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ORIGINAL ARTICLE

BAALC-associated gene expression profiles define *IGFBP7* as a novel molecular marker in acute leukemia

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Over expression of *BAALC* (brain and acute leukemia, cytoplasmic) predicts an inferior outcome in acute myeloid leukemia (AML) and acute lymphoblastic leukemia patients. To identify *BAALC*-associated genes that give insights into its functional role in chemotherapy resistance, gene expression signatures differentiating high from low *BAALC* expressers were generated from normal CD34$^+$ progenitors, T-acute lymphoblastic leukemia (T-ALL) and AML samples. The insulin-like growth factor binding protein 7 (*IGFBP7*) was one of the four genes (*CD34*, *CD133*, natriuretic peptide receptor C (*NPR3*), *IGFBP7*) coexpressed with *BAALC* and common to the three entities. In T-ALL, high *IGFBP7*-expression was associated with an immature phenotype of early T-ALL ($P<0.001$), expression of CD34 ($P<0.001$) and CD33 ($P<0.001$). Moreover, high *IGFBP7*-expression predicted primary therapy resistance ($P=0.03$) and inferior survival in T-ALL ($P=0.03$). *In vitro* studies revealed that IGFBP7 protein significantly inhibited the proliferation of leukemia cell lines (Jurkat cells: 42% reduction, $P=0.002$; KG1a cells: 65% reduction, $P<0.001$). In conclusion, *IGFBP7* was identified as a *BAALC* coexpressed gene. Furthermore, high *IGFBP7* was associated with stem cell features and treatment failure in T-ALL. In contrast to *BAALC*, which likely represents only a surrogate marker of treatment failure in acute leukemia, *IGFBP7* regulates the proliferation of leukemic cells and might be involved in chemotherapy resistance.

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Introduction

Understanding the molecular pathomechanisms in acute leukemia has dramatically improved over the last decades. Altered expression of genes implicated in signal transduction pathways, as well as transcription factors have been shown to have a critical role in leukemogenesis.[1–3] Identification of the molecular processes involved in normal hematopoiesis may help to gain further insights into the pathogenesis of the disease. Moreover, characterization of molecular changes may result in the design of new targeted therapies.[4]

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In a search for genes involved in leukemia, the human gene *BAALC* (brain and acute leukemia, cytoplasmic) located on human chromosome 8q22.3 was identified.[5] It was shown that over expression of *BAALC* was associated with an inferior outcome and chemotherapy resistance in adult patients with cytogenetically-normal acute myeloid leukemia (CN-AML), acute T-acute lymphoblastic leukemia (T-ALL) and B-precursor acute lymphoblastic leukemia (B-ALL).[5–10] Furthermore, *BAALC*-expression was associated with an immature leukemic phenotype in CN-AML and T-ALL, reflecting its role in early hematopoiesis. In normal hematopoiesis, studies have shown that *BAALC* is highly expressed in CD34$^+$ bone marrow (BM) cells and is downregulated with cell differentiation.[11] Although the prognostic value of *BAALC*-expression has been shown in CN-AML, T-ALL and recently in B-ALL, the function of *BAALC* remains unknown. So far, functional studies investigating the impact of *BAALC*-expression in leukemia cell lines and normal CD34$^+$ progenitors could not show a mechanistic role of *BAALC* on drug resistance, proliferation and differentiation.

The aim of this study was to identify genes correlated with *BAALC* to gain further insights into the *BAALC*-associated signaling pathways involved in the chemotherapy-resistant leukemia. Therefore, we compared *BAALC*-associated gene expression profiles (GEPs) of normal CD34$^+$ progenitors, T-ALL and CN-AML BM blast cells. Among the four *BAALC* coexpressed genes, we identified the human insulin-like growth factor binding protein 7 (*IGFBP7*) as a new candidate gene implicated in leukemia. *IGFBP7* has been reported to be involved in various human cancers, thus we further investigated its functional role in acute leukemia.

Materials and methods

Samples

Gene expression studies for *BAALC* and *IGFBP7* were performed in 219 adults with newly diagnosed T-ALL and in 230 adults with newly diagnosed B-ALL, registered on the German multicenter acute lymphoblastic leukemia study group 06/99 and 07/03 protocols.[12] *BAALC*- and *IGFBP7*-expression levels were further investigated in 23 patients with biphenotypic acute leukemia (BAL)/acute undifferentiated leukemia, 81 patients with acute myeloid leukemia (AML) and 44 patients with myelodysplastic syndrome. Furthermore, 14 BM samples from healthy donors were studied. All patients and donors gave written informed consent to participate in the study according to the Declaration of Helsinki. Patients and healthy donors' characteristics are summarized in Supplementary Table S1.

Immunophenotypic analyses of B-ALL, T-ALL and BAL/acute undifferentiated leukemia

Pre-treatment BM samples were collected centrally and enriched for the blast fraction by density gradient centrifugation (Ficoll-PaquePlus; Amersham Biosciences, Uppsala, Sweden). Immunophenotyping of fresh samples was centrally performed for all patients as previously described.[13] BAL classification was based on the European group for the immunological classification of leukemia scoring system.[14]

GEPs

CD34. To identify the genes that were coregulated with *BAALC* during lineage-specific differentiation, data from recently published oligonucleotide microarray (HG-U133A; Affymetrix, Santa Clara, CA, USA) studies were analyzed.[15] $CD34^+$ cells from healthy individuals were stimulated *in vitro* with erythropoietin, thrombopoietin, granulocyte and granulocyte macrophage colony-stimulating factor. For each of the lineages, cells were harvested for RNA extraction at days 0, 4, 7 and 11 and subsequently used for expression profiling. To identify the genes coregulated with *BAALC* the following criteria were applied: same shift in expression as *BAALC* at the four time points and in all lineages, with a correlation coefficient of $r \geqslant 0.95$.

