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High level of *CDK4* amplification is a poor prognostic factor in well-differentiated and dedifferentiated liposarcoma

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Summary. The amplification of *MDM2* and *CDK4* is the main molecular feature of well-differentiated liposarcomas (WDLS) and dedifferentiated liposarcomas (DDLS). Although the diagnostic usefulness of this molecular characteristic in liposarcomas has been investigated, its prognostic utility of quantitative gene level has not been explored. The aim of this study was to assess the prognostic significance of level of *CDK4* amplification in *MDM2*-amplified WDLS/DDLS. *MDM2* amplification in liposarcomas was confirmed by fluorescence *in situ* hybridization. The copy number of *MDM2* and *CDK4* was further determined by quantitative PCR (qPCR) and multiplex ligation-dependent probe amplification. Among 56 *MDM2*-amplified liposarcomas, 30 cases were assigned as WDLS, and 26 as DDLS. When liposarcomas were classified by qPCR-determined *CDK4* amplification levels, the high-*CDK4* group showed significantly poorer progression free survival (P=0.001) and disease specific survival (P=0.033) than the low-*CDK4* group. However, *MDM2* amplification level did not show prognostic significance. In WDLS/DDLS, the level of *CDK4* amplification was useful for prognosis prediction and precise stratification of patients for targeted therapy.

Key words: CDK4, Amplification, Liposarcoma, MDM2

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Introduction

Well-differentiated liposarcoma (WDLS) and dedifferentiated liposarcoma (DDLS), the most common subtypes of liposarcoma, are characterized by giant or ring chromosomes with amplification of the 12q13-15 region (Nilbert et al., 1995; Pedeutour et al., 1999; Fletcher et al., 2002). This amplified region includes the *MDM2* and *CDK4* oncogenes (Pedeutour et al., 1994; Dei Tos et al., 2000). MDM2 is known to promote oncogenesis by inactivating the tumor suppressor p53 (Momand et al., 1992). In addition, CDK4 also plays a role in tumorigenesis by regulating cell cycle

progression through the phosphorylation of the RB1 protein (Kato et al., 1993; Lee and Sicinski, 2006). Recently, knockdown of CDK4 and MDM2 was shown to inhibit proliferation of liposarcoma cell lines *in vitro* (Barretina et al., 2010). Therefore, the amplification and overexpression of MDM2 and CDK4 might play a crucial role in liposarcomagenesis.

Although gene amplification of *MDM2* or *CDK4* has been reported in several types of tumors, including malignant fibrous histiocytomas (MFHs), leiomyosarcomas, fibrosarcomas, chondrosarcomas, osteosarcomas, and malignant peripheral nerve sheath tumors (MPNSTs) (Nakayama et al., 1995; Kanoe et al., 1998; Bartel et al., 2001), their amplification levels in WDLS/DDLS are significantly higher compared with other tumors (Shimada et al., 2006). This molecular feature enabled us to identify WDLS/DDLS among other histologically similar tumors. Furthermore, a strong correlation between the expression of MDM2 and CDK4 by immunohistochemistry and their amplification status has been demonstrated, and immunostaining results of MDM2 and CDK4 were shown to be useful in distinguishing WDLS from other differentiated adipose tumors and DDLS from poorly differentiated sarcomas, suggesting that immunohistochemical detection of MDM2 and CDK4 overexpression may be a valuable diagnostic adjunct (Binh et al., 2005). Even though the values of *MDM2* and *CDK4* amplification and overexpression in the diagnosis of WDLS/DDLS have been evaluated (Thway et al., 2012), their prognostic utility has not been fully investigated. Italiano et al. (2009) have reported the clinical and biological significance of *CDK4* amplification in WDLS/DDLS. In their study, they compared the clinical characteristics of WDLS/DDLS with amplification of both *MDM2* and *CDK4* (*MDM2*$^+$/*CDK4*$^+$) with *MDM2* amplification and no *CDK4* amplification (*MDM2*$^+$/*CDK4*$^-$). However, there has been no study about the prognostic stratification between high and low *CDK4* amplification level in the *MDM2*+/*CDK4*$^+$ WDLS/DDLS. Therefore, one aim of this study was to assess the prognostic significance of *MDM2* and *CDK4* amplification levels in patients with WDLS/DDLS, as well as the correlation between this molecular feature and histological grade.

DDLS is usually defined as a high-grade tumor, in which dedifferentiated areas resemble MFH, fibrosarcoma, or high-grade myxofibrosarcoma in morphology (Coindre et al., 2010). However, tumors with low-grade dedifferentiation have been identified in DDLS and contain areas resembling fibromatosis or well-differentiated fibrosarcoma (Henricks et al., 1997). Furthermore, other distinctive histologic patterns, including meningothelial-like whorls, have been reported to be associated with DDLS (Fanburg-Smith and Miettinen, 1998; Kim et al., 2003; Thway et al., 2011). However, neither histologic grade nor the extent of dedifferentiation is known to be associated with the prognosis of DDLS patients (Henricks et al., 1997). Therefore, histologic grading of DDLS has not been emphasized even though a recent study in retroperitoneal liposarcoma patients demonstrated the prognostic value of histologic grade in DDLS (Mussi et al., 2008). In the present study, we also aimed to classify DDLS by histologic grade and to determine whether the grade of dedifferentiated areas affects patient outcome.

Materials and methods

Patient selection and histologic evaluation

Seventy cases of surgically resected WDLS and DDLS were retrospectively collected from Samsung Medical Center (Seoul, Korea) between 1995 and 2005. Informed consent and ethics approval were obtained for research purposes. All tumor specimens were fixed in buffered formalin and embedded in paraffin blocks. All available hematoxylin and eosin (H&E)-stained slides were reviewed by two pathologists (YLC and SEL) in a blinded manner. After review of H&E slides and analysis of *MDM2* amplification by fluorescence *in situ* hybridization (FISH), three cases were excluded due to revised diagnosis and 11 cases as unavailability of paraffin blocks or clinical information. Of the 56 liposarcomas that showed *MDM2* amplification, 30 cases were WDLS and 26 were DDLS. The mean number of slides reviewed per case was 8.3 (range: 4–28 slides).

