表2　テモゾロミドによる骨髄異形成症候群・白血病の報告

腫瘍	前治療	テモゾロミド回数	テモゾロミド開始から発病までの期間	病型	生存期間	文献
AA	RT, ACNU(400mg), IFN-β	4コース	8カ月	MDS	2カ月後死亡	20
GBM	RT	照射併用＋維持1コース	4カ月	ALL	5カ月生存	21
AO	RT, PCV(4コース), BCNU wafer	25コース	41カ月	MDS→AML	5カ月後死亡	22

AA: anaplastic astrocytoma, GBM: glioblastoma, AO: anaplastic oligodendroglioma, RT: radiotherapy, MDS: myelodysplastic syndroma, ALL: acute lymphoblastic leukemia, AML: acute myeloblastic leukemia.

30.3%という成績であった．これはテモゾロミド以前の成績の15%[17]，テモゾロミド単独による成績21%[18]と比べて優れたものであったが，grade 3以上のリンパ球数減少が24%の頻度で出現し，ニューモシスティス肺炎を1例に認めた他に約15%の症例で何らかの感染症（herpes zoster，尿路感染，上気道感染）を認めた．

他の方法はalternating weekly regimenあるいは1-week-on/1-week-offとよばれる方法で，150mg/m^{2}という用量を用いるものである[19]．64例の再発膠芽腫において6カ月無増悪生存率43.8%と報告され，リンパ球数減少の頻度は高いものの持続時間がthree weeks on and one week off法よりも短い傾向にあるとも報告されている．

D. 二次癌

テモゾロミドはアルキル化剤である．長期の暴露は二次癌，特に骨髄異形成症候群や白血病のリスクを高めることが知られている．テモゾロミドによると推測される骨髄異形成症候群・白血病は現在までに3例報告されている（表2）．テモゾロミドは開発・発売されてから日が浅いこと，また特に膠芽腫の場合は原病の生存期間中央値が1年でしかないことから，このような二次癌の報告はまだまだ少ないようであるが，今後，より生存期間の長い腫瘍型にも長期間投与される症例が増えてくると，二次癌の報告も増加していく可能性がある．

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Dysadherin Expression as a Significant Prognostic Factor and as a Determinant of Histologic Features in Synovial Sarcoma: Special Reference to its Inverse Relationship With E-cadherin Expression

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Abstract: Dysadherin is a cancer-associated cell membrane glycoprotein, which down-regulates E-cadherin and promotes metastasis. Synovial sarcoma is a very rare mesenchymal tumor that exhibits an epithelial profile. To confirm the diagnosis of synovial sarcoma, we evaluated several immunohistochemical markers, or detected SYT-SSX fusion gene transcript. We studied the clinicopathologic features in 92 synovial sarcoma patients and also assessed the immunohistochemical expression of dysadherin and E-cadherin to examine their possible association with histologic subtype and biologic behavior. Moreover, among 30 patients, for whom frozen materials were available, *dysadherin* mRNA expression was examined by reverse transcription-polymerase chain reaction and real-time quantitative reverse transcription-polymerase chain reaction analysis. Dysadherin-positive expression was significantly correlated with E-cadherin–reduced expression ($P = 0.0004$). Dysadherin-positive immunostaining was diffusely observed in the membranes of tumor cells in 30/68 (44%) patients with monophasic fibrous type and in 1/2 (50%) patients with poorly differentiated type. However, in biphasic tumors, dysadherin expression in the fibrous component was not diffusely observed, but often sporadically or focally observed [20/22 (91%) patients]. In addition, *dysadherin* mRNA expression in monophasic fibrous type was significantly higher than in biphasic type ($P = 0.0079$). Synovial sarcoma patients with dysadherin expression survived for a significantly shorter time than those without dysadherin expression ($P = 0.0006$). Patients with combined dysadherin-positive expression and E-cadherin–reduced expression had a significantly worse prognosis than those with other combinations of dysadherin and E-cadherin expression ($P = 0.0007$). SYT-SSX fusion gene transcript was detected in 39 patients. In our series, SYT-SSX fusion type was found to have no correlation with histologic subtype, prognosis, or dysadherin expression. In multivariate analysis, dysadherin immunopositivity ($P = 0.0411$) was an independent adverse prognostic factor, in addition to a high MIB-1 labeling index ($\geq 10\%$). We conclude that E-cadherin dysfunction by dysadherin is associated with reduced E-cadherin expression and morphologic change from epithelioid to spindle phenotype. Dysadherin expression is considered to be one of the determinants of histologic subtype in synovial sarcoma. Moreover, dysadherin expression is an excellent and independent prognostic indicator.

Key Words: synovial sarcoma, dysadherin, E-cadherin, prognosis, histologic features

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Synovial sarcomas are known to have 2 major forms, monophasic fibrous type, in which the tumors are composed of spindle cells, and biphasic type, in which the tumors contain both epithelial cells arranged in a glandular structure and spindle cells. In an effort to find the possible determinants of the histologic subtype of synovial sarcoma, studies have focused on the chimeric fusion transcripts, SYT-SSX1 and SSX2, that are caused by a characteristic chromosomal translocation, t(X;18)(p11;q11).[16] It has been demonstrated that there is a significant relationship between histologic subtype and the type of fusion transcript, although the mechanisms involved in epithelial differentiation in synovial

sarcoma remain unclear.[1,16,19] Dysadherin is a cancer-associated cell membrane glycoprotein, which down-regulates E-cadherin by a posttranscriptional mechanism and promotes metastasis.[15] In the present study, we assessed the immunohistochemical expression of dysadherin and E-cadherin in synovial sarcoma, and we examined their possible association with histologic subtype and biologic behavior. In addition, we also examined *dysadherin* mRNA expression in synovial sarcoma, and compared its expression with dysadherin immunohistochemical expression and histologic features. The aim of this study was to evaluate the influence of dysadherin on the progression of synovial sarcoma, with special emphasis on its histologic subtype, and to clarify the importance of dysadherin in the biologic behavior of synovial sarcoma.

MATERIALS AND METHODS

Cases were selected from among more than 12,000 cases of bone and soft-tissue tumors registered in the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan between 1955 and 2004. Because written informed consent had not been obtained, identifying information for all patients was removed before analysis for the purpose of strict privacy protection. There were 92 synovial sarcoma patients, suitable for immunohistochemical study. Histologic subtypes comprised 68 patients with monophasic fibrous type, 22 patients with biphasic type, and 2 patients with poorly differentiated synovial sarcoma. The monophasic fibrous type synovial sarcoma may resemble a number of other spindle cell neoplasms such as fibrosarcoma, leiomyosarcoma, malignant peripheral nerve sheath tumor, hemangiopericytoma, and spindle cell carcinoma. To confirm the diagnosis of synovial sarcoma, we evaluated several useful immunohistochemical markers such as cytokeratins (cytokeratin 7, cytokeratin 19, etc.), epithelial membrane antigen, S-100 protein, myogenic markers, bcl-2 protein and CD34,[26,27] or examined SYT-SSX fusion gene transcript. Frozen materials were available in 30 patients to detect SYT-SSX fusion gene transcript. Moreover, formalin-fixed paraffin-embedded materials were available to try detecting SYT-SSX fusion gene transcript in additional 43 patients. Clinical data for these 92 patients were collected from the medical records. Survival data were available for all 92 patients. Follow-up ranged from 1 to 278 months (mean, 56.2 mo). To assess the correlation between clinicopathologic factors and dysadherin expression, age, sex, the anatomic location of the tumor, tumor depth, size, glandularity (histologic subtype), mitotic rate, tumor necrosis, the presence of rhabdoid cells, SYT-SSX fusion type, FNCLCC (French Fédération Nationale des Centres de Lutte Contre le Cancer) grade and AJCC (American Joint Committee on Cancer) stage, were analyzed. The influence of these factors on prognosis has been previously reported.[26] As for histologic subtype, "presence of glandularity" implies biphasic type, whereas "absence of glandularity" includes monophasic fibrous type and poorly differentiated type. As for tumor depth, "superficial" tumor is located exclusively above the superficial fascia without invasion of the fascia, whereas "deep" tumor is located either exclusively beneath the superficial fascia, or superficial to the fascia with invasion of or through the fascia. As for tumor location, "distal" tumor occurs in the distal extremities (below knee or below elbow), whereas "proximal" tumor occurs in the proximal region of the extremities and in the trunk.

Immunohistochemistry

Immunohistochemical study was performed using mouse IgG monoclonal antibodies against dysadherin[15] (NCC-M53, 1:1000, National Cancer Center, Tokyo), E-cadherin (1:1000, BD Transduction Laboratories, San Jose, CA), and Ki-67 (MIB-1, 1:100, Dako, Grostrup, Denmark). Specimens are pretreated in an autoclave for dysadherin (20 min, 120°C) or in a microwave oven for E-cadherin and MIB-1 (20 min for both antibodies). Sections were incubated with the primary antibodies at 4°C overnight, followed by staining with a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan). Staining for dysadherin and E-cadherin was localized within the cell membranes. The pattern of dysadherin immunostaining in tumors was compared with that observed in the basal cells of normal skin tissue, endothelial cells and lymphocytes. Dysadherin immunoreactivity was evaluated in the fibrous component that was common to the 3 histologic subtypes, even in the biphasic type containing glandular components. Tumors were classified as dysadherin-positive when more than 50% of the cells in the fibrous component were stained.[2,24,34,35] Moreover, the proportion of dysadherin positive cells was evaluated semiquantitatively and classified into 4 groups: 0% to 9% (1+; sporadic), 10% to 29% (2+; focal), 30% to 49% (3+), and ≥ 50% (4+; diffuse). E-cadherin immunoreactivity has been described as preserved (positive) when more than 90% of the cells were stained within the membrane of the tumor cells.[29] The proportion of E-cadherin positive cells was also evaluated semiquantitatively and classified into 4 groups: 0% to 49% (1+), 50% to 69% (2+), 70% to 89% (3+), and ≥ 90% (4+). In biphasic types of synovial sarcoma, E-cadherin immunoreactivities were evaluated in both the fibrous and the glandular components, and if at least one component was described as preserved (positive), then that case was described as preserved (positive).[29] The MIB-1–labeling index was estimated as the percentage of Ki-67–positive cells based on a count of 1000 tumor cells within the tumor. Dysadherin and E-cadherin expression were evaluated independently by 3 of the authors (T.I., Y.O., and T.H.) without any knowledge of the clinical features in each case.

