Expression of Cyclin A in Soft Tissue Sarcomas Correlates with Tumor Aggressiveness

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ABSTRACT

Cyclins and cyclin-dependent kinases regulate the cell cycle. Cyclin A has a dual role in cell proliferation. It is essential in the S phase for DNA replication, and it is also involved in G2-M-phase transition, signifying actively dividing cells. The expression of cyclin A was determined by immunohistochemistry in paraffin sections of 126 soft tissue sarcomas. The median cyclin A score was 10.8% (range, 1–54%). Cyclin A expression correlated with the S-phase fraction, Ki-67 score, G2-M phase, and grade. It did not correlate with the size of the tumor. A high cyclin A score predicted a poor metastasis-free survival (P < 0.01) and a poor disease-specific overall survival (P = 0.01). We concluded that the expression of cyclin A is a powerful prognostic factor in soft tissue sarcoma. Moreover, the cyclin A score determines the fraction of tumor cells in the S phase and the G2 phase, which are the most sensitive cell cycle phases for current modalities of cancer treatment.

INTRODUCTION

Assessment of the proliferative cell fraction is extensively used to evaluate the tumor growth rate and to establish the prognosis of cancer patients. The counting of cells in mitosis to obtain a mitotic index is the traditional approach in surgical pathology (1, 2). More recently, flow cytometric DNA measurements to determine the proportion of cells in S phase have been widely used, as has immunohistochemical detection of the functionally still poorly defined antigens Ki-67 and proliferating cell nuclear antigen, the expression of which correlates with cell proliferation (3–8).

Our knowledge about the role of the cyclins and CDKs in the molecular regulation of the cell cycle has increased steadily. Immunohistochemical determination of the expression of various cyclins and CDKs in tumor cells has recently been applied to evaluate cancer growth (9–11).

Cyclins display oscillatory expression during the cell cycle. They regulate the activity of CDKs, and, together with CDKs, they form holoenzymes that phosphorylate regulatory substrates like the retinoblastoma proteins (pRb) and p107 (12, 13). Cyclin-CDK holoenzymes are also involved in essential phosphorylation reactions during DNA replication. Thus far, seven different mammalian cyclins are known, named cyclins A–G (14).

Cyclins are functionally divided into G1 cyclins and mitotic cyclins (14). G1 cyclins, e.g., cyclin C, D1–3, and E, are involved in the G1 to S-phase transition. Cyclins A and B are considered mitotic cyclins. Cyclin B regulates the G2 to M-phase transition. Thus far, cyclin A is the only characterized cyclin with a dual role in the cell cycle. It is required for DNA replication during the S phase, and it is also active at the initiation of mitosis (15, 16).

Studies on the prognostic impact of the expression levels of G1 cyclins in tumor tissue have revealed somewhat conflicting results. Amplification of the D1 cyclin gene or overexpression of the protein in breast cancers has been reported to predict an unfavorable outcome for the patient (17–23), although these findings have not been supported by other studies (24, 25). Overexpression of cyclin D1 that frequently associates with elevated expression of the CDK inhibitor p27Kip1 (26) has recently been reported to predict improved survival in breast cancer patients treated with hormone therapy (27, 28). The value of cyclin E as a prognostic cancer marker in the clinical setting is still controversial. Only small amounts of cyclin E are detected in normal breast tissue, and its expression in cancer cells has been reported as a negative prognostic factor (28).

There are very few reports on cyclin A as a marker of proliferative cell fraction in cancer. In two recent studies by Volm et al. (29, 30), tumor tissue negativity for cyclin A expression predicted a favorable outcome in non-small cell lung cancer patients. There are no previous studies on the correlation between cyclin A expression and the behavior of STS.

In this study, primary tumor samples from 133 STSs were stained immunohistochemically with monoclonal mouse antibodies to cyclin A. Our aim was to study whether cyclin A expression correlates with other proliferative indexes like the S-phase fraction, G2-M-phase fraction and Ki-67; with conventional prognostic factors (grade and size); and with the clinical outcome of individual patients.

PATIENTS AND METHODS

Patients. The patients were 145 adult patients treated for STS of the limbs or superficial trunk by the STS team at Helsinki University Central Hospital between August 1987 and May 1993. They were treated according to the team guidelines (31). In this study, we included patients with primary tumor SPF determined in the context of a previous study, with the exception of patients with dermatofibrosarcoma protuberans, a locally aggressive tumor that does not metastasize (4).