For DDLS, dedifferentiation was defined histologically as a region devoid of lipogenic differentiation occupying at least 10% of the tumor or two continuous low-power (x4 objective) microscopic fields (Henricks et al., 1997). Histologic grade was defined on the basis of cellularity, nuclear pleomorphism, mitosis, or necrosis. Dedifferentiated areas were classified into low-grade when the cellularity was similar to that of fibromatosis or low-grade fibrosarcoma, and low mitotic activity and atypia were observed without tumor necrosis. Dedifferentiated areas which displayed moderate-to-marked cellularity and pleomorphism, and appeared similar in morphology to MFH or pleomorphic sarcoma were designated as high-grade. Grade was assigned based on the highest grade present in reviewed slides. After all cases were graded, the pathologists met at a multiheaded microscope and reached consensus on one case for which there had not been agreement in the independent review. We included cases in which there was no adipocytic differentiation but in which the *MDM2* was highly amplified (more than 50 copies per cell) and could thus be considered as DDLS (Shimada et al., 2006). Myxoid/round cell liposarcoma and pleomorphic liposarcoma (a pleomorphic sarcoma characterized by numerous atypical lipoblasts admixed with pleomorphic areas) were excluded from this study.

Fluorescence in situ hybridization

FISH was performed on interphase nuclei of

formalin-fixed, paraffin-embedded (FFPE) tissue sections. Unstained 4 μm sections were placed on electrostatically charged slides (SuperFrost; Fisher Scientific, Hampton, NH, USA) and then hybridized with a *MDM2* (12q15) dual-color probe (Vysis, Downer's Grove, IL, USA) according to the manufacturer's instructions. Hybridized slides were reviewed on an Olympus IX-50 microscope (Olympus, Tokyo, Japan) at x100 magnification with oil immersion using a DAPI/green/red triple band-pass filter set. A minimum of 100 tumor nuclei per case were scored for evaluation. Amplification of *MDM2* was defined as a *MDM2*/CEP12 ratio ≥2.2 in 100 tumor cells (Cho et al., 2012).

Quantitative PCR analysis

FFPE tissues (50-100 mg) were scraped off blocks, deparaffinized, rinsed with absolute ethanol, and washed with TNE buffer (10 mM Tris, 100 mM NaCl, 1 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0). Tissues were incubated overnight at 55°C with proteinase K. DNA was purified using a Wizard DNA Clean-up system (Promega, Madison, WI, USA), rinsed with TE (10 mM Tris, 1 mM EDTA, pH 8.0) using minicolumns (Ultrafree-MC30; Millipore SA, Bedford, MA, USA), and then resuspended in 100 ml TE. PCR amplification was performed in duplicate in a 50 μl reaction mixture containing 300 nM of each primer, 200 nM of probe, and 200 nM of equal concentrations of dATP, dCTP, dGTP, dTTP in real-time PCR buffer. PCR was performed in an ABI Prism 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) (50°C for 2 min, then 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min). *CDK4* and *MDM2* gene copy numbers were calculated from a standard curve constructed from normal DNA amplification. The albumin gene (ALB) was used as the reference. The level of *MDM2* or *CDK4* amplification was calculated as the copy number of the target gene (*MDM2* or *CDK4*) /copy number of the reference gene (*ALB*). The cut-off value for the presence of amplification was 2.2 for *MDM2* and *CDK4*. We plotted all the levels of *CDK4* copy number, and 10.0 was found to be the best cutoff value for distinguishing high- and low-grade tumors. *MDM2* and *CDK4* were divided into two groups as follows: *MDM2*- and *CDK4*-high (≥10.0) and *MDM2*- and *CDK4*-low (<10.0).

Multiplex ligation-dependent probe amplification analysis

Multiplex ligation-dependent probe amplification (MLPA) analysis was performed according to the manufacturer's protocol using the P175-A1 Tumor-Gain MLPA Kit (MRC-Holland, Amsterdam, The Netherlands). This kit contains probes for each of 24 different genes that are frequently amplified in tumor cells including *MDM2*/*CDK4, MYCN*/*ALK, ERBB2*/*TOP2A, MYC, CCND1, CCND2, EGFR, FGFR1, RET,* and *MET*. Target DNA (200 ng) in 5 μl of 10 mM Tris (pH 8) and 0.1 mM EDTA was denatured for 5 min at 98°C and incubated with 3 μl of MLPA probes for 16 h at 60°C. The probes were ligated to the DNA and amplified by PCR. PCR products were then separated by capillary electrophoresis on an ABI model 3130 capillary sequencer (Applied Biosystems) using GeneScan-ROX 500 size standards (Applied Biosystems). The results were analyzed using GeneMapper software and MRC Coffalyzer MLPA software (MRC-Holland). Gene amplification or deletion were calculated and compared with control DNA from normal tissues. After normalization, each probe set was scored as follows: 0.01–1.00, deleted; 1.01–2.99, normal copy number; 3.00–3.99, copy number gain; ≥4.00, amplification. *MDM2* and *CDK4* scores were then used to create two groups depending on copy number: *MDM2*- and *CDK4*- high (≥7.0) and *MDM2*- and *CDK4*-low (<7.0).

