Elevated Expression Level of Survivin Protein in Soft-Tissue Sarcomas Is a Strong Independent Predictor of Survival

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ABSTRACT

Purpose: Survivin is a member of the inhibitor-of-apoptosis gene family and is known to be overexpressed in a number of tumor types. The aim of this study was to evaluate the prognostic value of survivin protein expression in tumor tissue extracts in a group of well-characterized soft-tissue sarcoma (STS) patients.

Experimental Design: In this investigation, malignant tissue samples from 63 STS patients as well as from a panel of tumor cell lines were investigated, with nonmalignant tissues serving as controls. The survivin protein level was quantified by a novel ELISA and by Western blot analysis. Results obtained by both methods were compared with clinopathological parameters regarding tumor grade and tumor entity, and they were then correlated to survival in a multivariate Cox regression model.

Results: High survivin levels were detected by ELISA and Western blot analysis in tumor tissue extracts and in lysates of tumor cell lines. None or only weak expression of survivin protein was found in nonmalignant cells and tissues. When comparing survivin values obtained by ELISA or Western blot, we found a significant correlation between both methods (P = 0.013, Pearson test). Our findings revealed that, in multivariate Cox regression analyses, survivin levels measured by ELISA and Western blot were significantly associated with tumor-related death in STS patients (P = 0.001, RR = 19.8, and P = 0.004, RR = 5.1, respectively). However, in a direct comparison of both survivin protein detection assays, we found a higher sensitivity and a stronger correlation to prognosis in survivin ELISA as compared with the Western blot assays. Furthermore, a higher tumor grade and more aggressive STS entity showed an elevated survivin protein expression level.

Conclusion: Altogether, an elevated survivin content in tumor tissue extracts has a significant and independent negative predictive value on the survival-rate of STS patients. This finding corresponds well to data obtained for the mRNA level of survivin, as shown previously (M. Kappler et al., Int. J. Cancer, 95: 360–363, 2001).

INTRODUCTION

Survivin, a member of the inhibitor-of-apoptosis protein family, represents a multifunctional protein that suppresses apoptosis and regulates cell division at the G2-M phase (reviewed in Refs. 1 and 2). It is a nuclear shuttle protein that is actively exported from the nucleus (3). Independently, survivin expression is also correlated to mitotic activity (4–6).

Survivin is strongly overexpressed in a vast majority of cancers, and it is one of the most tumor-specific human gene products (7). The potential utility of survivin overexpression in early diagnosis and as a prognostic marker of cancer is incontrovertible (1). A correlation between survivin detection and the outcome of the affected tumor patients was described for different carcinoma types including colorectal cancer (8–11), bladder cancer (12), lung carcinoma (13), breast cancer (14), esophageal cancer (15), pancreatic duct cell cancer (16), hepatocellular cancer (17), gastric cancer (18), and malignant glioma (19).

Survivin seems to have strong potential as a tumor marker for malignant solid tumors of mesodermal origin when correlated to tumor progression and prognosis. In addition to the studies performed on neuroblastoma by Adida et al. and others (20–23) we found that a high survivin transcript level significantly correlates with a poor outcome in adult STS patients (24). More recently, we have shown a significant correlation between the coexpression of the survivin mRNA and the telomerase reverse transcriptase mRNA and a highly increased risk of tumor-related death in the same group of STS patients (25).

Moreover, several reports describe therapeutic implications of the inhibition of survivin expression for different malignan-

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2 M. Ka., and M. Ko. contributed equally to the results of the study.

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4 The abbreviations used are: STS, soft-tissue sarcoma; Ab, antibody; RR, relative risk; WB, Western blot; RMS, rhabdomyosarcoma; LMS, leiomyosarcoma; MFH, malignant fibrous histiocytoma; FS, fibrosarcoma.
cies (1, 15, 26–32). Results from survivin promoter transfection experiments indicate that survivin protein expression appears to be regulated, at least in part, at the transcript level (33). Taken together, the biological and cancer-related findings for survivin implicate that this marker may be a universal anticancer-drug target (1).

In the present study, we investigated the survivin protein expression by quantitative WB analysis and, for the first time, quantitatively by ELISA. The data presented here indicate a prognostic impact of survivin protein content in tissue extracts of STS patients determined quantitatively by both ELISA and WB. Moreover, multivariate analyses of the survivin protein expression data support our previous finding of a strong predictive role of survivin at the transcript level (24). Therefore, survivin appears to be an independent and strong predictor of survival for patients suffering from STS both at the mRNA and at the protein expression level.

