Table 5. $p16^{INK4a}$ and $p14^{ARF}$ alterations in myxoid/round cell liposarcoma

				$p16^{INK4a}$				$p14^{ARF}$				
Number	**Age/sex**	**Location**	**Type**	**ME**	**HD**	**Mutation**	**IHC**	**ME**	**HD**	**Exon 1-β mutation**	**IHC**	**Prognosis**
M32-RC	49/F	Thigh	M/R	NA	(+)	NA	(−)	NA	(−)	Codon 40 GGC(Gly) ≫ GGA(Gly)	(+)	58M DOD
M43-RC	77/F	Retroperitoneum	M/R	NA	(+)	NA	(−)	(−)	(−)	(−)	(−)	9M DOD
M43-MX				(−)	(−)	(−)	(−)	(+)	(−)	(−)	(−)	
M49-MX	29/F	Thigh	M	NA	(+)	NA	(+)	(−)	(−)	(−)	(+)	NA
M104-MX	54/F	Buttock	M	NA	(+)	NA	(+)	(−)	(−)	(−)	(+)	105M NED
M91-MX	47/F	Thigh	M	NA	(+)	NA	(+)	NA	(−)	Codon 57 CIAO(Gly) ≫ AAG(Lys)	(+)	18M DOD
M71-MX	66/M	Thigh	M/R	NA	(+)	NA	(+)	(−)	(−)	(−)	(+)	55M NED
M58-RC	66/F	Retroperitoneum	M/R	NA	NA	Codon 28 GTG(Val) ≫ ATG(Met)	(+)	(−)	(−)	(−)	(+)	14M AWD
M39-RC	28/M	Thigh	M/R	NA	NA	Codon 23 GGT(Gly) ≫ AGT(Ser)	(−)	(+)	(−)	(−)	(−)	22M DOD
M105-RC	29/M	Upper arm	M/R	(−)	(−)	Codon 15 TGG(Trp) ≫ CAG(Gln)	NA	NA	(−)	(−)	(+)	NA
M36-MX	44/M	Abdominal cavity	M	(−)	NA	Codon 134 GCG(Ala) ≫ ACG(Thr)*	(+)	(−)	(−)	(−)	(+)	46M DOD
M25-MX	51/M	Thigh	M	NA	NA	Codon 134 GCG(Ala) ≫ ACG(Thr)*	(+)	NA	(+)	NA	(+)	139M DOD
M50-MX	67/M	Popliteal fossa	M	NA	NA	Codon 131 CGC(Arg) ≫ CAC(His)*	(+)	NA	NA	NA	(+)	108M NED
M47-MX	40/M	Thigh	M/R	(−)	(−)	Codon 68 GCG(Ala) ≫ ACG(Thr)*	(+)	(−)	(−)	(−)	(−)	38M NED
M47-RC				(−)	(−)	Codon 68 GCG(Ala) ≫ ACG(Thr)*	(−)	(−)	(−)	(−)	(−)	
M19-MX	30/F	Thigh	M	(−)	(−)	Codon 27 GAG(Glu) ≫ GAA(Glu)	(+)	(−)	(−)	(−)	(+)	249M NED
M72-MX	35/M	Thigh	M	(−)	(−)	Codon 115 GTG(Val) ≫ GTA(Val)*	(+)	(−)	(−)	(−)	(−)	51M NED
M26-RC	31/M	Shoulder	M/R	(−)	(−)	NA	(−)	(+)	(−)	NA	(−)	130M NED
M2-RC	43/M	Lower leg	M/R	(−)	(−)	(−)	(−)	(+)	(−)	(−)	(−)	22M AWD
M53-MX	56/F	Thigh	M	(−)	(−)	(−)	(+)	(+)	(−)	(−)	(+)	98M NED
M106-RC	44/M	Thigh	M/R	(−)	(−)	(−)	(+)	(+)	(−)	(−)	(−)	103M DOD
M63-MX	28/F	Thigh	M	(−)	(−)	(−)	(+)	(+)	(−)	(−)	(+)	111M NED
M9-MX	30/M	Abdominal wall	M/R	(−)	(−)	(−)	(+)	(+)	(−)	(−)	(+)	NA
M81-RC	44/M	Knee	M/R	NA	NA	(−)	(−)	NA	(+)	NA	(−)	27M NED
M30-MX	18/M	Cheek	M	NA	NA	NA	(+)	NA	(+)	NA	(+)	48M DOD
M45-MX	63/F	Thigh	M	(−)	(−)	(−)	(+)	NA	(+)	NA	(+)	31M DOD
M8-RC	53/F	Knee	M/R	(−)	(−)	(−)	(−)	(−)	(−)	Codon 42 CCG(Pro) ≫ CTG(Leu)	(+)	4M AWD
M64-MX	54/M	Retroperitoneum	M	(−)	(−)	(−)	(+)	(−)	(−)	Codon 85 CCT(Pro) ≫ TCT(Ser)	(+)	9M DOD
M35-MX	33/F	Lower leg	M	NA	NA	(−)	(+)	NA	(−)	Codon 80 GGG(Gly) ≫ GAG(Glu)	(+)	NA
M18-RC	27/F	Retroperitoneum	M/R	NA	NA	NA	(−)	NA	NA	Codon 82 CAG(Gln) ≫ TAG(stop)	(−)	NA
M80-MX	38/M	Thigh	M/R	(−)	(−)	(−)	(+)	(−)	(−)	Codon 3 TGC(Leu) ≫ TGT(Leu)	(+)	42M DOD
M67-RC	64/F	Chest wall	M/R	NA	NA	NA	(−)	NA	NA	Codon 30 TTG(Leu) ≫ TTA(Leu)	(−)	107M NED

MX, mysoil component; RC, round cell component; M/R, tumour with more than 5% of round cells; M tumour with less than 5% of round cells; NA, data not available; DOD died of disease; NED, no evidence of diseases; AWD, alive with disease; ME, methylation; HD, homozygous deletion, IHC, immunohistochemistry. Share with $p14^{ARF}$ gene mutation (exon2).

Table 6. *p53* mutations in 64 cases of myxoid/round cell liposarcoma

Case number	Age/Sex	Location	Type	Exon	Codon	Base change	IHC	Prognosis
M35-MX	33/F	Lower leg	M/R	7	242	TGC(Cys) ≫ TAC(Tyr)	(−)	NA
M36-MX	44/M	Abdominal cavity	M	6	214	CAT(His) ≫ TAT(Tyr)	(−)	46M DOD
M39-RC	28/M	Thigh	M/R	7	225	GTT(Val) ≫ GCT(Ala)	(−)	22M DOD
					247	AAC(Asn) ≫ AGC(Ser)		
M67-RC	19/M	Paravertebra	M/R	7	238	TGT(Cys) ≫ TAT(Tyr)	(−)	16M DOD
M41-RC	44/M	Thigh	M/R	6	219	CCC(Pro) ≫ CCT(Pro)	(+)	18M NED
M30-MX	18/M	Cheek	M	6	210	AAC(Asn) ≫ AAT(Asn)	(−)	48M DOD
M44-MX	57/F	Thigh	M	5	167	CAC(Gln) ≫ TAG(stop)	(−)	144M DOD
M83-RC	44/M	Retroperitoneum	M/R	8	282	CGG(Arg) ≫ CGA(Arg)	(−)	6M DOD

MX, myxoid component; RC, round cell component; M/R, tumour with >5% round cells; M, tumour with <5% round cells; IHC, immunohistochemistry; NA, data not available; DOD, died of disease; NED, no evidence of disease.

RC components that comprised >25% of the tumour was found to be an adverse prognostic factor by both univariate and multivariate analysis.

A few studies have evaluated the prevalence of p53 nuclear immunoreactivity in MLS/RCLS, regardless of the methods used [9,11,12,34]. Smith *et al* [34] demonstrated that only 2/30 tumours (6.7%) showed positive immunoreaction for p53, and this was only in the RC areas, using the antibody DO7. In contrast, Dei Tos *et al* [12] reported that accumulation of p53 protein was observed in all of the 21 cases examined, in both the MX and RC areas, using the same antibody. No authors have directly compared the frequency of p53 expression between MX and RC components. In the current study, the frequency of p53 expression in RC components was found to be significantly higher than that in MX components, although the antibody used in our study was different from that of previously described studies (Pab 1801). Moreover, p53 expression was more frequently observed in tumours with >5% RC components than in tumours with <5%, as Antonescu *et al* [9] have reported.

As for *p53* gene alterations, only two authors have analysed such alterations in this tumour [12,35]. Dei Tos *et al* [12] reported that aberrations of the *p53* gene were observed in 28.5% of cases, whereas Pilotti *et al* [35] showed that they were present in only 2.8% of cases, all of which demonstrated aggressive histological findings. In the current study, 12.5% of the cases examined had *p53* gene point mutations. In the series of Dei Tos *et al* [12] *p53* mutation was distributed

Table 7. Correlation between immunohistochemistry and gene alteration

		p53 IHC	+	−	
p53 mutation	+(*n* = 8)		1	7	*p* = 0.7058
	−(*n* = 66)		9	57	
p53 missense	+(*n* = 4)		0	4	*p* = 0.5522
mutation	−(*n* = 70)		10	60	
		MDM2 IHC	+	−	
MDM2 amp	+(*n* = 8)		5	3	*p* < 0.0001*
	−(*n* = 51)		1	50	
		p14 IHC	Red.	Pres.	
p14 methylation	+(*n* = 8)		4	4	*p* = 0.0176*
	−(*n* = 62)		7	55	
p14 HD	+(*n* = 4)		1	3	*p* = 0.5373
	−(*n* = 66)		11	55	
p14 mutation	+(*n* = 14)		5	9	*p* = 0.0971
	−(*n* = 52)		8	44	
p14 missense	+(*n* = 10)		2	8	*p* = 0.5438
mutation	−(*n* = 56)		11	55	
		p16 IHC	Red.	Pres.	
p16 HD	+(*n* = 6)		2	4	*p* = 0.401
	−(*n* = 48)		10	38	
p16 mutation	+(*n* = 10)		2	8	*p* = 0.6364
	−(*n* = 60)		13	47	
p16 missense	+(*n* = 8)		2	6	*p* = 0.5486
mutation	−(*n* = 62)		13	49	

IHC, immunohistochemistry; HD, homozygous deletion; Red, reduced; amp, amplification; Pres, preserved. * Statistically significant.

Table 8. Prognostic factors in myxoid/round cell liposarcoma

Variable	*p* Value on survival analysis: Univariate	Multivariate
Clinicopathological		
Age (years: ≤40 vs >40)	0.0165*	0.4771
Location (extremity or trunk vs others)	<0.0001*	0.0251*
Tumour size (≤5 cm vs >5 cm)	0.125	0.6072
Depth (superficial vs deep)	NE	
Round cell components (≤5% vs >5%)	0.0485*	
Round cell components (≤25% vs >25%)	0.039*	0.0113*
Necrosis (absent vs present)	0.0474*	0.1648
Mitosis (≤5/50 HPFs vs >5/50 HPFs)	0.2678	0.2997
Histological grade (FNCLCC) (I vs 2, 3)	0.0177*	0.0318*
AJCC stage (I, II vs III, IV)	0.0369*	0.0318*
Immunohistochemical		
p53 (cut-off 10%)	0.1692	0.0477*
MDM2 (cut-off 10%)	0.5138	0.1792
MIB-1 (cut-off 8.4%)	<0.0001*	0.0005*
p14 (preserved or reduced)	0.0338*	0.4266
p16 (preserved or reduced)	0.1627	0.2592
Molecular genetic		
p53 mutation (− vs +)	0.0328*	
p53 missense mutation (− vs +)	0.0111*	0.0036*
MDM2 amplification (− vs +)	0.3468	0.1792
p14 methylation (− vs +)	0.9646	0.9781
p14 HD (− vs +)	0.3785	NE
p14 mutation (− vs +)	0.5418	
p14 missense mutation (− vs +)	0.3958	0.1739
p16 HD (− vs +)	0.4424	0.3761
p16 mutation (− vs +)	0.27	
p16 missense mutation (− vs +)	0.1813	0.4536

* Statistically significant. NE, not evaluable.