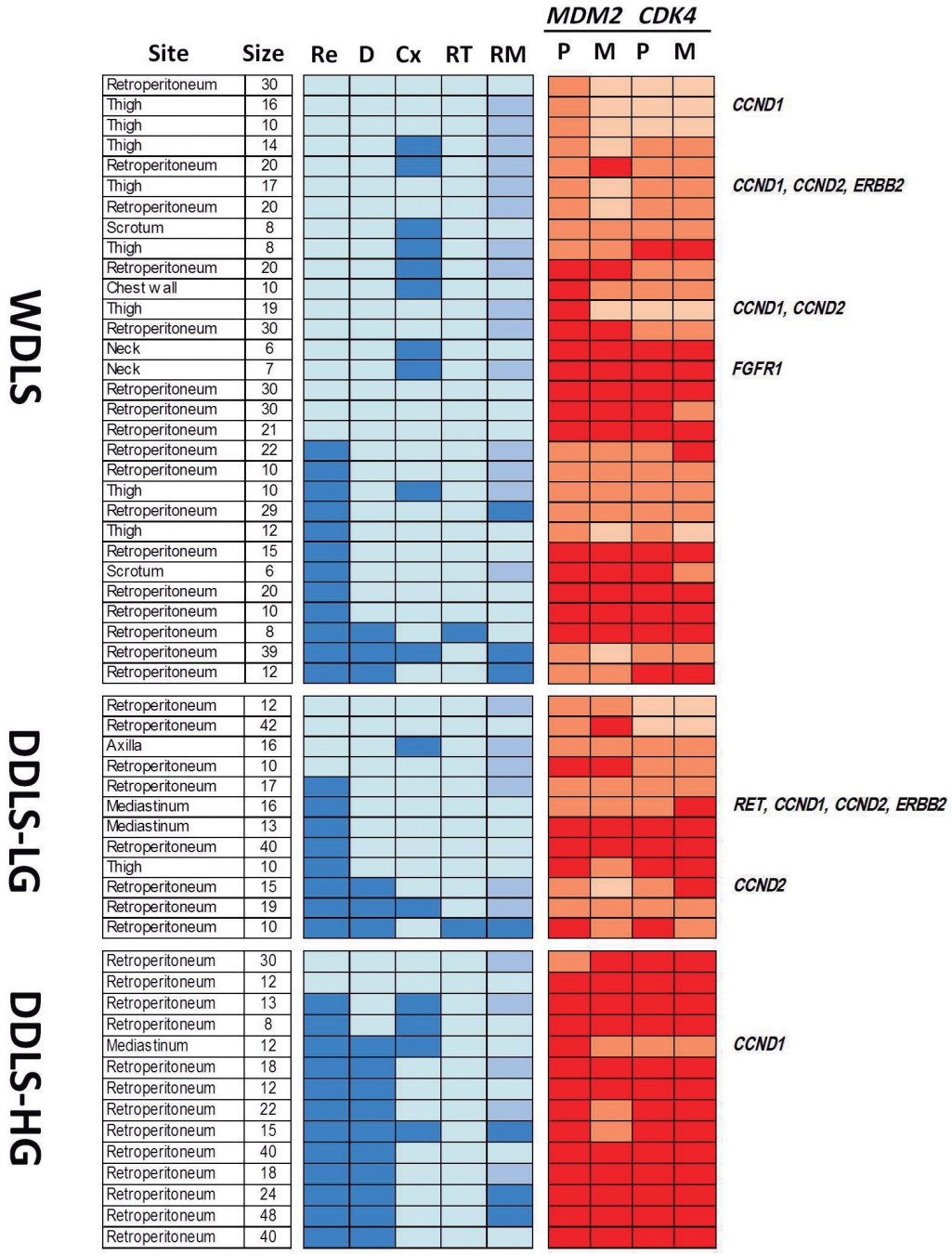
Statistical analysis

Clinical variables and follow-up information were obtained by chart review. Differences between groups were statistically analyzed using the Chi-square test or Mann-Whitney U test. Progression-free survival (PFS) and disease-specific survival (DSS) were estimated by the Kaplan-Meier method. PFS was defined as the time from the data of surgery until disease progression, death from liposarcoma or last patient contact. *DSS* was defined as the time from the data of surgery until death attributed only to the liposarcoma. Multivariate and univariate analyses were performed using Cox proportional hazard regression models (95% confidence interval [CI]). All statistical analyses were performed using SPSS 18.0 (Chicago, IL, USA) and R program (R.2.12.1). P-values were two-sided with a significance level of 0.05.

Results

Patient characteristics and histologic grading of WDLS/DDLS

Clinicopathologic data for the 56 patients are provided in Table 1 and Fig. 1. Patient age ranged from 27 to 76 years (mean: 52.6 years, median: 56.0 years). The male-to-female ratio was 1.2:1. The most frequent tumor location was the retroperitoneum (38 cases), followed by the thigh (9 cases), mediastinum (3 cases), neck (2 cases), scrotum (2 cases), axilla (1 case), and chest wall (1 case). Tumor size ranged from 3.0 to 48.0 cm (median: 35.0 cm). The follow-up period ranged from 2.0 to 175 months (median: 43 months). The recurrence rate was 57.1%, and the death rate was 28.6%. The *MDM2* amplification level was 2.05 to 59.0 (median: 26.68) by qPCR and 0.72 to 20.8 (median: 5.34) by MLPA. The *CDK4* amplification level was 1.32 to 44.8 (median, 7.75) by qPCR and 0.87 to 18.9



Re	Recur	No	Yes	
D	Death	No	Yes	
Cx	Chemotherapy	No	Yes	
RT	Radiotherapy	No	Yes	
RM	Resection margin	Negative	Microsroic	Macroscopic
P	qPCR	Negative	Low	High
M	MLPA	Negative	Low	High

Fig. 1. Summary of clinicopathologic variables and levels of *MDM2* and *CDK4* amplification by qPCR and MLPA in 56 cases of WDLS/DDLS. P and M represent the amplification levels of *MDM2* or *CDK4* by qPCR and MLPA, respectively. In addition to *MDM2* and *CDK4* amplification, other amplified genes quantified by the MLPA method are also listed.

Table 1. Clinical and molecular characteristics of 56 patients with WDLS/DDLS according to histologic grade.

