Survivin and its prognostic significance in pediatric acute B-cell precursor lymphoblastic leukemia

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ABSTRACT

Background and Objectives

Impaired apoptosis, mediated by members of the inhibitor of apoptosis proteins (IAP) family such as survivin, is thought to contribute to leukemic cell survival. In contrast to low expression of survivin in normal differentiated adult tissues, very high levels of survivin have been described in a number of different tumors. Overexpression of survivin was found to correlate with poor prognosis in a variety of cancers including hematologic malignancies. To date, however, there is no information available on the prognostic role of survivin in pediatric precursor B-cell acute lymphocytic leukemia (BCP-ALL), the most frequent malignancy in childhood.

Design and Methods

In a retrospective study including 66 pediatric patients we analyzed the impact of survivin protein levels on outcome in BCP-ALL.

Results

Survivin overexpression, with an up to ten-fold increase of the normal level, was detected in 65% of the leukemic samples in contrast to negligible expression in non-malignant hematopoietic cells. Despite considerable variety of expression levels in ALL cells, there was no association of survivin levels with established risk factors. However, patients suffering relapse of disease or death had significantly higher survivin expression than those with a favorable outcome. Overexpression of survivin is a significant prognostic marker for 3 year relapse free, event-free and overall survival, again independent of the established prognostic factors in ALL, such as age and leukocyte count at diagnosis as assessed in multivariate analysis.

Interpretation and Conclusions

Overexpression of survivin in BCP-ALL identifies patients with a high risk of early relapse. Upon confirmation in a prospective analysis, survivin expression may, in the future, serve to further refine treatment stratification with intensification of therapy in those patients prone to relapse.

Key words: apoptosis, BCP-ALL, prognosis, survivin.

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Deregulation of programmed cell death contributes to leukemogenesis as well as blast cell survival. Cells resistant to apoptosis are prone to accumulate genetic aberrations, acquire the capacity to survive independently from growth factor stimulation and escape from immune system control.[1] Apoptosis can be induced by two distinct pathways: the intrinsic mitochondrial pathway and a death-receptor-mediated extrinsic pathway.[2] Besides the primary response to chemotherapeutic agents, which is mainly facilitated by the mitochondrial pathway, immunological control mechanisms involving death-receptor-mediated apoptosis are critical for disease control and also influence treatment outcome.[3,4] Both apoptotic pathways commonly result in activation of the effector caspases 3 and 7. A group of apoptosis inhibitor molecules called inhibitor of apoptosis proteins (IAP) interact with these downstream caspases blocking their activation. One member of the IAP family is survivin.[2,5,6] Physiologically survivin is transiently expressed during embryonic development but barely detectable in normal, differentiated adult tissue.[7,8] Still even at this comparatively low level, in normal hematopoietic cells survivin has been shown to be involved in cell cycle control.[9] In contrast to expression in normal tissue, survivin has been found to be overexpressed in a number of different tumor tissues indicating that it has a role in carcinogenesis.[8,10] In these tumors, a high level of survivin expression correlates with poor outcome.[11-14] Similarly, in malignant hematologic diseases, overexpression of survivin correlates with reduced remission rates and survival in adult patients with acute myeloid and adult T-cell leukemia as well as diffuse large B-cell lymphoma.[15-17] In children, data on the prognostic role of survivin overexpression are only available for acute myeloid leukemia. In this disease overexpression is strongly correlated with poor overall survival.[18-20] Yet, while in adult acute lymphoblastic leukemia, variable survivin overexpression has been documented in a small scale study,[21] there is no information available on the prognostic relevance of increased survivin levels in B-cell precursor acute lymphoblastic leukemia (BCP-ALL), the most common pediatric malignancy.

Despite the very good overall prognosis of children with ALL, whose long-term survival is now 70-80%, treatment of relapsed disease remains a challenge.[22] Early identification of patients with a poor prognosis allows for the prospective evaluation of new consolidating treatment elements at an early stage of the disease. As survivin overexpression has been implicated as a poor prognostic marker in a variety of cancers including hematologic malignancies of the B-lineage, we assessed total survivin protein in blasts from 66 children with BCP-ALL. In contrast to other studies analyzing mRNA and qualitative protein expression, we chose to analyze total survivin protein due to its biological significance, stability of the target and ease of analysis to assess the prognostic impact of survivin in pediatric precursor B-ALL.