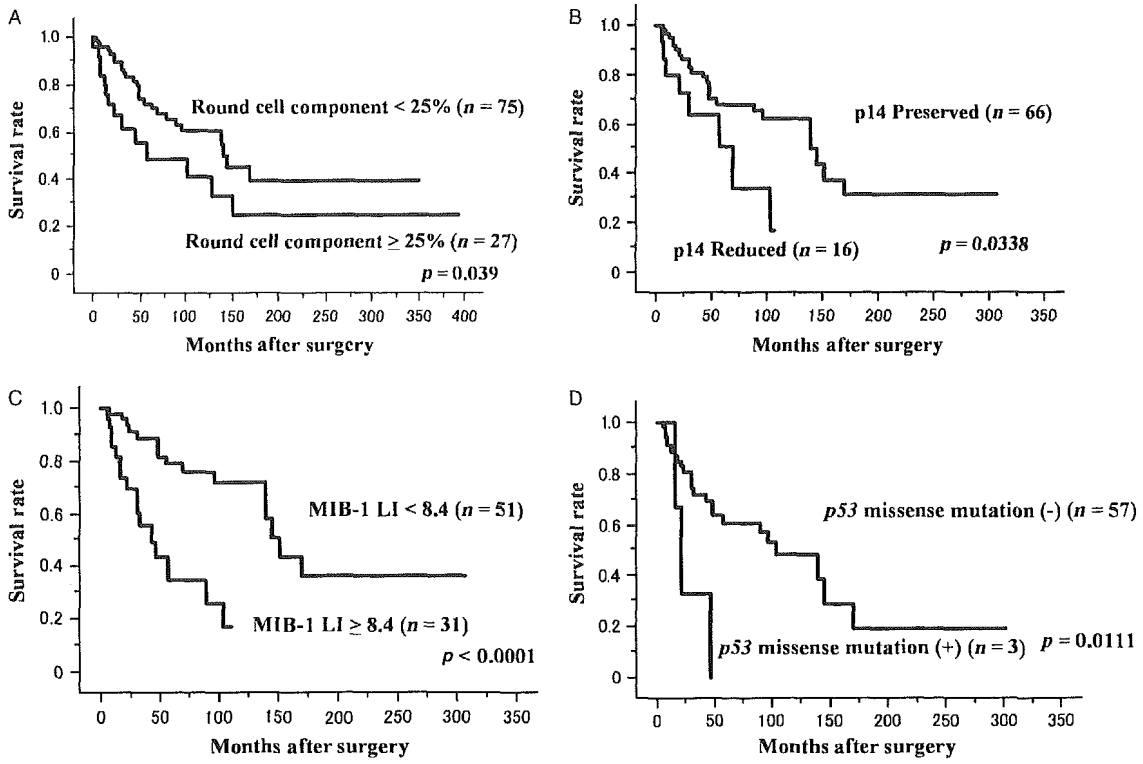

Figure 6. Kaplan–Meier curves for the patients with myxoid/round cell liposarcoma. (A) Curves for 75 patients with tumours containing <25% of round cell components and for 27 patients with tumours containing ≥ 25% round cell components (log rank $p = 0.039$). (B) Curves for 66 patients with tumours exhibiting preserved p14 expression and for 16 patients with reduced p14 expression (log rank $p = 0.0338$). (C) Curves for 51 patients with tumours exhibiting an MIB-1 LI of <8.4 and for 31 patients exhibiting an MIB-1 LI of ≥ 8.4 (log rank $p < 0.0001$). (D) Curves for three patients with tumours exhibiting *p53* gene missense mutation and 57 patients without *p53* missense mutation (log rank $p = 0.0111$)

equally in both the RC and MX components. On the other hand, in our current series, the frequency of *p53* point mutations in the RC components (21.5%) was higher than that in the MX components (6.8%), but this finding was not statistically significant. *p53* missense mutation was found to be one of the adverse prognostic factors by multivariate analysis. In sarcomas with specific translocation, such as synovial sarcoma [36], MLS/RCLS [9] and Ewing's sarcoma/PNET [37], p53 pathway alterations are a rather rare event, but when present they have been a strong prognostic factor. Our results support this phenomenon.

A few authors have reported homozygous deletion or hypermethylation of the $p16^{INK4a}$ gene and their correlation with loss of p16 protein and poor prognosis in bone and soft tissue sarcoma [19,21]. Kawaguchi *et al* [19] demonstrated that promoter hypermethylation of the $p16^{INK4a}$ gene correlated closely with decreased expression of the p16 protein and poor prognosis in soft tissue leiomyosarcoma.

In chondrosarcoma, van Beerendonk *et al* [23] showed that loss of p16 protein correlated significantly with high-grade tumours. In the current study, reduced expression of p16 was more frequent in RC components than in MX components and this reduced expression may play an important role in tumour progression in MLS/RCLS, as van Beerendonk *et al* [23] have demonstrated in chondrosarcoma. On the other hand, Olofsson *et al* [38] reported consistent immunohistochemical expression of p16 protein in MLS/RCLS. This discrepancy may be due to the use of different antibodies. They used polyclonal anti-p16 antibody, whereas we used a monoclonal antibody. Dei Tos *et al* [12] found $p16^{INK4a}$ gene mutations in 3/21 cases (14.3%) of MLS/RCLS and all the mutation cases were of MX histology. In our series, $p16^{INK4a}$ gene mutation was detected in 14.3% of the examined samples and it was observed in both MX and RC components.

Several studies have shown recently that $p14^{ARF}$ protein binds to the p53/MDM2 complex and inhibits the MDM2-mediated degradation of p53, which indicates that $p14^{ARF}$ is an upstream regulator of p53 through MDM2 [39,40]. In this study, reduced expression of p14 protein correlated significantly with p53 protein expression, which had no relationship to *p53* gene alteration, and which was therefore considered to be wild-type p53. We found that hypermethylation of the $p14^{ARF}$ gene correlated significantly with reduced p14 protein expression. In addition, promoter hypermethylation of $p14^{ARF}$ occurred independently of $p16^{INK4a}$ methylation status. Therefore, the main mechanism of inactivation of $p14^{ARF}$ may be hypermethylation of the promoter region. Furthermore, reduced expression of p14 was more frequently observed in RC components compared with MX components

and it was found to be an adverse prognostic factor by univariate analysis. In MLS/RCLS, reduced expression of p14 may have a larger contribution to make to its malignant progression, compared with reduced expression of p16. These deregulations of the p14–MDM2–p53 pathway have been reported in the progression of low-grade diffuse astrocytoma [25] or meningioma [26].

In conclusion, our results suggest that loss of p16INK4/p14ARF protein expression, especially repressed p14 protein, may be one of the important events during tumour progression in MLS/RCLS.

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Alterations of the *RB1* gene in dedifferentiated liposarcoma

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Dedifferentiated liposarcoma is a malignant adipocytic neoplasm containing a nonlipogenic sarcoma of variable histological grade that arises against the background of a pre-existing well-differentiated liposarcoma. The phenomenon of dedifferentiation is considered to be time-dependent, but the mechanism is not well known. The retinoblastoma protein, encoded by the *RB1* gene located at 13q14, is a key regulator of proliferation, development, and differentiation of certain cell types, including adipocytes. In the current study, we investigated the genetic alterations of the *RB1* gene, such as mutation (the essential promoter region and the protein-binding pocket domain; exons 20–24) and methylation of the promoter region, in addition to pRB expression and loss of heterozygosity (LOH) status, in two morphologically distinct areas (nonlipogenic dedifferentiated and well-differentiated components) in 27 patients. As a control, 11 undifferentiated high-grade pleomorphic sarcoma/pleomorphic malignant fibrous histiocytoma samples and 11 well-differentiated liposarcoma samples were also evaluated. Dedifferentiated components showed LOH (15/25; 60%) and abnormal retinoblastoma protein expression (18/27; 66.7%) more frequently than noted in the well-differentiated components (3/24; 12.5% and 9/27; 33.3%, respectively). Five and four out of the 27 dedifferentiated components harbored mutations and promoter methylation, respectively, whereas none of these alterations were seen in the well-differentiated components. These results suggest that retinoblastoma protein has a major role to play in dedifferentiation and that a 'two-hit' mechanism is involved in the altered retinoblastoma protein expression in dedifferentiated liposarcoma.
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Keywords: dedifferentiated liposarcoma; dedifferentiation; *RB1* gene

Dedifferentiated liposarcoma is defined as a neoplasm with a well-differentiated liposarcoma juxtaposed against areas of high-grade non-lipogenic sarcoma, or as a high-grade non-lipogenic sarcoma arising at precisely the same location that had previously been the primary site of well-differentiated liposarcoma (Figure 1a–c). Most of the dedifferentiated liposarcomas display extensive areas of high-grade dedifferentiation resembling malignant fibrous histiocytoma or high-grade fibrosarcoma, whereas some cases contain areas of only low-grade dedifferentiation.[1,2] The behavior of dedifferentiated liposarcoma is more aggressive than that of pure well-differentiated liposarcoma. Dedifferentiation is mostly considered a time-dependent phenomenon, and several reports have suggested an association between dedifferentiation and the altered expression of specific proteins, such as MDM2, P53, H-ras, β-catenin, and retinoblastoma protein (pRB).[3–7]

pRB negatively regulates the cellular G1/S transition of the proliferative cell cycle and is required for proper differentiation of certain cell types, including skeletal muscle and adipocytes.[8] Decreased expression of pRB in malignant mesenchymal tumors has been reported by some authors based on immunohistochemistry or Western blotting.[9–12] The retinoblastoma gene (*RB1*) at chromosome 13q14 was originally identified as the gene responsible for the development of retinoblastoma and has served as a prototype of human tumor suppressor genes. Biallelic inactivation of *RB1* is a hallmark not only of retinoblastoma, but has also been described in a variety of other tumors, including osteosarcoma

