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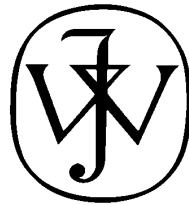
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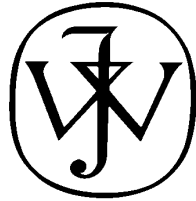
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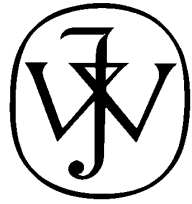
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## INCREASED SURVIVIN TRANSCRIPT LEVELS: AN INDEPENDENT NEGATIVE PREDICTOR OF SURVIVAL IN SOFT TISSUE SARCOMA PATIENTS

Matthias KAPPLER<sup>1</sup>, Thomas KÖHLER<sup>2,6</sup>, Christine KAMPF<sup>3</sup>, Petra DIESTELKÖTTER<sup>3</sup>, Peter WÜRL<sup>4</sup>, Marc SCHMITZ<sup>3</sup>, Frank BARTEL<sup>1</sup>, Christine LAUTENSCHLÄGER<sup>5</sup>, Ernst Peter RIEBER<sup>3</sup>, Hannelore SCHMIDT<sup>1</sup>, Matthias BACHE<sup>1</sup>, Helge TAUBERT<sup>1\*</sup> and Axel MEYE<sup>1</sup>

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**Survivin, a recently identified inhibitor of apoptosis protein (IAP), is expressed in diverse embryonic tissues and in various human cancers. We have investigated the quantitative expression of survivin mRNA by a sensitive TaqMan™-based RT-PCR assay in tissue samples from 94 patients with soft tissue sarcomas (STS). Survivin transcript levels were measured and normalized to GAPDH transcripts. By using a multivariate Cox regression analysis, we found an inverse correlation between the level of survivin mRNA (ratio >2 zmol survivin/amol GAPDH) and the rate of overall survival ( $p = 0.009$ , RR = 2.7). Survivin transcript variants as detected by qualitative RT-PCR analysis were revealed in 30 of 56 STS patients (64%). Only survivin  $\Delta$ Ex3 and/or full-length survivin variants but not survivin 2B were identified. Our results suggest that a higher level of survivin mRNA is an independent predictor of survival for STS patients.**

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**Key words:** survivin; quantitative RT-PCR; soft tissue sarcoma; multivariate analysis; TaqMan™

Survivin, a recently identified member of the inhibitor of apoptosis protein (IAP) family, is supposed to promote cell proliferation by interference with the apoptotic pathway. It was found to be expressed mainly in embryonic tissue and in the majority of tumor cells but was almost undetectable in normal adult tissues.<sup>1–3</sup> Three different survivin transcripts have been identified so far (survivin, survivin 2B and survivin  $\Delta$ Ex3). It has been shown that there is an elevated survivin expression in patients with pancreatic and prostate carcinoma,<sup>1</sup> neuroblastoma,<sup>4,5</sup> gastric carcinoma,<sup>6</sup> colorectal carcinoma,<sup>1,7,8</sup> lung cancer,<sup>1,9</sup> hepatocellular carcinoma,<sup>10</sup> breast carcinomas,<sup>1,11</sup> bladder cancer,<sup>12</sup> melanomas,<sup>13</sup> B-cell lymphoma<sup>14</sup> and in esophageal cancer.<sup>15</sup>

In our study, we investigated the survivin mRNA expression in STS entities by means of quantitative and qualitative RT-PCR techniques. We found that the total survivin mRNA level is inversely correlated with overall survival of STS patients. In line with the finding in neuroblastoma,<sup>4,5</sup> our study also provides evidence that the survivin mRNA level is, in addition to the well-known molecular genetic alterations of the tumor suppressor p53 and the murine-double minute gene 2,<sup>16–18</sup> of general importance as an independent negative prognostic factor for STS patients.

