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Altered Expression of Cell Cycle Regulatory Proteins in Benign and Malignant Bone and Soft Tissue Neoplasms

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Abstract. *Background: The binding of cyclins to cyclin-dependent kinases regulates cell proliferation. Overexpression of cyclins is believed to deregulate the cell cycle in human tumors. Here the expression of G1 cyclins D1 and D3, and of Ki-67 in a variety of bone and soft tissue sarcomas was assessed as compared to adjacent normal tissue and to a subset of leiomyomas. Materials and Methods: Twenty-nine human bone and soft tissue sarcomas were evaluated. Tissue sections from each case were subjected to immunostaining for cyclin D1, cyclin D3 and Ki-67 using the avidin-biotin complex method. Results: Cyclin D1 nuclear positivity was detected in 28% of sarcomas and in none of the leiomyomas. Cyclin D3 nuclear positivity was present in 62% of sarcomas and in none of the leiomyomas. Ki-67 nuclear staining was positive in 86% of sarcomas but in only 16% of leiomyomas. In addition, up-regulation of cyclin D1 was observed in leiomyosarcomas, pleomorphic sarcomas and gastrointestinal stromal tumors, but not in liposarcomas or osteosarcomas. Cyclin D3, however, was expressed in all of the sarcoma types including 2 out of 5 liposarcomas and 1 out of 4 osteosarcomas. The normal soft tissue adjacent to the tumors when present (10 cases) was negative for cyclin D1 and D3, and expressed Ki-67 in 5% of the cell nuclei. The expression of cyclin D3 was also noted in human sarcoma cell lines (SKLMS, MG63, SaOS-2 and HT1080) by Western blot. Conclusion: The higher expression of cyclin D1 and D3 and of Ki-67 in bone and soft tissue sarcomas, as compared to leiomyomas and peritumoral normal soft tissue, suggests that high cyclin expression may contribute to deregulation of the cell cycle in bone and soft tissue tumors. These data suggest a role of cyclins in the process of human sarcomagenesis.*

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Bone and soft tissue tumors are neoplastic proliferations of the extraskeletal, non-epithelial tissues of the body, exclusive of the reticuloendothelial system and glia. They are a very heterogeneous group of tumors which are classified according to the tissue of origin (*e.g.* muscle, fat, fibrous tissue, vessels and nerves) (1). They may arise in any location, but 40% of them arise in the lower extremities, 20% in the upper extremities, 10% in the head and neck, and 30% in the trunk and retroperitoneum (2). In general, the incidence of this type of tumor increases with age, however, specific types of sarcomas occur in certain age groups *e.g.* clear cell sarcoma of the kidney affects mainly pediatric patients after the first 6 months of life, while malignant fibrous histiocytoma (MFH) affects adult to elderly patients. Size, tumor stage, number of metastases and presence of necrosis are important diagnostic and prognostic indicators (3). The cause of most of these tumors is unknown, however a variety of physical and chemical factors, exposure to radiation, and inherited or acquired immunological defects have been implicated (4-6). Benign soft tissue tumors are about 100 times more common than their malignant counterpart (sarcomas) and are usually cured by surgical removal (7). Sarcomas on the other hand account for about 8,000 new cases diagnosed annually in the USA (8). Even when treated with surgery and/or other therapeutic approaches, such as radiation and/or chemotherapy, these malignant tumors have a poor outcome and are responsible for 2% of all cancer deaths (8).

In recent years, cytogenetic and molecular testing have provided significant insight into the biology of soft tissue tumors. Specific chromosomal abnormalities and genetic

alterations can now be used as diagnostic markers and also provide information about their genesis and prognosis (9).

Cell proliferation depends on cell cycle regulation and one of the most common molecular alterations detected in human soft tissue tumors are those of gene products regulating cell cycle progression. The major regulators of cell cycle progression include four cyclins (A, B, D and E). These cyclins interact with cyclin-dependent kinases to link signaling from growth factors to the cell cycle machinery. In particular, cyclin D together with cyclin-dependent kinases are responsible for the G1/S transition by phosphorylating the retinoblastoma protein, which then releases transcription factors important for the initiation of DNA replication (10,11).

Cyclin D is actually a family of three related proteins, cyclin D1, D2 and D3. Withdrawal of the mitogenic stimulus prior to the passage through the G1/S restriction point induces degradation of cyclin D by Ca^{++} activated calpain and terminates the progression through the cell cycle (12).

While the expression of cyclin D1 has been reported to be up-regulated in several neoplasms, including hematological malignancies (13), breast (14) and pancreatic carcinomas (14), and sarcomas (15, 16), reports of cyclin D3 overexpression in tumors are lacking.