Design and Methods

Patients

Sixty-six pediatric patients (1-17 years) with BCP-ALL diagnosed between May 1980 and March 2004 were included in the analysis. As normal controls bone marrow (n=5) and peripheral blood (n=5) samples from healthy donors were analyzed. The diagnosis of BCP-ALL was confirmed by morphological criteria as well as by immunophenotype with five patients diagnosed as having proB-ALL, 17 patients as having preB-ALL and 44 patients as having c-ALL. The median percentage of leukemic blasts in the bone marrow and blood samples was 87% (range 50-97%). All bone marrow samples and the majority of peripheral blood samples contained more than 80% blast i.e. only in 14% of peripheral blood samples, did the blasts content range from 50-80%. In these samples survivin expression levels were adjusted to the median blast cell count of our cohort as the survivin expression in normal peripheral blood was negligible.

The patients were treated according to the multicenter CoALL treatment protocols for childhood ALL.[22] The CoALL studies were all approved by the ethics committee and informed consent was obtained from parents or legal guardians of the patients at the time of enrolment. Table 1 summarizes the characteristics of the patient cohort. Low initial white blood cell (WBC) count was defined as leukocytes <25,000/μL, in contrast to a high initial WBC count with leukocytes ≥25,000/μL at diagnosis, which is in accordance to the definition used in the CoALL study protocols for stratification of patients into high and low risk groups. For 37 patients treated according to the CoALL protocols 06-97 and 07-03, the *in vitro* chemoresistance profile against prednisolone, vincristine and asparaginase, summarized as the PVA score, served as an additional risk parameter,[23] and for a subgroup of 41 patients cytogenetic data were available. The median follow-up of the studied patients was 31 months. For this reason and as the 3 year relapse-free survival in patients was comparable, irrespective of the applied treatment protocol as assessed by multivariate analysis, we focused our analysis primarily on that observation period when evaluating the impact of survivin protein level on survival.

Determination of survivin protein concentration

Mononucleated cells from blood samples or bone marrow were Ficoll-purified and immediately put into lysis buffer or cryopreserved in DMSO. Cryopreserved samples were thawed and washed prior to further analysis. The total amount of human survivin protein was determined by a sandwich enzyme-linked immunosorbent assay (ELISA) method (Duo Set, R&D, Minneapolis, USA). To this end a total of 1×10^6 primary ALL blasts, at a concentration of 1×10^7 cells/mL, were solubilized in lysis buffer containing 1mM EDTA; 0,5% triton-X100, 6M urea, 10 μg/mL leupeptin, 10 Mg/μL pepstatin, 10 μM

Table 1. Patients' characteristics for survivin protein measurement.

Patients	*N=66*
Male/female	36 (54.5%)/30 (45.5%)
Age (years)	
Median (range)	4.5 (1-17)
WBC count at diagnosis ($\times 10^6/\mu L$)	
Median (range)	12,500 (1,000-273,000)
Blast count (%)	
Median (range)	87 (50-97)
Immunophenotype	
c-ALL	N=44 (67%)
PreB-ALL	N=17 (26%)
ProB-ALL	N=5 (7%)
Cytogenetic analysis	
No abnormality	N=29 (44%)
TEL-AML	N=10 (15%)
BCR-ABL	N=2 (3%)
Not done	N=25 (38%)
PVA score	
3-6	N=28 (42%)
7-9	N=10 (15%)
Not done	N=28 (42%)
Risk group	
High risk	N=32 (48%)
Low risk	N=34 (52%)
Treatment outcome	
Relapse	N=22 (33%)
Death	N=18 (27%)

The table summarizes the patients´ characteristics with regard to the distribution of the classical risk parameters immunophenotype, age, initial leukocyte count, PVA score, genetic translocations as well as initial white blood cell (WBC) count. The criteria for prognostic classification into high risk (HR) and low risk (LR) patients at initial diagnosis include leukocyte count, age, genetic translocations and in vitro resistance to the critical chemotherapeutic agents prednisolone, vincristine and asparaginase (PVA score).