### MATERIAL AND METHODS

#### Tissue samples and histopathologic data

We examined 98 frozen tumor samples available from 94 adult STS patients (Institute of Pathology, University of Halle-Wittenberg, Halle, Germany, and Surgical Clinic I, University of Leipzig, Leipzig, Germany) by quantitative RT-PCR analysis (Table I). The patients ages ranged from 14–84 years (median age 54). Forty-six patients (43%) died from the tumor after an average of 25 months (range 2–201 months), whereas 48 patients (57%) are still alive after an average observation period (*i.e.*, after primary tumor resection) of 35 months (range 6–145 months). Further-

more, we analyzed mRNA expression in nontumor tissue in 22 of the 94 patients, in lymphocytes of 3 none-tumor patients and finally in 4 sarcoma cell lines (LMS6-93, US8-93, A-204, Saos-2). In 56 of the 94 STS patients studied, we investigated qualitatively the occurrence of survivin transcript variants (Table I).

#### RNA preparation, cDNA synthesis and semiautomated transcript analysis by quantitative fluorescence PCR

Total RNA was isolated from frozen tissue samples by using the RNeasy Mini Kit according to the manufacturer's instructions (QIAGEN, Hilden, Germany). For each cDNA synthesis, 1  $\mu$ g RNA per sample, 200 U Superscript™ II RNase H Reverse Transcriptase (RT) and 3  $\mu$ g random hexamer primers (GibcoBRL, Karlsruhe, Germany) were mixed. The RT reactions were run for 75 min at 42°C.

Survivin mRNA subsequences (77 bp), which do not overlap with the EPR-1 mRNA<sup>19</sup> and which are specific for all 3 survivin transcripts, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcripts (63 bp) were amplified from cDNA in duplicate experiments by ready-to-use PCR assays (Roboscreen®, Gesellschaft für molekulare Biotechnologie mbH, Leipzig, Germany) as previously described.<sup>16</sup>

#### Qualitative RT-PCR

In the qualitative RT-PCR for the detection of transcript variants, the RT reaction contained 1  $\mu$ g of total RNA, 1 $\times$  RT buffer (Promega, Mannheim, Germany), 25  $\mu$ M of each dNTP, 10 pmol of sequence-specific RT primer (5'-TTCCTCCCTCACTTCTACC-3'), and 12 U M-MLV RT RNase (Promega, Mannheim, Germany) in a final volume of 20  $\mu$ l. The RT reaction was incubated at 37°C for 1 hr. PCR amplification was performed on a PTC-100 cyclor (MJ Research/Biozym Diagnostic, Hess Oldendorf, Germany). Two microliters of cDNA were subjected to amplification in a 25  $\mu$ l mixture containing 0.8 U Ampli-Taq (Applied Biosystems, Weiterstadt, Germany), 0.04 U Pfu (Stratagene, La Jolla, CA), 1 $\times$  PCR buffer, 50  $\mu$ M

**Abbreviations:** amol, attomoles [ $10^{-18}$  moles]; FS, fibrosarcoma; GAPDH, glycine-aldehyde-3-phosphate dehydrogenase; IAP, inhibitor of apoptosis; LMS, leiomyosarcoma; LS, liposarcoma; MFH, malignant fibrous histiocytoma; MNT, malignant neural tumors; RMS, rhabdomyosarcoma; RR, relative risk; RT, reverse transcriptase; STS, soft tissue sarcoma; SyS, synovial sarcoma; zmol, zeptomoles [ $10^{-21}$  moles]

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TABLE I – SURVEY ON SURVIVIN mRNA EXPRESSION AND CLINICAL DATA FOR STS PATIENTS

STS patients	Total	LS	MFH	MNT <sup>1</sup>	LMS	RMS	FS	SyS	Other STS
Total	94	23	22	16 (3*)	10	8	6	6	3
Staging									
Stage I	15	11	0	0 (0*)	1	0	3	0	0
Stage II	40	9	9	8 (0*)	5	0	3	5	1
Stage III	30	3	12	6 (2*)	3	5	0	1	0
Stage IV	9	0	1	2 (1*)	1	3	0	0	2
Sex									
Female	51	11	15	9 (1*)	7	4	2	3	0
Male	43	12	7	7 (2*)	3	4	4	3	3
Survivin mRNA quantification	94								
≤2 zmol survivin/amol GAPDH	49	15	9	8 (1*)	3	4	4	5	1
>2 zmol survivin/amol GAPDH	45	8	13	8 (2*)	7	4	2	1	2
Survivin qualification	56								
No transcript detectable	20	12	3	1 (0*)	0	1	2	1	0
Survivin	21	3	6	3 (1*)	4	2	0	1	2
Survivin + survivin ΔEx3	15	1	5	2 (0*)	2	2	1	2	0

