

Chromosome-12 Copy Number Alterations and MDM2, CDK4 and TP53 Expression in Soft Tissue Liposarcoma

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Abstract. *Background:* Liposarcoma is a heterogeneous group of soft tissue sarcomas in which definitive prognostic parameters need to be identified. *Materials and Methods:* The series included 33 consecutive soft tissue (well-differentiated, WDLPS, n=19; and dedifferentiated, DDLPS, n=14) liposarcoma. *Clinicopathological variables* included age, gender, body location, degree of dedifferentiation and mitotic count. *The molecular analysis* included MDM2, CDK4 and TP53 expressions and chromosome-12 copy number alterations. *Results:* Centrally located (retroperitoneal, abdominal cavity or groin region) WDLPS had more dedifferentiation ($p=0.001$). Patients with DDLPS and a high mitotic rate died ($p=0.070$) or experienced recurrences ($p=0.029$) more frequently. Co-expression of MDM2/CDK4 ($p=0.001$) and TP53 accumulation ($p=0.017$) related to dedifferentiation but not to recurrence or death, both in WDLPS and DDLPS. DDLPS had higher centromeric chromosome-12 copy number than WDLPS ($p=0.013$), but this was unrelated to recurrence or death. *Conclusion:* Central location is a risk factor in WDLPS. Co-expression of MDM2/CDK4/TP53 and chromosome-12 alterations characterize DDLPS suggesting a link with dedifferentiation.

Liposarcoma (LPS) is the most common soft tissue sarcoma in adults (1) with five major subtypes recognized by the WHO classification (2). Recent molecular genetic data challenged the traditional morphological classification based on the

finding of ring or giant marker chromosomes that characterizes atypical lipomatous tumor/well-differentiated liposarcoma (ALT/WDLPS) (12q14-15 amplification) and dedifferentiated liposarcoma (DDLPS) (12q13-21 amplification); meanwhile, t(12;16)(q13;p11) would characterize myxoid liposarcoma (MXLPS) and round cell liposarcoma (RCLPS) with no specific molecular features defining pleomorphic liposarcoma (PLPS).

Clinically, ALT/WDLPS is considered an intermediate (locally aggressive) neoplasm, while DDLPS its malignant counterpart (2). Nonetheless, DDLPS is still an ill-defined category. Evans (3) and Kempson *et al.* (4) suggested that DDLPS is always a high-grade non-lipogenic sarcoma arising near or at the site of a previously removed ALT/WDLPS, and these authors, therefore, did not support including cases with low-grade dedifferentiation as addressed by others (2, 5). Molecular analysis has revealed that common cytogenetic markers for ALT/WDLPS-DDLPS (supernumerary rings and giant markers) represent amplification of MDM2, usually accompanied by amplification of the neighboring genes CDK4, SAS and HMGIC (2, 6, 7).

This paper reports on clinicopathological features of a sequential cohort series of ALT/WDLPS and DDLPS diagnosed, treated and followed-up in a single institution, with emphasis on their prognostic significance. The expression of MDM2, CDK4 and TP53 and chromosome-12 copy number alterations in these categories of LPS was included as a molecular approach to its biological characterization.

Materials and Methods

The study series included all ALT/WDLPS or DDLPS of soft tissues collected at the Virgen Macarena University Hospital (Seville, Spain), between 1974 and 2002, using pathology reports. The re-assessment of all LPS or pleomorphic sarcoma in the

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Key Words: Liposarcoma, immunohistochemistry, FISH, chromosome-12.

hospital database gave a total of 33 consecutive (19 ATL/WDLPS and 14 DDLPS) cases.

Clinical parameters recorded included tumor location: central (retroperitoneal, abdominal cavity or groin region) vs. peripheral (extremities) further subdivided into superficial (subcutaneous) and deep (non-subcutaneous) tumors. Patient's follow-up required a minimum of 2 years for inclusion and was calculated as the number of months from the date of the diagnostic surgical procedure to the date of the last visit or death. Tumor recurrence was defined as reappearance of the tumor after initial surgical treatment. Survival time was the period between diagnosis and cancer-related death. The end-point of our study was tumor recurrence and overall cancer-specific survival. Tumor size (cm) was defined as the largest diameter.

Pathological assessment. Hematoxylin-eosin stained slides including primary tumors and recurrences were evaluated without prior knowledge of the patient's clinical status. ALT/WDLPS and DDLPS were defined following WHO criteria (2) and included low vs. high grade of dedifferentiation. If a discrepancy occurred in grading, a pathology panel review round was organized to obtain a consensus diagnosis.

