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Prognostic Relevance of a Novel Proliferation Marker, Ki-S11, for Soft-Tissue Sarcoma

A Multivariate Study

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In 132 soft-tissue sarcomas and 52 benign soft-tissue tumors, cellular proliferation was examined by immunohistochemistry using monoclonal antibodies Ki-S11 (Ki-67 antigen) and Ki-S1 (topoisomerase IIα) and by flow cytometric analysis of the S-phase fraction (SPF). Malignant tumors were graded histologically according to the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC) system. Patient age, sex, tumor location, histological type, and DNA ploidy were considered as additional prognostic variables. Consistent immunoreactivity was seen in approximately 95% of the cases, and determination of SPF was possible in approximately 60%. Ki-S11 and Ki-S1 immunolabeling indices correlated in a linear manner. All proliferation parameters yielded significant differences between benign and malignant tumors. Ki-S11 and Ki-S1 immunoreactive scores also co-varied significantly with SPF, mitotic count, and histopathological grade. In univariate analysis, immunohistochemical proliferation indices, histopathological grade, mitotic count, and SPF were predictive of overall survival and the development of metastases. In multivariate analysis, immunolabeling scores of proliferation markers, grade, and SPF emerged as independent predictors of global survival and systemic progression. We conclude that the immunohistochemical assessment of proliferation, being more readily performable and more easily assessable than the equally relevant S phase fraction, may add appreciable information to the current prognostic models for soft-tissue sarcoma. (Am J Pathol 1997, 150:1997–2007)

The term soft-tissue sarcoma (STS) stands for a group of biologically and phenotypically heterogeneous aggressive tumors with a propensity for local recurrence and distant metastatic spread. Clinical outcome appears to depend on several factors with variable impact. Whereas the surgical margins are of capital importance for the local control of the disease,[1–4] the histopathological grade has emerged as the most relevant predictor of systemic progression and overall patient survival.[5–9] Several grading systems, based on morphological criteria such as cytology, patterns of tumor growth, gross or microscopic finding of necrosis, and mitotic count, have been proposed.[6,7,10–15] At least two of them, from the National Cancer Institute[6] and the Fédération Nationale des Centres de Lutte Contre le Cancer (FNCLCC),[7] were shown to be of high prognostic relevance and have gained widespread acceptance.[16] Nevertheless, the interpretation of morphological features inevitably harbors a part of subjectivity, which is reflected by an incomplete agreement between different observers.[17] Also, the method of mitosis counting is neither standardized nor well reproducible,[18,19] and the number of assessable mitotic figures does not always correlate with the actual

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proliferative activity of a tumor cell population.[20,21] In this regard, assessment of the growth fraction by immunohistochemistry using proliferation-specific antibodies might provide more precise and objective information and grant a better reproducibility.

The aim of the present retrospective study was therefore to investigate the prognostic relevance of the proliferative activity in adult STS. We present a novel monoclonal antibody to the Ki-67 antigen,[22,23] Ki-S11, which has the advantage of being well suited for Bouin-fixed material, and compare the immunostaining results with another proliferation marker generated in our laboratory, Ki-S1,[24–26] as well as the S-phase fraction (SPF) as determined by flow cytometry. The specificity of the antibody was verified by Western blot analysis, and its sensitivity was controlled by correlation of the immunolabeling scores with the signal intensity in Western blots using cellular lysates of the corresponding tumor samples. In addition, we investigated the relevance of various other parameters with a possible prognostic impact by both univariate and multivariate analysis. We will show that the immunohistochemical assessment of the proliferative activity provides complementary prognostic information and may help to improve the accuracy of current grading systems for STS.

Materials and Methods

Patients

A total of 184 cases of patients admitted between January 1, 1980, and December 31, 1989, from which fresh tissue samples were available, were retrieved from the files of the French Federation of Cancer Centers (FNCLCC). Of these, 132 cases were diagnosed as STS, the remainder as benign soft-tissue tumors. Of the 132 sarcoma patients, 71 had been admitted for primary surgery and 61 for local tumor recurrence, and 18 presented with metastatic disease at the time of diagnosis. From the collective, three groups were formed: patients with benign tumors, the totality of sarcoma patients (STST), and patients without assessable metastasis at the time of first surgery (STSM0). Complete clinical data could be obtained for all patients by review of the medical records. For all sarcoma patients, R0 or at least R1 tumor resection was verified. Median follow-up was 43.7 months (from 1 to 170 months).

Tumors

Hematoxylin-and-eosin-stained sections of the tumors were reviewed for diagnosis by the pathology subcommittee of the FNCLCC sarcoma group, which includes a mean number of seven pathologists. Whenever necessary, immunohistochemistry was performed for the confirmation of diagnosis or tumor typing according to the Enzinger and Weiss classification.[27] Benign tumors comprised mucoid pseudocysts (n = 1), inflammatory pseudotumors (n = 1), ossifying myositis (n = 1), palisading myofibroblastomas (n = 2), desmoid tumors (n = 16), benign fibrous histiocytomas (n = 1), fibromyxoid ossifying tumors (n = 4), lipomas (n = 8), angiolipomas (n = 2), angioleiomyomas (n = 1), hemangiomas (n = 1), neurofibromas (n = 8), and benign schwannomas (n = 6). Malignant tumors were graded according to the FNCLCC grading system,[7] which is based on three histological criteria, ie, tumor differentiation, mitotic count, and the extent of necrosis. A detailed survey of the diagnoses and corresponding tumor grades is given in Table 1. For further processing, paraffin-embedded samples were selected for the most representative and best preserved tumor tissue.

