including the alteration of *p53*, which is regulated by *DAP kinase*. Furthermore, to examine that *DAP kinase* promoter methylation or homozygous deletion is involved in the down-regulation of DAP kinase expression, we examined the expression of DAP kinase protein.

MATERIALS AND METHODS

Tumor Samples and DNA Extraction

Paraffin-embedded tissues from 29 cases and frozen materials from 16 cases, making a total of 45 LMSs of soft tissue that were registered in the soft-tissue tumor files of the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Japan, were used for the molecular analyses. Diagnoses of all cases were based on histological examinations with hematoxylin-eosin (HE) staining, and smooth muscle differentiation was confirmed by an immunohistochemically positive reaction of desmin, muscle-specific actin (HHF-35), and α-smooth muscle actin. In addition, the expression of c-kit, a gastrointestinal stromal tumor marker, was not recognized in any of the 45 cases. As for tumor location, the cases were divided into 2 groups: cases in which the tumors were located at intra-abdominal sites such as the retroperitoneum or abdominal cavity (abdominal type) and cases in which the tumors arose in extra-abdominal sites such as the skin, subcutis, and skeletal muscles (extra-abdominal type).[16]. The mitotic rate was evaluated by counting the number of mitotic figures in 10 high-power fields (HPFs). Survival data were available for 36 LMS cases, with a follow-up ranging from 14 to 258 (median, 46.0) months. Histological tumor grade was evaluated according to the grading system of the French Federation of Cancer Centers (FNCLCC).[17] If possible, cases were also evaluated according to the new American Joint Committee on Cancer (AJCC) staging system. Genomic DNA was purified by standard proteinase K digestion and phenol-chloroform extraction methods. To avoid as much as possible the contamination of normal tissue, microdissection was performed for formalin-fixed, paraffin-embedded tissues. As for the frozen materials, we confirmed that the normal tissue was not contaminated in each sample, referring to their concordant HE-staining slides.

Polymerase Chain Reaction–Single-Strand Conformation Polymorphism for *p53*

Mutation analysis of the *p53* gene was performed from exon 5 to exon 9. The primer sequences and conditions were as described elsewhere.[18] Polymerase chain reaction (PCR) products were electrophoresed through 2.0% agarose gel with ethidium bromide. The DNA band was cut from the gel and purified with a SUPREC tube (TAKARA Biomedicals, Japan), and the products were reamplified for 15 cycles. Single-strand conformation polymorphism (SSCP) was performed as described elsewhere.[18] To increase the quantity of DNA before sequencing, extra bands that seemed to be aberrantly migrating were excised from SSCP gel and were reamplified for 25 cycles under the same condition. The samples were analyzed for sequencing after the subsequent reaction.

Differential PCR Assay for *DAP Kinase*

The differential PCR method for homozygous deletions of *DAP kinase* was based on a reported method using *GAPDH* gene as an internal control.[8] DNA samples were added to a PCR mix with a total volume of 25 μL, containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 mM dNTP, 0.5 U Taq DNA polymerase, and 0.5 μM of each of the primers. The primer sequences and conditions were as described elsewhere.[8] Human genomic DNA (Clontech, Palo Alto, CA), which was confirmed to have the same base sequences as those in Genbank (accession no. AF000730-734), was used as a normal control for each PCR and the subsequent reactions. We also confirmed that there was no contamination in each PCR or in the subsequent reactions by using distilled water controls. After the amplification, 10 μL of PCR products were electrophoresed through 3.0% agarose gel. Homozygous deletion of the lesion of the *DAP kinase* CpG island examined was confirmed by the absence of the expected *DAP kinase* PCR product in the tumor DNA.

Bisulfite Modification and Methylation-Specific PCR for *DAP Kinase*

Bisulfite modification was performed by using the DNA modification kit (Intergen) according to the manufacturer's protocol. The modified DNA was added to a total volume of 20 μL containing 10 mM Tris-HCL (pH 8.3), 50 mM KCl, 2.0 mM $MgCl_2$, 25 mM of dNTP, 0.25 U Taq DNA polymerase, and 0.2 μM of each of the primers. The primer sequences and conditions were as described elsewhere.[8] As for positive control, DNA of the Raji cell line, which is known to show promoter hypermethylation of *DAP kinase* by MSP method, was used.[7] In addition, DNA of normal skeletal muscle was used as negative control. Each of the PCR products (10μL) was directly loaded onto 2% agarose gels, stained with ethidium bromide, and directly visualized under ultraviolet illumination.

Immunohistochemistry

Immunohistochemical analysis was performed by using rabbit IgG polyclonal antibodies against DAP kinase (1:100, Sigma Ltd, UK). After antigen retrieval by a microwave oven technique, the specimens were incubated at 4°C overnight with antibodies to these proteins, followed by staining with a streptavidin-biotin-peroxidase kit (Nichirei, Tokyo, Japan) and hematoxylin counterstaining. Normal lymphoid tissues were used as an external positive control for DAP kinase. As a negative control, the primary antibodies were omitted.

The extent of staining was graded as follows: 0, staining in <1% of tumor cells; 1, staining in 1% to 10%; 2, staining in 10% to 50% of tumor cells; 3, staining in >50% of tumor cells. The overall intensity of staining was also assessed as follows: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. When the final score was <4, we considered it to indicate decreased expression. When the tumor cells failed to stain in all areas or were stained in only some areas, we confirmed the staining of the admixed non-neoplastic cells as a positive control. The final score (range from 0 to 9) was obtained by multiplying the extent of staining with the intensity.

Statistical Analysis

Fisher's exact test was used to evaluate the association between 2 dichotomous variables. The survival correlations are illustrated with Kaplan-Meier curves, and survival analyses were performed by using the log-rank test. A P value of <0.05 was considered to indicate statistical significance.

TABLE 1. Clinicopathologic Parameters in 45 Cases of Leiomyosarcoma

Parameter	n
Age (yr)	
≥60	19
<60	26
Sex	
Male	24
Female	21
Location	
Extra-abdominal	27
Abdominal	18
Size (cm)	
≥5	26
<5	8
Unknown	11
Mitotic rate (per 10 HPFs)	
≤9	22
10–19	9
≥20	14
AJCC stage	
I	7
II	19
III	3
IV	5
Unknown	11
FNCLCC grading	
Grade 1	13
Grade 2	21
Grade 3	11

Abbreviations: HPFs, high-power fields; AJCC, American Joint Committee on Cancer; FNCLCC, French Federation of Cancer Centers.

RESULTS

Clinical and Histological Findings

The clinicopathologic data are summarized in Table 1. The ages of the patients ranged from 21 to 88 years (average, 58.5 years). Twenty-four patients were male and 21 were female. In 27 patients, the tumors occurred in extra-abdominal sites (13 in thigh, 3 in chest or abdominal wall, 3 in buttock, 2 in upper arm, lower leg, and 1 each in the neck, axilla, forearm, groin). In contrast, in 18 patients, the tumors were located in abdominal sites (9 in retroperitoneum, 5 in mesentery, and 4 in abdominal cavity). Tumor size could be determined in 34 cases. Twenty-six tumors were ≥5 cm in diameter, whereas 8 cases were measured as <5 cm. Histological subtype was as follows: 40 were classic type, 2 were pleomorphic type,[19] 2 were myxoid type,[20] and 1 was epithelioid type. The mitotic rate ranged from 1 to 48 per 10 high-power fields HPFs (mean, 15.1 per 10 HPFs). Twenty-two cases had less than 9 mitoses per 10 HPFs, whereas 9 had between 10 and 19 mitoses per 10 HPFs, and 14 had more than 20 mitoses per 10 HPFs. According to the FNCLCC grading system, 13 tumors were grade 1, 21 were grade 2, and 11 were grade 3. The AJCC stage was available in 34 cases. Seven cases were considered to be AJCC stage I, 19 cases were stage II, 3 were stage III, and 5 were stage IV.

p53 Mutation in Exons 5-9

p53 gene mutations were detected in 11 of the 45 LMS cases (24%) by PCR-SSCP analysis (Table 2). As for the type of base substitution, all 11 cases were missense mutations. There was no statistically significant correlation between *p53* mutation and clinicopathologic parameters.

Promoter Methylation and Homozygous Deletion of *DAP Kinase*

Hypermethylation of the *DAP kinase* gene was detected in 10 of 45 LMS cases (22%; Table 2). All 10 cases of LMS with promoter hypermethylation showed both ummethylated and methylated signals (Fig 1).

TABLE 2. *Death-Associated Protein Kinase* Alteration and *p53* Mutation in Soft-Tissue Leiomyosarcoma

Case	Sex	Age	Location	Size (cm)	MR	FNCLCC Grade	AJCC Stage	Follow-up (Result, mo)	DAP Kinase IHC	*DAP Kinase* Alteration	*p53* Mutation
L1	M	54	Thigh	15	39	3	II	DOD, 2	N	Methylation	−
L2	F	81	Retroperitoneum	6	31	3	II	DOD, 44	N	Methylation	+
L3	F	58	Mesenterium	NA	2	2	NA	NA	N	Methylation	−
L4	M	82	Retroperitoneum	9	14	3	IV	DOD, 33	D	HD	+
L5	F	72	Abdominal cavity	6	29	2	IV	NA	D	HD	−
L6	M	33	Thigh	5	5	1	III	DOD, 44	D	Methylation	−
L7	M	39	Abdominal cavity	8	1	1	II	NA	D	Methylation	−
L8	M	54	Thigh	7	3	2	II	AWD, 15	N	Methylation	−
L9	F	65	Thigh	7.5	40	3	II	DOD, 12	D	HD	+
L10	M	47	Neck	6	5	2	I	AWD, 12	N	Methylation	−
L11	F	61	Knee	4	4	2	II	NA	D	Methylation	−
L12	M	67	Knee	4	7	2	IV	AWD, 56	D	Methylation	+
L13	F	77	Upper arm	10	4	2	II	NA	D	Methylation	+
L14	M	55	Lower leg	NA	39	3	NA	DOD, 42	N	—	+
L15	F	88	Axilla	5	32	3	II	DOD, 65	N	—	+
L16	M	33	Thigh	5	5	1	III	DOD, 44	N	—	+
L17	M	75	Retroperitoneum	13.5	7	2	II	DOD, 4	N	—	+
L18	F	43	Retroperitoneum	4.5	14	2	I	AWD, 12	N	—	+
L19	M	39	Buttock	11	8	2	II	DOD, 2	N	—	+

Abbreviations: MR, mitotic rate per 10 high-power fields; IHC, immunohistochemistry; NA, not available; M, month; AWD, alive without disease; DOD, dead of disease HD, homozygous deletion; N; normal expression, D; Decreased expression

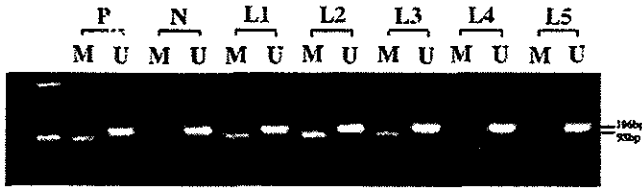

FIGURE 1. Methylation-specific polymerase chain reaction (PCR) analysis of *death-associated protein (DAP) kinase*. PCR products amplified by unmethylated (U) and methylated (M) specific primers. L1 to L3 shows hypermethylation of the *DAP kinase* gene promoter. P, positive control; N, negative control.