Assessment of immunohistochemistry. A representative paraffin block from each tumor was serially cut into 4 µm sections, de-waxed in xylene, rehydrated in graded ethanol, and washed for 5 min with phosphate-buffered saline. For antigen retrieval, sections were boiled in 10 mM citrate buffer (pH 6.0). Endogenous peroxidase was blocked by incubation of the slides for 30 min with 3% hydrogen peroxide in methanol. Sections were then incubated with primary antibodies at room temperature, incorporating positive and negative controls. Primary monoclonal antibodies were: MDM2 (diluted 1:50), CDK4 (diluted 1:200) and TP53 (diluted 1:100) (all from Novocastra, Newcastle-upon-Tyne, UK). Immunohistochemical staining was performed using the polymer-based EnVision system (Dako-Cytomation, Glostrup, Denmark). The reaction was visualized with diaminobenzidine as chromogen substrate solution (0.6 mg/ml in Tris-buffered saline, pH 7.6 with 12 ml 30% hydrogen peroxide) for 30 min at room temperature. Sections were counterstained with Mayer's hematoxylin, dehydrated and mounted as standard procedure.

All markers were quantitated using random fields delineated by a 1 cm² graded ocular grid attached to the eyepiece of the microscope. The regions were chosen inside highly-immunoreactive areas examined under high power magnification (x400) counting a mean of 1,000 cells/case. Cut-off values for each type of immunostaining were >5% for MDM2, CDK4 and TP53.

Quantitative and qualitative assessment of FISH. Centromeric chromosome-12 fluorescence-labeled probes (Vysis, Downers Grove IL, USA) were performed on 3-µm thick deparaffinized sections in ten cases (4 ALT/WDLPS and 6 DDLPS) following manufacturer's instructions for this probe. Signals were counted in at least 200 cells using the recommended filters (Nikon E600, Kingston, UK) and imaging was performed with an image analysis system (Cytovision, Applied Imaging, UK).

Statistical analyses. Statistical analyses were carried out using the Statistical Package for the Social Sciences (SPSS, v. 12.0; SPSS Inc, Chicago, IL, USA). The Chi-square test and Fisher's exact test were applied for relationships between categorical and dichotomous variables, and the Mann-Whitney non-parametric test was used to

Table I. Demographic and clinico-pathological data on 33 consecutive soft tissue LPS.

	ALT/WDLPS	DDLPS	p-value
Gender			
Male	15 (79%)	7 (50%)	
Female	4 (21%)	7 (50%)	
Age: mean (range)	63.5 (38-89)	64.8 (54-81)	
Location			0.0027*
Central (%)	8 (42%)	13 (93%)	
Retroperitoneum	4 (50%)	11 (84%)	
Groin	3 (37%)	1 (8%)	
Abdominal cavity	1 (13%)	1 (8%)	
Peripheral (%)	11 (58%)	1 (7%)	
Superficial	2 (8%)	0 (0%)	
Deep	9 (92%)	1 (100%)	
Mean tumor size (cm)	11.5	19.6	
Tumor recurrence (%)	4 (21%)	6 (46%)	
Cancer related death (%)	0 (0%)	7 (54%)	0.0005*

ALT/WDLPS: well-differentiated liposarcoma; DDLPS: dedifferentiated liposarcoma; *Fisher's exact test.

compare categorical with continuous variables. For the above comparisons, $p < 0.05$ was considered statistically significant.

Results

Subtypes in ALT/WDLPS were "lipoma-like" (90%) followed by sclerosing and inflammatory types (5% each) (Figure 1). The predominant well-differentiated component in DDLPS was sclerosing (57%) or "lipoma-like" (36%). The transition from differentiated to dedifferentiated components was abrupt in 63% of cases and progressive in 37%; dedifferentiated component was low-grade (6, 43%) and high-grade (8, 57%) (Figure 1); in the latter, the most common subtype was "MFH-like". In two cases, the dedifferentiated component was focal osteosarcoma or chondrosarcoma, respectively. Two retroperitoneal tumors contained focal "meningotheial-like" areas within areas of low-grade dedifferentiation (Figure 1).