Table 1. *Survey of the Histological Tumor Types and Corresponding FNCLCC Grades of the Total Sarcoma Collective (n = 130)*

Histological type	Grade 1	Grade 2	Grade 3	Total
MFH	3	21	21	45
Fibrosarcoma	2	1	1	4
Liposarcoma	17	15	3	35
Leiomyosarcoma	0	6	4	10
Synovial sarcoma	0	2	4	6
Others	1	8	11	20
Unclassified	0	3	7	10
Total	23	56	51	130

In two cases, grading could not be performed (drill biopsies).

Antibodies

Monoclonal antibody Ki-S11 was generated by somatic hybridization of splenocytes from BALB/c mice immunized with nuclear extracts from L428 cells with P_3x63-Ag.8653 mouse myeloma cells as described before.[28] Immunoreactive clones were selected by screening on normal tissues, subcloned, and expanded.

Reactivity of the paraffin-embedded material was tested with the proliferation-specific antibodies Ki-S5,[21] MIB-1 (Dianova, Hamburg, Germany), Ki-S1,[21,24] and Ki-S11 by comparison with the immunolabeling in snap-frozen samples of the same tumor tissue.

Western Blot Analysis

Tumor tissue from snap-frozen samples was mashed with a scalpel, weighed, dissolved in a lysis buffer composed of 4 mol/L urea, 2.5% sodium dodecyl sulfate (SDS), 100 mmol/L Tris, pH 6.8, and 5 mmol/L EDTA (all reagents purchased from Merck, Darmstadt, Germany) and stirred overnight at 4°C. After addition of 500 μl of buffer (composition as above but with omission of SDS), the probes were disrupted by sonication (80 bursts) and stored as 500-μl aliquots. The protein content was determined by means of the BCA protein assay (Pierce Chemicals, Rockford, IL) according to the distributor's instructions. Each aliquot was then supplemented with 20 μl of β-mercaptoethanol, and proteins were denatured for 30 minutes at 60°C. Subsequently, 60, 30, and 15 μg of protein from each tumor were separated by SDS-polyacrylamide gel electrophoresis (5 to 10% gels) and transferred to polyvinylidene difluoride membranes (Millipore, Eschborn, Germany) by semi-dry blotting for 50 minutes. Correct protein loading was verified by Coomassie Brilliant Blue staining. Primary antibodies (Ki-S11 and Ki-S1, cell culture supernatants diluted 1:100 in phosphate-buffered saline (PBS) supplemented with 1% bovine serum albumin (BSA), and antibody Ki-67 (Dianova), diluted 1:30) were admixed with 10% Tris buffer (1 mol/L), pH 8.0, and incubated on the membranes overnight. After four washes with PBS/BSA, the membranes were incubated successively with biotinylated goat anti-mouse antiserum (DAKO, Glostrup, Denmark) diluted 1:2000 and streptavidin-biotin complex diluted 1:100, for 1 hour each, and washed each time as above. The reaction was visualized by chemiluminescence exposition of roentgen films (ECL, Amersham, Poole, UK).

Immunohistochemistry

Immunohistochemistry was performed as described before.[21] Briefly, 4-μm sections were cut from Bouin-fixed, paraffin-embedded tissue specimens, mounted on 3-aminopropyl-triethoxysilane-coated slides, and routinely processed. For antigen retrieval, the sections were immersed in 0.01 mol/L citric acid, pH 6.0, and heated in a microwave oven (Toshiba) for 15 minutes at the highest power setting. Endogenous peroxidase activity was blocked by 3% (v/v) hydrogen peroxide in methanol for 5 minutes. After rinsing with PBS, primary antibodies were incubated on the sections for 30 minutes at room temperature, and the immunoreaction was enhanced using the streptavidin-biotin complex and rabbit anti-mouse antibody.

After scanning the slides at low magnification to determine the most evenly labeled tissue areas, a minimum of 1000 tumor cells were counted at high power, and the number of labeled nuclei was calculated as a percentage of the total cell count.

DNA Measurements

Cellular DNA content was assessed as described elsewhere.[29] Briefly, snap-frozen tissue samples were mechanically disintegrated and resuspended in 0.5 ml of PBS. Two nuclear suspensions of each tumor with adjusted cell concentrations were stained with propidium iodide. Human peripheral blood lymphocytes added previously to one of them served as diploid standards. Flow cytometric analysis of DNA content was performed on a FACSCAN flow cytometer (Becton Dickinson, Mountain View, CA). A coefficient of variation of 2% or less, as measured at the base of the diploid peak of GO/G1 peripheral blood lymphocytes, was considered a prerequisite for tumor sample analyses, which were all performed under equivalent conditions. The DNA index of a cell population was determined by dividing the modal channel position of the aberrant G0/G1 peak by the modal channel position of the diploid peak. The fractions of cells in GO/G1, S, and G2/M phases of the cell cycle were calculated by means of the RFIT mathematical model of the flow cytometer-integrated software. This analysis was submitted to restrictive conditions, ie, less than 15% cell debris, a major percentage of aneuploid cells in aneuploid tumors, and a coefficient of variation of <5% for each aneuploid peak. Multiploidy (more than one nondiploid stemline) also precluded cell cycle analysis.