Promoter hypermethylation was detected in 4 of 13 cases with decreased immunohistochemical expression of DAP kinase (31%). Homozygous deletion of the *DAP kinase* gene was detected in 3 of 45 LMS cases (7%; Table 2; Fig 2). All 3 cases with homozygous deletion revealed decreased expression of DAP kinase protein. A statistically significant correlation between *DAP kinase* alteration and/or *p53* mutation and high FNCLCC grade was observed (P = 0.0244; Table 3). Furthermore, cases with *DAP kinase* alteration and/or *p53* mutation showed a statistically significant correlation with poor prognosis (Fig 3). There was no statistical correlation between *DAP kinase* methylation and clinicopathologic parameters or prognosis (data not shown).

Immunohistochemical Analysis of DAP Kinase

To evaluate whether *DAP kinase* alteration is involved in a decrease of DAP kinase expression because promoter methylation or homozygous deletion of *DAP kinase* in 13 of 45 cases studied (29%) were detected, we examined the expression of DAP kinase protein by immunohistochemistry. Decreased expression of DAP kinase protein was recognized in 13 of 45 LMS cases (29%; Fig 4A-C). Seven of 13 cases (54%) with decreased expression of DAP kinase protein revealed promoter methylation or homozygous deletion of *DAP kinase*, and the methylation status or homozygous deletion of its gene showed a close correlation with decreased DAP kinase expression (P = 0.0300; Table 4). A correlation between the DAP kinase immunoreaction and clinicopathologic parameters was not observed (data not shown).

DISCUSSION

The *death-associated protein kinase* (*DAP kinase*) gene was initially isolated as a positive mediator of INF-γ–induced apoptosis and is associated with the cell cytoskeleton.[1-3] Primary mechanisms have been postulated for inactivation of the *DAP kinase* gene: primarily homozygous deletion and promoter hypermethylation. Homozygous deletion of the *DAP kinase* gene has been reported in invasive pituitary tumors based on the method of multiplex PCR assay,[8] having been observed

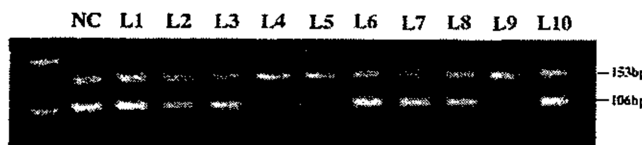

FIGURE 2. Differential polymerase chain reaction (PCR) for *death-associated protein (DAP)* kinase homozygous deletion. L4, L5, and L9 display the absence of PCR products of the *DAP kinase* gene.

TABLE 3. Correlation Between *p53* Mutation, *DAP Kinase* Alteration, and Clinicopathologic Parameters in Leiomyosarcoma (n = 45)

Parameter	*p53* Mutation and/or *DAP Kinase* Alteration +	*p53* Mutation and/or *DAP Kinase* Alteration −	*P* Value*
Age (yr)			
≥60 (n = 19)	8	11	>0.9999
<60 (n = 26)	11	15	
Sex			
Male (n = 24)	11	13	0.7636
Female (n = 21)	8	13	
Location			
Extra-abdominal (n = 27)	11	16	>0.9999
Abdominal (n = 18)	8	10	
Size (cm) (n = 34)			
≥5 (n = 26)	13	13	0.6933
<5 (n = 8)	3	5	
Mitotic rate (per 10 HPFs)			
≥20 (n = 14)	5	9	0.7460
<20 (n = 31)	14	17	
AJCC stage (n = 34)			
I and II (n = 26)	13	13	0.6933
III and IV (n = 8)	3	5	
FNCLCC grading			
1 (n = 13)	2	11	0.0244†
2 and 3 (n = 32)	17	15	

Abbreviations: DAP, death-associated protein; HPFs, high-power fields; AJCC, American Joint Committee on Cancer; FNCLCC, French Federation of Cancer Centers.
*Fisher's exact test.
†Statistically significant ($P < 0.05$).

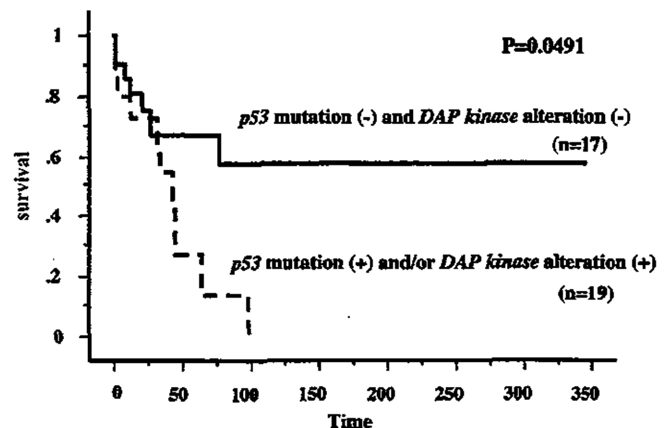

FIGURE 3. Overall survival classified according to *death-associated protein (DAP) kinase* alteration or *p53* mutation. Patients with *DAP kinase* promoter methylation or homozygous deletion and/or with *p53* mutation have a poor prognosis (P = 0.0491).

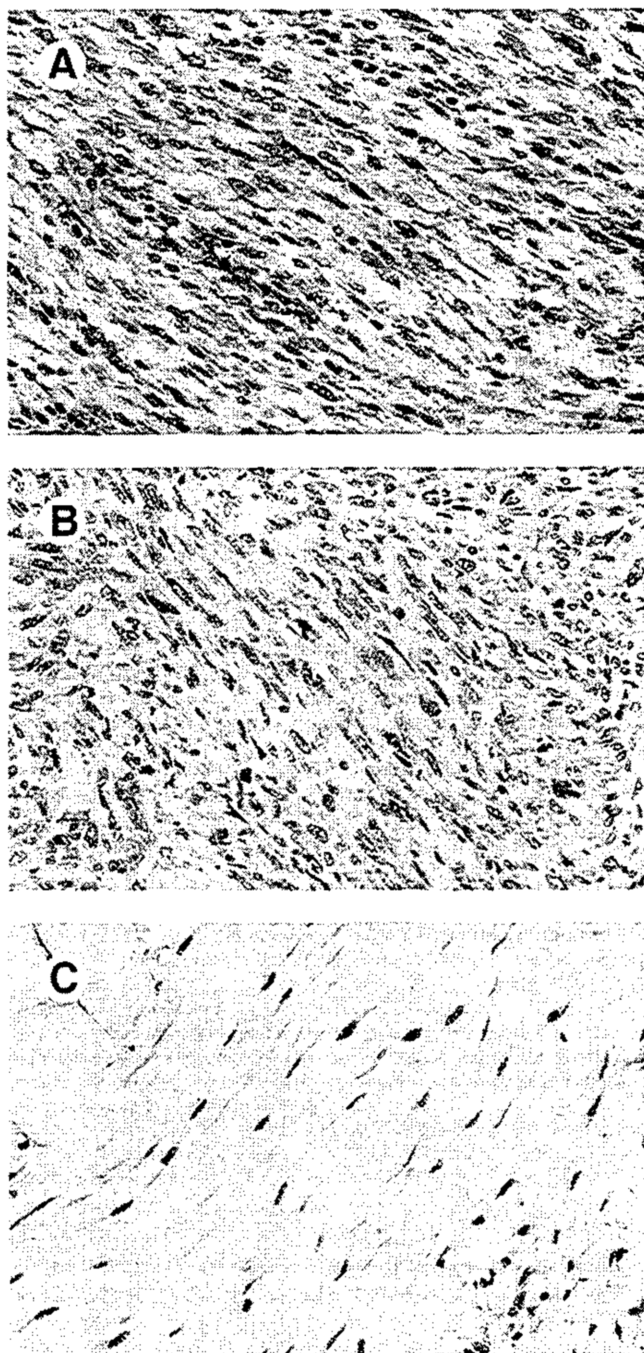

FIGURE 4. Immunohistochemical staining of death-associated protein (DAP) kinase proteins. (A) Immunohistochemistry showing cytoplasmic staining in the majority of tumor cells. This case had no homozygous deletion or hypermethylation. (B) A loss of DAP kinase expression was observed in the majority of tumor cells, but (C) surrounding normal tissue showed strong cytoplasmic positivity. This case showed promoter methylation of the *DAP kinase* gene (L6).

in 4 of 11 (36%) cases with a loss of DAP kinase expression. In the present study, although homozygous deletion was detected in only 3 of 45 LMS cases (7%), all of these cases showed decreased expression of DAP kinase. Methylation of either the 5' regulatory region or discrete regions of CG dinucleotides called *CpG islands* is an important mechanism of transcriptional repression. Methylation of the 5' CpG island of *DAP kinase* has been described in various types of cancers, with the range of methylation varying according to the tumor type.[4,5,7,8] It has been reported elsewhere that promoter hypermethylation is associated with down-regulation of DAP kinase expression in cancer cells and primary malignancies.[4,5,7] In our study, the methylation status of *DAP kinase* alone had no correlation with decreased expression of DAP kinase protein. Simpson et al[9] have described that promoter hypermethylation or homozygous deletion of *DAP kinase* correlates with an absence of the DAP kinase protein, indicating that the methylation status or homozygous deletion has a close association with down-regulation of the DAP kinase protein. In our study, *DAP kinase* methylation or homozygous deletion was detected in 7 of 13 cases (54%) with decreased expression of DAP kinase protein; our analysis also revealed a close correlation between *DAP kinase* methylation or homozygous deletion and immunohistochemical expression of DAP kinase. These results indicate that inactivation of the *DAP kinase* by promoter hypermethylation or homozygous deletion may be associated with a silencing of its expression in soft-tissue LMS.

A decrease or loss of *DAP kinase* expression has been described in many types of carcinomas and other malignancies, and it is thought to lead to inactivation of this critical apoptotic pathway in tumorigenesis.[4,5,7] *DAP kinase* also participates in apoptosis induced by other ligands such as *TNF-α* and *Fas*.[21] The expression of p53 and bcl-2, as positive and negative regulators of apoptosis, respectively, has been recognized in various types of sarcomas, and these apoptosis-related factors appear to play an important role in tumor progression.[13] On the other hand, it previously has been reported that expression of Fas and Fas-ligand is less frequent, indicating that the Fas system does not play an essential role in sarcomas.[22,23] In our study, DAP kinase expression was relatively frequent in tumor cells (71%), and 13 of 45 LMS cases (29%) displayed decreased DAP kinase expression by immunohistochemical analysis; however, its expression status had no correlation with clinicopathologic parameters in this tumor. Further study is necessary to elucidate the role of apoptosis-related factors in sarcomas, including DAP kinase.

TABLE 4. Correlation Between DAP Kinase Immunoreactivity and *DAP Kinase* Methylation or Homozygous Deletion (n = 45)

DAP Kinase Methylation or Homozygous Deletion	DAP kinase IHC	
	Decreased	Normal
+	7	6*
−	6	26

Abbreviations: IHC, immunohistochemistry; DAP, death-associated protein.

*Statistically significant, $P = 0.0300$ (decreased vs. normal IHC groups for positive *DAP kinase* methylation or homozygous deletion.