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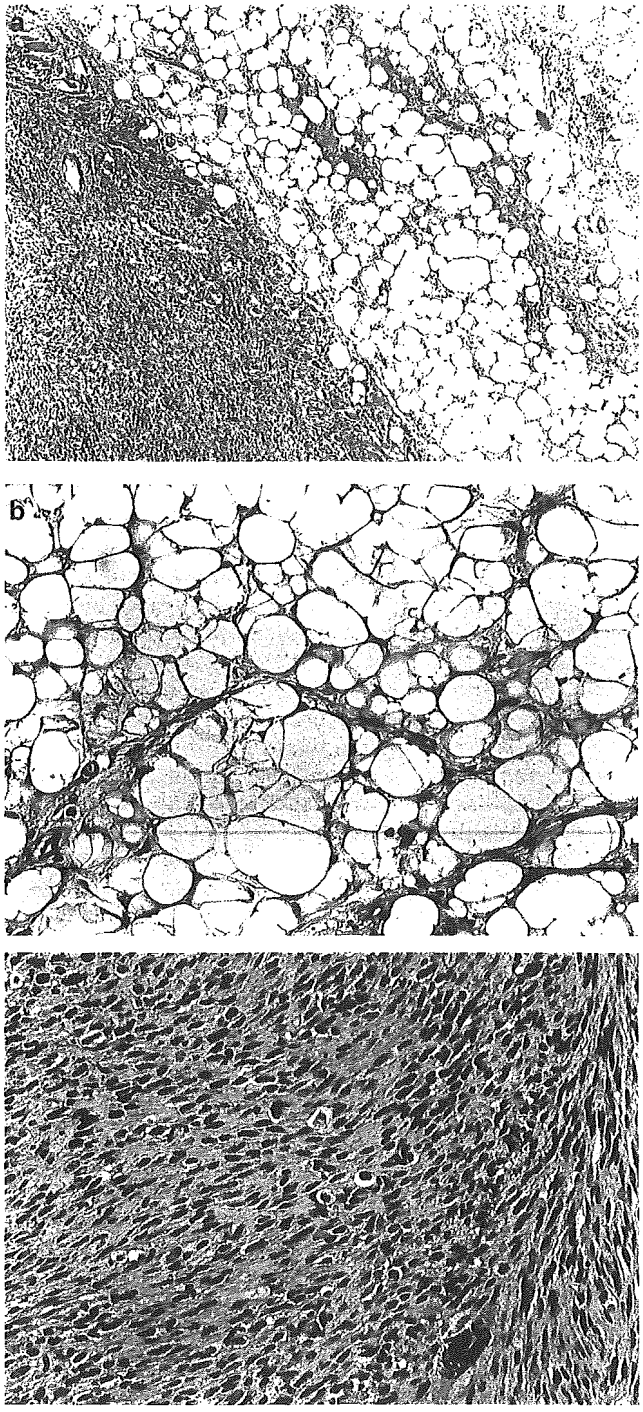

Figure 1 (**a–c**) Dedifferentiated liposarcoma. (**a**) There is an abrupt transition from the well-differentiated area to the high-grade sarcoma component (Case D25). (**b**) Well-differentiated component (Case D22). (**c**) Dedifferentiated component (Case D22).

and malignant fibrous histiocytoma.[13,14] Schneider-Stock *et al* analyzed *RB1*-loss of heterozygosity (LOH) in 11 dedifferentiated liposarcoma patients and concluded that *RB1*-LOH plays an important role in the tumor progression of well-differentiated liposarcoma to dedifferentiated liposarcoma;[7] however, whether or not there are alterations to the other allele in this tumor has not been previously ascertained. In the present study, we first performed LOH analysis using five microsatellite markers at 13q12–q14 in 27 cases of dedifferentiated liposarcoma. Then, mutation and methylation analysis screening for genetic changes of the *RB1* gene was performed, in addition to immunohistochemical analysis.

Materials and methods

Patients and DNA Extraction

In all, 27 patients with dedifferentiated liposarcoma were included in this study. As a control, 11 cases of well-differentiated liposarcoma and 11 cases of malignant fibrous histiocytoma were used. Recently, some authors have suggested that most retroperitoneal malignant fibrous histiocytomas have the possibility of actually being dedifferentiated liposarcomas.[15,16] Accordingly, we selected those cases of malignant fibrous histiocytoma where the tumors arose only in the extremities or the trunk. The diagnosis of all the malignant fibrous histiocytomas was confirmed by the panels of immunohistochemical study, since malignant fibrous histiocytoma is defined as an undifferentiated pleomorphic sarcoma which shows no distinct line of differentiation. All the cases were collected from among the soft-tissue sarcomas that had been registered in the Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Japan, between 1982 and 2004. In all, 12 cases of dedifferentiated liposarcoma had been examined in previous mutation analyses.[5,6] All the materials were fixed in 10% formaldehyde and then embedded in paraffin. Separate blocks from two morphologically distinct components of dedifferentiated liposarcoma were selected for both immunohistochemical and molecular studies. Clinicopathological data of the 27 patients with dedifferentiated liposarcoma, the 11 patients with well-differentiated liposarcoma, and the 11 patients with malignant fibrous histiocytoma are listed in Tables 1 and 2, respectively. The clinical data of these patients were obtained from their medical records.

Genomic DNA was isolated from all the cases using standard proteinase K digestion and phenol/chloroform extraction, and was used for the following molecular analysis.

LOH Analysis

Tumor and normal DNA samples were subjected to PCR using primers for the following five dinucleotide microsatellite markers at 13q12–14: D13S175, D13S153, D13S233, D13S1293, and D13S1312. D13S153 is located within intron 2 of the *RB1* gene. Primer sequences were obtained by use of the NCBI UniSTS database (http://www.ncbi.nlm.nih.gov/).

Table 1 Clinicopathologic features of 27 cases of dedifferentiated liposarcoma

Case	*Age/Sex*	*Location*	*De novo or secondary*	[a]*Well-differentiated component Histology*	*Dedifferentiated component Histology/grade*
D1	66/M	Retroperitoneum	*De novo*	Lipoma-like	[b]S-P/high
D2	56/M	Retroperitoneum	*De novo*	Sclerosing	S-P/high
D3	59/M	Retroperitoneum	*De novo*	Lipoma-like	S-P/high
D4	62/M	Retroperitoneum	*De novo*	Lipoma-like	S-P/high
D5	51/M	Retroperitoneum	*De novo*	Lipoma-like	S-P/high
D6	72/M	Retroperitoneum	*De novo*	Lipoma-like	S-P/high
D7	53/F	Retroperitoneum	*De novo*	Lipoma-like	S-P/high
D8	60/M	Retroperitoneum	*De novo*	Lipoma-like	S-P/high
D9	73/M	Retroperitoneum	Secondary	Lipoma-like	S-P/high
D10	42/F	Retroperitoneum	*De novo*	Lipoma-like	S-P/high
D11	74/M	Retroperitoneum	*De novo*	Lipoma-like	S-P/high
D12	28/F	Retroperitoneum	*De novo*	Lipoma-like	S-P/high
D13	32/F	Retroperitoneum	Secondary	Lipoma-like	S-P/high
D14	61/M	Retroperitoneum	*De novo*	Lipoma-like	[c]myxoid/high
D15	76/M	Retroperitoneum	*De novo*	Lipoma-like	S-P/high
D16	52/F	Retroperitoneum	*De novo*	Lipoma-like	S-P/high
D17	47/F	Retroperitoneum	*De novo*	Lipoma-like	S-P/high
D18	45/M	Retroperitoneum	*De novo*	Lipoma-like	low-grade
D19	52/M	Mediastinum	*De novo*	Lipoma-like	myxoid/high
D20	66/M	Mesenterium	*De novo*	Lipoma-like	S-P/high
D21	64/M	Groin	*De novo*	Lipoma-like	S-P/high
D22	70/M	Abdominal wall	Secondary	Lipoma-like	S-P/high
D23	81/M	Back	*De novo*	Lipoma-like	S-P/high
D24	82/F	Thigh	*De novo*	Lipoma-like	S-P/high
D25	55/M	Lower leg	*De novo*	Lipoma-like	[d]inflammatory/high
D26	64/M	Thigh	Secondary	Lipoma-like	S-P/high
D27	69/M	Thigh	*De novo*	Lipoma-like	fibromatosis/low

[a]Well-differentiated component includes primary well-differentiated liposarcoma in secondary dedifferentiated liposarcoma.
[b]Storiform-pleomorphic type malignant fibrous histiocytoma.
[c]Myxoid type malignant fibrous histiocytoma.
[d]Inflammatory type malignant fibrous histiocytoma.

The forward primer was end-labeled with 6-carboxyfluorescein (6-FAM) at the 5′ end. The procedure was carried out according to a method described previously.[17,18] The data which were processed using GeneScan software (Applied Biosystems) were compared between the tumor and normal DNA for each patient. Informative cases were defined as when the heterozygous alleles were identified within normal DNA. LOH was defined as when an allelic imbalance was observed (the detected allele of the tumor DNA was less than 50% of that of the corresponding normal DNA). Reproducibility was confirmed by 2–4 independent PCR amplifications for each sample.

Mutation Analysis by PCR-SSCP

Mutation analysis was performed for the essential promoter region (encompassing nucleotides −300 to −174) and for the protein-binding pocket domain (exons 20–24) of the *RB1* gene. PCR was performed in a final reaction volume of 20 μl containing 100 ng of template DNA, 1.5 mM $MgCl_2$, 1 × PCR buffer (Applied Biosystems, Foster City, CA, USA), 0.25 mM of dNTP mix, 0.5 μM each of sense and antisense primer, and 1 U of Gold Taq polymerase (Applied Biosystems). DNA sequences were amplified for 40 cycles. Primer sequences and annealing temperature are listed in Table 3. Human genomic DNA (Clontech, Palo Alto, CA, USA) was used as a positive control for each PCR and for all the subsequent reactions. SSCP was performed using a gel containing 12.5% acrylamide (GenePhor™, Amersham Pharmacia Biotech, Uppsala, Sweden) and DNA fragment analyzer (GenePhor™, Amersham Pharmacia Biotech), and then the DNA bands were visualized by a DNA Silver Staining Kit (GenePhor™, Amersham Pharmacia Biotech). To increase the quantity of mutant DNA prior to sequencing, the extra bands that seemed to be aberrantly migrating were excised from the SSCP gel and re-amplified using the same primers. The sequence data were obtained using ABI Prism 310 Collection Software, and were analyzed using Sequencing Analysis and Sequence Navigator Software.

Methylation-Specific PCR for the Promoter Region of the *RB1* Gene

Bisulfite conversion was performed with 1 μg of genomic DNA and the reagents provided with Intergen's CpGenome DNA Modification Kit (Inter-

Table 2 Clinicopathologic features and analysis results of well-differentiated liposarcoma and malignant fibrous histiocytoma

[a]Case	Age/ Sex	Location	Histology	LOH					Mutation	Methylation	[b]IHC
				D13S175	*D13S1293*	*D13S1312*	*D13S153*	*D13S233*			
W1	41/F	Retroperitoneum	Lipoma-like	n	n	–	n	+	–	–	3
W2	59/F	Retroperitoneum	Lipoma-like	–	–	–	–	n	–	–	3
W3	57/M	Groin	Lipoma-like	–	n	n	–	n	–	–	3
W4	51/M	Chest wall	Lipoma-like	–	n	n	–	n	–	–	3
W5	41/M	Buttock	Lipoma-like	–	n	–	–	n	–	–	3
W6	63/F	Thigh	Lipoma-like	–	n	–	–	–	–	–	2
W7	73/F	Thigh	Lipoma-like	n	–	–	–	n	–	–	3
W8	73/M	Thigh	Lipoma-like	n	–	n	n	–	–	–	2
W9	73/F	Thigh	Lipoma-like	n	–	n	–	n	–	–	3
W10	79/F	Thigh	Lipoma-like	n	n	n	–	n	–	–	2
W11	47/F	Thigh	Lipoma-like	–	–	n	–	n	–	–	3
M1	62/F	Groin	Storiform-pleomorphic	–	–	n	+	+	–	–	2
M2	54/M	Back	Storiform-pleomorphic	+	+	n	n	+	–	+	1
M3	52/F	Thigh	Storiform-pleomorphic	n	+	n	–	+	–	–	1
M4	75/F	Thigh	Storiform-pleomorphic	n	n	n	+	+	–	+	2
M5	52/F	Thigh	Myxoid	n	n	+	n	+	–	–	2
M6	76/F	Thigh	Storiform-pleomorphic	n	+	n	+	+	–	+	1
M7	69/F	Thigh	Storiform-pleomorphic	n	–	n	–	n	–	+	2
M8	66/F	Thigh	Storiform-pleomorphic	n	–	n	–	n	–	–	3
M9	68/F	Thigh	Storiform-pleomorphic	n	–	n	–	–	–	–	3
M10	59/M	Thigh	Storiform-pleomorphic	–	–	n	–	n	–	–	2
M11	69/F	Abdominal wall	Storiform-pleomorphic	–	–	n	–	–	–	–	2

[a]W1–11: well-differentiated liposarcoma, M1–11: malignant fibrous histiocytoma.
[b]Immunohistochemistry.

