Modern Pathology (2006) 19, 106–114
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www.modernpathology.org

DNA hypermethylation status of multiple genes in soft tissue sarcomas

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The aberrant methylation of promoter CpG islands is known to be a major inactivation mechanism of tumor-related genes. To determine the clinicopathological significance of gene promoter methylation in soft tissue sarcomas, we examined the promoter methylation status of 10 tumor-related genes in 65 soft tissue sarcomas and 19 adjacent non-neoplastic tissues by methylation-specific PCR. The methylation frequencies of tumor-related genes tested in soft tissue sarcomas were 17 (26%) for *RASSF1A*, 11 (17%) for *DAP kinase*, 10 (15%) for *MGMT*, nine (14%) for *GSTP1*, eight (12%) for *PTEN*, six (9%) for *p16* and *hMLH1*, five (8%) for *hMSH2*, two (3%) for *p14*, and one (2%) for *RB*. Promoter methylation of these genes was not recognized in non-neoplastic tissues. All those cases of soft tissue sarcoma that had *MGMT* methylation, with the exception of one case of malignant peripheral nerve sheath tumor, showed large tumor size ($\geq$10 cm) or recurrence. Moreover, eight of 10 cases with *MGMT* methylation revealed high American Joint Committee on Cancer stage. Seven of 10 cases (70%) with *MGMT* methylation showed a loss of MGMT expression by immunohistochemistry. In addition, *MGMT* methylation status had a statistically significant correlation with a loss of MGMT expression ($P = 0.014$). In conclusion, although methylation of tumor-related genes was a relatively rare event in soft tissue sarcomas, methylation was tumor-specific. Of 10 tumor-related genes, cases with *MGMT* methylation had a tendency to be aggressive behavior. Moreover, *MGMT* methylation was closely associated with a loss of MGMT expression. Although our findings need to be extending to a large series, promoter methylation of tumor-related genes is likely to have an association with the pathogenesis of soft tissue sarcomas. Furthermore, *MGMT* methylation may be associated with tumor aggressiveness and the inactivation of *MGMT* gene.
Modern Pathology (2006) **19,** 106–114. doi:10.1038/modpathol.3800502; published online 21 October 2005

Keywords: soft tissue sarcoma; promoter hypermethylation; methylation-specific PCR; tumor-related genes

The pathogenesis of soft tissue sarcoma is unclear; however, some soft tissue sarcomas, such as synovial sarcoma and myxoid liposarcoma, frequently show specific balanced translocations as the sole cytogenetic anomaly, and their plausible contribution to pathogenesis has been suggested. On the other hand, soft tissue sarcomas without specific balanced translocations, such as leiomyosarcomas, malignant fibrous histiocytomas, and malignant peripheral nerve sheath tumors, are characterized by genetic changes, including complex karyotypic changes and extensive heterogeneity;[1–4] however, its frequencies are low.

The aberrant methylation of promoter-associated CpG islands has emerged as a distinct molecular pathway leading to cellular malignant transformation. Recent studies have demonstrated that the silencing of tumor suppressor genes by promoter hypermethylation is a common feature among many types of malignancies.[5–7] It has been proposed that DNA methylation provides an alternate pathway to gene deletion or mutation. Some genes, such as the cell cycle inhibitor *p16*, are hypermethylated across many tumor types, for example, colorectal, lung, and breast carcinomas,[8–10] as well as bone and soft tissue sarcomas.[11–18] According to recent studies demonstrating the role of methylation status in pathogenesis, we hypothesized that epigenetic abnormalities, particularly DNA hypermethylation, may play a primary role in the pathogenesis of soft

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Received 28 April 2005; revised and accepted 08 September 2005; published online 21 October 2005

tissue sarcomas in particular; here, we focused especially on cases lacking specific balanced translocation. However, it should be noted that the target genes inactivated by DNA methylation in soft tissue sarcomas remains largely unknown, and no global profile of CpG island methylation in soft tissue sarcomas has yet emerged.

