Insulin-Like Growth Factor Type 1 Receptor Expression Correlates to Good Prognosis in Highly Malignant Soft Tissue Sarcoma

Jan Åhlén,[1,2,3,4] Johan Wejde,[4] Otte Brosjö,[3] Anette von Rosen,[2] Wen-Hui Weng,[1] Leonard Girnita,[4] Olle Larsson,[2] and Catharina Larsson[1]

[1]Departments of Molecular Medicine, [2]Surgery, [3]Orthopedics, and [4]Oncology and Pathology, Karolinska University Hospital, Stockholm, Sweden

ABSTRACT

Purpose: **To evaluate known and suggested prognostic markers, especially insulin-like growth factor type 1 receptor (IGF-1R), in highly malignant soft tissue sarcomas (STS).**

***Experimental Design*: A cohort of 101 patients with primary STS of high malignancy grade was studied with respect to development of metastasis, local recurrence, and survival during a minimum of 5 years follow-up. All tumors were analyzed by immunohistochemistry for expression of Ki-67, p53, p27, Bcl-2, IGF-1R, and microvessel density. The traditional clinical variables size, malignancy grade (3 or 4), necrosis, mitotic frequency, infiltrative tumor growth, vascular invasion, depth, and surgical margins were also evaluated.**

Results: **A significant association was shown between high expression of IGF-1R and favorable outcome. Among STS with positive IGF-1R immunoreactivity, cases with high expression (76-100% positive cells) had the best outcome, whereas cases with the lowest expression (1-25% positive cells) had the worst. As expected, large tumor size (>11 cm), presence of necrosis, high mitotic count, intralesional surgery, and deep location were all significantly associated with poor outcome, both in univariate and multivariate analyses. No difference in outcome was observed between cases of malignancy grade 3 versus 4, whereas the included and more objective variables necrosis and mitotic count were found to be reliable prognostic markers.**

Conclusion: **IGF-1R expression is a common feature of highly malignant STS. Further elucidation of the role of IGF-1R and the IGF system in STS may both provide a basis for development of new prognostic tools in STS, as well as shed light on the basic mechanisms of the STS development.**

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Requests for reprints: Jan Åhlén, Department of Molecular Medicine, CMM L8:01, Karolinska University Hospital, SE-171 76 Stockholm, Sweden. Phone: 46-8-51773616; Fax: 46-8-51776180; E-mail: jan.ahlen@kirurgi.ki.se.

INTRODUCTION

Soft tissue sarcomas (STS) are a heterogeneous group of rare malignant tumors originating from mesenchymal tissue (1). The 5-year overall survival for STS patients is about 63% to 76% (2–4). Histopathologic malignancy grading has been shown to be of great prognostic value. Specifically, the 5-year survival is much better (85-96%) for cases with low-grade STS (grades 1 and 2), as compared with only 45% to 55% for cases with high-grade STS (grades 3 and 4; refs. 2, 5, 6). The most important variables in the histopathologic malignancy grading include tumor size, depth, necrosis, and vascular invasion as well as cellularity and cellular atypia (1–3). For patients with low-grade sarcoma where the prognosis is fairly good, surgery alone is the standard treatment, whereas in high-grade sarcoma where the prognosis is poor the question arises whether the patient should receive adjuvant therapy or not. Still 45% to 55% survive and the natural course of each individual cannot be reliably predicted at the time of surgery. To approach this question, we have evaluated known and suggested prognostic markers in a series of 101 cases consisting of only highly malignant STS followed-up for at least 5 years.

The insulin-like growth factor type 1 receptor (IGF-1R) is a cell membrane receptor that is activated by its ligands IGF-1 and IGF-2 and has an effect on cell proliferation, differentiation, and prevention of apoptosis (8, 9). After ligand binding, downstream signals are sent to the cell leading to either mitogenic and antiapoptotic effects, or to induction of differentiation with subsequent growth arrest, differentiation and cell death (10). Ewing's sarcoma cells with dominant negative IGF-1R exhibit increased apoptosis, inhibition of tumorigenicity, and enhanced chemosensitivity (11). Since IGF-1R has been shown to be involved in malignant transformation (9, 10), and IGF-1R is frequently overexpressed in human cancer, development of IGF-1R–directed cancer therapy has been initiated.

The present study was aimed to describe the expression of IGF-1R in highly malignant STS and evaluate its possible prognostic value. Furthermore, we also compared the histopathologic grading 3 versus 4 and evaluated the included and more objective traditional clinical variables size, necroses, mitotic frequency, infiltrative tumor growth, vascular invasion, depth, and surgical margins as well as immunohistochemical analyses focused on antigens involved in the proliferation, differentiation, angiogenesis, and apoptotic process (Ki-67, p53, Bcl-2, p27, and Factor VIII for estimation of vessel density).

MATERIAL AND METHODS

Cell Lines. The murine cell lines R− and P6 were kindly provided by Dr. Renato Baserga, Thomas Jefferson University Philadelphia, and have been described previously (12). The R− line is IGF-1R negative and was derived from fibroblasts of an IGF-1R knockout mouse embryo. The P6 line is a derivative of NIH-3T3 that overexpresses human IGF-1R. The cells were cultured in DMEM supplemented with 5% to 10% fetal bovine serum in the presence of G-418 (Promega, Madison, WI).

Patients and Tumor Material. All STS patient who had their primary surgery during the period 1985 to 1993 at the orthopedic department, Karolinska University Hospital and subsequently followed at the same unit were identified from the database. From this group, we selected only patients that were treated for highly malignant STS (grade 3 or 4), were without metastases at the time of diagnosis, and had not received any preoperative or postoperative adjuvant treatment. The histopathologic samples from these 128 patients were then re-evaluated by an experienced histopathologist who was without knowledge of the clinical course. The tumors were thereby classified according to established histopathologic criteria (13), and malignancy grading was determined on a four-graded scale (2, 14). Moreover, mitotic count, necrosis, vascular invasion, and infiltrative growth pattern outside the tumor capsule were separately recorded. Patients who after re-evaluation was classified as having osteosarcoma or Ewing sarcoma (n = 9), low malignancy grade STS (n = 3), or a non-STS (n = 6) were excluded from the study. Similarly, patients from whom histopathologic samples were missing (n = 7) or follow-up data were not available (n = 3) were also excluded. Thus, after the evaluation 101 patients with a primary STS of high malignancy grade remained who were included in the study (Table 1).

The 101 patients included 47 males (47%) and 54 females (53%) with a mean age at diagnosis of 62 years (range = 5-90 years). All patients had been operated on with curative intent and the surgical margins were re-evaluated from the surgical report and histopathologic assessment of margins. The primary tumors were located in the lower extremities in 66 of the cases. Of the remaining tumors, 16 were located in the upper extremities, 9 in the pelvic area, and 10 in the trunk or abdominal wall. The histopathologic re-evaluation of STS diagnosis showed that the material consisted of 13 different entities including malignant fibrous histiocytoma (MFH, n = 65), liposarcoma (n = 16), malignant peripheral nerve sheath tumor (n = 4), synovial sarcoma (n = 3), fibrosarcoma (n = 3), alveolar STS (n = 3), leiomyosarcoma (n = 2), mesenchymoma (n = 1), rhabdomyosarcoma (n = 1), spindle cell sarcoma (n = 1), angiosarcoma (n = 1), and high grade sarcoma NOS (n = 1).