	Total (n=56)	WDLS (n=30)	DDLS (n=26)		P-value
			DDLS-LG (n=12)	DDLS-HG (n=14)	
Age [years] (Median, range)	56 (27-76)	56 (30-76)	57 (27-72)	63 (34-75)	0.517
M:F	30:26	12:18	7:5	11:3	0.054
Size [cm] (Median, range)	35.0 (6.0-48.0)	21.0 (3.0-39.0)	11.0 (10.0-42.0)	35.0 (8.0-48.0)	0.211
F/U [months] (Median, range)	43 (2-175)	83 (2-155)	78 (7-175)	28 (3-84)	0.059
Progression (%)	32 (57.1%)	12 (40.0%)	8 (66.7%)	12 (85.7%)	**0.013**
Death (%)	16 (28.6%)	3 (10.0%)	3 (25.0%)	10 (71.4%)	**<0.001**
Resection margin					0.553
Negative+Micro	49 (87.5%)	27 (90.0%)	11 (91.7%)	11 (78.6%)	
Macro	7 (12.5%)	3 (10.0%)	1 (8.3%)	3 (21.4%)	
Site					**0.042**
Periphery	18 (32.1%)	13 (43.3%)	4 (33.3%)	1 (7.1%)	
Peritoneum	38 (67.9%)	17 (56.7%)	8 (66.7%)	13 (92.9%)	
Gene Amplification					
MDM2 (qPCR) (Median, range)	26.68 (2.05-59.0)	6.45 (2.05-42.7)	11.10 (3.82-25.9)	29.26 (8.64-59.0)	**<0.001**
MDM2 (MLPA) (Median, range)	5.34 (0.72-20.8)	4.07 (1.08-17.7)	5.74 (0.72-18.4)	8.03 (4.73-20.8)	**0.033**
CDK4 (qPCR) (Median, range)	7.75 (1.32-44.8)	5.95 (1.32-44.8)	8.60 (2.10-21.6)	25.66 (5.01-41.1)	**<0.001**
CDK4 (MLPA) (Median, range)	5.33 (0.87-18.9)	4.79 (0.87-15.8)	5.13 (1.02-18.7)	13.64 (6.28-18.9)	**<0.001**

M: male, F: female, SD: standard deviation, F/U: follow-up, WDLS: well-differentiated liposarcoma, DDLS-LG: low-grade dedifferentiated liposarcoma, DDLS-HG: high-grade dedifferentiated liposarcoma, MLPA: multiplex ligation-dependent probe amplification. Statistically significant P-values (P<0.05) are shown in bold.

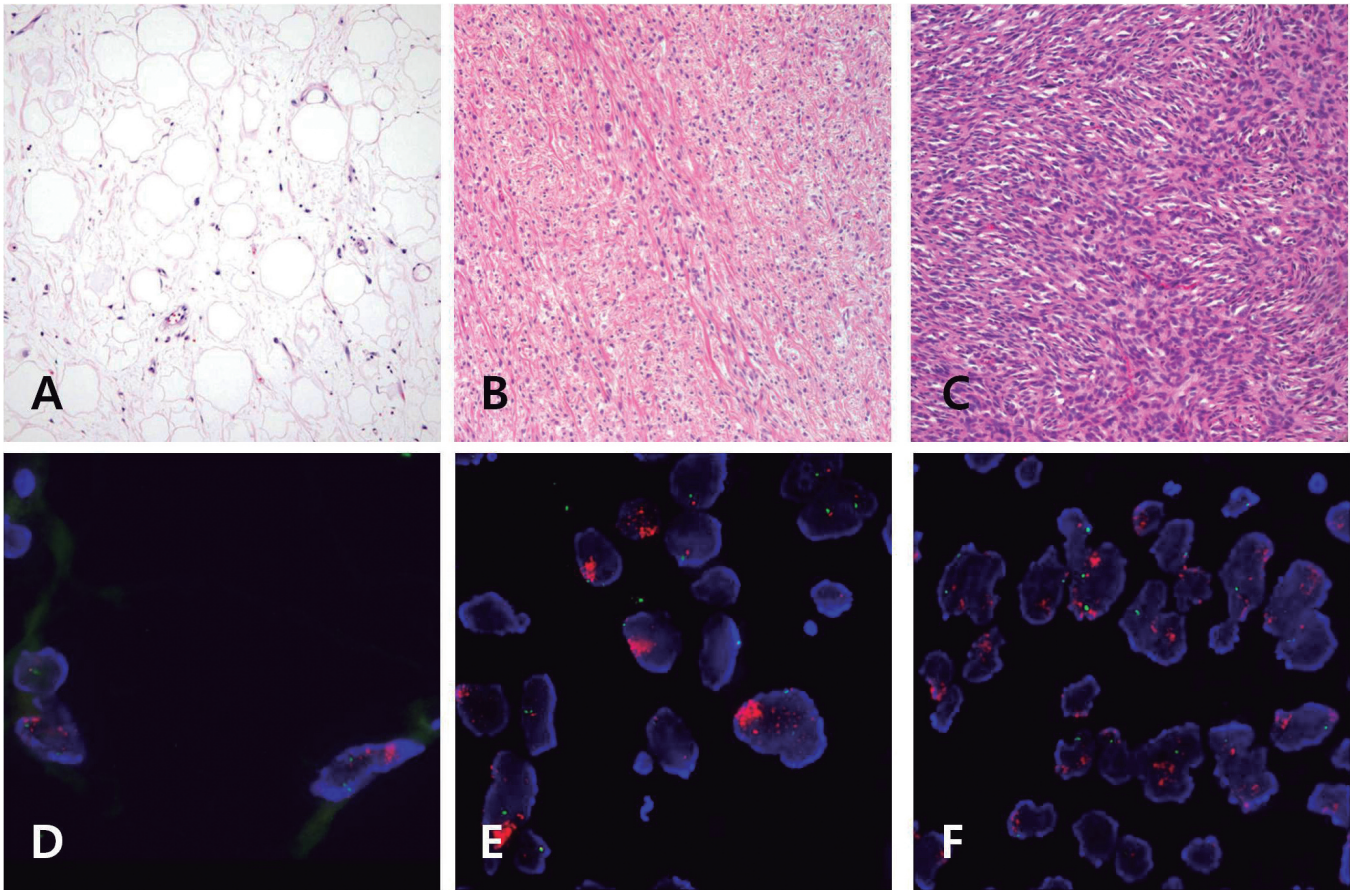


Fig. 2. Representative histologically-confirmed cases of WDLS **(A)**, DDLS-LG **(B)**, and DDLS-HG **(C)** (H&E, x 200). **A.** WDLS, scattered atypical cells and adipocytes. **B.** DDLS-LG, low-grade spindle cell proliferative lesions very similar to fibromatosis. **C.** DDLS-HG, high-grade pleomorphic sarcomatous lesions very similar to fibrosarcoma. Representative photomicrograph of the dual-color FISH analysis of the *MDM2* gene (red) and centromere 12 (green) in WDLS **(D)**, DDLS-LG **(E)**, and DDLS-HG **(F)** cases. **D.** WDLS, two tumor cell nuclei showing *MDM2* amplification with clusters of *MDM2* gene copies. **E.** DDLS-LG, scattered tumor cell nuclei showing *MDM2* amplification with clusters of *MDM2* gene copies. **F.** DDLS-HG, the majority of the tumor cell nuclei showing *MDM2* amplification with clusters of *MDM2* gene copies.