Clinical and outcome information is reported Table I. DDLPS was mainly centrally located ($p=0.0027$), but was heterogeneous in the 19 ALT/WDLPS. Follow-up ranged from 26 to 122 months. Progression time for dedifferentiation could not be evaluated, since 100% of DDLPS displayed dedifferentiation at the time of diagnosis; 21% (n=4) of ALT/WDLPS recurred, compared to 46% (n=6) of DDLPS; mean disease-free intervals were 41 months and 28 months, respectively. None of the patients with ALT/WDLPS died during follow-up, whereas 7 (54%) out of 13 DDLPS patients died of cancer (mean survival time of 47 months, $p=0.0005$).

Centrally-located ALT/WDLPS displayed greater recurrence than peripheral tumors (37% vs. 9%) ($p=0.13$). Dedifferentiation was more frequent ($p=0.001$) in centrally-located than in peripheral tumors (65% vs. 8%).

Table II. Expression profiles of MDM2, CDK4 and TP53 in well-differentiated and dedifferentiated LPS according to anatomic location.

Body location	MDM2	TP53	CDK4	MDM2+TP53	MDM2+CDK4
ALT/WDLPS (n=19)	11 (58%)	7 (37%)	6 (31%)	7 (37%)	4 (21%)
Central (n=8)	5 (62%)	3 (37%)	3 (37%)	3 (37%)	2 (25%)
Retroperitoneum (n=4)	3 (75%)	2 (50%)	2 (50%)	2 (50%)	2 (50%)
Abdominal cavity (n=1)	0 (0%)	0 (0%)	1(100%)	0 (0%)	0 (0%)
Groin (n=3)	2 (66%)	1 (33%)	0 (0%)	1 (33%)	0 (0%)
Peripheral (n=11)	6 (54%)	4 (36%)	3 (27%)	4 (36%)	2 (18%)
Superficial (n=2)	1 (50%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Deep (n=9)	5 (55%)	4 (44%)	3 (33%)	4 (44%)	2 (22%)
DDLPS (n=14)	12(86%)	11(78%)	13 (93%)	11 (78%)	11 (78%)
Central (n=13)	11(85%)	10 (77%)	12 (92%)	10 (77%)	10 (77%)
Retroperitoneum(n=11)	9 (82%)	8 (73%)	11 (100%)	8 (73%)	9 (82%)
Abdominal cavity (n=1)	1 (100%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)
Groin (n=1)	1 (100%)	1 (100%)	0 (0%)	1 (100%)	0 (0%)
Peripheral (n=1)	1 (100%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)
Superficial (n=0)	–	–	–	–	–
Deep (n=1)	1 (100%)	1 (100%)	1 (100%)	1 (100%)	1 (100%)
ALT/WDLPS vs. DDLPS <i>p</i> -value*	0.085	0.017	0.0004	0.017	0.001

ALT/WDLPS: Well-differentiated liposarcoma; DDLPS: dedifferentiated liposarcoma; *Fisher's exact test.

No significant differences in outcomes (recurrence or death) were observed as a function of the high-grade vs. low-grade dedifferentiation in DDLPS. Fifty per cent (4 out of 8) of patients with high-grade dedifferentiation recurred and 62% (5 out of 8) died of the disease. The rates of recurrence and death in patients with low-grade dedifferentiation were 40% (2 out of 5). DDLPS patients with a mitotic index of $\geq 10 \times 10$ HPF had more recurrences ($p=0.029$) or higher death-rates ($p=0.070$).

Immunohistochemical findings for MDM2, CDK4 and TP53 are shown in Table II; all were in the nucleus, except CDK4 which was also present in the cytoplasm. Overexpression of MDM2 ($p=0.085$), CDK4 ($p=0.0004$) and TP53 ($p=0.017$) was more common in DDLPS than in ALT/WDLPS (Figure 2). Similarly, co-expression of MDM2/CDK4 ($p=0.001$) and MDM2/TP53 ($p=0.017$) was also higher in DDLPS. Protein expressions as a function of clinical outcome and tumor location had no association with ALT/WDLPS or DDLPS.

FISH analysis in cases of ALT/WDLPS (n=4) and DDLPS (n=6) is displayed in Table III. Most cells in ALT/WDLPS had two centromeric chromosome-12 copies, but occasionally had >4 copies; by contrast, more than 4 copies were common in DDLPS tumor cells ($p=0.013$) (Figure 2). Tumor recurrence and death-rate was greater, but not significantly, in patients with more than 4 copies of chromosome-12 (recurrence, $p=0.643$; death, $p=0.512$).

Discussion

Recent developments in molecular biology have considerably improved our knowledge of liposarcoma allowing new

Table III. FISH analysis for chromosome-12 copy number alterations in soft tissue LPS.