T-ALL. GEPs of 86 T-ALL samples were generated from raw data obtained from the microarray innovations in leukemia multicenter study.[16] RNA isolation, labeling and hybridization using HG-U133 Plus 2.0 microarray (Affymetrix) were performed as described previously.[17] For data analyses, samples were divided into quartile (Q1–4) groups according to *BAALC*-expression levels represented by the median of the two probe sets (218899_s_at and 222780_s_at). The low *BAALC* group was defined with expression levels in Q1 to Q3 ($n = 64$) and the high *BAALC* group with expression levels in Q4 ($n = 22$). This grouping was based on the previous study that classified *BAALC*-expression into three lower quartiles (Q1–Q3) and the upper quartile (Q4), with Q4 showing a clinically distinct inferior outcome compared with the remaining quartiles.[8] Upregulated genes between the high and low *BAALC* groups were defined by a minimum expression change of threefold and probe sets called Present (P) by the Affymetrix data analysis in at least 75% of the samples. Statistical significance was calculated by the nonparametric *t*-test with a *P*-value $\leqslant 0.05$. Our data analyses were performed with the GeneSpring software version 4.2 (Silicon Genetics; Redwood City, CA, USA).

AML. Langer *et al.*[9] described a *BAALC*-associated GEP in 50 adult CN-AML patients using HG-U133 Plus 2.0 microarray (Affymetrix). In this study, high and low *BAALC*-expression groups were differentiated by the median *BAALC*-expression levels. They identified 149 genes upregulated (fold-change $\geqslant 1.3$; $P \leqslant 0.05$) in the high *BAALC*-expression group. These genes were selected for comparison with the Affymetrix data used in our study.

RNA extraction and real-time reverse transcriptase-PCR for BAALC *and* IGFBP7

Isolation of total RNA from mononuclear cells of pre-treatment BM samples was carried out using TRIzol reagent (Invitrogen, Karlsruhe, Germany) according to the manufacturers' instructions. Complementary DNA was synthesized using 500 ng of total RNA and Avian Myeloblastosis Virus reverse transcriptase (RT-AMV; Roche, Mannheim, Germany) at 50 °C for 60 min in the presence of RNase inhibitor (RNasin; Roche, Mannheim, Germany). *BAALC* mRNA expression levels were determined by quantitative real-time reverse transcriptase-PCR (real-time quantitative PCR) as previously described.[6] Expression analysis of *IGFBP7* was performed using primers IGFBP7-forward: CATCACCCAGGTCAGCAAG and IGFBP7-reverse: TCACAG CTCAAGTACACCTG, with an IGFBP7-probe: 5′-FAM-TGCGA GCAAGGTCCTTCCATAGT-TAMRA-3′. Final concentrations of primers and the probe were chosen on the basis of optimization experiments. Multiplex PCR was performed with glucose phosphate isomerase (*GPI*), as a housekeeping gene,[5] in duplicates. Amplification procedures and expression analyses were performed similar to those applied for *BAALC*. In all samples, amplification of *GPI* reached the threshold within 30 cycles. Positive and negative controls were included in all assays. *BAALC*- and *IGFBP7*-expression levels were normalized to KG1a-expression levels.

Cell lines and cell culture

The human myeloblastic cell line KG1a and the human T-cell leukemia cell line Jurkat were purchased from the DSMZ (Braunschweig, Germany) and maintained in RPMI1640 culture medium (GIBCO Invitrogen, Karlsruhe, Germany) with the recommended supplements. Recombinant human IGFBP7 (rhIGFBP7) was obtained from PreproTech GmbH (Hamburg, Germany) and was diluted in phosphate-buffered saline with 0.1% bovine serum albumin.

Cell proliferation assays

To determine the effect of rhIGFBP7 on KG1a and Jurkat cells, changes in cell number and cell proliferation were detected. The total number of viable cells was assessed by trypan blue exclusion on days 0, 2, 4 and 7 after rhIGFBP7 treatment. KG1a and Jurkat cells were cultured with 100 µg per ml rhIGFBP7 and the corresponding control (phosphate-buffered saline 0.1% bovine serum albumin). Changes in cell proliferation were determined using the cell proliferation reagent WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium). (Roche Diagnostics GmbH, Mannheim, Germany). Cells were plated in triplicate wells in a 96-well plate with 100 µl medium with different concentrations of rhIGFBP7 (0–100 µg/ml). Separate plates were cultured over 7 days with changes of the appropriate media. On days 0, 2, 4 and 7, the proliferation assays were performed according to the manufacturers' protocol. Reduction of the WST-1 dye, indicating mitochondrial activity, was determined by optical density absorption analyses at 450 nm using a Dynatech ELISA plate reader (Dynatech International, Chantilly, VA, USA).