(median: 5.33) by MLPA.

Based on the histologic grade, we divided *MDM2*-amplified liposarcomas into three groups: 1) the WDLS group with no dedifferentiated area, 2) the DDLS-LG group with at least 25% dedifferentiated area and low-grade, and 3) the DDLS-HG group with at least 25% dedifferentiated area and high-grade (Table 1 and Fig. 2). Of the 56 cases, 30 were classified as WDLS, 12 were DDLS-LG, and 14 were DDLS-HG. Differences in age (P=0.517), sex ratio (P=0.054), tumor size

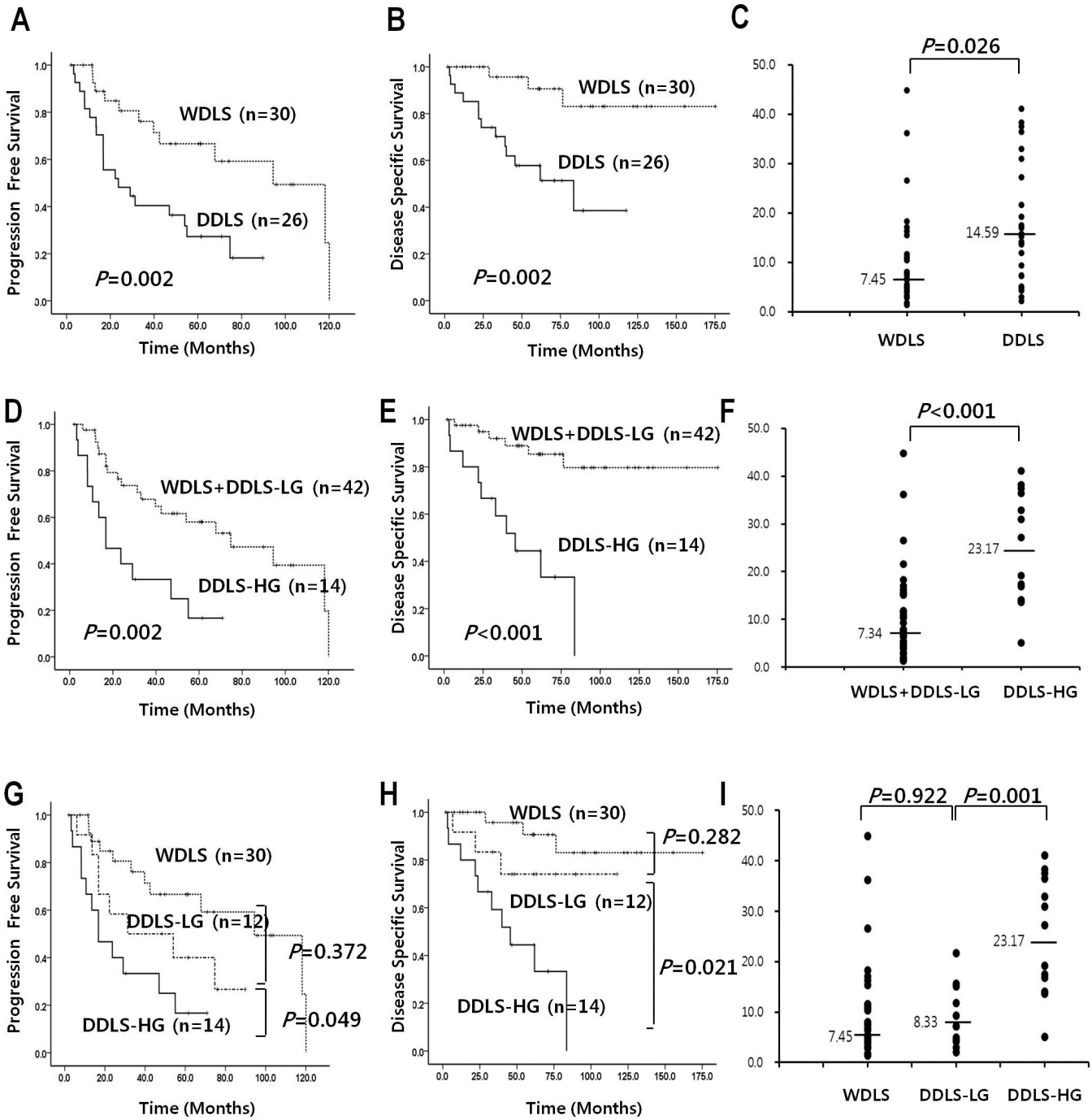


Fig. 3. Subgroup analysis according to histologic grade of *MDM2*-amplified liposarcoma. Survival curves for PFS and DSS, and *CDK4* amplification levels of patients with WDLS/DDLS after classification into the following groups: 1) WDLS and DDLS **(A, B, C)**; 2) WDLS with DDLS-LG and DDLS-HG **(D, E, F)**; 3) WDLS, DDLS-LG, and DDLS-HG **(G, H, I)**.

Table 2. Univariate analysis of disease-specific survival and progression-free survival in 56 patients with WDLS/DDLS.

Prognostic factors	Disease-specific survival No. of events Hazard ratio (95% CI)	P-value	Progression-free survival No. of events Hazard ratio (95% CI)	P-value
CDK4 (qPCR)				
Low	4/27		11/27	
High	13/29	**0.044**	21/29	**0.003**
	3.21 (1.032-9.968)		3.57 (1.550-8.23)	
MDM2 (qPCR)				
Low	5/24		11/24	
High	12/32	0.106	21/32	**0.029**
	2.55 (0.82-7.91)		2.427 (1.094-5.386)	
Grade				
WDLS	3/30		12/30	
DDLS-LG	3/12	0.226	8/12	0.108
	2.69 (0.54-13.53)		2.19 (0.843-5.388)	
DDLS-HG	11/14	**<0.001**	12/14	**<0.001**
	11.31 (3.018-40.64)		5.03 (2.086-12.14)	
Grade				
Low	6/42		20/42	
High	11/14		12/14	
	3.156 (1.598-6.232)	**0.001**	4.909 (2.048-11.77)	**<0.001**
Resection margin				
Negative+Micro	11/49		25/49	
Macro	6/7	**<0.001**	7/7	**<0.001**
	5.95 (2.45-14.43)		5.26 (2.37-11.67)	
Post-op RT				
Done	5/16		7/16	
Not done	12/40	0.896	25/40	0.065
	1.062 (0.428-2.636)		1.98 (0.959-4.091)	
Site				
Periphery	1/18		7/18	
Peritoneum	16/38	**0.047**	25/38	**0.027**
	2.230 (1.101-5.753)		2.107 (1.089-4.077)	