Table 3 Oligonucleotides used for PCR amplification

Primer name	*Sense 5′–3′*	*Antisense 5′–3′*	*Annealing (°C)*	*Size (bp)*
Sequence analysis				
SRB1[a]	cgccccagttccccacaga	ggcaactgagcgccgcgt	60	104
RB20	tctacttgtaattcaaaatgaac	gagaaggtgaagtgcttgat	56	230
RB21-1	attctgactacttttacatc	aagatccttgtatgctgtta	48	125
RB21-2	ccttaaattcaaaatcattg	aaatgagatcaaatgaattacc	52	138
RB22-1	agaaaagaaaatctaaaggtag	tgcatgaagaccgagttat	52	170
RB22-2	ctataactcggtcttcatgc	ttggtggacccattacatta	52	115
RB23-1	taatgtaatgggtccaccaa	tatagatgttccctccagga	58	151
RB23-2	acaagtttcctagttcacc	tcaaaataatccccctctca	53	175
RB24	gaatgatgtatttatgctca	ttcttttatacttacaatgc	48	165
Methylation-specific PCR				
RB1M	gggagtttcgcggacgtgac	ccgcccgacaactaaacg	58	78
RB1U	gggagttttgtggatgtgat	ctcccccacccaacaacta	58	83

[a]Essential promotor region.

gen, New York, NY, USA). Methylation-specific PCR was performed to determine the DNA methylation status of CpG islands of the promoter region of the *RB1* gene. Primer pairs were designed according to criteria described previously.[19] The composition of PCR mixes was the same as that of the mutation analysis described above. PCR of bisulfite-treated template DNA was carried out for 35 cycles. To ensure PCR amplification of the methylated *RB1* promoter sequence after modification, we methylated genomic DNA *in vitro* with the CpG methylase enzyme Sss-I (New England BioLabs, Beverly, MA, USA). This DNA was then subjected to sodium bisulfite modification as described above and served as a positive control. In addition, DNA of normal skeletal muscle was used as a negative control.

pRB Immunohistochemistry

Immunohistochemistry was performed using the anti-pRB mouse monoclonal antibody (clone G3–245, which recognizes *RB1* exons 9–12; PharMingen, San Diego, CA, USA; 1:1000). According to the

previously published criteria,[20] the intensity and pattern of pRB nuclear staining were used to separate the cases into one of three groups. Group 1 comprised patients whose tumors had minimal or undetectable nuclear staining (<20% of tumor cells), and these were considered to be pRB-negative. Patients whose tumors were stained in a heterogeneous pattern (20–80% of tumor cells) were classified as Group 2. The staining of Group 3 was strongly positive with a homogenous pRB nuclear immunoreactivity (>80% of tumor cells). Vascular endothelial cells in each specimen were used as an internal positive control.

Statistical Analysis

We performed Fisher's exact test to assess the correlation among various factors. A *P*-value of less than 0.05 was considered to be statistically significant.

Results

Clinical Findings

The distribution of clinicopathologic characteristics is outlined in Table 1. Patients with dedifferentiated liposarcoma ranged from 28 to 82 years of age (mean, 59.7 years), whereas those with well-differentiated liposarcoma and malignant fibrous histiocytoma ranged from 41–79 years (mean, 59.7 years) and from 52–76 years (mean, 63.8 years), respectively. Dedifferentiated liposarcoma showed a male predominance of 20 men to seven women, whereas well-differentiated liposarcoma was almost evenly distributed. In all, 18 cases of dedifferentiated liposarcoma occurred in the retroperitoneum, one case each occurred in the mediastinum and mesenterium, and the others occurred in accessible sites, including the thigh (three cases), abdominal wall (one case), back (one case), groin (one case), and lower leg (one case). Histologically, 23 cases of dedifferentiated component showed a proliferation of atypical spindle-shaped and pleomorphic cells arranged in fascicles or in a storiform pattern, mimicking storiform-pleomorphic type malignant fibrous histiocytoma. Two and one cases had the pathologic features of myxoid malignant fibrous histiocytoma and inflammatory malignant fibrous histiocytoma, respectively. One case consisted of elongated slender spindle-shaped cells of a uniform appearance surrounded by abundant collagen, resembling fibromatosis (Case D27, Figure 2). In all, 23 cases were *de novo* tumors, whereas the others showed secondary dedifferentiation.

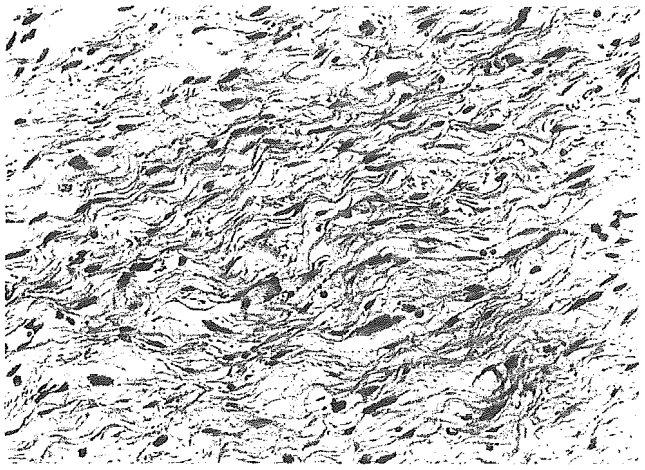

Figure 2 One case consisted of elongated slender spindle-shaped cells of uniform appearance surrounded by abundant collagen, resembling fibromatosis (Case D27).

LOH on Chromosome 13q12–14

As shown in Table 4, LOH analysis at the 13q12–14 locus was performed in 27 patients. Two cases showed noninformative findings for all five markers. LOH for one or more markers was found in 15 out of 25 (60%) cases with dedifferentiated component and in three out of 24 (12.5%) cases with well-differentiated component. Representative examples of LOH are depicted in Figure 3. The frequency of LOH in dedifferentiated components was significantly higher than that in well-differentiated components ($P=0.0009$). All the LOH-positive well-differentiated components also demonstrated LOH positivity with concomitant dedifferentiated components. Most cases with dedifferentiated component showed LOH from 13q12 to 13q14, while only one case with well-differentiated component showed LOH at two or more markers (Case D17). Among the control cases, LOH for one or more markers was found in six out of 11 malignant fibrous histiocytomas (54.5%), but in only one out of 11 well-differentiated liposarcomas (9.1%) (Table 2).

Mutation of Exons 20–24 and the Promoter Region of the *RB1* Gene

The results of mutational analysis are summarized in Table 4. SSCP analysis followed by DNA direct sequencing revealed five missense mutations in 27 dedifferentiated components (18.5%, Figure 4a–b). None of the cases with well-differentiated component showed any sequence changes, and none of the cases had mutations within the essential promoter region. In four out of the five cases with *RB1* mutation, we detected LOH at the *RB1* intragenic marker (D13S153). The remaining one case with *RB1* mutation was not informative at D13S153, although this case did show LOH for the other markers (Case D24).

Table 4 Results of molecular and immunohistochemical analysis in dedifferentiated liposarcoma

Case	*LOH*										*Mutation*		*Methylation*		*Immunohistochemistry*	
	[a]*WDC*					[b]*DDC*										
	D13S175	*D13S1293*	*D13S1312*	*D13S153*	*D13S233*	*D13S175*	*D13S1293*	*D13S1312*	*D13S153*	*D13S233*	*WDC*	*DDC*	*WDC*	*DDC*	*WDC*	*DDC*
D1	–	–	n	–	n	+	–	n	+	n	–		–	–	3	3
D2	–	–	–	–	–	–	–	–	–	–	–		–	–	3	3
D3	–	–	–	–	n	+	+	+	+	n	–		–	+	3	1
D4	[c]n	–	n	–	n	n	–	n	–	n	–		–	–	3	3
D5	n	–	+	–	–	n	+	+	+	+	–		–	–	3	1
D6	n	–	–	–	–	n	–	–	–	–	–		–	–	3	2
D7	–	–	n	+	–	+	+	n	+	+	–		–	–	3	1
D8	–	n	n	–	n	+	n	n	+	n	–	codon775 agg>aag;Arg>Lys	–	–	2	2
D9	[d]x	x	x	x	x	+	+	n	–	–	x		x	–	3	3
D10	–	n	–	–	–	+	n	+	+	+	–	codon761 atg>ata;Met>Ile	–	–	3	1
D11	–	–	–	–	n	–	–	–	–	n	–		–	–	3	3
D12	–	–	n	–	–	–	–	n	–	–	–		–	–	3	3
D13	–	–	n	–	–	–	+	n	+	–	–	codon821 aca>ata;Thr>Ile	–	–	2	2
D14	–	n	–	–	–	–	n	–	–	–	–		–	–	3	3
D15	n	–	–	–	–	n	+	+	+	+	–		–	–	3	2
D16	–	–	n	–	–	–	+	n	+	+	–		–	–	3	2
D17	n	+	+	+	+	n	+	+	+	+	–		–	–	1	1
D18	x	x	x	x	x	n	n	n	n	n	x		x	–	1	1
D19	–	–	n	–	–	+	+	n	+	+	–	codon811 agt>aat;Ser>Asn	–	–	3	2
D20	–	n	–	–	–	+	n	+	+	–	–		–	+	3	2
D21	+	–	n	–	–	+	+	n	+	+	–		–	–	3	2
D22	n	n	n	n	n	n	n	n	n	n	–		–	–	2	2
D23	n	n	n	–	n	n	n	n	–	n	–		–	+	3	3
D24	n	–	n	n	–	n	+	n	n	+	–	codon665 ctt>ttt;Leu>Phe	–	–	2	1
D25	–	–	n	–	n	–	–	n	–	n	–		–	–	3	3
D26	–	–	n	–	–	–	–	n	–	–	–		–	+	1	1
D27	–	–	–	–	–	–	–	–	–	–	–		–	+	3	1

[a]Well-differentiated component.
[b]Dedifferentiated component.
[c]Not informative
[d]Not available.

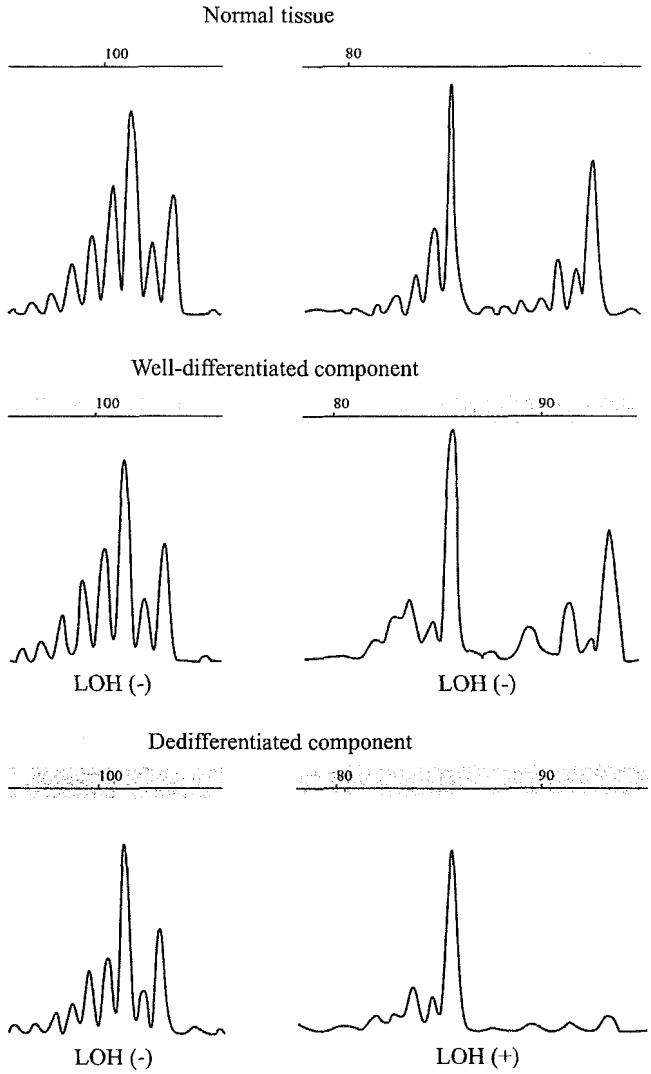

Figure 3 Representative example of the results of LOH analysis showing LOH and retention cases at D13S233 (left, Case D6; right, Case D7).