PMSF, and 3 μg/mL aprotinin. All samples were stored briefly in this lysis buffer before analysis. Ninety-six-well plates were coated with capture antibody directed against total survivin overnight. Thereafter 100 μL of the leukemia samples or survivin standards were added. For detection of survivin protein, a biotinylated survivin antibody was used, which bound horseradish-peroxidase coupled streptavidin. Twenty minutes after addition of H_2O_2 and tetramethylbenzidine as color reagents, optical density was determined at 450nm in a microplate reader set. The average total survivin protein concentration of duplicate measurements was calculated on the basis of a standard curve.

Statistical analysis

The Mann-Whitney U-test was used for statistical comparisons of survivin expression between two subgroups of the cohort of patients, while the Kruskal-Wallis test was applied to the same end when comparing more than two subgroups. The Kaplan-Meier method was used to analyze time-dependent outcome parameters such as survival. Differences between the resulting survival curves of patients with high and low survivin expression were calculated using the log-rank test. Relapse-free survival (RFS) delineated survival in complete remission. In this case relapse was the event and surviving patients who had not suffered from relapse were censored at last follow-up or death. Event-free survival (EFS) was defined as survival in complete remission. Here relapse and death were considered events and surviving patients were censored at last follow-up. For overall survival (OS) analyses, death was the event and surviving patients were censored at last follow-up. Censored patients were mathematically removed from the curve as indicated by tick marks in the presented graphs. All of the statistical analyses were done with the SPSS software program (version 12). A p-value of <0.05 was considered to indicate statistical significance.

Results

Overexpression of survivin protein in precursor B-ALL blasts of children and its association with classical risk factors

The aim of this study was to assess survivin protein expression levels with regard to the potential role of this protein as a prognostic marker in precursor B-ALL, the most common childhood malignancy. Survivin expression was negligible in peripheral blood cells (PB) and homogeneously low in normal bone marrow (BM) with median levels of 0 pg/mL (range: 0-77 pg/mL) in PB (n=5) and 71 pg/mL (45-305 pg/mL) in BM (n=5). In contrast, survivin protein expression was significantly elevated in the BCP-ALL blasts median (407 pg/mL; range, 0-2765 pg/mL; p=0.0001). Median survivin expression levels were comparable between the different immunophenotypes being 511 pg/mL (range, 0-1472 pg/mL) in the most immature proB-ALL blasts, 356 pg/mL (range, 47-2765 pg/mL) in preB-ALL and 379 pg/mL (range, 0-2157 pg/mL) in c-ALL. Thus, with respect to blast immaturity, we could not identify significant variation of the survivin expression level in childhood BCP-ALL (Figure 1A). Besides immunophenotype, age, leukocyte count at diagnosis, cytogenetic aberrations and *in vitro* chemoresistance are used as risk factors for the stratification of patients into high risk and low risk groups. Taking all this information together, there was a trend towards a higher median level of survivin expression (575 pg/mL; range 0-2765 pg/mL) in the high risk group than that in the low-risk group (345 pg/mL, range 0-1589 pg/mL) (p=0.06). However, when looking at the various risk factors individually no significant difference in survivin expression levels could be identified. In detail, there was no statistical difference of survivin expression level between the groups older (582 pg/mL; range, 256-1602 pg/mL) and younger than 10 years (398 pg/mL; range, 0-2765 pg/mL) (p=0.151) or patients presenting with low initial leukocyte counts (333 pg/mL; range, 0-1589 pg/mL) in contrast to patients with high initial leukocyte counts (644 pg/mL; range, 0-2765 pg/mL) (p=0.079). In 37 patients treated according to the CoALL 06-97 or following protocol, the *in vitro* chemoresistance profile of the