WDLS: well-differentiated liposarcoma, DDLS-LG: low-grade dedifferentiated liposarcoma, DDLS-HG: high-grade dedifferentiated liposarcoma, RT: radiation therapy, CI: confidence interval. Statistically significant P-values (P<0.05) are shown in bold.

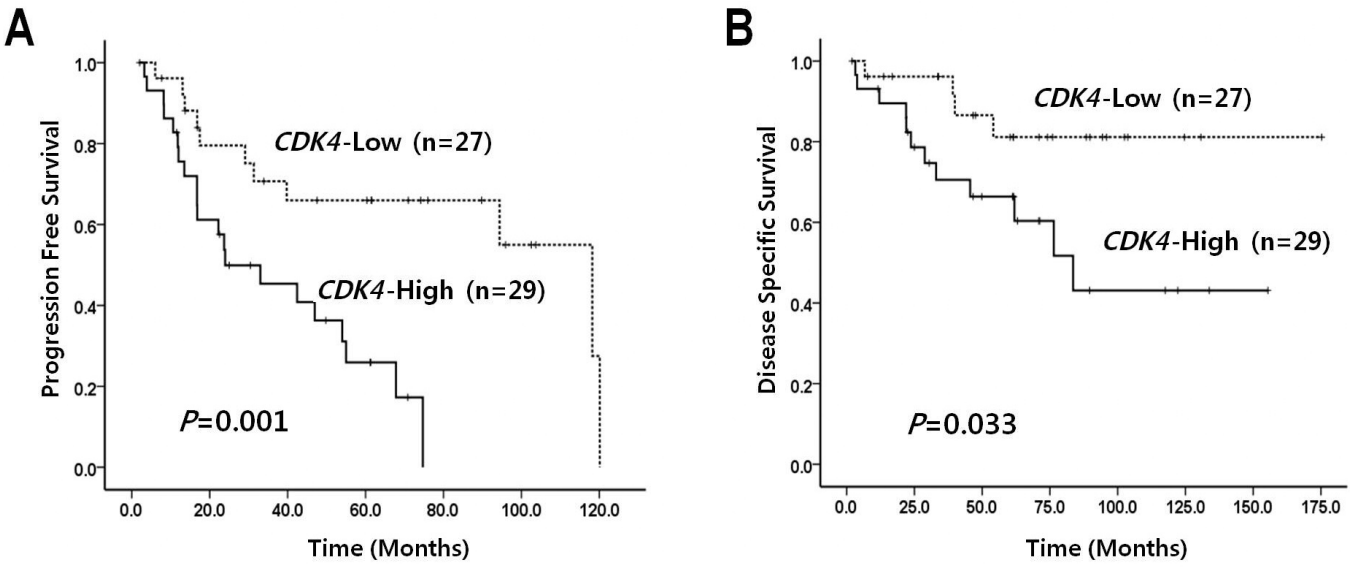


Fig. 4. Survival curves for PFS **(A)** and DSS **(B)** of patients with *MDM2*-amplified WDLS/DDLS according to *CDK4* amplification levels. PFS and DSS were significantly different between the *CDK4*-high group and the *CDK4*-low group.

(P=0.211), resection margin status (P=0.553) and follow-up duration (P=0.059) among these three groups were not statistically significant. However, the recurrence and death rates were significantly different among the three groups, with the highest rates observed in the DDLS-HG group (P=0.013 and P<0.001, respectively). The *MDM2* and *CDK4* amplification levels were statistically different among the three groups (P<0.05). DDLS-HG showed higher levels of *MDM2* and *CDK4* amplification by both qPCR and MLPA compared with WDLS and DDLS-LG.

Survival analysis of the subgroups by histologic grade

To assess the association between histologic grade and survival of patients with WDLS/DDLS, subgroup

Table 3. Multivariate analysis of disease-specific survival and progression-free survival in 56 patients with WDLS/DDLS.

	Disease-specific survival		Progression-free survival	
Prognostic factors	Hazard ratio (95% CI)	P-value	Hazard ratio (95% CI)	P-value
CDK4 (qPCR)				
Low				
High	2.19 (1.42-23.5)	**0.044**	3.08 (1.03-10.81)	**0.048**
MDM2 (qPCR)				
Low				
High	1.35(0.144-12.5)	0.794	1.22 (0.45-2.39)	0.655
Grade				
WDLS				
DDLS-LG	5.28 (0.84-33.4)	0.076	2.42 (0.87-6.71)	0.091
DDLS-HG	8.65 (1.41-45.4)	**0.020**	1.83 (0.69-4.86)	0.223
Resection margin				
Negative+Micro				
Macro	2.94 (1.28-12.11)	**0.022**	2.86 (1.13-6.30)	**0.026**
Post-op RT				
Done				
Not done	0.43 (0.12-1.57)	0.200	0.949 (0.34-2.59)	0.918
Site				
Periphery				
Peritoneum	5.34 (0.58-49.13)	0.139	2.61 (0.880-7.73)	0.084

WDLS: well-differentiated liposarcoma, DDLS-LG: low-grade dedifferentiated liposarcoma, DDLS-HG: high-grade dedifferentiated liposarcoma, RT: radiation therapy, CI: confidence interval. Statistically significant P-values (P<0.05) are shown in bold.

