# Referral patterns, prognostic models and treatment in soft tissue sarcomas

Seinen, Johanna Magda

# Chapter 5

## Prognostic value of proliferation in pleomorphic soft tissue sarcomas:

## a new look at an old measure

Seinen JM, Jönsson M, Bendahl PO, Baldetorp B, Rambech E,
Åkerman M, Rydholm A, Nilbert M, Carneiro A

Human Pathology 2012 Dec;43(12):2247-54

## Abstract

*Objectives.* Through proliferation has repeatedly shown a prognostic role in sarcomas, it has not reached clinical application.

*Methods.* We performed a comprehensive evaluation of the prognostic role of 5 proliferation measures in a large series of soft tissue sarcomas of the extremities and the trunk wall.

*Results.* One hundred ninety-six primary soft tissue sarcomas of the extremities and the trunk wall were subjected to DNAflow cytometry for quatification of S-phase fraction and to immunohistochemicalevaluation of Ki-67, Top2a, p21, and p27Kip1. In univariate analysis, positive expression of Ki-67 (hazard ratio = 4.5, CI = 1.6-12.1), Top2a (hazard ratio = 2.2, CI = 1.2-3.5) and high S-phase fraction (hazard ratio = 1.8, CI = 1.2-3.7) significantly correlated with risk for metastasis. When combined with currently used prognostic factors, Ki-67, S-phase fraction and Top2a fraction contributed to refined identification of prognostic risk groups.

*Conclusion.* Proliferation, as assessed by expression of Ki-67 and Top2a and evaluation of S-phase fraction and applied to statistical decision-tree models, provides prognostic information in soft tissue sarcomas of the extremity and trunk wall.
Though proliferation contributes independently to currently applies prognosticators, its role is particularly strong when few other factors are available, which suggests a role in preoperative decision-making related to identification of high-risk individuals who would benefit from neoadjuvant therapy.

## Introduction

Soft tissue sarcomas (STS) of the extremities and the trunk wall comprise a heterogeneous group of rare malignant tumors with diverse genetic aberrations, morphological features, and clinical behavior. In STS of the extremity and trunk wall, surgery with wide margin is the cornerstone of therapy, usually combined with radiotherapy, which is considered the standard approach in nearly all high-grade tumors and low-grade ones operated with marginal margin. Still, metastases develop in about one-third of the patients, most of whom will die from their disease.

Several studies have correlated proliferation measures to development of distant metastasis as well as to overall survival in STS. [1-4] However, these findings have not gained clinical applicability, which is explained by small and heterogeneous materials, use of different proliferation measures and uncertainty about the impact of proliferation when additional prognostic markers are considered.

The Ki-67 antigen, also referred to as Ki-67, is present in the nucleus during all active phases of the cell cycle (G1, S, G2, and mitosis) and is strictly associated with proliferative potential. [5] High Ki-67 expression, which has been defined as expression in more than 10–30% of the cells, has been correlated to overall survival in soft tissue sarcoma. [3,6-12] The impact of Ki-67 has, however, been reported to vary according to histologic type, and Ki-67 has in some studies lost its prognostic significance when other prognostic factors, for example, malignancy grade and necrosis, have been taken into account. [7,8,13] Topoisomerase 2 alpha (Top2a) cleaves and re-ligates double-stranded DNA, is essential for cell division and accumulates in cells throughout the cell cycle with peak levels prior to mitosis. Top2a represents a molecular target of anthracyclines and has been broadly studied as a prognostic and predictive marker in a number of tumor types, for example, in breast cancer; still its role in STS remains unclear. [14]

Cyclin-dependent kinase inhibitor 1 (p21) is a member of the KIP family of cyclin-dependent kinase (Cdk) inhibitors, which also includes the cyclin-dependent kinase inhibitor 1B (p27Kip1). p21 and p27Kip1 have dual roles in inducing cell cycle arrest through CDK2 inhibition and act as oncoproteins when located in the cytoplasm. Loss or inactivation of p21 has been reported in solid tumors, and in STS, low levels of p21 have been reported to correlate with low grade. [12,15]

Reduced expression of p27Kip1 has been associated with poor prognosis in epithelial cancers of, for example, the esophagus, the breast, and the prostate, but their role in sarcomas is controversial. [1,4,16-19]

Proliferation, assessed by flow cytometric analysis of S-phase fraction, has also been reported to represent an independent prognostic factor in STS. [2,20,21] The prognostic impact of S-phase has been reported to be equivalent to Ki-67 at low cut-offs with a weaker prognostic effect and discriminative power at higher cut-offs. [21]

The aim of our study was to explore the interactions between clinical variables (size, necrosis, grade and vascular invasion) and proliferation markers (S-phase fraction, Ki-67, Top2a, p21, and p27Kip1) and their impact on prognosis. To analyze how the covariates interact we used the Classification and Regression Tree Analysis (CART) technique, which uses recursive partitioning to generate prognostic subgroups. CART analysis identifies specific combinations of covariates associated with a given risk for metastasis and has been applied in diagnostic and prognostic classification in solid tumors. [22-24]