In this study, the expressions of cyclin D1 and D3 were tested for their relationship with the expression of the Ki-67 antigen. Ki-67 has been found to be a statistically significant marker of rapid tumor growth in leiomyosarcomas (18) and a Ki-67 index higher than 20% seems to be a strong independent prognostic factor for metastasis in pleomorphic soft tissue sarcomas (19). Furthermore, it was observed that Ki-67 expression correlated with tumor grade of soft tissue sarcomas (20).

We report the expression of cyclin D1 and cyclin D3 in a group of human sarcomas as compared to the adjacent normal soft tissue and to a set of benign neoplasms of the soft tissue. The expression of cyclin D1 and cyclin D3 was compared to that of Ki-67 as a marker of cell proliferation.

Materials and Methods

This study was carried out in accordance with the research protocol approved by the Institutional Review Board at the Moffitt Cancer Center and Research Institute.

Tissue samples and patient data. This study includes 36 patients who underwent resection of their bone and soft tissue neoplasms at the H. Lee Moffitt Cancer Center & Research Institute (HLMCC), Tampa, FL, USA between 2004 and 2006. The neoplasms included 4 bone sarcomas, 25 soft tissue sarcomas and 6 benign soft tissue neoplasms (uterine leiomyomas). In addition, corresponding normal adjacent soft tissue was present for evaluation in 10 of the 29 sarcomas and in all of the leiomyomas.

Pertinent clinical data for the 29 sarcoma patients were compiled and included patient age, sex, tumor size and location. Pertinent pathological data were tabulated including histological tumor type and histological grade (Table I). The cases were randomly selected and the H&E slides of all cases were reviewed independently by two pathologists (MB, DC) for confirmation of the histopathological findings and selection of blocks for immunostaining.

Immunohistochemistry. Sections from the resected tumors were submitted for histological examination. Each sample was fixed in 10% neutral buffered formalin for 9 h. After fixation, the tissue samples were processed into paraffin blocks. The primary antibodies used in this study were rabbit monoclonal (D1) and mouse monoclonal (D3 and Ki-67) antibodies raised against the corresponding antigens of human origin in paraffin-embedded sections. Immunohistochemical staining was performed on a Ventana BenchMark XT (Tucson, AZ, USA) automated slide stainer, using 4-μm thick paraffin sections from each of the representative tumor blocks selected. The sections were deparaffinized, rehydrated and incubated with 3% H_2O_2 to block endogenous peroxidase. Following antigen unmasking using proprietary CC1 solution for 60 min online (standard) at 100˚C, the sections were incubated with antibodies to cyclin D1 (proprietary dilution; Cell Marque, Rocklin, CA, USA), D3 (1:20 dilution; Novocastra, Norwell, MA, USA) and Ki-67 (proprietary dilution; Ventana, Tucson, AZ, USA). The incubation times were 32 min for cyclin D1, 2 hours for cyclin D3 and 32 minutes for Ki-67, according to the manufacturer's instructions. The sections were then subjected to biotin block using a Ventana Endogenous Biotin Kit (Ventana, Tucson, AZ, USA). A solution of 3,3'-diaminobenzidene tetrahydrochloride (Sigma, St. Lois, MO, USA) was used as a chromogen followed by the addition of sodium azide and 20 μl of H_2O_2 in 100 ml of Tris-HCl (50 μM, pH 7.6). After light counterstaining with Harris' hematoxylin, the sections were examined under light microscopy.

Interpretation of immunohistochemical findings. The sections stained for Ki-67, cyclin D1 and cyclin D3 protein were independently evaluated by both pathologists (MB, DC) without prior knowledge of the clinicopathological data. These evaluations were made in the most representative and viable areas of the stained tumor sections. Both qualitative and semi-quantitative results were determined for each case based on nuclear/cytoplasmic expression of the proteins.

The tumors were considered cyclin D1 and D3 proteins-positive if ≥10% tumor cells showed unequivocal immunostaining. This cut-off was used in order to avoid interpreting cases with borderline / focal immunostain (≥10% tumor cells) as positive. Such an approach has previously been utilized by other experienced investigators for good reproducibility and clinical correlations in similar studies (21). For Ki-67, tumors were considered positive if >5% of the tumor cells stained. All three immunostains were nuclear in location. Any difference of interpretation was resolved by joint immunohistochemical review by the two pathologists.

All of the above studies were performed with appropriate positive controls. For cyclin D1 breast cancer tissue was used, and for cyclin D3 and Ki-67 tonsil tissue was used as per the manufacturer's instructions. The negative control included tissue sections incubated with normal rabbit serum.