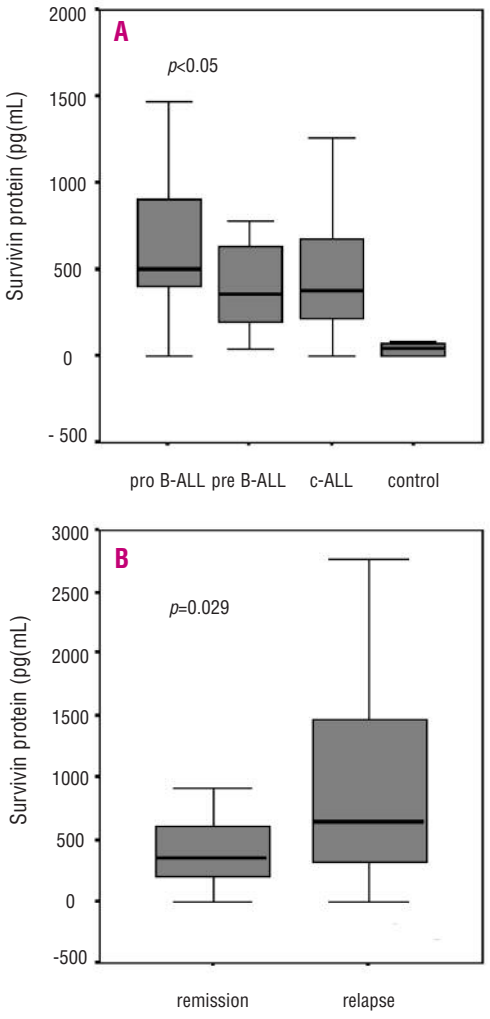


Figure 1. Survivin expression in BCP-ALL cells is significantly higher than in normal tissue. Survivin expression in different phenotypes of BCP–ALL cells in comparison to that in normal peripheral blood and bone marrow as assessed by ELISA. Survivin expression was significantly higher in the malignant cells than in the normal controls ($p<0.001$) [Figure 1A]. Survivin expression was also compared in patients with and without relapse and differed significantly ($p=0.029$) [Figure 1B].

blasts, summarized by the so-called PVA score, was known. Blasts with high *in vitro* chemoresistance did not differ from chemosensitive blasts in terms of their survivin protein content (328 pg/mL; range, 0-2765 pg/mL in patients with PVA<7 vs 398 pg/mL; range, 216-630 pg/mL in patients with PVA≥7) ($p=0.589$). Independently from the karyotype, in the blasts of those patients tested for genetic translocations (n=41) the survivin levels in *TEL-AML* positive patients were equal to those in patients with a normal karyotype (346 pg/mL; range, 0-630 pg/mL in *TEL-AML* positive vs 333 pg/mL; range, 0-1602 pg/mL in patients with blasts without translocations) ($p=0.272$). There were only two patients with *BCR-ABL* positive blasts and both had high survivin levels. None of the patients carried a *MLL*-rearrangement as infants younger than 1 year of age were not included in the CoALL protocols.

Overexpression of survivin protein in precursor B-ALL blasts of children and its relevance for treatment outcome

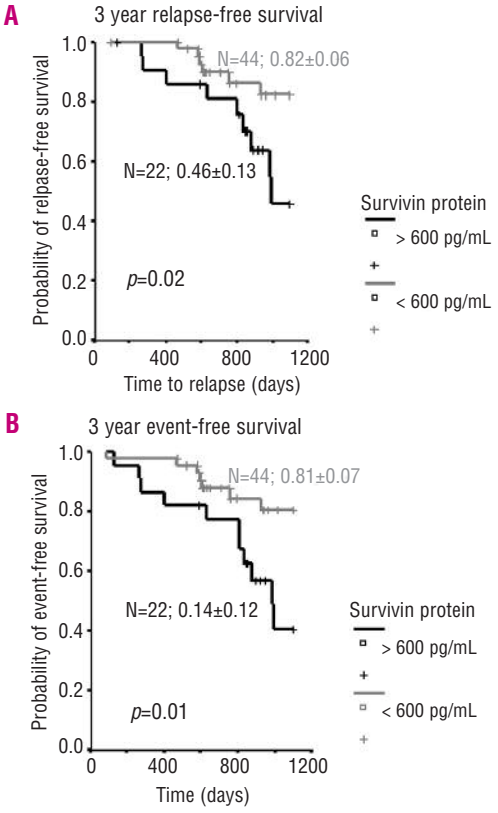
While no association was found between the survivin expression level and the above mentioned classical risk factors, expression differed significantly between patient subgroups depending on the treatment outcome. Thus, patients suffering relapse had significantly higher survivin expression levels (636 pg/mL; range, 0-2765 pg/mL) compared to those remaining in complete remission (346 pg/mL; range, 0-1515 pg/mL) ($p=0.029$) (Figure 1B). Moreover, blasts from patients who died of disease during the observation period exhibited significantly higher survivin levels (777 pg/mL; range, 71-2765 pg/mL) than blasts from surviving patients (346 pg/mL, range, 0-1573 pg/mL) ($p=0.012$). Thus, median survivin levels in patients dying from relapsed leukemia were twice as high as median levels in surviving patients.