The follow-up data have been collected until October 2001 or until the patients' death, and the occurrence of metastasis and/or local recurrence was recorded (Table 1). For the analyses, the patients were divided into the following groups depending on the different kind of recurrent disease: Patients who had no evidence of disease during follow-up (DFS) were used as a reference group in calculations of local recurrence and metastasis; Patients who developed local recurrence but no metastases during follow-up (Only Lrec); Patients with metastases but no local recurrence during follow-up (Only Met); and patients who had developed both local recurrence and metastases during follow-up (Lrec + Met). In the Lrec + Met group, the date of the first event was used in the statistical analyses. Separate calculations were also done for the group of patients who developed local recurrence irrespective of metastases during follow-up (All Lrec) and the patients with metastases irrespective of local recurrence during follow-up (All Met). Furthermore, the group of patients who were without evidence of disease at the end of follow-up but may have had a local recurrence or in one case metastasis (NED) was used as reference in calculation of death from or with disease (DOD).

In addition, for the Western analyses, fresh frozen tissue samples of STSs were collected from patients operated at the orthopaedic department Karolinska University Hospital, representing different histopathologic entities including leiomyosarcoma grade 3 (n = 1), clear cell sarcoma (n = 1); liposarcoma grade 3 (n = 1), synovial sarcoma (n = 1), MFH grade 3 (n = 1), and MFH grade 4 (n = 1).

Immunohistochemical Analyses. Paraffin-embedded tissue from the primary tumor was available for all 101 cases, and the single most representative tumor tissue block was used for immunohistochemical analyses. The following dilutions and antibodies were used for the antigen detection: 1:40 of anti human-Bcl-2 oncoprotein (Dakopatt, Copenhagen, Denmark), 1:100 of DO-1 for p53 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), 1:150 of Mib-1 for Ki-67 (Immunotech nuclear antigen), 1:500 of Clone F8/86 for anti-human von Willebrand Factor (Factor VIII; Dakopatt), 1:1,000 of Kip-1 for p27 (Transduction Laboratories, Newington, NH), and 1:1,000 of polyclonal of an antibody to the IGF-1R α-subunit (N20 Santa Cruz Biotechnology). In control experiments, slides from 10 of the cases were incubated with another polyclonal antibody against the IGF-1R β-subunit (H60 Santa Cruz). The immunohistochemical staining was done according to the standard ABC technique (Elite Standard Kit, Vector, Burlingame, CA). Paraffin sections of 4 μm were deparaffinized, rehydrated, and pretreated with citrate buffer at pH 6 in a microwave for 20 minutes. After rinsing, the endogenous peroxidase activity was blocked by treatment with 0.5% hydrogen peroxide for 30 minutes. The sections were then rinsed and incubated with blocking serum (1% bovine serum albumin) for 20 minutes. The primary antibody was applied and sections were incubated overnight in a moist chamber at +8°C. A biotinylated anti-mouse immunoglobulin G, used as secondary antibody was incubated for 30 minutes, followed by rinsing and the avidin-biotin complex for another 30 minutes. The peroxidase reaction was developed using 3,3-diaminobenzidine for 6 minutes and nuclear counterstaining was done with Mayers hematoxylin. TBS (pH 7.4) was used for rinsing between the steps. For IGF-1R, the same method for immunostaining was done as for the other antibodies with the exception that the 20 minutes pretreatment with citrate buffer was omitted and biotinylated anti rabbit immunoglobulin G was used as a secondary antibody. Paraffin sections from tissues known to be negative or positive for the respective markers were analyzed in parallel as negative and positive controls.

For Bcl-2, p53, Ki-67, and p27 the degree of staining was calculated as the proportion of positive cells using a 10 × 10 grid in 10 high power fields (200×). For each high power field the total number of positive and negative cells were determined by counting all positively stained cells in two or more representative rows and multiplied to 10 × 10. The scorings were done by one independent observer for all cases and by two independent observers for randomly selected cases. In the evaluation of vessel density, 10 fields (200×) with the most intensive neovascularization (hotspot) were evaluated, and all vessels stained by Factor VIII were counted. The IGF-1R staining was evaluated by three independent observers using a semiquantitative approach by which the

