Overexpression of Cyclin D1 Is Associated with Poor Prognosis in Extremity Soft-Tissue Sarcomas

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ABSTRACT

Binding of G1 cyclins to cyclin-dependent kinases leads to phosphorylation of the retinoblastoma protein and progression through G1 and S phases of the cell cycle. Overexpression of cyclins is thought to deregulate this process and has been noted in many human malignancies. This study was conducted to assess patterns of expression and potential gene amplification of the G1 cyclins in 84 patients affected with extremity soft-tissue sarcomas. Sixty cases were primary tumors, whereas the remaining 24 cases were locally recurrent lesions. There were 58 high-grade and 26 low-grade tumors. Immunohistochemical analyses were conducted using antibodies to cyclins D1, E, and A. Southern blot analysis was performed on DNA available from 53 of 84 patients with a cyclin D1-specific probe. Cyclin D1 overexpression was noted in 23 of 79 informative cases (29%), whereas cyclin E was found overexpressed in 26 of 80 cases (33%) and cyclin A overexpression was observed in 9 of 81 cases (11%). Overexpression of cyclins D1, E, or A each correlated significantly with high tumor grade (P <0.05). On multivariate analysis, neither cyclin E nor cyclin A were significant predictors of outcome. However, overexpression of cyclin D1 was significantly associated with worse overall survival for the entire group as well as in the subset of high-grade lesions (P <0.05), notwithstanding the relatively short follow-up time (mean, 2.4 years). Nevertheless, the presence of a significant association between laboratory data and outcome implies that the study is adequately powered. Furthermore, none of the cases demonstrated CCND1 gene amplification. These data support the concept that cyclin D1 overexpression determines the evolution of a particularly aggressive subset of these lesions.

INTRODUCTION

Cyclins are the regulatory components of the catalytic complexes that control cell cycle progression. The G1 cyclins include the members of the cyclin D family (D1, D2, and D3), cyclin E, and cyclin A. D-type cyclins bind to Cdk4 and Cdk6, whereas cyclins E and A are associated with Cdk2. Phosphorylation of the retinoblastoma protein by these complexes leads to the release of a variety of transcription factors, usually represented by E2F family members, and is considered the major driving event in the transition from G1 to S phase of the cell cycle (1, 2).

CCND1 was the first gene of the cyclin family to be implicated in neoplastic transformation. Located on chromosome 11q, it was initially noted to undergo frequent amplification in breast cancers (1). Furthermore, PRAD1, a gene overexpressed in parathyroid adenomas, was found to result from a chromosomal translocation that placed the regulatory sequences for the parathyroid hormone gene adjacent to the coding sequences for cyclin D1 (3, 4). Since then, amplification and/or overexpression of cyclin D1 has been noted in a diverse group of tumors, including cancers of the breast, lung, esophagus, and colorectum (5). Transgenic mice engineered to overexpress cyclin D1 in breast tissue develop ductal hyperplasia, which eventually progresses to mammary neoplasia (6). Similar phenomena occur in cyclin E transgenics (7), and overexpression of this regulator has been noted in breast, gastrointestinal, endometrial, and ovarian cancers (8–12). Cyclin A alterations were first noted in hepatocellular carcinomas (13) and have also recently been identified in prostate tumors (14).

Data on alterations of cyclin expression in soft-tissue sarcomas is limited. Maelandsmo et al. (15) examined 109 human sarcoma samples and found increased levels of cyclin D1 mRNA in 37 cases. However, previous reports did not examine clinicopathological correlates or the association between cyclin overexpression and survival in soft-tissue neoplasms. In the present study, we limited our analysis to a group of extremity soft-tissue tumors that have been clinically and pathologically well characterized. Using a combination of genetic assays and immunohistochemistry, we identified subsets of these tumors that overexpress cyclins D1, E, and/or A. The purpose of this study was to elucidate whether cyclin overexpression correlated with outcome in patients with extremity soft-tissue sarcoma.