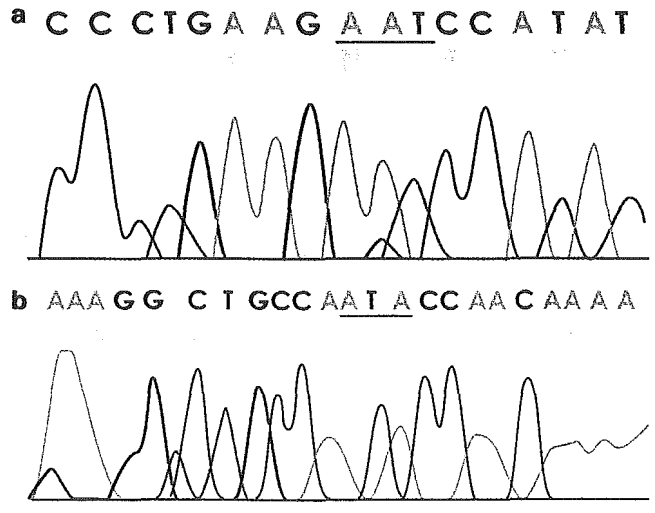

Figure 4 (**a**, **b**) Sequencing results for exon 23 of the *RB1* gene. (**a**) AAT (mutant) signals can be observed at codon 811 (Case D19). (**b**) ATA (mutant) signals can be observed at codon 821 (Case D13).

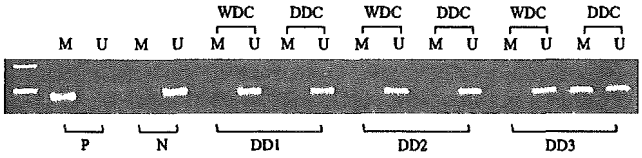

Figure 5 Methylation status of the promoter region of the *RB1* gene by methylation-specific PCR. PCR products amplified by unmethylated (U) and methylated (M) specific primers. Dedifferentiated component of Case D3 shows hypermethylation of the *RB1* gene promoter. P, positive control; N, negative control.

RB1 Promoter Hypermethylation

Methylated and unmethylated control DNA showed the expected fragment sizes of 78 and 83 bp, respectively. *RB1* promoter hypermethylation was detected in four out of 27 (14.8%) dedifferentiated components, whereas no hypermethylation was detected in any of the well-differentiated components (Figure 5). In the control cases, promoter hypermethylation was detected in four out of 11 (36.4%) malignant fibrous histiocytomas. No hypermethylation was detected in any of the well-differentiated liposarcomas.

pRB Expression

Immunohistochemical results in dedifferentiated liposarcoma are listed in Table 4. Alterations in pRB expression were observed in 18 of the dedifferentiated components (66.7%) and in nine of the well-differentiated components (33.3%) among the 27 patients, on the basis of minimal or heterogeneous staining (Groups 1 and 2, Figure 6a–b). In all, 15 LOH-positive dedifferentiated components showed significantly decreased expression (Groups 1 and 2, $P=0.009$). This correlation was not observed in well-differentiated component. All the dedifferentiated components with *RB1* mutation and four out of the five dedifferentiated components with *RB1* promoter hypermethylation showed decreased expression.

Discussion

Dedifferentiated liposarcoma is one of the subtypes of liposarcoma, which is the most common soft-tissue sarcoma in adults, and it occurs in the retroperitoneum, the abdominal cavity, and the lower extremities. The term 'dedifferentiation' is defined as the abrupt transition of a low-grade, well-differentiated sarcoma to high-grade morphology, mostly resembling malignant fibrous histiocytoma either in the primary tumor (*de novo*) or in local recurrence; this seems to occur in well-differentiated liposarcoma in about 12% of the cases.[21] Although most of the dedifferentiated liposarcomas display extensive areas of high-grade dedifferentiation, some cases have been described as having only

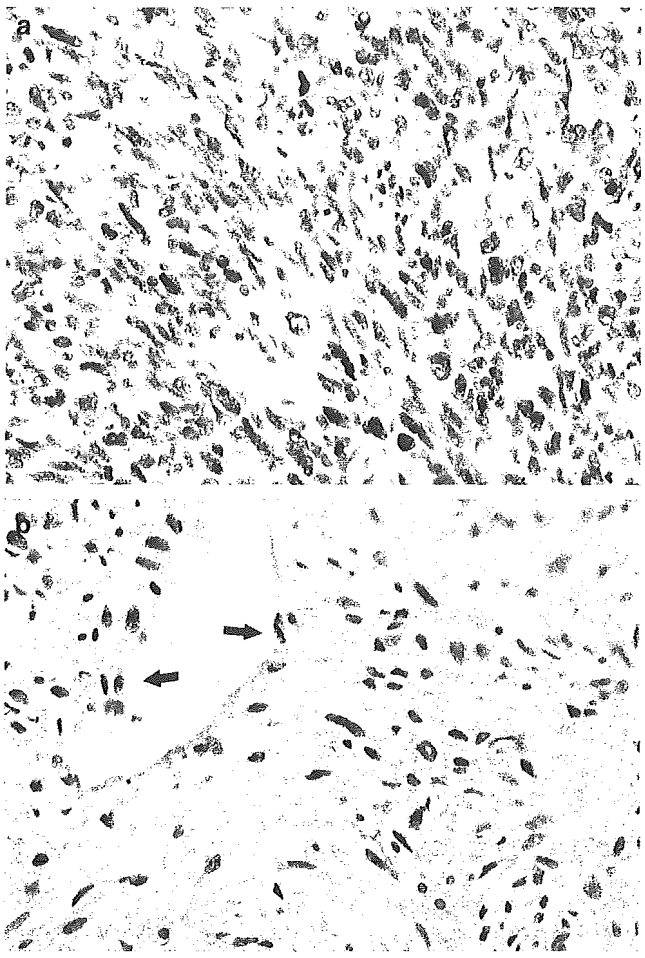

Figure 6 (**a**, **b**) Immunohistochemical staining of pRB. (**a**) Positive staining for pRB in dedifferentiated component (Group 3, Case D1). (**b**) Almost all tumor cells are negative for pRB in the dedifferentiated component (Group 1, Case D3). Note that vascular endothelial cells show positive staining (arrow).

areas of low-grade dedifferentiation resembling fibromatosis or well-differentiated fibrosarcoma.[1,2] In this study, one case showed low-grade dedifferentiation. The behavior of dedifferentiated liposarcoma is more aggressive than that of pure well-differentiated liposarcoma, with a local recurrence rate of 41–75%, a distant metastasis rate of 9–20%, and a disease-related mortality rate of 30–50%,[1,2,22] and low-grade dedifferentiation is not associated with an improved outcome.[1]

Cytogenetic, CGH, FISH, and microarray analyses have revealed supernumerary ring or giant marker chromosomes containing amplified DNA sequences in the 12q13–15 in dedifferentiated liposarcoma identical to those detected in well-differentiated liposarcoma.[23–26] It is therefore suggested that well-differentiated liposarcoma and dedifferentiated liposarcoma compromise one subgroup with a broad spectrum in morphology and biologic behavior; however, the mechanism of dedifferentiation is not well known. Chibon *et al* and Coindre *et al* have recently demonstrated that most of the malignant fibrous histiocytomas which develop in the retroperitoneum have these alterations.[15,16] Dedifferentiation is mostly considered a time-dependent phenomenon, and several reports have suggested the association between dedifferentiation and the altered expression of specific proteins. Overexpression of the proteins including MDM2 and CDK4, which are encoded by the genes located in the 12q13–15 region, is well described in liposarcoma, and dedifferentiated components exhibit a high level of MDM2-positive inmmunoreactivity in dedifferentiated liposarcomas.[3,4] Hostein *et al* investigated the amplification level of *MDM2* and *CDK4* using quantitative real-time PCR, and higher-level amplification was observed in dedifferentiated liposarcomas than in well-differentiated liposarcomas.[27] Other than these genes, Sakamoto *et al* suggested that alteration of the *β-catenin* and *H-ras* gene is involved in the dedifferentiation in dedifferentiated liposarcoma.[5,6] Schneider-Stock *et al* investigated *RB1*-LOH in two microdissected components of 11 dedifferentiated liposarcoma patients, and *RB1*-LOH was detected in all the dedifferentiated components, using four intragenic *RB1* markers and restriction fragments analysis.[7] In our present study, LOH for one or more markers out of five microsatellite markers at 13q12–14 was found in 15 out of 25 (60%) cases with dedifferentiated component and in three out of 24 (12.5%) cases with well-differentiated component. We detected LOH at D13S153, one of the five microsatellite markers located within intron 2 of the *RB1* gene, in 13 out of 24 (54%) dedifferentiated components and in two out of 23 (8.7%) well-differentiated components. LOH was more frequently found in dedifferentiated components than in well-differentiated components, and this result is consistent with that of Schneider-Stock *et al*, although the LOH rate is different (54 *vs* 100% in dedifferentiated component and 8.7 *vs* 0% in well-differentiated component). This difference may be explained by the number of cases, different primers, different analyzing procedures, or a different judging standard of LOH. Most dedifferentiated components showed a wide range of LOH from 13q12 to q14. Although it has not been ruled out that other genes in the region of 13q, such as *BRCA2*, are involved in the tumor progression of well-differentiated liposarcoma to dedifferentiated liposarcoma, in the current study we investigated the alteration of the *RB1* gene, one of the best characterized tumor suppressor genes.

The *RB1* gene, located on the long arm of chromosome 13, is one of the best-characterized tumor-suppressor genes, and its inactivation has been noted in a variety of human tumors. Biallelic inactivation of *RB1* is a hallmark not only of retinoblastoma, but has also been described in a variety of other tumors. Chibon *et al* reported that *RB1* mutations and/or homozygous deletions were found in seven out of 34 malignant fibrous histiocytomas, in addition to frequent (78%) losses of the

13q14–q21 region.[14] In the present study, we performed sequence analysis for the essential promoter region and the protein-binding pocket domain and observed *RB1* missense mutations in five out of 27 (18.5%) dedifferentiated components, whereas no mutation was detected in the corresponding well-differentiated components. All the cases with *RB1* mutation were LOH-positive and showed decreased pRB expression. The mutation rate of dedifferentiated component was almost equivalent to that of malignant fibrous histiocytoma reported by Chibon *et al*, but our control malignant fibrous histiocytoma cases showed no mutation whatsoever. This was perhaps because our malignant fibrous histiocytoma cases did not include cases which occurred in the retroperitoneum, or may simply have been because the number of our malignant fibrous histiocytoma cases was relatively small.