High survivin expression as a negative risk factor for 3-year survival

When using the maximum expression level of normal hematopoietic cells (305 pg/mL) as a cut-off to determine overexpression, 43 (65%) of the studied ALL samples were found to overexpress survivin. Expression levels varied considerably in these patients with a median overexpression level of 600 pg/mL and a range of 311-2765 pg/mL. For the survival analysis the whole patient cohort was thus divided into two groups of patients: those with survivin expression above the median overexpression level (>600 pg/mL) and those with survivin expression below the median overexpression level (<600 pg/mL), which is in keeping with other studies in acute myeloid leukemia (AML) and adult T-cell leukemia (ATL)patients in whom only very high expression of survivin indicated a poor prognosis.[17,36,37,40]

Of note, the clinical course of the two subgroups differed with respect to relapse-free (RFS), event-free (EFS) and overall survival (OS) which were significantly better in the group with low expression of survivin during an observation period of 3 years as assessed by Kaplan-Meier analysis (RFS 0.82+0.06 vs 0.46+0.13; $p=0.02$; EFS 0.81+0.07 vs 0.41+0.12; $p=0.01$; OS 0.98+0.002 vs 0.70+0.11; $p=0.005$). These results are depicted in Figure 2A-C. Moreover, when analyzing the impact of survivin expression level on survival within the clinically defined low and high-risk patient groups separately, survivin overexpression was only discriminative in the low-risk group. Thus, in the low-risk group, patients with very high survivin expression had a significantly worse 3- year RFS (RFS in low risk patients with high survivin expression 0.0+0.0 vs RFS in low-risk patients with low survivin expression 0.82+0.08; $p=0.019$).

We performed Cox regression analysis to test the impact of survivin against each of the alternative prognostic markers such as PVA score (n=38) and genetic abnormalities (n=39) individually. High survivin expression is an



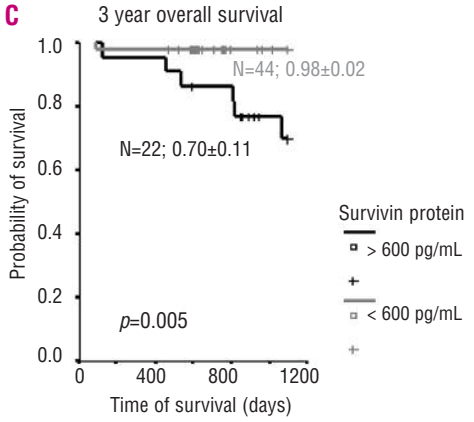


Figure 2. Overexpression of survivin is associated with worse RFS, EFS and OS. The graphs show the 3 year RFS (A), EFS (B) and OS (C) of patients with very high survivin expression above the median in overexpressing cells (>600 pg/mL) compared to patients with low or negative survivin expression (<600 pg/mL).

independent risk factor for poor RFS not only when tested against leukocyte counts and age but also against PVA score. However, as PVA score and genetic evaluation were only available for about half of the patients, confidence intervals were large due to small sample sizes. Moreover, when comparing the classical risk factors initial leukocyte count and age, which were available for all patients with survivin expression, in a multivariate analysis using a Cox regression model, overexpression of survivin was revealed as an independent risk parameter for 3 year RFS (p=0.04), EFS (p=0.02) and OS (p=0.05) (Table 2). Thus our data document for the first time that survivin overexpression is an independent risk factor for early relapses in pediatric precursor B-ALL.