Table 1 Summary of the 101 STS cases studied

Variable		Total	Only Met		Lrec + Met		Only Lrec		DFS		Total
			Total	Dead	Total	Dead	Total	Dead	Total	Dead	Dead
Total		101 (65, 16)	41 (21, 9)	40 (21, 8)	12 (9, 1)	12 (9, 1)	13 (10, 1)	4 (3, 1)	35 (25, 5)	6 (5, 1)	62 (38, 11)
Clinical											
Sex	Female	54 (30, 8)	21 (8, 5)	21 (8, 5)	8 (5, 1)	8 (5, 1)	8 (5, 1)	3 (2, 1)	17 (12, 1)	2 (1, 1)	34 (16, 8)
	Male	47 (35, 8)	20 (13, 4)	19 (13, 3)	4 (4, 0)	4 (4, 0)	5 (4, 0)	1 (1, 0)	18 (13, 4)	4 (4, 0)	28 (22, 3)
Depth	S.c.	22 (15, 2)	4 (1, 1)	3 (1, 0)	1 (0, 0)	1 (0, 0)	3 (3, 0)	1 (1, 0)	14 (11, 1)	4 (4, 0)	9 (7, 0)
	I.m.	45 (26, 10)	19 (9, 5)	19 (9, 5)	4 (4, 0)	4 (4, 0)	8 (5, 1)	2 (1, 1)	14 (8, 4)	2 (1, 1)	27 (15, 7)
	Extracompartmental	26 (17, 3)	12 (6, 2)	12 (6, 2)	6 (4, 1)	6 (4, 1)	2 (2, 0)	1 (1, 0)	6 (5, 0)	0 (0, 0)	19 (11, 3)
Size (cm)	≤6	32 (22, 4)	3 (1, 1)	3 (1, 1)	5 (3, 1)	5 (3, 1)	3 (2, 0)	0 (0, 0)	21 (16, 2)	4 (4, 0)	12 (8, 2)
	7-11	23 (17, 3)	9 (6, 2)	9 (6, 2)	1 (1, 0)	1 (1, 0)	5 (5, 0)	2 (2, 0)	8 (5, 1)	1 (0, 1)	13 (9, 3)
	>11	33 (21, 6)	19 (11, 3)	19 (11, 3)	4 (4, 0)	4 (4, 0)	5 (3, 1)	2 (1, 1)	5 (3, 2)	1 (1, 0)	26 (17, 4)
Surgical margin	Wide	44 (26, 8)	17 (7, 4)	17 (7, 4)	3 (2, 0)	3 (2, 0)	6 (4, 1)	2 (1, 1)	18 (13, 3)	6 (5, 1)	28 (15, 6)
	Marginal	31 (19, 6)	10 (3, 4)	9 (3, 3)	4 (4, 0)	4 (4, 0)	5 (4, 0)	0 (0, 0)	12 (8, 2)	0 (0, 0)	13 (7, 3)
	Intralesional	17 (12, 1)	7 (5, 0)	7 (5, 0)	4 (2, 1)	4 (2, 1)	2 (2, 0)	2 (2, 0)	4 (3, 0)	0 (0, 0)	13 (9, 1)
Histopathological											
Malignancy grade	Grade 3	38 (22, 7)	12 (5, 3)	12 (5, 3)	8 (5, 1)	8 (5, 1)	6 (5, 0)	0 (0, 0)	12 (7, 3)	2 (1, 1)	22 (11, 5)
	Grade 4	63 (43, 9)	29 (16, 6)	28 (16, 5)	4 (4, 0)	4 (4, 0)	7 (5, 1)	4 (3, 1)	23 (18, 2)	4 (4, 0)	40 (27, 6)
Necroses	Yes	77 (51, 12)	35 (18, 8)	34 (18, 7)	9 (7, 1)	9 (7, 1)	11 (8, 1)	4 (3, 1)	22 (18, 2)	4 (3, 1)	51 (31, 10)
	No	24 (14, 4)	6 (3, 1)	6 (3, 1)	3 (2, 0)	3 (2, 0)	2 (2, 0)	0 (0, 0)	13 (7, 3)	2 (2, 0)	11 (7, 1)
Mitosis	0-2 per HPF	21 (10, 5)	5 (1, 2)	5 (1, 2)	2 (0, 1)	2 (0, 1)	4 (4, 0)	0 (0, 0)	10 (5, 2)	0 (0, 0)	7 (1, 3)
	3-9 per HPF	30 (22, 5)	14 (9, 3)	13 (9, 2)	4 (4, 0)	4 (4, 0)	3 (2, 1)	2 (1, 1)	9 (7, 1)	0 (0, 0)	19 (14, 3)
	>10 per HPF	48 (32, 6)	21 (10, 4)	21 (10, 4)	6 (5, 0)	6 (5, 0)	6 (4, 0)	2 (2, 0)	15 (13, 2)	6 (5, 1)	35 (22, 5)
Growth pattern	Infiltrative	89 (59, 12)	37 (20, 7)	36 (20, 6)	10 (7, 1)	10 (7, 1)	12 (9, 1)	4 (3, 1)	30 (23, 3)	6 (5, 1)	56 (35, 9)
	Pushing	7 (3, 2)	2 (0, 1)	2 (0, 1)	1 (1, 0)	1 (1, 0)	0 (0, 0)	0 (0, 0)	4 (2, 1)	0 (0, 0)	3 (1, 1)
Vascular invasion	Yes	11 (6, 0)	6 (3, 0)	6 (3, 0)	0 (0, 0)	0 (0, 0)	1 (1, 0)	0 (0, 0)	4 (2, 0)	0 (0, 0)	6 (3, 0)
	No	84 (55, 16)	33 (17, 9)	32 (17, 8)	12 (9, 1)	12 (9, 1)	11 (8, 1)	4 (3, 1)	28 (21, 5)	6 (5, 1)	54 (34, 11)
Immunohistochemical											
Ki-67	≤10% positive cells	10 (3, 4)	4 (1, 2)	4 (1, 2)	0 (0, 0)	0 (0, 0)	2 (1, 1)	1 (0, 1)	4 (1, 1)	0 (0, 0)	5 (1, 3)
	>10% positive cells	69 (46, 9)	26 (13, 5)	26 (13, 5)	11 (9, 0)	11 (9, 0)	10 (8, 0)	3 (3, 0)	22 (16, 4)	3 (2, 1)	43 (27, 6)
p53	≤40% positive cells	68 (41, 12)	30 (15, 7)	30 (15, 7)	7 (4, 1)	7 (4, 1)	7 (5, 1)	3 (2, 1)	24 (17, 3)	5 (4, 1)	45 (25, 10)
	>40% positive cells	19 (14, 4)	5 (2, 2)	4 (2, 1)	4 (4, 0)	4 (4, 0)	3 (3, 0)	0 (0, 0)	7 (5, 2)	0 (0, 0)	8 (6, 1)
p27	≤30% positive cells	45 (31, 5)	17 (10, 2)	16 (10, 1)	4 (4, 0)	4 (4, 0)	8 (5, 1)	3 (2, 1)	16 (12, 2)	2 (2, 0)	25 (18, 2)
	>30% positive cells	45 (25, 11)	18 (7, 7)	18 (7, 7)	8 (5, 1)	8 (5, 1)	4 (3, 0)	1 (1, 0)	15 (9, 3)	3 (2, 1)	30 (15, 9)
Bcl-2	≤30% positive cells	84 (53, 15)	32 (16, 8)	31 (16, 7)	10 (7, 1)	10 (7, 1)	11 (8, 1)	4 (3, 1)	31 (22, 5)	5 (4, 1)	50 (30, 10)
	>30% positive cells	8 (4, 1)	4 (1, 1)	4 (1, 1)	2 (2, 0)	2 (2, 0)	1 (1, 0)	0 (0, 0)	1 (0, 0)	0 (0, 0)	6 (3, 1)
Vessel density	≤30 vessels per HPF	67 (39, 13)	30 (13, 8)	29 (13, 7)	10 (7, 1)	10 (7, 1)	7 (5, 1)	3 (2, 1)	20 (14, 3)	4 (3, 1)	46 (25, 10)
	>30 vessels per HPF	23 (17, 2)	7 (5, 1)	7 (5, 1)	2 (2, 0)	2 (2, 0)	4 (3, 0)	0 (0, 0)	10 (7, 1)	1 (1, 0)	10 (8, 1)
IGF-1R	≤50% positive cells	69 (42, 14)	31 (14, 8)	31 (14, 8)	9 (7, 1)	9 (7, 1)	7 (5, 1)	3 (2, 1)	22 (16, 4)	3 (2, 1)	46 (25, 11)
	>50% positive cells	18 (12, 1)	4 (4, 0)	4 (4, 0)	2 (1, 0)	2 (1, 0)	4 (3, 0)	0 (0, 0)	8 (4, 1)	2 (2, 0)	8 (7, 0)

NOTE: (x, x), figures in parenthesis represent MFH and liposarcoma.
Abbreviation: HPF, high power field.

proportion of positive cells were scored in 25% intervals giving either negative tumors (0% positive cells) or positive tumors (1-25%, 26-50%, 51-75%, or 76-100% positive cells). In addition, the STSs with positive IGF-1R expression were also evaluated for relative staining intensity on the cellular level.

Western analyses of IGF-1R. Total protein was extracted from the six fresh frozen STSs and the two cell lines R− and P6 and used for Western analyses using standard methods (15, 16). The tumors were cut in pieces, suspended in freshly prepared homogenization buffer as described (16), and centrifuged at 14,000 × g for 10 minutes at 4°C. Protein extracts from tumors and cells were dissolved in a sample buffer containing 0.0625 mol/L Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, bromphenol blue, and DTT. Fifty micrograms of each sample was electrophoresed in parallel with molecular weight markers (Bio-Rad) in 7.5% SDS PAGE gels, and transferred overnight to nitrocellulose filters (Amersham Pharmacia, Piscataway, NJ). After blocking for 1 hour at room temperature in a solution of 5% skimmed milk powder and 0.02% Tween 20 in PBS (pH 7.5), the filters were incubated with the primary antibody for 1 hour at room temperature. Two different polyclonal rabbit antibodies targeted against the α-subunit of IGF-1R (N20, Santa Cruz Biotechnology) and against the β-subunit of IGF-1R (C20, Santa Cruz Biotechnology) were used at dilution 1:500. After washing in PBS, the filters were incubated for 1 hour with a biotinylated secondary antibody (1:500; Amersham Pharmacia), then with streptavidin-labeled horseradish peroxidase (1:500), whereupon the detection was made by exposure to Hyperfilm-enhanced chemiluminescence (Amersham Pharmacia). As a control of loading and protein quality, the filters were reincubated with a monoclonal anti-â-Actin (1:1000; Sigma, St. Louis, MO), followed by labeled anti-mouse horseradish peroxidase (1:1,000) and detected.