MATERIALS AND METHODS

Clinical and Pathological Data. The cohort analyzed consisted of well-characterized adult extremity soft-tissue sarcomas from 84 patients treated at MSKCC between March 1993
and February 1996. Tissue sections of each specimen were stained with H&E and examined microscopically by a single reference pathologist (J. M. W.). All cases were reviewed to evaluate histopathological diagnosis, tumor grade, and quality of the tissue. Pediatric (<16 years of age) and bone tumors, as well as those of uncertain histopathology, were excluded. In addition, only primary or locally recurrent tumors were examined; no metastatic lesions were included in this study.

The median age of the cohort was 53 years (range, 16–90 years). There were 60 primary tumors and 24 local recurrences. The most common histological subtype was malignant fibrous histiocytoma (n = 25; 30%), followed by liposarcoma (n = 21; 25%) and fibrosarcoma (n = 14; 17%). All tumors were deep lesions, i.e., below the level of muscular fascia. The majority of tumors (n = 58; 69%) were high-grade as determined by cellularity, extent of necrosis, cytological atypia, and number of mitoses seen in the specimen (16). The size distribution of the tumors was as follows: <5 cm (n = 13; 16%), ≥5 cm but <10 cm (n = 39; 46%), and ≥10 cm (n = 32; 38%).

Median follow-up for the entire group at the time of correlating laboratory results and clinicopathological data was 2.4 years. Disease-free and overall survival were defined as time from primary tumor resection to first recurrence (either local or distant) or death from disease, respectively. The mean overall survival for patients who had primary tumors resected at MSKCC (n = 60) was 3.9 years, with an actuarial 3-year survival of 81%. The mean disease-free survival for patients with primary tumors operated on at MSKCC was 2.7 years (median, 3.6 years), with an actuarial 3-year disease-free survival of 52%.

Tissue and Immunohistochemistry. Tumor samples were obtained fresh after surgical resection, embedded in a cryopreservative solution (OCT Compound; Miles Laboratories, Elkhart, IN), snap-frozen in liquid nitrogen, and stored at −70°C. Five micron frozen sections were fixed in formalin and used for immunohistochemical analysis (17). Tissues and cell lines, which were known to have amplification and/or overexpression of each of the cyclins, were used as positive controls. Sections were first incubated with 10% normal horse serum for 30 min, and then primary antibodies were applied for 1–2 h. Mouse monoclonal antibodies to human cyclin D1 (Ab-3, clone DCS-6; Oncogene Research Products, Cambridge, MA) and cyclin A (clone BF683; PharMingen, San Diego, CA) were used at 5 μg/ml. Anti-cyclin E rabbit purified antisera was used at a dilution of 1:250 (a gift from Dr. A. Koff, MSKCC). Using similar conditions, the mouse monoclonal antibody MlgS-Kpl and rabbit preimmune serum were used as negative controls. Samples were then incubated with biotinylated antimouse or antirabbit immunoglobulins at 1:500 or 1:1000 dilution (Vector Laboratories, Inc., Burlingame, CA), followed by avidin-biotin peroxidase complexes (1:25; Vector Laboratories, Inc.) for 30 min. Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain. Nuclear immunoreactivities were classified as a continuum data (undetectable levels or 0% to homogenous staining or 100%). The cutoff value of <5% positive tumor nuclei was selected based on previous reports and the observation that high proliferative compartments in normal tissues, such as the germinal center of lymph nodes and crypt of colonic mucosa, displayed <1% cyclin D1 positive cells. In addition, statistical analysis revealed 5% positive tumor cell nuclei to be an appropriate standard (see below). Tumors were then grouped into two categories defined as follows: group A (0% or undetectable staining to <5% nuclear immunoreactivity in tumor cells) and group B (neoplasms with ≥5% tumor cells with nuclear staining; see statistical section).