Discussion

In this retrospective analysis of a cohort of 66 patients with precursor B-ALL treated according to CoALL protocols, we identified overexpression of survivin protein as a strong predictive marker for relapse. To date, there are only a few reports available on survivin expression in ALL. In these studies, the numbers of patients are low and the methods to determine survivin expression patterns differ considerably with a preference for analysis at the mRNA level.[21,24-28] At the mRNA level, alternative splice variants have been identified with variable anti-apoptotic potency and distinct cellular localization patterns.[21,30,50] Survivin itself, as well as the pro-apoptotic survivin2B, is predominantly found in the cytoplasm, whereas the anti-apoptotic survivin δExon3 localizes to the nucleus.[31,32] Although the cytosolic fraction seems to dominate over the nuclear fraction in most cell types, survivin is able to shuttle between the two compartments.[33] Cellular localization of survivin influences the relative ratio between survivin, downstream caspases and stabilizing proteins. A high concentration of survivin protein is thought to block the distal apoptosis pathway by indirectly inhibiting activation of the effector caspase 3. Survivin interacts with the pro-apoptotic SMAC molecule and thereby prevents effective removal of the caspase 3 inhibiting IAP.[34] As a consequence of elevated survivin levels, free active caspase 3 does not reach the threshold level to trigger apoptosis.[35] Given that the cytoplasmic and nuclear distribution of survivin was described as heterogenous in a small set of ALL samples,[24] we opted to measure total cellular

Table 2. Multivariate analysis.

	Relative risk in Cox regression analysis for 3-year RFS, EFS and OS					
	RFS		*EFS*		*OS*	
	Relative Risk (95%CI)	*p value*	*Relative Risk (95%CI)*	*p value*	*Relative Risk (95%CI)*	*p value*
Survivin (< median)	0.31 (0.10-0.95)	0.04	0.31 (0.11-0.84)	0.02	0.11 (0.01-1.00)	0.05
Age (<10 years)	0.37 (0.11-1.28)	0.12	0.38 (0.12-1.15)	0.09	0.34 (0.05-2.35)	0.28
Leukocyte count (<25,000/μL)	0.91 (0.28-2.98)	0.87	0.97 (0.33-2.86)	0.95	0.50 (0.09-2.95)	0.45

A multivariate analysis was assessed to determine the impact of prognostic parameters for risk of relapse as well as risk of events or death within the first 3 years.

survivin protein, comprising both fractions, in order to assess the prognostic impact of survivin overexpression in ALL in a large cohort of 66 children suffering from precursor B-ALL. In contrast to the sparse data on the clinical relevance of survivin expression in ALL, larger clinical studies are available in both pediatric and adult AML pointing towards a prognostic role of survivin in acute leukemia.[17,36,37] These studies also documen-ted that the methods used for survivin detection might be the reason for a certain variability of results. In children with AML high survivin levels, assessed by western blot analysis, were associated with poor overall survival defining survivin as a negative prognostic marker.[18] In contrast, when employing immunohistochemistry for detection of cytoplasmic survivin in adult AML, using a low cut-off of 5% to define survivin positivity, positivity was not predictive of a poor outcome.[17,37] These studies underscore the importance of quantitative determination of survivin levels to document overexpression of the anti-apoptotic protein as the prognostically relevant parameter. To this end a methodology such as the ELISA used in our study, which is suitable for the analysis of large numbers of samples due to ease of application and standardization, may prove advantageous. In our 66 ALL patients, expression of survivin protein above the background level in normal hematopoietic cells was detected in two thirds of patients and displayed considerable variability. In mature B-cell malignancies such as chronic lymphocytic leukemia (CLL), elevated survivin levels are preferably found in the proliferative pool that supplies the accumulation compartment and not in non-cycling CLL cells.[38,39] In chronic myeloid leukemia (CML) high survivin levels in blast crisis also characterize leukemic cells with a high proliferative capacity. Similarly in ATL, survivin mRNA levels are higher in acute disease than in chronic disease[24] implying an association with the enhanced proliferative capacity of acute ATL blasts resulting in short survival in these patients.[40] Thus survivin overexpression may help to identify patients with a more aggressive form of malignant disease. As in diffuse large B-cell lymphoma, in which survivin also serves as a negative prognostic marker,[16,38,39] we were able to identify elevated total survivin protein as a strong risk factor for relapse in children with precursor B-ALL. In keeping with the observation that survivin overexpression is a negative prognostic parameter in immature as well as mature B-cell malignancies,[16] there was no difference in survivin levels with regard to the maturity of the B cell precursor blasts, i.e. between pro-, pre- and c-ALL. Furthermore, in our ALL cohort, there was no correlation between survivin overexpression and standard clinical risk factors such as age, leukocyte count, cytogenetic aberrations or *in vitro* chemoresistance.