## Materials and methods

*Patient and tumor characteristics*

The study was approved by the Lund University Ethics Committee. Adult patients (>16 years) with primary, non-metastatic STS of the extremities or the trunk wall who were referred before surgery and from whom paraffin-embedded tumor tissue was available were selected. All patients were treated at the Southern Sweden Sarcoma Centre in Lund between 1980 and 2003 and patient data were identified in the Scandinavian Sarcoma Group registry. The study included three common histologic subtypes during the time period, that is, undifferentiated pleomorphic sarcoma (UPS), pleomorphic leiomyosarcoma and pleomorphic liposarcoma. In total, 203 patients were eligible, 7 of which were excluded due to incomplete data, nonrepresentative paraffin-embedded tumor blocks, or technical issues, which left 196 STS for analysis (Table 1).

| Tabel 1 Patient clinicopathological characteristics | | |
|---|---|---|
| **Clinicopathologic characteristics** | **Total cohort (n = 196, %)** | **Number with metastasis (n = 70)** |
| Age at diagnosis, years | | |
| Median (mean) | 69 (66) | - |
| Sex | | |
| Female | 86 (44) | - |
| Male | 110 (56) | - |
| Tumor size | | |
| <5 cm | 56 (28) | 9 |
| ≥5 cm | 140 (72) | 61 |
| Tumor depth | | |
| Subcutaneous | 59 (30) | 17 |
| Deep | 137 (70) | 53 |
| Vascular invasion | | |
| Absent | 149 (76) | 40 |
| Present | 47 (24) | 30 |
| Tumor necrosis | | |
| Absent | 77 (39) | 14 |
| Present | 119 (61) | 56 |
| Grade | | |
| Low (I-II) | 21 (11) | 1 |
| High (III-IV) | 175 (89) | 69 |
| Histology | | |
| UPS | 93 (47) | 37 |
| Leiomyosarcoma | 66 (34) | 25 |
| Liposarcoma | 37 (19) | 8 |
| Local recurrence | | |
| No | 144 (74) | 45 |
| Yes | 52 (26) | 25 |
| Postoperative radiotherapy | | |
| No | 139 (71) | 41 |
| Yes | 57 (29) | 29 |
| Adjuvant chemotherapy | | |
| No | 187 (95) | 67 |
| Yes | 9 (5) | 3 |

Follow-up included clinical examination and chest radiographs every 3 months for the first 2 years and twice yearly thereafter. No patient was lost to follow-up. The median follow-up for survivors was 6,5 (2-26) years. During follow-up, 52 (26%) patients developed local recurrence, of which 25 also developed metastasis. The metastasis rate was 35%, and occurred most commonly to the lungs with a median time to metastasis of 9 months. The median follow-up for patients free from metastases was 6.4 years, but because of non-proportional hazards

for most prognostic factors, 5-year survival was used for analysis. No patient received preoperative chemotherapy or radiotherapy and all patients underwent primary surgery.

Local treatment was classified as carrying a high risk for local recurrence (marginal surgical margin without postoperative radiotherapy (RT) or intralesional margin irrespective of RT) in 11% of the patients or a low risk for local recurrence (wide margin with or without RT or marginal margin with RT) in 89%. [25] Radiotherapy was given postoperatively to 57 (29%) patients and adjuvant chemotherapy was administered to 9 (5%) patients.

Histopathologic diagnosis was made by a sarcoma pathologist (M.Å.). In total, 93 tumors were classified as UPS, 66 as leiomyosarcoma and 37 as liposarcoma. Tumor characteristics, including localization, size, depth, vascular invasion, and necrosis were obtained from the pathology reports and from the SSG database (Table 1). Necrosis was defined as the presence of amorphous cellular debris, usually associated with a neutrophil polymorphonuclear cell response and was dichotomized as present/absent. Vascular invasion was defined as tumor cells surrounded by an endothelial lining. Tumour cells had to be adherent to the luminal aspect of the vessel wall or, if free-floating, had to be associated with adherent fibrin, red blood cells, or leucocytes. Vascular invasion was classified as present/absent. Depth was defined in relation to the deep fascia and tumor depth was classified as deep/subcutaneous. Malignancy grading was based on a 4-tiered scale used in Scandinavia [26], with grades III-IV corresponding to grade 3 of the three-tiered scale of the Fédération Nationale des Centres de Lutte Contre le Cancer grading system [27] and with 90% being high grade [28]. Compared to a population-based study in STS [29], our cohort was comparable as regards tumor size (8 cm versus 7 cm), grade (89%), depth (70%) and metastasis rate (35%), which implies that our STS series is clinically representative.

*Proliferation measures*

S-phase fraction from DNA flow cytometric analysis was calculated from frozen or paraffin-embedded archival tissue using a parametric method and the ModFit LT software, as described elsewhere. [30]

*Immunohistochemical stains and evaluations*

Monoclonal antibodies against Ki-67 (clone M7240), Top2a (clone M7186), p21 (clone M7202), and p27Kip1 (M7203) antibodies from Dako, Glostrup, Denmark) were used for immunohistochemical stains of 4-µm sections.

Heat-induced antigen retrieval was performed prior to staining. Staining was visualized with the EnVision Detection System (Dako, Glostrup, Denmark). A sarcoma pathologist selected one representative tumor block from each tumor. Two observers, who were blinded to the clinical data, assessed all slides. In half of the cases, the 2 observers were A.C. and J.S., and in another half, A.C. and M.J. Evaluations of stained tumor nuclei were based on 5 high-power fields, with an area of 0.28 $mm^2$, in the region of the tumor slide with the greatest density of staining. The ratio of positive nuclei (independent of intensity was recorded semi quantitatively as <5%, 6%-10%, 11%-20%, 21%-30%, 31%-40%, 41%-50% and >50%. All cases with interobserver discordance (≈10%) in the highest score obtained were re-evaluated simultaneously by the two observers together and a consensual score was attributed. No significant difference in the number of discordant cases was observed between the two pairs of observers.