DNA Isolation and Southern Blot Hybridization. Tissue for DNA extraction was available in sufficient quantity for Southern blotting in 53 of 84 tumors (39 primary tumors, 14 local recurrences). DNA was extracted using the Oncor Nonorganic DNA Extraction Kit (Oncor, Inc., Gaithersburg, MD) and was digested overnight with EcoRI. After electrophoresis on a 0.9% agarose gel, samples were vacuum-transferred to nylon hybridization membranes (Sure Blot; Oncor, Inc.). Southern blot analysis for CCND1 amplification was performed using a 1.3-kb cDNA probe labeled with 32P-CTP using the Klenow fragment of Escherichia coli DNA polymerase I. Hybridization was performed overnight at 42°C in Hybrisol I solution (Oncor, Inc.), and membranes were washed twice with 0.1 × SSC/0.1% SDS at 65°C for 1 h. Blots were then analyzed on a phosphorimager to quantify signal intensities. Membranes were then stripped and reprobed with a 1.3-kb cDNA probe for β-actin to control for sample loading. Tumors were designated as having CCND1 amplification if there were at least three copies of this gene per copy of β-actin.

Statistical Analysis. Associations between clinicopathological parameters and laboratory data were studied using Fisher’s exact test (18). Statistical analysis revealed 5% positive tumor cell nuclei to be the most appropriate standard for this cohort of soft-tissue sarcomas. Survival analysis was performed by the method of Kaplan and Meier (19), and statistical significance (P ≤0.05) was evaluated by log rank testing for univariate analysis (20) and by the Cox regression model for multivariate analysis (21).

RESULTS

Neither histopathology nor tumor size affected disease-free or overall survival in this study. High tumor grade was a significant factor associated with decreased overall survival (P = 0.05). In addition, high tumor grade also predicted earlier disease recurrence (either local or distant; P <0.05; Fig. 1).

Overexpression of each of the three cyclins examined in our study was noted in a subset of the extremity sarcomas. Tumors with ≥5% positive nuclei were classified as overexpressing the protein (group B; Fig. 2). Only tumors with adequate tissue for immunohistochemistry were included in the analysis. Increased levels of cyclin D1 were found in 23 of 79 samples (29%); cyclin E was overexpressed in 26 of 80 samples (33%); and cyclin A was overexpressed in 9 of 81 samples (11%; see Table 1). No significant relationship was noted between histological type and overexpression of each of the cyclins. There was a clear association between high tumor grade and overexpression of each cyclin (P < 0.05). However, no correlation was noted with tumor size.

We next examined the effect of cyclin overexpression on patient outcome. For this analysis, only the patients with pri-
Primary tumors were examined \( (n = 60) \). Overexpression of cyclin E \( (17 \text{ of } 58 \text{ informative primary tumors; } 29\%) \) did not affect survival (the denominator is \(<60\) due to the preservation artifact in some of the samples, which precluded interpretable staining). However, overexpression of either cyclin D1 \( (18 \text{ of } 57 \text{ primary tumors; } 32\%) \) or cyclin A \( (7 \text{ of } 59 \text{ primary tumors; } 12\%) \) was significantly associated with decreased overall survival on univariate analysis \( (P = 0.02 \text{ and } P = 0.05, \text{ respectively; Fig. 3}) \).

Subsequent multivariate analysis including tumor grade, size, cyclin D1, and cyclin A revealed that only cyclin D1 remained a significant predictor of overall survival in the cohort of patients studied \( (\text{see Table 1}) \). When only the high-grade lesions were examined \( (n = 44) \), there was a trend toward worse overall survival in patients with high levels of cyclin D1 \( (n = 17; P = 0.08) \). Only one of the low-grade lesions analyzed showed cyclin D1 overexpression. Metastasis-free survival was also significantly decreased in patients whose tumors overexpressed cyclin D1. These patients had a mean time to distant recurrence of 2.0 years compared with 3.2 years for those without overexpression \( (P <0.05) \).

Patients with sarcomas overexpressing combinations of the three cyclins were analyzed to see if they had a worse prognosis \( (\text{see Table 1}) \). Less than one-third of the tumors overexpressing cyclin D1 overexpressed another cyclin as well. Although the sample size was limited, co-overexpression of cyclin D1 with either cyclins A or E did not worsen prognosis compared with patients who expressed cyclin D1 alone. Similarly, if cyclin D1-expressing lesions are excluded, patients with overexpression of both cyclin A and E did not have a worse outcome than patients with tumors with no cyclin overexpression or with overexpression of either cyclin by itself. Thus, no additive detrimental effect on prognosis seems to exist due to overexpression of combinations of cyclins within a single lesion.