RT-PCR and Real-time Quantitative RT-PCR to Detect *Dysadherin* mRNA

Frozen materials were also available for 30 patients with synovial sarcoma (20 patients with monophasic

fibrous type, 8 patients with biphasic type, and 2 patients with poorly differentiated type). Two synovial sarcoma cell lines (HS-SY-II,[39] and SYO-1[17]) were generously provided by the coauthors for this study. HS-SY-II was established from monophasic fibrous type, whereas SYO-1 was established from biphasic type synovial sarcoma. Total RNA was prepared, using Trizol Reagent (Invitrogen Corp, Carlsbad, CA) according to the manufacturer's protocol. Five micrograms of RNA in each sample were used for the subsequent reverse transcription. Sequences of specific pairs of primers were as follows: *dysadherin* (upper primer: 5'-AGA GCA CCA AAG CAG CTC AT-3'; lower primer: 5'-GGG TCT GTC TGG ACG TCT GT-3'; product size, 85 bp). The PCR products were electrophoresed in 2.0% agarose gel and visualized with ethidium bromide. cDNA from the Li-7 human hepatoma cell line was used as a positive control for *dysadherin* expression, and PLC/PRF/5 was used as a negative control.[15]

Real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) (TaqMan PCR) for *dysadherin* was performed using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) and predeveloped TaqMan assay reagents of human *dysadherin* and *GAPDH*. The PCR reaction was carried out according to the manufacturer's protocol.[13] The standard curve was constructed with serial dilutions of the cDNA samples of Li-7. All reactions of the samples were triplicated and the data were averaged from the values obtained in each reaction. To determine the mRNA levels of *dysadherin*, we used an mRNA expression index (EI), which is a relative mRNA expression level standardized by using the internal housekeeping gene, *GAPDH*. The *dysadherin* mRNA EI was calculated as follows [in arbitrary units (AU)]: mRNA EI = (copy numbers of *dysadherin* mRNA/copy numbers of *GAPDH* mRNA) × 1000 AU.

Statistical Analysis

The survival curves were analyzed by the Kaplan-Meier method and the outcome of the different groups of patients was compared by the log-rank test. The Cox proportional hazards regression with the stepwise procedure was used in multivariate analysis of survival data. The correlations between dysadherin and E-cadherin immunoreactivity and glandularity were evaluated using the Mann-Whitney *U* test. The correlation between dysadherin and E-cadherin expression was evaluated using Kendall rank correlation coefficient. Fisher exact test was used to evaluate the association between 2 dichotomous variables. The correlations between each group and *dysadherin* mRNA expression were determined by using the Mann-Whitney *U* test. Probability values of less than 0.05 were considered as significant.

RESULTS

The clinicopathologic findings of 92 patients with synovial sarcoma are summarized in Table 1. Sixty patients were affected at the proximal extremities or trunk, whereas 32 patients had a tumor at the distal extremities. Eighty-one tumors were deeply situated, and 11 were superficially situated. Histologically, 22 patients were categorized as presence of glandularity (biphasic type) and 70 patients as absence of glandularity (monophasic fibrous type and poorly differentiated type). Rhabdoid cells were detected in 9 patients, but were absent in 83 patients. Among the 30 patients, for whom frozen materials were available, 20 patients demonstrated SYT-SSX1 fusion type, whereas 10 patients demonstrated SYT-SSX2 fusion type. Among the 43 patients, for whom only formalin-fixed paraffin-embedded materials were available, high quality and quantity of total RNA suitable for RT-PCR analysis could be extracted in 15 patients. Fusion type was detected in 9 out of these 15 patients. Eight patients demonstrated SYT-SSX1 fusion type, whereas 1 patient demonstrated SYT-SSX2 fusion type.

Immunohistochemical Findings

The results of immunohistochemical study for dysadherin and E-cadherin are summarized in Table 2. As for glandularity, there was a strong relationship between higher dysadherin expression and absence of glandularity ($P = 0.0002$), and a strong relationship between higher E-cadherin expression and presence of glandularity ($P < 0.0001$). Dysadherin-positive staining was diffusely ($\geq 50\%$) and strongly observed in the membranes of tumor cells in 30/68 (44%) patients with monophasic fibrous type (Fig. 1B) and in 1/2 (50%) patients with poorly differentiated type. E-cadherin membranous expression was reduced (negative) in 56/68 (82%) patients with monophasic fibrous type (Fig. 1C) or in 2/2 (100%) patients with poorly differentiated type. In biphasic tumors, dysadherin expression in the fibrous component was not diffusely ($\geq 50\%$) observed, but often sporadically (0% to 9%) or focally (10% to 29%) observed [20/22 (91%) patients]

TABLE 1. Clinicopathologic Parameters in 92 Cases of Synovial Sarcoma

Age (y)		Sex		Location		Depth		Size (cm)		Glandularity	
≤20	19	Male	35	Proximal	60	Deep	81	≤5	35	Present	22
> 20	73	Female	57	Distal	32	Superficial	11	> 5	57	Absent	70
Mitotic Rate (per 10 HPFs)		**Tumor Necrosis (%)**		**Rhabdoid Cells**		**Chimera Gene (39 Patients)**		**FNCLCC Grade**		**AJCC Stage**	
≤15	59	< 50	74	(+)	9	SYT-SSX 1	28	Grade 2	41	Stage II	37
> 15	33	≥ 50	18	(−)	83	SYT-SSX 2	11	Grade 3	51	Stage III	42
										Stage IV	13

TABLE 2. Immunohistochemical Results for Dysadherin and E-cadherin

	Histologic Subtype	
	Glandularity (Absent) (n = 70)	Glandularity (Present) (n = 22)
	Monophasic (n = 68) and Poorly (n = 2)	Biphasic (n = 22)
Dysadherin		
≥ 50% (4+) (diffuse)	31 (1)*	0
30%-49% (3+)	7	2
10%-29% (2+) (focal)	14 (1)*	8
0%-9% (1+) (sporadic)	18	12
E-cadherin		
≥ 90% (4+)	12	20
70%-89% (3+)	4	1
50%-69% (2+)	12	0
0%-49% (1+)	42 (2)*	1

$P = 0.0002$ by Mann-Whitney U test [between dysadherin expression and glandularity (histologic subtype)].
$P < 0.0001$ by Mann-Whitney U test [between E-cadherin expression and glandularity (histologic subtype)].
*Poorly differentiated type.

(Fig. 1E) (Table 2). However, in the glandular component, dysadherin and E-cadherin were frequently coexpressed in 20/22 (91%) patients (Figs. 1E, F). The correlations between dysadherin immunoreactivity and E-cadherin immunoreactivity (inverse relationship) are summarized in Table 3. Kendall τ value was calculated to be −0.339 ($P < 0.0001$, by Kendall rank correlation coefficient), indicating a significant negative correlation between dysadherin expression and E-cadherin expression. The correlations between dysadherin and E-cadherin immunoreactivity and clinicopathologic parameters are summarized in Table 4. There was a strong relationship between absence of glandularity and dysadherin-positive expression ($P < 0.0001$), and a strong relationship between presence of glandularity and E-cadherin—preserved (positive) expression ($P < 0.0001$). Dysadherin-positive expression was also significantly correlated with a high age (> 20 y), a large tumor size (> 5 cm), and a high MIB-1 labeling index (≥ 10%). E-cadherin–preserved (positive) expression was significantly correlated with a low mitotic rate (≤ 15 per 10 HPFs), a low MIB-1 labeling index (< 10%) and a low FNCLCC grade (grade 2). Other parameters including the chimera gene (SYT-SSX fusion type) had no statistically significant correlation with dysadherin or E-cadherin expression.

Dysadherin mRNA Expression by RT-PCR and Real-time Quantitative RT-PCR

The specificity of RT-PCR was confirmed and visualized by the presence of *dysadherin* mRNA in the Li-7 cell line and the absence of *dysadherin* mRNA in the PLC/PRF/5 cell line (Fig. 2). *Dysadherin* mRNA expression by RT-PCR in synovial sarcoma was observed in all 3 histologic subtypes, varying in density (Fig. 2). Monophasic fibrous type tumors showed a higher *dysadherin* mRNA expression compared with biphasic type tumors.

The comparison between histologic subtypes and *dysadherin* mRNA EIs by real-time quantitative RT-PCR is summarized in Figure 3. *Dysadherin* mRNA EIs in monophasic fibrous type (median, 568.25 AU) were significantly higher than those in biphasic type (median, 56.07 AU) ($P = 0.0079$). In addition, the HS-SY-II cell line established from monophasic fibrous type revealed a higher *dysadherin* mRNA EI (286.10 AU) compared with the SYO-1 cell line established from biphasic type (60.86 AU). A comparison between dysadherin immunoreactivity and *dysadherin* mRNA EIs is summarized in Figure 4. Parallel to the immunohistochemical results, dysadherin-positive patients showed statistically significant higher *dysadherin* mRNA EIs (median, 912.32 AU) compared with those seen in dysadherin-negative cases (median, 81.66 AU) ($P = 0.0001$).

Prognostic Value of Dysadherin Expression

Patients with dysadherin immunoreactivity survived for a significantly shorter time than those without dysadherin immunoreactivity ($P = 0.0006$; Fig. 5). Patients with diffuse dysadherin immunoreactivity (4+; ≥ 50%) had the worst survival, whereas patients with sporadic dysadherin immunoreactivity (1+; 0% to 9%) had the best survival ($P = 0.0068$; Fig. 6). The greater the proportion of dysadherin positive cells, the worse the prognosis turned out to be. Patients with combined dysadherin-positive expression and E-cadherin–reduced (negative) expression had a significantly worse prognosis than those with other combinations of dysadherin and E-cadherin expression ($P = 0.0007$; Fig. 7). In univariate analysis, dysadherin-positive expression, E-cadherin–reduced (negative) expression, large tumor size (> 5 cm), absence of glandularity, massive tumor necrosis (≥ 50%), the presence of rhabdoid cells, a high MIB-1 labeling index (≥ 10%), a high FNCLCC grade (grade 3), and a high AJCC stage (stages III and IV) were all significantly correlated to a worse overall survival rate (Table 5). Multivariate analysis revealed that dysadherin immunopositivity ($P = 0.0411$) and a high MIB-1 labeling index (≥ 10%) ($P < 0.0001$) were independent poor prognostic