FS, fibrosarcoma; LMS, leiomyosarcoma; LS, liposarcoma; MFH, malignant fibrous histiocytoma; MNT, malignant neural tumors; RMS, rhabdomyosarcoma; STS, soft tissue sarcoma; SyS, synovial sarcoma.<sup>1</sup>The 3 neuroblastoma samples are marked by an asterisk in parentheses.

of each dNTP and 25 pmol of each primer. The PCR conditions were initial denaturation for 3 min at 95°C, followed by 35 cycles of denaturation at 95°C for 1 min, annealing for 1 min at 60°C and extension at 72°C for 2.5 min. The primers for amplification of survivin were forward primer 4N (5'-ATGGGTGCCCGACGTT-GCCCCCT-3') and reverse primer 5C (5'-TCAATCCATGGCAGC-CAGCTGCTC-3'). The identity of the PCR products was confirmed by direct sequencing (ABI373; Applied Biosystems) (data not shown).

Statistical analysis

The Cox regression model, which was used to estimate a correlation between survivin overexpression and survival, was adjusted to the prognostic effects of staging, tumor entity, tumor localization and type of tumor resection. Kaplan-Meier analysis was performed to study a correlation between high survivin transcript levels and survival of patients without diagnosed recurrences. A probability of  $p < 0.05$  was defined as significant and the relative risk (RR) was calculated. The statistical analyses were carried out using SPSS software version 9.0 (SPSS, Chicago, IL).

RESULTS

Survivin transcript levels and correlation to survival

To determine the survivin mRNA expression in tissue samples of 94 STS patients, real-time and quantitative RT-PCR analyses were performed. Detectable levels were found in 77 patients (82%). The median transcript ratios were calculated as 1.83 zmol survivin mRNA/amol GAPDH mRNA (range 0–102, mean 9.4) for all 98 STS samples derived from 94 STS patients, as 0 (range 0–3.8, mean 0.5) for 22 samples of adjacent nontumor tissue (muscle), as 1.0 (range 0.9–1.1, mean 1.0) for leukocytes derived from 3 blood samples, and as 8.9 (range 4.5–16.1, mean 9.7) for 4 sarcoma cell lines. Survivin mRNA was never detected in surrounding nontumorigenic muscle tissue when the STS sample itself was negative for survivin mRNA.

Data from tumor tissues and normal muscle tissues were used to define an arbitrary cut point for survivin expression of 2 zmol survivin mRNA/amol GAPDH mRNA, since the survivin mRNA levels in normal muscle tissue and mononuclear blood cells were always below this threshold with the exception of 2 cases (Table I, Fig. 1). By multivariate Cox regression analysis, we found that an elevated level of survivin mRNA expression (*i.e.*, ratio >2 zmol/amol) significantly correlated with a poor prognosis in STS patients ( $p = 0.009$ , RR = 2.7) (Fig. 2).

Moreover, when focusing Kaplan-Meier analysis exclusively on STS patients without recurrences (57 of 94 patients), 29 patients

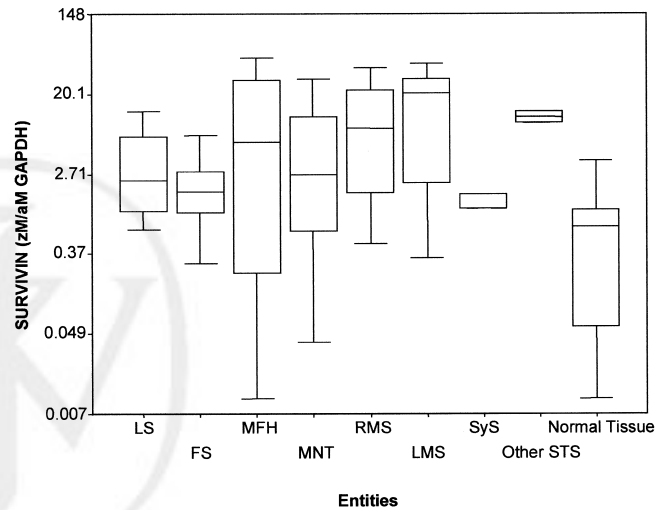


FIGURE 1 – Box plot of quantitative survivin data in relation to the entities of the tumor samples. RNA samples from nontumor tissue (muscle tissues adjacent to the tumor) and mononuclear blood cells of healthy donors served as controls. For both patients with multiple tumors, only the first one was considered in the figure.