Cell line evaluation. The expression of cyclin D1 and cyclin D3 was also evaluated using western blot in the following human sarcoma

Table I. *Clinical pathological features.*

Case	Age (years)	Gender	Tumor location	Tumor size (cm)	Diagnosis	Broder's tumor grade
1	75	M	Thigh (L)	3.0	Pleomorphic sarcoma	High
2	84	F	Chest wall (L)	8.5	Undifferentiated sarcoma	High
3	61	F	Uterus	8.5	Pleomorphic sarcoma	High
4	86	M	Thigh (R)	8.4	Leiomyosarcoma	Intermediate
5	61	M	Calf (R)	8.0	Leiomyosarcoma	Intermediate
6	67	F	Thigh (R)	7.7	Leiomyosarcoma	Intermediate
7	74	F	Retroperitoneum	13.0	Leiomyosarcoma	High
8	36	M	Groin (R)	8.3	Osteosarcoma	High
9	60	F	Uterus	21.0	Leiomyosarcoma	High
10	14	M	Femur (R, distal, bone)	19.0	Osteosarcoma	High
11	77	F	Lung (L, upper lobe)	5.0	Leiomyosarcoma	High
12	74	F	Femur (L, distal, bone)	9.8	Osteosarcoma	High
13	37	F	Femur (L, distal, bone)	8.0	Pleomorphic sarcoma	High
14	66	M	Acetabulum (bone)	5.5	Osteosarcoma	Intermediate
15	69	F	Retroperitoneum	30.0	Liposarcoma	High
16	45	M	Knee (R)	7.0	Liposarcoma	High
17	40	M	Chest wall (L)	6.7	Liposarcoma	Low
18	71	M	Pelvis	12.0	Liposarcoma	Low
19	45	F	Thigh (R)	9.5	Liposarcoma	Low
20	77	M	Stomach	14.0	GIST	High
21	46	M	Sigmoid colon	8.0	GIST	High
22	42	M	Colon	1.0	GIST, recurrent	High
23	80	F	Small bowel	4.5	GIST	Low
24	84	F	Stomach	8.3	GIST	Low
25	58	F	Stomach	1.0	GIST	Low
26	59	M	Small bowel	12	GIST	High
27	79	M	Stomach	2.5	GIST, recurrent	High
28	73	M	Stomach	2.0	GIST	Low
29	33	F	Stomach	7.4	GIST, recurrent	High

cell lines: SKLMS (leiomyosarcoma), MG63 and SaOS-2 (osteosarcoma) and HT1080 (fibrosarcoma). All of the cell lines were procured from the American Type Culture Collection (ATCC).

Preparation of cell extracts and Western blotting. Growing cells were maintained in Dulbecco's modified essential medium supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37˚C in a humidified incubator containing 5% CO_2. The cells cultures were rinsed twice in ice-cold phosphate-buffered saline, harvested by scraping and collected by centrifugation. The pellets were resuspended in lysis buffer (50 mM HEPES (pH 7.5), 100 mM NaCl, 2 mM EDTA, 0.5% NP-40, 10% glycerol, 0.1 mM sodium orthovanadate, 0.5 mM NaF, 0.1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml of leupeptin and 1 mM dithiothreitol), vortexed three times during 30 min incubation on ice. Insoluble material was removed by centrifugation. Cell extracts (100 μg) were boiled in Laemmli buffer (20% glycerol, 3% sodium dodecyl sulfate (SDS), 4% β-mercaptoethanol, 0.5% bromophenol blue) and run on 11% SDS-polyacrylamide gels. Resolved proteins were electrophoretically transferred to nitrocellulose membranes. The protein bound membranes were first blocked in PBST (phosphate-buffered saline plus 0.1% Tween 20) containing 5% instant milk for 1 h at room temperature and then incubated with antibody in PBST containing 5% milk for 16 h in a cold- room. Proteins recognized by the antibody were detected by enhanced chemiluminescence using a horseradish peroxidase-coupled secondary antibody as specified by the manufacturer (Pierce, Rockford, IL, USA). For the Western blot we used a cyclin D3 (monoclonal) antibody purchased from BD Transduction Laboratories (BD Biosciences, San Jose, CA, USA) and a cyclin D1 (polyclonal) antibody generated against a KLH conjugated peptide (EVEEEAGLACTPTDVRDVDI), corresponding to the *C*-terminus of cyclin D1.

Statistical analysis. Statistical analysis for the scores was performed for each group. The Wilcoxon Rank Sum test was used to compare expression of cyclin D1, cyclin D3 and Ki-67 between sarcomas *versus* leiomyomas, sarcomas *versus* normal tissues (adjacent to tumors), and leiomyomas *versus* normal tissues (adjacent to tumors). The Bonferroni-Holm correction for multiple testing was carried out to adjust the *p*-values using SAS software (Cary, N.C. USA).