Apoptosis in human malignancies, including sarcomas, has been described in relation to progression, tumor grade, and cell proliferative activity.[24-26] In sarcomas, with regard to the apoptosis-related factors, some investigators have reported that bcl-2 expression or the bcl-2–bax ratio may be considered useful in determining the prognosis.[14,15] Loss of *DAP kinase* expression is known to be associated with the process of metastasis.[27] Previous investigators have described that the loss of *DAP kinase* by promoter hypermethylation is associated with tumor aggressiveness in carcinoma cell lines and primary malignancies.[9-11] In the present study, although cases with *DAP kinase* alteration tended to have short survival, we found no significant association with poor survival in soft-tissue LMS.

DAP kinase was recently characterized as an upstream regulator of *p53*.[12] The *p53* gene product can promote apoptosis in response to a variety of stimuli.[28] This regulatory function of *p53* may be lost by deletion and frequent mutation. In soft-tissue LMS, *p53* mutation have been reported in 17% to 43% studied.[16,29] Similarly, our current study showed *p53* mutation in 11 of 45 cases (24%). In addition, *DAP kinase* alteration and/or *p53* mutation had a close association with high histological grade and poor prognosis. Although further detailed analysis of a large number of cases is necessary, the alteration of *DAP kinase* and/or *p53* mutation may have an important role in tumor progression of soft-tissue LMS.

In conclusion, although *DAP kinase* alteration was relatively rare, *DAP kinase* alteration and/or *p53* mutation may associate with the parameters of aggressive behavior in soft-tissue LMSs. Furthermore, promoter methylation or homozygous deletion was shown to play an important role in inactivation of the *DAP kinase* gene; however, further studies of DAP kinase expression related to various genetic alterations are necessary.

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E-cadherin mutation and Snail overexpression as alternative mechanisms of E-cadherin inactivation in synovial sarcoma

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We have recently reported frequent E-cadherin gene mutations in synovial sarcoma (SS), suggesting mutational inactivation of E-cadherin as a potential mechanism of spindle cell morphology in SS, a spindle cell sarcoma that shows areas of glandular epithelial differentiaton in some cases (biphasic SS) and only pure spindle cell morphology in most cases (monophasic SS). However, the mechanism of downregulation of E-cadherin in SS remains unknown. To further address this issue, we analysed the mechanisms of E-cadherin silencing in 40 SS. Genetic and epigenetic changes in the E-cadherin gene, and the expression level of its transcriptional repressor Snail were examined by polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP), methylation-specific PCR, and real-time quantitative PCR, respectively. Expression of E-cadherin was examined by RT–PCR and immunohistochemistry. We also examined ELF3, a transcription factor associated with epithelial differentiation in SS in a previous cDNA microarray, by RT–PCR. E-cadherin and ELF3 transcripts were detected, respectively, in 27/40 (67.5%) and in 25/40 (62.5%) of SS, and these epithelial-related genes were almost always coexpressed. Hypermethylation of the promoter of the E-cadherin gene was detected in five cases (12.5%) in SS; however, E-cadherin was silenced at mRNA level in only one of the five cases. E-cadherin missense mutations were observed in five cases (12.5%) of SS. In SS, all five cases with E-cadherin missense mutations had the SYT-SSX1 fusion and were monophasic tumors, suggesting a relationship between the SYT-SSX fusion type and E-cadherin missense mutation ($P = 0.07$). E-cadherin mRNA expression in SS was associated with reduced Snail expression level ($P = 0.03$). E-cadherin membranous expression was observed in 14/40 (35.0%) of SS, and was also correlated with SYT-SSX1 fusion type and biphasic histology. ELF3 was confirmed to be more highly expressed in biphasic than monophasic SS by real-time quantitative PCR. These results suggest that in SS the loss of E-cadherin expression occurs either by Snail trans-repression or by inactivating mutations. Thus, E-cadherin downregulation is associated with the loss or absence of glandular epithelial differentiation in certain SS.
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Keywords: E-cadherin; Snail; CpG methylation; mutation; ELF3; synovial sarcoma; epithelial–mesenchymal transition

Introduction

Synovial sarcomas (SS) account for approximately 10% of all soft-tissue sarcomas, and typically arise from the para-articular region in adolescents and young adults, but have no relationship to synovium (Weiss and Goldblum, 2001). The origin of SS is uncertain, since the normal cellular counterpart for this tumor is unknown. Some studies support the idea that SS arises from a mesenchymal stem cell with a capacity for epithelial differentiation (Noguchi *et al.*, 1997; Yakushiji *et al.*, 2000). SS is known to have two major forms. Monophasic tumors, in which the tumors are entirely composed of spindle cells with or without solid epithelial areas, are distinguished from biphasic tumors that contain epithelial cells arranged in glandular formation in a background of spindle cells. SS contains a t(X;18)(p11.2;q11.2) representing the fusion of *SYT* on the chromosome 18 with either *SSX1* or *SSX2* (or rarely with *SSX4)* on the X chromosome (Clark *et al.*, 1994; Crew *et al.*, 1995; Skytting *et al.*, 1999). It has been shown that almost all of the biphasic tumors contain the SYT—SSX1 fusion, whereas tumors with SYT-SSX2 rarely exhibit biphasic histology (Ladanyi *et al.*, 2002). Therefore, it has been suggested that subtle functional differences between SYT-SSX1 and SYT-SSX2 may

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impact on epithelial differentiation in these sarcomas (Ladanyi, 2001); however, this model awaits experimental confirmation.

The cell–cell adhesion molecule E-cadherin has been shown to perform important functions in embryogenesis and tissue architecture by forming intercellular junction complexes and establishing cell polarization (Takeichi, 1991, 1993; Overduin et al., 1995; Shapiro et al., 1995; Hirohashi, 1998). The extracellular domain of E-cadherin acts as a molecular zipper that mediates cell–cell adhesion, whereas the cytoplasmic tail is linked to the actin cytoskeleton via catenins (Ozawa et al., 1990; Kemler, 1993). It has been suggested that the loss of E-cadherin expression is involved in the invasive and metastatic properties of neoplastic cells (Takeichi, 1991, 1993; Mayer et al., 1993; Hirohashi, 1998). In addition, downregulation of E-cadherin has also been reported to occur during epithelial–mesenchymal transitions, the process of cellular morphological changes in epithelial cells from epithelial features to a more fibroblastic and flattened phenotype (Batlle et al., 2000; Cano et al., 2000; Poser et al., 2001; Savagner, 2001; Blanco et al., 2002; Hajra et al., 2002; Locascio et al., 2002; Nieto, 2002; Rosivatz et al., 2002; Thiery, 2002).

There are several mechanisms by which E-cadherin function can be impaired. E-cadherin inactivation caused by mutations has been reported in various tumors and cancer cell lines (Oda et al., 1994; Berx et al., 1995; Soares et al., 1997; Guilford et al., 1998; Machado et al., 1999; Saito et al., 1999, 2001; Endo et al., 2001). Promoter inactivation attributed to hypermethylation has been observed in human breast, gastric, thyroid, bladder and prostate cancers as well as in certain leukemias (Graff et al., 1995, 1998, 2000; Hennig et al., 1995; Hiraguchi et al., 1998; Melki et al., 2000; Tamura et al., 2000; Bornman et al., 2001). Promoter hypermethylation is by far the predominant mechanism, and gene mutation is very rare except in specific subtypes of breast and gastric cancer.

The Snail family of zinc-finger transcription factors occupies a central role in morphogenesis, as its members are essential for mesoderm formation in several organisms from flies to mammals (Nieto et al., 1994; Sefton et al., 1998; Carver et al., 2001; Nieto, 2002; Thiery, 2002). Recently, the transcription factor Snail has been shown to be a direct repressor of E-cadherin gene expression by binding to its proximal promoter in epithelial tumor cells (Batlle et al., 2000; Cano et al., 2000). The Snail family of transcription factors has previously been shown to be expressed in fibroblasts and implicated in the control of epithelial–mesenchymal transitions during embryonic development (Batlle et al., 2000; Cano et al., 2000). Furthermore, it has also been demonstrated that endogenous Snail proteins are present in invasive human carcinoma cell lines and in tumors in which E-cadherin expression has been lost with associated acquisition of a fibroblastic phenotype (Batlle et al., 2000). In addition, Snail can mediate significant negative regulation of E-cadherin expression in gastric and breast carcinomas as well as malignant melanoma (Poser et al., 2001; Hajra et al., 2002; Rosivatz et al., 2002).

Another gene implicated in control of epithelial differentiation, ELF3, is from the ETS family of transcription factors. A large number of genes expressed in the epithelium contain consensus binding sites for ETS transcription factors in their promoter or enhancer regions (Scott et al., 1994; Gambarotta et al., 1996; Rodrigo et al., 1999). A recent expression profiling study of SS revealed ELF3 (E74-like factor-3), an ets-family transcription factors (Oettgen et al., 1997), as one of the differentially expressed genes between monophasic and biphasic tumors (Allander et al., 2002), raising the possibility that ELF3 might have an important role to play in epithelial differentiation of SS, specifically that overexpression of ELF3 might be associated with glandular epithelial differentiation in SS.

Recently, E-cadherin expression has been studied in sarcomas, and found to be largely restricted to SS (Sato et al., 1999; Saito et al., 2000; Laskin and Miettinen, 2002; Yoo et al., 2002). We recently reported frequent E-cadherin gene mutations in SS and demonstrated the mutational inactivation of E-cadherin as a potential mechanism of spindle cell morphology in SS (Saito et al., 2001). A putative cause–effect relationship between E-cadherin inactivation and histologic subtype in SS was suggested based on similar observations in specific subtypes of gastric and breast cancers (Berx et al., 1995; Machado et al., 1999).

As part of our overall effort to better understand epithelial differentiation in SS, we studied possible mechanisms of E-cadherin silencing in SS. We demonstrate here different modes of E-cadherin inactivation in SS, and find that the loss of E-cadherin expression correlates with histologic and molecular diversity in SS.