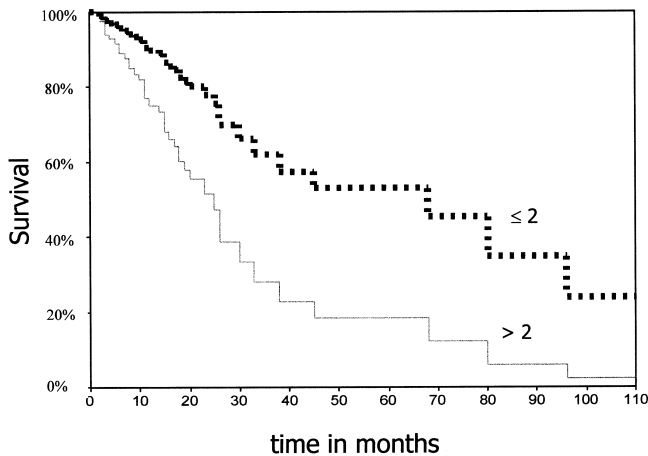
showed intratumoral survivin mRNA overexpression and died on average 29 months earlier than the 28 patients who had low survivin transcript levels (*i.e.*, ratio ≤ 2 zmol/amol) ( $p = 0.025$ ).

Among the STS subtypes investigated in our study, LMS and MFHs in particular showed survivin transcript overexpression (ratio >2) in a high percentage (70% and 59%, respectively) (Table I).

From 2 STS patients, multiple tumor tissue samples were available for survivin mRNA determination. One patient suffering from fibrosarcoma developed 3 STS recurrences. The survivin/GAPDH ratios increased successively from 1.7 (2nd recurrence, 12 years after primary tumour resection) to 3.6 (2 years later) to 29 (6 years later) in the tumor samples obtained from the recurrences. The other patient developed 3 diffuse and primary malignant peripheral nerve sheath tumors in which a survivin ratio of 29 (first surgical resection in 1994), 0.1 and 102 (both resected 1 year later) was detected. This patient died 3 months after the ratio of 102 was determined.

F1

F2



**FIGURE 2** – Multivariate Cox model for survivin mRNA overexpression and survival in STS patients. Overall survival rates of patients with a survivin mRNA expression  $\leq 2$  (zmol survivin/amol GAPDH) (dashed line;  $n = 41$ ) and patients with a survivin mRNA expression  $> 2$  (zmol survivin/amol GAPDH) (solid line;  $n = 38$ ) are significantly different ( $p = 0.009$ ). A relative hazard of 2.7 is associated with survivin mRNA expression  $> 2$  (zmol survivin/amol GAPDH) in a multivariate Cox model adjusted to staging, to tumor entity, tumor localization and type of tumor resection (patients in stage I group were excluded because no patient died in this group).

#### Detection of survivin transcript variants in STS

Recently, 3 survivin transcript variants have been described: survivin (426 bp), survivin  $\Delta$ Ex3 lacking exon 3 (411 bp) and survivin 2B (495 bp).<sup>20</sup> In 56 (60%) of 94 STS patients, expression of different survivin transcript variants were identified. In 36 (64%) of these STS samples, the survivin transcript was detected (Fig. 3). Fifteen (42%) of these cases additionally showed expression of the shorter transcript variant survivin  $\Delta$ Ex3. The survivin 2B transcript variant was not detected in any of the investigated STS samples. As revealed by Cox regression analysis, no correlation between the occurrence of certain transcript variants and outcome of the STS patients was found (data not shown).

#### Detection of antisurvivin antibodies

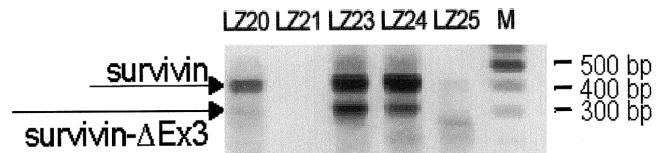
Prompted by a high percentage of STS patients showing intratumoral survivin transcript overexpression, we screened sera of STS patients. The titer of antisurvivin antibodies in the sera of 83 STS patients tested was low and did not significantly differ from that measured in sera of healthy blood donors (data not shown).