Statistical Analyses. Correlations between clinical, immunohistochemical, histopathologic variables and outcome during follow-up were analyzed by the Kaplan-Meier survival test, and the differences were calculated with the log-rank test. For each variable evaluated, a series of cutoff values were tested. The cutoff that gave the best Ps is presented in the tables and used in the

statistical analyses. Cox proportional hazard regression model was used for multivariate analyses and correlation analyses were done with Spearman rank order test. All calculations were done in Statistica 6.0 software, and $P < 0.05$ was accepted as significant. Separate analyses for some of the variables in the tumors diagnosed as MFH have been previously published (17).

RESULTS

In this study, 101 primary highly malignant STSs were characterized regarding clinical, histopathologic, and immunohistochemical variables and the findings were then evaluated in relation to the survival and the occurrence of local recurrence and distant metastasis during follow-up (Tables 1 and 2). No major difference in clinical course was observed between the different histopathologic entities except that patients with liposarcoma tended to have a somewhat slower course to recurrence. All cases have therefore been analyzed together, regardless of the histopathologic type of STS. All patients were followed from surgery for at least 5 years (mean = 92 months, median = 90, range = 60-131) or until their death. For all patients, the 5-year overall survival was 51%, and at the end of follow-up, 45% of the patients were still alive. There were 53 patients with distant metastases and of these were 32 (78%) diagnosed within 2 years after the primary surgery (mean = 17 months, median = 14, range = 1-62). Local recurrence without distant metastasis was diagnosed in 13 of the patients, and nine of the patients in this group were without evidence of disease at the end of follow-up (mean follow-up = 57 months, median = 64, range = 17-107). Both local recurrence and distant metastasis were found in 12 patients and all in this group died within the follow-up time. The remaining 35 patients remained disease-free during follow-up.

Association of Size, Site, Surgical Margin, Necrosis, and Mitosis with Prognosis. With regard to the clinical variables, tumor depth, size, and surgical margin were all associated with the outcome (Table 3; Fig. 1). Patients with deeply or i.m. located tumors more frequently developed metastases ($P = 0.006$) and were accordingly significantly correlated to death from disease ($P = 0.005$, Fig. 1*B*). In contrast, s.c. tumors had the best prognosis and were also significantly associated with smaller size at diagnosis as compared with non-subcutanous

Table 2 Parameters studied in relation to 5-year survival in the 101 STS

Parameter		Total no.	5-year follow-up		
			DOD		NED
			Total (%)	(MFH, LPS)	Total (MFH, LPS)
Clinical					
Sex	Female	54	26 (48)	(13, 5)	28 (17, 3)
	Male	47	22 (48)	(17, 2)	24 (17, 6)
Depth	S.c.	22	5 (23)	(2, 0)	17 (13, 2)
	Extracompartmental	26	15 (60)	(10, 2)	10 (6, 1)
	I.m.	45	21 (47)	(12, 4)	24 (14,6)
Size (cm)	≤6	32	6 (19)	(2, 2)	26 (20, 2)
	7-11	23	10 (43)	(9, 1)	13 (8, 2)
	>11	33	23 (70)	(15, 3)	9 (5, 3)
Surgical margin	Wide	44	20 (47)	(10, 3)	23 (15, 5)
	Marginal	31	9 (29)	(5, 2)	22 (14, 4)
	Intralesional	17	11 (65)	(8, 1)	6 (4, 0)
Histopathological					
Malignancy grade	Grade 3	38	16 (43)	(8, 3)	21 (13, 4)
	Grade 4	63	32 (51)	(22, 4)	31 (21, 5)
Necrosis	Yes	77	41 (54)	(26, 7)	35 (24, 5)
	No	24	7 (29)	(4, 0)	17 (10, 4)
Mitosis	0-2 per HPF	21	6 (30)	(1, 2)	14 (8, 3)
	3-10 per HPF	30	14 (47)	(12, 1)	16 (10, 4)
	>10 per HPF	48	27 (56)	(16, 4)	21 (16, 2)
Growth pattern	Pushing	7	2 (29)	(1, 0)	5 (2, 2)
	Infiltrative	89	44 (50)	(27, 7)	44 (31, 5)
Vascular invasion	Yes	11	5 (45)	(3, 0)	6 (3, 0)
	No	84	42 (51)	(26, 7)	41 (28, 7)
Immunohistochemical					
Ki-67	≤10% positive cells	10	3 (30)	(1, 1)	7 (2, 3)
	>10% positive cells	69	35 (51)	(22, 5)	33 (23, 4)
p53	≤40% positive cells	68	33 (49)	(18, 6)	35 (23, 6)
	>40% positive cells	19	8 (44)	(6, 1)	10 (7, 3)
Vessel density	≤30 per HPF	67	36 (53)	(19, 7)	31 (19, 6)
	>30 per HPF	23	8 (35)	(7, 0)	15 (10, 2)
Bcl-2	≤30% positive cells	84	38 (46)	(24, 6)	45 (28, 9)
	>30% positive cells	8	5 (62)	(2, 1)	3 (2, 0)
p27	≤30% positive cells	45	20 (44)	(15, 1)	25 (16, 4)
	>30% positive cells	45	23 (52)	(11, 6)	21 (13, 5)
IGF-1R	≤50% positive cells	69	36 (52)	(21, 7)	33 (21, 7)
	>50% positive cells	18	5 (29)	(4, 0)	12 (7, 1)

NOTE: (*x*, *x*), figures in parenthesis represent MFH and LPS.
Abbreviations: NED, no evidence of disease at the end of follow-up; DOD, death from or with disease; LPS, liposarcoma; HPF, high power field.

Table 3 Association between clinical, histopathological, and immunohistochemical variables and outcome during follow-up