Results

Clinical pathological data. The patients with sarcomas included 14 females and 15 males, aged 14-86 years (mean 61 years). The tumor size was between 1 and 30 cm (mean

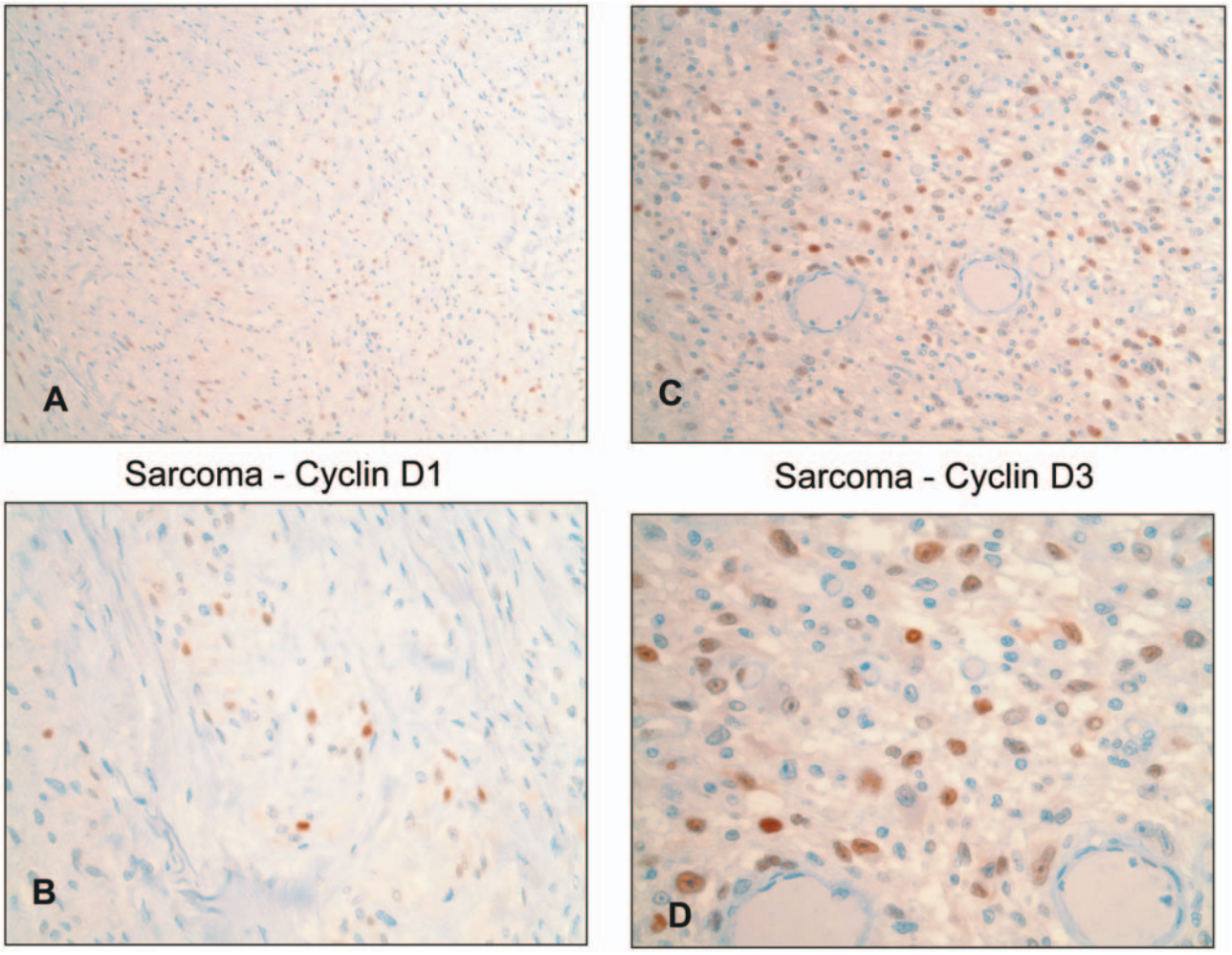

Figure 1. *Expression of cyclin D1 and D3 in sarcomas. A leiomyosarcoma exhibiting focal immunoreactivity for cyclin D1 (A, x100; B, x400) and strong nuclear cyclin D3 positivity in approximately 40% of the tumor cells (C, x100; D, x400).*

8.8 cm). Eighteen tumors were high grade, 4 intermediate and 7 low grade. The tumors included 6 leiomyosarcomas, 4 pleomorphic undifferentiated sarcomas (malignant fibrous histiocytomas), 4 osteosarcomas, 5 liposarcomas and 10 malignant gastrointestinal stromal tumors (GIST) (Table I).

Immunohistochemistry. Cyclin D1 protein expression was detected in the nuclei of 8 of the 29 sarcomas (Figure 1A, B) and in none of the benign neoplasms (Figure 2A, B). Cyclin D3 was found in the nuclei in 18 out of 29 sarcomas (Figure 1C, D), and in none of the 6 leiomyomas (Figure 2C, D). Ki-67 nuclear positivity was identified in 25 out of 29 sarcomas (Figure 3A, B), but in only 1 of the 6 leiomyomas (Table II).