### DISCUSSION

The sensitive quantitative RT-PCR study for STS samples from 94 patients indicates a significant correlation between an increased survivin mRNA expression (ratio  $> 2$ ) and a poor prognosis evaluated by Cox regression analysis. This result is in accordance with earlier findings that indicate that level of survivin is correlated with poor survival in different carcinomas, *i.e.*, colorectal carcinoma,<sup>7</sup> breast carcinoma<sup>11</sup> and B-cell lymphoma<sup>14</sup> where survivin protein was detected, and colorectal carcinoma,<sup>8</sup> lung carcinoma<sup>9</sup> and esophageal cancer<sup>15</sup> where survivin mRNA was determined. Survivin expression in tumor cell nuclei, however, seems to be predictive of favorable prognosis in gastric cancer patients.<sup>21</sup>

The finding of a relatively high survivin transcript overexpression for LMS and MFHs, both entities with well-known aggressive biologic behaviour, suggests an association between aggressiveness of STS subgroups and increased survivin transcript levels (Fig. 1, Table I).

In accordance with the recently published findings obtained using Northern blot analysis in neuroblastoma,<sup>5</sup> we detected an



**FIGURE 3** – Survivin transcript variants in STS samples. Representative survivin (426 bp) and survivin  $\Delta$ Ex3 (411 bp) mRNA expression pattern in different STS samples (LZ 23, LZ 24) detected by qualitative RT-PCR. Note the single expression of full-length survivin transcript in sample LZ 20. LZ 21/LZ225 are survivin-negative STS samples. M marks a 100 bp ladder.

increased survivin mRNA level in 2 of 3 neuroblastoma samples (see also Table I). In 2 of 7 patients with neurogenic sarcomas (as MNT), high survivin levels were associated with an amplification of the survivin chromosomal region (17q25) detected by comparative genomic hybridization analyses (H. Schmidt, unpublished data).

Moreover, the survivin transcript level data for 2 STS patients with multiple tissue samples suggest that survivin expression levels may increase in STS patients with recurrences with time.

The expression of survivin in normal tissues is currently being debated. On one hand, no survivin expression was detected in skin<sup>13</sup> and most of normal tissues examined.<sup>3</sup> On the other hand, several recent studies have described survivin gene expression also in normal tissue, such as skin, endometrium, endothelial cells and normal blood lymphocytes.<sup>22–25</sup> By sensitive quantitative RT-PCR analysis, we found that low levels of total survivin mRNA are detectable in normal tissue either adjacent to STS tumor cells (*e.g.*, muscle) or in lymphocytes of blood donors. Further studies are necessary to find out whether expression of survivin in normal tissue is due to mitotically active cells.

Using qualitative RT-PCR in the majority of STS samples studied, the shorter transcript variant survivin  $\Delta$ Ex3 and/or survivin variant were identified but not the variant survivin 2B. This is in line with the finding that survivin 2B is only expressed at very low levels in normal tissue<sup>26</sup> and in human renal cell carcinomas.<sup>20</sup> In our study, the occurrence of the transcript variants did not correlate with patients' outcome. However, in neuroblastoma, a high expression of the survivin variant correlated with a poor prognosis.<sup>4,5,26</sup> As expected, the sensitivity of this qualitative RT-PCR assay to detect survivin transcript variants (60%) was lower than for the quantitative RT-PCR assay (82%).

Recently, occurrence of antisurvivin antibodies has been reported in sera of lung cancer and colorectal cancer patients.<sup>27</sup> So, we screened 83 sera from these STS patients for antisurvivin antibodies. In contrast to findings in other tumors, our preliminary data do not recommend antisurvivin antibodies as suitable diagnostic or prognostic markers in STS.

In summary, our results clearly indicate that high expression of survivin mRNA is a strong independent prognostic marker for STS patients. Together with other independent predictors such as the p53 tumor-suppressor gene and the murine-double minute gene 2,<sup>16–18</sup> it might essentially contribute to the prognostic assessment of STS patients. In addition, overexpression of survivin transcripts in the tumor tissue in a high percentage of STS patients renders the IAP member survivin a candidate target in immunotherapy and/or gene therapy for STS patients.

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