Formalin-fixed, paraffin-embedded histological samples were available for immunohistochemical staining in 133 cases. In seven cases, cyclin A staining was repeatedly unsuccessful.

The pretreatment characteristics of the 126 patients included in this study are shown in Table 1. All patients referred to the center due to a primary tumor (n = 94) or local recurrence (n = 25) received similar local treatment according to the treatment protocol of the STS team of Helsinki University Central Hospital (31). Twelve patients were given chemotherapy as part of primary treatment, seven of whom had either rhabdomyosarcoma or extraskeletal Ewing’s sarcoma. Ten patients had hematological metastases, and two patients had lymph node metastases at the time of primary diagnosis.

The Helsinki STS team is part of the Scandinavian Sarcoma Group, which applies a four-grade histological malignancy grading system. For grading, a combination of the histological type and a combination of histopathological parameters, including necrosis, vascular invasion, cellularity, mitotic activity, and nuclear pleomorphism, are used.

The median follow-up time of living patients (n = 73) was 74 months.
classification, as described previously (3). We separated tumors known to have specific chromosomal changes, e.g., synovial sarcomas, extraskeletal Ewing’s sarcomas, myxoid liposarcomas, and round cell liposarcomas. The remaining tumors formed a mixed group of which pleomorphic sarcomas constituted the largest proportion. The stratification is shown in Table 1. In the miscellaneous sarcoma group, three sarcomas with specific chromosomal changes were included because they were single cases of alveolar rhabdomyosarcoma, extraskeletal myxoid chondrosarcoma, and clear cell sarcoma (32, 33).

Methods. Primary tumor samples were collected irrespective of the status at referral. In seven cases, no primary tumor material was available, so material from the local recurrence was used. A section adjacent to the flow cytometric sample was evaluated by light microscopy. Seventy-four samples contained more than 75% tumor cells, and 23 cases contained less than 50% tumor cells. Sample deparfination was done in xylene, and rehydration was done in alcohol to distilled water. Antigen demasking was carried out by heating the samples in a microwave oven (850 W) in citric acid buffer (pH 6; 0.1M) four times for 5 min. Treatment with 1.6% methanol-peroxidase was used to inhibit endogenous peroxidase activity. For immunohistochemistry, the specimens were incubated overnight at room temperature with a 1:100 ratio of diluted mouse monoclonal antibody: human cyclin A (Novoceastra). The binding of the primary antibody was detected by a peroxidase-conjugated secondary antibody using the Vectastain ABC kit (Vector Laboratories, Inc.). The sections were counterstained with hematoxylin. Immunohistochemical demonstration of cyclin A expression is shown in Fig. 1. We used hyperplastic tonsil tissue as

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Fig. 1. Immunohistochemical demonstration of cyclin A expression in grade 3 fibrosarcoma.

(range, 23–447 months). The longest follow-up times were encountered in patients referred to the center for recurrence. Eight patients died disease-free.

In the statistical analysis, we stratified the cases according to genetic classification, as described previously (3). We separated tumors known to have specific chromosomal changes, e.g., synovial sarcomas, extraskeletal Ewing’s sarcomas, myxoid liposarcomas, and round cell liposarcomas. The remaining

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Fig. 2. Correlation between cyclin A and the S-phase fraction and linear regression curves in diploid and nondiploid tumors.
positive and negative controls for cyclin A stainings. The primary antibody was omitted in the negative controls.

The Ki-67 staining procedure was similar to that for cyclin A. We used a mouse antihuman monoclonal Mib-1 antibody (PharMingen) diluted 1:500 (3).

In flow cytometric measurements, a modification of the basic Hedley method was used (34, 35). The sections (100 μm) from the paraffin blocks were placed in fine-mesh bags that were inserted into cassettes and then dewaxed and rehydrated in a tissue processor. Enzymatic digestion was done with Subtilisin Carlsberg (Sigma Protease type 24), and all centrifugation steps were omitted, resulting in nuclei suspensions with extremely low amounts of clumped nuclei and debris. The nuclei were stained with 4’, 6-diamidino-2-phenylindole (final concentration, 5 μmol) and analyzed using a PAS 2 flow cytometer. To confirm the ploidy of near-diploid populations, the DNA content of identified nuclei was measured by using a static image analysis system. Tumors were considered diploid if only one G1 peak was present. For determination of the DNA index in grossly aneuploid cell populations, the peak of the lowest DNA content was assumed to represent diploid cells. For determination of the percentage of cells in the S phase of the cell cycle, the MultiCycle program with the sliced-nuclei option for background subtraction developed by Rabinowich was used (Phoenix Flow System, San Diego, CA). In 13 cases, the G2-M-phase peak was not quantifiable because of overlap with multiple aneuploid G1 peaks.