DNA replication assays

For DNA replication assays KG1a cells were seeded in triplicates in a 96-well plate with a conditioned medium (100 µg per ml rhIGFBP7), and cultured over 4 days. BrdU was added to a final concentration of 1 µM. After incubation for 24 h, DNA synthesis was analyzed with the cell proliferation ELISA, BrdU (Roche Diagnostics GmbH, Mannheim, Germany) using colorimetric detection, according to the manufacturers' instructions. Newly synthesized BrdU-DNA was determined by

optical density absorption at 405 nm, correction wavelength at 490 nm using the ELISA reader.

Apoptosis and senescence assays

To examine the induction of apoptosis followed by rhIGFBP7 treatment, KG1a cells were cultured in conditioned media with 10 µg per ml rhIGFBP7. Cells were stained for Annexin V-phycoerythrin using the phycoerythrin Annexin V apoptosis detection kit (BD Pharmingen, Heidelberg, Germany) on days 0, 4, 7, 9, 11, 15 and 17. Senescence-associated β-galactosidase activity was detected as described[18] with slight modifications. KG1a and Jurkat cells were cultured with rhIGFBP7 (0–100 µg/ml) for up to 15 days. Cells were harvested at specific time points (10 µg per ml rhIGFBP7: days 4, 7, 9, 11, 15 and 17; 50–100 µg per ml rhIGFBP7: days 4 and 7), washed with phosphate-buffered saline, centrifuged and cell numbers were adjusted to 1×10^6 cells/ml. A total of 100 µl was transferred to one well in a 96-well plate and centrifuged. Cells were incubated with the staining solution for 6, 12, 16 and 24 h at 37 °C in a humidified atmosphere and analyzed for the development of blue color using a microscope.

Statistical methods

For correlation of *IGFBP7* mRNA expression levels in T-ALL patients (German multicenter acute lymphoblastic leukemia 06/99 and 07/03 protocols) with clinical and molecular features, patient samples were divided into a *IGFBP7* low group with expression levels in Q1 to Q3 ($n=158$) and a *IGFBP7* high group with expression levels in Q4 ($n=53$), similar to the *BAALC*-expression grouping as described above.

Clinical follow-up data were available from 207 T-ALL patients with a median follow-up time of 35.8 months (range: 3.2–84.7 months) for alive patients. Complete remission was assessed after the completion of induction chemotherapy. Overall survival (OS) was calculated using the Kaplan–Meier method, and the log-rank test was used to compare differences between survival curves. OS was measured from the protocol on study date until the date of death of any cause. Patients undergoing stem cell transplantation in first remission were excluded for this analysis.

The statistical difference between values of two independent groups was tested by the nonparametric Mann–Whitney *U*-test. A *P*-value $\leqslant 0.05$ (two-sided) was considered to indicate a significant difference. Spearman's rank correlation coefficient was used to measure the correlation between *BAALC*- and *IGFBP7*-expression. All calculations were performed using the SPSS software version 17 (SPSS, Chicago, IL, USA) and GraphPad Prism software version 5 (GraphPad Software, La Jolla, CA, USA).

Results

BAALC-*associated GEP of CD34$^+$ progenitors, T-ALL and CN-AML samples*

BAALC is highly expressed in undifferentiated normal CD34$^+$ cells and continuously downregulated during hematopoietic differentiation. On the basis of the published oligonucleotide microarray analysis,[15] we identified 239 genes that showed a significant positive correlation ($r \geqslant 0.95$) to *BAALC* in their expression during *in vitro* differentiation (at all four different time points and in the three different lineages, induced by erythropoietin, thrombopoietin and granulocyte and granulocyte macrophage colony-stimulating factor). These genes are summarized in Supplementary Table S2.

To generate a *BAALC*-associated gene expression signature in adult T-ALL, 86 T-ALL samples were grouped according to their *BAALC*-expression levels into quartiles Q1–Q4, as described in the design and methods section, and were defined as high *BAALC* (Q4) and low *BAALC* (Q1–Q3) groups. We identified 72 upregulated probe sets corresponding to 56 unique genes, hypothetical genes/proteins and open reading frames in the high *BAALC* compared with the low *BAALC* group. For a complete list of the 72 differentially expressed probe sets see Supplementary Table S3.

To explore a common and lineage-independent *BAALC* signature, we compared the lists of the *BAALC*-associated genes in normal CD34$^+$ progenitors, the *BAALC*-associated GEP in T-ALL and the upregulated genes in the high *BAALC*-expression group in CN-AML reported by Langer.[9] Figure 1a shows the comparison of these three *BAALC*-associated GEPs and their overlap between the entities; genes in the intersections are listed in Supplementary Table S4.

A total of five genes and one open reading frame (*BAALC*, *CD34*, *CD133*, natriuretic peptide receptor C (*NPR3*), *IGFBP7* and C5orf23) were common to the three *BAALC*-associated GEPs. As a proof of principle, *BAALC* was among these genes (represented by the probe set 218899_s_at). Two genes (*CD34* and *CD133*) have been associated with hematopoietic stem cells.[19,20] Interestingly, the human gene *NPR3*, *IGFBP7*, as well as the open reading frame (C5orf23) were also highly expressed in CD133$^+$ hematopoietic progenitors.[19,20]

As the insulin-like growth factor (IGF) signaling has been shown to have a role in leukemia,[21,22] *IGFBP7* was of particular interest and was chosen for further investigations. When we explored the GEP data, specifically with respect to *IGFBP7*, a strong correlation of *IGFBP7* and *BAALC*-expression quartiles in T-ALL was observed (Figure 1b). Moreover, in normal CD34$^+$ cells, *IGFBP7*-expression was continuously downregulated during erythroid, granulocytic and megakaryocytic differentiation similar to *BAALC*[11] (Supplementary Figure S1).