*Statistical analysis*

Immunohistochemical expression of the cell cycle markers was evaluated as discrete variables. For each tumor, the highest score in 5 evaluable fields was considered and the immunohistochemical scores were dichotomized at the median into positive and negative. In addition, the clinicopathological variables size, vascular invasion, necrosis, depth, and grade were analyzed as dichotomous variables with predefined cut-off value for size. All analyses were based on the 196 patients for whom all data on proliferation markers, clinicopathological variables, and follow-up were complete. Metastasis-free survival was used as endpoint for all analyses, and metastasis-free survival rates were calculated according to the Kaplan-Meier method.

Pairwise associations between the variables were analyzed using the ϕ coefficient of correlation. The null hypothesis of equal prognostic effect for a given marker in different histotypes and in different treatments, and postoperative treatment was evaluated using a Cox model with a term for the interaction between the variable of interest and the marker.

The log-rank test was used to test for equality of survival curves. Hazard ratios (HRs) with 95% CI are presented as effect measures and were calculated using the Cox proportional hazards model in univariate and multivariate analyses. Proportional hazards assumptions were verified systematically both graphically and using Schoenfeld test. [31]

To further explore the interactions between the significant clinical variables identified in univariate analysis and their impact on risk for metastasis, CART analysis was used. This method uses recursive partitioning to assess the effect of specific variables on the risk of metastasis, thereby generating a tree-structured model. The analysis was performed using a Stata implementation of CART (cart.ado) by Putten. [32] In order to protect against over fitting, we used adjusted P values and defined an upper bound for the P values ($P_{stop}$ = .01) and a minimum number of patients within a group ($n_{min}$ = 10).

All statistical calculations were performed using Stata 11 (StataCorp 2009; Stata Statistical Software: Release 11; StataCorp LP, College Station, TX).

## Results

*Proliferation measures and their prognostic importance of proliferation*
Immunohistochemical expression (Fig. 1) was detected for all proliferation markers with variable expression between the markers and the tumor subtypes (Fig. 2). The median expression was 10% for Ki-67, 20% for Top2a, 5% for p21, and 50% for p27, and the median S-phase fraction was 10%.

Correlations between the proliferation markers were higher (0.27-0.53) for Ki-67-Top2a and Ki-67-S-phase fraction than for Ki-67-p21, Top2-p21, p21-S-phase fraction, and Top2a-S-phase fraction (0.14-0.37) (Table 2). Correlations to prognostic factors were stronger (0.31-0.44) between Ki-67-necrosis as well as Ki-67-grade and Top2a-necrosis than between (0.28-0.29) Top2a-grade and p21-necrosis (Table 2).

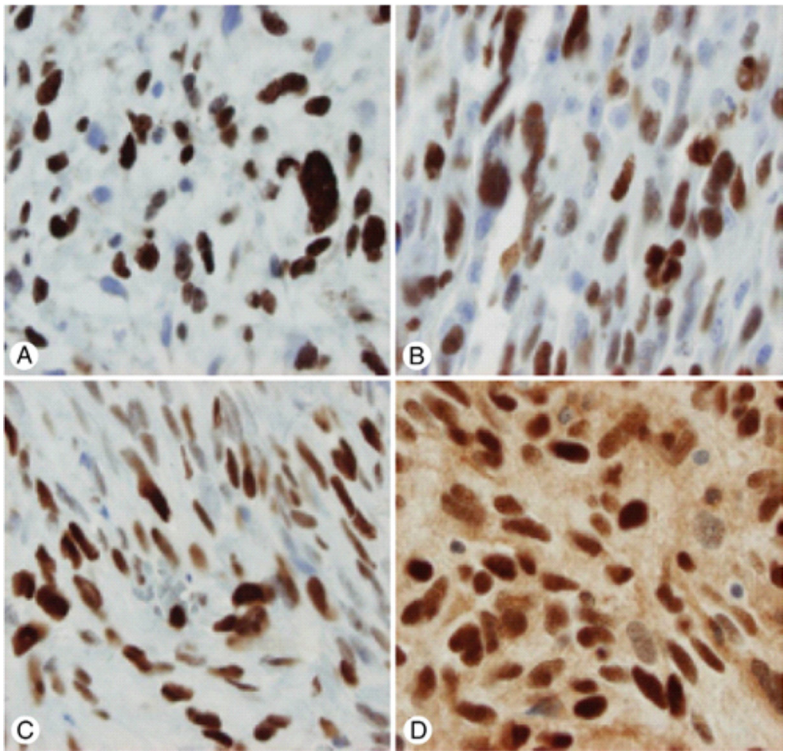


Figure. 1 Representative photographs of immunohistochemical stainings with high expression of Ki-67 (A); Top2a (B); p21 (C); p27Kip1 (D) (all photographs at x40 amplification in Olympus BX45 microscope).

