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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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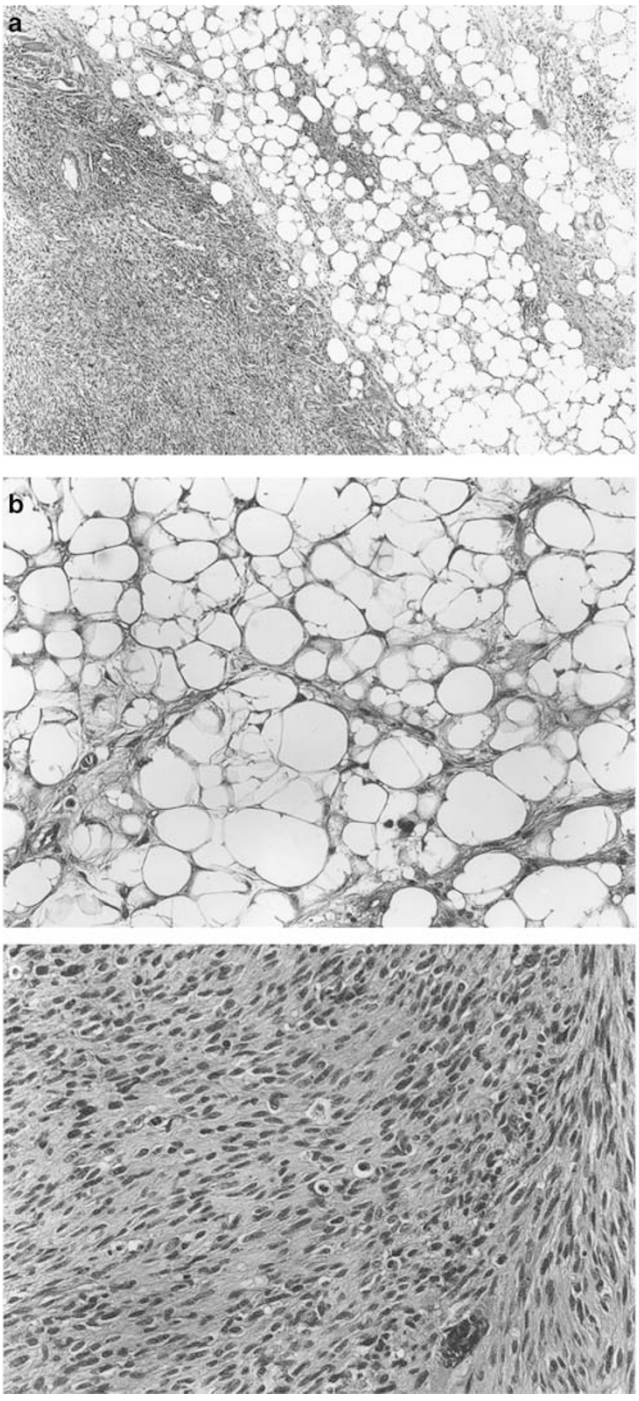

Figure 1 (**a–c**) Dedifferentiated liposarcoma. (**a**) There is an abrupt transition from the well-differentiated area to the high-grade sarcoma component (Case D25). (**b**) Well-differentiated component (Case D22). (**c**) Dedifferentiated component (Case D22).

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.

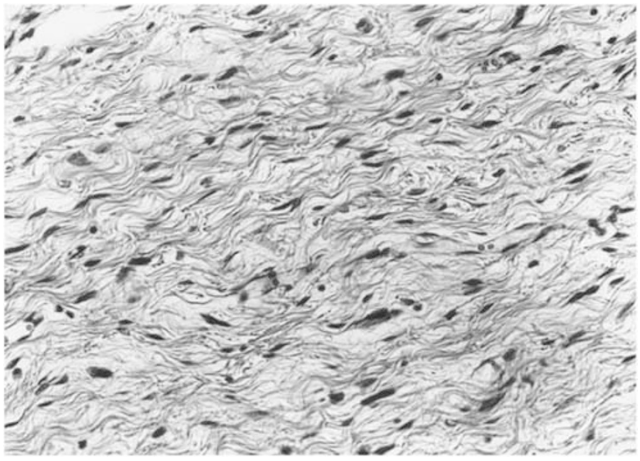

Figure 2 One case consisted of elongated slender spindle-shaped cells of uniform appearance surrounded by abundant collagen, resembling fibromatosis (Case D27).

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.