To explore the role of DNA methylation in soft tissue sarcomas, we selected 10 tumor-related genes, the frequently silenced by aberrant methylation in a number of tumor types, and examined the methylation status for the following genes by methylation-specific PCR: *RASSF1A*, *p16*, *p14*, *RB*, *hMLH1*, *hMSH2*, *MGMT*, *GSTP1*, *PTEN*, and *death-associated protein kinase* (*DAP kinase*). We also analyzed the correlation between the methylation status of these genes and the clinicopathologic features known to be important for the prognosis of cases of soft tissue sarcoma.

Materials and methods

Tumor Samples and DNA Extraction

Snap-frozen tumor samples from 65 soft tissue sarcoma cases and 19 adjacent non-neoplastic tissues were obtained from the 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 from the tumors in order to exclude the surrounding normal tissue, and the samples were immediately frozen in liquid nitrogen and stored at -80°C. Diagnosis in each of the 65 cases was based on light microscopic examination of hematoxylin—eosin-stained paraffin blocks. Smooth muscle differentiation of leiomyosarcomas was confirmed by immunohistochemical positive reaction for desmin, muscle-specific actin, and alpha smooth muscle actin. Malignant peripheral nerve sheath tumors showed positive immunoreactivity for S-100 protein, and solitary fibrous tumors were revealed for CD34. One case of fibrosarcoma was positive for vimentin but negative for myogenic markers, S-100 protein, CD34, cytokeratins, and epithelial membrane antigen. In addition, no SYT-SSX fusion gene was recognized in this case. Histologic diagnosis of the 65 cases of soft tissue sarcoma included this study revealed 32 cases of malignant fibrous histiocytoma (pleomorphic type, 24 cases; myxoid type, eight cases), 17 cases of malignant peripheral nerve sheath tumor, 13 cases of leiomyosarcoma, two cases of solitary fibrous tumor (one of which was malignant), and one case of fibrosarcoma. Five of 17 malignant peripheral nerve sheath tumor cases occurred in association with neurofibromatosis type 1. As regards the staging of primary tumors, cases were also evaluated according to the new American Joint Committee on Cancer (AJCC) staging system.[19] Genomic DNA was purified using standard proteinase K digestion and phenol/chloroform extraction methods.

Bisulfite Modification and Methylation-Specific PCR

Bisulfite modification was performed using a DNA modification kit (Intergen). DNA (1 μg) in a volume of 100 μl was denatured by NaOH 0.2 M for 10 min at 37°C. Salmon sperm DNA (1 μg) (Sigma) was added as a carrier before modification. In all, 550 μl of freshly prepared 3 M sodium bisulfite at pH 5 was added and mixed with the samples, which were then incubated at 50°C for 16 h. Modified DNA was purified using Wizard DNA purification resin according to the manufacturer's instructions (Promega). Samples were eluted into 50 μl of water, and modification was completed by NaOH 0.3 M treatment for 5 min at room temperature, followed by ethanol precipitation. The DNA was resuspended in water and used immediately or stored at -20°C. The sequences of the primers and annealing temperatures are summarized in Table 1. Negative control samples without DNA were included for each set of PCR. Each PCR product (10 μl) was directly loaded onto 2% agarose gel, stained with ethidium bromide, and directly visualized under UV illumination.

Immunohistochemistry

Immunohistochemical analysis was performed using mouse IgG monoclonal antibodies against MGMT (1:50, Santa Cruz Biotechnology, USA). Sections of formalin-fixed, paraffin-embedded tissue (4 μm thick) were deparaffinized in xylene and dehydrated in ethanol. After dehydration, the endogenous peroxidase was blocked by methanol containing 3% H_2O_2 for 30 min. For staining with the above antibody, specimens were pretreated with citrate buffer (0.01 mol/l citric acid, pH 6.0) four times, and each pretreatment was carried out for 5 min at 100°C in a microwave oven. Sections were incubated with the primary antibody at 4°C overnight, followed by staining with a streptavidin–biotin–peroxidase kit (Nichirei, Tokyo, Japan). The sections were reacted in a 3,3′ diaminobenzidine, peroxytrichloride substrate solution, counterstained with hematoxylin and mounted. MGMT expression was considered as lost when the distribution of stained cells amounted to less than 10% of the tumor cells.[28]

Statistical Analysis

Fisher's exact test was used to evaluate the association between two dichotomous variables. A *P*-value of <0.05 was considered to indicate statistical significance.