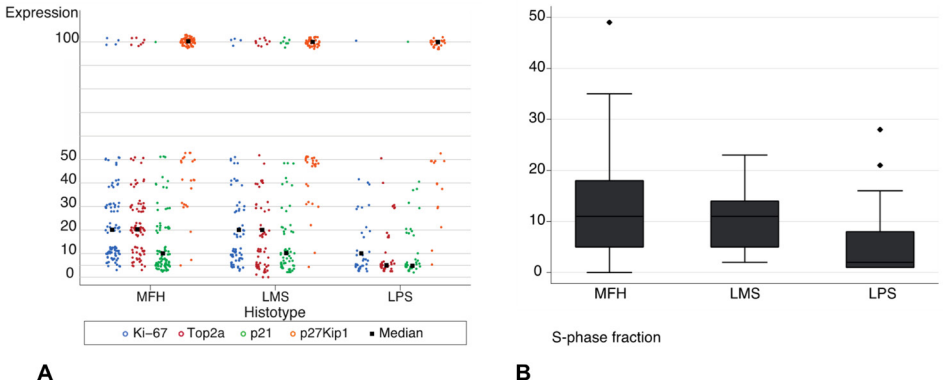


Figure. 2 A, Scatter plot of Ki67, Top2a, p21, and p27Kip expression by histotype (to avoid overplotting, random noise was added to the data). B, Boxplot of S-phase fraction, by histotype.

**Table 2. Correlation coefficient (Cramer φ) for proliferation markers and other prognostic factors**

| Variable | Ki-67 | Top2a | p21 | S-phase | Size | Depth | Grade | VI | Necrosis |
|---|---|---|---|---|---|---|---|---|---|
| Ki-67 | | | | | | | | | |
| Top2a | 0.53* | | | | | | | | |
| p21 | 0.27* | 0.27* | | | | | | | |
| S-phase | 0.40* | 0.37* | 0.14* | | | | | | |
| Size | 0.18* | 0.11 | 0.10 | 0.007 | | | | | |
| Depth | 0.03 | 0.005 | 0.05 | 0.09 | 0.41* | | | | |
| Grade | 0.37* | 0.28* | 0.13* | 0.17* | 0.26* | 0.04 | | | |
| Vascular invasion | 0.20* | 0.10 | 0.17* | 0.06 | 0.28* | 0.05 | 0.19 | | |
| Necrosis | 0.44* | 0.31* | 0.29* | 0.26* | 0.27* | 0.23* | 0.41* | 0.53* | |

Abbreviations: VI, vascular invasion
* Significant correlation

*Univariate analysis*

Univariate analysis of S-phase fraction and proliferation markers revealed a statistically significant correlation to development of metastases for high S-phase fraction (HR =1.8, CI = 1.2-3.7), high Ki-67 expression (HR = 4.5, CI =1.6-12.1) and high Top2a expression (HR = 2.2, CI = 1.2-3.5), whereas expression of p21 and p27Kip1 did not significantly predict metastasis (Table 3). The findings remained significant after adjustment for type of local treatment (data not shown). Size (<5 cm or ≥5 cm), vascular invasion, necrosis, tumour depth, and malignancy grade also significantly predicted risk of metastasis, whereas histotype did not (Table 3).

*Multivariate analysis and CART analysis*

Bivariate models were used to test the independent prognostic role of the markers that showed significant correlations in univariate analysis (Supplementary Table 1).

Ki-67 expression independently predicted development of metastases also after adjustment for size or vascular invasion. When size, malignancy grade, necrosis, and vascular invasion were taken into account together, the prognostic ability of Ki-67 expression disappeared, whereas S-phase fraction remained significant after adjustment for size or vascular invasion but not after adjustment for necrosis, grade or the SIN (size, vascular invasion, necrosis) prognostic model used in Scandinavia. Top2a remained significant only after adjustment for size.

| Table 3. Univariate analysis (n = 196) | | | |
|---|---|---|---|
| **Variable** | **HR** | **95% CI** | ***P*** |
| Ki-67 (positive expression) | 4.4 | 1.6 - 12.1 | 0.004 |
| Top2a (positive expression) | 1.9 | 1.2 - 3.5 | 0.02 |
| p21 (positive expression) | 0.4 | 0.2 - 0.9 | 0.18 |
| P27Kip1 (positive expression) | 0.9 | 0.4 - 1.6 | 0.76 |
| S-phase fraction (positive expression) | 2.1 | 1.2 - 3.7 | 0.008 |
| Size (≥5cm) | 3.7 | 1.8 - 7.6 | <0.001 |
| Vascular invasion (present) | 3.5 | 2.1 - 5.7 | <0.001 |
| Necrosis (present) | 3.5 | 2.0 - 6.4 | <0.001 |
| Depth (deep) | 1.8 | 1.0 - 3.2 | 0.04 |
| SIN (high risk[a]) | 4.0 | 2.3 - 7.3 | <0.001 |
| Grade (high grade) | 9.9 | 1.4 - 71.0 | 0.02 |

Abbreviation: CI, confidence interval.
[a]High risk SIN is defined as patients with two or three risk factors among size, vascular invasion, and necrosis

To explore the interactions between the biomarkers evaluated and more established prognostic factors, in metastasis prediction, we performed CART analysis. Herein, the prognostic factors size, vascular invasion, necrosis, depth, histotype, and malignancy grade provided a basis to which the proliferation markers Ki-67, Top2a, and S-phase fraction were added one at a time. Ki-67 and S-phase fraction created branches in the prognostic tree, which implicates an added value to commonly used prognostic markers (Fig. 3).