Expression of IGFBP7 *and* BAALC *in hematological malignancies*

To confirm the generated GEP data, we also analyzed *BAALC* and *IGFBP7*-expression in T-ALL samples ($n=30$) that had sufficient material available for real-time quantitative PCR verification. As shown in Supplementary Figures S2 A-B, the results of microarray and real-time quantitative PCR studies strongly correlated for *BAALC*- ($r=0.84$; $P<0.001$), as well as for *IGFBP7*-expression levels ($r=0.84$; $P<0.001$).

Thus far, no comprehensive studies have yet determined the role of *IGFBP7* in hematological malignancies. To gain further insights into its expression pattern, we investigated *IGFBP7*-expression levels by real-time quantitative PCR in different hematological malignancies including 611 patients and healthy controls (Figure 2).

In myelodysplastic syndrome patients ($n=44$), the median expression of *IGFBP7* (1.19) was not significantly different compared with that in normal BM (1.03; $P=0.36$). However, in the myelodysplastic syndrome high-risk group (classification according to the International prognostic scoring system[23]), *IGFBP7* was significantly higher expressed compared with healthy controls (median expression myelodysplastic syndrome high-risk: 1.9; median expression normal BM: 1.03; $P=0.02$). Contrastingly, for the remaining International prognostic scoring system risk groups, no significant changes in *IGFBP7*-expression were observed. *IGFBP7*- and *BAALC*-expressions were strongly correlated in these samples ($r=0.52$; $P<0.001$).

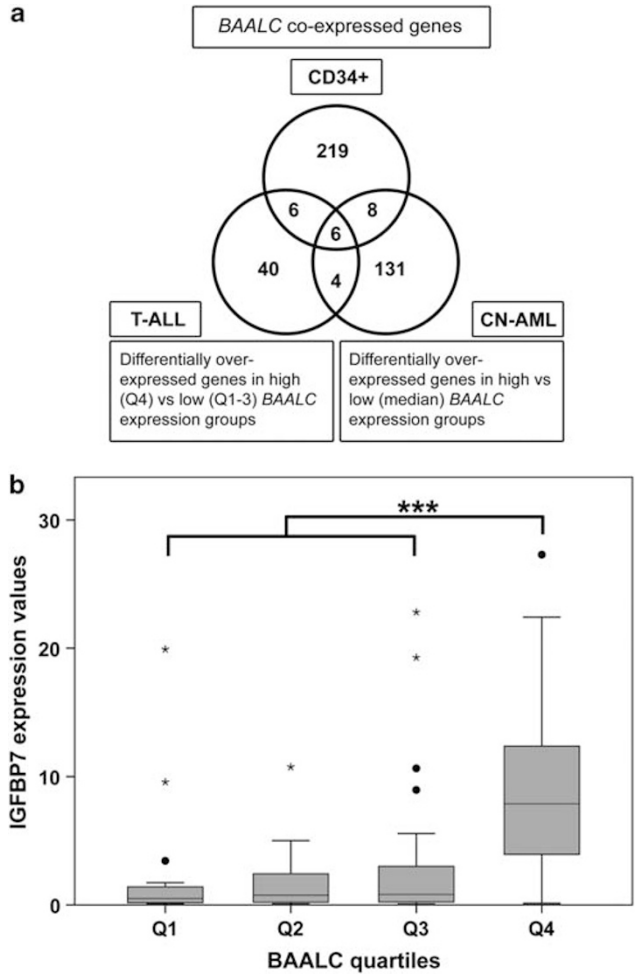

Figure 1 Brain and acute leukemia, cytoplasmic (*BAALC*)-associated gene expression profiling. (**a**) Venn diagram showing the gene overlap among *BAALC*-associated gene expression profiles (GEPs) in $CD34^{+15}$ progenitors, T-acute lymphoblastic leukemia (T-ALL) and cytogenetically normal acute myeloid leukemia (CN-AML).[9] (**b**) Affymetrix-based correlation between *BAALC* groups with low *BAALC*-expression (Q1–Q3) and high *BAALC*-expression (Q4) and insulin-like growth factor binding protein 7 (*IGFBP7*) expression in T-ALL ($n=86$). *x* axis: *BAALC*-expression groups (mean expression of the two probe sets: 218899_s_at and 222780_s_at); *y* axis: normalized *IGFBP7*-expression values (mean of the two probe sets: 201162_at and 201163_s_at). A statistical significance of *IGFBP7*- and *BAALC*-expression groups is shown by ***$P<0.001$ (*IGFBP7*-expression in low *BAALC* vs *IGFBP7*-expression in high *BAALC*; Mann–Whitney *U*-test).

There was no significant difference in the median expression levels of *IGFBP7* in acute lymphoblastic leukemia when compared with healthy controls (T-ALL: 0.62 vs 1.03, $P=0.15$; B-ALL: 0.76 vs 1.03, $P=0.14$). However, in T-ALL (expression range: 0–79.1) and B-ALL (expression range: 0.04–42.96) *IGFBP7* showed a more heterogeneous expression pattern compared with normal BM (expression range: 0.6–2.27). A strong correlation between *IGFBP7*- and *BAALC*-expression levels was observed for T-ALL ($r=0.56$; $P<0.001$) and B-ALL ($r=0.34$; $P<0.001$).