Parameter	Overall survival	Death from disease	Only met	All met	Disease-free survival
Clinical					
Sex	0.99 (0.35, 0.010, 0.51)	0.75 (0.65, 0.032, 0.51)	0.83 (0.30, 0.08, 0.69)	0.99 (0.48, 0.09, 0.56)	0.82 (0.45, 0.06, 0.90)
Depth, not s.c.	0.065 (0.09, 0.15, 0.94)	0.005 (0.003, 0.19, 0.94)	0.006 (0.008, 0.46, 0.48)	0.006 (0.002, 0.48, 0.83)	0.003 (0.002, 0.41, 0.67)
Size ≤ 6 cm	0.0017 (0.006, 0.46, 0.39)	0.00016 (0.0002, 0.61, 0.39)	0.00002 (0.00008, 0.41, 0.18)	0.00045 (0.0006, 0.50, 0.36)	0.00005 (0.00005, 0.43, 0.37)
>11 cm	0.0005 (0.002, 0.87, 0.087)	0.0001 (0.0004, 0.68, 0.087)	0.00005 (0.0006, 0.68, 0.016)	0.00013 (0.0007, 0.74, 0.07)	0.00015 (0.001, 0.58, 0.02)
Intralesional surgery	0.033 (0.049, 0.49, 0.72)	0.039 (0.017, 0.47, 0.72)	0.73 (0.11, NS, 0.76)	0.1 (0.053, 0.92, 0.65)	0.17 (0.10, 0.99, 0.97)
Histopathological					
Malignancy grade 4	0.33 (0.20, 0.77, 0.77)	0.48 (0.44, 0.96, 0.77)	0.73 (0.96, 0.56, 0.48)	0.75 (0.69, 0.53, 0.74)	0.96 (0.70, 0.43, 0.65)
Presence of necroses	0.029 (0.24, 0.04, 0.27)	0.025 (0.17, 0.066, 0.27)	0.024 (0.27, 0.047, 0.11)	0.06 (0.27, 0.042, 0.32)	0.01 (0.16, 0.03, 0.13)
Mitotic count > 2 per HPF	0.005 (0.002, 0.95, 0.19)	0.017 (0.008, 0.93, 0.19)	0.073 (0.13, 0.56, 0.047)	0.023 (0.009, 0.56, 0.17)	0.11 (0.30, 0.47, 0.055)
Infiltrative growth pattern	0.31 (0.36, 0.54, 0.74)	0.4 (0.52, 0.52, 0.74)	0.33 (0.20, 0.43, 0.73)	0.57 (0.61, 0.44, 0.79)	0.21 (0.27, 0.38, 0.71)
Vascular invasion	0.33 (0.42, NS, 0.63)	0.6 (0.69, NS, 0.63)	0.92 (0.64, NS, 0.59)	0.82 (0.90, NS, 0.72)	0.68 (0.92, NS, 0.32)
Immunohistochemical					
Ki-67 > 10% positive cells	0.19 (0.30, 0.84, 0.20)	0.36 (0.42, 0.95, 0.20)	0.89 (0.89, 0.81, 0.17)	0.42 (0.57, 0.081, 0.23)	0.55 (0.77, 0.66, 0.07)
p53 ≤ 40% positive cells	0.15 (0.33, 0.18, 0.40)	0.34 (0.68, 0.27, 0.40)	0.6 (0.53, 0.43, 0.33)	0.6 (0.77, 0.43, 0.35)	0.73 (0.90, 0.37, 0.37)
p27 > 30% positive cells	0.49 (0.83, 0.29, 0.48)	0.55 (0.85, 0.41, 0.48)	0.75 (0.75, 0.47, 0.91)	0.37 (0.79, 0.72, 0.38)	0.97 (0.70, 0.69, 0.83)
Bcl-2 > 30% positive cells	0.49 (0.51, 0.40, 0.83)	0.42 (0.48, 0.38, 0.83)	0.16 (0.21, 0.72, 0.84)	0.35 (0.42, 0.67, 0.88)	0.27 (0.15, 0.73, 0.55)
Vessel density ≤ 30 vessels per HPF	0.023 (0.21, 0.22, 0.14)	0.037 (0.24, 0.27, 0.14)	0.12 (0.62, 0.38, 0.23)	0.64 (0.40, 0.40, 0.10)	0.09 (0.51, 0.34, 0.11)
IGF-1R ≤ 50% positive cells	0.023 (0.47, 0.12, 0.058)	0.02 (0.31, 0. 21, 0.058)	0.093 (0.98, 0.23, 0.016)	0.044 (0.41, 0.2, 0.06)	0.11 (0.76, 0.18, 0.053)

NOTE: *P*s were calculated with log-rank test from the difference between the curves in the Kaplan-Meier survival plot.
Figures in the columns represent All tumors (MFH, liposarcoma, and non-MFH/liposarcoma).
Abbreviation: HPF, high power field; NS, not significant.

tumors (mean = 4.8 versus 11.7 cm; $R = 0.47$; $P < 0.001$). Size was significantly correlated to outcome and two separately limits were found to give the most significant results. A large tumor size (>11 cm) was a powerful prognostic predictor of poor outcome with significant association both regarding shorter overall survival ($P = 0.00048$), development of metastases ($P = 0.00017$), and death in disease ($P = 0.0001$). Similarly, small tumor size (≤6 cm) was a good marker for improved outcome with better overall survival ($P = 0.0016$), lower risk for metastases ($P = 0.00045$), and lower risk of death from disease ($P = 0.00016$, Fig. 1*A*). In agreement with previous reports (3), a cutoff level of 8 cm was also associated with the outcome. Significant association were found both for shorter overall survival ($P = 0.03$), development of metastases ($P = 0.009$), and dead in disease ($P = 0.01$). Surgical margin was also found to be of prognostic importance in that intralesional surgery was significantly associated with shorter overall survival ($P = 0.033$) and death of disease ($P = 0.039$, Fig. 1*D*).

Among the different histopathologic characteristics of the primary tumor, necroses and mitotic count had both an impact on the clinical outcome (Table 3). Patients with presence of necroses in their tumors developed distant metastases more frequently ($P = 0.024$). These patients consequently had shorter overall survival ($P = 0.029$) and a higher risk of death from disease ($P = 0.025$, Fig. 1*F*). Mitotic count >2 per high power field was similarly associated with metastatic disease ($P = 0.023$), shorter overall survival ($P = 0.005$), and death of disease ($P = 0.017$, Fig. 1*E*). None of the remaining histopathologic variables (i.e., malignancy grade 3 versus 4, infiltrative growth pattern, and vascular infiltration) was associated with the outcome.

Association between IGF-1R Expression and Prognosis. Immunohistochemical stainings were done for analyses of p53, Bcl-2, p27, Ki-67, and IGF-1R expression levels as well as to estimate vessel density following staining of the endothelial cells with Factor VIII (Tables 1-3). Vessel density showed a large variation between the individual tumors, ranging from 3 to 75 vessels per high power field with a mean of 21. If only the highest count of vessels within one tumor was regarded, the number of vessels ranged from 5 to 147 with a mean of 37 vessels per high power field. Ki-67 showed an overall high level of expression and 69 tumors (87%) had more than 10% positive nuclei (mean = 42% and range = 0-88%). Similarly, the vast majority of the tumors expressed p27 with a mean of 35% positive cells (range = 0-100%). p53 was expressed in approximately half of the tumors with a mean of 20% positive cells (range = 0-100%). Only 15 tumors (16%) showed Bcl-2 expression in more then 5% of the

cells (range = 0-100%). IGF-1R expression was evident in 42 (48%) of the tumors, and of these were 18 (20%) detected with >50% positive cells.

High IGF-1R expression was found to be significantly correlated with favorable outcome. Of the other tested immunohistochemical markers, microvessel density was found to be inversely proportional to both death of disease ($P = 0.037$) and overall survival ($P = 0.023$), whereas neither of Ki67, p53, p27, or Bcl-2 showed significant association for any of the end criteria.