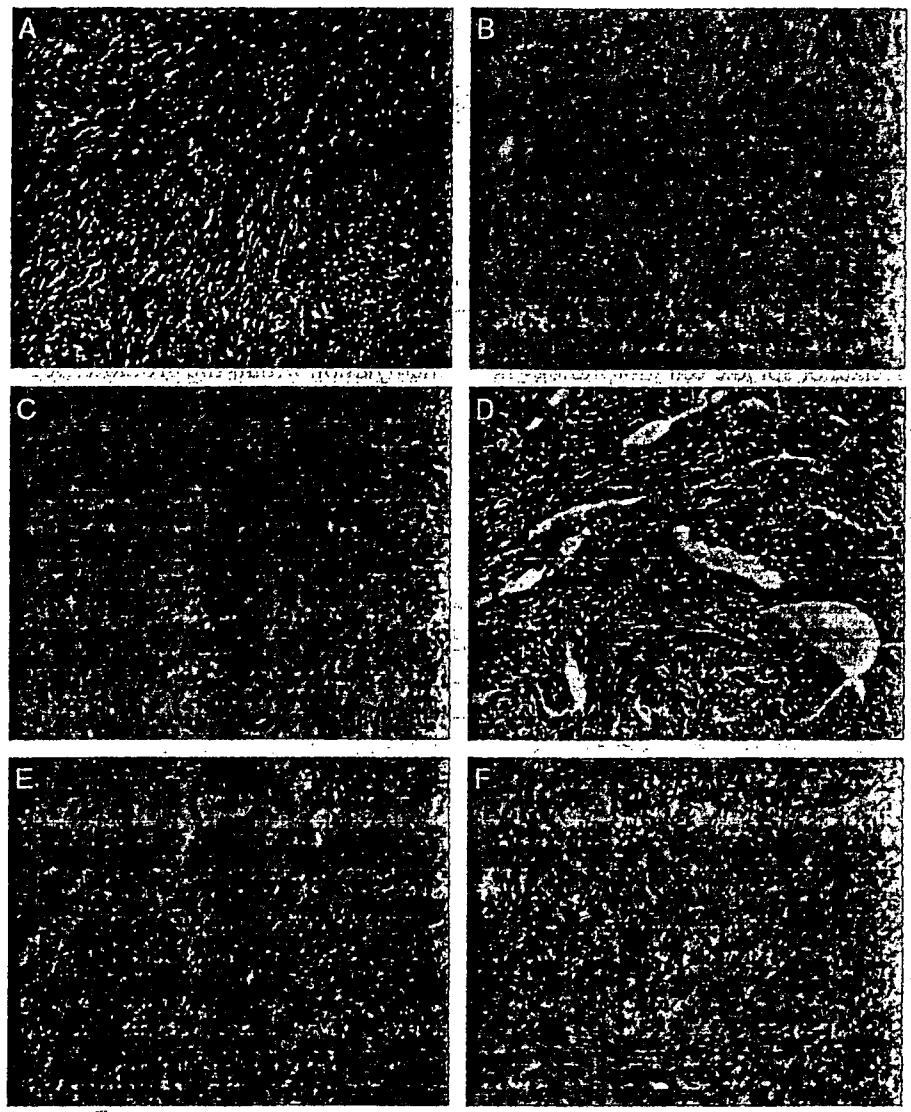

FIGURE 1. The hematoxylin and eosin staining of a monophasic fibrous synovial sarcoma (A). Relatively plump spindle or small ovoid tumor cells arranged in fascicles (A). In monophasic fibrous type, dysadherin-positive staining was diffusely (≥ 50% positive cells) observed in the membranes of tumor cells in 30/68 (44%) patients (B). E-cadherin membranous expression was reduced (negative) in 56/68 (82%) patients and aberrational nuclear staining for E-cadherin was often detected (C). The hematoxylin and eosin staining of a biphasic synovial sarcoma (D). The fibrous component composed by plump spindle or small ovoid cells admixed with variably sized epithelioid glandular component (D). In biphasic type, dysadherin expression in the fibrous component was not diffusely (≥ 50% positive cells) observed [0/22 (0%) patients], but often sporadically (0% to 9% positive cells) or focally (10% to 29% positive cells) observed [20/22 (91%) patients] (E). However, in the glandular component, dysadherin (E) and E-cadherin (F) were frequently coexpressed in 20/22 (91%) patients.

factors (Table 5). Other clinicopathologic prognostic factors were not significant.

DISCUSSION

Dysadherin is a cancer-associated cell membrane glycoprotein, which down-regulates E-cadherin by a posttranscriptional mechanism and promotes metastasis.[15] Dysadherin is expressed in a variety of cancer cells and malignant melanoma cells,[2,15,24,25,34,35] however, it has never been reported in the field of sarcomas.

Synovial sarcoma is a mesenchymal tumor that has an epithelial profile, with cell shapes varying from epithelioid to spindle phenotype. Recent reports have demonstrated E-cadherin expression in sarcomas with epithelioid features, such as synovial sarcoma, especially in the glandular structures of biphasic type.[20,29,32,44] A reduced E-cadherin expression by genetic and epigenetic alterations in the E-cadherin gene has been shown to cause cellular morphologic changes in epithelial cells, from epithelioid features to a more spindle phenotype.[3,6,42] Initially, we identified mutational inactivation

TABLE 3. Correlation Between Immunoreactivity of Dysadherin and E-cadherin (Inverse Relationship)

	E-cadherin			
Dysadherin	≥ 90% (4+)	70%-89% (3+)	50%-69% (2+)	0%-49% (1+)
≥ 50% (4+) (diffuse)	3	3	3	22
30%-49% (3+)	5	0	1	3
10%-29% (2+) (focal)	7	2	2	11
0%-9% (1+) (sporadic)	17	0	6	7

$P < 0.0001$ by Kendall rank correlation coefficient.
Kendall τ value was calculated to be −0.339, indicating a significant negative correlation between dysadherin expression and E-cadherin expression.

of E-cadherin as a correlate of spindle cell morphology in synovial sarcoma, being noted in 12/49 (25%) of tumors.[30] In addition, silencing of E-cadherin by CpG hypermethylation within its promoter region has also been reported in other carcinomas such as breast, gastric, urinary bladder, and thyroid carcinomas and several carcinoma cell lines.[7,8,12,14,40] In our previous study, CpG methylation within the promoter region was also found to occur in 5/40 (13%) of synovial sarcomas. However, E-cadherin was silenced at the mRNA level in only 1 of the 5 tumors.[31] In the current study, dysadherin-positive immunostaining was diffusely and strongly observed in the membranes of spindle-shaped tumor cells in 30/68 (44%) patients with monophasic fibrous type and in 1/2

TABLE 4. Correlation Between Dysadherin and E-cadherin Immunoreactivity and Clinicopathologic Parameters

	Dysadherin			E-cadherin		
Variables	(+)	P	(−)	(+)	P	(−)
Age (y)		0.027*			0.589	
≤20 (n = 19)	2		17	8		11
> 20 (n = 73)	29		44	24		49
Sex		> 0.999			0.181	
Male (n = 35)	12		23	9		26
Female (n = 57)	19		38	23		34
Location		> 0.999			> 0.999	
Proximal (n = 60)	20		40	21		39
Distal (n = 32)	11		21	11		21
Depth		0.498			0.742	
Deep (n = 81)	26		55	29		52
Superficial (n = 11)	5		6	3		8
Size (cm)		0.012*			0.261	
≤5 (n = 35)	6		29	15		20
> 5 (n = 57)	25		32	17		40
Glandularity		< 0.0001*			< 0.0001*	
Present (n = 22)	0		22	20		2
Absent (n = 70)	31		39	12		58
Mitotic rate (per 10 HPFs)		0.107			0.0006*	
≤15 (n = 59)	16		43	28		31
> 15 (n = 33)	15		18	4		29
Tumor necrosis (%)		0.163			> 0.999	
< 50 (n = 74)	22		52	26		48
≥ 50 (n = 18)	9		9	6		12
Rhabdoid cells		0.057			0.488	
(+) (n = 9)	6		3	2		7
(−) (n = 83)	25		58	30		53
MIB-1 labeling index (%)		0.004*			0.016*	
< 10 (n = 50)	10		40	23		27
≥ 10 (n = 42)	21		21	9		33
Chimera gene		0.7388			0.7129	
SYT-SSX 1 (n = 28)	11		17	11		17
SYT-SSX 2 (n = 11)	5		6	3		8
FNCLCC grade		0.2689			0.0155*	
Grade 2 (n = 41)	11		30	20		21
Grade 3 (n = 51)	20		31	12		39
AJCC stage		0.071			0.1855	
Stage II (n = 37)	8		29	16		21
Stages III and IV (n = 55)	23		32	16		39

*Statistically significant.

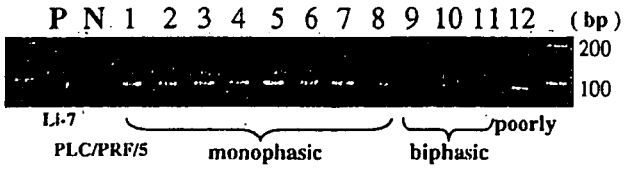

FIGURE 2. Results of RT-PCR to detect *dysadherin* mRNA expression using frozen samples. P: positive control (Li-7 cell line); N: negative control (PLC/PRF/5 cell line); lanes 1 to 8: monophasic fibrous type; lanes 9 to 11: biphasic type; lane 12: poorly differentiated type. *Dysadherin* mRNA expression was observed in all 3 histologic subtypes varying in density. Monophasic fibrous type tumors showed higher *dysadherin* mRNA expression compared with biphasic type tumors.

(50%) patients with poorly differentiated type. Whereas, E-cadherin membranous expression was frequently reduced (negative) in 56/68 (82%) patients with monophasic fibrous type and in all 2/2 (100%) patients with poorly differentiated type. In addition, dysadherin-positive expression was found to be significantly correlated with E-cadherin–reduced (negative) expression ($P = 0.0004$). Some previous studies have demonstrated a significant positive correlation between dysadherin and E-cadherin expression in tongue carcinoma,[24] but other studies have failed to demonstrate such correlation in pancreatic, colorectal, or gastric carcinoma.[2,34,35] In synovial sarcoma, it seems that dysadherin plays a most important role in the down-regulation of E-cadherin and in the demonstration of spindle cell morphology, compared with other already known genetic and epigenetic mechanisms. Interestingly, in biphasic tumors, dysadherin expression in the fibrous component was not diffusely observed, but often sporadically or focally observed [20/22 (91%) patients]. However, in the glandular component, dysadherin and E-cadherin were frequently coexpressed in 20/22 (91%) patients. In biphasic tumors, we consider that dysadherin expression in the fibrous component might be much important and significant for prognosis as compared with dysadherin expression in the glandular component, because in the glandular component, function of dysadherin would be counteracted and weakened by frequent coexpression of E-cadherin. These findings are similar to those of a previously reported study dealing with thyroid papillary carcinoma[33] in that dysadherin and E-cadherin coexpression was frequently observed in more than 38/51 (75%) tumors at the cell-cell boundaries. When papillary carcinoma is classified with

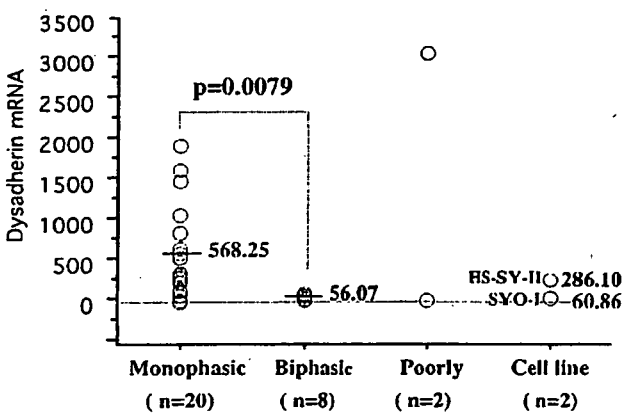

FIGURE 3. Scattergram of *dysadherin* mRNA EIs in synovial sarcoma according to histologic subtypes (monophasic fibrous, biphasic, and poorly differentiated type) and cell lines. *Dysadherin* mRNA EIs in monophasic fibrous type (median, 568.25 AU) were significantly higher than those in biphasic type (median, 56.07 AU) ($P = 0.0079$). The HS-SY-II cell line established from monophasic fibrous type revealed a higher *dysadherin* mRNA EI (286.10 AU) compared with the SYO-1 cell line established from biphasic type (60.86 AU).