Statistics

The following variables were considered for their prognostic value: patient age at presentation, sex, tumor location, histological type, tumor grade, mitotic count, SPF, ploidy status, and Ki-S11/Ki-S1 immunolabeling scores. Relationships between variables were analyzed by means of the Spearman rank correlation coefficient, the Wilcoxon matched pairs test, and nonparametric Kruskal-Wallis analysis of variance. Survival curves and metastasis-free interval were computed by the Kaplan-Meier method using the BMDP 1L software. Date of diagnosis was considered as the time of origin. All deaths, regardless of their cause, were considered as events. Significance levels of the differences between curves

were calculated by the log-rank test. Hierarchy among prognostic variables was established by multivariate analysis using the Cox model and a stepwise method (program BMDP 2L). The confidence level for statistical significance was set at $P < 0.01$. All variables were analyzed as binary variables. For multivariate analysis with respect to overall survival and the occurrence of metastases, only the STSM0 patient collective was taken into account, and factors achieving statistical significance in univariate analysis ($P < 0.01$) were considered exclusively.

Results

Clinical Data

The male/female ratio was approximately 1:1 in the two patient collectives. Among the patients with benign soft-tissue tumors, 25 (48.1%) were male and 27 (51.9%) female; in the group with STS, there were 65 males and 67 females (49.2 and 50.8%, respectively). Patient age ranged from 15 to 76 years in the benign tumor group (mean 45.7 ± 15.4) and from 18 to 92 years (mean 56.1 ± 16.1) in the STS group. After exclusion of the 32 patients who presented with metastatic disease at the time of initial surgery, there remained 54 (54%) males and 46 (46%) females. Mean age in this group (M0) was 55.6 ± 15.7 years (ranging from 20 to 85 years).

Tumor locations in the STST group were head and neck (n = 4), upper limbs (n = 11), lower limbs (n = 54), and trunk (n = 61); of the latter, 32 were superficially located and 29 deep seated. In two cases, no corresponding clinical information could be obtained.

The 5-year survival probability in the STST collective was 54% with a median survival of 62 months; local recurrence occurred in 59 patients (44.7%), 47 (35.6%) developed metastases, and 50 (37.9%) died of the disease. Corresponding data in the STSM0 group were 5-year survival probability of 64.6%, local recurrence in 34 cases (34%), and 25 (25%) disease-related deaths; 25 (25%) of these patients ultimately developed metastases.

Antibody Characteristics

Screening of Ki-S11 on normal tissues had revealed a distribution pattern highly reminiscent of that of the Ki-67 protein. In accordance with this observation, Western blot analysis of tissue lysates revealed a double protein band at approximately 395 and 345 kd, which was likewise recognized by the monoclonal antibodies Ki-67 and Ki-S5 (Figure 1). In addition,

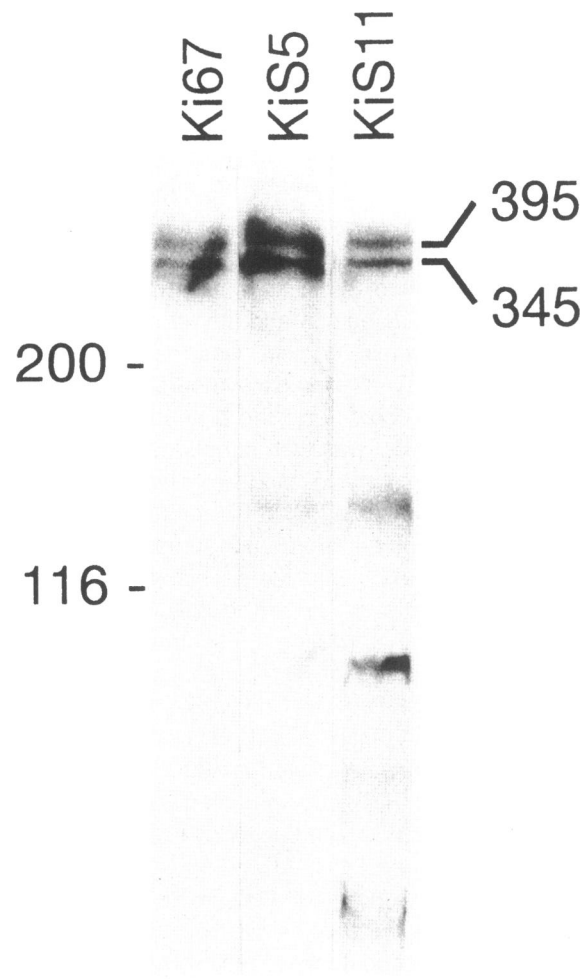

Figure 1. *Comparative Western blot analysis of crude extracts from L428 cells . Like the original Ki-67 antibody[22] and Ki-S5,[21,45] Ki-S11 recognizes a double band at 395 and 345 kd corresponding to the Ki-67 protein. The additional bands at approximately 130 and 80 kd probably represent degradation products containing the Ki-S11 binding site.*