Results

SYT-SSX fusion type and expression of E-cadherin and ELF3

First, SYT-SSX fusion type in SS was analysed. SYT-SSX1 and SYT-SSX2 fusions were detected in 24 tumors (60%) and 16 tumors (40%), respectively. SYT-SSX fusion type showed the same association with histology previously well described in larger studies (Ladanyi et al., 2002). The 24 SYT-SSX1 tumors included 16 monophasic SS and eight biphasic SS, whereas the 16 SYT-SSX2 tumors included 15 monophasic but only one biphasic case ($P = 0.06$; Fisher's exact test). Next, endogenous E-cadherin and ELF3 expression were examined. E-cadherin mRNA expression was observed in 27 out of 40 cases (67.5%: monophasic, 18/30; biphasic, 9/9; poorly differentiated, 0/1) of SS. ELF3 mRNA expression was observed in 25 out of 40 cases of SS (62.5%: monophasic, 16/30; biphasic, 9/9; poorly differentiated, 0/1), and almost completely correlated with that of E-cadherin ($P = 0.0013$ by Fisher's exact test; Table 1, and Figure 1). By RT–polymerase chain reaction (PCR), there was no correlation between

Table 1 Summary of pathologic and molecular findings in SS

	SYT-SSX		*Epithelial nest*	*E-cadherin status*				*ELF3* mRNA exp.	*Snail exp. level*[c]
SS#[a]	*Fusion type*	*Histology*		*MSP*[b]	*Mutation*	*mRNA exp.*	*Membranous exp.*		
Kyushu Univ. 22	1	B		(−)		(+)	(+)	(+)	2.2
Kyushu Univ. 14	1	B		(−)		(+)	(+)	(+)	161.58
Kyushu Univ. 19	1	B		(−)	Silent	(+)	(+)	(+)	37.61
MSKCC 63	1	B		(+)		(+)	(+)	(+)	10.2
MSKCC 85	1	B		(−)		(+)	(+)	(+)	6.33
MSKCC 90	1	B		(+)		(+)	(+)	(+)	2.3
MSKCC 75	1	B		(−)		(+)	(+)	(+)	184.49
MSKCC 51	1	B		(−)		(+)	(+)	(+)	6.81
Kyushu Univ. 17	1	M	(+)	(−)	Silent	(+)	(+)	(+)	13.47
Kyushu Univ. 9	1	M	(+)	(−)		(+)	(+)	(+)	0.67
Kyushu Univ. 50	1	M	(+)	(−)		(+)	(+)	(+)	17.71
MSKCC 71	1	M	(−)	(−)		(+)	(+)	(−)	74.77
Kyushu Univ. 36	1	M	(+)	(−)	Missense	(+)	(−)	(+)	14.09
Kyushu Univ. 37	1	M	(−)	(−)	Silent	(+)	(−)	(+)	1.2
Kyushu Univ. 2	1	M	(+)	(−)	Missense	(+)	(−)	(+)	276.54
Kyushu Univ. 16	1	M	(−)	(−)	Missense	(+)	(−)	(+)	12.67
Kyushu Univ. 1	1	M	(−)	(−)		(+)	(−)	(+)	39.49
MSKCC 111	1	M	(+)	(−)		(+)	(−)	(+)	27.65
Kyushu Univ. 47	1	M	(−)	(+)	Missense	(−)	(−)	(−)	10.24
Kyushu Univ. 38	1	M	(+)	(−)	Missense	(−)	(−)	(−)	3.79
Kyushu Univ. 35	1	M	(−)	(−)		(−)	(−)	(−)	6.2
Kyushu Univ. 7	1	M	(−)	(−)		(−)	(−)	(−)	575.64
MSKCC 68	1	M	(−)	(−)		(−)	(−)	(+)	51.66
MSKCC 114	1	M	(−)	(−)		(−)	(−)	(−)	1178.98
Kyushu Univ. 45	2	B		(−)		(+)	(+)	(+)	6.95
MSKCC 109	2	M	(−)	(−)		(+)	(+)	(+)	11.72
Kyushu Univ. 13	2	M	(−)	(−)		(+)	(−)	(+)	3.28
Kyushu Univ. 46	2	M	(−)	(−)		(+)	(−)	(+)	10.53
Kyushu Univ. 8	2	M	(+)	(−)		(+)	(−)	(+)	15.15
MSKCC 70	2	M	(−)	(+)		(+)	(−)	(−)	13.84
MSKCC 65	2	M	(−)	(−)		(+)	(−)	(−)	3.48
MSKCC 112	2	M	(−)	(−)		(+)	(−)	(−)	6.18
MSKCC 123	2	M	(+)	(+)		(+)	(−)	(−)	9.56
MSKCC 64	2	M	(−)	(−)		(−)	(−)	(+)	30.61
MSKCC 78	2	M	(−)	(−)		(−)	(−)	(+)	3271.13
MSKCC 12	2	M	(−)	(−)		(−)	(−)	(−)	15.08
MSKCC 98	2	M	(−)	(−)		(−)	(−)	(−)	12.38
MSKCC 121	2	M	(−)	(−)		(−)	(−)	(−)	22.89
MSKCC 141	2	M	(−)	(−)		(−)	(−)	(−)	54.22
Kyushu Univ. 43	2	P	(−)	(−)		(−)	(−)	(−)	619.97

[a]SS numbers from Kyushu University are identical to those in the previous paper (Saito *et al.*, 2001). [b]MSP: methylation-specific PCR. [c]Snail exp. level: (Snail/GAPDH) × 1000

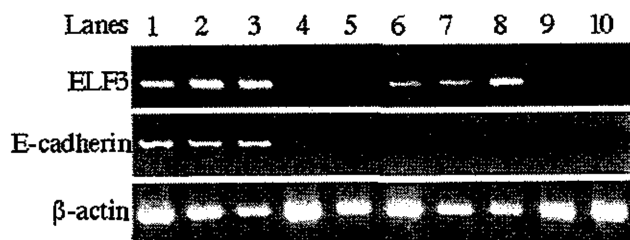

Figure 1 ELF3 and E-cadherin mRNA expression by RT–PCR. Lanes 1–5: synovial sarcoma, lanes 6–10: other spindle cell sarcomas. In SS, ELF3 and E-cadherin expression were completely correlated. In other spindle cell sarcomas, ELF3 expression was detected, but E-cadherin expression was not observed in any of the cases

SYT-SSX fusion type and E-cadherin or ELF3 mRNA expression in SS. These positive RT–PCR results for E-cadherin and ELF3 expression were confirmed to be derived from tumor cells by immunohistochemistry for the former or negative results in corresponding normal tissues where these tissues were available for the latter.

E-cadherin gene mutations in SS with SYT-SSX1 fusion type

We have recently shown that the E-cadherin gene is inactivated in a subset of SS, and that this event might be a determinant of histological features in SS (Saito *et al.*, 2001). This analysis was extended to the present series of 40 SS cases. Five of the 40 SS cases contained E-cadherin missense mutations (12.5%, Table 1). All five SS cases with E-cadherin missense mutations had the SYT-SSX1 fusion transcript (Fisher's exact test; $P = 0.07$, Table 2). SYT-SSX1 are more likely to show biphasic histology than SYT-SSX2 tumors (Ladanyi, 2001), but interestingly the five SYT-SSX1 SS cases with

Table 2 Correlation between SYT-SSX fusion type and E-cadherin missense mutation

	SYT-SSX fusion type	
E-cadherin	*1*	*2*
Missense mut.	5	0
Others	19	16

$P = 0.07$ (Fisher's exact test)

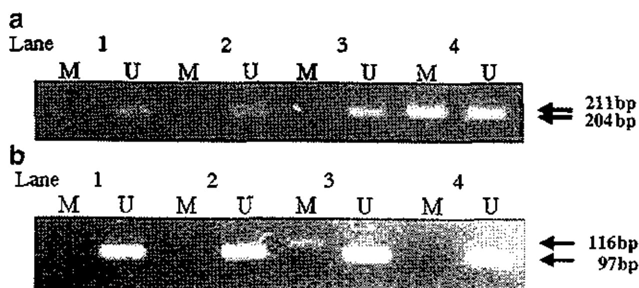

Figure 2 (a) MSP for E-cadherin gene by using primer pair for island 3 in SS. PCR products derived from methylated allele can be observed only in lane 4 (lane 4: MSKCC-SS90). (b) MSP for E-cadherin gene by using primer pair for island 1 in SS. PCR products derived from methylated allele can be observed only in lane 3 (lane 3: Kyushu-Univ.-SS47)

E-cadherin missense mutations were all monophasic tumors.

Methylation of CpG islands in the E-cadherin promoter region is rare in SS

Next, we investigated the possibility that aberrant methylation of the CpG island flanking the 5′ transcriptional start site on the E-cadherin gene was responsible for its lack of expression in SS. Promoter hypermethylation of the E-cadherin gene was detected in only five out of 40 cases (12.5%) at least with one of two primer sets (Figure 2a and b). Among five cases with promoter hypermethylation, only one was negative for E-cadherin mRNA expression (Table 1). Two of the remaining four cases with E-cadherin promoter hypermethylation were of biphasic histology. Methylation status of the E-cadherin gene did not correlate with E-cadherin mRNA expression in SS.

Inverse correlation between E-cadherin expression and Snail expression in SS

First, the correlation between Snail expression level and epithelial-related gene (E-cadherin or ELF3) expression in SS was examined. When SS were divided into two groups according to epithelial gene mRNA expression (E-cadherin or ELF3), E-cadherin-positive SS cases showed statistically significant lower Snail expression than negative cases (Figure 3, $P = 0.027$, Mann–Whitney's *U*-test). However, there were no significant correlations between the histologic subtype (biphasic vs others) and Snail expression, or between SYT-SSX fusion type and Snail expression in SS ($P = 0.29$ and $P = 0.98$, respectively).

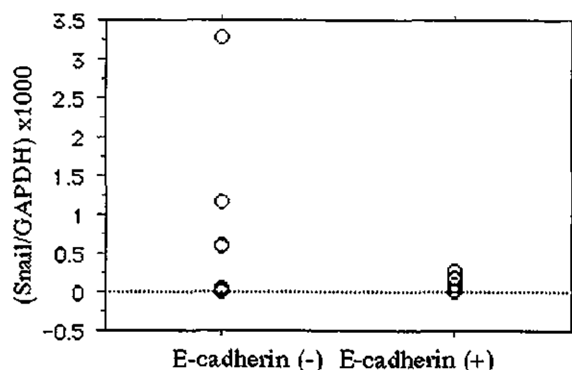

Figure 3 Scattergram of Snail mRNA expression levels in SS according to E-cadherin mRNA expression. The value of Snail mRNA expression was standardized to that of GAPDH mRNA expression. Snail mRNA level in SS with E-cadherin mRNA expression was significantly lower than those without E-cadherin mRNA expression ($P = 0.03$, Mann–Whitney *U*-test)

Table 3 Correlation between SYT-SSX fusion type and E-cadherin membranous expression

	SYT-SSX fusion type	
E-cadherin	*1*	*2*
Membranous exp. (+)	12	2
Membranous exp. (−)	12	14

$P = 0.0199$ (Fisher's exact test)

Correlation of E-cadherin membranous expression with SYT-SSX1 fusion type and biphasic histology in SS

E-cadherin membranous staining was observed immunohistochemically in 14 of 40 SS cases (35.0%: monophasic fibrous, 5/30; biphasic, 9/9; poorly differentiated, 0/1). Some cases showed not membranous but nuclear expression of E-cadherin. E-cadherin membranous expression correlated with biphasic histology in SS ($P < 0.0001$). In addition, E-cadherin membranous expression correlated with SYT-SSX1 fusion type (Table 3; $P = 0.02$ by Fisher's exact test), as might be expected given the association of SYT-SSX fusion type and histology. There was no correlation between E-cadherin membranous expression and Snail mRNA expression level ($P = 0.27$).

Higher expression of ELF3 in biphasic SS compared to monophasic SS

To independently validate previous cDNA microarray data that first identified the preferential expression of ELF3 in biphasic tumors (Allander *et al.*, 2002), quantitative real-time RT–PCR for ELF3 was performed using a separate set of 73 cases of SS. This revealed that ELF3 was more highly expressed in

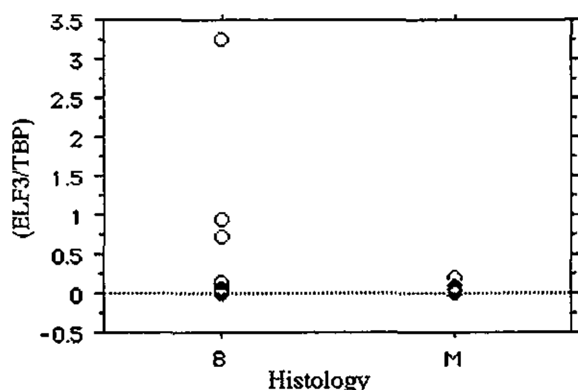

Figure 4 Scattergram of ELF3 mRNA expression levels in SS according to histology (biphasic vs monophasic). The value of ELF3 mRNA expression was standardized to that of TBP mRNA expression. ELF3 mRNA level in biphasic SS was significantly higher than those of monophasic tumors ($P = 0.04$, Mann–Whitney U-test)

biphasic SS than in monophasic tumors ($P = 0.04$ by Mann–Whitney U-test, Figure 4). However, there was no correlation between SYT-SSX fusion type and ELF3 expression level.