The published GEP data were generated on CN-AML samples.[9] We further investigated *IGFBP7*-expression in an independent set of 81 AML samples including different cytogenetic subtypes. The median expression of *IGFBP7* in these AML patients was significantly higher compared with normal BM controls (2.79 vs 1.03; $P<0.001$) and showed a more homogenous expression pattern compared with T-ALL. For these AML samples, *IGFBP7* did not correlate with *BAALC*-expression ($r=0.05$; $P=0.70$).

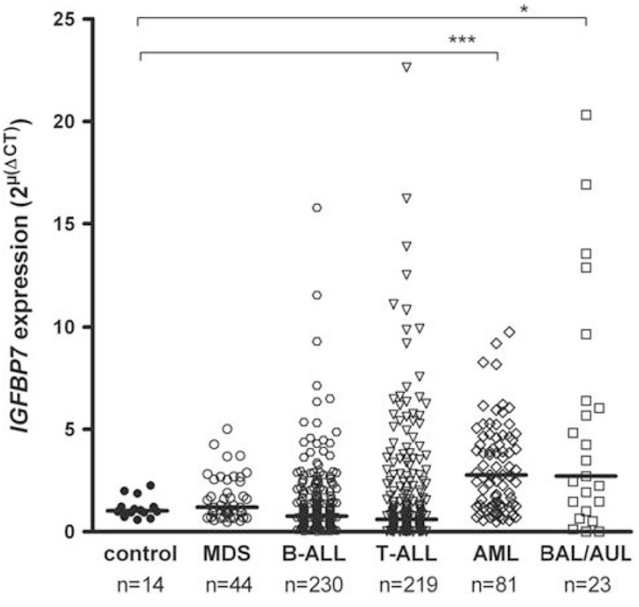

Figure 2 Expression of *IGFBP7* in hematological malignancies. Real-time quantitative PCR was performed in 611 samples. For each entity the median expression is shown by a horizontal bar. Expression outliers are not included in this graph; however, they are included in the statistical analyses. Significance values compared with control are as follows: *$P\leqslant0.05$, ***$P\leqslant0.001$ (Mann–Whitney *U*-test). AUL, acute undifferentiated leukemia; MDS, myelodysplastic syndrome.

In BAL/acute undifferentiated leukemia, *IGFBP7* was significantly higher expressed (BAL/acute undifferentiated leukemia median: 2.71 vs control median: 1.03; $P=0.02$) and *IGFBP7*-expression correlated with *BAALC* ($r=0.47$; $P=0.03$).

In leukemia cell lines, the undifferentiated acute myeloblastic cell line KG1a showed a lower expression of *IGFBP7* compared with the median expression of AML patient samples (0.92 vs 2.79) whereas the human T-cell leukemia line Jurkat lacked *IGFBP7*-expression.

Association of IGFBP7 *with clinical and molecular features in T-ALL*

As T-ALL samples showed the highest variation in *IGFBP7*-expression, we further verified the role of *IGFBP7* in T-ALL. Of the 219 T-ALL patients analyzed for *IGFBP7*-expression by real-time quantitative PCR, clinical follow-up data were available for 207 patients. Patients were assigned to low and high *IGFBP7*-expression groups as described in the statistical section. There were no significant differences between patients with low and high *IGFBP7*-expression with regard to the initial white blood cell count, age and gender. Like *BAALC*,[8] high expression of *IGFBP7* was also associated with a more immature phenotype of early T-ALL, higher expression of CD34 and aberrant expression of the myeloid antigen CD33 (Table 1). Patients with high *IGFBP7* showed a lower complete remission rate (87 vs 95%; $P=0.08$) compared with patients with low *IGFBP7*-expression and a higher rate of refractory disease after induction therapy (11 vs 3%; $P=0.03$; Table 1). Furthermore, high *IGFBP7* expression was associated with an inferior OS (4-year OS IGFBP7 high: 42% (s.e. +/−9.5) vs IGFBP7 low: 55% (s.e. +/−5.3); $P=0.03$; Figure 3).

Table 1 Molecular characteristics and outcome with respect to *IGFBP7*-expression in T-ALL

		IGFBP7 *low* *(Q1–3)* *(n = 158)*	IGFBP7 *high (Q4)* *(n = 53)*	P-*value*
Early T-ALL	No.	23	33	<0.001
	(%)	(15)	(62)	
Thymic T-ALL	No.	108	14	
	(%)	(68)	(26)	
Mature T-ALL	No.	27	6	
	(%)	(17)	(11)	
CD34 expression (surface), %	Mean	15	36	<0.001
	Range	0–95	0–97	
CD33 expression (surface), %	Mean	3	24	<0.001
	Range	0–96	0–98	
BAALC-expression group (mRNA)[a]	High/total	18/133	26/46	<0.001
	(%)	(13.6)	(56.4)	
Complete remission	No. of patients/total	144/151	39/45	0.08
	(%)	(95)	(87)	
Refractory disease	No. of patients/total	4/148	5/44	0.03
	(%)	(3)	(11)	
Relapse	No. of patients/total	31/144	5/39	0.26
	(%)	(22)	(13)	

Abbreviations: *BAALC*, brain and acute leukemia, cytoplasmic; *IGFBP7*, insulin-like growth factor binding protein 7; T-ALL, T-lymphoblastic leukemia.
[a]Patients' samples were divided into quartile (Q) groups according to *BAALC*-expression and were defined as low *BAALC*, with expression levels in Q1 to Q3 ($n = 133$), and as high *BAALC*, with expression levels in Q4 ($n = 46$).