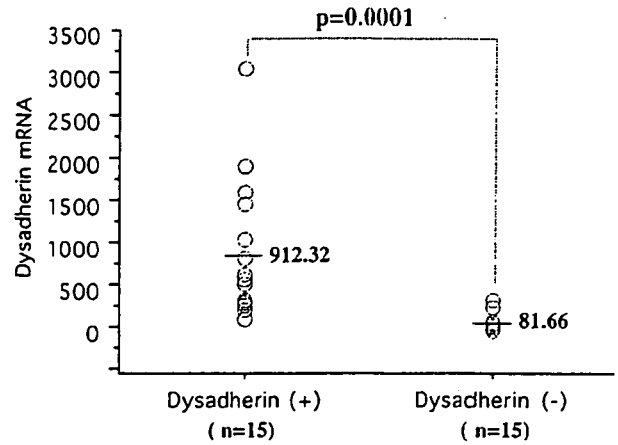

FIGURE 4. Scattergram of *dysadherin* mRNA EIs in synovial sarcoma according to dysadherin immunoreactivity. Dysadherin-positive patients showed statistically significant higher *dysadherin* mRNA EIs (median, 912.32 AU) compared with those seen in dysadherin-negative patients (median, 81.66 AU) ($P = 0.0001$).

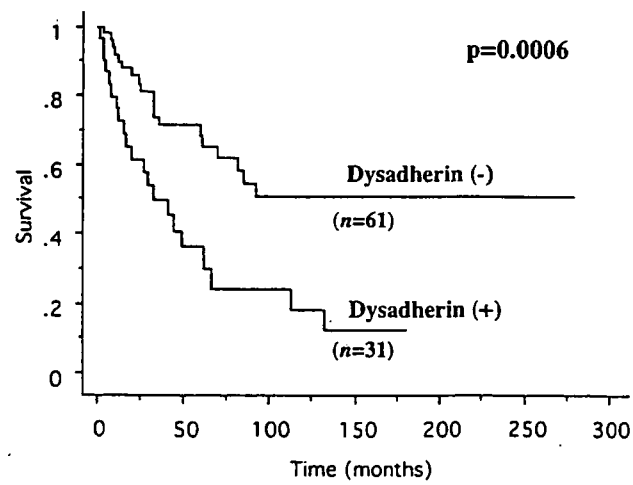

FIGURE 5. Survival of patients with and without dysadherin immunoreactivity. The survival of patients with dysadherin immunoreactivity (≥ 50% positive cells) was significantly worse than that of patients without dysadherin immunoreactivity ($P = 0.0006$).

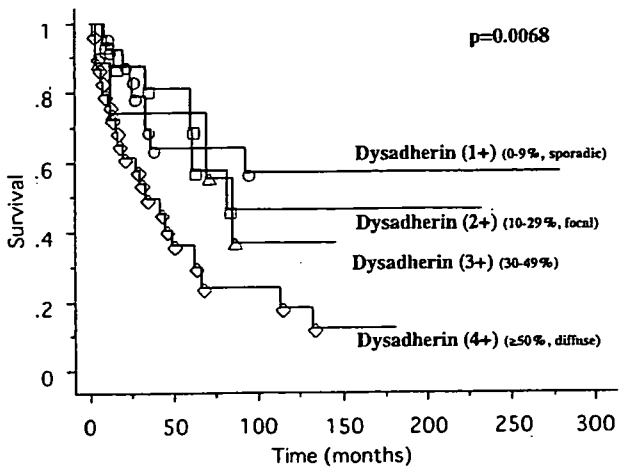

FIGURE 6. Survival in relation to the proportion of dysadherin positive cells. Patients with diffuse dysadherin immunoreactivity (4+; ≥ 50%) had the worst survival, whereas patients with sporadic dysadherin immunoreactivity (1+; 0% to 9%) had the best survival ($P = 0.0068$). The greater the proportion of dysadherin positive cells, the worse the prognosis turned out to be.

respect to the occurrence of undifferentiated carcinoma as a secondary carcinoma, then dysadherin expression is found to be significantly higher in papillary carcinoma with undifferentiated carcinoma components than in papillary carcinoma without undifferentiated carcinoma components.[33] It has been hypothesized that the biphasic type of synovial sarcoma is similar to "papillary carcinoma without an undifferentiated carcinoma component" with preserved (positive) E-cadherin expression and low dysadherin expression, whereas monophasic fibrous type synovial sarcoma is similar to "papillary carcinoma with an undifferentiated carcinoma component" with reduced (negative) E-cadherin expression and high dysadherin expression (Fig. 8). In synovial sarcoma, biphasic type tumors with epithelioid glandular components would then transform into spindle-shaped monophasic type tumors in accordance with the increased dysadherin expression and reduced E-cadherin expression (Fig. 8). In fact, by real-time quantitative RT-PCR analysis of 30 frozen samples, *dysadherin* mRNA Els in monophasic fibrous type were found to be significantly higher than those in biphasic type ($P = 0.0079$). Parallel to the immunohistochemical results, dysadherin-positive

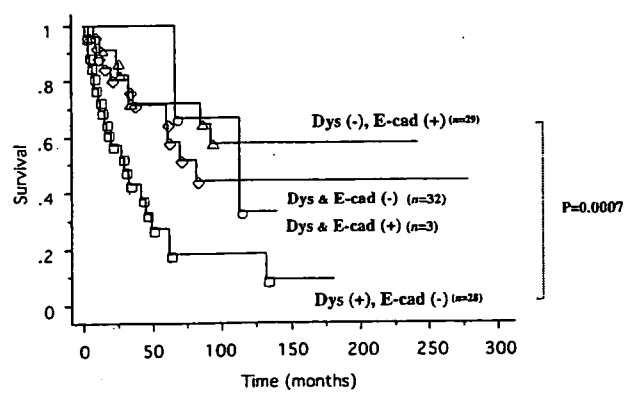

FIGURE 7. Survival in relation to the combined dysadherin/E-cadherin status. Patients with dysadherin immunoreactivity and reduced E-cadherin expression had the worst survival, whereas patients with negative dysadherin immunoreactivity and preserved E-cadherin expression had the best survival. Dys (+), dysadherin immunoreactivity; Dys (−), negative dysadherin immunoreactivity; E-cad (+), preserved E-cadherin expression; E-cad (−), reduced E-cadherin expression.

TABLE 5. Survival Analysis in 92 Cases of Synovial Sarcoma

Variables	*P* Value on Survival Analysis: Univariate	Multivariate
Dysadherin (+; ≥ 50%)	0.0006*	0.0411*
E-cadherin (−; < 90%)	0.0217*	0.1133
Age (> 20 y)	0.0860	0.1996
Sex (male)	0.0680	0.1456
Location (proximal)	0.2167	0.2208
Depth (deep)	0.0926	0.1219
Size (> 5 cm)	0.0069*	0.4898
Glandularity (absent)	0.0369*	0.6246
Mitotic rate (> 15 per 10 HPFs)	0.2672	0.8057
Tumor necrosis (≥ 50%)	0.0170*	0.2799
Rhabdoid cells (+)	0.0249*	0.1638
MIB-1 labeling index (≥ 10%)	< 0.0001*	< 0.0001*
FNCLCC grade (grade 3)	0.0014*	0.4198
AJCC stage (stages III and IV)	0.0096*	0.2647

*Statistically significant.

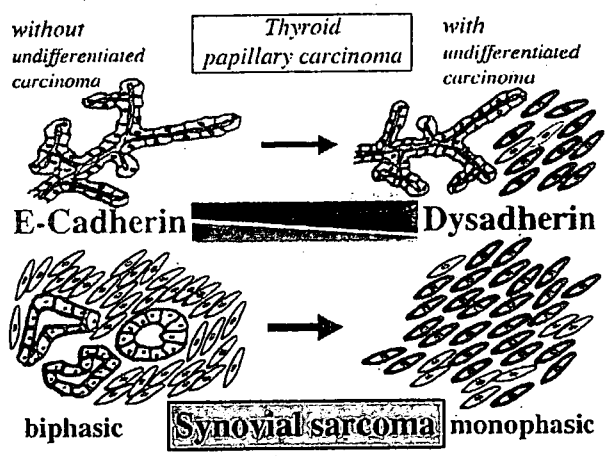

FIGURE 8. Histologic transformation model of thyroid papillary carcinoma and synovial sarcoma according to inverse expression between dysadherin and E-cadherin. In thyroid papillary carcinoma (PC),[33] "PC without undifferentiated carcinoma components" transforms into "PC with undifferentiated carcinoma components" in accordance with the increased dysadherin expression and reduced E-cadherin expression. Likewise, in synovial sarcoma, biphasic type tumors with epithelioid glandular components transform into spindle-shaped monophasic type tumors in accordance with the increased dysadherin expression and reduced E-cadherin expression.

patients showed statistically significant higher *dysadherin* mRNA EIs compared with those seen in dysadherin-negative patients ($P = 0.0001$). In biphasic tumors, *dysadherin* mRNA EI and dysadherin protein expression would be lower compared to monophasic fibrous type and E-cadherin down-regulation might be incomplete, resulting in preserved epithelioid morphology and glandular structures.

Dysadherin is a member of FXYD family (FXYD5) and its cDNA encodes 178 amino acids, including a putative signal sequence, an O-glycosylated extracellular domain, a single transmembrane domain, and a short cytoplasmic tail.[15] The FXYD5 gene for dysadherin is located at chromosome 19 (19q12-q13.1), however, interaction between FXYD5 gene for dysadherin and SYT-SSX fusion gene still remains unclear.