The *RB1* gene harbors a small (almost 600 bp) CpG island that encompasses the essential promoter region. Experimental data have shown that *in vitro* methylation of the *RB1* promoter region reduced pRB expression.[28] Unilateral retinoblastoma and some brain tumors show loss of pRB expression which is associated with aberrant methylation of the CpG island within the *RB1* promoter region;[29–31] however, the methylation status of the *RB1* gene in soft-tissue sarcoma has not been described. *RB1* promoter hypermethylation was detected in four out of 27 (14.8%) dedifferentiated components, whereas no hypermethylation was detected in any of the well-differentiated components. In contrast to the results of mutation analysis, three out of the four cases with hypermethylation had no LOH.

pRB, encoded by the *RB1* gene, is a key regulator of proliferation, development, and differentiation of certain cell types. pRB is phosphorylated and dephosphorylated synchronously with the cell cycle. The phosphorylation of pRB by the cyclin D1/CDK4(CDK6) complex in the late G1 phase results in the release of nuclear proteins and transcription factors, including the E2F family, thereby initiating the expression of genes critical for transition into the S phase of the cell cycle. It has been proved that MDM2 interacts physically and functionally with pRB and, as with p53 protein, inhibits the pRB regulatory function.[32] Using immunohistochemical methods, the present study demonstrated the expression of pRb to be abnormal in 18 dedifferentiated components and in nine well-differentiated components, results which are similar to those observed in a previous study.[7] These results suggest that pRB plays a role in dedifferentiation from well-differentiated liposarcoma to dedifferentiated liposarcoma, through the alteration of the coding gene *RB1*, or through the interaction of CDK4 or MDM2, which are considered to be expressed at a higher level in dedifferentiated component than in well-differentiated component, as described above. pRB has been shown to promote adipocyte differentiation by enhancing the DNA-binding and transactivation activity of C/EBPβ, and/or by inhibiting Ras signaling to suppress the activation of the ERK1/2 MAPKs.[33,34] These findings support our hypothesis.

In summary, we investigated the genetic alterations of the *RB1* gene, in addition to the LOH status of 13q12–q14 and pRB expression, in two morphologically distinct areas (dedifferentiated component and well-differentiated component) in 27 dedifferentiated liposarcoma patients. LOH and abnormal pRB expression were observed more frequently in the dedifferentiated component than in the well-differentiated component. Five and four out of the 27 dedifferentiated component samples harbored mutations and promoter methylation, respectively, whereas the well-differentiated components showed no such alterations. These results suggest that pRB plays a role in 'dedifferentiation', and that 'two-hit' mechanism is involved in the altered pRB expression in dedifferentiated liposarcoma.

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Involvement of P-glycoprotein and MRP1 in resistance to cyclic tetrapeptide subfamily of histone deacetylase inhibitors in the drug-resistant osteosarcoma and Ewing's sarcoma cells

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Despite recent improvements in multimodal therapies for osteosarcoma (OS) and Ewing's family of tumors (EFTs), the prognosis of relapsed cases remains very poor because of the resistance to chemotherapy. Histone deacetylase inhibitors (HDACIs), including members of the cyclic tetrapeptide family such as FK228 and apicidin, are novel antitumor agents that can induce cell cycle arrest and apoptosis in various cancer cells. HDACIs also exhibit potent antitumor effects on OS and EFTs. However, to date there have been no studies to our knowledge reporting the effects of HDACIs on drug-resistant OS and EFTs. Here, we demonstrated that FK228 and apicidin exhibited strong resistance in doxorubicin-resistant clones of OS and EFTs expressing P-glycoprotein (P-gp) and multidrug resistance-associated protein 1 (MRP1) and that P-gp and MRP1 might play a crucial role in the resistance mechanism to FK228 and apicidin. A P-gp inhibitor (verapamil) and an MRP1 inhibitor (MK571) could independently reverse the resistance to FK228 and apicidin in the drug-resistant clones. Moreover, the combination of verapamil and MK571 could enhance HDACI-induced cell number reduction in drug-resistant clones to a similar extent as that in their parental clones. Although these findings suggest the difficulty in treating drug-resistant tumors expressing P-gp and/or MRP1 with these HDACIs, the combination of P-gp and MRP1 inhibitors might reverse the resistance to the HDACIs in the treatment of those tumors. Because HDACIs are potent and promising antitumor drugs and seem to be close to clinical use, it is necessary to pay attention to the resistance mechanisms against HDACIs.

Key words: HDAC inhibitor; FK228 (Depsipeptide, FR901228); apicidin; P-gp; MRP1

Histone deacetylase inhibitors (HDACIs) are novel and promising antitumor agents. Previous studies demonstrated that HDACIs can activate transcription of specific genes via the accumulation of histone acetylation and subsequently cause a variety of phenotypic changes, including cell cycle arrest, morphologic reversion of transformed cells, differentiation and apoptosis.[1–3] A number of HDACIs exert antitumor effects on several cancers and are under clinical trials. Among them, FK228, a cyclic tetrapeptide acting as an HDACI, has been reported to show strong antitumor effects on various types of malignant tumors.[4] FK228 is under phase II clinical trials for relapsed or refractory cases of leukemia and advanced or metastatic colorectal cancer. Apicidin is a novel HDACI and another member of the cyclic tetrapeptide family with a potent broad spectrum of antiproliferative activity against various cancer cell lines,[5,6] and its structure is related to FK228.

Osteosarcoma (OS) and Ewing's family of tumors (EFTs) are the two most frequent primary malignant bone tumors in childhood and adolescence. Despite marked improvements in the multimodal therapies and outcome, systemic relapses are observed in approximately 30–40% of cases and prognosis is very poor.[7–10] It has been reported that there was no significant difference in overall survival between cases with or without second-line chemotherapy after first disease recurrence in OS.[11,12] These results strongly suggest that recurrent tumors become resistant to drugs used in chemotherapy. The major cause of multidrug resistance (MDR) is attributed to efflux pumps that reduce intracellular drug concentration. The efflux pumps are identified as ATP-binding cassette (ABC) transporters characterized with their homologous ATP-binding domains, including P-glycoprotein (Pgp), an *MDR1* gene product, and multidrug resistance-associated protein 1 (MRP1).[13,14] Because key drugs in chemotherapy for OS and EFTs, including adriamycin (Dox),[15–17] are the substrates of Pgp and MRP1,[18] drug resistance in OS and EFTs might be related to these efflux pumps.

A recent study demonstrated that FK228 exhibited potent antitumor effects on OS.[19] We have also demonstrated that HDACIs induced p21 expression in EFT cells, which was inhibited by EWS-Fli1 fusion protein, and in addition we showed that the induced p21 led EFT cells to growth arrest at the G1 phase in the cell cycle.[20–22] However, to date, there are no studies reporting the effects of HDACIs on the multidrug-resistant characteristics of OS and EFTs. In our present study, we established drug-resistant clones of OS and EFTs expressing P-gp and MRP1 (ADR clones) and investigated the effects of cyclic tetrapeptide HDACIs including FK228 and apicidin on the drug-resistant OS and EFT cells. The results demonstrated that the drug-resistant clones exhibited strong resistance to FK228 and apicidin and that the resistance was partially reversed by the P-gp inhibitor verapamil or the MRP1 inhibitor MK571, suggesting that P-gp and/or MRP1 might play a role in the resistance to FK228 and apicidin. In addition, we combined both P-gp and MRP1 inhibitors with the HDACIs and further analyzed the resistance mechanisms against FK228 and apicidin.

Material and methods

Reagents

Adriamycin/doxorubicin (Dox) and FK228 were obtained from Kyowa Hakko (Tokyo, Japan) and Fujisawa (Osaka, Japan), respectively. Apicidin, verapamil and MK571 were purchased from Calbiochem (San Diego, CA), Sigma Chemical (St. Louis, MO) and Alexis Biochemicals (Lausen, Switzerland), respectively.

Cell lines and culture conditions

Human OS cell lines MNNG/HOS and MG63, obtained from the American Type Culture Collection (Manassas, VA), were cultured in Dulbecco's modified Eagle's medium (DMEM) (Nissui

Abbreviations: ADR, adriamycin-resistant; ApiR, apicidin-resistant; Dox, doxorubicin; EFTs, Ewing's family tumors; FKR, FK228-resistant; HDAC, histone deacetylase; MDR, multidrug resistance; MRP1, multidrug resistance-associated protein 1; OS, osteosarcoma; PARP, poly-ADP-ribose polymelase; P-gp, P-glycoprotein.

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Pharmaceuticals, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) 100 μg/ml penicillin and 100 μg/ml streptomycin. Human EFTs cell lines WE-68 and VH-64, kindly provided by Dr. Frans van Valen (Westfalische-Wilhelms-University, Munster, Germany), were cultured in RPMI 1640 (Invitrogen) supplemented with 10% FBS 100 μg/ml penicillin and 100 μg/ml streptomycin. Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2 in air.

Establishment of the drug-resistant OS and EFTs clones

MNNG/ADR and MG63/ADR (originally called MNNG/HOS/DXR1000 and MG63/DXR1000, respectively) were established and characterized in our laboratory.[23] The drug-resistant OS and EFT clones were isolated after multiple steps of selection in the presence of increasing concentrations of Dox. Dox concentration was increased from 2.5 to 100 ng/ml (2.5, 5, 10, 25, 50, 100 ng/ml). The clones selected at 100 ng/ml were repurified and designated as WE-68/ADR, VH-64/ADR, respectively. ADR clones were maintained in conditioned medium with 100 ng/ml Dox.

Chemosensitivity assay

For the chemosensitivity assay, 3×10^3 cells were seeded in 96-well plates. After 24 hr incubation, various concentrations of Dox, FK228 and apicidin were added to the media, in the presence or absence of 10 μM verapamil or 50 μM MK571. After 48 hr incubation, the number of viable cells in each well was measured using the CellTiter-Glo™ Luminescent Cell Viability Assay (Promega, Madison, WI), according to the manufacturer's protocol. Before the use of ADR clones in this assay, ADR clones were cultured in culture medium without Dox for 10 days. The chemosensitivity assay was carried out in triplicate and repeated at least 3 times.

Flow cytometry

Cells treated with FK228 and apicidin were harvested and fixed with 70% ethanol for 30 min at 4°C. Then cells were centrifuged and resuspended in PBTB (PBS containing 0.1% Tween 20 and 0.05% bovine serum albumin) with 10 μg/ml RNase A and 50 μg/ml propidium iodide. Alterations in cell distribution were analyzed using an Epics-XL flow cytometer (Beckman Coulter, Fullerton, CA). Cell proportions were analyzed using the EXPO32 Software (Beckman Coulter). For each sample, 10,000 events were scored.

Western blot analysis

Western blot analyses were carried out as described[24] with several modifications. Cells were harvested and solubilized in a Nonidet P-40-based lysis buffer (20 mM Tris (pH 7.4), 250 mM NaCl, 1.0% Nonidet P-40, 1 mM EDTA, 50 mg/ml leupeptin and 1 mM phenylmethylsulfonyl fluoride). After incubation on ice for 5 min, cell lysates were clarified by centrifugation at 16,100*g* for 30 min at 4°C. The protein quantity was determined using Bradford protein assays (Bio-Rad, Hercules, CA) and fractionated on precast 4–12% gradient MOPS polyacrylamide gels (NOVEX, San Diego, CA). After transfer to nitrocellulose membranes, membranes were pretreated with TBS containing 5% dry milk and 0.05% Triton X-100 (TBST) for 1 hr at room temperature and then incubated with antibodies to acetylated histone H3 (Upstate Biotechnology, Lake Placid, NY), cleaved-PARP (Promega), actin (BD Pharmingen, San Diego, CA), C219 for P-gp and MRPr1 for MRP1 (Alexis Biochemicals) for 1 hr at room temperature. After several washes in TBST, the filters were treated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) at room temperature for 1 hr. After a final wash with TBST, immunoreactivity of the blots was detected using an enhanced chemiluminescence (ECL) detection system (Amersham, Buckinghamshire, UK).