Significantly higher survivin protein levels in patients who suffered relapse suggest that high survivin levels confer a survival advantage to blasts by inhibiting programmed cell death. Indeed, in our ALL cohort, in multivariate analysis overexpression of survivin was an independent risk factor for relapse associated with significantly inferior RFS. The impact of an additional independent risk factor may well be more pronounced in low-risk patients, as indicated in our analysis. Patients classified as high risk by standard parameters are treated more aggressively than low-risk patients which may explain why the negative effect of survivin overexpression in the high-risk group is masked in the intensified treatment arm. In addition, the numbers of patients in the analysis of subgroups were small which may also influence the results.

The fact that survivin acts as an inhibitor of effector caspases 3/7 and blocks the mutual downstream events of both apoptosis pathways[4] renders survivin a key factor in the response to chemotherapy. Most chemotherapeutic agents induce cell death in a mitochondria-dependent manner, yet death receptor-mediated signals can also be involved. In ALL however, we were not able to determine a correlation between overexpression of survivin and *in vitro* chemoresistance to prednisolone, vincristine and asparaginase, assessed in about one third of the patients. Beyond the limitation of a small number of patients, the lack of a correlation between *in vitro* chemoresistance and elevated survivin levels may be due to chemotherapy-induced triggering of alternative caspase-independent apoptosis pathways as described for other B-cell malignancies.[41,42] Of interest, chemotherapeutic agents, such as daunorubicin, are themselves also known to modulate survivin expression.[43]

Survivin, by conferring a proliferative advantage to malignant cells expressing high levels of the anti-apoptotic protein, may, however, not only function as a risk factor discriminating patients with poor prognosis but also serve as a therapeutic target with the aim of specifically eliminating those cells that drive the disease and trigger relapse.[44] In addition to therapeutic blockage of survivin activity by antisense oligonucleotides or pharmacological inhibitors such as resveratrol,[45,46] survivin may also be a target for immunotherapy since discrimination of malignant cells from normal tissue is critical for immunological control.[47] Besides leukemia-specific antigens, shared antigens that are overexpressed in different malignancies but not in normal tissue represent potential targets for anti-leukemic effector mechanisms.[8,48] Expression of survivin is up to ten-fold higher in ALL blasts than in normal peripheral blood and bone marrow. The efficacy of survivin-specific cytotoxic T cells has already been shown for both ALL as well as CLL.[49]

In summary, this is the first report documenting overexpression of survivin protein in ALL blasts in an entirely pediatric population of patients. As in a variety of other cancers and hematologic malignancies, in B-cell precursor ALL high survivin levels are indicative of a poor prognosis. In our retrospective analysis, overexpression of survivin was an independent risk factor for inferior RFS, EFS and OS. Upon prospective confirmation of the prognostic significance of survivin in a larger ALL population, elevated survivin levels may in future be used to refine treat-

ment stratification, with the purpose of intensifying therapy in those patients prone to relapse. Survivin may even serve as a therapeutic target itself. Targeting the cell fraction with a definitive survival advantage might prove an effective strategy to specifically eradicate treatment-resistant blasts as the cellular source of relapse.

Authors' Contributions

TA: designed and performed the research, analyzed and interpreted the data, created the figures and tables and wrote the paper; MS: performed the research, analyzed and interpreted the data and critically revised the article; GE: analyzed and interpreted the data and critically revised the article; RM: performed the statistical analysis and critically revised the article; RW: performed the statistical analysis and revised the article critically; SG: provided materials and edited the article; LHJ: ALL diagnostic and critically revised the article; HH: ALL diagnostics and critically revised the article; UG: provided materials and critically revised the article; GJ-S: provided materials, interpreted data and critically revised the article; CM: analyzed and interpreted the data and critically revised the article; DD: designed the research, interpreted the data, created the figures and tables and wrote the paper. All authors: final approval of the submitted manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

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