SYT-SSX fusion type has been reported to be correlated with the epithelial morphology in synovial sarcoma. It is tempting to speculate that the target genes of the SYT-SSX protein, which is thought to function as an aberrant transcriptional regulator, are associated with epithelial differentiation, because the SYT-SSX2 fusion is almost exclusively found in monophasic synovial sarcoma, whereas biphasic synovial sarcoma usually contains the SYT-SSX1 fusion.[1,16,19] In the current study using 39 samples, SYT-SSX fusion type showed no statistically significant correlation with epithelial differentiation, dysadherin expression or E-cadherin expression. This result might be due to the small size of the samples in the current study, compared with other multi-institutional retrospective studies.

With regard to the prognosis, SYT-SSX fusion type was also found to have no statistically significant correlation with overall survival in this study. In a multi-institutional retrospective study, Ladanyi et al[19] reported that SYT-SSX fusion type seemed to be the single most significant prognostic factor by multivariate analysis, whereas Guillou et al[9] revealed that histologic grade, but not SYT-SSX fusion type, was the most significant prognostic factor. Several factors have been shown to be variably associated with rapid tumor-related death, including a high patient age,[4,22,26] large tumor size,[4,5,10,21–23,28,36,41] vascular invasion,[41] invasion of bone and neurovascular structures,[21,41] poorly differentiated histology,[4,10,22,38] high mitotic rate,[10,11,22,26,28,36,41] presence of tumor necrosis,[4,10,26,28,38,41] male sex, and truncal tumor location.[41] Alterations of some cell-cycle regulators (p27,[18] p53, Rb, cyclin A and D1), a high MIB-1 labeling index,[37] elevated insulinlike growth factor-1 receptor expression,[43] coexpression of hepatocyte growth factor and c-MET,[27] and aberrant β-catenin expression[10,30] have also been shown to be correlated with reduced survival. In the present study, dysadherin-positive expression, E-cadherin–reduced (negative) expression, large tumor size (> 5 cm), absence of glandularity, massive tumor necrosis (≥ 50%), the presence of rhabdoid cells, a high MIB-1 labeling index (≥ 10%), a high FNCLCC grade (grade 3) and a high AJCC stage (stages III and IV) were all found to be significantly correlated to a worse overall survival rate. Multivariate analysis revealed that dysadherin immunopositivity ($P = 0.0411$) and a high MIB-1 labeling index (≥ 10%) ($P < 0.0001$) were independent poor prognostic factors.

In conclusion, dysadherin is diffusely and frequently expressed in synovial sarcoma, especially in monophasic fibrous type and poorly differentiated type, whereas it is not diffusely but often sporadically or focally observed in the fibrous component of biphasic type. E-cadherin dysfunction by dysadherin is associated with reduced E-cadherin expression and histologic change from epithelioid to spindle-shaped morphology. Dysadherin expression is considered to be one of the determinants of histologic subtype in synovial sarcoma. Moreover, dysadherin expression is an excellent and independent prognostic indicator, as assessed by univariate and multivariate survival analysis.

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Silencing of tissue factor pathway inhibitor-2 gene in malignant melanomas

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To identify tumor-suppressor genes inactivated by aberrant methylation of promoter CpG islands (CGIs) in human malignant melanomas, genes upregulated by treatment of cells with a demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), were searched for using oligonucleotide microarrays in melanoma cell lines, HMV-I, MeWo and WM-115. Seventy-nine known genes with CGIs were identified as being upregulated (≥16-fold), and 18 of them had methylation of their putative promoter CGIs in 1 or more of 8 melanoma cell lines. Among the 18 genes, *TFPI-2*, which is involved in repression of the invasive potential of malignant melanomas, was further analyzed. Its expression was repressed in a melanoma cell line with its complete methylation, and was restored by 5-aza-dC treatment. It was unmethylated in cultured neonatal normal epidermal melanocyte, and was induced by ultraviolet B. In surgical melanoma specimens, *TFPI-2* methylation was detected in 5 of 17 metastatic site specimens (29%), while it was not detected in 20 primary site specimens (0%) ($p = 0.009$). By immunohistochemistry, the 5 specimens with promoter methylation lacked immunoreactivity for TFPI-2. The results showed that *TFPI-2* is silenced in human malignant melanomas by methylation of its promoter CGI and suggested that its silencing is involved in melanoma metastasis.

Key words: TFPI-2; silencing; DNA methylation; malignant melanoma; oligonucleotide microarray

The incidence of malignant melanoma has been steadily increasing in Western countries over the past few decades.[1] It is one of the tumors with the worst prognosis for its tendency to metastasize and its resistance to conventional chemotherapy in advanced stages. Although it is recognized that therapies based on specific molecular targets can be very effective for some cancers,[2,3] the molecular pathogenesis of malignant melanomas has only been partially elucidated. Known genetic alterations include mutational activations of *N-ras* (5–37.5%)[4,5] and *B-RAF* (80%),[6] and inactivations of *CDKN2A* (11–44%)[7,8] and *PTEN* (14%)[9] by chromosomal deletions. On the other hand, contrary to the initial expectation that epigenetic aberrations would be infrequent in malignant melanomas, increasing numbers of genes are shown to be inactivated by methylations of their promoter CpG islands (CGIs), including *CDKN2A* (32%),[10] *RASSF1A* (41–50%),[11] *TIMP3*,[12] *PRDX2* (8%)[13] and *TSPY* (5/5 in males).[14]

Genome-wide screening for aberrant methylation is useful to identify genes with epigenetic aberrations, and various techniques have been developed.[15] Most techniques are based on the methylation status of genomic DNA, including methylation-sensitive-representational difference analysis (MS-RDA) and restriction landmark genomic scanning.[15] We previously applied MS-RDA to 3 melanoma cell lines, MeWo, WM-266-4 and MMAc, and identified silencing of *PRDX2*,[13] a negative regulator of PDGF signaling.[16] Another technique developed by Suzuki *et al.* utilizes a demethylating agent, 5-aza-2′-deoxycytidine (5-aza-dC), and a microarray to identify genes reexpressed by demethylation.[17] This chemical genomic screening is simple and effective in identifying genes silenced in cell lines.[18] Since only a minor fraction of tumor-related genes are finally utilized as molecular targets, we need to determine more genes involved in melanomas, and chemical genomic screening is a choice.

In the present study, we performed a chemical genomic screening using 3 human melanoma cell lines, HMV-I, MeWo and WM-115. These cell lines were selected because they had different origins and frequencies of methylation[19]: HMV-I, MeWo and WM-115 originated from a primary lesion of genital mucosa, a metastatic lesion of a lymph node, and a primary skin lesion, respectively, and exhibited frequent, infrequent and intermediately frequent gene silencing, respectively.

Material and methods

Cell lines, tumor samples and nucleic acid extraction

Human melanoma cell lines, MeWo, VMRC-MELG, A2058, C32TG and GAK were obtained from the Health Science Research Resources Bank (Sennan, Japan); G361, SK-Mel-28 and HMV-I from the Cell Resource Center for Biomedical Research Institute of Development (Sendai, Japan); COLO 679 and MMAc from RIKEN BioResource Center (Tsukuba, Japan); WM-266-4 and WM-115 from the American Type Culture Collection (Rockville, MD) and cultured normal human epidermal melanocyte (HEM) from Cascade Biologics (Portland, OR).

Thirty-seven surgical melanoma specimens, 20 from primary sites and 17 from metastatic sites, were obtained from 37 patients in Stages III and IV determined by the American Joint Committee on Cancer with informed consents at Tsukuba University Hospital and The University of Tokyo Hospital. Thirty-three specimens were fixed in formalin and embedded in paraffin and 4 (Cases 6, 7, 32, 33) were fresh-frozen. Five lymph nodes specimens were obtained from 5 nonmelanoma skin cancer cases. For DNA extraction from paraffin-embedded melanoma specimens, specimens were sliced into 40-μm-thick tissue sections, deparaffinized and then dissected by a fine needle. After incubation in a lysis buffer (50 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.5% Tween-20, 200 mg/ml of proteinase K) at 55°C over night, DNA was extracted by the phenol/chloroform procedure. From cultured cells and fresh-

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TABLE I – LIST OF GENES WHOSE PROMOTER CpG ISLANDS WERE METHYLATED IN MELANOMA CELL LINES

Number	Gene	Description	Accession number	Chromosomal location	Region analyzed by MSP	Methylation incidence
1	*ADFP*	adipose differentiation-related protein	NM_001122	9p21	19917683-804[1]	1/8
2	*ANGPTL4*	angiopoietin-like 4	NM_139314	19p13.3	8334705-842	2/8
3	*APM2*	adipose specific 2	NM_006829	10q23.2	88718121-207	5/8
4	*CLDN3*	claudin 3	NM_001306	7q11.23	72822580-716[1]	2/8
5	*F2RL1*	coagulation factor II (thrombin) receptor-like 1	NM_005242	5q13	7615463-583	1/8
6	*FKBP1B*	FK506 binding protein 1B	NM_004116	2p23.3	24125969-6063	1/8
7	*GREM1*	gremlin 1 homolog, cysteine knot superfamily	NM_013372	15q13-q15	30797292-442	1/8
8	*GSTM4*	glutathione S-transferase M4	NM_147148	1p13.3	110000074-223	1/8
9	*IRX4*	iroquois homeobox protein 4	NM_016358	5p15.3	1935993-6112[1]	1/8
10	*ISYNA1*	myo-inositol 1-phosphate synthase A1	NM_016368	19p13.11	18410001-168[1]	1/8
11	*MT1K*	metallothionein 1K	NM_005950	16q13	55259557-678[1]	3/8
12	*PDLIM4*	PDZ and LIM domain 4	NM_003687	5q31.1	131621019-118	4/8
13	*RRAD*	Ras-related associated with diabetes	NM_004165	16q22	65516994-7100[1]	2/8
14	*SPINT2*	serine protease inhibitor, Kunitz type, 2	NM_021102	19q13.1	43446926-7042	2/8
15	*TAC1*	tachykinin, precursor 1	NM_003182	7q21-q22	97199101-201	6/8
16	*TFPI-2*	tissue factor pathway inhibitor 2	NM_006528	7q22	93358033-228[1,2]	1/8
17	*TPM2*	tropomyosin 2 (beta)	NM_213674	9p13.2-p13.1	35680034-298[1]	2/8
18	*WARP*	von Willebrand factor A domain-related protein	NM_022834	1p36.33	1360665-798	2/8

[1]Positions in the reverse strand.–[2]Region analyzed by the new set of primers, which is shown as "MSP 2 in Figure 1*a*."

frozen specimens, DNA was extracted by the phenol/chloroform procedure or a QuickGene DNA tissue kit S (FUJIFILM, Tokyo, Japan). Total RNA was extracted from the cultured cells using ISOGEN (NIPPON GENE, Tokyo, Japan).