Statistical analysis

For data on chemosensitivity assay (% cell death), results were represented as means ± SD from 3 independent experiments with triplicate wells. Data were analyzed by repeated measure ANOVA with Scheffe post-hoc test. Statistical analyses were performed using the StatView J-5.0 software (SAS Institute, Cary, NC). *p*-values < 0.01 were considered to be significant.

Results

Cross-resistance to FK228 and apicidin in ADR clones of OS and EFTs

We initially investigated whether FK228 and apicidin could reduce the viability of ADR clones as well as their parental clones. After ADR and parental clones of OS (MNNG and MG63) and EFTs (WE-68 and VH-64) were treated with medium containing various concentrations of Dox, FK228 and apicidin for 48 hr, the number of viable cells was analyzed. The chemosensitivity assay revealed that FK228 and apicidin as well as Dox did not affect the growth of ADR clones at concentrations sufficient to suppress growth of the parental clones (Fig. 1). ADR clones exhibited a relatively high level of resistance to Dox (14.1- to 74.0-fold resistance), to FK228 (309- to 610-fold resistance) and to apicidin (2.9- to 25.5-fold resistance) (Table I). These data indicate that FK228 and apicidin showed cross-resistance in ADR clones.

A number of studies have demonstrated that HDACIs cause a variety of phenotypic changes, such as cell cycle arrest and apoptosis.[1–3] Therefore, we next performed flow cytometric analysis to define cell cycle profiles of ADR and parental clones treated with FK228 or apicidin. Treatment with 6 ng/ml FK228 or 4 μM apicidin for 24 hr caused the accumulation of populations in the sub-G1 fraction in the parental VH-64 but not in VH-64/ADR (Fig. 2*a*). The percentage of sub-G1 fraction was increased from 2.3% to 29.2% by 6 ng/ml FK228 treatment and to 38.7% by 4 μM apicidin treatment in VH-64 but was not increased in VH-64/ADR (Fig. 2*b*). Similar results were also obtained when the other parental and ADR clones were treated with FK228 or apicidin (data not shown).

We further examined proteolytic cleavage of poly (ADP-ribose) polymerase (PARP) as another marker of apoptosis in ADR and parental clones. The 116 kDa PARP is specifically cleaved to produce an 85 kDa fragment during apoptosis. Western blot analysis using the antibody specific to 85 kDa fragment of the cleaved PARP demonstrated that FK228 and apicidin induced PARP cleavage dose-dependently in the parental clones. However, treatment with HDACIs exhibited faint or weak induction of the cleaved PARP in ADR clones (Fig. 2*c*). These data indicate that ADR clones might be resistant to the induction of apoptosis by FK228 and apicidin.

Reduction of the accumulation of acetylated histone H3 by FK228 and apicidin in ADR clones

Previous studies showed that inhibition of HDAC activity is closely related to the induction of apoptosis by HDACI treatment in various cancer cells, including APL and myelomonocytic leukemia.[6,25,26] Therefore, we next examined the acetylation status of histone H3 (Ac-H3) to investigate whether FK228 and apicidin show the same inhibitory effects on HDACI activities in ADR clones as in the parental cells. After treatment with FK228 and apicidin for 24 hr, whole cell lysates from cells were extracted and subjected to Western blot analysis. The results indicated that FK228 and apicidin didn't increase the level of Ac-H3 in ADR clones compared to the case in the parental clones, consistent with the results in the apoptosis experiments shown in Figure 2 (Fig. 3). These data suggest that FK228 and apicidin could not exhibit strong HDACI activities in ADR clones as they did in their parental cells.

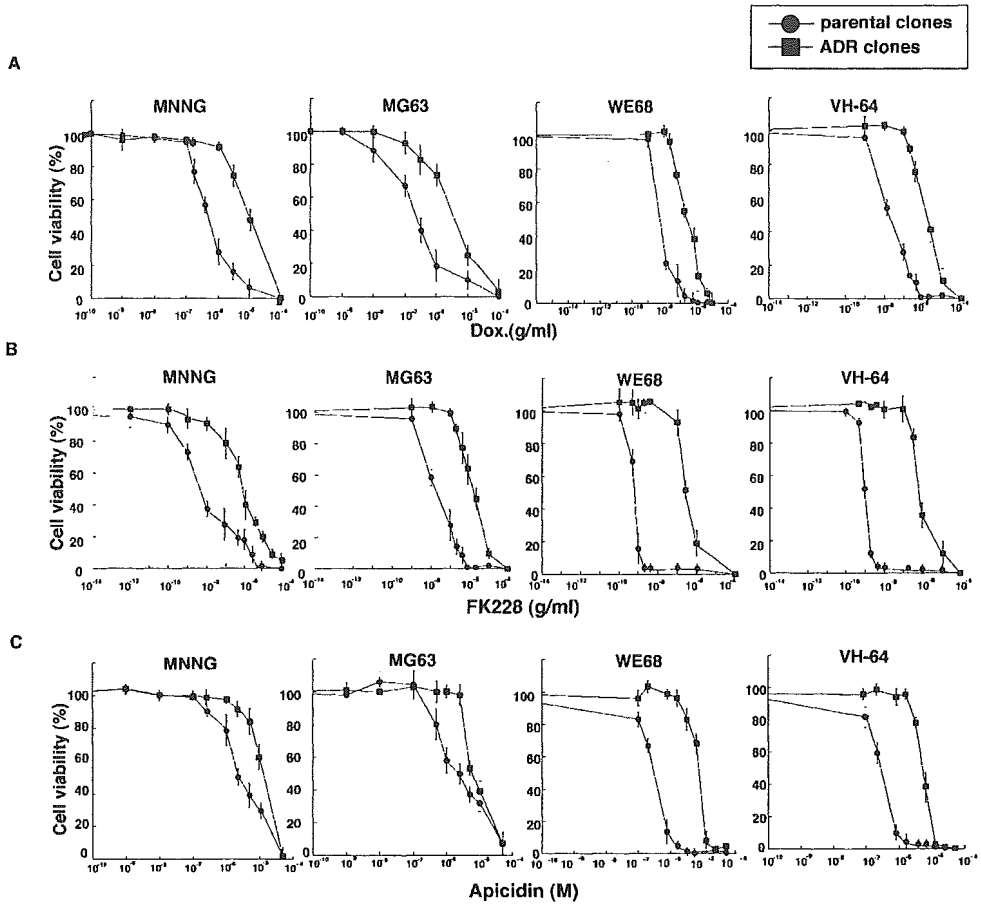

FIGURE 1 – Cross-resistance of ADR clones of OS and EFTs to FK228, apicidin and Dox. The parental clones and the ADR clones of MNNG, MG63, WE-68 and VH-64 (circles, parental clone; squares, ADR clone) were treated with various doses of the reagents [(a) Dox; (b) FK228; (c) apicidin) for 48 hr. Percentages of viable cells were determined by the CellTiter-Glo™ Luminescent Cell Viability Assay. The data represent the means of three separate experiments performed in triplicate; bars represent SD.

TABLE I – DRUG SENSITIVITY OF THE PARENTAL AND ADR CLONES TO VARIOUS ANTICANCER AGENTS

Clones	IC_{50}[1]			Fold resistance[2]		
	Dox (ng/ml)	FK228 (ng/ml)	Apicidin (μM)	Dox	FK228	Apicidin
MNNG	430	2.3	3.0			
MNNG/ADR	7,200	930	12.4	16.7	404	4.1
MG63	220	2.3	2.5			
MG63/ADR	3,100	710	7.3	14.1	309	2.9
WE-68	6.2	0.81	0.31			
WE-68/ADR	250	380	7.9	40.3	469	25.5
VH-64	19	1.0	0.29			
VH-64/ADR	1,400	610	5.2	74.0	610	17.9

[1]Drug concentration that inhibited cell survival by 50%.–[2]Fold resistance: IC_{50} of ADR clones/IC_{50} of parental clones.

Effects of verapamil, a Pgp inhibitor, on the resistance to FK228 and apicidin in ADR clones

It is well known that the major cause of multidrug resistance is attributed to efflux pumps. Therefore, we hypothesized that the reduction of HDACI activities in ADR clones would result from the reduction of intracellular concentrations of the drugs caused by the efflux pumps including P-gp, a *MDR1* gene product. Western blot analysis using P-gp antibody revealed that all tested ADR clones strongly expressed P-gp. On the other hand, the expression of P-gp was not observed in the parental clones (Fig. 4*a*). We next examined the effects of verapamil, a P-gp inhibitor, on the resistance of ADR clones to FK228 and apicidin, using the chemosensitivity assay. In MNNG/ADR, 10 μM verapamil significantly enhanced FK228-induced cell number reduction (reduction in cell number from 19.8% to 60.9%) and apicidin-induced cell number reduction (reduction in cell number from 16.9% to 45.2%). Significant enhancement of the effects of FK228 and apicidin on VH-64/ADR cells was also observed (Fig. 4*b*). Western blot analysis using anti-cleaved-PARP and Ac-H3 antibody revealed that the induction of both apoptosis and acetylation of histone H3 in ADR clones were much more increased in the presence of verapamil than in the absence of verapamil (Fig. 4*c,d*). These results indicate that resistance to FK228 and apicidin of ADR clones was reversed by verapamil, suggesting that FK228 and apicidin might be the substrates of Pgp.

Effects of MK571, an MRP1 inhibitor, on the resistance to FK228 and apicidin in ADR clones

Enhancement of the effects of FK228 and apicidin on growth inhibition and induction of acetylation in ADR clones by verapamil remained partial (Fig. 4*b*). Treatment with high doses of verapamil also failed to enhance both apoptosis and accumulation of Ac-H3 induced by FK228 and apicidin in ADR clones to the level of the enhancement in the parental clones (data not shown). Taken together, these results suggest that a mechanism other than Pgp

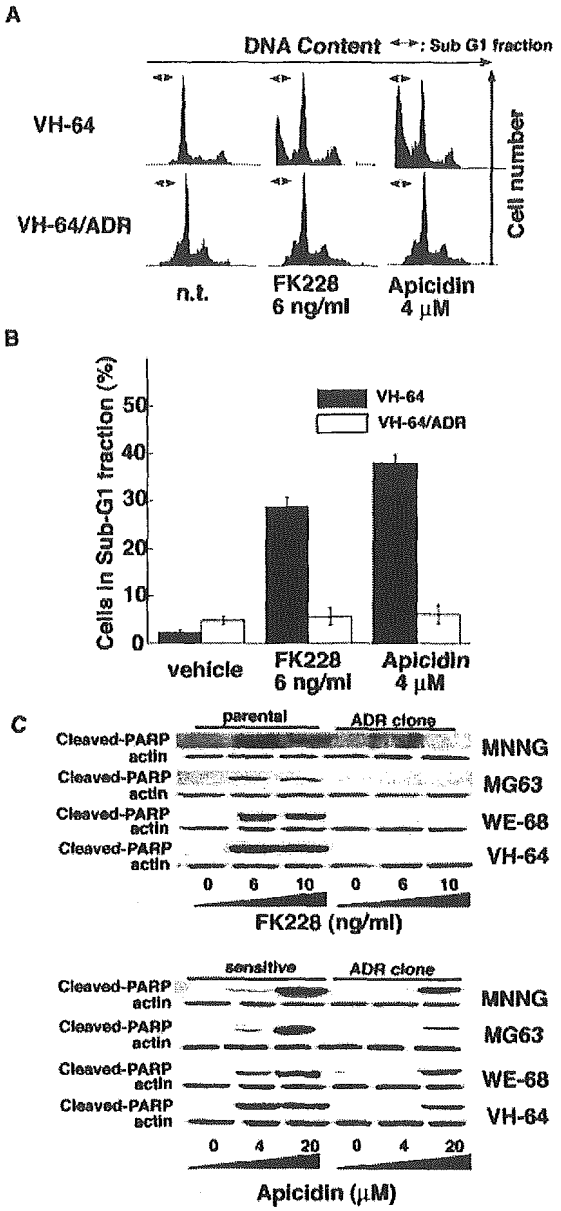

FIGURE 2 – FK228- and apicidin-induced apoptosis in the parental and ADR clones. (*a*) Cells were incubated with 6 ng/ml FK228 and 4 μM apicidin for 24 hr and fixed in 70% ethanol. After staining with PI, the apoptotic DNA content was analyzed by flow cytometry. (*b*) The percentage of sub-G1 fraction was determined by flow cytometry (black bars, parental clones; white bars, ADR clones). The data represent the means of three separate experiments performed in triplicate; bars represent SD. (*c*) After the cells were incubated with various concentrations of FK228 and apicidin for 24 hr, whole cell lysates from these cells were subjected to Western blot analysis using cleaved-PARP and actin antibody.

might play a role in the resistance to FK228 and apicidin of ADR clones.

