23.0	++	-	+/W	++	24.5
ACC MESO 4			EM	58.3	++	-	++/S	++	92.8
ACC MESO 1			SM	57.2	++	-	++/S	+	74.6
NCI-H2452			SM	76.3	++	-	++/S	++	109.2

AI, apoptotic index; BM, biphasic mesothelioma; EM, epithelioid mesothelioma; F, female; M, male; NI, not informative; S, strong; SM, sarcomatoid mesothelioma; W, weak

[a]Details of immunohitochemical scoring in mesothelioma described in methods

Table 2. Apoptotic index in mesothelioma, lung carcinoma, gastric adenocarcinoma and colon adenocarcinoma

	No. of cases	Apoptotic index			
		Range	Mean	SD	p-value[a]
Mesothelioma	32	0 - 41.9	17.6	11.1	
Epitheliod mesothelioma	21	0 - 34.9	15.2	9.9	
Sarcomatoid mesotehlioma	9	5.0 - 41.9	23.5	12.9	0.063[b]
Biphasic mesothelioma	2	11.1 - 23.0	17.1	8.4	
Lung carcinoma	10	10.0 - 57.8	28.6	15.8	0.018[c]
Gastric adenocarcinoma	10	6.5 - 57.0	30.1	16.4	0.009[c]
Colon adenocarcinoma	10	12.8 - 71.3	31.4	17.3	0.005[c]

[a] Student t- test

[b] Epithelioid mesothelioma versus sarcomatoid mesothelioma

[c] Versus mesothelioma

Table 3. Apoptotic index and cleaved caspase-3 index in mesothelioma

	No. of cases	Range	Mean	SD	r	p-value*
Apoptotic index	32	0 - 41.9	17.6	11.3	0.373	0.036
Cleaved caspase-3 index	34	0 - 36.5	14.7	10.6		

* Pearson's correlation used 32 cases, except for 2 cases as not informative of apoptosis.

DISCUSSION

In the present study, the mean apoptotic index (AI) in mesothelioma tissue was 17.6, which is similar to those reported previously in mesothelioma[6, 16]. With similar techniques, mean AI in various other human malignancies of lung, stomach and colon were 28.6, 30.1 and 31.4, respectively (Table 2). The mean AI in non-small cell lung carcinoma has been previously reported to be as low as 13.9[9] or 20.7[12]. This discrepancy of AI in our result may be due to the difference in technique or the kit used for the TUNEL method. Therefore, the mean AI in mesothelioma is significantly lower than that in other malignancies, suggesting the more aggressive nature of mesothelioma.

Caspase-3 is an inactive proenzyme and its cleavage by other upstream proteases such as active caspase-8 and caspase-9 leads to its active form, called cleaved caspase-3[13, 18]. IAPs inhibit the proteolytic cleavage of caspase proforms caspase-3 and caspase-9[8, 11, 17] and/or directly inhibits activated caspases[4]. In the present study, we used two different anti-caspase-3 and anti-cleaved caspase-3 antibodies. The former can detect full length caspase-3 (35 kD) and the large fragment of caspase-3 resulting from cleavage (17 kD), and the latter can specifically detect the large fragment (17/19 kD). Mesothelioma
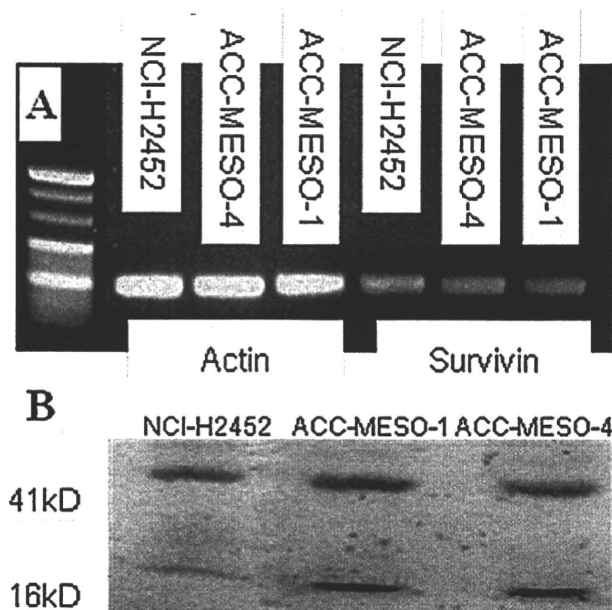

Fig. 2. (A) Electrophoresis of RT-PCR product of survivin mRNA and actin mRNA amplification shows 121 bp and 120 bp fragments. (B) Western blot analysis shows expression of survivin protein (16 kD) and actin (41 kD) in all three of the mesothelioma cell lines.