The normal soft tissue adjacent to the tumors when present (10 cases) was negative for cyclin D1 and D3, and expressed Ki-67 in <5% of the cell nuclei.

Table II. *Immunohistochemical results.*

	Sarcomas	Leiomyomas	Normal soft tissue
D1	8/29	0/6	0/10
D3	18/29	0/6	0/10
D1+/D3+	6/29	0/6	0/10
D1–/D3–	9/29	6/6	10/10
Ki-67	25/29	1/6	0/10

When comparing the expression of cyclin D1 and D3 proteins it became apparent that the two proteins were differentially expressed in some types of the sarcomas studied. For example, cyclin D3 was found in 18/29 of the sarcomas investigated, but cyclin D1 was present in only 8/29 of them. Only 6/29 tumors were positive for both

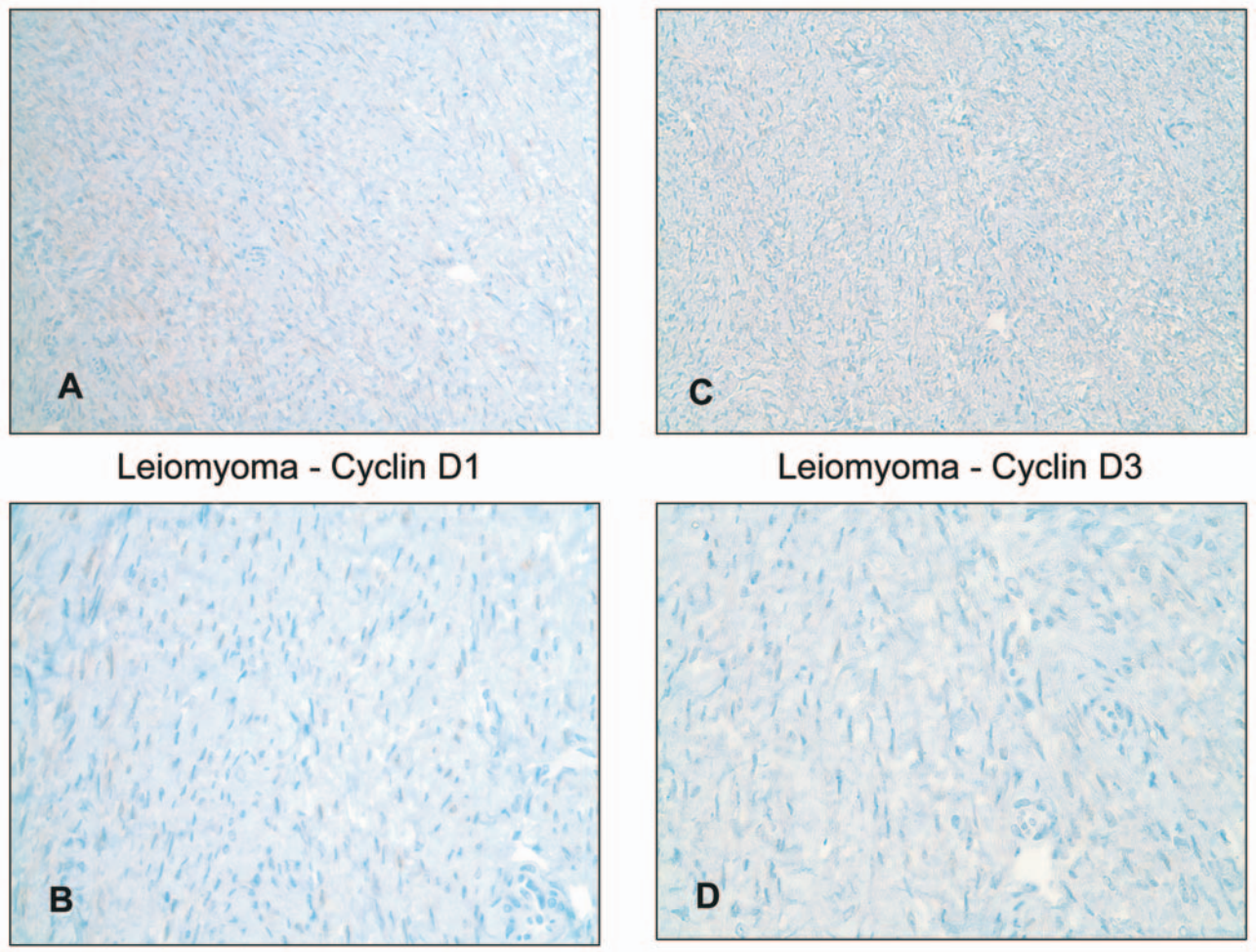

Figure 2. *Expression of cyclin D1 and D3 in leiomyomas. A leiomyoma showing lack of staining for both cyclin D1 (A, x100; B, x400) and D3 (C, x100; D, x400).*

cyclins (Table II: D1+/D3+), while 9/29 tumors were negative for both cyclins (Table II: D1–/D3–). When the type of sarcoma was considered, it became apparent that cyclin D3 protein was expressed in 9 of 10 GISTs evaluated (Figure 4C, D), but cyclin D1 expression was negative in 7 of them (Figure 4A, B). In addition, cyclin D1 was found to be expressed in leiomyosarcomas and in malignant fibrous histiocytomas, but in none of the liposarcomas or osteosarcomas studied. Cyclin D3, however, was found to be expressed in 2 of 5 liposarcomas and in one of 4 osteosarcomas.