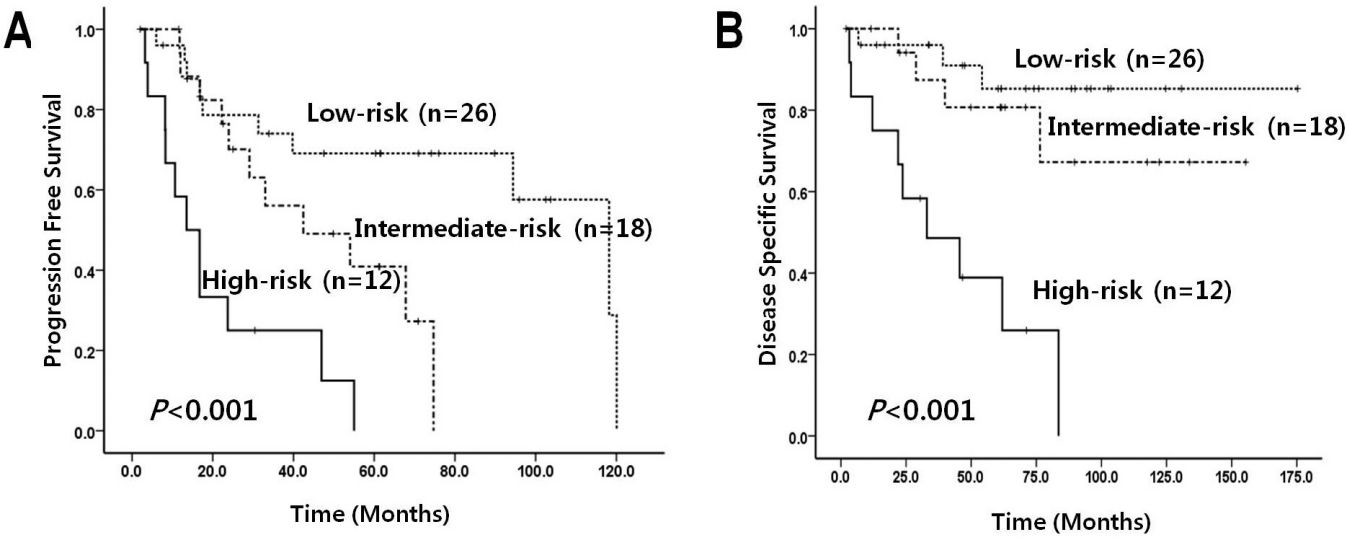


Fig. 5. Survival curves for PFS **(A)** and DSS **(B)** of patients with *MDM2*-amplified WDLS/DDLS according to recurrence risk. The three risk groups according to *CDK4* amplification levels and histologic grade showed well-discriminated survival outcomes (PFS and DSS).

analysis by histologic grade was performed. Subgroup analysis based on the existing classification for WDLS/DDLS showed that patients with DDLS had a significantly shorter PFS (P=0.002) and DSS (P=0.002) than those with WDLS (Fig. 3A,B). *CDK4* amplification levels in DDLS were significantly higher than those in WDLS (P=0.026) (Fig. 3C).

Patients with DDLS-HG had a significantly poorer outcome (P=0.002 for PFS, P<0.001 for DSS) than patients with WDLS or DDLS-LG when *MDM2*-amplified liposarcomas were divided into two groups: one group of DDLS-HG and the other group of WDLS and DDLS-LG (Fig. 3D,E). DDLS-HG showed a significantly higher mean *CDK4* amplification level than that of the other group (P<0.001) (Fig. 3F).

Finally, when *MDM2*-amplified liposarcomas were divided into three groups (WDLS, DDLS-LG, and DDLS-HG), there were significant survival differences between DDLS-LG and DDLS-HG (P=0.049 for PFS, P=0.021 for DSS) whereas the differences between WDLS and DDLS-LG were not statistically significant (P=0.372 for PFS, P=0.282 for DSS) (Fig. 3G,H). *CDK4* amplification levels were also significantly higher in the DDLS-HG group than in the DDLS-LG group (P=0.001), but there was no significant difference in *CDK4* amplification between the WDLS and DDLS-LG groups (P=0.922) (Fig. 3I).

Survival analysis according to MDM2 and CDK4 amplification levels

The levels of *MDM2* and *CDK4* amplification according to qPCR and MLPA were highly correlated (Spearman correlation coefficient =0.907 for *MDM2*, P<0.001; 0.874 for *CDK4*, P<0.001). The patients were divided into two groups based on a cut-off of 10.0 for *MDM2* and *CDK4* amplification in qPCR. The *CDK4*-low group showed significantly better PFS (P=0.001) and DSS (P=0.033) than the *CDK4*-high group (Fig. 4). The *MDM2*-low group also showed significantly better PFS (P=0.025), but not DSS (P=0.094). When MLPA data for *MDM2* and *CDK4* were divided into two groups according to a cut-off of 7.0, only the *CDK4*_MLPA-low group showed significantly better PFS (P=0.001) and DSS (P=0.027) than the *CDK4*_MLPA-high group. In addition, when patients in the WDLS with DDLS-LG group were further divided into *CDK4*-low and -high group, patients in the *CDK4*-high group had a shorter DFS compared with the *CDK4*-low group (data not shown).

Classification of disease progression risk groups by CDK4 amplification level and histologic grade

On the basis of the associations detected between *CDK4* amplification levels and histologic grade and the survival of patients with WDLS or DDLS, patients were classified into three disease progression risk groups according to *CDK4* amplification levels and histologic grade. The low-risk group was defined as WDLS or DDLS-LG with low *CDK4* amplification levels, the high-risk group as DDLS-HG with high *CDK4* amplification levels, and the intermediate-risk group as WDLS or DDLS-LG with high *CDK4* amplification levels or DDLS-HG with low *CDK4* amplification levels. Of 56 cases, 26 (46.4%) were classified into the low-risk group, and the intermediate-risk and high-risk groups comprised 18 (32.1%) and 12 (21.4%) cases, respectively. This disease progression risk classification revealed significantly different survival outcomes among the groups (P<0.001) (Fig. 5). Progression-free survival analysis of the risk groups showed well-discriminated survival outcomes, indicating that disease progression risk grouping according to *CDK4* amplification levels and histologic grade is efficient for discriminating patients.