Discussion

Reduced E-cadherin expression has been shown to cause cellular morphological changes in epithelial cells, resulting in a more fibroblastic and flattened phenotype (Frixen *et al.*, 1991; Vleminckx *et al.*, 1991; Batlle *et al.*, 2000; Cano *et al.*, 2000; Poser *et al.*, 2001; Savagner, 2001; Blanco *et al.*, 2002; Hajra *et al.*, 2002; Locascio *et al.*, 2002; Nieto, 2002; Rosivatz *et al.*, 2002; Thiery, 2002). Recent reports have demonstrated E-cadherin expression in some sarcomas, mainly SS, particularly in the glandular structures of biphasic SS (Sato *et al.*, 1999; Saito *et al.*, 2000; Laskin and Miettinen, 2002; Yoo *et al.*, 2002). The spindle cell component of SS often shows histological features that closely resemble those of other spindle cell sarcomas such as MFH, LMS and MSNST, although spindle cell sarcomas other than SS rarely exhibit morphological epithelial differentiation. We recently identified mutational inactivation of E-cadherin as a correlate of spindle cell morphology in SS (Saito *et al.*, 2001). These findings suggested the hypothesis that the silencing of epithelial-related genes, such as the E-cadherin gene, may be responsible for the acquisition or maintenance of the spindle cell morphology in SS.

ELF3 was recently identified as one of the genes differentially expressed between monophasic and biphasic SS (Allander *et al.*, 2002). A large number of genes expressed in diverse epithelia are known to contain consensus binding sites for ETS transcription factors in their promoter or enhancer regions, including E-cadherin (Scott *et al.*, 1994; Gambarotta *et al.*, 1996; Rodrigo *et al.*, 1999). Therefore, we examined E-cadherin and ELF3 expression in SS to evaluate their correlates. As expected, E-cadherin expression was almost completely correlated with that of ELF3 in SS. E-cadherin was confirmed by immunohistochemistry to be expressed in the tumor cells of SS with E-cadherin transcripts. However, we could not confirm the histologic distribution of ELF3 in SS, because an antibody to ELF3 currently is not available. Therefore, it remains formally possible that the ELF3 transcripts detected in SS are derived from non-neoplastic elements, although we performed RT–PCR by extracting RNAs from which paired normal tissues were available, and obtained negative results.

In some tumors, putative cause–effect relationships between the histological type and E-cadherin inactivation by mutation or promoter hypermethylation have been proposed (Berx *et al.*, 1995; Machado *et al.*, 1999; Saito *et al.*, 2001). We therefore examined silencing of the E-cadherin gene by genetic and epigenetic changes as a potential mechanism for its inactivation in SS. E-cadherin missense mutation was detected in five cases of SS leading to the inactivation of the E-cadherin gene and resulting in the monophasic histology. E-cadherin promoter methylation was observed in five cases (12.5%) in SS, but in only one of which E-cadherin expression was silenced. Of four SS cases with E-cadherin mRNA expression in spite of E-cadherin promoter methylation, two were monophasic and another two were biphasic tumors. Our finding of unmethylated bands in all of the samples with promoter methylation of the E-cadherin gene (Figure 2) raises the possibility that the methylation status of E-cadherin might be heterogeneous within individual monophasic tumors. Alternatively, methylation status might be different in the different cell types (spindle cells, epithelial cells) in biphasic tumors.

In addition to silencing by genetic and epigenetic changes, transcriptional repression has emerged as another mechanism of downregulation of E-cadherin expression. The transcription factor Snail has been shown to be expressed by fibroblasts and some epithelial tumor cells, and in turn blocks E-cadherin gene expression by binding directly to the E-boxes present on the proximal E-cadherin promoter (Batlle *et al.*, 2000; Cano *et al.*, 2000). Although many studies have documented an inverse relationship between endogenous E-cadherin expression and endogenous Snail expression (Poser *et al.*, 2001; Hajra *et al.*, 2002; Nieto, 2002; Rosivatz *et al.*, 2002; Thiery, 2002), there are no published data in the field of sarcomas. In this study, we employed quantitative real-time PCR to examine the expression level of Snail transcription factor, because at present no antibodies are available for immunohistochemical analysis for this gene product. We found statistically lower expression of Snail in SS with E-cadherin mRNA expression compared to those without. The data are consistent with transcriptional repression of E-cadherin by Snail in SS, but this remains to be confirmed at the functional level. E-cadherin expression may be regulated by molecular switching through Snail, directing the differentiation of SS tumor cells to an epithelial phenotype. It should also be noted that it is

equally plausible that this inverse correlation between E-cadherin and Snail in SS is indirect (i.e. SS tumor cells with epithelial characteristics will tend to express reduced levels of mesenchymal-associated proteins such as Snail). Another possible level of control of E-cadherin expression is through chromatin-mediated effects such as chromatin modification and chromatin remodeling (Brown, 2003).

Another correlate of the epithelial morphology in SS cells is SYT-SSX fusion type. It is tempting to speculate that 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 SS, whereas biphasic SS usually contain the SYT-SSX1 fusion (Kawai *et al.*, 1998; Antonescu *et al.*, 2000; Ladanyi *et al.*, 2002). Recent microarray-based expression profiling studies of SS have identified genes that appear differently expressed between SS and other SCS or monophasic and biphasic histology (Allander *et al.*, 2002; Nagayama *et al.*, 2002; Nielsen *et al.*, 2002; Lee *et al.*, 2003; Segal *et al.*, 2003). ELF3, for example, was identified as one of the genes differentially overexpressed in biphasic cases, compared to monophasic tumors (Allander *et al.*, 2002). We therefore compared E-cadherin expression and ELF3 transcript levels with the SYT-SSX fusion type, to evaluate them as potential epithelial-specific downstream target genes of SYT-SSX. However, there was no relationship between their expression at the mRNA level and SYT-SSX fusion type. The meaning of ELF3 expression in SS is currently unknown, but its higher expression level in biphasic SS suggests some role in epithelial glandular differentiation. However, it is of interest to note that all SS cases with E-cadherin missense mutations contained the SYT-SSX1 fusion, and this association was marginally statistically significant. This finding might provide a key to the question as to why SS with the SYT-SSX1 fusion can show either a monophasic or biphasic phenotype.

The morphological transitions of SS cells are reminiscent of epithelial–mesenchymal transitions in other biological systems. It is unclear whether the loss of E-cadherin expression is a passive result of differentiation or whether it might play a more causative role in the differentiation of spindle cell morphology in SS. A previous report illustrated that disruption of E-cadherin function in mammalian epiblast cells at the primitive streak *in vitro* caused the cells to acquire phenotypic characteristics of the mesoderm (fibroblastic morphology) (Burdsal *et al.*, 1993). The inverse correlation between Snail and E-cadherin suggests the possibility of transcriptional repression of E-cadherin by Snail in SS, as has been shown in epithelial malignancies. In addition, it has been also shown that the epithelial–mesenchymal transition can indeed be a reversible process, and that it occurs several times during embryonic development (Nieto *et al.*, 1994; Sefton *et al.*, 1998; Auersperg *et al.*, 1999; Cheng *et al.*, 2001). In fact, E-cadherin re-expression has been found to occur in the metastatic process where the migratory cells need to establish attachment to their destination tissue (Mareel *et al.*, 1991; Bukholm *et al.*, 2000; Graff *et al.*, 2000). These findings may suggest that the loss of E-cadherin expression plays a causative role in the acquisition of spindle cell morphology in SS, being in agreement with the previous finding of mutational inactivation of E-cadherin gene in some cases of monophasic SS (Saito *et al.*, 2001). Taken into account the present E-cadherin data, we propose a model of morphologic differentiation in SS (Figure 5). In spite of still poorly understood differences in the transcriptional targets of SYT-SSX1 and SYT-SSX2, SS cases with the SYT-SSX1 fusion transcript have a greater likelihood of epithelial differentiation. However, some SS cases with the SYT-SSX1 fusion transcript escape epithelial differentiation by downregulating E-cadherin either by Snail-mediated transcriptional repression or through E-cadherin inactivating mutations. Among biphasic SS, additional events may be necessary for SS tumor cells with E-cadherin protein expression to show glandular differentiation. An example of this may be the remodeling of the extracellular matrix (Saito *et al.*, 2002b). Recently, Slug, ZEB1, SIP1 (ZEB2) and E12/47 were also found to downregulate E-cadherin expression through direct binding to single or paired E-boxes (Comijn *et al.*, 2001; Perez-Moreno *et al.*, 2001; Guaita *et al.*, 2002; Bolos *et al.*, 2003). Therefore, the possibility remains that downregulation of E-cadherin occurs via these non-Snail-related silencers. Furthermore, we should note that overactivity or overexpression of transcription factors positively mediating epithelial differentiation might also play a role in glandular epithelial differentiation in SS.

Finally, we should briefly discuss the conflicting data regarding clinical behavior and E-cadherin expression and SYT-SSX fusion type in SS. It is described that patients with SYT-SSX1 tumors present more often with metastatic disease and poor clinical course (Kawai *et al.*, 1998; Ladanyi *et al.*, 2002), whereas we reported

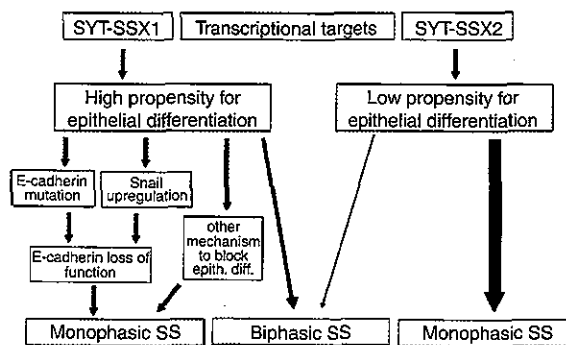

Figure 5 Proposed model of epithelial differentiation pathways in SS. Owing to putative subtle differences in transcriptional targets of SYT-SSX1 and SYT-SSX2, SS cases with the SYT-SSX1 fusion protein may have a greater propensity or potential for epithelial differentiation. Some SS cases with the SYT-SSX1 fusion transcript may escape epithelial differentiation by downregulating E-cadherin either by Snail-mediated transcriptional repression or through E-cadherin inactivating mutations. The thickness of the arrows is proportional to the percentage of cases in each pathway

favorable survival rate in patients with preserved E-cadherin expression (Saito *et al.*, 2000). Actually, almost all biphasic tumors that tend to show preserved E-cadherin expression are known to have SYT-SXX1 fusion transcript, although data on fusion type were not available in all of our original cases (Saito *et al.*, 2000). We confirmed this correlation between SYT-SSX fusion type and E-cadherin membranous expression in the present study. The aforementioned discrepancy may be explained by the fact that biphasic histology had a statistically significant favorable prognostic impact in the series from Kyushu University after further follow-up ($P=0.02$) (Saito *et al.*, unpublished data). The impact of monophasic vs biphasic histology on survival is known to be inconsistent among different prognostic analyses (Kawai *et al.*, 1998; Machen *et al.*, 1999; Lewis *et al.*, 2000; Trassard *et al.*, 2001). This might affect the significance of E-cadherin expression on survival rate in our previous report where the significance of E-cadherin expression disappeared by multivariate analysis (Saito *et al.*, 2000).