**MATERIALS AND METHODS**

**STS Patients and Clinical Data.** In this study, tumor tissue samples from 63 adult patients with histologically verified STS were included. Tumor patients and tissue samples were part of two studies on the evaluation of the survivin transcript level in STS patients that were described previously (24, 25).

The patients’ age ranged from 22 to 83 years (mean age, 58 years). Twenty-nine patients died from the tumor after a mean of 25 months (range, 2–96 months), whereas 34 patients were alive after a mean observation period of 36 months (range, 4–107 months after primary tumor resection). Of the tumor samples 13 (20.6%) were grade 1, 29 (46.0%) were grade 2, and 21 (33.3%) were grade 3. The tumors were staged according to the Union International contre Cancrum (UICC) system (Table 1). All of the primary tumors (n = 41) were untreated before primary tumor resection. Of 16 recurrences, 6 were treated by radiotherapy and 8 by chemotherapy. Of these, three got both therapies. Of six metastases, two were treated by both radiotherapy and chemotherapy, and one received only chemotherapy. Representative clinical data of the STS patients are summarized in Table 1.

**Table 1** Patient/tumor characteristics and survivin quantification

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cases</th>
<th>ELISA + (% above cutoff)</th>
<th>WB + (% above cutoff)</th>
<th>Tumor-related death (%)</th>
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<tr>
<td><strong>Sex</strong></td>
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<tr>
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<td>27</td>
<td>19 (70%)</td>
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<td>Female</td>
<td>36</td>
<td>29 (81%)</td>
<td>25 (69%)</td>
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<td><strong>Tumor type</strong></td>
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<td></td>
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<tr>
<td>Primary tumors</td>
<td>41</td>
<td>29 (71%)</td>
<td>26 (63%)</td>
<td>16 (39%)</td>
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<tr>
<td>Recurrences</td>
<td>16</td>
<td>13 (81%)</td>
<td>9 (56%)</td>
<td>9 (56%)</td>
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<tr>
<td>Metastases</td>
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<tr>
<td>Liposarcoma</td>
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<td>9 (50%)</td>
<td>7 (39%)</td>
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<td>3</td>
<td>2 (66%)</td>
<td>2 (66%)</td>
<td>1 (33%)</td>
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<td>MFH</td>
<td>14</td>
<td>12 (86%)</td>
<td>12 (86%)</td>
<td>8 (57%)</td>
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<td>RMS</td>
<td>5</td>
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<td>5 (45%)</td>
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<td>I</td>
<td>10</td>
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<td>II</td>
<td>29</td>
<td>22 (76%)</td>
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<td>III</td>
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<td>13 (77%)</td>
<td>10 (59%)</td>
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<tr>
<td>IV</td>
<td>7</td>
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<td>5 (71%)</td>
<td>6 (86%)</td>
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<td><strong>Tumor localization</strong></td>
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<td>Extremities</td>
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<td>Abdomen/Retroperitoneum</td>
<td>20</td>
<td>17 (85%)</td>
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<tr>
<td>Radical (R0)</td>
<td>39</td>
<td>31 (80%)</td>
<td>25 (64%)</td>
<td>13 (33%)</td>
</tr>
<tr>
<td>Not radical (R1)</td>
<td>24</td>
<td>17 (71%)</td>
<td>14 (58%)</td>
<td>16 (67%)</td>
</tr>
<tr>
<td><strong>Patients follow-up</strong></td>
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<tr>
<td>Alive</td>
<td>34</td>
<td>21 (62%)</td>
<td>17 (50%)</td>
<td>17 (50%)</td>
</tr>
<tr>
<td>Dead</td>
<td>29</td>
<td>27 (93%)</td>
<td>22 (76%)</td>
<td>29 (93%)</td>
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</tbody>
</table>

*Cutoff, 0.5 ng/mg protein.

*Cut-off, 0.1 (ratio survivin/β-actin).

*mal., malignant.
Survivin Expression Strongly Correlates to Prognosis

of healthy donors by standard Ficoll-Hypaque gradient technique.

Five tissue samples from nonmalignant muscle (from 5 of the 63 STS patients) as well as 5 tissue samples from nontumor patients (normal lung, spleen, stomach, breast, and thyroid gland; Table 2) collected during surgery, were inspected by a pathologist, snap-frozen, and stored in liquid nitrogen until further use.