For quantification of immunostaining, we chose the tumor area with the highest density of positive nuclear staining. To calculate the percentage of positively stained nuclei, an ocular grid of 100 (10×10) squares was used at 10×40 magnification. All positive nuclei from this area were counted. To estimate the number of negative nuclei in the same area of 100 squares, three different rows of 10 squares were counted, and the mean score was multiplied by 10. In the case of tumors of scarce cellularity, several fields were evaluated, and negative nuclei were counted from the whole grid area of 100 squares. The percentage of positive nuclei was counted by dividing the number of the positive-stained cells by the whole number of cells in the same area. All samples were scored by two independent observers (R. L. H. and T. O. B.). In the case of more than a 5% interobserver difference in the result, the sample was rescorded by the two investigators together.

SPFs were available for all patients, G2-M-phase fractions were available for 110 patients, and Ki-67 was available for 116 patients.

Statistical Methods. Statistical analyses were done with the Macintosh SPSS computer program. With the exception of ploidy, correlations between different variables were calculated with the Spearman rank order correlation test. The correlation between ploidy and cyclin A was calculated with the Mann-Whitney U test.

The Cox regression model was used in the MFS and DSOS analysis as well in the multivariate analysis. In the Cox regression model analysis, cyclin A and size were used as continuous variables, and grade was classified into four categories. Genetic classification was used to stratify the material in the DSOS and MFS analyses.

The prognostic value of the different cut points of cyclin A was investigated by splitting the material by 10% percentiles (i.e., cut points defining 10% of the patients in the low-risk group and 90% of the patients in the high-risk group, 20% of patients in the low-risk group and 80% of patients in the high-risk group and so forth). For each cut point, the RR of death and 95% CIs were calculated by Cox regression analysis.

Survival curves were calculated by the Kaplan-Meier model.

RESULTS

The median value of cyclin A was 10.75% (range, 1.0–54.0%). The medians and ranges of cyclin A by grade, histotype, and genetic classification are shown in Table 2.

The cyclin A staining score correlated with the histological grade (P < 0.01; r = 0.46), ploidy (P < 0.01), SPF (P < 0.01; r = 0.71), G2-M-phase (P < 0.01; r = 0.56), and Ki-67 (P < 0.01, r = 0.74).
Cyclin A positivity did not correlate with tumor size ($P = 0.1$). Scattergrams of the correlation with SPF and Ki-67 are presented in Figs. 2 and 3. There was a significant difference between diploid and nondiploid tumors in the slopes and intercepts of the linear regression curves of SPF on cyclin A. The regression equation for diploid tumors was $\text{SPF} = 1.42 \times (\text{cyclin A}) + 1.31$, with a 95% CI of 1.10–1.74 for the slope and $-0.99$ to 3.61 for the intercept on the $Y$ axis, whereas the equation for nondiploid tumors was $\text{SPF} = 0.61 \times (\text{cyclin A}) + 9.56$, with a 95% CI of 0.36–0.86 for the slope and 5.19–13.9 for the intercept.

When analyzed as a continuous variable, lower cyclin A values predicted longer MFS and DSOS ($P = 0.02$ and $P = 0.01$, respectively). The result was the same when a genetic stratification was used in MFS and DSOS analyses ($P < 0.01$ and $P = 0.01$, respectively). When analyzed separately for the four different grades, only grade 4 showed an association between DSOS and cyclin A ($P = 0.09$; RR = 1.03). When analyzed separately in superficial and deep tumors, a high cyclin A score had a negative prognostic value in deep tumors ($P = 0.02$), but not in superficial tumors ($P = 0.3$).