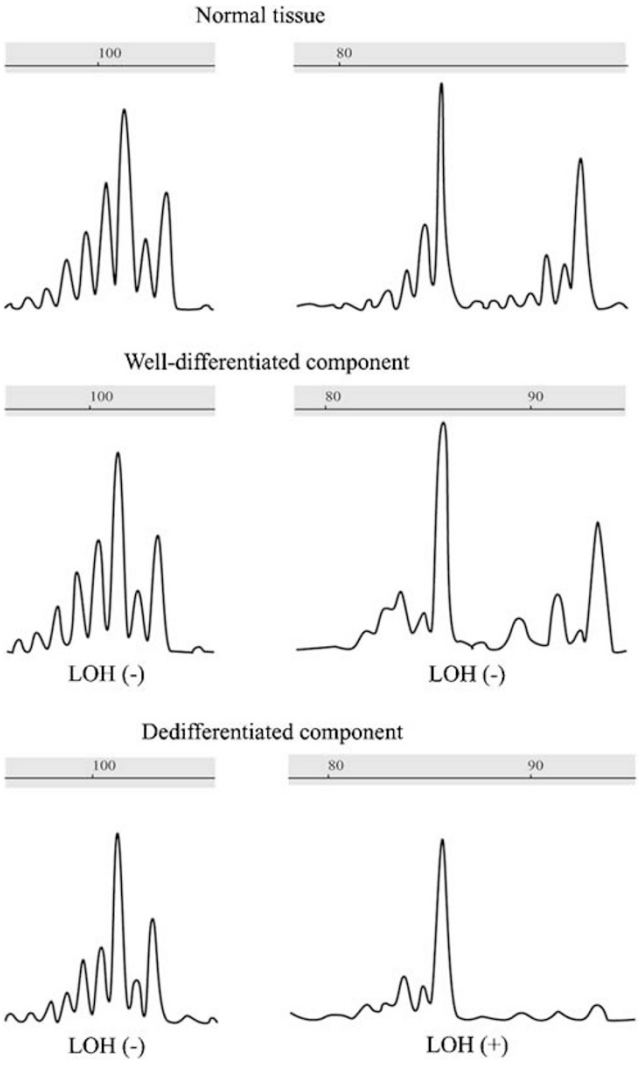

Figure 3 Representative example of the results of LOH analysis showing LOH and retention cases at D13S233 (left, Case D6; right, Case D7).

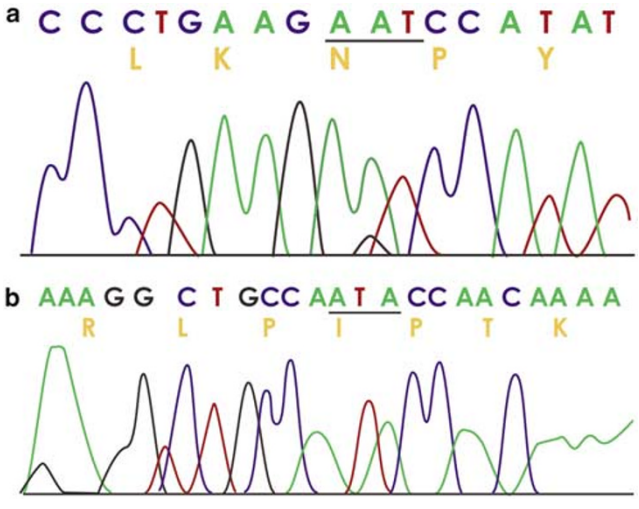

Figure 4 (**a**, **b**) Sequencing results for exon 23 of the *RB1* gene. (**a**) AAT (mutant) signals can be observed at codon 811 (Case D19). (**b**) ATA (mutant) signals can be observed at codon 821 (Case D13).

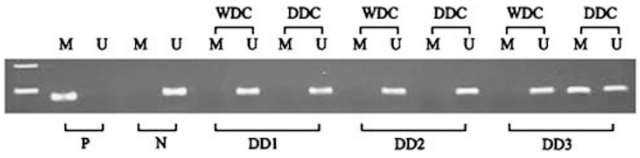

Figure 5 Methylation status of the promoter region of the *RB1* gene by methylation-specific PCR. PCR products amplified by unmethylated (U) and methylated (M) specific primers. Dedifferentiated component of Case D3 shows hypermethylation of the *RB1* gene promoter. P, positive control; N, negative control.

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

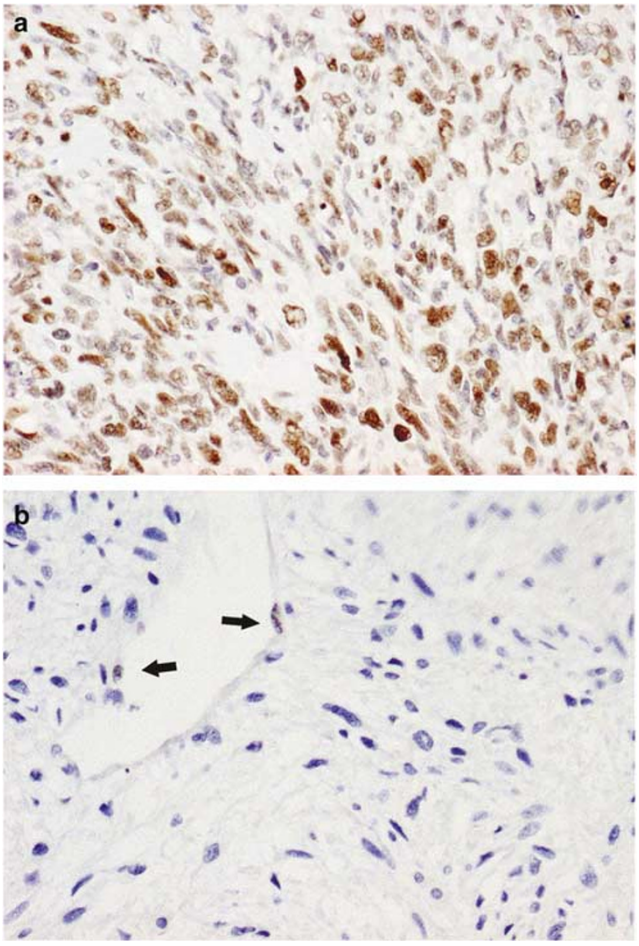

Figure 6 (**a**, **b**) Immunohistochemical staining of pRB. (**a**) Positive staining for pRB in dedifferentiated component (Group 3, Case D1). (**b**) Almost all tumor cells are negative for pRB in the dedifferentiated component (Group 1, Case D3). Note that vascular endothelial cells show positive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