Results

Clinical and Histological Findings

The clinicopathologic data for 65 soft tissue sarcoma cases are summarized in Table 2. According

Table 1 Primers used for the analysis of methylation of 10 tumor-related genes

Determination	*Primer sequence (5′ to 3′)*		*AT (°C)*	*References*
	Sense	*Antisense*		
RASSF1A				
M	GTGTTAACGCGTTGCGTATC	AACCCCGCGAACTAAAAACGA	60	[20]
U	TTTGGTTGGAGTGTGTTAATGTG	CAAACCCCACAAACTAAAAACAA	60	
DAP kinase				
M	GGATAGTCGGATCGAGTTAACGTC	CCCTCCCAAACGCCG	55	[21]
U	GGAGGATAGTTGGATTGAGTTATTGTT	CAAATCCCTCCCAAACACCAA	55	
MGMT				
M	TTTCGACGTTCGTAGGTTTTCGC	GCACTCTTCCGAAAACGAAACG	59	[7]
U	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTCTTCCAAAAACAAAACA	59	
GSTP1				
M	TTCGGGGTGTAGCGGTCGTC	GCCCCAATACTAAATCACGACG	59	[22]
U	GATGTTTGGGGTGTAGTGGTTGTT	CCACCCCAATACTAAATCACAACA	59	
PTEN				
M	GTTTGGGGATTTTTTTTTCGC	AACCCTTCCTACGCCGCG	60	[23]
U	TATTAGTTTGGGGATTTTTTTTTTGT	CCCAACCCTTCCTACACCACA	60	
p16				
M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA	65	[17]
U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA	65	
hMLH1				
M	ACGTAGACGTTTTATTAGGGTCGC	CCTCATCGTAACTACCCGCG	59	[24]
U	TTTTGATGTAGATGTTTTATTAGGGTTGT	ACCACCTCATCATAACTACCCACA	59	
hMSH2				
M	TCGTGGTCGGACGTCGTTC	CAACGTCTCCTTCGACTACACCG	59	[25]
U	GGTTGTTGTGGTTGGATGTTGTTT	CAACATCTCCTTCAACTACACCA	59	
p14				
M	GTGTTAAAGGGCGGCGTAGC	AAAACCCTCACTCGCGACGA	60	[26]
U	TTTTTGGTGTTAAAGGGTGGTGTAGT	CACAAAAAACCCTCACTCACAACAA	60	
RB				
M	GGGAGTTTCGCGGACGTGAC	ACGTCGAAACACGCCCCG	55	[27]
U	GGGAGTTTTGTGGATGTGAT	ACATCAAAACACACCCCA	55	

M: methylated; U: unmethylated; AT: anneling temperature.

to the case breakdown, 29 patients were affected in thigh, seven in buttock, six in retroperitoneum, four each in upper arm and knee, three in forearm, two in elbow, and one in each of the following: chest wall, scapular region, axilla, posterior mediastinum, sacral region, pelvic cavity, inguinal region, ankle, finger, and spinal cord. The AJCC stage was evaluable in 50 cases. Two cases were considered to be AJCC stage I, 30 cases were stage II, 16 cases were stage III, and two cases were stage IV.

Frequency of Promoter Hypermethylation of Tumor-Related Genes in Soft Tissue Sarcomas

The frequency of methylation of the 10 tumor-related genes in 65 soft tissue sarcomas is detailed in Figure 1. Among 65 soft tissue sarcomas, the methylation percentages (in descending order) were as follows: 17 (26%) for *RASSF1A*, 11 (17%) for *DAP kinase*, 10 (15%) for *MGMT*, 9 (14%) for *GSTP1*, 8 (12%) for *PTEN*, 6 (9%) for *p16* and *hMLH1*, 5 (8%) for *hMSH2*, 2 (3%) for *p14*, and 1 (2%) for *RB* (Table 3). All cases of soft tissue sarcoma with promoter hypermethylation showed both ummethylated and methylated signals (Figure 2). As regards the subtypes of soft tissue sarcoma, no statistically significant correlation was found between the subtypes of soft tissue sarcoma and the methylation status of these tumor-related genes. Most tumors (42 of 65, (65%)) showed promoter methylation in at least one of the 10 tumor-related genes. In all, 13 tumors (20%) displayed hypermethylation of three or more genes. Promoter hypermethylation of the 10 tumor-related genes examined was not observed in any of the non-neoplastic tissues examined.

