

# 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  $\beta$ -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  $\beta$ -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  $\mu$ g total RNA using

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**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	GGGGATTGCGTTTTTTGTTTTTC	CATACCGACTCTACGCCCTA	109	40-45	62
Unmethylated	GTTTTTTGTTTGTGGGGTT	ATAAAAATACACACCACCTC	109	40-45	62
SFRP2 promoter					
Methylated	GGGTTGTAGCGTTTCGTTTC	ACCCGCTCTCTTCGCTAAAT	113	40-45	60
Unmethylated	GGGTTGTAGTGTTTTGT	ACCCACTCTCTTCACTAAAT	113	40-45	56
SFRP4 promoter					
Methylated	GTTTTTTGTTTGTGGGGTTC	ATAAAAATACGCACCGCCTC	133	40-45	58
Unmethylated	GTTTTTTGTTTGTGGGGTT	ATAAAAATACACACCACCTC	133	40-45	54
$\beta$ -catenin exon3					
$\beta$ -cat1	AAAGTAACATTTCCAATCTACTAATGC	CTGTGGTAGTGGCACCAGAA	163	40	60
$\beta$ -cat2	GAATCCATTCTGGTGCCACT	TGACTTTTCAGTAAGGCAATGAAAA	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  $\mu$ g 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  $\mu$ l 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 <sup>a</sup>
	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

<sup>a</sup>Statistical analysis of log transformed 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.

***β-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 β-catenin gene, which contains the consensus GSK-3β 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<sub>2</sub> (all

purchased from Invitrogen Corp.). The cells were seeded at 10<sup>5</sup> 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<sup>5</sup> 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-protein 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 (Scitrope, Tokyo, Japan). The primary polyclonal antibodies were anti-*WIF-1* (1:1000; Cell Signaling Technologies, Danvers, MA, USA) and β-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 <sup>a</sup>
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

<sup>a</sup>Statistical analysis of log transformed data by t-test with Benjamini and Hochberg correction.

**Immunohistochemistry.** Immunohistochemistry for the detection of WIF-1 expression was performed using 3- $\mu$ m 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<sub>2</sub>O<sub>2</sub> in PBS for 30 min, and then the tissue sections were incubated with anti-WIF-1 antibody (1:1000; Cell Signaling

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

<sup>a</sup>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.

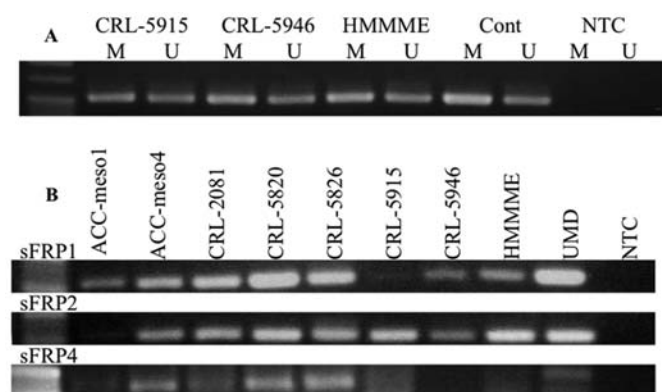


Figure 2. Methylation-specific PCR analysis in mesothelioma cell lines. Mesothelioma cell lines show *WIF-1* (A) and *SFRP1*, 2 and 4 promoter methylation [(B), only methylated bands are shown]. M, methylated DNA product amplified with methylation-specific primers; U, unmethylated DNA product amplified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

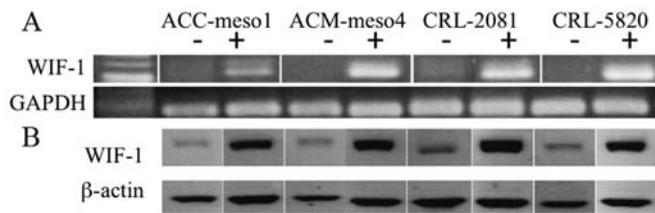


Figure 3. Real-time RT-PCR shows amplification of WIF-1 mRNA in a 5-aza-2dC treated mesothelioma cell line compared to amplification of GAPDH mRNA in cell lines treated either with or without 5-aza-2dC. The PCR product from real-time RT-PCR was electrophoresed in a 2% agarose gel followed by ethidium 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 mesothelioma 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-