Treatment with 5-aza-2′-deoxycytidine and ultraviolet-B irradiation

For 5-aza-2′-deoxycytidine (5-aza-dC) treatment, melanoma cells were seeded at a density of 2×10^5–6×10^5 cells/10-cm dish on day 0 in RPMI 1640 (Invitrogen, Carlsbad, CA), exposed to a medium containing 1 μM 5-aza-dC (Sigma-Aldrich, St Louis, MO) for 48 hr on days 1 and 3 and harvested on day 5. All cell lines showed mild growth suppression on day 5, compared to untreated cells. Growth curve of HMV-I cells and their morphology after 5-aza-dC treatment are shown in Supplementary Figure 1.

For ultraviolet-B (UVB) irradiation, HEM was seeded at a density of 1.5×10^6 cells/10-cm dish on day 0 in Medium 254 (Cascade Biologics, Mansfield, UK), irradiated at doses of 0, 10 and 20 mJ/cm^2 on day 1 and harvested on day 2. HEMs were irradiated uncovered in phosphate-buffered saline (PBS) to UV generated by TL20W/12 tubes (Philip, Eindhoven, the Netherlands) at 0.51 mW/cm^2, monitored by a UV radiometer (Model UVR 3036/S2, Topcon, Tokyo, Japan).

Oligonucleotide microarray analysis

Oligonucleotide microarray analysis was performed using GeneChip Human Genome 133 Plus 2.0 (Affymetrix, Santa Clara, CA) with 54,000 probe sets, 47,400 transcripts from 39,000 genes. From 7 μg of total RNA, double-stranded cDNA was synthesized and biotin-labeled cRNA was prepared using a BioArray High-Yield RNA transcript labeling kit (Enzo, Farmingdale, NY). Twenty microgram of labeled cRNA was fragmented, and hybridized to GeneChips. Hybridization signals were scanned, processed using GeneChip Operating Software and normalized so that the average of all the genes on a GeneChip was 500. The *p*-values for differential expression (Change *p*-value) were calculated in each probe by statistical algorithms based on the Wilcoxon Signed-Rank test. The "Change *p*-values" of 0.003 and 0.997, respectively, were used as thresholds to define genes with increased and decreased expression.

Methylation-specific PCR and bisulfite sequencing

DNA samples (500 ng each) digested by *Bam*HI were denatured in 0.3 N NaOH at 37°C for 15 min. The samples in 3.6 N sodium bisulfite (pH 5.0) and 0.6 mM hydroquinone underwent 15 cycles of 30-sec denaturation at 95°C and 15-min incubation at 50°C. They were desalted with the Wizard DNA Clean-Up system (Promega, Madison, WI), and desulfonated in 0.3 N NaOH. DNA was ethanol-precipitated and dissolved in 20 μl of TE buffer.

Methylation-specific PCR (MSP) was performed with a primer set specific to the methylated or unmethylated sequence (M or U set), using 1 μl of the sodium bisulfite-treated DNA. Primers were designed in the 5′ regions of the reported transcription initiation site of candidate genes (Supplementary Table I). DNA methylated with *Sss*I methylase (New England Biolabs, Beverly, MA) and DNA amplified by GenomiPhi DNA amplification kit (GE Healthcare, Little Chalfont, UK) were used to determine specifically amplified conditions for M and U sets.[20] Minimum cycles to obtain visible bands with positive control samples were determined for each primer set and 4 cycles were added for test samples. A further 4 cycles were added for paraffin-embedded samples that were degraded.

For bisulfite sequencing, 1 μl of the sodium bisulfite-treated DNA was used for PCR with the primers common to methylated and unmethylated DNA sequences (Supplementary Table I). The PCR products were cloned into a pCR4-TOPO cloning vector (Invitrogen) and 10 clones or more were cycle-sequenced for each sample.

Quantitative real-time reverse transcription-PCR

Total RNA was treated with DNase I (Ambion, Austin, TX), and cDNA was synthesized from 1 μg of total RNA using a Superscript II kit (Life Technologies, Rockville, MD). Quantitative real-time reverse transcription-PCR (RT-PCR) was performed with the SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) and a 7,300 Real Time PCR Systems (Applied Biosystems, Foster City, CA). The number of molecules of a specific gene in a sample was measured by comparing its amplification with that of standard samples, which contained 10^1 to 10^8 copies of the gene. The mRNA quantity of each gene was normalized with that of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). The primers and PCR conditions are shown in Supplementary Table II.

Immunohistochemical analysis

Immunohistochemical staining of TFPI-2 was performed using a mouse monoclonal antibody against human TFPI-2 (clone 28Aa) as the primary antibody.[21] Formalin-fixed and paraffin-embedded sections were sliced at 4 μm thickness, deparaffinized and heated in 10 mM citrate buffer (pH 6.0) for 20 min at 95°C. After blocking, the sections were incubated with the primary antibody at a dilution of 100-fold at 4°C for 1 hr, and then with the second anti-

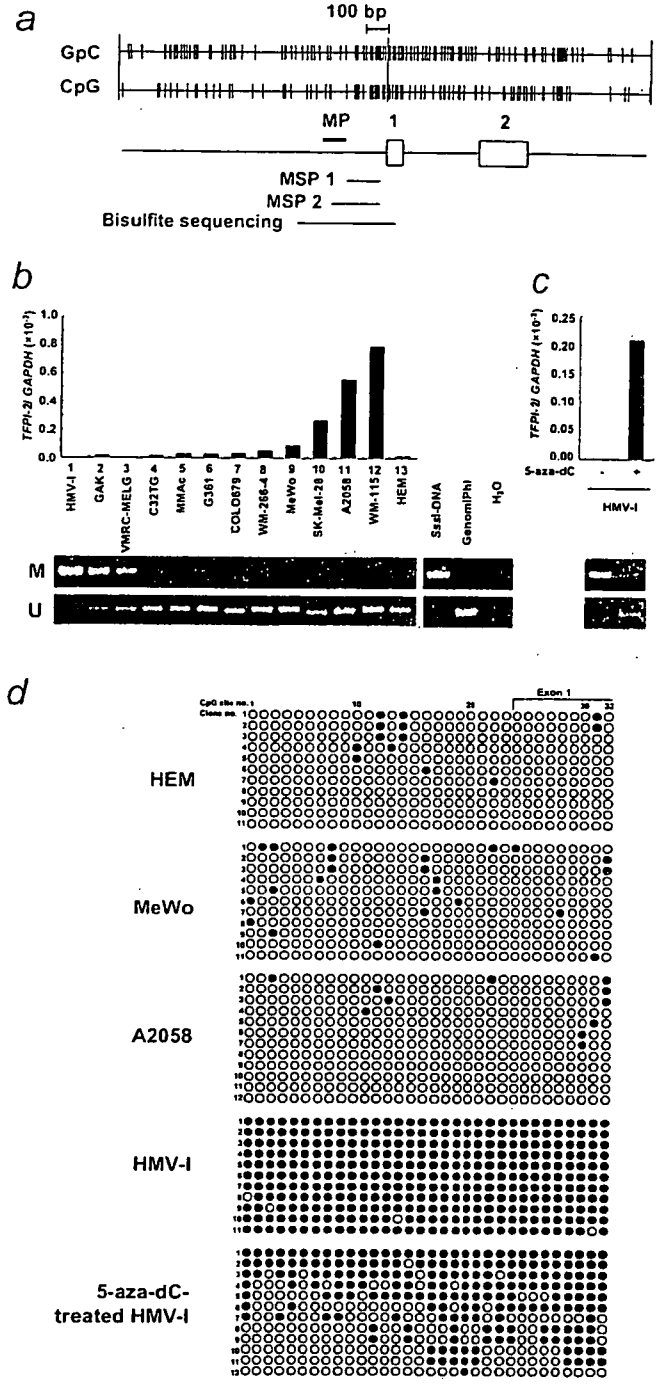

FIGURE 1 – (a) The genomic structure of *TFPI-2* in its promoter region. Vertical ticks show individual GpC sites (top) and CpG sites (bottom). The regions shown are those analyzed by the initial MSP (MSP 1), the second MSP (MSP 2) and the bisulfite sequencing. MP: minimal promoter region. (*b*) *TFPI-2* expression and methylation of its promoter CGI. *TFPI-2* expression and methylation were analyzed by quantitative RT-PCR and MSP, respectively, in 12 melanoma cell lines (Lanes 1–12) and HEM (Lane 13). M, primers specific to methylated DNA; U, primers specific to unmethylated DNA. As control fully methylated and fully unmethylated DNA, genomic DNA methylated using *Sss*I-methylase and genomic DNA amplified using phi29 DNA polymerase (GenomiPhi DNA), respectively, were used. HMV-I did not have unmethylated DNA molecules, and lacked *TFPI-2* expression. (*c*) *TFPI-2* reexpression after 5-aza-dC treatment was confirmed by quantitative RT-PCR. (*d*) Methylation of the *TFPI-2* promoter CGI shown by bisulfite sequencing. Thirty-two CpG sites were analyzed in HEM, MeWo, A2058, HMV-I, and HMV-I after 5-aza-dC treatment. For each sample, 10 or more clones were sequenced. Closed and open circles show methylated and unmethylated CpG sites, respectively.

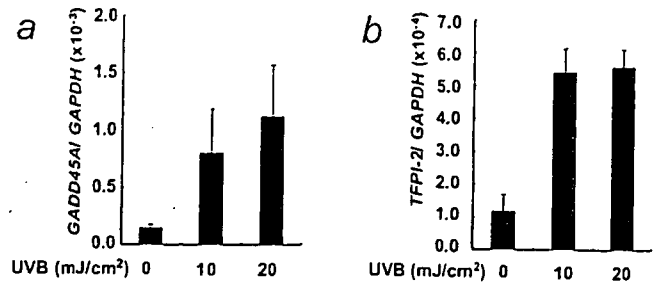

FIGURE 2 – *TFPI-2* expression after irradiation of HEMs to UVB. (*a*) *GADD45A* induction in response to UVB irradiation was first confirmed. Bars, SD. (*b*) *TFPI-2* expression was found to be induced by UVB 10 and 20 mJ/cm^2 irradiation. Bars, SD.