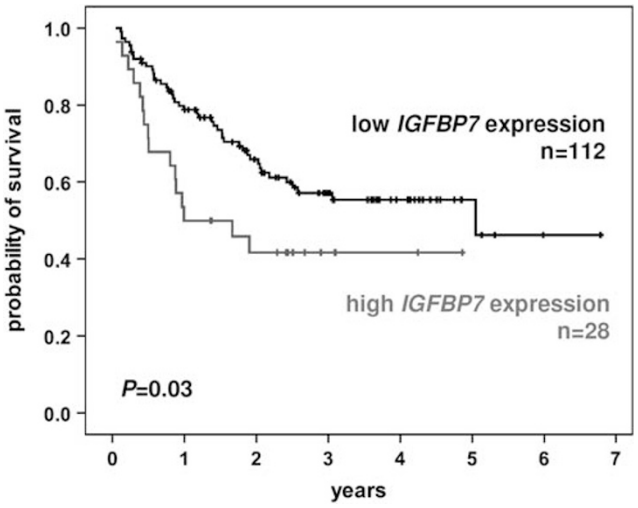

Figure 3 Kaplan–Meier analysis of overall survival (OS) in T-ALL with respect to *IGFBP7* expression. T-ALL patients ($n = 140$; German multicenter acute lymphoblastic leukemia 06/99 and 07/03 protocols) with high *IGFBP7* expression showed a significantly inferior OS compared with low *IGFBP7* patients ($P = 0.03$; log-rank test).

Effects of rhIGFBP7 on proliferation, apoptosis and senescence

To determine the functional role of IGFBP7 in leukemia, the cell lines KG1a and Jurkat were treated with rhIGFBP7 using varying concentrations (0–100 μg/ml). As shown in Figure 4a, treatment with rhIGFBP7 resulted in a significant reduction of cell growth in both cell lines compared with untreated cells (KG1a growth inhibition: 52%; $P = 0.004$ and Jurkat growth inhibition: 39%; $P = 0.048$).

The effect of rhIGFBP7 on cellular proliferation was further confirmed using the WST-1 proliferation assay. Addition of rhIGFBP7 significantly reduced the proliferation of KG1a and Jurkat cells in a dose-dependent manner (Figure 4b). To explore cell cycle changes, we assessed rhIGFBP7-treated KG1a cells with the BrdU proliferation ELISA. rhIGFBP7 led to a 26% reduction of DNA replication, S-phase (Figure 4c). These results show that IGFBP7 has an important role in the regulation of proliferation in leukemic cells.

In addition, we examined whether the rhIGFBP7-mediated inhibition of proliferation was also caused by apoptosis or senescence, as described in other cancers.[24,25] rhIGFBP7 (10 μg/ml) did not induce apoptosis in KG1a cells, as determined by Annexin V-phycoerythrin staining. With concentrations up to 100 μg/ml increases in senescent cells were not detectable in KG1a and Jurkat cells (data not shown).

Regulation of *IGFBP7* mRNA expression has not yet been investigated in leukemia. As the autocrine-secreted IGFBP7 protein may impact on its transcriptional regulation, we investigated whether *IGFBP7* underlies an autoregulation. Treatment of KG1a cells with rhIGFBP7 protein led to a 30% increase in *IGFBP7* mRNA levels (Figure 5). Thus, a positive feedback mechanism may regulate *IGFBP7*-expression in leukemia. These data suggest that *IGFBP7* suppresses cell proliferation and growth without the induction of apoptosis or senescence in leukemic cells.

Discussion

Recent studies have highlighted the prognostic impact of *BAALC*-expression in CN-AML, T-ALL and B-ALL. However, the function of *BAALC* in hematopoiesis and leukemogenesis remains unknown.[6,8,9] Therefore, characterization of *BAALC* and its related pathways may provide more insights into the pathology of the *BAALC*-associated chemoresistance and treatment failure in acute leukemia. These studies may reveal new molecular target genes and might result in more tailored therapy approaches.

To identify the genes that are coactivated with *BAALC*, we first explored *BAALC*-associated gene expression signatures common to normal $CD34^+$ progenitors, T-ALL and CN-AML. Using this approach, we discovered four *BAALC* coexpressed genes namely *CD34*, *CD133*, *NPR3* and *IGFBP7*. Interestingly, these genes associated with high *BAALC*-expression were also upregulated in undifferentiated hematopoietic cells.[19,20] *CD34* and *CD133* are known markers characterizing hematopoietic progenitor cells.[26–28] *NPR3* belongs to the family of natriuretic peptides involved in vascular, renal and endocrine effects regulating the blood pressure and extracellular fluid volume.[29] Evidence of its involvement in carcinogenesis and leukemogenesis has not yet been reported. *IGFBP7* was of particular interest, as IGFBPs have already been linked to cancer and leukemia.[21,30,31] Moreover, the observation that *IGFBP7* was coexpressed with *CD34* and *CD133* in normal progenitors suggested that *IGFBP7* might have a role in early hematopoiesis and leukemogenesis.