Vascular invasion was the strongest predictor and thus determined the first branching, where after Ki-67 was the major stratifying factor in the remaining 149 tumors (Fig. 3A). Estimated prediction curves were generated for each group created by the CART analysis (Fig. 3B). Inclusion of S-phase fraction yielded 5 subsets with vascular invasion determining the first split, followed by necrosis, and tumor size. In tumors <5 cm S-phase fraction contributed to the final branching (Fig. 3D). When Top2a was considered only vascular invasion, necrosis and size determined the structure of the tree, generating 4 terminal subsets with different risk for metastasis (Fig. 3C). Therefore, Top2a expression was not a significant prognostic factor in CART analysis including all the other variables.

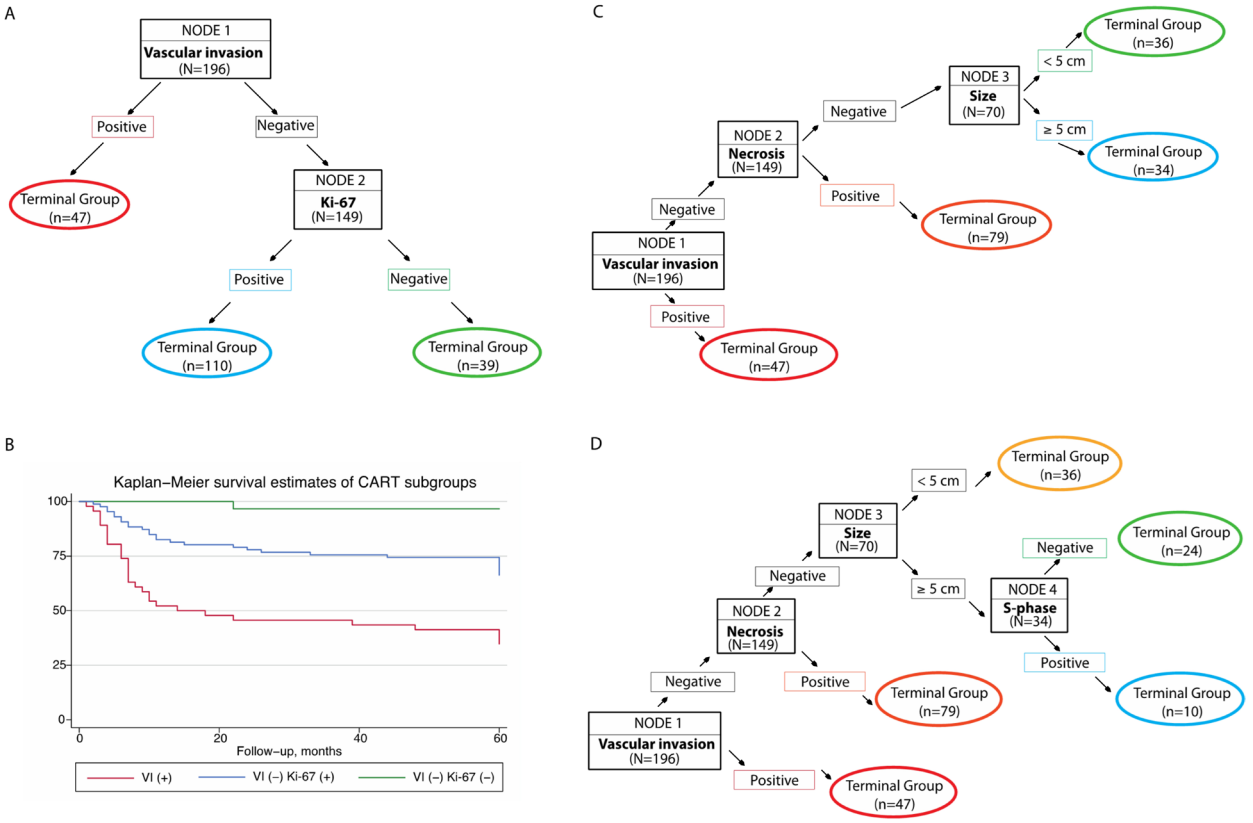


Figure. 3 Default classification tree generated in the presence of vascular invasion, size, deep, necrosis, histotype, grade, and Ki-67 (A), Top2a (C) and S-phase fraction (D). Squares represent non-terminal groups and ellipses terminal subgroups, in which the number of patients is also indicated (n). For the tree issued with Ki-67, Kaplan-Meier survival estimates, according to the generated risk groups are presented (B).