As illustrated in Fig. 2, tumors with positive IGF-1R staining showed a cytoplasmic signal in varying proportions of the cells. The proportion of positive cells was scored in 25%

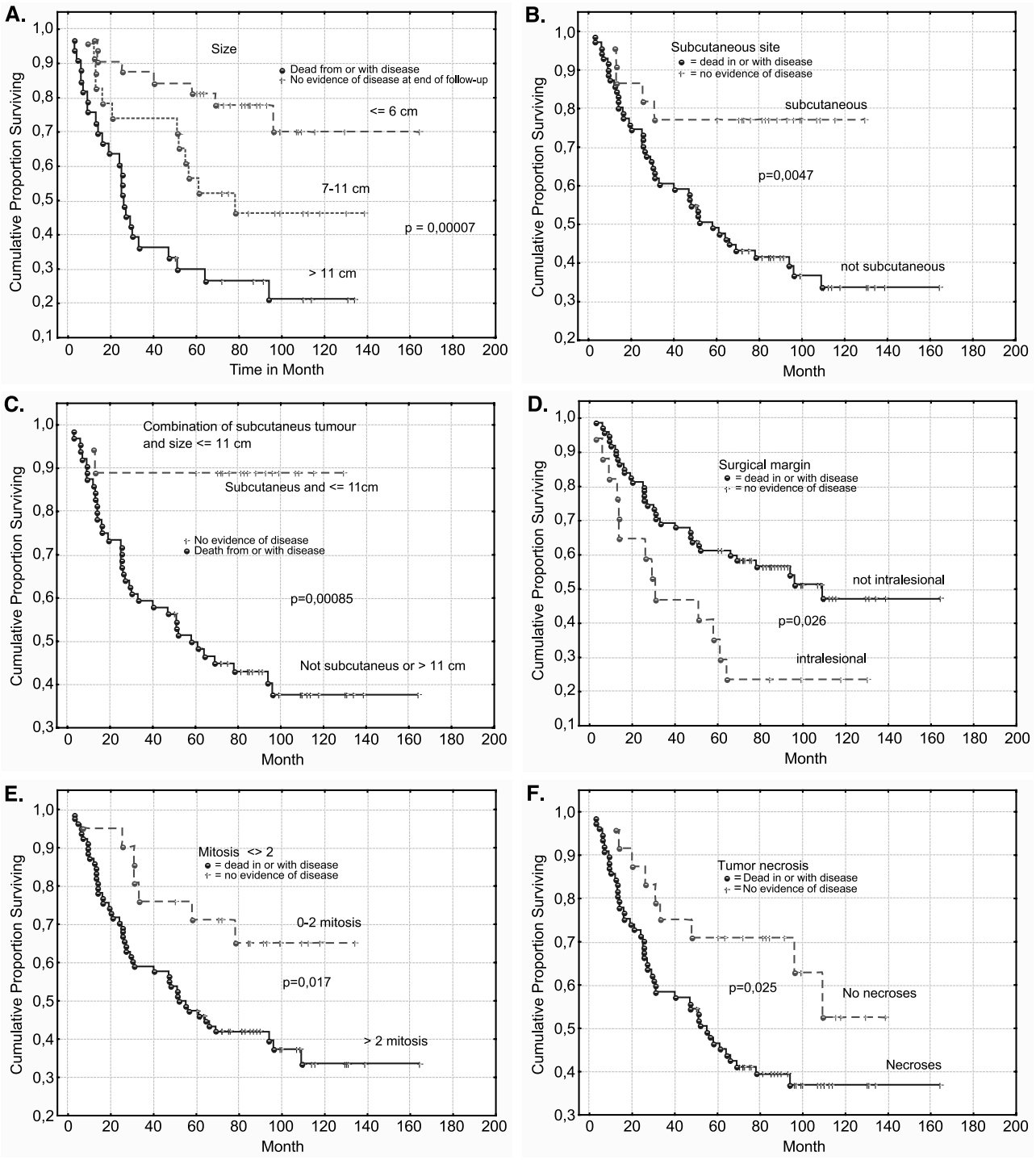

Fig. 1 Kaplan-Meier plots illustrating significant association between survival and (*A*) tumor size, (*B*) tumor site, (*C*) combination of size and site, (*D*) surgical margin, (*E*) number of mitosis, and (*F*) presence of necrosis.

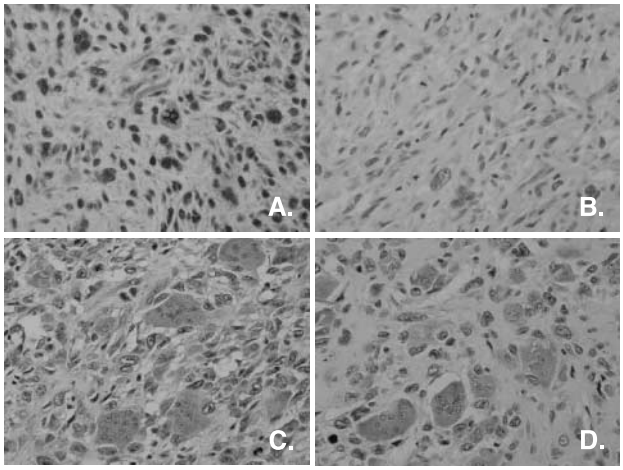

Fig. 2 Examples of immunohistochemical detection of IGF-1R protein expression using antibodies (*A-C*) IGF-1R-α/N20 and (*D*) IGF-1R-β/H60. STSs: tumors with (*A*) high expression and (*B*) negative expression. *C* and *D,* one STS with similar staining using the two different IGF-1R antibodies. *A,* almost all cells are positive with a strong staining in the individual cells; *C,* sample with intermediate level of staining and lower frequency of stained cells.

intervals giving either negative tumors (0% positive cells) or positive tumors (1-25%, 26-50%, 51-75%, or 76-100% positive cells). When cases with positive and negative IGF-1R staining were compared as a whole, no difference in outcome was revealed (Fig. 3*A*). However, cases with high expression (>50% positive cells) showed significantly better survival and lower occurrence of metastases as compared with cases with no or lower expression levels (<50% positive cells; Fig. 3*B* and *C*). Specifically, high IGF-1R expression was significantly associated with less death in disease ($P = 0.02$) and better overall survival ($P = 0.023$). The same pattern was also observed when the cases were further subdivided according to quartiles of expression level (i.e., cases with 76-100% positive cells had the best and cases with 0-25% positive cells had the worst outcome; Fig. 3*D*). The prognostic impact of the other cutoff values were also evaluated which showed that tumors with >25% positive cells were significantly associated to less death in disease ($P = 0.04$), whereas no significant association to overall survival was found. The STSs with positive expression of IGF-1R were also evaluated for relative staining intensity of the individual tumor cells. Although the staining intensities were found to vary, this was not found to have an impact on the patient's outcome during follow-up.

The results for the different immunohistochemical markers were each combined with IGF-1R and compared with the clinical outcome. This revealed two significant associations with suggested additive effect as compared with IGF-1R alone (Fig. 3). Hence, high IGF-1R expression combined with low Bcl-2 expression, and high IGF-1R expression combined with high p27 expression were each significantly associated with less death of disease ($P = 0.007$ and $P = 0.012$).

In verification experiments, the expression detected with the antibody IGF-1R-α/N20 were compared with two other IGF-1R antibodies in Western and immunohistochemical analyses. Comparable staining patterns were observed by immunohistochemistry in the 10 STSs analyzed with IGF-1R-α/N20 and IGF-1R-β/H60 (Fig. 2*C* and *D*). To confirm the specificity and sensitivity of the N20 antibody used for the above immunohistochemical stainings, we did a Western blot analysis to compare it with another established antibody to IGF-1R (C20). C20 recognizes epitopes in the β-subunit of the receptor. We included also samples from cells overexpressing human IGF-1R (P6) and IGF-1R negative cells (R−) as positive and negative controls. Figure 4 shows that both antibodies immunoreacted adequately with the positive and negative controls. All six STS cases (isolated from six different samples) were positive but to various extents. As can be seen, there was a good correlation of the signal intensities between the two IGF-1R antibodies. These data confirms that the N20 antibody is both specific and sensitive for the IGF-1R.