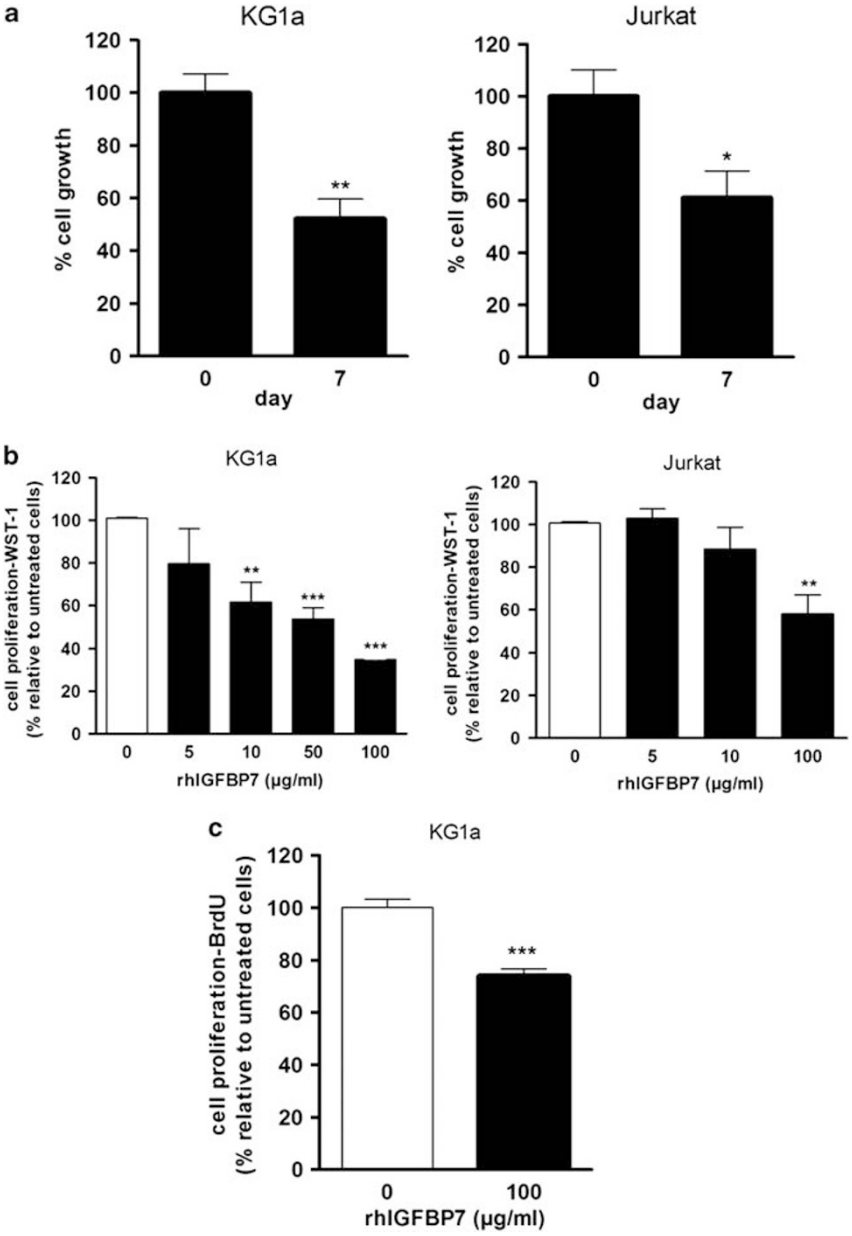

Figure 4 Effects of rhIGFBP7 on proliferation of leukemia cell lines. (**a**) KG1a and Jurkat cells were incubated for 7 days with 100 µg per ml rhIGFBP7. The number of viable cells was counted using the trypan blue dye and values were given as percent cell growth compared with untreated cells. The mean of two independent experiments is shown. (**b**) Dose-dependent effects of rhIGFBP7 were determined using the WST-1 proliferation reagent. KG1a and Jurkat cells were incubated for 7 days with rhIGFBP7 (0–100 µg/ml). The mean optical density (OD) values corresponding to the non-treated control were taken as 100%. The results were expressed as percentages of the OD of treated vs untreated control cells. For each concentration, independent experiments were performed in triplicates. (**c**) Effect of rhIGFBP7 on proliferation was determined by BrdU incorporation. KG1a cells were incubated for 4 days with 100 µg per ml rhIGFBP7. Data are expressed as percentage of the untreated control. Two experiments were performed in triplicates. All results are expressed as means ± s.e.m. vs control (0 µg per ml rhIGFBP7). The asterisks indicate statistical significance $*P \leqslant 0.05$, $**P \leqslant 0.01$, $***P \leqslant 0.001$ (two-tailed unpaired *t*-test).

IGF-signaling, including IGF-1 and IGF-2, together with their receptors, binding proteins and the proteolytic enzymes have an important role in various human cancers.[21,22,32–35] IGFBPs, as carriers of IGFs, prolong their half-life and modulate IGF availability and activity.[36] IGFBPs also mediate IGFs' independent biological functions.[37] IGFBP7 is a secreted 31 kDa protein, which is also known as IGFBP-related protein 1, MAC25, tumor-derived adhesion factor (TAF), angiomodelin and prostacyclin-stimulating factor.[38] Unlike the other family members (that is, IGFBP1–6), IGFBP7 binds to IGFs with lower affinity and has been implicated in tumor growth suppression and induction of apoptosis.[24,25,39] It was also shown that *IGFBP7* stimulates the proliferation of glioma cells.[40] In colorectal cancer, high expression of *IGFBP7* was correlated with poor prognosis.[41] Contrastingly, high *IGFBP7* expression was associated with a favorable prognosis in breast cancer.[42] The nature of these functional as well as prognostic differences remains unknown. The expression pattern and various biological functions of *IGFBP7* likely depend on the cell type and might also be different in normal cells and their malignant counterparts.