In conclusion, we report here that E-cadherin mRNA expression in SS is associated with reduced Snail expression level, consistent with the possibility of the transcriptional repression of E-cadherin by Snail in SS. Hypermethylation of the promoter region of the E-cadherin gene and E-cadherin missense mutation were observed equally in 12.5% of SS, and the latter being found only in monophasic tumors with SYT-SSX1 fusion gene. These results indicate that the possible regulation of E-cadherin expression by Snail as well as its silencing by E-cadherin missense mutation may have an important role in understanding the phenotypic heterogeneity of SS.

Materials and methods

Tumor samples

Snap-frozen tumor samples from 20 cases of SS were obtained from a collection of soft-tissue tumors registered in the Department of Anatomic Pathology, Pathological Sciences, Graduate School of Medical Sciences Kyushu University, Japan. Fresh tumor samples were carefully dissected in order not to include the surrounding normal tissue, and the samples were frozen in liquid nitrogen immediately and stored at −80°C. In addition, 20 frozen samples of SS were obtained from Department of Pathology, Memorial Sloan-Kettering Cancer Center (MSKCC), NY, USA under a protocol approved by the MSKCC Institutional Review Board. Diagnosis in each case was based on conventional light microscopic examination and molecular detection of SYT-SSX fusion gene by RT-PCR, and finally 40 cases of SS were prepared. Some of the SS cases used in this study were the same as in our previous study (Saito *et al.*, 2001) except one case in which the SYT-SSX fusion transcript could not be detected by RT-PCR, which was therefore excluded from the present study. Histological subclassification of SS was made according to the presence of an epithelial glandular component. Of the 40 cases, 30 were thus classified as monophasic, nine were classified as biphasic, and the single remaining case was classified as a poorly differentiated SS. A poorly differentiated SS was included within the monophasic group for statistical analysis. All of the SS cases were analysed for SYT-SSX fusion type using previously described specific primers (Kawai *et al.*, 1998), and thereby confirmed to have either SYT-SSX1 or SYT-SSX2.

RT-PCR for E-cadherin and ELF3

Total RNA was extracted using TRIZOL Reagent (GIBCO BRL, Tokyo, Japan) according to the manufacturer's protocol. In all, 5 μg of RNA from each sample was used for the subsequent reverse transcription. After the reaction, RNase treatment was performed to eliminate RNA. Sequences of specific pairs of primers were as follows: E-cadherin (upper primer: 5′-GAC GCG GAC GAT GAT GTG AAC-3′; lower primer: 5′-TTG TAC GTG GTG GGA TTG AAG A-3′), ELF3 (upper primer: 5′-CTG AGC AAA GAG TAC TGG GAC TGT C-3′; lower primer: 5′-CCA TAG TTG GGC CAC AGC CTC GGA GC) and β-actin (upper primer: 5′-AGG CCA ACC GCG AGA AGA TGA CC-3′; lower primer: 5′-GAA GTC CAG GGC GAC GTA GCAC-3′). Each PCR product was obtained after 40 cycles of amplification with an annealing temperature of 56.3°C. The PCR products were electrophoresed on a 2.0% agarose gel and visualized with ethidium bromide.

Polymerase chain reaction-single-strand conformation polymorphism (PCR-SSCP) and mutational analysis of E-cadherin gene

Genomic DNA was extracted from each frozen specimen as previously described (Saito *et al.*, 2002a). Some of the mutational analyses of E-cadherin in SS were previously performed (Saito *et al.*, 2001). Six sets of intronic primers were used for genomic DNA screening of the E-cadherin gene from exon 4 to exon 9. The primer sequences were the same as those aforementioned (Berx *et al.*, 1995; Saito *et al.*, 1999). PCR and subsequent SSCP were carried out as described previously (Saito *et al.*, 2001).

Methylation-specific PCR (MSP) analysis of the promoter region of the E-cadherin gene

Bisulfite conversion was performed with 1 μg of genomic DNA, using the reagents provided in the CpGenome DNA modification Kit (Intergen, NY, USA). This process converts unmethylated cytosine residues to uracil, whereas methylated cytosine residues remain unchanged. MSP was performed to determine the DNA methylation status of CpG islands of the promoter region of the E-cadherin gene. MSP is based on the principle that the DNA sequences of methylated and unmethylated genomic regions differ after bisulfite conversion, and are thus distinguishable by sequence-specific PCR primers (Herman *et al.*, 1996). The two pairs of primers (Islands 1 and 3) used in this study were as previously described (Graff *et al.*, 1997).

Quantitative TaqMan-based RT–PCR analysis of Snail expression

Real-time quantitative RT–PCR for Snail (GenBank: NM005985) mRNA level was also performed for each sample using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Oligonucleotide primers and TaqMan probes were designed as follows: forward primer, 5′-ACCAC-TATGCCGCGCTCTT-3′, reverse primer, 5′-GGTCGTAGG GCTGCTGGAA-3′, and probe, 5′-FAM (6-carboxy-fluorescein)-TCGTCAGGAAGCCCTCCGACCC-TAMRA (6-carboxy-tetramethyl-rhodamine)-3′ (Sugimachi *et al.*, 2003).

Each primer was placed on a different exon to avoid amplification of contaminating genomic DNA. A set of primers and probe for the internal housekeeping gene, glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) (TaqMan GAPDH control reagent kit) was purchased from Perkin–Elmer Applied Biosystems. For the construction of the standard curve, a serial 1:10 dilution of plasmid DNA containing each target cDNA was analysed, and copy numbers of the target cDNA were estimated by standard curves. All the reactions for standard samples and samples of patients were performed in triplicate, and the data were averaged from the values obtained in each reaction. The obtained data were standardized by using data from the internal housekeeping gene, GAPDH.

Immunohistochemistry for E-cadherin

Formalin-fixed, paraffin-embedded tissue sections from 40 SS cases corresponding to those from which RNA was extracted were processed for immunohistochemical staining using a primary monoclonal antibody against E-cadherin (diluted at 1:1000; Transduction Laboratories), as described previously (Saito *et al.*, 2000). Subcellular localization (membrane, cytoplasm, or nuclear) was also noted.

Quantitative TaqMan-based RT–PCR analysis of ELF3 expression

To validate our previous microarray result (Allander *et al.*, 2002), a separate set of 73 cases of SS was prepared from the Department of Pathology, MSKCC, NY, USA under a protocol approved by the MSKCC Institutional Review Board. This subset contained 54 of monophasic tumors and 19 of biphasic tumors. Real-time PCR for ELF3 was performed using iCycler (BioRad, USA). The quantity of ELF3 expression was normalized by using TATA-box binding protein (TBP) as an endogenous control gene. The sequences for primers and probes used were as follows: ELF3-FP, 5′-GCATATTTTCTTGCTGCCAGTCT-3′; ELF3-RP, 5′-ACC ACGGCACTGATTTTCAGTT-3′; ELF3-probe, 5′-ACTGT TCTTCACTCTCTTGGCTCCTGTGCA-3′, TBP-FP, 5′-GC ATATTTTCTTGCTGCCAGTCT-3′; TBP-RP, 5′-ACCACG GCACTGATTTTCAGTT-3′; TBP-probe, 5′-ACTGTTCTT-CACTCTCTTGGCTCCTGTGCA-3′. Standard plasmids were generated by cloning cDNA fragments of ELF3 and TBP into the pCRII TOPO plasmid (Invitrogen).

Statistical analysis

The χ^2 test was used to assess the correlation between various parameters. A two-tailed P-value less than 0.05 by Fisher's exact test was considered as significant. Comparison between the Snail mRNA expression level and E-cadherin expression was evaluated using Mann–Whitney U-test. A P-value <0.05 was considered to be statistically significant.

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Minireview

The role of nuclear Y-box binding protein 1 as a global marker in drug resistance

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Abstract

Gene expression can be regulated by nuclear factors at the transcriptional level. Many such factors regulate *MDR1* gene expression, but what are the sequence elements and transcription factors that control the basal and inducible expression of this gene? The general principles through which transcription factors participate in drug resistance are now beginning to be understood. Here, we review the factors involved in the transcriptional regulation of the *MDR1* gene. In particular, we focus on the transcription factor Y-box binding protein 1 and discuss the possible links between Y-box binding protein 1 expression and drug resistance in cancer, which are mediated by the transmembrane P-glycoprotein or non-P-glycoprotein. [Mol Cancer Ther 2004;3(11):1485-92]

Introduction

Drug export from cells is mediated through a group of proteins belonging to the ATP binding cassette family of transporters. The 170-kDa transmembrane protein P-glycoprotein (PGP), which is encoded by the multidrug resistance 1 (*MDR1*) gene, is a representative example of an ATP binding cassette transporter. PGP consists of two membrane-spanning domains and two nucleotide binding domains and has been reported to affect the pharmacokinetics of drugs by limiting the rate at which they are absorbed (1-5). Various molecules are targeted by drug treatments for cancer; however, PGP expression is responsible for resistance to the widest range of anticancer drugs (6, 7).

The expression of *MDR1*/PGP in human malignant cancers is expected to play a critical role in limiting their sensitivity to anticancer agents. Therefore, the determination of *MDR1* gene expression levels, along with studies of the regulatory mechanisms of this gene, will be useful in developing tailor-made therapeutic strategies for cancer patients.

The partial sequence of the human *MDR1* gene was first reported in the 1980s (8), and its complete sequence, including clustered CpG sites that are not associated with a TATA box, is now known (9). Within the *MDR1* promoter sequence, a GC box forming a Sp1 site and an inverted CCAAT (ATTGG) site for Y-box binding protein 1 (YB-1) or nuclear factor Y (NF-Y) binding both play key roles in *MDR1* gene expression (10).

MDR1 gene expression is often observed in recurrent cancers and appears after the chemotherapeutic treatment of various human malignancies. In cultured human cancer cells, the *MDR1* promoter was activated by both PGP targeting drugs (vincristine and doxorubicin) and non-PGP-targeting drugs (5-fluorouracil and etoposide; ref. 11). In addition, treatment with retinoic acids and other differentiating agents resulted in enhanced expression of the *MDR1* gene product PGP (12). Expression of the *MDR1* gene was also up-regulated by heat shock, arsenate, and serum starvation in cultured human cancer cells (13-16). Consistent with these findings, *MDR1* gene expression was markedly induced by anticancer agents (17); the gene promoter was also activated in response to both anticancer agents and UV light (18, 19). These results show that *MDR1* gene expression is highly susceptible to various environmental stimuli (Table 1) and might therefore be stress responsive (11).

This review focuses on the molecular mechanism of the transcriptional regulation of human *MDR1*/PGP and the role of YB-1 expression in the acquisition of drug resistance.