Protein Extraction. Cultured cells were harvested from monolayer cultures by trypsinization, resuspended in PBS, and pelleted by centrifugation. Cell pellets were stored at −80°C until needed. Cells were resuspended in Laemmli buffer and disrupted by sonication on ice. Cell lysates were incubated at 93°C for 10 min and centrifuged at 4°C for 20 min at 10,000 × g, and the supernatant was used for survivin protein quantification by ELISA and WB analyses.

Tissue extracts were prepared from frozen tissues by a standard extraction protocol (34). Briefly, 10–20 slices of 40–50 μm in thickness were cut, and the total protein was extracted by solubilizing with Laemmli buffer. The protein content of cell lysates and tissue extracts was determined using the Lowry protein assay (Sigma, Deisenhofen, Germany).

Survivin WB. Thirty μg of total protein from each cell lysate or tissue extract were separated on a 12.5% polyacrylamide/SDS gel (Minigel systems; Biometra, Göttingen, Germany). After protein transfer (235 milli Ampere for 1 h) to a polyvinylidene difluoride membrane (Millipore, Eschborn, Germany), the membranes were incubated in blocking buffer for 1 h and then incubated with rabbit anti-human survivin Ab (dilution 1:1000, clone AF886; R&D Systems, Wiesbaden, Germany) overnight at 4°C. After washing, the membranes were incubated with a horseradish peroxidase-labeled goat antirabbit IgG (1:2000 dilution; DAKO, Glostrup, Denmark) for 1 h at room temperature. For protein detection, membranes were incubated with ECL-substrate for 1 min (AmershamPharmacia Biotech, Freiburg, Germany) and exposed to X-ray film (Biomain; Kodak, Braunschweig, Germany). The band intensities were digitalized and quantified using the ImagemasterVDS version 3.0 software (ImagemasterVDS; AmershamPharmacia). For normalization, the band intensity of survivin protein was related to that of β-actin, which was run in parallel blots. We used a constant protein amount of the p53-mutated cell line RD (ATCC CCL 136) as an internal standard on each blot to normalize the exposure conditions. The survivin:β-actin ratios were calculated only for the STS patients. In the WB analyses of the cell lines, we applied a scoring system, because defined numbers of cells were analyzed. The cell lines were classified with regard to their survivin content into four categories: no (score 0), weak (score 1), moderate (score 2), or strong (score 3) survivin expression.

Survivin ELISA. Human survivin antigen was determined quantitatively using a commercially available survivin ELISA kit (DuoSet IC-ELISA; R&D Systems) according to the manufacturer’s instructions with some modifications. Briefly, 96-well immunoassay plates (MaxiSorp; Nunc, Wiesbaden, Germany) were coated with 100 μl/well rabbit anti-human survivin Ab (0.4 μg/ml) overnight at 4°C, followed by a blocking step. Plates were incubated with 100 μl/well of test samples and standards diluted in sample buffer [PBS containing 1% (w/v) BSA and 1 M urea (pH 7.6)] for 2 h at room temperature.

Two-fold serial dilutions of recombinant human survivin, covering a concentration range of 62.5–4000 pg/ml were used as standards. All of the cell lysates and tissue extracts were analyzed in duplicate, diluted 1:60 and 1:250, respectively (coefficient of variation <8%). After incubation with 100 μl/well of biotinylated rabbit anti-human survivin Ab (100 ng/ml; 2 h, room temperature) streptavidine-conjugated horseradish peroxidase was added for 20 min at room temperature. The peroxidase reaction was initiated by the addition of 100 μl/well of 3,3′,5,5′-tetramethylbenzidine/H2O2 (K&P Laboratories, Gaithersburg, MD) and stopped after 30 min by the addition of 50 μl/well of 0.5 M H2SO4. The absorbance was measured at 450 nm with a multichannel microtiter plate reader (Titertek MS2; ICN, Eschwege, Germany). Survivin levels were normalized to total protein content and expressed as ng per mg of protein.

The standard curve was linear in the range of 62.5–4000 pg/ml (mean absorbance, 0.080–1.750 units, respectively). The analytical sensitivity of the assay was found to be 60 pg/ml (calculated from background absorbance: mean rate ± 5D).