Little is known about the gene expression levels of the components of the IGF system, and their functional role in

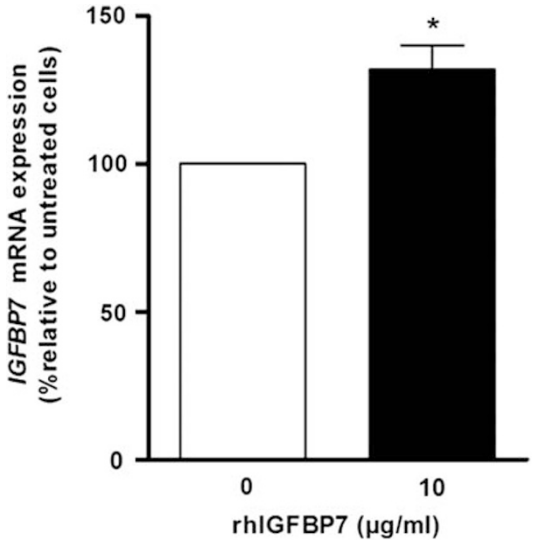

Figure 5 Expression of *IGFBP7* after treatment with rhIGFBP7. To determine the effect of rhIGFBP7 on *IGFBP7* mRNA expression levels, KG1a cells were cultured with 10 µg per ml rhIGFBP7 over 9 days. RNA was isolated using TRIzol reagent and *IGFBP7*-specific real-time quantitative PCR was performed. Data are expressed as percentage of the untreated control. Three independent experiments were performed. The mean percentage of change in expression (± s.e.m.) is shown. The asterisk indicates statistical significance. $*P \leqslant 0.05$ (two-tailed unpaired *t*-test).

hematopoiesis and leukemia. To gain insights into the function of *IGFBP7* in hematopoiesis, we explored the expression levels of *IGFBP7* during *in vitro* lineage-specific hematopoietic differentiation and observed downregulation of *IGFBP7* with the onset of maturation. These results suggest that *IGFBP7* might have a role in hematopoietic differentiation and might be a marker of early progenitor cells. We found that *IGFBP7* was aberrantly expressed in subsets of hematological malignancies and strongly correlated to *BAALC*-expression implicating its role in leukemia. Moreover, high *IGFBP7* was associated with the expression of early progenitor antigens CD34 and CD133 and a more immature phenotype of early T-ALL.

Studies in CN-AML, T-ALL and B-ALL have associated high *BAALC*-expression with chemotherapy resistance and inferior survival.[8–10] Similar to *BAALC*, elevated *IGFBP7*-expression was associated with a lower response rate to induction therapy and an inferior OS in T-ALL patients. Therefore, aberrant *IGFBP7*-expression might have a role in drug resistance and may consequently contribute to a poorer outcome. Interestingly, aberrantly elevated IGFBP7 protein levels were found in the cerebrospinal fluid of children with acute lymphoblastic leukemia[43] and thus, IGFBP7 might translate into a more aggressive subtype of acute lymphoblastic leukemia.

To further explore the functional role of *IGFBP7*, addition of rhIGFBP7 to leukemia cell lines led to a reduction of proliferation in a dose-dependent manner, as determined by WST-1 assay. We postulate that inhibition of proliferation by rhIGFBP7 without increased apoptosis and senescence leads to a reduced sensitivity to cytostatic drugs and thereby contributing to drug resistance. This is supported by the clinical data showing that T-ALL patients with high *IGFBP7*-expression have a lower response rate and an inferior outcome.

The underlying mechanisms of *IGFBP7* function and its downstream signaling is unknown. In various malignancies, it was proposed that *IGFBP7* regulates different cell types through the mitogen-activated protein kinase as well as through the phosphoinositide 3-kinase/AKT pathway.[25,40,44,45] Although acute stimulation of mitogen-activated protein kinases was associated with cell proliferation, continuously increased levels of the phosphorylated mitogen-activated protein kinase have been associated with growth arrest and impaired differentiation.[46,47] Thus, our findings of a decreased proliferation and an increase in *IGFBP7* mRNA levels after treatment with rhIGFBP7 suggest that sustained IGFBP7 secretion might lead to a chronic increase of phosphorylated mitogen-activated protein kinase, which subsequently blocks proliferation.

By performing a comprehensive analysis of *BAALC*-associated GEP, we identified *IGFBP7* as a new candidate gene involved in leukemia. We showed aberrant expression of *IGFBP7* in adult leukemia and its correlation to chemotherapy resistance. *IGFBP7* resembles *BAALC* characteristics. However, in contrast to *BAALC*, which may only be a surrogate marker for treatment failure in acute leukemia, *IGFBP7* may have a functional role in leukemogenesis and might contribute to the molecular mechanisms of drug resistance. The finding that the addition of rhIGFBP7 suppresses proliferation, suggests that IGFBP7 might promote resistance to cell cycle-specific cytotoxic agents by a decrease in replicative activity in these cells. Further studies are needed to characterize the molecular mechanisms of IGFBP7 function in leukemic cells.

Conflict of interest

The microarray innovations in leukemia study was in part supported by Roche Molecular Systems. TH is a part owner of the MLL Münchner Leukämie Labor GmbH. The other authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Leukemia website (http://www.nature.com/leu)