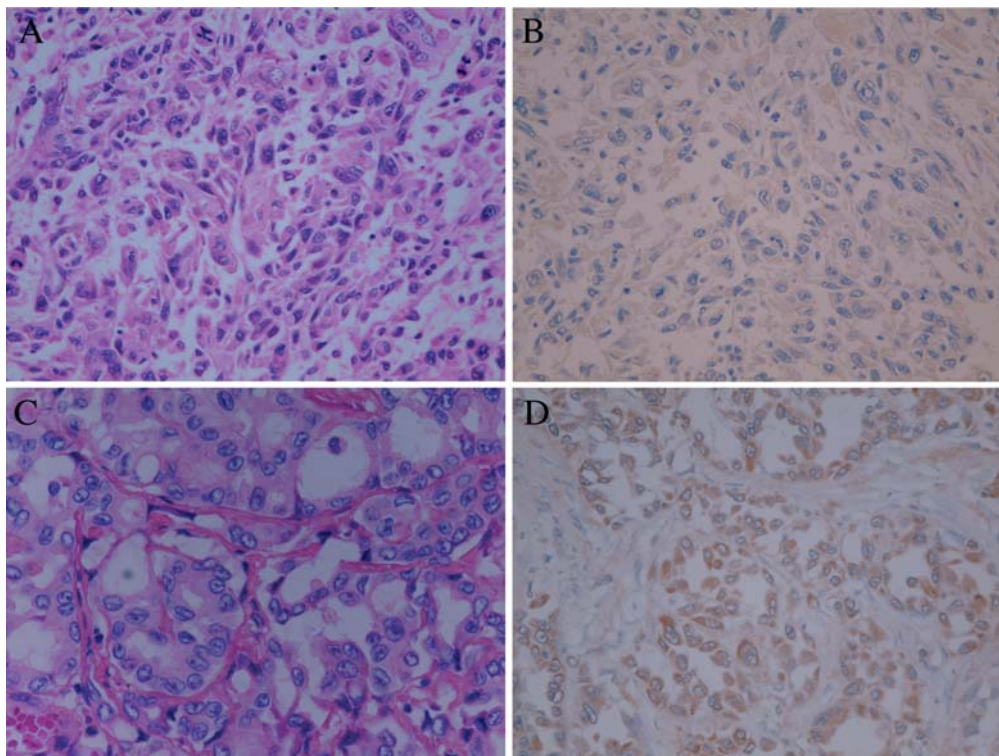


Figure 4. Immunohistochemistry. Sarcomatoid mesothelioma (A) with *WIF-1* promoter methylation shows no WIF-1 immunoreactivity (B). Epithelioid mesothelioma (C) without *WIF-1* promoter methylation shows WIF-1 immunoreactivity in the cytoplasm of tumor cells (D).

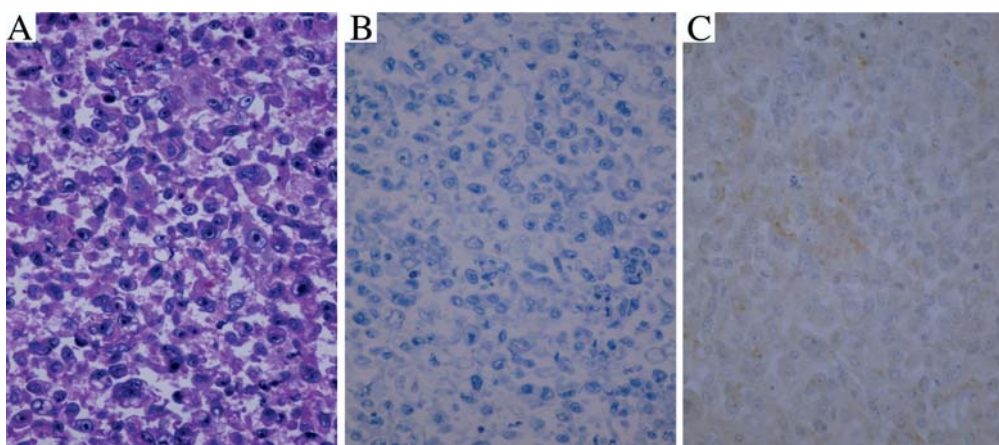


Figure 5. Immunohistochemistry. Mesothelioma cells, ACC-Meso1 (A) that had no expression of WIF-1 (B) prior to treatment with demethylating agents show membranous and cytoplasmic expression of WIF-1 after 5-aza-2dC treatment (C).

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

Sample ID	Age	Gender	Histology	Site	Methylation				β-catenin mutation	Immunoreactivity score	
					WIF-1	SFRP1	SFRP2	SFRP4		WIF-1	β-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				β-catenin mutation	Immunoreactivity score	
					WIF-1	SFRP1	SFRP2	SFRP4		WIF-1	β-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.

***$\beta$ -catenin expression and mutation.***  $\beta$ -catenin nuclear immunoreactivity 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-3 $\beta$  phosphorylation sites of exon 3 of  *$\beta$ -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- $\beta$ -catenin (canonical), planar cell polarity and Wnt-Ca<sup>2+</sup> pathways (non-canonical) are known; canonical pathway being studied in details. We analyzed the expression of  $\beta$ -catenin in mesothelioma tissue and cell lines. We found the cytoplasmic expression of  $\beta$ -catenin in most of the tumor cells without its accumulation in nucleus. In addition, we could not detect any mutation in GSK-3 $\beta$  phosphorylation sites of exon 3 of  *$\beta$ -catenin* gene, suggesting that mesothelioma did not involve the canonical pathway. Microarray analysis of this study did not reveal  *$\beta$ -catenin* alteration. Instead, we found alteration of PLC and CaMKII, genes involved in Wnt-Ca<sup>2+</sup> 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<sup>2+</sup> 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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