Table 2 Clinicopathologic parameters in 65 soft tissue sarcomas

Clinicopathological characteristics	*Cases*
Median age (range)	59.6 (1–93)
Sex	
Male	28
Female	37
Location	
Thigh	29
Buttock	7
Retroperitoneum	6
Upper arm	4
Knee	4
Forearm	3
Elbow	2
Others	10
Median size, cm (range)	8.2 (2–26)
Tumor type	
Primary tumors	50
Local recurrences	15
Histological subtype	
Malignant fibrous histiocytoma	32
Malignant peripheral nerve sheath tumor	17
Leiomyosarcoma	13
others	3
AJCC stage[a]	
I and II	32
III and IV	18

[a] $n = 50$.

Correlation with Clinical Parameters

The correlation between methylation status and the clinicopathological parameters is given in Table 4. We excluded *p14* and *RB* genes from the analysis because the frequency of methylation in these genes was very low. All those cases with *MGMT* methylation, with the exception of one case of malignant peripheral nerve sheath tumor, showed large tumor size (≥ 10 cm) or recurrence (Table 5). Moreover, eight of 10 cases with *MGMT* methylation revealed high AJCC stage. With regard to other tumor-related genes, there was no relationship between methylation status and clinicpathologic parameters. Although survival data were available for 38 cases, no patients with *MGMT* methylation showed an association with poor survival statistically because some cases in which the follow-up interval was too short were included in these series. With regard to *p16* methylation, although we reported previously that *p16* methylation was observed in 22% of the leiomyosarcoma cases examined,[17] in the current study, *p16* methylation was also detected in three out of 13 leiomyosarcoma cases (23%). Leiomyosarcoma appeared with a higher frequency than the other spindle-shaped sarcomas (6%); however, no statistically significant correlation was observed in this regard. With regard to the cases of malignant peripheral nerve sheath tumor, no differences were observed between sporadic and neurofibromatosis type-1-associated cases. There was no correlation of methylation status with tumor grade, patients age, and tumor cytogenetics.

Immunohistochemical Analysis of MGMT

The inactivation of *MGMT*-involved promoter methylation was examined here because almost those cases with *MGMT* methylation showed large tumor size and high AJCC stage, which are known as factors of tumor aggressiveness in cases of soft tissue sarcoma. Thus, we investigated the expression of MGMT protein using immunohistochemical analysis. In 50 cases, paraffin-embedded tissues were available for analysis; 10 of these cases were found to have *MGMT* methylation, whereas the remaining 40 cases lacked *MGMT* methylation. Of the 50 soft tissue sarcoma cases, 13 (26%) showed a loss of MGMT expression. Seven of the 10 cases (70%) with *MGMT* methylation showed a loss of MGMT expression, on the contrary, six of the 40 soft tissue sarcoma cases (15%) without *MGMT* methylation revealed a loss of MGMT expression (Figure 3a and b). There was a statistically significant correlation between *MGMT* methylation and a loss of MGMT expression ($P = 0.014$) (Table 6).

Discussion

The pathogenesis remains unaccounted for in soft tissue sarcomas without specific balanced translocations, such as leiomyosarcomas, malignant fibrous histiocytomas, and malignant peripheral nerve sheath tumors. In these cases, genetic changes such as chromosomal deletions and mutations have been reported;[1–4] however, such changes are relatively rare, and associations with other mechanisms have been considered in this context. An epigenetic factor, that is, transcriptional silencing by the hypermethylation of CpG islands in the promoter region, is becoming recognized as a common mechanism for the inactivation of tumor suppressor genes in human malignancies.[29,30] Recently, the growing list of genes inactivated by promoter hypermethylation has provided a reason to examine the epigenetic alteration of multiple tumor-related genes in various types of malignancy. In the present study, we analyzed the methylation characteristics of patients with soft tissue sarcoma and investigated possible associations between aberrant methylation and clinical characteristics; to this end, we examined the methylation status of 10 tumor-related genes in patients with soft tissue sarcoma.