Southern blot analysis on DNA extracted from 53 cases was conducted to assess if cyclin D1 overexpression was accompanied by \( CCND1 \) amplification. Genomic DNA was di-
gested with EcoRI and probed with a 1.3-kb CCND1 cDNA probe resulting in three fragments of 4.0, 2.2, and 2.0 kb. After normalization against β-actin signals, none of the lesions examined were found to have amplification of CCND1 (Fig. 4). This included 12 sarcomas that were determined immunohistochemically to overexpress the gene product. Ten of these 12 tumors displayed 20% nuclear labeling for cyclin D1.

**DISCUSSION**

We found increased nuclear levels of cyclins D1, E, and A in different subsets of extremity soft-tissue sarcomas. However, only high cyclin D1 levels predicted poorer survival on multivariate analysis. Although the follow-up time was relatively short (mean, 2.4 years), the presence of a significant association between laboratory data and outcome implies that the study is adequately powered. The lack of accompanying CCND1 amplification is consistent with data from Maelandsmo et al. who reported gene amplification in only 4 of 109 (4%) human sarcomas they studied, despite the fact that 37 (34%) demonstrated elevated cyclin D1 mRNA transcripts (15). Thus, overexpression likely occurs at the transcriptional, translational, and/or posttranslational levels in these tumors. A similar phenomenon occurs in melanomas, as well as colorectal and uterine cancers, tumor types for which low incidences of CCND1 amplification but high frequencies of cyclin D1 overexpression (22) has been reported.

In contrast to cyclin D1, few human tumors have been identified with amplification and/or overexpression of cyclin A. Alterations of cyclin A have been found in hepatocellular cancers (13), and a recent report has demonstrated an association between overexpression and increased rates of disease relapse in patients with prostate cancer (14). Elevated nuclear levels of cyclin A were found in 11% of the extremity soft-tissue sarcomas analyzed in our study. All of these tumors were high-grade lesions (P <0.05). A significant survival disadvantage for patients with cyclin A-overexpressing tumors was noted on uni-

**Table 1** Results of cyclin immunohistochemistry

Overexpression of one or more of the cyclins was correlated with tumor grade and effect on overall survival in patients with extremity soft-tissue sarcoma.

<table>
<thead>
<tr>
<th>Cyclin(s)</th>
<th>Status</th>
<th>n</th>
<th>Median fraction of nuclei labeled</th>
<th>Correlation(^a) with high-grade</th>
<th>Univariate analysis of effect on survival</th>
<th>Multivariate analysis of effect on survival(^b)</th>
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<tr>
<td>D1</td>
<td>Negative</td>
<td>56</td>
<td>1% (0-3%)</td>
<td>P &lt; 0.05</td>
<td>P = 0.02</td>
<td>P = 0.05</td>
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<tr>
<td></td>
<td>Positive</td>
<td>23</td>
<td>20% (5-40%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>Negative</td>
<td>52</td>
<td>0% (0-3%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>26</td>
<td>20% (5-80%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>Negative</td>
<td>72</td>
<td>0%</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>9</td>
<td>10% (5-20%)</td>
<td>P &lt; 0.05</td>
<td>P = 0.05</td>
<td>P = NS</td>
</tr>
<tr>
<td>A + D1</td>
<td>Negative</td>
<td>76</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A + E</td>
<td>Negative</td>
<td>75</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>5</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>D1 + E</td>
<td>Negative</td>
<td>74</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Positive</td>
<td>5</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>A + D1 + E</td>
<td>Negative</td>
<td>77</td>
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<tr>
<td></td>
<td>Positive</td>
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\(^a\) Univariate chi square analysis.
\(^b\) With tumor grade and size as covariates.
\(^c\) Compared with lesions overexpressing cyclin D1 only.
\(^d\) Compared with lesions not overexpressing cyclin D1.