Ki-S11 stained two protein bands near 130 and 80 kd, the first of which also yielded a weak signal with Ki-S5. A Western blot of the same cell extracts with MIB-1 showed several bands within the same range (data not shown). To compare the tissue immunoreactivity with the actual expression of the antigenic protein, we selected four tumors with different immunolabeling scores (three STSs and one benign tumor) and performed a Western blot analysis with the antibodies Ki-S1 and Ki-S11 using different protein quantities of tumor cell lysates. Tumors with high immunostaining indices yielded a significantly stronger signal with both antibodies, and the signal intensity was proportional to the quantity of applied protein (Figure 2). Again, in samples C and D, minor protein bands were stained by Ki-S11, which were apparently unrelated to the immunoreactivity of the

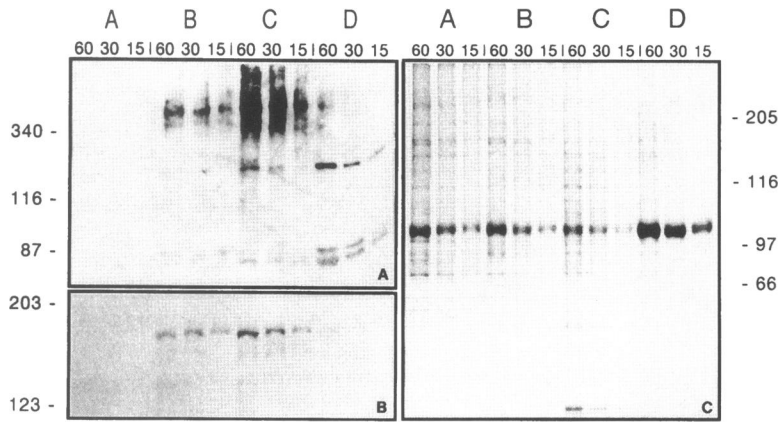

Figure 2. *Western blot experiment using whole lysates from fresh-frozen tumor tissue (60, 30, and 15 μg of protein, respectively) with Ki-S11* (**A**) *and Ki-S1* (**B**). *Correct protein loading is verified by Coomassie Brilliant Blue staining* (**right**). **Lane A,** *benign desmoid tumor with approximately 1% immunoreactive cells;* **lane B,** *leiomyosarcoma with 45% immunoreactivity,* **lane C,** *MFH with 70% immunoreactivity;* **lane D,** *round-cell liposarcoma with an immunolabeling index of approximately 5%. The signal intensity for both the Ki-67 antigen and topoisomerase IIα is proportional to the immunolabeling indices, which were virtually equivalent for Ki-S11 and Ki-S1 in these samples.*

tissue samples. Indeed, nonspecific cross-reactivity was never observed. Figure 3 illustrates the immunostaining of paraffin sections by Ki-S11 and Ki-S1.

In a preliminary test series, including 10 benign and 20 malignant tumors, only the antibodies Ki-S1 and Ki-S11 were found to yield consistent results. Virtually identical immunolabeling scores were observed in fresh and paraffin-embedded material. With Ki-S5 and MIB-1, the immunostaining of paraffin sections was often weak, and a substantial percentage of tissue samples showed no reactivity. Consequently, the two former antibodies were exclusively used in the present study. All sections were evaluated by at least two independent pathologists. Interobserver agreement as to immunolabeling scores averaged 95%.

Tumor Characteristics

The number of mitotic figures per 10 high-power fields (HPF) ranged from 0 to 100 (mean 16 ± 17) in the STST group and from 0 to 90 (mean 16 ± 16) in the STSM0 subgroup. In benign tumors, the mitotic count ranged from 0 to 6 (mean, 1 ± 1.5).

The SPF could be determined in 83 (62.9%) of all STS cases, corresponding to 57 (57%) in the MO group and in 46 (88.5%) of the benign tumor patients. Failure to calculate SPF was mainly related to the high rate of multiploidy in the STS collective and to technical causes in a minor percentage of cases. Mean values, standard deviations, and ranges are summarized in Table 2. The ploidy status was assessable in all cases. Forty-eight (92.3%) of the benign tumors were diploid, two (3.9%) near diploid, and two (3.8%) aneuploid. In the STST group, there were 37 diploid (28.0%), 14 hypodiploid (10.6%), and 5 near-diploid tumors (3.8%); the remaining 76 tumors (57.6%) were aneuploid. Among the STSM0 tumors, 28 (28%) were diploid, 10 (10%) hypodiploid, 5 (5%) near diploid, and 67 (67%) aneuploid.