Transcriptional Regulation of the Human *MDR1* Gene

Many studies have shown the involvement of various *cis*-acting elements in *MDR1* gene expression, suggesting

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Table 1. Transcriptional regulation of the *MDR1* gene in human cell lines

Transcription factor	Inducers	References
NF-Y	None	(40)
	Sodium butylate	(43)
	Trichostatin A	(42)
Spl	None	(45)
YB-1	UV light	(30, 32)
	Anticancer agents	(31)
Nuclear factor-interleukin-6	Phorbol ester	(52, 78)
EGR1	Phorbol ester	(45, 46)
HSF1	Heat shock	(15, 55)
20-kDa protein	Serum starvation	(16)
Transcription factor 4/β-catenin	None	(56)
Human T-cell lymphotrophic virus-1 Tax	Virus infection	(79)
SXR	Digoxin	(80)
MDR1 promoter-enhancing factor 1/RNA helicase A	None	(59, 60)
Nuclear factor-κB	Daunomycin	(58)
p53	None	(49–51)

pleiotropic mechanisms (10). As shown in Table 1, several transcription factors are expected to play critical roles in the basal expression of the *MDR1* promoter in addition to stimulus-induced activation.

Y-Box Binding Protein 1

Many reports on the factors associated with drug resistance have shown a plausible association of YB-1 with drug resistance both in cultured cancer cells and in numerous clinical human tumor samples.

YB-1 is a member of the cold shock domain (CSD) protein family, which is found in the cytoplasm and nucleus of mammalian cells. It has pleiotropic functions in the regulation of gene transcription and translation, DNA repair, drug resistance, and cellular responses to environmental stimuli (20–22). The structures of YB-1 and two other members of the CSD family, hdbpA (23) and Contrin/hdbpC (24), are presented in Fig. 1A. The *YB-1* gene, which is located on chromosome 1p34 (25, 26), contains eight exons spanning 19 kb of genomic DNA (Fig. 1B). The 1.5-kb mRNA encodes a 43-kDa protein comprising three domains: a variable NH_2-terminal tail domain (A/P domain), a highly conserved nucleic acid binding CSD, and a COOH-terminal tail domain (B/A repeat; refs. 27–29). The A/P domain (amino acids 1-51) seems to be involved in transcriptional regulation, whereas the CSD domain and part of the B/A repeat (amino acids 51-205) function in binding the Y-box (inverted CCAAT box) or double-stranded DNA. Most of the COOH-terminal region of the B/A repeat domain (amino acids 129-324) is thought to bind ssDNA or RNA, and part of this region (amino acids 129-205) is involved in dimerization.

We identified YB-1 as a transcription factor that binds to the inverted CCAAT box of the *MDR1* promoter (30). Decreased expression of YB-1, resulting from the introduction of YB-1 antisense expression constructs into cancer cells, markedly reduced the activation of the *MDR1* gene by DNA-damaging agents (31).

YB-1 is normally present in the cytoplasm, although it is translocated to the nucleus when cells are exposed to anticancer agents, hyperthermia, or UV light irradiation (19, 32, 33). YB-1 is often overexpressed in malignant cells and its expression is regulated by both the proto-oncogene product c-Myc and the tumor suppression gene product p73 (25, 34). The COOH-terminal tail domain seems to play a key role in the localization of YB-1 to either the cytoplasm or the nucleus (32). Studies have shown that cell cycle–specific nuclear translocation is mediated by cooperation of the CSD and COOH-terminal tail domain (35) and that the nuclear translocation of YB-1 requires wild-type p53 (36). The introduction of antisense RNA into human cancer cell lines,[10] and the targeted disruption of one Y-box allele in chicken DT40 cells (37) both inhibited growth. By contrast, the targeted disruption of one allele of the *YB-1* gene in mouse ES-1 cells had no effect on the growth rate (38).

Nuclear Factor Y

The CCAAT box is among the most ubiquitous DNA elements in both forward and reverse orientation. NF-Y is the major transcription factor recognizing the CCAAT box (39). This heteromeric protein is composed of three subunits, NF-YA, NF-YB, and NF-YC, all of which are necessary for DNA binding (Fig. 1A). Mutation and/or deletion of the CCAAT box have been shown to result in a significant loss of *MDR1* promoter activity (40). It has been reported that both the inverted CCAAT box and the GC box are required for activation of the *MDR1* promoter by UV light, and NF-Y, not YB-1, is thought to be the factor regulating the *MDR1* gene (41). However, these findings are not consistent with the results discussed above. The YB-1 protein is abundant and localized in the cytoplasm; however, when the effect of YB-1 overexpression on *MDR1* promoter activity was evaluated in human cancer KB cells, it was unclear whether the nuclear YB-1 content was increased. As YB-1 is known to repress translation, increased levels of cytoplasmic YB-1 might inhibit the translation of luciferase mRNA. Further studies are required to resolve this issue. Treatment with a histone deacetylase inhibitor (trichostatin) induced a marked increase in the amount of *MDR1* mRNA, although this drug-induced increase was inhibited in dominant-negative NF-Y mutants (42). NF-Y therefore seems to regulate *MDR1* gene expression through an interaction with p300/CBP-associated factor, which shows histone acetylation activity. NF-Y might also be responsible for the sodium butyrate–induced *MDR1* gene up-regulation in colon cancer cells (43). This transcription factor therefore plays a pivotal role in *MDR1* gene expression. Recently, the antitumor agent HMN-176, which interacts with NF-YB, has been shown to inhibit *MDR1* gene expression and to restore chemosensitivity to MDR cells (44).

[10] K. Kohno and M. Kuwano, unpublished data.

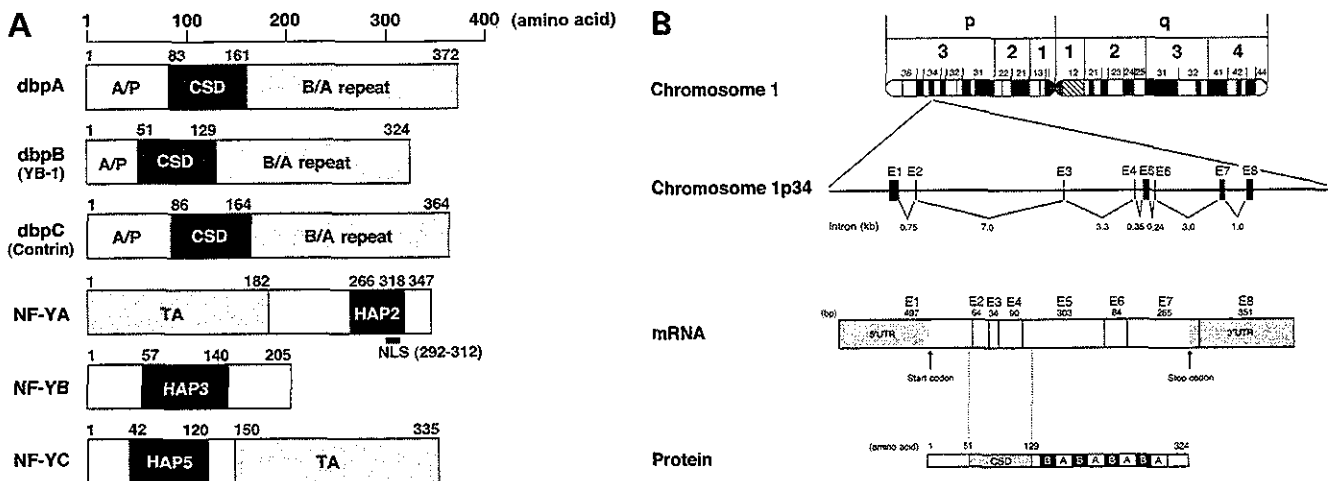

Figure 1. **A,** protein structure and functional domains of hdbpB/YB-1, hdbpA, hdbpC, NF-YA, NF-YB, and NF-YC. *A/P*, alanine and proline domain, residues 1-82, 1-50, and 1-85 in hdbpA, hdbpB/YB-1, and Contrin/hdbpC, respectively. *CSD*, residues 83-161, 51-129, and 86-164. *B/A repeat*, basic and acidic amino acid, residues 162-372, 130-324, and 165-364. The CSD domains of the three genes are highly homologous. Of the three subunits of NF-Y, NF-YB and NF-YC contain histone folding motifs homologous to the yeast transcription factors *HAP3* and *HAP5*, respectively. NF-YA contains a domain homologous to *HAP2*, which interacts with NF-YB and NF-YC, and the heterotrimer of NF-Y binds to DNA. Both NF-YA and NF-YC contain glutamine-rich domains and activate transcription. **B,** general structure of the genomic DNA, mRNA, and protein product of *YB-1*. The gene is mapped at chromosome 1p34 and has eight exons (*E1, E2, E3, E4, E5, E6, E7,* and *E8*). The YB-1 protein consists of 324 amino acids. *B,* basic amino acid clusters; *A,* acidic amino acid clusters.

Sp1 and Early Growth Response Element 1

The introduction of mutations in the GC-rich region −59 to −45 (G region) of the *MDR1* promoter markedly decreased its activity as a result of the transcription factor Sp1 (40, 45). Sp1 was first cloned and identified as a transcription factor specifically bound to the GC box of the SV40 promoter. A GC box is found in the promoter region of many eukaryotic genes. The Sp1 family is involved in various cellular functions including proliferation, apoptosis, differentiation, and neoplastic changes. As the early growth response element 1 (EGR1) binding motif partially overlaps with the Sp1 binding sites, it is conceivable that they mutually influence *MDR1* gene expression in a competitive manner (45). Treatment with phorbol ester induced the expression of both *EGR1* and *MDR1* genes in human leukemia cells (46). However, the expression of *EGR1* alone did not enhance *MDR1* promoter activity. Coexpression of the oncosuppressor gene *WT1* resulted in the inhibition of *MDR1* promoter activation by EGR1 or phorbol ester (47). Therefore, the direct binding of WT1 to the GC box might compete with Sp1 to down-regulate the *MDR1* gene. These findings suggest that interactions between EGR1 and WT1 might play a key role in *MDR1* promoter activation.

p53

Mutant p53 has been shown to enhance *MDR1* promoter activity in mouse cells; this was reversed by wild-type p53 (14, 48). By contrast, stimulation of the *MDR1* promoter by wild-type, but not mutant, p53 was shown in several human p53-null cancer cell lines. The *MDR1* promoter region −39 to +53 is responsible for this p53-mediated activation (49), whereas the region −189 to +133 is thought to be responsible for negative regulation by wild-type p53 (50). In addition, p53 has been reported recently to bind directly to a novel binding element (−72 to −40) within the *MDR1* core promoter and to repress its promoter activity (51).

Nuclear Factor-Interleukin-6

The treatment of human monocytic cells with phorbol ester enhanced *MDR1* promoter activity through interaction with nuclear factor-interleukin-6, which is a CCAAT/enhancer binding protein family member. This study also revealed that the mitogen-activated protein kinase pathway activates nuclear factor-interleukin-6 (52). In addition, CCAAT/enhancer binding protein β has been shown recently to transactivate the *MDR1* promoter by interaction with the Y-box (53).

Heat Shock Factor

MDR1 promoter activation in response to arsenate or heat shock seems to be mediated through a heat shock element in the −178 to −165 region. An additional region at −136 to −76 has also been proposed as a critical heat shock element for the heat shock response (15, 54), although no direct binding of heat shock factor to this region has been shown. Recently, Vilaboa et al. (55) reported that infection with adenovirus carrying heat shock transcription factor 1 cDNA increased the levels of *MDR1* mRNA and PGP.