Case	Histological subtype	% cells with >4 copies of chromosome-12	Recurrence	Death
WDLPS	sclerosing	1%	no	no
WDLPS	inflammatory	1%	yes	no
WDLPS	lipoma-like	0%	no	no
WDLPS	lipoma-like	2%	no	no
DDLPS	myxofibrosarcoma	2%	no	no
DDLPS	pleomorphic MFH	40%	no	no
DDLPS	pleomorphic MFH	30%	yes	yes
DDLPS	pleomorphic MFH	20%	yes	yes
DDLPS	myxofibrosarcoma	3%	no	no
DDLPS	pleomorphic MFH	29%	no	yes

DDLPS vs. WDLPS ($p=0.013$), Fisher's exact test; >4 copies and recurrence, $p=0.643$, Fisher's exact test, >4 copies and death, $p=0.512$, Fisher's exact test. WDLPS: well-differentiated liposarcoma; DDLPS: dedifferentiated liposarcoma; MFH: malignant fibrous histiocytoma.

classifications and nomenclature. ALT/WDLPS is a category that clearly benefits most from new developments. Some controversy persists concerning prognosis, which is strongly influenced by location and time (8-10). Our study confirms a greater incidence of both recurrences and dedifferentiation in centrally-located ALT/WDLPS and – to a lesser extent – in peripheral WDLPS located deep in the extremities. Terminological discrepancies are common in ALT/WDLPS and DDLPS. Until 1997, the definition of dedifferentiation in LPS corresponded only to dedifferentiated chondrosarcoma and was based on the development of a high-grade non-