Ki-S1 immunoreactivity was seen in 49 (94.2%) of the benign tumors and 125 (94.7%) of the sarcomas, amounting to 97 (97%) in the STSM0 subgroup. Ki-S11 reacted with 126 (95.5%) of the sarcomas (98 (98%) in the STSM0 subgroup) and 46 (88.5%) of the benign tumors. Negative samples lacking an internal positive control (eg, lymphocytes or endothelia with proliferative activity) were disregarded. For median values as well as the 25th and 75th percentiles of the different tumor groups and malignancy grades, with an additional distinction between malignant fibrous histiocytomas (MFHs) and other histological types (see Table 2).

Table 2. *Median Values and 25th and 75th Percentiles (in Parentheses) of Ki-S11 and Ki-S1 Immunolabeling Indices, Mitotic Count (MC), SPF in Relation to Tumor Grades and Histological Type (MFH/Other Histology)*

	Ki-S11 (%)	Ki-S1 (%)	MC/10 HPF	SPF (%)
BST	5 (1–10)	5 (1–10)	0 (0–1)	1.30 (0.7–1.8)
STS				
G1	10 (5–23.75)	10 (5–23.75)	2 (1–3.5)	1.90 (1.15–2.8)
G2	25 (15–35)	25 (15–35)	8 (4–14)	2.65 (1.7–4.5)
G3	40 (30–50)	40 (30–50)	28 (21–36.5)	9.05 (3.5–12.9)
MFH	35 (25–45)	37.5 (25–50)	17 (6.7–2.5)	8.5 (4.2–12.5)
Non-MFH	20 (10–35)	25 (15–35)	10 (3–21)	2.6 (1.6–4.5)

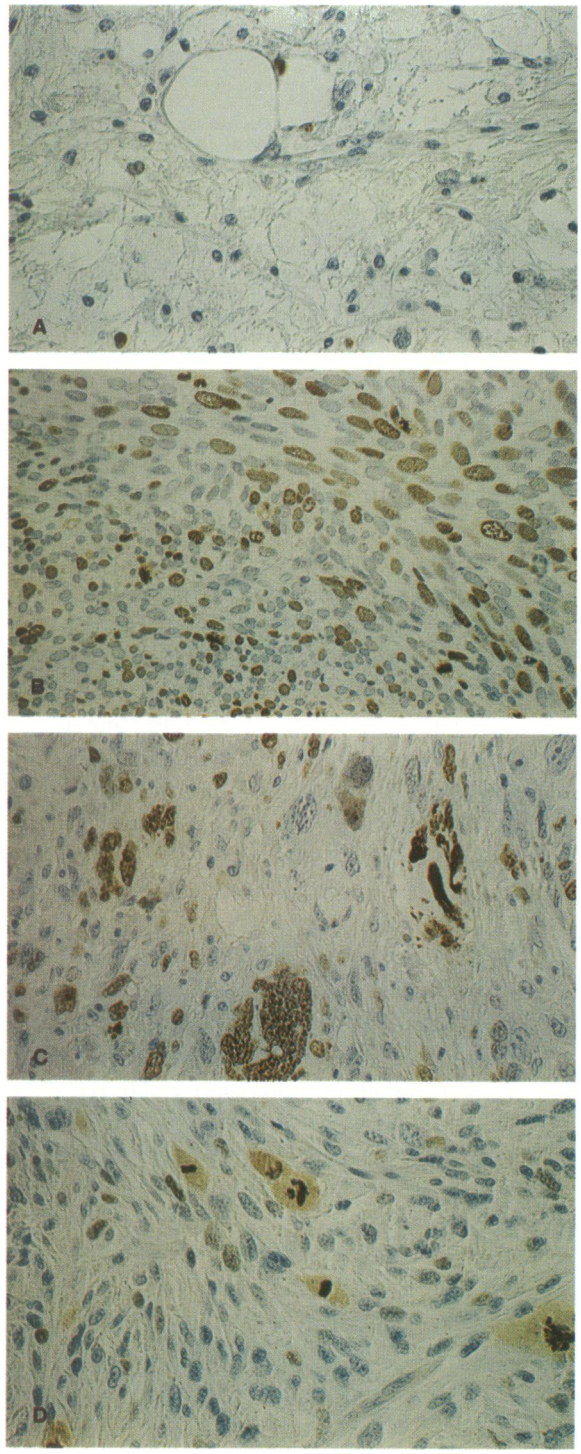

Figure 3. A: *Low-grade myxoid liposarcoma with less than 5% Ki-S11-positive cells.* B: *In this case of poorly differentiated sarcoma, a high percentage of tumor cells are reactive with Ki-S11.* C: *The bizarre giant cells of MFH do not represent degenerative changes but retain a proliferative potential as shown by Ki-S11 labeling; a highly atypical mitotic figure is seen at right.* D: *During mitosis, a finely punctate nucleoplasmic staining is characteristic of topoisomerase IIα (Ki-S1). Streptavidin-biotin complex technique; hematoxylin counterstain; magnification, ×350.*