The profile of promoter methylation for the genes differs for each tumor types, providing a tumor-type and gene specific profile. In cancers, some genes, such as p16, are methylated across many tumors, on the contrary, the methylation of certain genes reflects their very specific involvement in selected

	Diagnosis	*RASSF1A*	*DAPk*	*MGMT*	*GSTP1*	*PTEN*	*p16*	*hMLH1*	*hMSH2*	*p14*	*RB*	Sum
Tumor												
1	MFH	■	■		■	■						4
2	MFH	■										1
3	MFH			■	■				■			3
4	MFH											0
5	MFH											0
6	MFH				■							1
7	MFH	■		■				■				3
8	MFH											0
9	MFH					■						1
10	MFH											0
11	MFH	■	■						■			3
12	MFH											0
13	MFH			■								1
14	MFH											0
15	MFH	■		■			■					3
16	MFH											0
17	MFH											0
18	MFH										■	1
19	MFH											0
20	MFH				■							1
21	MFH	■										1
22	MFH											0
23	MFH	■							■	■		3
24	MFH (rec.)				■		■					2
25	MFH (rec.)	■			■							2
26	MFH (rec.)											0
27	MFH (rec.)											0
28	MFH (rec.)	■										1
29	MFH (rec.)			■								1
30	MFH (rec.)											0
31	MFH (rec.)	■										1
32	MFH (rec.)								■			1
33	MPNST	■	■	■		■		■				5
34	MPNST											0
35	MPNST					■						1
36	MPNST							■				1
37	MPNST			■		■		■				3
38	MPNST		■									1
39	MPNST	■		■	■							3
40	MPNST											0
41	MPNST		■									1
42	MPNST					■						1
43	MPNST		■			■						2
44	MPNST											0
45	MPNST		■									1
46	MPNST	■										1
47	MPNST											0
48	MPNST											0
49	MPNST (rec.)											0
50	LMS											0
51	LMS	■		■				■				3
52	LMS		■		■			■				3
53	LMS											2
54	LMS						■					1
55	LMS											0
56	LMS	■		■			■					3
57	LMS					■						1
58	LMS						■					1
59	LMS (rec.)	■										1
60	LMS (rec.)		■									1
61	LMS (rec.)	■							■	■		3
62	LMS (rec.)		■									1
63	SFT											0
64	Malignant SFT											0
65	Fibrosarcoma (rec.)											0
Normal tissue												
2	MFH											0
3	MFH											0
18	MFH											0
19	MFH											0
22	MFH											0
23	MFH											0
25	MFH (rec.)											0
26	MFH (rec.)											0
30	MFH (rec.)											0
31	MFH (rec.)											0
32	MFH (rec.)											0
33	MPNST											0
46	MPNST											0
48	MPNST											0
52	LMS											0
59	LMS (rec.)											0
62	LMS (rec.)											0
63	SFT											0
65	Fibrosarcoma (rec.)											0

MFH; Malignant fibrous histiocytoma, MPNST; Malignant peripheral nerve sheath tumor, LMS; Leiomyosarcoma, SFT; Solitary fibrous tumor
rec.; recurrence

Table 3 Frequency of 10 tumor-related genes methylation in soft tissue sarcomas ($n=65$)

Genes	*Subtypes of soft tissue sarcomas*				*Total*
	Malignant fibrous histiocytoma (n = *32*)	*Malignant peripheral nerve sheath tumor* (n = *17*)	*Leiomyosarcoma* (n = *13*)	*Others* (n = *3*)	
RASSF1A	10 (31%)	3 (18%)	4 (31%)	0	17 (26%)
DAP kinase	2 (6%)	5 (29%)	4 (31%)	0	11 (17%)
MGMT	5 (16%)	3 (18%)	2 (15%)	0	10 (15%)
GSTP1	6 (19%)	1 (6%)	2 (15%)	0	9 (14%)
PTEN	2 (6%)	5 (29%)	1 (8%)	0	8 (12%)
p16	2 (6%)	0	3 (23%)	1 (33%)	6 (9%)
hMLH1	1 (3%)	3 (18%)	2 (15%)	0	6 (9%)
hMSH2	4 (13%)	0	1 (8%)	0	5 (8%)
p14	1 (3%)	0	1 (8%)	0	2 (3%)
RB	1 (3%)	0	0	0	1 (2%)