Prognostic factors associated with disease-free survival and overall survival

The clinicopathologic factors associated with PFS and DSS on univariate analyses were *CDK4* amplification levels, histologic grade, and resection margin status (Table 2). When multivariate analyses were performed, independent prognostic factors for DSS were *CDK4* amplification levels (P=0.044; hazard ratio [HR], 2.19; 95% CI, 1.42–23.5), histologic grade (P=0.020; HR, 8.65; 95% CI, 1.41–45.4) and resection margin status (P=0.022; HR, 2.94; 95% CI, 1.28–12.11) (Table 3). *CDK4* amplification levels and resection status remained significant independent prognostic factors of PFS.

Discussion

WDLS and DDLS share common molecular cytogenetic abnormalities, such as the amplification of chromosome 12q13-15, which contains the *MDM2*, *HMGA2*, and *CDK4* genes. However, *CDK4* amplification is not always detected in WDLS/DDLS, whereas *MDM2* and *HMGA2* are consistently amplified in these tumors (Italiano et al., 2008, 2009).

In the present study, we found that patients of *MDM2*-amplified liposarcoma with high *CDK4* amplification levels had a significantly poorer clinical outcome than those with low *CDK4* amplification levels. The previous study showed a significantly higher local recurrence rate in patients with *CDK4*-amplifed WDLS/DDLS than in patients with *CDK4*-nonamplified WDLS/DDLS (Italiano et al., 2009). Although there was no subgroup analysis based on the level of *CDK4* amplification in *CDK4*-amplifed WDLS/DDLS (*MDM2*$^+$/*CDK4*$^+$), their findings are consistent with our results. Given the role of CDK4 in regulating cell cycle progression and the effect of CDK4 inhibition on the proliferation of liposarcoma cell lines, *CDK4*

amplification is likely to play a crucial role in the progression of liposarcoma and thus may be associated with poor patient prognosis.

In line with previous studies, our subgroup analysis according to the histologic grade of *MDM2*-amplified liposarcoma showed that the recurrence rate was higher in DDLS than WDLS (Singer et al., 2003; Dalal et al., 2006). This finding is most likely explained by the aggressive nature and metastatic potential of DDLS with dedifferentiation. However, differences in *CDK4* amplification levels and overall survival between low-grade (WDLS and DDLS-LG) and high-grade (DDLS-HG) groups were greater than those between WDLS and DDLS. In addition, when patients were divided into three groups (WDLS, DDLS-LG, and DDLS-HG), *CDK4* amplification levels and survival outcomes between DDLS-LG and DDLS-HG showed more discriminating results than between WDLS and DDLS-LG. Our findings indicate that the biological behavior of DDLS-LG may be distinct from that of DDLS-HG, and that subgroup classification of DDLS by histologic grade can reflect the prognosis of patients with liposarcoma, suggesting the prognostic value of histologic grade in DDLS. However, our results are in contrast to a previous report showing no association between histologic grade in DDLS and clinical outcome (Henricks et al., 1997). The reason for this discrepancy between our study and this previous study is unclear but may be due to the small number of patients with low-grade DDLS in both studies. Therefore, further research with larger sample sizes is required to confirm the association between histologic grade and survival outcome in DDLS.

It was recently found that specific copy number alterations are associated with genomic complexity and distinct morphology (11q23-24) or outcome of patients with DDLS (19q13) (Crago et al., 2012). Copy number alterations in a specific region in addition to histologic subtype grading may be used to predict the prognosis of DDLS. The classification of risk groups for WDLS/DDLS recurrence according to *CDK4* amplification and histologic grade was well correlated with patient outcome in this study. This result suggests the utility of *CDK4* amplification levels and histologic grading in the stratification of patients with WDLS/DDLS. Furthermore, both *CDK4* amplification levels and histologic grade were found to be independent prognostic factors for overall survival of patients with *MDM2*-amplified liposarcoma in this study, thus confirming their prognostic utility in WDLS/DDLS.

The identification of novel molecular targets for more effective therapy in WDLS/ DDLS is required because current systemic chemotherapy in WDLS/DDLS is inadequate (Crago and Singer, 2011). Therefore, the search for molecular targets in WDLS/DDLS is underway, and drugs targeting several proteins including MDM2 and CDK4 are currently in clinical trials (National Institutes of Health). By demonstrating an association between *CDK4* amplification and patient prognosis, our findings lend additional support to the possibility of CDK4 as a promising target for the treatment of WDLS/DDLS. For example, a CDK4 inhibitor might be effective for treating liposarcoma with *CDK4* amplification. For this reason, the recognition of *CDK4* amplification in liposarcomas of the same histologic grade may be important for identifying patients who will benefit from specific therapies and for predicting patient prognosis. The amplified gene targeting therapy, including HER2-targeting trastusumab in HER2-positive breast cancer, has been successful. Gullo et al has reported that level of *HER2* gene amplification was a predictive factor of response to trastuzumab-based therapy in patients with HER2-positive metastatic breast cancer (Gullo et al., 2009). For accurate patient selection, the level of *CDK4* amplification might be a predictive factor for CDK4-inhibitor therapy in WDLS/DDLS.

Many different methods have been used for detecting copy number changes in genomic DNA, including FISH, Southern blotting, qPCR, multiplex amplifiable probe hybridization (Armour et al., 2000), MLPA (Schouten et al., 2002), multiplex amplicon quantification (Suls et al., 2006), array technology, and massive parallel sequencing. In this study, we analyzed the quantitative level of *MDM2* and *CDK4* amplification by both qPCR and MLPA, and observed high agreement between the two approaches. The MLPA test was found to be accurate, even for the detection of polysomic cases, and was easier to perform and less expensive than FISH (Bravaccini et al., 2012). Moreover, MLPA is quantitative, not observer-dependent, and provides single-step multiple-gene information for deciding the best therapeutic approach. However, our results showed that for cut-off discrimination of *CDK4* amplification levels, qPCR provided a wider range of levels with more power to divide groups in the analysis.

In conclusion, we demonstrated the independent prognostic significance of level of *CDK4* amplification and histologic grade in WDLS/DDLS. Our results suggest that *CDK4* amplification levels and histologic grade in WDLS/DDLS may be useful for the prediction of patient outcome and stratification of patients for targeted therapy. This further provides the evidence that molecular genetics based on specific genetic abnormalities, as well as morphology, might be crucial for the precise classification of liposarcoma for targeted therapy.

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