Statistical Methods. The Cox’s proportional hazards regression model, which was used to estimate the correlation between survivin protein expression and survival, was adjusted for the prognostic effects of staging, tumor entity, tumor localization, and type of tumor resection. A probability of P < 0.05 was defined as significant, and the RR was calculated. The optimal cutoff point for discriminating low-risk and high-risk STS patients in ELISA was determined by log-rank analysis.

The bivariate correlation between the continuous data obtained from both methods of survivin detection was estimated by the Pearson test. The difference in the continuous data among categorized groups was evaluated using the Mann-Whitney test and Kruskal-Wallis test (more than two variables). All of the statistical tests with the continuous data of ELISA and WB analyses were made only with data sets from STS patients (37 of 63) whose tumors had detectable survivin expression levels.

RESULTS

Survivin Expression in Tumor Cell Lines and Nonmalignant Cells and Tissues. All of the cancer cell lines derived from mesenchyme or from epithelium were found to be positive for survivin measured by ELISA or WB (Table 2).

The survivin protein concentration determined by ELISA in tumor cell lysates ranged from 2.8 to 32.6 ng per mg of protein (mean, 11.7 ng/mg; Table 2). Similarly, a high (score 2 or 3) survivin content was also observed by WB in 11 of 14 tumor cell lysates (Table 2). Remarkably, high ELISA quantification data significantly correlated with score 2 or 3 in WB analysis (P = 0.048; Kruskal-Wallis test).

In contrast to this finding, in the fibroblast cell line analyzed and in lymphocyte preparations from four different healthy donors, we found only weak or no survivin expression, respectively (Table 2). Furthermore, 8 of 10 tissue samples of nonmalignant origin had survivin expression rates in ELISA below the detection limit. However, in ELISA, survivin was...
Survivin Expression in STS Tissue Extracts and Correlation to Clinical Data.

In ELISA tests, we defined a cutoff point of 0.5 ng survivin per mg of protein (mean, 3.8 ng; range, 0–18.4 ng) and in WB, a cutoff point of 0.1 relative units survivin:relative units H9252-actin (mean, 1.24; range, 0–8.4).

In the ELISA-based quantification, 48 (76%) of 63 samples were above the cutoff level. In WB analyses 39 (61%) of 63 STS samples had detectable survivin levels. Comparing the survivin protein values obtained by ELISA and WB in tissue extracts of 63 STS patients, we observed a significant correlation between the continuous data of both detection methods (P = 0.013, Pearson test).

We found a strong association between high survivin expression detected by ELISA and high tumor grade (P = 0.004; Kruskal-Wallis test) but not when it was detected by WB analysis. The tendency of statistical association was conserved between tumor staging and protein level quantified by ELISA (P = 0.03; Kruskal-Wallis test).

Moreover, we found that more aggressive STS entities like RMS, LMS, or MFH and FS contain much more survivin protein than less aggressive STS-like liposarcoma (Table 1).

Interestingly, for the histological STS subtypes, significant differences were found between ELISA survivin values in liposarcoma versus (a) myogenic sarcoma (RMS and LMS, P = 0.012; Mann-Whitney test) or versus (b) the subgroup including MFH and FS (P = 0.02; Mann-Whitney test; Table 1).

Survivin protein expression and tumor-related survival were analyzed in a multivariate Cox regression adjusted to the prognostic effects of staging, tumor entity, tumor localization, and type of tumor resection. In ELISA, an elevated level of survivin (0.5 ng per mg of protein) correlated significantly with a poor prognosis in STS patients (P = 0.001; Fig. 1). The RR of tumor-related death was 19.8. In addition, in WB analyses, we found that survivin content (0.1 relative units survivin:relative units H9252-actin) also significantly correlated with a poor prognosis (P = 0.004, RR = 5.1; Figs. 2 and 3).
vivin ELISA seems to be more sensitive than survivin WB ($P = 0.022$; Table 3).

**DISCUSSION**

In the present study, we describe the detection of survivin protein expression in tumor-derived cell lines, in tumor tissue samples of 63 STS patients, and in nonmalignant cells as well as normal tissue samples as controls, independently using two methods: WB assay and, for the first time, ELISA. Moreover, we evaluated the prognostic value of survivin protein expression in tumor tissue extracts for a group of well-characterized STS patients with a known survivin mRNA level (24).