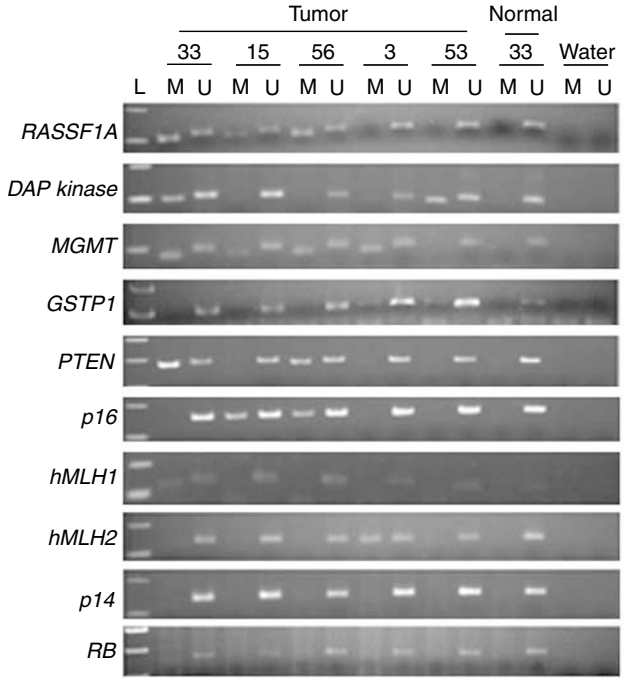

Figure 2 Methylation status of the promoter region of 10 tumor-related genes as determined by methylation-specific PCR. PCR products were amplified by unmethylated (U) and methylated (M)-specific primers.

tumor types. Of the 10 genes studied, we found that *RASSF1A* gene was methylated at 26% of soft tissue sarcoma cases examined. Harada *et al*[16] also reported that *RASSF1A* methylation was frequently seen in pediatric tumors, including some types of sarcoma (40%). On the other hand, the frequencies of other nine genes methylation (*DAP kinase*, *MGMT*, *GSTP1*, *PTEN*, *p16*, *hMLH1*, *hMSH2*, *p14*, and *Rb*) were low if ever methylated. Seidel *et al*[18] reported that *RASSF1A* methylation was more frequent in leiomyosarcoma compared to other type soft tissue sarcomas. Our result had no association between methylation status and the subtype of soft tissue sarcomas. In the current study, more than half of tumors examined (65%) showed promoter methylation in at least one of the 10 tumor-related genes. In addition, methylation was absent in corresponding nonmalignant tissue, indicating that methylation was tumor-specific. These results suggest that promoter methylation may play an important role with the pathogenesis of soft tissue sarcomas.

MGMT is a DNA repair protein that remove mutagenic and cytotoxic adducts from O6-guanine in DNA.[31] Although the deletion or mutation of the *MGMT* gene is rare, frequent methylation of this gene is associated with the gene silencing demonstrated in human cancers.[32–35] As regards sarcomas, a previous report demonstrated that promoter hypermethylation of the *MGMT* gene was a rare event.[16] Similarly, the present study also revealed that *MGMT* methylation occurred in 10 out of 65 soft tissue sarcoma cases (15%). Although the number of methylated cases is relatively low, nine of 10 cases with *MGMT* methylation showed large tumor size ($\geq$10 cm) or recurrence, and eight cases revealed high AJCC stage. In addition, seven of 10 cases (70%) with *MGMT* methylation showed a loss of MGMT expression, and *MGMT* methylation was closely correlated with a loss of MGMT expression. Although the presence of loss of heterozygosity (LOH) should be checked, these results indicate that the epigenetic inactivation of *MGMT* may play an important role in tumor aggressiveness in the case of soft tissue sarcomas. In this study, three cases with promoter methylation showed preserved

Figure 1 Methylation profiles of 10 tumor-related genes in 65 soft tissue sarcomas and 19 adjacent normal tissues. A filled box (black box) indicates that promoter methylation was detected by methylation-specific PCR and an open box (white box) indicates that no methylation was detected.