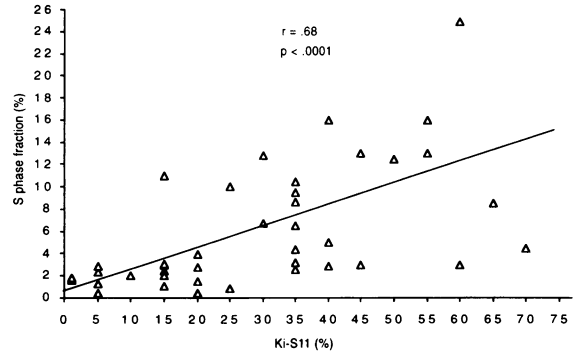

Figure 4. *Scatterplot diagram illustrating a linear and highly significant correlation between the S phase fraction and the Ki-S11 immunolabeling index.*

Correlations

Correlations were calculated separately for the three tumor groups. Overall, an excellent correlation was found between Ki-S1 and Ki-S11 immunolabeling scores ($r = 0.889$ in benign tumors, $r = 0.881$ in STST, and $r = 0.857$ in STSM0, $P < 0.0001$). Ki-S1 indices also correlated well with the SPF in STS ($r = 0.683$, $P < 0.0001$) and the STSM0 subgroup ($r = 0.755$, $P < 0.0001$), whereas they co-varied to a much lesser extent in benign tumors ($r = 0.325$, $P = 0.0337$). Comparison of Ki-S11 indices with the SPF yielded similar results ($r = 0.321$, $P = 0.0407$ in BST; $r = 0.662$ and 0.677 in STST and STSM0, respectively, $P < .0001$). Figure 4 illustrates the respective values of SPF and Ki-S11 labeling in the STSM0 group in a scattergram with corresponding regression function. The correlation of the immunolabeling scores with the mitotic count also achieved high statistical significance (for Ki-S1, $r = 0.533$ and 0.528 in the STST and STSM0 groups, respectively, and 0.494/0.482 for Ki-S11, $P < 0.0001$). A significant correlation was also found between Ki-S11 and Ki-S1 labeling indices and the tumor grade as well as DNA ploidy. The results are summarized in Table 3.

Survival Analysis

Overall and relapse-free survival probabilities in relation to all potential prognostic factors were computed by Kaplan-Meier analysis for the STST and STSM0 groups. The differences between the survival curves were evaluated by the Mantel-Haenszel log-rank test to determine statistical significance levels. The results are summarized in Table 4. With regard to Ki-S1 and Ki-S11 immunoreactive scores, cutoff points at 20 and 40% immunoreactive cells were selected according to the results of previous studies.[26,30,31] In the STST collective, low Ki-S1 and Ki-

Table 3. *Correlations between Ki-S11 and Ki-S1 Immunolabeling Indices, Mitotic Count, SPF, Histological Type (MFH/Other), Tumor Grade, and DNA Ploidy Status in Benign Soft Tissue Tumors (BST) and Sarcomas (STS)*

	Significance level (P value)						
	Ki-S11	Ki-S1	MC	SPF	Type	Grade	Ploidy
Ki-S11							
BST		<0.0001	<0.001	0.09			
STS		<0.001	<0.001	<0.0001	0.0029	<0.001	0.0003
Ki-S1							
BST	<0.0001		0.012	0.032			
STS	<0.001		<0.001	<0.0001	0.0009	<0.001	0.0003

S11 scores were associated with a significantly improved overall survival (Figure 5), whereas no statistically significant differences were found by comparison of the classes with 20 to 40 and >40% proliferating cells. Similar results were found in the STSM0 collective, and immunolabeling scores ≥ 20% were highly predictive of the development of metastases (Figure 5). Concerning overall survival, FNCLCC grade, mitotic count, and SPF were also of high prognostic relevance. In addition, FNCLCC grade 3 and a mitotic count of ≥20 were significantly predictive of metastatic tumor expansion, whereas aneuploidy and an SPF of ≥4% reflected a mere trend. All other parameters did not amount to statistical significance (Table 4).

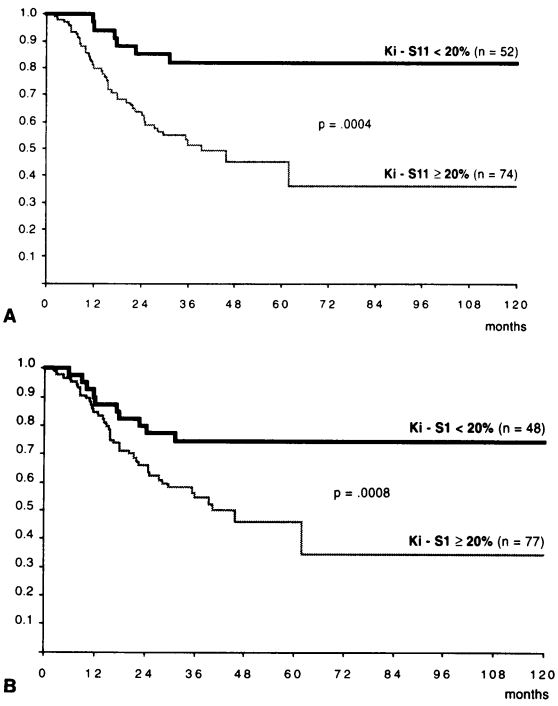

Figure 5. *Kaplan-Meier analysis of cumulative overall survival in the STST group (132 observations).* **A:** *A Ki-S11 immunolabeling index of ≥20% is associated with a significantly poorer prognosis.* **B:** *Similar results are found for Ki-S1.*