Prognostic Variables in Relation to STS Histiotype. The 101 STS were also subgrouped according to histiotype giving three groups with large enough sample sizes to allow separate analyses (i.e., MFH, $n = 65$; liposarcomas, $n = 16$; and STSs outside these categories, $n = 20$). Evaluation of the clinical variables in relation to outcome in the different subgroups confirmed the detected associations for most variables in the MFH group (Table 3) as previously reported in part (17). Large tumor size was significantly associated to metastases and short disease free survival in the non-MFH/liposarcoma group (Table 3). For the histopathologic variables, it can be noted that the prognostic impact of mitotic count was confirmed in the MFH group. Furthermore, the presence of necroses was significantly associated to poor survival and development of metastases in the liposarcoma group. With regard to the immunohistochemical markers studied IGF-1R expression was significantly associated to metastases in the non-MFH/liposacoma, whereas this was not found in the entire material.

Correlation Test and Multivariate Analyses. The clinical, histopathologic, and immunohistochemical variables evaluated in this study were all pairwise compared in correlation analyses. This revealed that mitotic count was positively correlated to Ki-67 ($R = 0.44$, $P < 0.001$) as expected and also to malignancy grade ($R = 0.36$, $P = 0.0001$). IGF-1R expression was weakly correlated to Bcl-2 expression ($R = 0.28$, $P = 0.009$).

In the multivariate analyses, we found that depth, size, necroses, mitosis, and IGF-1R expression all were independent factors significantly associated to both dead in disease and overall survival. Vessel density was in this regression model only significant for overall survival, whereas Ki-67 was significant for dead in disease. When tumor size and s.c. location were calculated together a very strong and independent association was observed for both death in disease ($P = 0.0008$, Fig. 1*C*) and overall survival ($P = 0.029$).

DISCUSSION

In this cohort consisting of only highly malignant STS, the factors size, depth, necrosis, mitotic count, and surgical margin were each found to have significant impact on the outcome during follow-up. These findings and the fact that the patients were all recorded and similarly treated in the same surgical unit during a limited time period support the accuracy and the representativity of the material studied. In fact, the distinction of

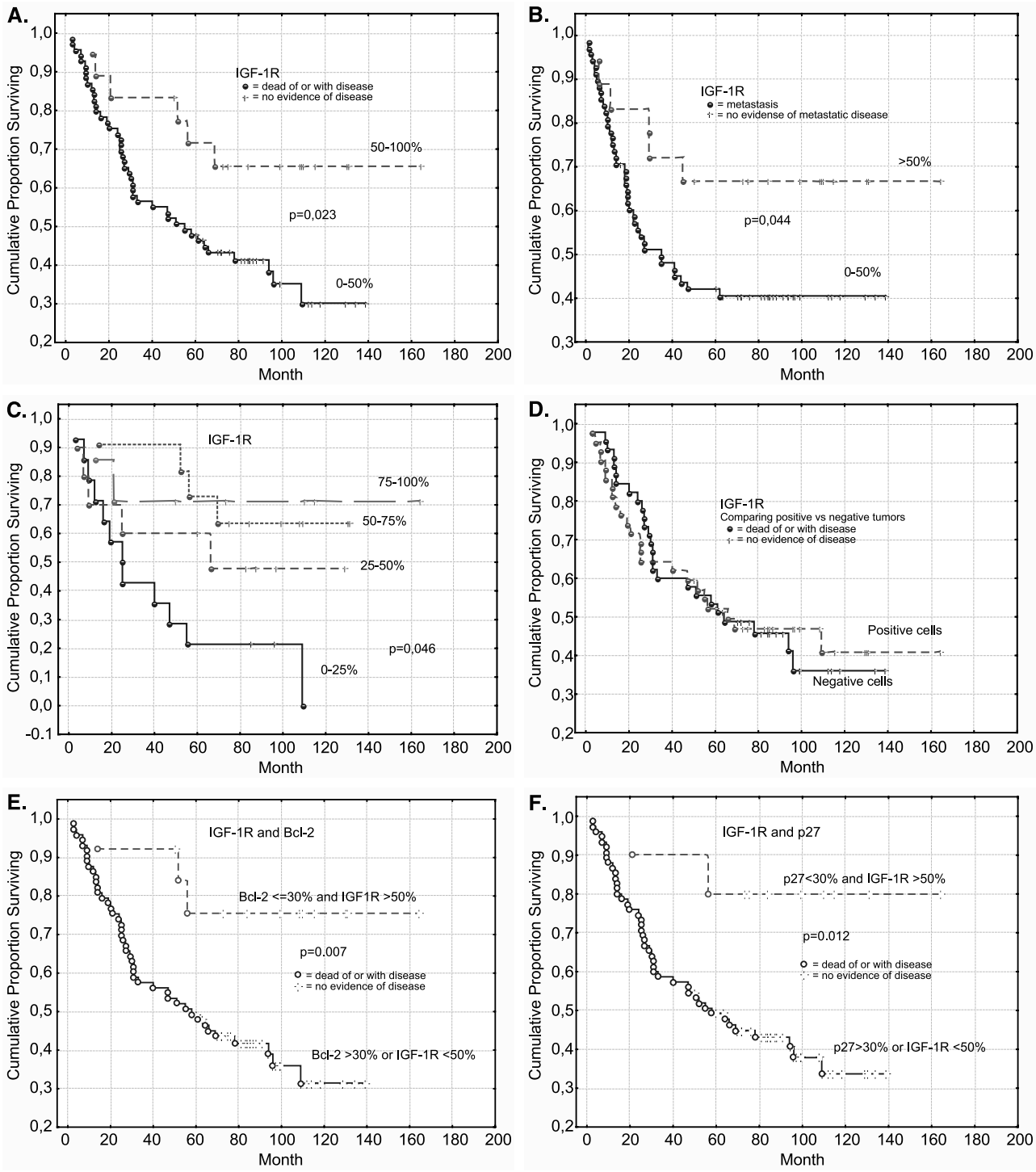

Fig. 3 Kaplan-Meier plots illustrating significant association between expression level of IGF-1R and (*A*, *C*, and *D*) survival and (*B*) occurrence of metastasis during follow-up, as well as between survival and IGF-1R expression combined with (*E*) Bcl-2 expression or (*F*) p27 expression.

tumor size at the limits of 6 and 11 cm were very strong prognostic markers for good and poor outcome. In previous reports, a cutoff level at 8 cm has been suggested (3), which was confirmed in the present study. However, the prognostic associations were less significant as compared with the 6 and 11 cm limits. Malignancy grading that as a whole is known to be a good prognostic marker, could not distinguish the prognosis in highly malignant STS of grade 3 versus grade 4, whereas the more objective and in judgment of grade included variables, necroses, and mitotic count could. This suggests that in clinical practice, distinction of tumors of low-malignant and high-malignant grade or a three-grade scale would be more relevant