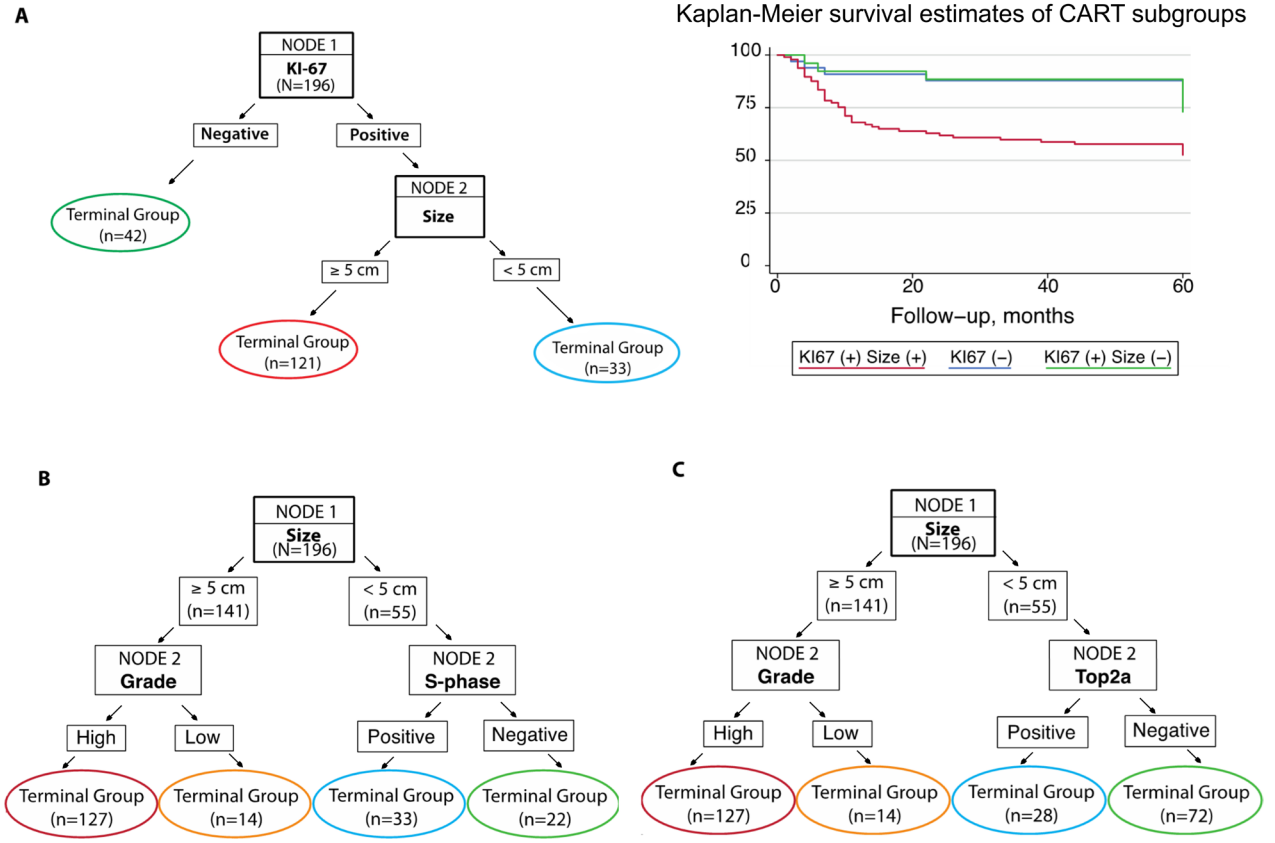


Figure. 4 Classification tree generated considering size, histotype, grade, and Ki-67 (A), S-phase fraction (B) and Top2a (C). Kaplan-Meier survival estimates of the CART subgroups are also presented for Ki-67 (A). Squares represent non-terminal groups and ellipses terminal subgroups, in which the number of patients is also indicated (n).

In the preoperative setting, limited prognostic information is available and generally includes size, histotype, and grade. In order to reflect the prognostic value in this setting, we added Ki-67, Top2a, and S-phase fraction to these factors one at the time (Fig. 4). In the presence of tumor size, histotype did not provide significant prognostic information, whereas high expression of Ki-67, Top2a, S-phase, and grade did. Ki-67 was the strongest prognostic factor (Fig. 4A) splitting the patients in two groups, where after size further divided the subset with high Ki-67 expression. This partition resulted in three subsets with different metastasis rates (Fig. 4A). In the presence of Top2a or S-phase fraction, size was the strongest prognostic factor. Hereafter, Top2a and S-phase fraction were the most important in small tumors, whereas grade determined risk in large tumors (Fig. 4B and C).

## Discussion

We determined the prognostic strength of 5 major proliferation markers in a large STS cohort including common subtypes, that is, leiomyosarcoma, pleomorphic liposarcoma and UPS. Positive correlations were identified for several proliferation markers and prognostic markers with the strongest correlations for Ki-67-Top2a, Ki-67-S-phase, and Ki-67-necrosis (Table 2). This supports observations on correlations between Ki-67 and S-phase fraction, mitotic rate, grade, and necrosis. [7,10,11,27,33] The prognostic impact of the proliferation markers did in univariate analysis identify S-phase, Top2a, and Ki-67 as prognostic markers. The prognostic significance of histotype in STS is controversial. [6,9,34-36] Among pleomorphic sarcomas, myogenic differentiation has also been reported as an adverse prognostic factor [37], but in our data set, we could not find a significant interaction between Ki-67 and histotype (data not shown), therefore suggesting that proliferation could be a useful prognostic factor in pleomorphic sarcomas as a group.

Proliferation markers have not reached clinical application in STS, which largely depends on failure to integrate these markers into currently used prognostic systems, and to distinguish their contribution from that of other prognostic factors, for example, age, tumor size, depth, histologic type, malignancy grade, and necrosis.

There is therefore a need to integrate different markers and to determine their impact in specific subgroups. CART analysis visualizes interaction between survival covariates and allows identification of groups with similar prognosis and has

been applied in diagnostic and prognostic classification in several solid tumor types. [22-24]

Vascular invasion was a major prognostic predictor in pleomorphic STS, but in two thirds of the tumors without vascular invasion, CART analysis identified Ki-67 expression as a major prognostic determinant (Fig. 3) and demonstrated that S-phase fraction contributed to prognostic stratification, following vascular invasion and necrosis in large STS.