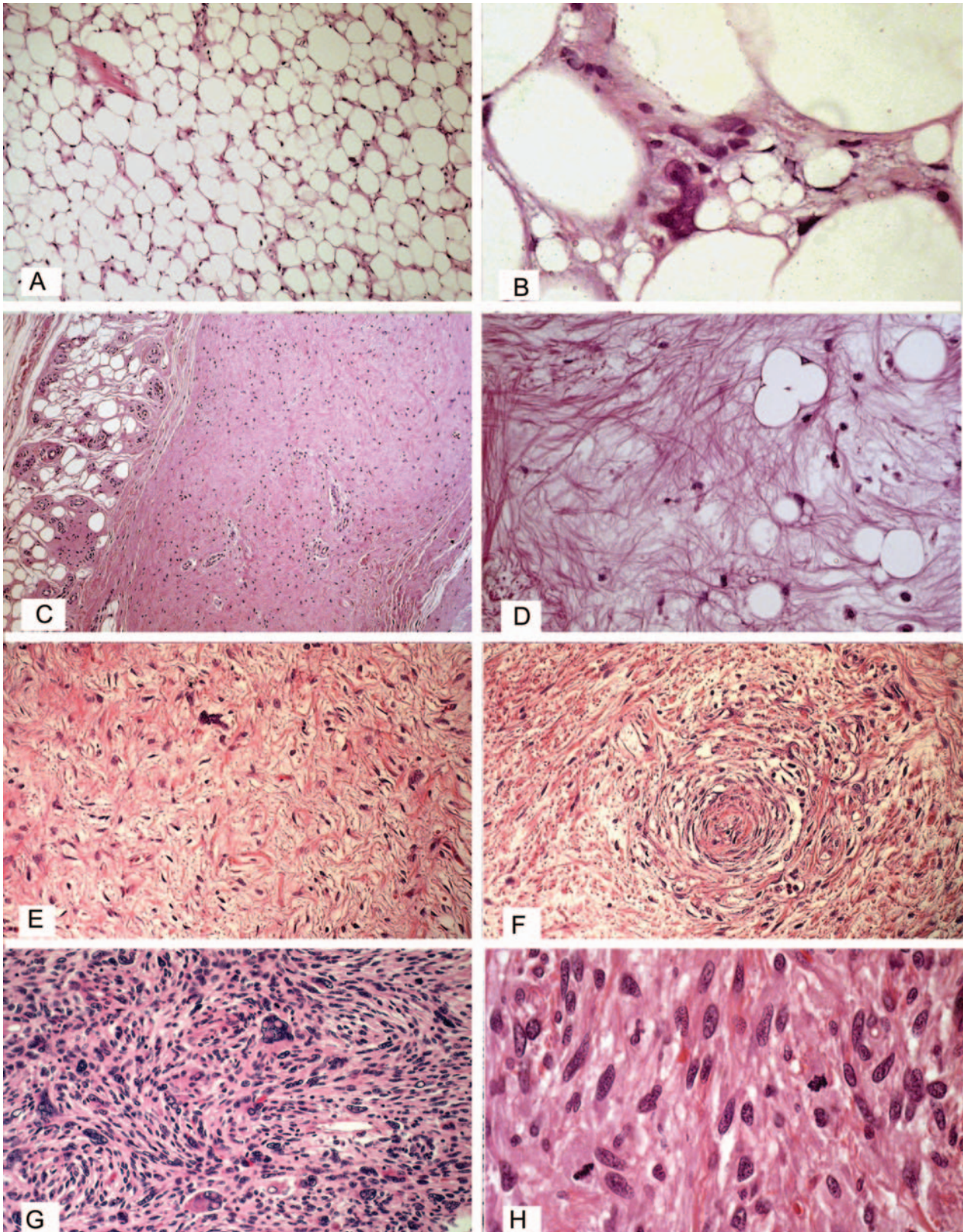


Figure 1. A and B: Well-differentiated liposarcoma, lipoma-like type. C and D: Well-differentiated liposarcoma, sclerosing type. E and F: Dedifferentiated liposarcoma, low-grade component. G and H: Dedifferentiated liposarcoma, high-grade component.