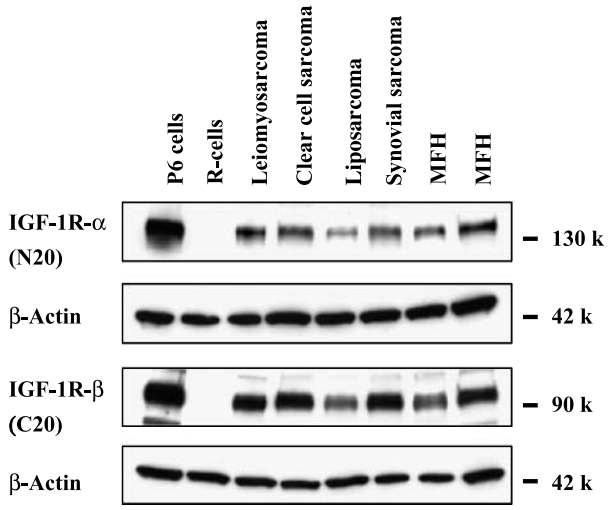

Fig. 4 Western blot analyses of IGF-1R protein expression using antibodies IGF-1R-α/N20 (*top*) and IGF-1R-β/C20 (*bottom*). Expression of IGF-1R is detected with both antibodies in the positive control and in all STSs studied. The P6 cell line with overexpression of IGF-1R served as a positive control and the R− cell line with IGF-1R knockout as a negative control. Reincubation of the filters with a β-actin antibody confirmed appropriate protein amounts in each lane.

and that the objective variables included, necrosis, and mitotic count are more reliable. In addition to these already known prognostic markers, a significant association was shown between high expression of IGF-1R and favorable outcome, a finding that may have potential predictive impact.

The immunohistochemical markers studied were selected because of their documented importance for tumor growth or because of their suggested value as prognostic markers in STS in previous studies. p53 contributes to tumor suppression by inducing both cell cycle arrests (18) and apoptosis (19, 20), and Bcl-2 is a regulatory protein acting as an inhibitor of apoptosis (21). The significance of p53 and Bcl-2 as prognostic markers is still controversial (22–30). The p27 protein is a major regulator of G_1-S transition of the cell cycle (31, 32) and has been shown to be an independent prognostic factor in patients with synovial sarcoma (33) and leiomyosarcoma (34). Ki-67 (35) antigen correlates with the growth fraction of the tumor and high-level of Ki-67 expression have been shown to correlate with poor outcome (22, 36–38). We could in the present study not find any correlation to prognosis, metastasis, or local recurrence for any of these markers. The lack of significant association between expression levels of p53, Bcl-2, p27, and Ki-67 and clinical outcome most likely reflects the malignant phenotype and strong growth potential in the group of highly malignant STS studied. It is therefore not surprising that proteins promoting or reflecting growth of the primary tumor were highly expressed in most of the cases studied (Table 1). Angiogenesis has been suggested to be of importance for the tumor growth beyond a certain size as well as for tumor invasion and tumor dissemination (39). Neovascularization measured as vessel density has been associated with malignancy in many tumor types (40, 41). Thus far, there have not been any convincing results of vessel density as a prognostic factor in STS (42, 43). We found an inverse association to survival but no association to metastasis or local recurrence. Taken together, the reports on vessel density in STS suggest that this marker is of unreliable value as a prognostic marker in this tumor group. More studies have to be done to clarify the role of angiogenesis in STS.

The IGF system of ligands, receptors, and soluble binding proteins offers excellent candidates for involvement in local and distant tumor spreading. Normally, the IGF system is involved in the intrauterine and postnatal development, and most likely the development of all tissues and organs is regulated more or less by the IGF system (44). The demonstration of IGF-1R involvement in malignant transformation (9, 10), and the frequent detection of IGF-1R overexpression in human cancer have fired the efforts to develop IGF-1R directed cancer therapy. Possible routes in this respect would include, e.g., antisense molecules, antibodies, dominant-negative IGF-1R and tyrosine kinase inhibitors, and targeted radionuclides (45). Until date, antisense molecules (46), antibodies (47, 48), dominant-negative IGF-1R (9), and tyrosine kinase inhibitors (49–51) have been applied for these purposes and shown to cause massive apoptosis of tumors cells and abrogation of tumor development *in vitro* and/or *in vivo.* Actually, some of these studies have shown antitumor activity after blocking IGF-1R activity in sarcomas, including STS (11, 16, 51).

In this study, a positive IGF-1R expression was revealed in approximately half of the cases by immunohistochemistry. The N20 antibody used in the immunohistochemistry targets the extracellular α-subunit of IGF-1R, and its accuracy was verified using two different approaches. In paraffin sections, cytoplasmic immunoreactivity was revealed in all positive cases. Immunohistochemistry done on 10 of the same STSs using a second IGF-1R antibody (H60) revealed the same type of staining pattern. One of the cases is illustrated in Fig. 2, whereby the identical morphology of the positively stained cells is evident (Fig. 2*C* and *D*). In Western analyses, the IGF-1R-α/N20 antibody revealed a protein product of expected size in primary STSs and in cells overexpressing IGF-1R, whereas no product was detected in IGF-1R knockout cells. Application of a second IGF-1R antibody (C20) verified the result.

The accuracy of the detection system has also been previously shown in parallel analysis of RNA expression by reverse transcription-PCR and protein expression by Western and immunohistochemistry using the same antibody (52, 53). Taken together with the available literature, the present findings suggest that IGF-1R expression is a common feature of STS in general. In our study, high IGF-1R expression was associated with better survival and low development of metastases. A similar pattern has been shown in other malignancies as well (54), whereas in some malignancies the opposite situation is found with high IGF-1R expression in cases with metastatic disease (52, 55–57). The increased expression of IGF-1R observed here could per se reflect both reduced and increased receptor activity depending on the functional status of the receptor, the level of available ligands, and whether the ligand binding interferes with antibody binding. The level of receptor activity is of obvious importance for interpreting the association between high IGF-1R expression and good prognosis. This could be due to a higher secretion of IGF-1 or IGF-2 in the cases with more aggressive disease leading to increased occupation and activation of the IGF-1R, which in turn leads to increased internalization of ligand-receptor complex (58). An

alternative mechanism is that Mdm2 modulates the IGF-1R expression in the tumors. Recently it was shown that Mdm2 causes down-regulation of IGF-1R (15) and it is well known that increased Mdm2 expression is associated with a more malignant phenotype in many STSs (58, 59).

The weak correlation observed between Bcl-2 and IGF-1R expression ($R = 0.28$, $P = 0.009$) could indicate an activation of the apoptotic signaling pathway. If so, the IGF-1R expression would reflect activation of the maturation process in the positively stained cases. On the other hand, it is possible that the IGF-1R over expression is a compensatory effect following low activity of ligand-receptor interaction. Both these possibilities would be in agreement with the better prognosis observed for patients with high expression levels.

In conclusion, we show that the traditional clinical variables necroses, mitotic count, intralesional surgery, and tumor localization are independent factors significantly associated to both death of disease and overall survival, whereas the differentiation between malignancy grade 3 and 4 seem less meaningful. Importantly, the distinction of tumors smaller than 6 am or larger than 11 cm has very strong prognostic value. The significant association shown between high expression of IGF-1R and favorable outcome imply that further elucidation of the role of IGF-1R and the IGF system in STS may aid in future prognostication of STS, as well as shed light on the basic mechanisms of the STS development.

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