Increasing use of preoperative therapy motivates development of factors that can be determined in diagnostic biopsies, which excludes most of the currently applied markers, for example, vascular invasion and necrosis that need to be evaluated in surgical specimens. We therefore sought to address a prognostic model only taking into account factors proliferation markers in conjunction with preoperatively available clinicopathological markers, that is, tumor size, histotype and grade.

Herein, Ki-67 was a major prognostic factor (Fig. 4A). These findings suggest that clinical prognostic classification could consider as high risk only tumors with positive Ki-67 expression and large size. Albeit not included in the initial node, both Top2a and S-phase fraction could contribute to establish risk groups by dividing the small size tumors into two groups (Fig. 4B, 4C), hereby suggesting that in complement to size, Ki-67 should be considered for preoperative prognostication. In conclusion, proliferation markers are independent prognostic factors in pleomorphic STS. In particular, assessment of Ki-67 expression and S-phase fraction combined with currently used prognostic factors contributes

Proliferation in pleomorphic soft tissue sarcoma to refined risk stratification that may allow preoperative application for refined selection of high-risk patients for neoadjuvant therapies.

## References


1. Goto Y, Kawauchi S, Ihara K, et al. The prognosis in spindle-cell sarcoma depends on the expression of cyclin-dependent kinase inhibitor p27(Kip1) and cyclin E. Cancer Sci 2003;94:412-7.
2. Gustafson P, Ferno M, Akerman M, et al. Flow cytometric S-phase fraction in soft-tissue sarcoma: prognostic importance analysed in 160 patients. Br J Cancer 1997;75:94-100.
3. Heslin MJ, Cordon-Cardo C, Lewis JJ, et al. Ki-67 detected by MIB-1 predicts distant metastasis and tumor mortality in primary, high grade extremity soft tissue sarcoma. Cancer 1998;83:490-7.
4. Oliveira AM, Nascimento AG, Okuno SH, Lloyd RV. p27(kip1) protein expression correlates with survival in myxoid and round-cell liposarcoma. J Clin Oncol 2000;18:2888-93.
5. Scholzen T, Gerdes J. The Ki-67 protein: from the known and the unknown. J Cell Physiol 2000;182:311-22.
6. Choong PF, Akerman M, Willen H, et al. Expression of proliferating cell nuclear antigen (PCNA) and Ki-67 in soft tissue sarcoma. Is prognostic significance histotype-specific? APMIS 1995;103:797-805.
7. Choong PF, Akerman M, Willen H, et al. Prognostic value of Ki-67 expression in 182 soft tissue sarcomas. Proliferation—a marker of metastasis? APMIS 1994;102:915-24.
8. Drobnjak M, Latres E, Pollack D, et al. Prognostic implications of p53 nuclear overexpression and high proliferation index of Ki-67 in adult soft-tissue sarcomas. J Natl Cancer Inst 1994;86:549-54.
9. Hoos A, Stojadinovic A, Mastorides S, et al. High Ki-67 proliferative index predicts disease specific survival in patients with high-risk soft tissue sarcomas. Cancer 2001;92:869-74.
10. Huuhtanen RL, Blomqvist CP, Wiklund TA, et al. Comparison of the Ki-67 score and S-phase fraction as prognostic variables in soft-tissue sarcoma. Br J Cancer 1999;79:945-51.
11. Rudolph P, Kellner U, Chassevent A, et al. Prognostic relevance of a novel proliferation marker, Ki-S11, for soft-tissue sarcoma. A multivariate study. Am J Pathol 1997;150:1997-2007.
12. Sabah M, Cummins R, Leader M, Kay E. Immunoreactivity of p53, Mdm2, p21(WAF1/CIP1) Bcl-2, and Bax in soft tissue sarcomas: correlation with histologic grade. Appl Immunohistochem Mol Morphol 2007;15:64-9.
13. Jensen V, Sorensen FB, Bentzen SM, et al. Proliferative activity (MIB-1 index) is an independent prognostic parameter in patients with high-grade soft tissue sarcomas of subtypes other than malignant fibrous histiocytomas: a retrospective immunohistological study including 216 soft tissue sarcomas. Histopathology 1998;32:536-46.
14. Press MF, Sauter G, Buyse M, et al. Alteration of topoisomerase II-alpha gene in human breast cancer: association with responsiveness to anthracycline-based chemotherapy. J Clin Oncol 2011;29:859-67.
15. DeiTos AP, Piccinin S, Doglioni C, et al. Molecular aberrations of the G1-S checkpoint in myxoid and round cell liposarcoma. Am J Pathol 1997;151:1531-9.
16. Antonescu CR, Leung DH, Dudas M, et al. Alterations of cell cycle regulators in localized synovial sarcoma: a multifactorial study with prognostic implications. Am J Pathol 2000;156:977-83.
17. Hayashi E, Yasui A, Oda K, et al. Loss of p27(Kip1) accelerates DNA replication after partial hepatectomy in mice. J Surg Res 2003;111:196-202.
18. Kawauchi S, Goto Y, Ihara K, et al. Survival analysis with p27 expression and apoptosis appears to estimate the prognosis of patients with synovial sarcoma more accurately. Cancer 2002;94:2712-8.
19. Kawauchi S, Goto Y, Liu XP, et al. Low expression of p27(Kip1), a cyclin-dependent kinase inhibitor, is a marker of poor prognosis in synovial sarcoma. Cancer 2001;91:1005-12.
20. Collin F, Chassevent A, Bonichon F, et al. Flow cytometric DNA content analysis of 185 soft tissue neoplasms indicates that S-phase fraction is a prognostic factor for sarcomas. French Federation of Cancer Centers (FNCLCC) Sarcoma Group. Cancer 1997;79:2371-9.
21. Huuhtanen RL, Blomqvist CP, Wiklund TA, et al. S-phase fraction of 155 soft tissue sarcomas: correlation with clinical outcome. Cancer 1996;77:1815-22.