Transcription Factor 4/β-Catenin

Transcriptional profiles produced using cDNA microarrays in human colon cancer cell lines identified the *MDR1* gene as the target of transcription factor 4/β-catenin. Seven transcription factor 4/β-catenin binding sites were in the promoter region between −2,030 and +31 (56).

Nuclear Factor-κB

The hepatocarcinogen 2-acetylaminofluorene was shown to activate the *MDR1* gene in human hepatoma cells and the induction of *MDR1* by 2-acetylaminofluorene was mediated by a nuclear factor-κB binding site located around −6 kb (57). Another group showed that the inhibition of nuclear factor-κB reduced levels of *MDR1* mRNA and PGP expression and that nuclear factor-κB transactivated the *MDR1* promoter in human colon cancer HCT15 cells (58). This study identified a nuclear factor-κB binding site in the first intron.

MDR1 Promoter-Enhancing Factor 1/RNA Helicase A

MDR1 promoter-enhancing factor 1 has been shown to bind to the CCAAT sequence causing up-regulation of the *MDR1* gene (59). RNA helicase A has also been reported to bind to the CCAAT box as a member of the *MDR1* promoter-enhancing factor 1 complex (60). Overexpression of RNA helicase A enhanced the expression of both the *MDR1* promoter-reporter construct and endogenous PGP.

Clinical Implications of PGP Expression and Nuclear Translocation of YB-1

PGP triggers resistance to a wide range of anticancer agents including *Vinca* alkaloids, anthracyclines, epipodophyllotoxins, and taxols (7). In addition, YB-1 plays a role in limiting the drug sensitivity of cancer cells by increasing the expression of PGP and other proteins. Immunohistochemical studies of YB-1 expression in the nuclei of untreated primary breast cancers showed an almost complete association between nuclear YB-1 and PGP expression in 9 of 27 cases (Table 2; ref. 61). Studies of clinical specimens have also shown an association between YB-1 and PGP in osteosarcoma (62), synovial sarcoma (63), breast cancer (64, 65), ovarian cancer (66–68), and prostate cancer (Table 2; ref. 69). Figure 2 shows examples of the presence and absence of YB-1 and PGP in clinical samples of osteosarcoma and synovial sarcoma based on the results of immunohistochemical analyses with anti-YB-1 and anti-PGP antibodies.

Table 2. The association of nuclear expression of YB-1 with PGP-mediated and/or non-PGP-mediated drug resistance in human malignancies

Tumor type	Malignant characteristics	References
Ovarian cancer	PGP* ↑	(66)
	PGP* ↑	(67)
	Cisplatin resistance	(68)
Breast cancer	PGP ↑	(61)
	PGP* ↑	(64)
	Drug resistance	(65)
Osteosarcoma	PGP ↑	(62)
Synovial sarcoma	PGP* ↑	(63)
Prostate cancer	PGP* ↑	(69)

*These studies also reported a significant correlation between nuclear YB-1 expression and disease progression or prognosis.

There was a significant correlation between the nuclear expression of YB-1 and the presence of PGP in 69 cases of osteosarcomas (62). A recent study confirmed that YB-1 expression was specifically associated with the overexpression of PGP rather than with three other ATP binding cassette transporters: MRP1, MRP2, and MRP3 (63). By contrast, no association was observed between YB-1 and PGP expression in colon cancers (70). It remains unclear whether YB-1 is directly involved in the transcriptional regulation of PGP in human malignancies. Nevertheless, measurements of the expression of YB-1 and PGP could suggest treatment modalities for individual cancer patients. Recently, we showed that coexpression of YB-1 and PGP correlated with poor prognosis in epithelial ovarian cancer (67). The expression of *MDR1* is augmented in cancerous areas in breast cancer and other tumors, resulting in drug resistance. Furthermore, the presence of YB-1 in the nuclei of cancer cells is closely associated with the clinical outcome. YB-1 could therefore be a useful indicator of malignancy as well as a promising target for cancer therapy. We recommend the use of non-PGP-targeting drugs against malignant tumors with nuclear YB-1 expression.

Clinical Implications of Nuclear Localization of YB-1: Drug Resistance to non-PGP-Targeting Drugs

As described above, YB-1 is translocated to the nucleus in response to various environmental stresses including UV light, anticancer agents, heat, and infection in cultures of cancer cells (21). YB-1 was shown to be overexpressed in cisplatin-resistant cell lines, and antisense YB-1 RNA triggered the augmentation of sensitivity to cisplatin, mitomycin C, UV light, and hydrogen peroxide (30, 38). YB-1 associates with p53 (71) and proliferating cell nuclear antigen (72), both of which modulate DNA repair, cell cycle, transcription, and drug sensitivity. Moreover, wild-type p53 is required for the nuclear translocation of YB-1, which in turn inhibits p53-induced cell death (36). However, it remains unclear how reduced YB-1 expression increases resistance to non-PGP-targeting DNA-damaging agents such as cisplatin and mitomycin C. Potential mechanisms might include a reduction in the YB-1 interaction with proliferating cell nuclear antigen, which is necessary for nucleotide excision repair, or in the interaction with p53. However, pleiotropic drug resistance to DNA-interacting drugs (e.g., aphidicolin, hydroxyurea, cytarabine, etoposide, doxorubicin, and mafosfamide) is associated with the increased expression of YB-1 and 19 other genes that are involved in DNA replication, repair, and stress responses (73).

Nuclear expression of YB-1 was reported to be a prognostic factor in ovarian serous adenocarcinoma (66). It was also associated with cisplatin resistance in ovarian cancer cell lines, and expression levels were increased at some sites of ovarian cancer recurrence (68). This pattern was seen in 7 of 21 serous adenocarcinomas, 2 of 7 clear cell

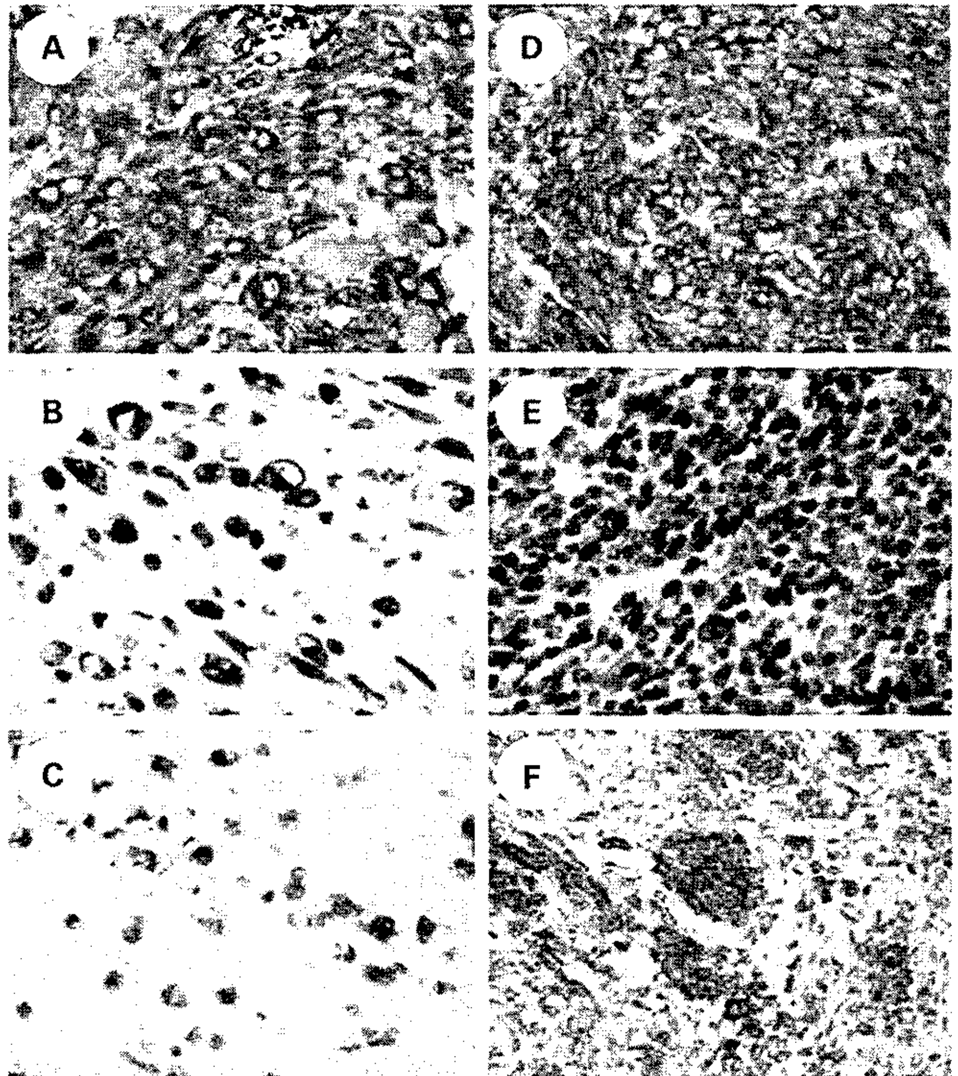

Figure 2. Immunohistochemical detection of nuclear and cytoplasmic YB-1 in osteosarcoma and synovial sarcoma. Antibodies were used against YB-1 (**A**, **B**, **D**, and **E**) or PGP (**C** and **F**). Osteosarcoma is shown with cytoplasmic YB-1 expression (**A**), nuclear YB-1 expression (**B**), and PGP expression (**C**). Synovial sarcoma is shown with cytoplasmic YB-1 expression (**D**), nuclear YB-1 expression (**E**), and PGP expression (**F**). The patient in **D** showed no evidence of disease 131 months after surgery. The patient in **F** died of lung metastasis 8 months after the initial surgery.

adenocarcinomas, and 1 of 4 mucinous adenocarcinomas (Table 2). There was also a positive correlation between the nuclear expression of YB-1 and poor prognosis in synovial sarcoma (63).

Analysis of the clinical relevance of YB-1 expression in the cytoplasm or nucleus in 83 cases of breast cancer, after a median follow-up of 61 months, revealed that the 5-year relapse rate was 66% in patients with high YB-1 expression who received postoperative chemotherapy (65). By contrast, none of the patients with low YB-1 expression experienced relapse. Taken together, these findings indicate that the overexpression and nuclear expression of YB-1 have a predictive value in some human malignancies, both with and without postoperative chemotherapy.

An investigation of 588 genes associated with mouse lung tumor progression revealed that 19 were differentially expressed between lung adenoma and adenocarcinoma; YB-1 was one of these candidate lung tumor progression genes (74). Overexpression of YB-1 was observed in >90% of anaplastic thyroid carcinomas, whereas it was absent in normal follicles and other pathologic tumor types. These findings suggested the involvement of YB-1 in the anaplastic transformation of thyroid carcinoma (75). YB-1 expression induced a strong cellular resistance to malignant transformation through the phosphatidylinositol 3-kinase pathway possibly through the inhibition of protein synthesis that is required for the phosphatidylinositol 3-kinase- or Akt-induced oncogenic transformation (76).

Conclusion

The ancestral protein YB-1 modulates cell growth, apoptosis, drug resistance, DNA repair, transcription, and translation as a pleiotropic regulator. YB-1 overexpression or nuclear YB-1 expression might play a key role not only in the acquirement of PGP-mediated drug resistance but also in sensitivity to non-PGP-targeting chemotherapeutic agents. YB-1 in the nucleus modulates drug resistance to PGP-targeting and non-PGP-targeting drugs in cancer cells