Survival curves were calculated using the cut point value of 6% for cyclin A. Thirty-seven (28%) patients had tumors with a cyclin A value $\leq 6\%$. In the group of miscellaneous sarcomas, of 96 cases 18 (19%) had cyclin A $\leq 6\%$. The cut point value of 6% for cyclin A was selected because it divided the material into equal proportions, as in our previous studies on SPF and Ki-67, in which cut point values of 10% for Ki-67 and 3.9% for SPF were used (3). The survival curves are thus comparable. With a cut point of 6%, the estimated 5-year MFS was 74% and 56%, respectively ($P = 0.04$), in tumors with low and high cyclin A values (Fig. 4). The corresponding DSOS figures were 78% and 59% ($P = 0.07$; Fig. 5). High cyclin A values predicted a poorer survival, irrespective of the cut point; however, a better discrimination value was obtained with the more extreme values than with values close to the median. Fig. 6 shows the RRs and 95% CIs by different cut point values. The RR of metastasis and death per 10%
increase in cyclin A was greater in diploid (RR = 1.6 and 95% CI = 1.1–2.3 and RR = 1.6 and 95% CI = 1.1–2.4, respectively) than in nondiploid tumors (RR = 1.2 and 95% CI = 0.8–1.7 and RR = 1.3 and 95% CI = 0.9–1.8, respectively).

In the MFS multivariate analysis, the cyclin A value did not reach significance when analyzed together with grade and size; only size reached statistical significance.

**DISCUSSION**

This is the first study to show that the expression of cyclin A correlates with tumor behavior in STS. A low cyclin A score predicted favorable MFS and DSO5. We have previously studied SPF and Ki-67 as prognostic markers in STS (3, 4). Tumor grade is the standard prognostic factor in STS. However, the pathologist must be familiar with the special histological features of each subtype of STS to succeed in grading (36). Due to the rarity of STS, the reproducibility of the grade is rather poor (37, 38). New, more easily standardized, reproducible prognostic tools would be of help to evaluate the prognosis of STS patients in addition to the tumor grade.

In the present study, cyclin A correlated strongly to all other proliferative indices: SPF, Ki-67, and G2-M phase. A similar correlation has also been found in studies on breast (39) and liver cancer (40). In flow cytometry, the cells are not visualized as in immunohistochemistry. Thus, SPF analyzed by flow cytometry may also include nontumor cells; thus, the results are not exactly directly comparable with the immunohistochemical results.

Only two previous studies have evaluated the prognostic implication of cyclin A in cancer (Volm et al., Refs. 29 and 30). In these studies on non-small cell lung cancer, Volm et al. compared survival differences between patients who had totally negative cyclin A staining in their primary tumors and all of the patients with positive staining. Patients with cyclin A activity in their primary tumors had a worse outcome than the patients with cyclin A-negative tumors. The authors also described a scoring system for cyclin A staining that takes into account the percentage of stained cells and the staining intensity, but the results for different scores were not reported. In view of our findings, it is unnecessary to score intensity. We had only seven samples that were interpreted as “negative” or rather “unsuccessful.” Some of these were also negative for Ki-67 staining. The poor quality/adequate fixation of the samples was the obvious cause of failure in at least some of these cases.

After having passed the restriction point in the late G1, the cell is no longer sensitive to external mitogen regulators (41). Cyclin A is expressed after the point at which only intracellular factors guide the cell’s way toward division. It should be kept in mind that malignant cells may display an extended or unscheduled expression of cyclin A in vivo, as reported previously in tumor cell lines (42). However, also in the latter part of the cell cycle, cell cycle arrests that affect tumor growth may occur for various reasons (43, 44).

It is known that cancer cells in different phases of the cell cycle have different sensitivities to treatment procedures. In this respect, it is essential to understand the phases from the beginning of the S phase, because interference with the DNA replication is one of the most important principles of chemotherapy (45). By determining the different cyclins, the fractions of cells in these phases can be described. Cyclin A determines the fraction of cells in the S phase-G2 phase. The effect of doxorubicin on non-small cell lung cancer cell lines has been studied in vitro. Cyclin A-negative cells were significantly (P = 0.026) more resistant to doxorubicin than cyclin A-positive ones (29). To date, no studies have investigated cyclin A as a predictive marker of therapy response. Cyclin D1 can activate estrogen receptors directly in the absence of estrogen, and this activation is not inhibited by antiestrogens (46). However, in clinical studies, cyclin D1 overexpression in breast cancer patients predicts good responses to hormonal therapy (27, 47).

Several prognostic factors besides the grade are available to evaluate the prognosis of the STS patient, but the situation is still unclear as to which of these factors is most beneficial. Despite its significant association with outcome in univariate analyses, cyclin A lost its significance in multivariate analysis, in which tumor size was the strongest prognostic variable, with more impact than both proliferation rate and grade. The fact that tumor size is still the strongest recognized determinant of outcome clearly illustrates the problem with the application of biological markers in vivo. Based on the present study, the cyclin A score is easy to determine and offers an additional prognostic tool. Cyclin A could also possibly be used to predict the response to therapy.

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