TABLE II – METHYLATION STATUS AND IMMUNOREACTIVITY OF TFPI-2 IN SURGICAL SPECIMENS

Specimen number	Origin	Methylation status	Immunoreactivity
1	Skin metastasis	M	–
2	Skin metastasis	M/U	–
3	Skin metastasis	M	–
4	Lung metastasis	M	–
5	Lymph node metastasis	M/U	–
6	Skin metastasis	U	NA
7	Skin metastasis	U	NA
8	Lung metastasis	U	+
9	Lymph node metastasis	U	+
10	Lymph node metastasis	U	+
11	Lymph node metastasis	U	+
12	Lymph node metastasis	U	–
13	Lymph node metastasis	U	–
14	Lymph node metastasis	U	–
15	Lymph node metastasis	U	–
16	Lymph node metastasis	U	NA
17	Lymph node metastasis	U	NA
18	Primary	U	+
19	Primary	U	+
20	Primary	U	+
21	Primary	U	+
22	Primary	U	+
23	Primary	U	+
24	Primary	U	+
25	Primary	U	–
26	Primary	U	–
27	Primary	U	–
28	Primary	U	–
29	Primary	U	–
30	Primary	U	–
31	Primary	U	NA
32	Primary	U	NA
33	Primary	U	NA
34	Primary	U	NA
35	Primary	U	NA
36	Primary	U	NA
37	Primary	U	NA

–, negative immunoreactivity for TFPI-2; +, positive immunoreactivity for TFPI-2; NA, not analyzed. Methylation of the *TFPI-2* promoter CGI was observed specifically in metastatic site specimens, not in primary site specimens ($p = 0.009$). No significant differences were seen in immunoreactivity between primary and metastatic specimens ($p > 0.05$, χ^2 test).

body (biotinylated anti-mouse IgG antibody) at a dilution of 250-fold at room temperature for 40 min. Melanin color was removed by placing the slides in 0.5% sodium azide at 4°C for 3–5 days. The binding of the second antibody was visualized by a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin. As a negative control, absence of staining without the primary antibody was confirmed. As a positive control, staining of vascular epithelium was confirmed.[22]

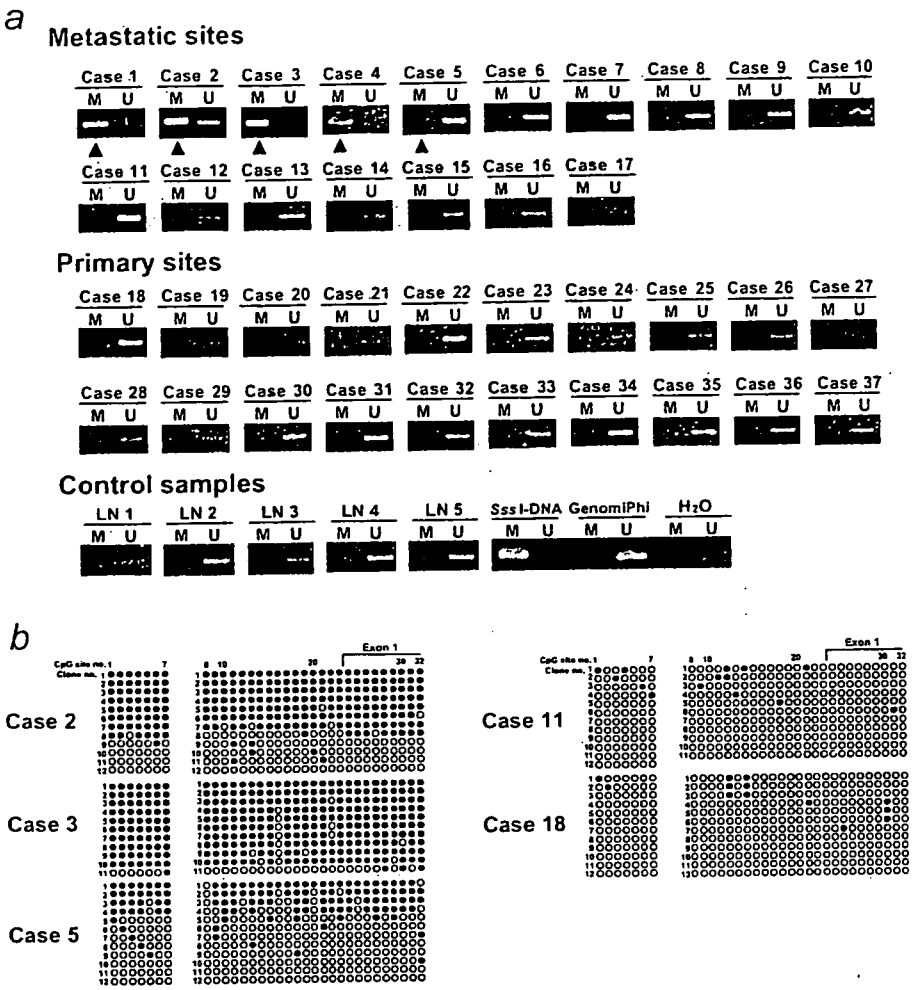

FIGURE 3 – Methylation statuses of the *TFPI-2* promoter CGI in surgical melanoma specimens. (*a*) MSP of the *TFPI-2* promoter CGI in 37 surgical melanoma specimens and 5 normal lymph nodes (LN). *Sss*I-DNA: fully methylated DNA; GenomiPhi: fully unmethylated DNA; M: primers specific to methylated DNA; and U: primers specific to unmethylated DNA. Arrowheads show bands obtained with primers specific to methylated DNA. (*b*) Bisulfite sequencing of the *TFPI-2* promoter CGI in representative surgical melanoma specimens. Cases 2, 3 and 5 had methylation by MSP, and cases 11 and 18 did not. Thirty-two CpG sites were analyzed in 2 separate PCR due to sample degradation. For each sample, 10 or more clones were sequenced. Closed and open circles show methylated and unmethylated CpG sites, respectively.

Statistical analysis

The differences in the incidences between the primary sites and metastatic sites were compared by a χ^2 test.

Results

Chemical genomic screening and identification of 18 genes with promoter methylation

Oligonucleotide microarray analyses were performed using RNA obtained from human melanoma cells with and without 5-aza-dC treatment. HMV-I, MeWo and WM-115 cell lines had 107, 112 and 16 genes, respectively, that (*i*) had signal intensities of 50 or less in nontreated cells and 100 or more in treated cells, and (*ii*) showed an increase of 16-fold or more. Genomic structures and chromosomal localizations were analyzed for these genes and 38, 35 and 6 genes (total 79 genes), respectively, were found to have CGIs in their 5′ regions, and not located on chromosome X, which harbors many normally methylated genes.[23]

Methylation statuses of the putative promoter CGIs of these 79 genes were analyzed by MSP in HEM and 8 melanoma cell lines, including HMV-I, MeWo and WM-115. Eighteen of them were found to be completely methylated (without unmethylated DNA molecules) in 1 or more of the 8 melanoma cell lines, but not in HEM (Table I).

Methylation and expression analysis of the 18 genes, and identification of TFPI-2

mRNA expression of the 18 genes was analyzed in 12 melanoma cell lines and HEM by quantitative RT-PCR, and association between methylation and transcriptional repression was examined. It was found that 9 genes were not expressed in HEM, that 7 genes (*APM2, FKBP1B, GSTM4, ISYNA1, PDLIM4, TFPI-2* and *TPM2*) were expressed in HEM and consistently repressed in melanoma cell lines with complete methylation, and that 2 genes (*ADFP* and *MT1K*) were expressed even in melanoma cell lines with complete methylation.

Among the 7 genes that had consistent association between methylation and transcriptional repression, *TFPI-2* was known to possess growth-suppressive functions[24–28] and to be silenced in gliomas, choriocarcinomas, pancreatic cancers and lung cancers.[28–31] Therefore we decided to focus on *TFPI-2* as a candidate tumor-suppressor gene silenced in malignant melanomas.

TFPI-2 minimal promoter activity was reported between −224 and −139 from the transcription initiation site[32] (Fig. 1*a*), and the methylation status of the region was confirmed by new sets of MSP primers (Fig. 1*b*). The same results with the initial screening were obtained. *TFPI-2* was completely methylated in HMV-I, partially methylated (both methylated and unmethylated DNA detected) in GAK, VMRC-MELG and C32TG, and completely unmethylated in the other 8 melanoma cell lines and HEM. *TFPI-2* was not expressed in HMV-I, but was expressed in most melanoma cell lines with unmethylated DNA molecules. Reexpression of *TFPI-2* by treatment of HMV-I with 5-aza-dC was confirmed by quantitative RT-PCR analysis (Fig. 1*c*). Further, the results by MSP were confirmed by bisulfite sequencing (Fig. 1*d*). The promoter region of *TFPI-2* was densely methylated in all DNA molecules in HMV-I, while it was unmethylated in 2 melanoma cell lines (MeWo and A2058) with high *TFPI-2* expression and HEM.

These results showed that *TFPI-2* was silenced by methylation of its promoter CGI in a melanoma cell line.

TFPI-2 *induction in response to UVB irradiation*

In a steady-state culture, HEM expressed *TFPI-2* mRNA only at a low level. It is generally difficult to distinguish genes that are functional in a tissue, even with low expression levels, such as *Rb*, *BRCA1* and *HIF-1*,[33–35] from genes that are not functional in a tissue and have little expression.[36] To make the distinction, it was considered informative to examine whether or not *TFPI-2* expression can be induced in response to cellular stress. Therefore, HEMs were irradiated with increasing doses of UVB. The effect of UVB irradiation was confirmed by observing induction of *GADD45A* mRNA expression (Fig. 2*a*). It was found that *TFPI-2* expression was induced at 4.2-fold after treatment with 10 mJ/cm^2 and 4.6-fold after that with 20 mJ/cm^2 in 3 independent treatments (Fig. 2*b*).

TFPI-2 *methylation and loss of expression*

Methylation of the *TFPI-2* promoter CGI was analyzed in 37 surgical melanoma specimens (17 metastatic site and 20 primary site specimens) and 5 normal lymph nodes (negative controls) by MSP. Methylation was detected only in 5 of the 17 metastatic site specimens (29%), while none in the primary site specimens (p = 0.009, χ^2 test) (Table II and Fig. 3*a*). Further, the result was confirmed by bisulfite sequencing in 5 representative specimens (Fig. 3*b*).

Immunohistochemical analysis of TFPI-2 was then performed on 26 surgical melanoma specimens, including the 5 specimens with methylated DNA and 21 specimens without methylated DNA. The remaining 11 of the 37 specimens were excluded due to excessive melanin deposition even after demelanization or unavailability of tissue sections. Five of the 5 specimens with methylated DNA and 10 of the 21 specimens without lacked immunoreactivity for TFPI-2 (representative results in Fig. 4). The remaining 11 specimens had TFPI-2 immunoreactivity (52%).