Table 4 Correlation between methylation status and clinicopathological parameters in 65 soft tissue sarcomas

	RASSF1A	DAP kinase	MGMT	GSTP1	PTEN	p16	hMLH1	hMSH2
Age (years)								
≥60 ($n=36$)	11 (31%)	4 (11%)	7 (19%)	7 (19%)	3 (8%)	4 (11%)	3 (8%)	4 (11%)
<60 ($n=29$)	6 (21%)	7 (24%)	3 (10%)	2 (7%)	5 (17%)	2 (7%)	3 (10%)	1 (3%)
Gender								
M ($n=28$)	9 (32%)	5 (18%)	6 (21%)	3 (11%)	4 (14%)	4 (14%)	5 (18%)	4 (14%)
F ($n=37$)	8 (22%)	6 (16%)	4 (11%)	6 (16%)	4 (11%)	2 (5%)	1 (3%)	1 (3%)
Tumor size (cm)								
≥10 ($n=19$)	7 (37%)	4 (21%)	8 (42%)	1 (5%)	2 (11%)	2 (11%)	4 (21%)	3 (16%)
<10 ($n=46$)	10 (22%)	7 (15%)	2 (4%)	8 (17%)	6 (13%)	4 (9%)	2 (4%)	2 (4%)
Tumor type								
Primary ($n=50$)	12 (24%)	9 (18%)	9 (18%)	7 (14%)	8 (16%)	5 (10%)	6 (12%)	3 (6%)
Recurrece ($n=15$)	5 (33%)	2 (13%)	1 (7%)	2 (13%)	0	1 (7%)	0	2 (13%)
AJCC stage[a]								
I and II ($n=32$)	5 (16%)	5 (16%)	1 (3%)	4 (13%)	6 (19%)	4 (13%)	2 (6%)	2 (6%)
III and IV ($n=18$)	7 (39%)	4 (22%)	8 (44%)	3 (17%)	2 (11%)	1 (6%)	4 (22%)	1 (6%)

[a]$n=50$.

Table 5 Clinicopathologic findings of 10 soft tissue sarcoma cases with *MGMT* methylation ($n=10$)

Cases	*Age (years)*	*Sex*	*Location*	*Tumor size (cm)*	*Tumor type*	*Histological subtype*	*AJCC stage*
Case 3	62	M	Thigh	10	Primary	Malignant fibrous histiocytoma	III
Case 7	93	M	Upper arm	21	Primary	Malignant fibrous histiocytoma	III
Case 13	81	F	Buttock	11	Primary	Malignant fibrous histiocytoma	III
Case 15	67	M	Retroperitoneum	10	Primary	Malignant fibrous histiocytoma	III
Case 29	87	F	Forearm	8	Recurrence	Malignant fibrous histiocytoma	NA
Case 33	27	F	Buttock	15	Primary	Malignant peripheral nerve sheath tumor	III
Case 37	47	M	Knee	15	Primary	Malignant peripheral nerve sheath tumor	III
Case 39	77	F	Mediastinum	4	Primary	Malignant peripheral nerve sheath tumor	II
Case 51	84	M	Thigh	16	Primary	Leiomyosarcoma	III
Case 56	55	M	Retroperitoneum	26	Primary	Leiomyosarcoma	III

NA: not available.

expression of the MGMT protein. The present findings suggest the possibility that *MGMT* methylation occurred in only one allele. In addition, another explanation could be that the MSP is too sensitive and that the methylation will concern only a subclone of the tumor.

The frequency of the promoter methylation of *p16* was observed in 0–35% of cases studied in soft tissue and bone sarcomas, including those included in our previous report.[11–18] Furthermore, we previously demonstrated that *p16* methylation played an important role in the inactivation of *p16* gene in soft tissue leiomyosarcomas.[17] In the current study, the promoter hypermethylation of *p16* was observed in three out of 13 leiomyosarcoma cases (23%), and this was found to be a relatively frequent event, in comparison to the incidence of such hypermethylation associated with other types of soft tissue sarcomas (6%); however, in this regard, no statistically significant correlation was observed.