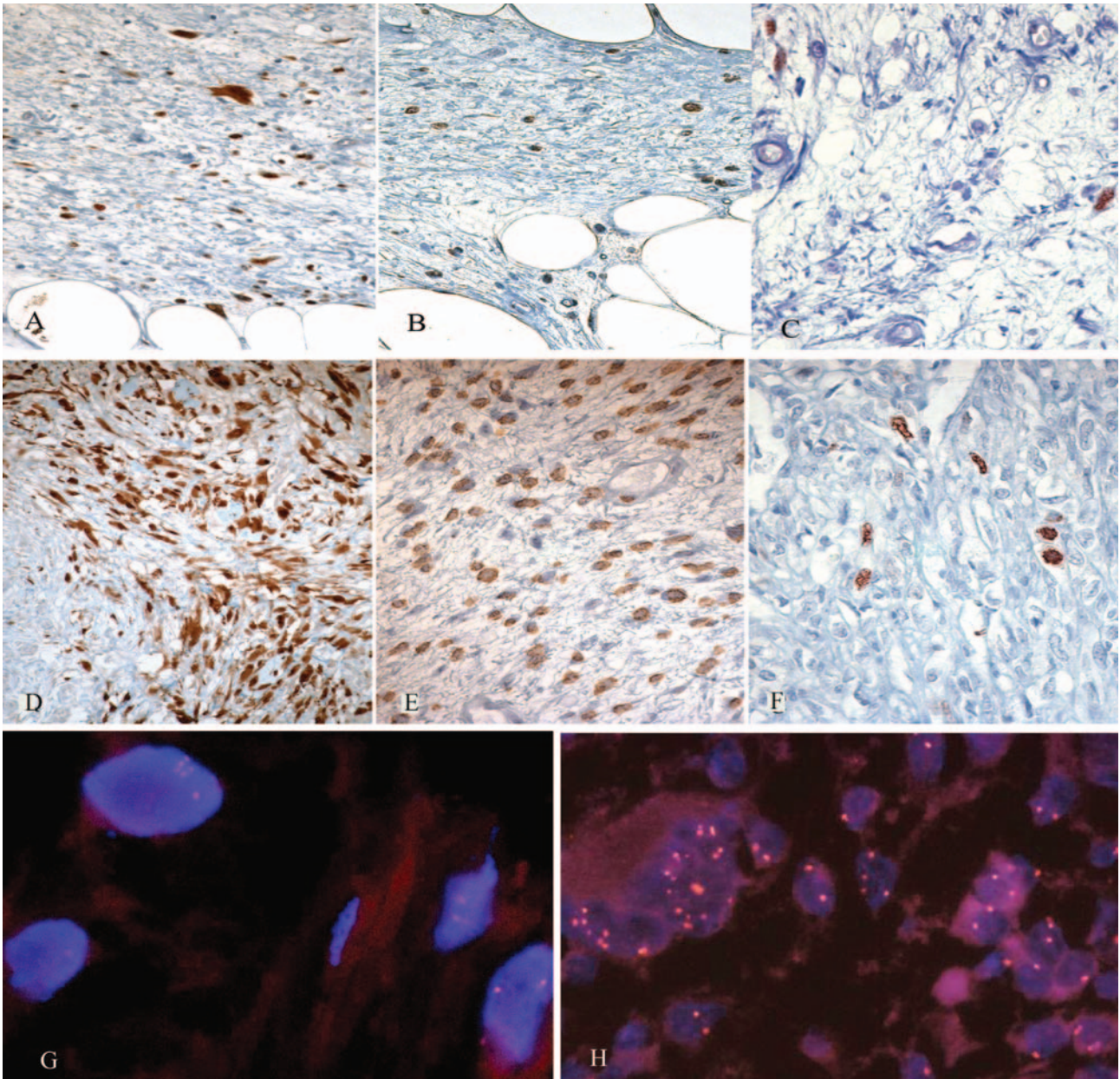


Figure 2. Well-differentiated liposarcoma, sclerosing type: A: Anti-CDK4 monoclonal antibody, x40; B: Anti-MDM2 monoclonal antibody, x40; C: Anti-TP53 monoclonal antibody, x40. Dedifferentiated liposarcoma, high-grade component, D: Anti-CDK4 monoclonal antibody, x40; E: Anti-MDM2 monoclonal antibody, X40; F: Anti-TP53 monoclonal antibody, x40. Fluorescent in situ hybridization. G: Well-differentiated liposarcoma, lipoma-like with six chromosome-12 signals x100. H: Dedifferentiated liposarcoma, high-grade showing a high number of chromosome-12 signals. x100.

lipogenic sarcoma near or at the site of a previously-removed ALT/WDLPS (3). In 1997, Henricks *et al.* (5) expanded the definition of DDLPS to include low-grade non-lipogenic tumors (low-grade dedifferentiated liposarcoma), a criterion included in the current WHO classification (2). Major criticism of the low-grade dedifferentiation concept argues that this type of tumor is a cellular form of ALT/WDLPS and that "low-grade" cases by Henricks *et al.* (5), whose prognoses

were similar to that of high-grade tumors, were just poorly-sampled DDLPS (4, 9). In the cases reported hereby, 6 of which were classified as low-grade dedifferentiation and 8 as high-grade dedifferentiation, the biological behavior of low-grade dedifferentiation-bearing tumors resembled more that of DDLPS than that of ALT/WDLPS. Thus, although this patient group was relatively small, the authors suggest that low-grade dedifferentiation should be classified as a less-

aggressive form of DDLPS rather than as a special or cellular form of ALT/WDLPS.

Although there is no agreement on the prognostic value of the mitotic index in DDLPS, in agreement with McCormick *et al.* (10), a higher recurrence and death-rate was found in patients with ≥ 10 mitoses. Concerning the expression of TP53, MDM2 and CDK4, greater ($p=0.001$) co-expression of MDM2/CDK4 was found in DDLPS (78%) than in ALT/WDLPS (21%), which might be due to greater amplification of *MDM2* and *CDK4* genes in chromosome-12q14-15, a finding recently challenged (11-13). Expression of MDM2 and TP53 was also more common in DDLPS than in ALT/WDLPS, similar to Adachi *et al.* (14), who found 48% and 29% of MDM2 and TP53 in ALT/WDLPS and 86% and 57%, in DDLPS, respectively. Co-expression of MDM2 and TP53 was associated with tumor location in our series, as well as in the study of Pilotti *et al.* (15), who found MDM2/TP53 co-expression only in centrally-located tumors and suggested that dedifferentiation of peripheral ALT/WDLPS might occur using a mechanism different from that in central tumors. In the current study, the unique peripheral DDLPS displayed a MDM2/P53 profile similar to that of centrally-located DDLPS, which does not support the "site-dependent" hypothesis by Pilotti *et al.* (15). Our findings on chromosome-12 alterations agreed with those reported by Tsuji *et al.* (16), who noted that numerical aberrations were more common in dedifferentiated areas.

Conclusion

Low-grade dedifferentiation provides intermediate risk between ALT/WDLPS and patients with high-grade dedifferentiation. Co-expression of proteins MDM2/CDK4/TP53 and high number of centromeric chromosome-12 copies are more common in DDLPS than in ALT/WDLPS but have limited prognostic significance. Our study supports that tumor location is a key clinical prognostic parameter in ALT/WDLPS and that, in some cases, low-grade dedifferentiation might represent a stage in ALT/WDLPS evolving into high-grade non-lipogenic sarcoma.

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Received August 16, 2006
Accepted September 19, 2006