Multivariate Analysis

Multivariate analysis was performed for the STSM0 collective exclusively. A Ki-S11 score of ≥20% emerged as the most significant predictor of shortened overall survival, followed by an SPF of ≥4% and histology of MFH. As to the development of metastases, the most relevant prognosticator was FNCLCC grade 3, followed by a high Ki-S11 index and histology of MFH. When SPF was removed from the model (94 observations), only the Ki-S1 immunolabeling index appeared to influence overall survival, whereas the FNCLCC grade 3 was found the leading independent predictor of metastatic spread, followed by a Ki-S1 score of ≥20%. Relative risks, confidence intervals, and *P* values are summarized in Table 5.

Discussion

Current strategies for the clinical management of STSs are largely dependent on the prediction of their biological behavior. Despite the low incidence of this type of neoplasia, multiple studies have endeavored in elaborating reliable prognostic criteria (see Ref. 8 and references therein). Several clinical characteristics, eg, tumor location, tumor size, and patient sex and age, appear to influence local recurrence, metastatic spread, and survival rates.[8] Nevertheless, histopathological criteria, such as tumor type, degree of differentiation, cellularity, nuclear atypia, mitotic activity, and the amount of necrosis, are likely to yield more consistent prognostic information.[6,32] By combining several of those parameters, histopathological grading appears to improve the prognostic accuracy irrespective of the grading system. However, a recent study comparing the two most widely accepted grading systems (National Cancer Institute and FNCLCC) demonstrated a significantly better correlation with the clinical outcome for the latter[9] and suggested its preferential use for predicting the

Table 4. *Univariate Analysis of the Influence of Tumor Type, Patient Age and Sex, Histopathological Grade, S Phase Fraction, Mitotic Count, and Ki-S11/Ki-S1 Labeling Indices on the Development of Metastases and Overall Survival in the STST and STSMO (*) Collectives*

	P Value	
	Metastases	Overall survival
Histology (MFH/others)	0.3977	0.723
	0.6801*	0.6418*
Age (<50 years/≥50 years)	0.2739	0.7665
	0.2101*	0.4175*
Sex	0.52	0.9917
	0.1908*	0.097*
Grade	7×10^{-7}	0.004
	1×10^{-5}*	0.016*
SPF (<4%/≥4%)	0.035	0.0017
	0.1806*	0.01605*
Mitotic count (<10/≥20/10 HPF)	<0.0001	0.005
	<0.0001*	0.0047*
DNA polidy	0.017	0.2224
	0.064*	0.469*
Ki-S11 LI (<20/≥20%)	0.0005	0.0004
	0.0032*	0.0016*
Ki-S1 LI (<20/≥20%)	0.0008	0.0008
	0.0001*	0.0001*

prognosis of STS. Accordingly, the FNCLCC grade was exclusively used in the present study.

Notwithstanding their predictive value, current prognostic models might profit from further refinement, eg, the complementary application of biopotential markers. We therefore investigated the relevance of immunohistochemical proliferation markers and the flow cytometric analysis of DNA content in relation to the histopathological grade and a panel of other potential prognostic variables. The growth fraction was assessed by means of monoclonal antibodies reactive with two different antigens the proliferation specificity of which is amply documented.[22,28,33]

Antibody Ki-S11 recognizes the Ki-67 protein,[22,23] as verified by staining of the characteristic 395- and 345-kd bands in Western blot analysis (Figure 1). Although we cannot definitely exclude a cross-reactivity with an unrelated protein, we believe that the additional bands at approximately 130 and 80 kd represent degradation products of the main antigen. Considering the high sensitivity of the Ki-67 protein to protease digestion,[23] which is due to the presence of numerous PEST sequences[33] and accounts for the rapid disappearance of the antigen from cells leaving the cycle, those are an expected finding. The fact that they were not recognized by the antibody Ki-67 may be explained by a lower overall sensitivity of the latter.[34] Also, it is probable that Ki-S11 binds to a different epitope, which would be consistent with its exceptional reactivity in Bouin-fixed tissue. Because enhancement of Ki-67 antigenicity requires the association of the protein with DNA,[35] the proteolytic fragments apparently do not influence the histological immunolabeling scores, as documented in Figure 2, lane D. Accordingly, immunoreactivity scores on paraffin-embedded material correlated well with the signal intensity in immunoblots using whole lysates of snap-frozen tissue of the same tumors (Figure 2). Comparable results were obtained with Ki-S1, an antibody to topoisomerase IIα,[28,36] as shown by the signal intensity of a 170-kd band in Western blot analysis using the same tissue samples (Figure 2B).