Table III. *Statistical analysis: Group comparison*

	p-value*		
Groups	Cyclin D1	Cyclin D3	Ki-67
Sarcomas *vs*. leiomyomas	0.0208	0.0026	0.0174
Sarcomas *vs*. normal	0.0061	<0.0001	<0.0001
Normal *vs*. leiomyomas	1.0	1.0	1.0

*Wilcoxon rank sum test, adjusted for Bonferroni-Holm correction.

Cell line evaluation. When compared to the immunohistochemical data from the tumor tissue samples, the cell lines revealed similar distribution in the expression of cyclin D1 and D3. The Western blot analysis showed that the leiomyosarcoma and the fibrosarcoma cell lines (SKLMS and HT1080, respectively) expressed comparable amounts of cyclin D1 and cyclin D3 (Figure 5). Interestingly, both osteosarcoma cell lines revealed expression of cyclin D3 but not of cyclin D1.

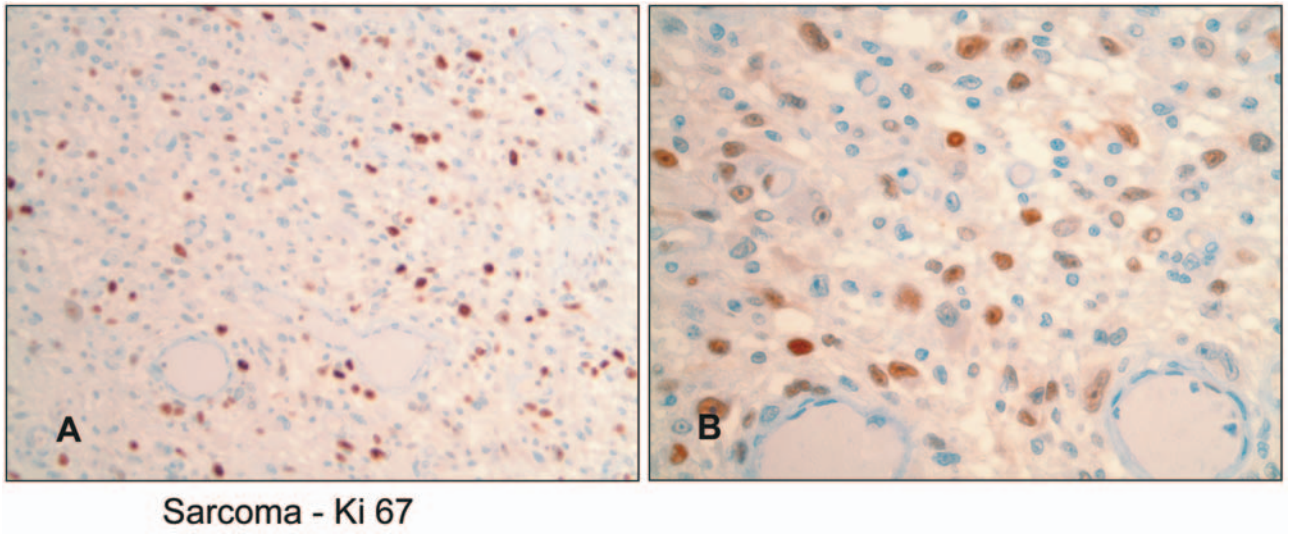

Figure 3. *Expression of Ki-67 in leiomyosarcomas. A leiomyosarcoma showing Ki-67 nuclear positivity in approximately 10-15% of the tumor cells (A, x100; B, x400).*