In 76% (48 of 63) of patients investigated, an elevated expression of survivin protein was found by ELISA. High survivin expression correlated with poor prognosis in STS patients ($P = 0.001$, RR = 19.8; Fig. 1). Additionally in 61% (39 of 63) of patients, detectable amounts of survivin were found by WB. This elevated expression of survivin was again correlated with a poor prognosis in STS patients ($P = 0.004$, RR = 5.1; Fig. 2). Hence, we demonstrate that the prognostic value of survivin detection by both methods (ELISA and WB) for this STS patient group was significantly associated with a worse outcome.

The ELISA assay is significantly more sensitive in detecting survivin protein expression than the WB analysis ($P = 0.022$, McNemar test, Table 3). This could be explained with the different detection limits. Another reason for differences between the two protein detection assays could be the use of different Abs, which recognize different antigen epitopes and may differ in their sensitivities. Although the results of ELISA and WB were comparable ($P = 0.013$, Pearson test), the strongest prognostic statement can be made using the more sensitive ELISA ($P = 0.001$, Fig. 1). The associated RR factor found for ELISA (RR = 19.8) was much higher than that for the survivin transcript level (RR = 2.7; Ref. 24). To date, no other single molecular marker with such a strong correlation to prognosis has been described for STS patients.

Survivin is also correlated with a poor survival in many other tumor types, e.g., colorectal carcinoma (8), breast carcinoma (14), B-cell lymphoma (35), neuroblastoma (22), and colorectal carcinoma (8).
gliomas (19). To our knowledge, only one report has been published recently that shows a correlation between the WB-based survivin protein detection and the survival of glioma patients (19).

Most clinical investigations published to date used immunohistochemical methods or WB for survivin protein detection, but these techniques are relatively time-consuming and do not allow a high-throughput-screening (10, 12, 14, 15, 35–37). In this study we show for the first time that the application of the survivin ELISA is well-suited for the measurement of survivin content in cell lysates and tissue extracts and provides additional prognostic information for STS patients.

The sensitive ELISA shows that survivin protein is not expressed in 8 of 10 normal tissue types. However, a detectable, but relatively low survivin content was found in tissue extracts of stomach and spleen specimens. This finding could be explained with the assumption that these proliferative active tissues express all proteins, including others and survivin of course, which are necessary for a correct chromosomal segregation. Our observation supports a direct correlation between survivin expression level and the proliferative activity of each cell type (22, 38). This is in accordance with our finding that high-grade tumors are associated with an elevated expression of survivin protein (P = 0.004, Kruskal-Wallis test).

Survivin is widely expressed in fetal tissues, but becomes restricted during development, and appears to be negligibly expressed in the majority of adult tissues (1). Survivin protein participates in the regulation of chromosome segregation and within the spindle checkpoint pathway (1, 5). The importance of survivin for a correct segregation shall be stressed by mentioning the binding of different proteins: e.g., microtubules (y-tubulin; Ref. 39), incenP and aurora (40, 41), cdk4 (42), p34<sup>cdc2</sup> (43), and p21<sup>med</sup> (44).

The central role of survivin in apoptosis and cell cycle regulation suggests that survivin might be a target for tumor therapy. Different therapeutic approaches have been described. Phosphorylation of survivin could be prevented e.g., by application of p34<sup>cdc2</sup> inhibitors such as flavopyridol (1, 45), which, therefore, represent attractive candidates in counteracting survivin in sarcoma. In a gene therapeutic approach, the application of an adenovirus expressing mutant survivin (T34A) inhibited proliferation and induced apoptosis in tumor cell lines in vitro, whereas normal cells of mesenchymal origin including fibroblasts, muscle cells, and endothelial cells were not affected (31).

In conclusion, a high survivin expression at the mRNA (24) and at the protein level is a significant and independent prognostic factor for STS patients. But before a clinical application of survivin as a predictive marker is feasible, prospective randomized studies should be performed.

**ACKNOWLEDGMENTS**

We express our gratitude to Dr. Udo Bilkenroth for providing frozen nonmalignant tissues and Diana Pinkert (Institute of Pathology, Martin-Luther-University Halle-Wittenberg) for providing protein samples of the nonmalignant tissues. We thank Antje Zobjack (Institute of Pathology, Technical University Dresden) for her excellent technical assistance and Claire Burns-Klein for revising the manuscript.

**REFERENCES**


**Table 3** ELISA and WB compared by the McNemar test

The comparison of the sensitivity of ELISA and WB by the McNemar test showed a significant difference (P = 0.022).

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<th>ELISA Below cutoff</th>
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