22. Bartley AN, Thompson PA, Buckmeier JA, et al. Expression of gastric pyloric mucin, MUC6, in colorectal serrated polyps. Mod Pathol 2010;23:169-76.
23. Hess KR, Abbruzzese MC, Lenzi R, et al. Classification and regression tree analysis of 1000 consecutive patients with unknown primary carcinoma. Clin Cancer Res 1999;5:3403-10.
24. Zhai R, Chen F, Liu G, et al. Interactions among genetic variants in apoptosis pathway genes, reflux symptoms, body mass index, and smoking indicate two distinct etiologic patterns of esophageal adenocarcinoma. J Clin Oncol 2010;28:2445-51.
25. Enneking WF, Spanier SS, Goodman MA. A system for the surgical staging of musculoskeletal sarcoma. Clin Orthop Relat Res 1980;153:106-20.
26. Angervall L, Kindblom LG, Rydholm A, Stener B. The diagnosis and prognosis of soft tissue tumors. Semin Diagn Pathol 1986;3:240-58.
27. Coindre JM, Terrier P, Guillou L, et al. Predictive value of grade for metastasis development in the main histologic types of adult soft tissue sarcomas: a study of 1240 patients from the French Federation of Cancer Centers Sarcoma Group. Cancer 2001;91:1914-26.
28. Fletcher CDM, Unni KK, Mertens F. World Health Organization classification of tumours. Pathology and genetics of tumours of soft tissue and bone. Lyon: IARC Press; 2002. Page 17.
29. Gustafson P. Soft tissue sarcoma. Epidemiology and prognosis in 508 patients. Acta Orthop Scand Suppl 1994;259:1-31.
30. Gustafson P, Baldetorp B, Ferno M, Akerman M. Prognostic implications of various models for calculation of S-phase fraction in 259 patients with soft tissue sarcoma. Br J Cancer 1999;79:1205-9.
31. Schoenfeld D. Partial Residuals for The Proportional Hazards Regression Model. 1982; 69: 239-241.
32. van Putten W. Classification And Regression Tree analysis (CART) with Stata. Stata Users Group; 2002.
33. Jensen V, Hoyer M, Sorensen FB, et al. MIB-1 expression and iododeoxyuridine labelling in soft tissue sarcomas: an immunohistochemical study including correlations with p53, bcl-2 and histological characteristics. Histopathology 1996;28:437-44.
34. Engellau J. Prognostic factors in soft tissue sarcoma. Tissue microarray for immunostaining, the importance of whole-tumor sections and time-dependence. Acta Orthop Scand Suppl 2004;75 2 p preceding table of contents-52, backcover.
35. Hasegawa T, Yamamoto S, Yokoyama R, et al. Prognostic significance of grading and staging systems using MIB-1 score in adult patients with soft tissue sarcoma of the extremities and trunk. Cancer 2002;95:843-51.
36. Ludwig JA. Personalized therapy of sarcomas: integration of biomarkers for improved diagnosis, prognosis, and therapy selection. Curr Oncol Rep 2008;10:329-37.
37. Fletcher CD, Gustafson P, Rydholm A, Willen H, Akerman M. Clinicopathologic re-evaluation of 100 malignant fibrous histiocytomas: prognostic relevance of subclassification. J Clin Oncol 2001 Jun 15;19:3045-50.

## Supplementary data

**Table 1: Patient clinicopathological characteristics**

| Clinicopathologic characteristics | Total cohort (N=196, %) | Number with metastasis (n=70) |
|---|---|---|
| Age at diagnosis, years | | |
| Median (Mean) | 69 (66) | - |
| Sex | | |
| Female | 86 (44) | - |
| Male | 110 (56) | - |
| Tumor size | | |
| < 5 cm | 56 (28) | 9 |
| ≥ 5 cm | 141 (72) | 61 |
| Tumor depth | | |
| Subcutaneous | 59 (30) | 17 |
| Deep | 137 (70) | 53 |
| Vascular invasion | | |
| Absent | 149 (76) | 40 |
| Present | 47 (24) | 30 |
| Tumor necrosis | | |
| Absent | 77 (39) | 14 |
| Present | 119 (61) | 56 |
| Grade | | |
| Low (I-II) | 21 (11) | 1 |
| High (III-IV) | 175 (89) | 69 |
| Histology | | |
| UPS | 93 (47) | 37 |
| Leiomyosarcoma | 66 (33) | 25 |
| Liposarcoma | 37 (19) | 8 |
| Local recurrence | | |
| No | 144 (74) | 45 |
| Yes | 52 (26) | 25 |
| Postoperative radiotherapy | | |
| No | 139 (71) | 41 |
| Yes | 57 (29) | 29 |
| Adjuvant chemotherapy | | |
| No | 187 (95) | 67 |
| Yes | 9 (5) | 3 |

HR: Hazard ratio; CI, confidence interval
*High risk SIN defined as patients with two or three risk factors among size, vascular invasion and necrosis

# PART III

**Isolated limb perfusion**