Both proliferation markers co-varied with the mitotic count and the SPF as determined by flow cytometry ($P < 0.001$) and correlated with the FNCLCC tumor grade ($P < 0.0001$). In univariate analysis, they were predictive of both overall survival and the development of metastases. In a multivariate model including SPF, a Ki-S11 index of ≥20% emerged as the most significant independent predictor of overall survival ($P = 0.0088$), followed by the SPF ($P = 0.012$) and histology of MFH ($P = 0.027$). Development of metastases was influenced by the histopathological grade ($P = 0.0011$), the Ki-S11 label-

Table 5. *Multivariate Analysis Concerning Overall Survival and Metastatic Spread*

	Overall survival			Metastatic spread		
	RR	95% CI	*P* value	RR	95% CI	*P* value
Model including SPF (52 observations)						
Histology (MFH)	0.47	0.23–0.91	.027	.47	0.23–0.96	0.0407
Grade	n.s.			3.13	1.57–6.21	0.0011
SPF ≥ 4%	2.32	1.19–4.52	.012	n.s.		
Ki-S11 ≥ 20%	2.61	1.27–5.38	.0088	2.58	1.18–5.67	0.0174
Ki-S1 ≥ 20%	n.s.			n.s.		
Omission of SPF (94 observations)						
Grade	n.s.			6.07	2.51–14.6	0.0079
Age > 50 years	n.s.			.094	0.011–0.77	0.27
Ki-S11 ≥ 20%	n.s.			n.s.		
Ki-S1 ≥ 20%	5.08	1.52–16.88	6.1×10^{-5}	n.s.		

RR, relative risk; CI, confidence interval; n.s., not selected.

ing index ($P = 0.0174$), and histology of MFH ($P = 0.0407$). When SPF was removed from the model to circumvent the concomitant reduction of case numbers, a Ki-S1 immunoreactive score of ≥20% was the sole independent factor with respect to overall survival ($P = 0.0079$), whereas the histopathological grade alone was predictive of the development of metastases ($P = 6.1 \times 10^{-5}$).

Although both Ki-S11 and Ki-S1 scores were statistically significant in univariate analysis, the selection of either factor in the multivariate model was influenced by the inclusion or omission of SPF. Possibly, a closer co-variability of SPF and Ki-S1 may account for this shift in the statistical weight. However, the same cutoff level having been arbitrarily selected for both antibodies instead of two individual cutoff points to optimize the results, small deviations of the respective labeling indices to either side of the 20% limit may be responsible for the different statistical significance in the two collectives. Also, besides reflecting the proliferative activity of tumors, topoisomerase IIα expression levels may modulate the sensitivity of tumor cells to chemotherapeutic drugs and thus have an additional prognostic impact.[37] Although one of the immunohistochemical proliferation markers emerged as an independent prognostic factor in either statistical model, these observations suggest that the assessment of different proliferation-associated antigens may improve the prognostic information.

Apparently, metastatic spread and lethal tumor progression are separate processes depending on different biological parameters. The intrinsic growth potential of a tumor, which equally affects the local and the distant situation, logically accounts for the rapidity of its progression and thus may influence the survival time. Metastatic dissemination, on the other hand, is more likely to depend on the expression of adhesion molecules that may facilitate vascular invasion and of histocompatibility antigens that may help to elude immunological host defense mechanisms. Rather than being associated with a growth advantage, these properties may be linked to a particular phenotype that may be mirrored by histological differentiation, thus by the FNCLCC grade.

Although the mitotic count was reported to be an independent prognostic factor in several studies,[14,38,39] mitotic count was not selected in our multivariate model. This may be due to various factors influencing the assessability of mitotic figures in fixed material.[18,19,40] Also, rather than the number of actual cell divisions, the global proliferative potential of a tumor, ie, the growth fraction, may be apt to reflect its biological behavior. So far, few studies have been devoted to the immunohistochemical assessment of proliferation in soft-tissue tumors.[20,41–44] They all demonstrate a significant correlation between proliferation rates and overall as well as metastasis-free survival in univariate models, but multivariate analyses are wanting.

Flow cytometric DNA analyses of STS have yielded conflicting results. Although many studies have reported an association of the ploidy status with the clinical course,[45–50] other authors found no such correlation.[51–53] The prognostic impact of SPF was tested in three studies only[48,53,54] and has likewise led to controversial conclusions. In keeping with the observations of Becker et al,[54] we determined by both univariate and multivariate analysis a prognostic significance of SPF for overall survival, whereas aneuploidy was not predictive of mortality or the occurrence of metastases. Although both flow cytometry and immunohistochemistry proved to be prognostically relevant, our data suggest the latter might be a more significant independent prognosticator. At least, considering the limitation that flow cytometry requires fresh material for a reliable analysis while being successful only in a limited number of cases, immunohistochemistry may usefully replace this method in larger series.

We therefore conclude that, concerning the risk of mortality, the proliferative activity of STS provides prognostic information beyond the histopathological grade, which nevertheless remains the most reliable predictor of metastasis development. Owing to its specificity and outstanding sensitivity also in Bouin-fixed material, Ki-S11 may become a useful tool for the investigation of the proliferative activity in tumors. As this study seems to be a pioneer concerning multivariate analysis of proliferation markers in STSs, additional investigations are needed to confirm our results. In particular, it might be of interest to examine the significance of these parameters in relation to different histological types of STS, which may have an influence on the clinical outcome as well.[32] Also, the combination of histopathological grading and proliferation indices might contribute to improve the accuracy of predictions concerning patient survival and might be helpful for the selection of therapeutic strategies.

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