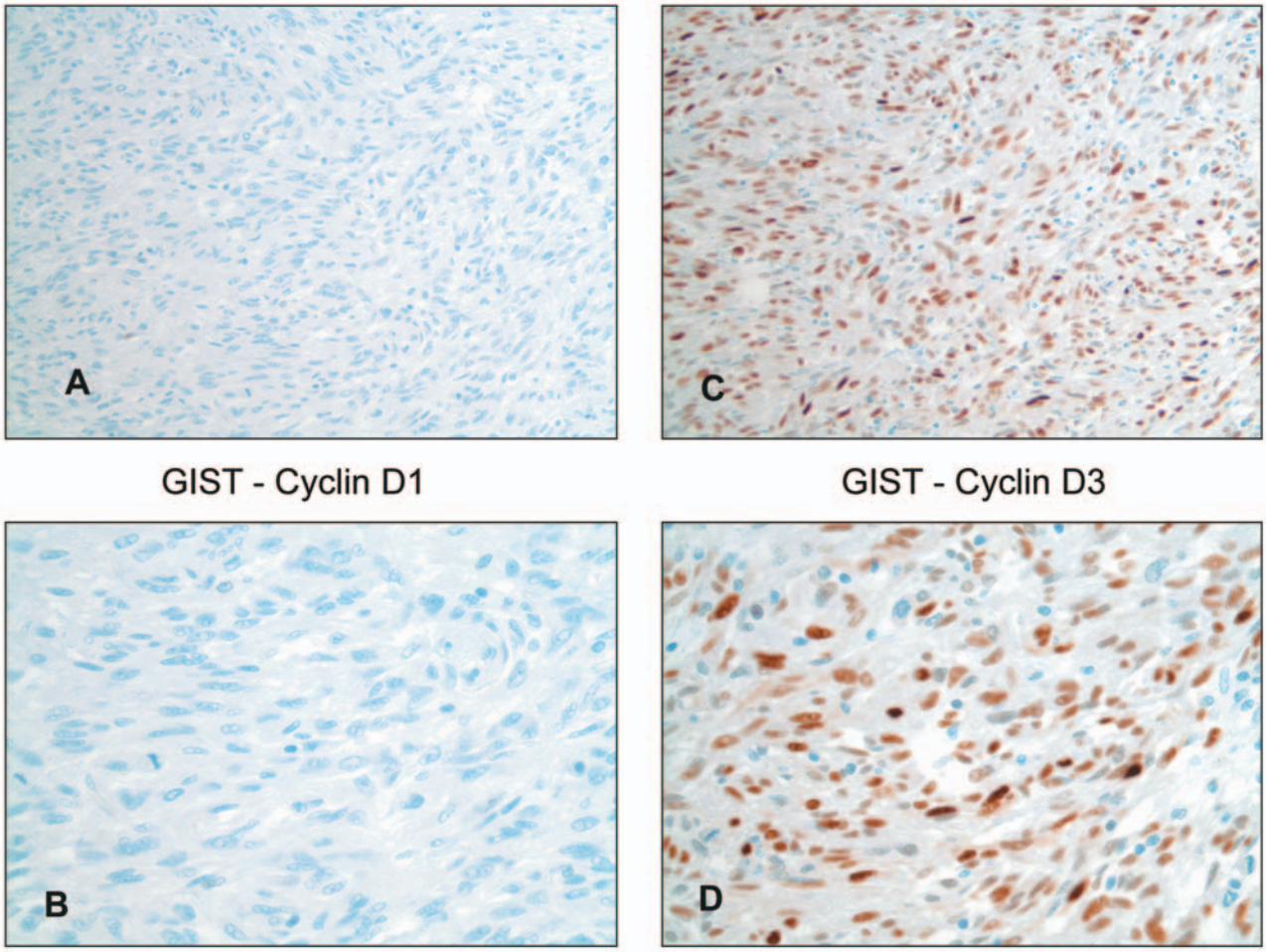

Figure 4. *Expression of cyclin D1 and D3 in GISTs. A gastrointestinal stromal tumor negative for cyclin D1 (A, x100; B, x400), but exhibiting cyclin D3 nuclear positivity in a large percentage of the tumor cells (C, x100; D, x400).*

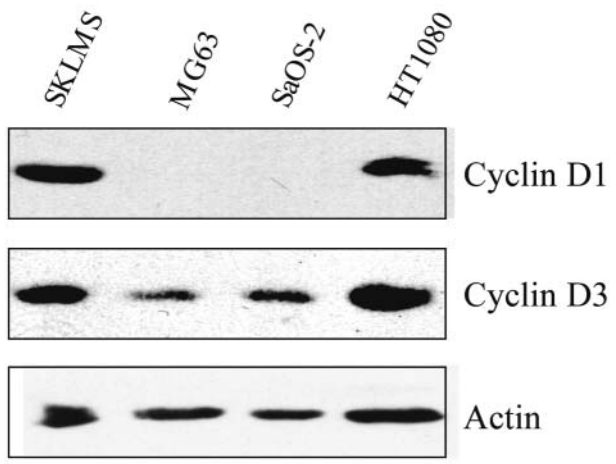

Figure 5. *Expression of cyclin D1 and cyclin D3 in sarcoma cell lines. Western blot analysis of cyclin D1 and D3 protein expression in sarcoma cell lines, as compared to the actin control. SKLMS: leiomyosarcoma; MG 63 and SaOS-2: osteosarcomas; HT1080: fibrosarcoma.*

Statistical analysis. The results of the statistical analysis are reported in Table III. The data indicate that the expression of cyclin D1, cyclin D3 and Ki-67 in sarcomas was greater than that in leiomyomas, and also than that in normal tissues (adjacent to tumors), for all three markers. All of these differences were statistically significant. However, no statistically significant differences were observed in the expression of cyclin D1, cyclin D3 or Ki-67 between normal tissues (adjacent to tumors) and leiomyomas.