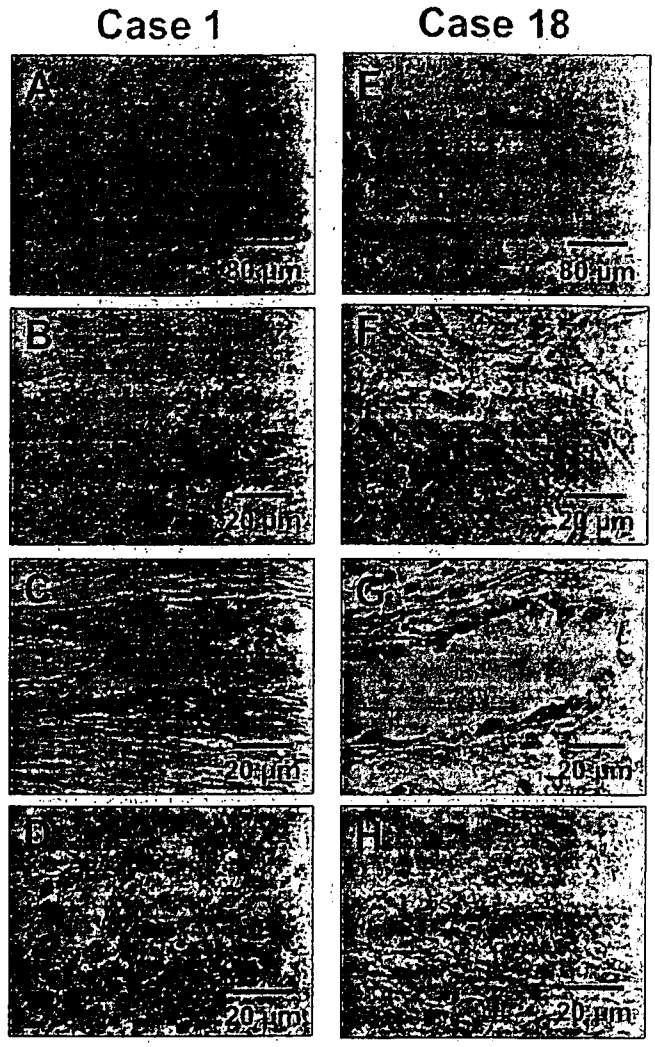

FIGURE 4 – Representative results of TFPI-2 immunohistochemical analysis in surgical melanoma specimens. Case 1 with methylated DNA showed no immunoreactivity (*a*,*b*) while case 18 without methylated DNA showed it (*e*,*f*). Staining of the vascular endothelium on the same slices was confirmed as positive controls (*c*,*g*). Absence of immunoreactivity was confirmed without anti-TFPI-2 antibody as negative controls (*d*,*h*).

Discussion

TFPI-2 silencing in human malignant melanomas was identified by a genome-wide screening using demethylation by 5-aza-dC and oligonucleotide microarray analysis. Transcriptional silencing of *TFPI-2* by promoter methylation has been recently reported in glioma,[29] choriocarcinoma,[30] pancreatic ductal adenocarcinoma[28] and nonsmall cell lung cancer.[31] In malignant melanoma, silencing by promoter methylation has not been reported, but TFPI-2 is known to suppress the invasiveness of amelanotic melanomas.[24] Our finding that methylation of the *TFPI-2* promoter CGI was present in 5 of 37 surgical melanoma specimens (13.5%) showed that silencing by promoter methylation is one of the mechanisms for *TFPI-2* inactivation in malignant melanomas.

TFPI-2, also known as placental protein (PP5) and matrix-associated serine protease inhibitor (MSPI), is a 32 kDa Kunitz-type serine proteinase inhibitor and an extracellular matrix protein that inhibits plasmin.[37] Since plasmin is involved in activation of matrix metalloproteinases (MMPs),[38] TFPI-2 can inhibit MMPs, which are known to be involved in tumor progression.[39] In various malignancies, such as choriocarcinoma, glioma, prostate cancer and lung cancer, tumor-suppressive functions of TFPI-2 have been demonstrated.[25,26,40,41] Additionally, Konduri *et al.* demonstrated that cell invasion and migration in melanomas can also be repressed by *TFPI-2* introduction.[24] In the present study, methylation of the *TFPI-2* promoter CGI was observed exclusively in metastatic site specimens (p = 0.009), and it was suggested that silencing of *TFPI-2* is one of the mechanisms for melanoma metastasis.

Distinction between a tumor-suppressor gene and a gene that has been methylated as a secondary event of tumorigenesis is important when a gene that appears to be silenced by promoter methylation is identified.[15] Tumor-suppressor genes tend to have low expression levels in the normal steady-state, but are often induced in response to cellular stresses.[33–35] At the same time, genes with little transcription in a tissue, most of which are not functional in the tissue, tend to be methylated in tumor cells.[36] For the distinction, in the present study, induction of *TFPI-2* expression in response to UVB irradiation, possibly through MAP kinase signaling activation,[42] was examined in HEMs.[43] *TFPI-2* expression was found to be induced by UVB, which is one of the most common causes of malignant melanomas and also induces apoptosis.[44] This suggested that *TFPI-2* could have a tumor-suppressive function and exert it through its roles in responding to cellular stresses, including induction of apoptosis.[27]

In the screening process of silenced genes, 2 genes (*ADFP* and *MT1K*) were found to be expressed even in melanoma cell lines with complete methylation. This was considered to be due to temporarily assigned transcription start sites in the database, or due to the presence of unidentified alternative promoters. Such inconsistency is usually solved by experimental identification of the exact

transcription start sites and promoters. However, since these 2 genes were expressed anyway, they are not targets of this study and such experiments were not performed here.

In our previous study, using MS-RDA with 3 methylation-sensitive restriction enzymes, *Hpa*II, *Sac*II and *Nar*I, putative promoter CGIs of 5 genes derived from MeWo were found to be completely methylated in at least 1 of 13 melanoma cell lines, but not in HEM.[13] However, among the 5 genes, none was expressed in HEM. On the other hand, in the present study, using a chemical genomic screening, we identified 5 genes whose putative promoter CGIs were completely methylated in 1 or more of the 8 melanoma cell lines, but unmethylated in HEM. Among the five, 2 genes (*AMP2* and *TPM2*) were expressed in HEM and were considered to be candidate tumor-suppressor genes. There was no overlap between the 5 genes identified by MS-RDA and the 5 genes identified by chemical genomic screening. These results suggested that different genes are identified by the 2 different approaches.

In conclusion, it was demonstrated for the first time in this study that *TFPI-2* is silenced by methylation of its promoter CGI in malignant melanomas. The specific presence of its methylation in metastatic sites suggested that its silencing is one of the mechanisms for melanoma metastasis.

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In breast carcinoma dysadherin expression is correlated with invasiveness but not with E-cadherin

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Reduction/loss of E-cadherin is associated with the development and progression of many epithelial tumours. Dysadherin, recently characterised by members of our research team, has an anti-cell–cell adhesion function and downregulates E-cadherin in a post-transcriptional manner. The aim of the present study was to study the role of dysadherin in breast cancer progression, in association with the E-cadherin expression and the histological type. We have selected ductal carcinoma, which is by far the most common type and lobular carcinoma, which has a distinctive microscopic appearance. Dysadherin and E-cadherin expression was examined immunohistochemically in 70 invasive ductal carcinomas, no special type (NST), and 30 invasive lobular carcinomas, with their adjacent *in situ* components. In ductal as well as in lobular carcinoma dysadherin was expressed only in the invasive and not in the *in situ* component, and this expression was independent of the E-cadherin expression. Specifically, all 10 (100%) Grade 1, 37out of 45(82.2%) Grade 2 and six out of 15 (40%) Grade 3 invasive ductal carcinomas showed preserved E-cadherin expression, while 'positive dysadherin expression' was found in six out of 10 (60%) Grade 1, 34 out of 45(75.5%) Grade 2 and all 15 (100%) Grade 3 neoplasms. None of the 30 infiltrating lobular carcinomas showed preserved E-cadherin expression, while all the 30 infiltrating lobular carcinomas exhibited 'positive dysadherin expression'. Dysadherin may play an important role in breast cancer progression by promoting invasion and, particularly in lobular carcinomas, it might also be used as a marker of invasion.
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Recent advances into molecular pathology of breast cancer have refined diagnostic accuracy and classification systems of the most common malignant neoplasm of women, rendering personalised therapy more possible. Today, there is a plethora of molecular genetic data that indicate differences in pathogenesis between the various types of breast carcinomas and thus support their categorisation, to the patient benefit. The demonstration of lack of E-cadherin expression in lobular neoplasms has had a sound impact with practical applications (Mastracci *et al*, 2005) In about half of lobular carcinomas, loss of E-cadherin involves genetic changes, that is loss of heterozygosity (LOH) at 16q22.1, while in the other half epigenetic events are involved (Knudsen and Wheelock, 2005; Mastracci *et al*, 2005). Ductal carcinomas, on the other hand, express E-cadherin, albeit in reduced levels and/or in abnormal cellular locations (Knudsen and Wheelock, 2005). Reduction/loss of E-cadherin has been associated with the development and progression of many epithelial neoplasms. Aberrant E-cadherin expression (heterogeneous, cytoplasmic, or absent) has been detected immunohistochemically in several cancers, including head and neck carcinoma, gastric adenocarcinoma, lobular breast carcinoma, lung cancer, colorectal carcinoma, prostate adenocarcinoma, pancreatic, and bladder cancer (Becker *et al*, 1994; Hirohashi, 1998; Chang *et al*, 2002; Charalabopoulos *et al*, 2002, 2004; Hirohashi and Kanai, 2003; Mastracci *et al*, 2005; Massarelli *et al*, 2005). In the vast majority of these neoplasms such expression has been associated with poor differentiation, increased metastatic potential and poor prognosis. In breast the scenario is more complicated, since lobular carcinoma, that typically does not express E-cadherin has a more favourable outcome than ductal carcinoma, which in general expresses E-cadherin. Furthermore, there are contradictory data on the possible association between E-cadherin expression and high-grade tumours with increased metastatic potential (Oka *et al*, 1993; Charpin *et al*, 1997; Heimann *et al*, 2000; Gillett *et al*, 2001; Parker *et al*, 2001; Elzagheid *et al*, 2002; Gupta *et al*, 2003; Knudsen and Wheelock, 2005; Rakha *et al*, 2005). It is clear that there are pieces missing from the puzzle of adhesion molecules and breast carcinoma.

Recently, the cloning and characterisation of dysadherin (FXYD5), a cell membrane glycoprotein that has an anti-cell–cell adhesion function and downregulates E-cadherin in a post-transcriptional manner has been reported, by members of our research team. This novel cancer-associated protein has been detected in head and neck, tongue, oesophageal, gastric, colorectal,

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