Fig. 2 Photomicrographs of selected cases of extremity soft-tissue sarcomas studied using immunohistochemical staining with antibodies to cyclin D1. A, tumor sample with a lack of nuclear staining with the mouse monoclonal antibody DCS-6 (Ab-3) to cyclin D1. B, the heterogeneous nuclear immunoreactivities of anticyclin D1 antibody in an aggressive high-grade sarcoma (×400).
Fig. 3 A, Kaplan-Meier analysis of survival in patients with primary extremity soft-tissue sarcomas based on cyclin D1 overexpression (n = 57; immunohistochemistry was uninformative in 3 tumors for cyclin D1 and 1 tumor for cyclin A). Patients with no cyclin D1 overexpression had a much better overall survival than patients with cyclin D1 overexpression on univariate analysis (median survival, 4.2 years versus 2.9 years, respectively; P = 0.02). B, cyclin A overexpression also predicted poor overall survival on univariate analysis (n = 59; P = 0.05). No difference in overall survival was noted in patients with tumors overexpressing cyclin E (data not shown).

Fig. 4 Representative Southern blots of DNA from four high-grade extremity soft-tissue sarcomas probed for CCND1. Tumors are malignant fibrous histiocytomas (A and B), a leiomyosarcoma (C), and a synovial sarcoma (D). Although none demonstrated CCND1 amplification when compared with a β-actin control, B was found to overexpress the gene product on immunohistochemical analysis with 10% of the nuclei demonstrating reactivity.

variate analysis; nevertheless, this did not retain statistical significance when tumor grade and cyclin D1 overexpression were included in a multivariate analysis.

In general, there is little data to support a role for cyclin A abnormalities in human cancer. Hypothetically, its ability to prevent E2F-mediated transactivation when bound to CDK2 (23) may play an inhibitory role in neoplasia, especially if overexpressed. This premise is supported by recent studies in transgenic mice. In contrast to a similar experiment using cyclin D1 (24), mice that overexpressed cyclin A in breast tissue demonstrated nuclear karyotypic abnormalities, suggestive of a disorder at the G2-M transition, as well as significantly increased numbers of apoptotic cells in the mammary glands, but no tumorigenesis was noted (25).

Unlike cyclin A, overexpression of cyclin E in the breast tissue of transgenic mice eventually leads to the development of tumors (7). This is consistent with data demonstrating that both cyclins D1 and E are prognostic indicators in women with breast cancer.
cancer (5, 9, 26). However, this does not seem to be the case with extremity soft-tissue sarcomas. There was no significant change in overall survival in the subset of patients with primary tumors with cyclin E overexpression (29%: P = 0.55), although these were all high-grade lesions (P < 0.05). Given the complete lack of association with survival, the latter finding may be a secondary phenomenon due to the rapidly proliferating nature of these high-grade sarcomas. Interestingly, experiments with breast cancer cell lines have shown that cells with constitutive overexpression of INK4A/CDKN2, a potent inhibitor of cyclin D/Cdk4 and Cdk6 complexes, may be able to bypass this block of the retinoblastoma pathway through cyclin E overexpression and resultant activation of cyclin E/Cdk2 (26). Thus, our data may attest to a crucial, nonredundant role for cyclin D1/Cdk4 activation in a subset of aggressive extremity sarcomas.

The observation of significantly decreased overall survival in patients with tumors overexpressing cyclin D1 was remarkable because follow-up on this study was relatively short (median, 2.4 years). In fact, the Kaplan-Meier curve had not yet reached median survival (Fig. 1A).

Cyclin D1 overexpression seems to define a biologically more aggressive subset of extremity soft-tissue sarcomas. Even when we limited our analysis to the subset of high-grade lesions, cyclin D1 overexpression showed still a trend toward worse overall survival (P = 0.08). Only a single low-grade lesion had elevated cyclin D1 levels. On the basis of this data, we conclude that determination of cyclin D1 overexpression in extremity soft-tissue sarcomas may confer information over and above the clinicopathological factors of tumor grade, size, and depth. In clinical practice, patients with high-risk lesions (high-grade, deep, >10 cm in size) are often treated with multimodality therapy, which may include surgery, chemotherapy, and/or radiation therapy. Despite optimal treatment, approximately 50% of these patients will eventually die of their disease (16). Cyclin D1 overexpression may be helpful in identifying those cases early in their course and allowing their selection for trials of novel therapeutic agents such as vaccines and/or cytokine therapies.

REFERENCES


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S H Kim, J J Lewis, M F Brennan, et al.