Discussion

In this study we show the significant increase in cyclin D1 and D3 protein expression in a variety of human bone and soft tissue sarcomas as compared to the adjacent normal soft tissue and to a subset of benign soft tissue neoplasms (uterine leiomyomas). The proliferative rate, measured by Ki-67, was, as expected, higher in malignant sarcoma as compared to normal soft tissue and benign soft tissue neoplasms.

The cyclin D1 gene, *CCND1*, is located on chromosome 11q13 and is amplified in a variety of tumors (22). Interestingly, some malignancies are characterized by chromosomal translocations, *e.g.* t(11;14)(q13;32) in lymphoproliferative disorders, causing the rearrangement of *CCND1* and consequent overexpression of cyclin D1 protein (13, 15, 23). In sarcomas, accumulation of cyclin D1 has been described in the absence of *CCND1* locus rearrangements (24, 25). It has been shown that the synthesis of cyclin D1 protein may also be induced by activation of transcription factors and it may not require *de novo* mRNA synthesis. This supports the view that in some tumors, including sarcomas, cyclin D1 protein up-regulation may be mediated by transcriptional or post transcriptional mechanisms (23, 26). Whatever the mechanism, accumulated cyclin D1 will bind and activate the cyclin-dependent kinases CDK4 and CDK6, leading to phosphorylation of the retinoblastoma protein and release of the transcriptional activator E2F. E2F will eventually induce transcription and expression of important cell cycle regulatory proteins associated with the transition from G-1 to the S-phase of the cell cycle (27).

Prior reports have shown that overexpression of cyclin D1 in soft tissue sarcoma of extremities is associated with poor prognosis (16). In our study, we show that the expression of cyclin D1 was identified in 8/29 cases and that of cyclin D3 in 18/29 cases. In addition, we observed high expression of cyclin D1 in leiomyosarcomas and malignant fibrous histiocytomas, but not in liposarcomas or osteosarcomas. This finding is in agreement with previous reports describing overexpression of cyclin D1 as a rare event in liposarcomas (28) and osteosarcomas (29). Both cyclin D1 and cyclin D3 expression was higher in malignant soft tissue tumors as compared to the adjacent normal counterpart suggesting a pathogenetic role of these proteins in sarcomagenesis. In addition, the lack of expression of cyclin D1 and D3 proteins in leiomyomas supports the view that these tumors are not a precursor of the malignant counterpart.

Cyclin D3 is another regulator of the G1 check-point of the cell cycle and has sequence homology with cyclin D1 and similar function (30, 31). However, cyclin D3 seems to have additional functions depending on the cell system: in the fetus, for example, cyclin D3 is involved in the developmental phases of specific organs and has been linked to cell differentiation (32); conversely, in normal adult tissues, cyclin D3 seems to be expressed in terminally differentiated cells and in the proliferative compartment of lymphoid tissues (33).

The observation of cyclin D3 expression in a large proportion of sarcomas is a novel finding. A few previously published studies have reported cyclin D3 expression only in a small percentage of human breast, colorectal, head and neck and uterine carcinoma. When present, the expression of cyclin D3 in these tumors was described as heterogeneous and scanty (34). In one study, the investigators reported strong and diffuse cyclin D3 immunostain in gastrointestinal stromal tumors (35). We observed a similar finding here, where the majority of the GIST tumors exhibited strong and diffuse cyclin D3 expression. This finding is in agreement with published data describing up-regulation of cyclin D3 by stem cell factor/c-kit (36).

In contrast to cyclin D1, the cyclin D3 antibody was found in a fraction of liposarcomas and osteosarcomas, suggesting that cyclin D1 and D3 may be differentially expressed in sarcoma subtypes. A similar finding was observed while investigating the expression of cyclin D1 and D3 in liposarcoma and osteosarcoma cell lines, using Western blot analysis.

Expression of cyclins D1 and D3 was associated with the expression of the antigen Ki-67 in the same tumoral tissues. The Ki-67 antigen identifies proliferating cells (cycling cells present in G1-, G2-, M- and S-phases of the cell cycle) (37-39). In this study, significant Ki-67 positivity (>5%) was identified in the majority of the tumors (25/29) but in none of the peritumoral normal tissues, and was associated positively with cyclin D3 expression. Ki-67 positivity was not related to cyclin D1 expression.

Conclusion

The high expression of cyclin D1 and D3 and of Ki-67 in human bone and soft tissue sarcomas found in this study suggests that these proteins may play a pivotal role in sarcomagenesis.

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