In conclusion, the present findings indicated that the frequency of promoter hypermethylation of tumor-related genes was not high in spindle-shaped sarcomas, that is, in cases without specific balanced translocations such as those of malignant fibrous histiocytoma, leiomyosarcoma, and malignant peripheral nerve sheath tumor. Methylation was absent in the corresponding nonmalignant tissues examined, confirming that methylation was tumor-specific. Among the 10 tumor-related genes studied, cases with *MGMT* methylation showed large tumor size and high AJCC stage. In addition, *MGMT* methylation was closely correlated with a loss of MGMT expression. These findings indicate that *MGMT* methylation may play an important role in tumor aggressiveness of soft tissue sarcoma and the inactivation of *MGMT* gene; however, a length follow-up will be required to determine whether methylation is a predictive factor for survival. Although the sample size should be increased in further studies of this nature, the present results suggest that the promoter methylation of tumor-related genes may be associated with the pathogenesis of soft tissue sarcomas.

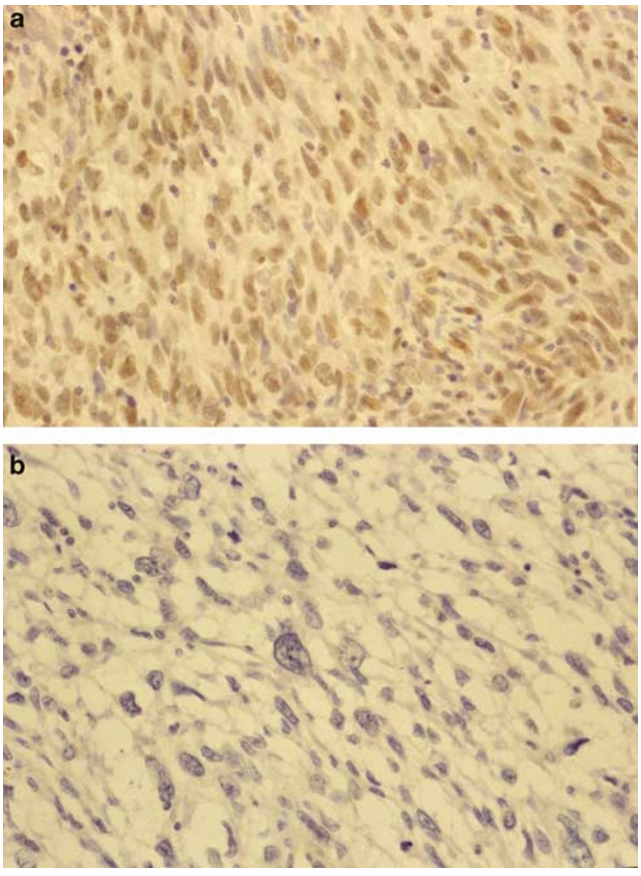

Figure 3 Immunohistochemical staining of MGMT. (**a**) Immunohistochemistry showing nuclear staining in the majority of tumor cells (Case 40, malignant peripheral nerve sheath tumor). No promoter methylation of the *MGMT* gene was detected in this case. (**b**) A loss of MGMT expression was recognized in the majority of tumor cells. This case was revealed as having promoter methylation of *MGMT* (Case 15, malignant fibrous histiocytoma).

Table 6 Correlation between *MGMT* methylation and MGMT expression in soft tissue sarcomas ($n=50$)

MGMT methylation	*MGMT expression*		P-*value*
	Loss	*Normal*	
+	7	3	$P=0.014$*
−	6	34	

Fisher's exact test.
*Statistically significant.

Acknowledgements

We are grateful to Miss Y Nouzuka and Miss N Tateishi for their excellent technical assistance. The English used in this manuscript was revised by KN International. Grant sponsors: This work was supported in part by a Grant-in-Aid for Scientific Research (C) (no. 15590304) from the Japan Society for the Promotion of Science, Tokyo, Japan.

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