Another transmembrane glycoprotein, MRP1, has been characterized as a drug exporter belonging to the ATP-binding superfamily.[27] The significant expression of MRP1 was also reported in OS cell lines.[23,28] We therefore examined the expression of MRP1 in OS and EFTs ADR clones. Western blot analysis showed that the expression levels of MRP1 in ADR clones were higher than those in their parental clones, whereas all parental clones except MG63 did not express MRP1 (Fig. 5*a*). To investigate the significance of MRP1 in FK228 and apicidin-induced cell number reduction in ADR clones, we applied an MRP1 inhibitor, MK571. When ADR clones were cotreated with 100 ng/ml FK228 or 3.2 μM apicidin and 50 μM MK571, FK228-induced cell number reduction (reduction in cell number from 19.8% to 57.6%) and apicidin-induced cell number reduction (reduction in cell number from 16.9% to 43.0%) were significantly increased in MNNG/ADR cells by MK571 treatment. In VH-64/ADR cells, treatment with MK571 also significantly enhanced cell number reduction induced by FK228 (reduction in cell number from 6.35% to 34.4%) and apicidin (reduction in cell number from 8.94% to 37.2%) (Fig. 5*b*).

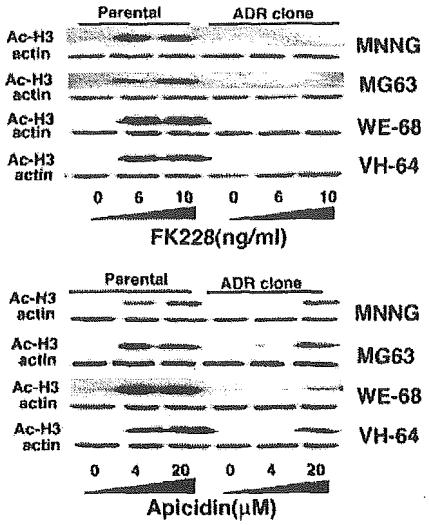

FIGURE 3 – Effect of FK228 and apicidin on acetylation of histone H3 in the parental and ADR clones. Cells were incubated with various concentrations of FK228 and apicidin for 24 hr. The whole cell lysates were isolated, and the accumulation of acetylated histone H3 was examined by Western blot analysis using antibodies against acetylated histone H3 (17 kD). The actin blot was performed as loading control.

We also examined the effects of MK571 on the level of apoptosis and the accumulation of Ac-H3 induced by FK228 and apicidin. Western blot analysis revealed that the expression levels of both cleaved-PARP and Ac-H3 in MK571-treated ADR clones were much higher than those in the cells treated with FK228 and apicidin alone (Fig. 5*c,d*). Despite the presence of MK571, FK228 and apicidin could not induce the same levels of apoptosis and the accumulation of Ac-H3 in ADR clones as those in the parental clones, *i.e.*, MK571 could partially reverse the resistance of ADR clones to FK228 and apicidin. Taken together, these results suggested that FK228 and apicidin were, at least in part, substrates of MRP1.

Effects of the combination of verapamil and MK571 on the resistance of ADR clones to FK228 and apicidin

We next examined the effects of the combination of verapamil and MK571 on drug activities of FK228 and apicidin in ADR clones. We treated P-gp(+)/MRP1(+) MNNG/ADR and VH-64/ADR clones and P-gp(−)/MRP1(−) MNNG and VH-64 parental clones with 100 ng/ml FK228 and 3.2 μM apicidin in the presence of 10 μM verapamil and/or 50 μM MK571. In both ADR clones, the chemosensitivity assay revealed that the combination of verapamil and MK571 further enhanced FK228- and apicidin-induced cell number reduction more than verapamil alone or MK571 alone. The combination enhanced the levels of FK228- and apicidin-induced cell number reduction in MNNG/ADR clones to almost the same extent of those in the parental MNNG cells (Fig. 6). The combination also enhanced FK228- and apicidin-induced cell number reduction in VH-64/ADR clones to approxi-

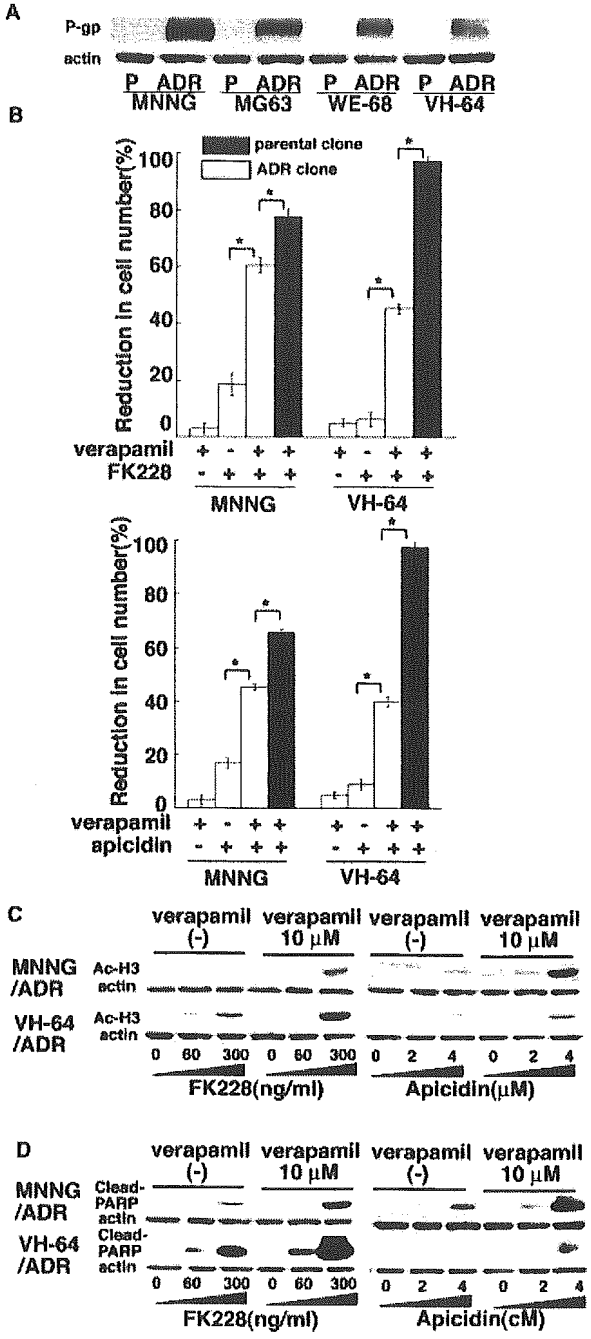

FIGURE 4.

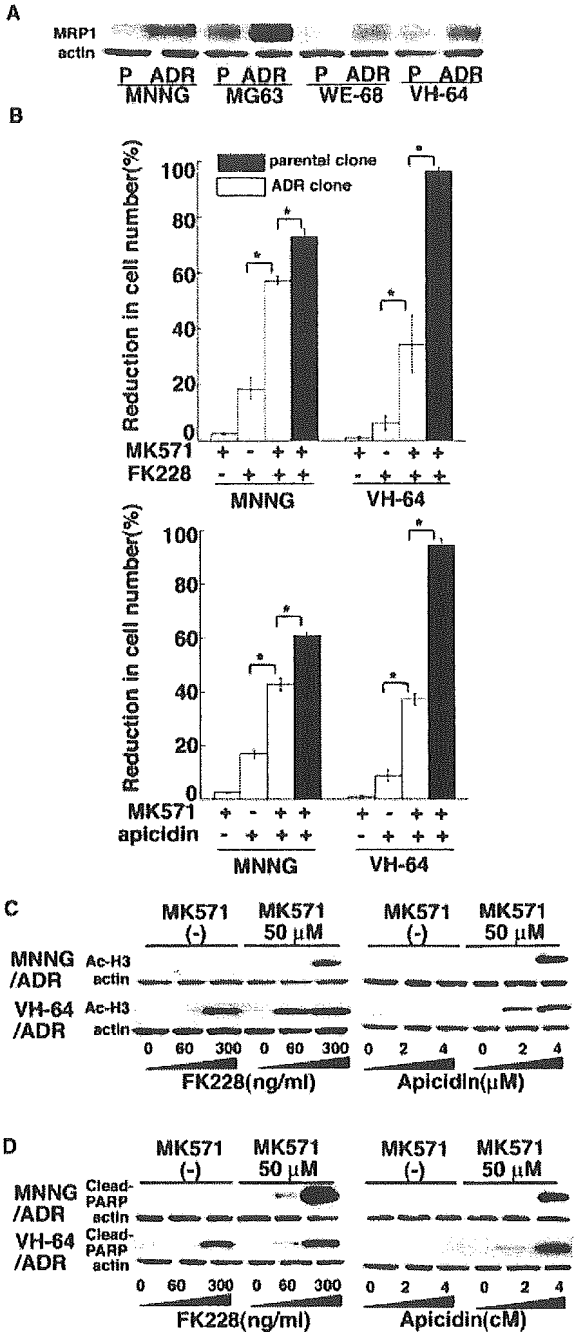

FIGURE 5.

mately 70–80% of those in parental VH-64 (Fig. 6). These results indicate that the combination of verapamil and MK571 exhibited more reversal of the resistance to FK228 and apicidin in ADR clones than verapamil alone or MK571 alone, suggesting that P-gp and MRP1 independently serve as the resistance mechanisms against FK228 and apicidin.

Discussion

In our present study, we found that FK228 and apicidin showed cross-resistance to ADR clones expressing Pgp and MRP1. We demonstrated that FK228 and apicidin exhibited strong resistance in a chemosensitivity assay and that both HDACIs could not induce apoptosis and the accumulation of Ac-H3 in ADR clones as in the parental clones. HDACIs are novel and promising antitumor agents that cause cell cycle arrest, morphologic reversion of transformed cells, differentiation and apoptosis by activation of transcription of specific genes.[1–3] HDACIs might be effective in the case of relapsed tumors, which show resistance to drugs used in first-line chemotherapy, because of their different mechanisms of action from the current antitumor drugs. In fact, several studies reported that HDACIs show good antitumor effects on drug-resistant tumors.[29,30] Since the major cause of multidrug resistance is attributed to ABC transporters, such as Pgp and MRP1, we constructed doxorubicin-resistant (ADR) clones of OS and EFTs expressing P-gp and MRP1 and examined the effects of FK228 and apicidin on the clones. Our results clearly showed that FK228 and apicidin showed strong cross-resistance in ADR clones expressing P-gp and MRP1.