High VLA-4 expression is associated with adverse outcome and distinct gene expression changes in childhood B-cell precursor acute lymphoblastic leukemia at first relapse

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The online version of this article has a Supplementary Appendix.

ABSTRACT

Background
Resistance to therapy and subsequent relapse remain major challenges in the clinical management of relapsed childhood acute lymphoblastic leukemia. As the bone marrow environment plays an important role in survival and chemotherapy resistance of leukemia cells by activating different signaling pathways, such as the VLA-4 and PI3K/Akt pathways, we studied the prognostic and biological impact of *VLA-4* expression in leukemia cells from children with relapsed B-cell precursor acute lymphoblastic leukemia and its influence on the sensitivity of the leukemia cells to drugs.

Design and Methods
VLA-4 expression was quantified by real-time polymerase chain reaction in leukemia cells from 56 patients with relapsed acute lymphoblastic leukemia enrolled in the ALL-REZ BFM 2002 trial of the Berlin-Frankfurt-Münster study group. Gene expression changes related to *VLA-4* expression were investigated by microarray-based mRNA profiling. The effect of VLA-4 signaling on proliferation and drug resistance was studied in co-cultures of leukemia and stromal cells.

Results
High expression of *VLA-4* at first relapse was associated with adverse prognostic factors, poor molecular response to therapy and significantly worse probabilities of event-free and overall survival. *VLA-4* expression was an independent prognostic parameter. Comparing gene expression profiles of leukemia cells with high *versus* low *VLA-4* expression, we identified 27 differentially expressed genes primarily involved in the PI3K/Akt, ephrin and Rho GTPase pathways. Blocking of VLA-4 signaling in combination with cytarabine treatment abolished the growth supportive effect of stromal cells.

Conclusions
Our results show that high *VLA-4* expression is a marker of poor prognosis and a potential therapeutic target in children with relapsed acute lymphoblastic leukemia and confirm that cellular interactions and biological effects related to VLA-4 play a decisive role in the survival of leukemia cells and response to therapy. *(ClinicalTrials.gov identifier: NCT00114348)*

Key words: VLA-4, acute lymphoblastic leukemia, stromal cells, prognosis markers.

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Introduction

The survival rate of children at their initial diagnosis of acute lymphoblastic leukemia (ALL) has improved to about 80%.[1,2] However, the overall outcome of children with relapsed disease remains poor at less than 40%.[3] The main predictors of outcome in relapsed ALL are duration of first remission, immunophenotype of the leukemia cells, site of relapse[4] and molecular response to therapy (minimal residual disease, MRD).[5] Based on these biological and clinical risk factors, three main risk groups with significantly different event-free survival rates (standard risk: S1, intermediate risk: S2 and high risk: S3/S4) were defined in the ALL relapse trial of the Berlin-Frankfurt-Münster (BFM) study group: ALL-REZ BFM 2002. High-risk parameters are short duration of first remission (very early/early time point of relapse), T-cell ALL and bone marrow involvement. In fact, in more than 80% of childhood ALL the site of first relapse is the bone marrow.[4,6] Bone marrow stromal cells have been shown to protect normal and malignant hematopoietic cells from damaging events, including drug-induced cell death.[7-12]

Normal hematopoiesis is regulated by the adhesive interaction of hematopoietic cells with the bone marrow microenvironment, as well as by growth factors and cytokines expressed by different types of bone marrow cells.[12] The bone marrow microenvironment consists of stromal cells and extracellular matrix proteins such as fibronectin, collagen and laminin. Among these extracellular matrix proteins, fibronectin has been shown to support survival and proliferation of hematopoietic stem cells and progenitor cells through their fibronectin receptors such as very late antigen-4 (VLA-4; ITGA4; $\alpha_4\beta_1$). α_4 integrins act as both adhesive and signaling receptors by interacting with their ligands, fibronectin and vascular cell adhesion molecule-1 (VCAM-1) expressed by stromal cells.[13] In hematopoietic malignancies (e.g. acute myeloid leukemia), VLA-4-mediated signaling has been shown to support the survival of leukemia cells through the phosphatidylinositol-3-kinase (PI3K)/Akt/Bcl2 signaling pathway.[14-17] Furthermore, PI3K signaling has a central role in several decisive processes for cancer progression, including metabolism, growth, survival and motility.[18,19] Given the importance of the bone marrow environment for leukemia cell development and survival and considering the poor outcome of children with relapsed ALL, we hypothesized that high expression of VLA-4 in leukemia cells could be associated with an adverse outcome in relapsed childhood ALL. We, therefore, investigated the clinical and prognostic impact of *VLA-4* expression in bone marrow leukemia cells from 56 children with B-cell precursor (BCP) ALL at diagnosis of first relapse. Subsequently, gene expression changes related to *VLA-4* expression were investigated by microarray-based mRNA profiling, and the effect of VLA-4 signaling on leukemia cell survival and drug resistance was studied in co-cultures of leukemia cells and bone marrow stromal cells.

Design and Methods

Patients and samples

VLA-4 expression was determined retrospectively in bone marrow samples from 56 children and adolescents obtained at diagnosis of first relapse of BCP-ALL with bone marrow involvement. The study was confined to relapses of BCP-ALL because BCP-ALL represent the majority of childhood ALL (85%). Bone marrow aspirates were selected to contain more than 75% leukemia cells based on morphological evaluation of bone marrow smear preparations. All patients (n=56) were enrolled and 51 patients were treated according to the relapse trial ALL-REZ BFM 2002 protocol approved by the Institutional Review Board of the Charité-Universitätsmedizin Berlin. Patients were included, in accordance with the above mentioned criteria, from the start of 2002 until the end of 2003.[20] The total cohort of patients registered in the ALL-REZ BFM trial during the same time period was compared to the cohort of patients analyzed for *VLA-4* mRNA expression with respect to frequencies of clinical and therapeutic parameters to assess the representativeness of the group, and showed no selection bias (Table 1). Written informed consent was obtained from patients or guardians prior to treatment.

Methods and statistical analysis

Detailed information about the quantification of *VLA-4* mRNA by real-time polymerase chain reaction (QRT-PCR) analysis, VLA-4 protein assessment by FACS and immunocytochemistry (ICC), gene expression analysis, cell culture experiments (cell lines, cell culture, western blot analysis, proliferation and adhesion assays) and the statistical analysis are provided in the *Online Supplementary Design and Methods*.

Results

VLA-4 expression is associated with clinical features and outcome at first relapse

As a prerequisite for our mRNA-based study we analyzed the correlation between *VLA-4* mRNA expression and protein level. We, therefore, compared both expression levels in five BCP-ALL cell lines and in 11 patients' samples, by flow cytometry, ICC and QRT-PCR. The comparative protein-mRNA expression analysis showed that the relative *VLA-4* mRNA expression correlated well with protein level (R^2=0.76), allowing us to study the impact of VLA-4 expression in leukemia cells by QRT-PCR (*Online Supplementary Figure S1*).

VLA-4 expression was determined by QRT-PCR in 56 samples of bone marrow leukemia cells. Expression levels of *VLA-4* ranged from 1.0 to 148.1 in relation to the reference gene *ABL1*. We compared expression levels of *VLA-4* in relevant clinical and biological subgroups of ALL relapse (Table 1 and *Online Supplementary Figure S2*). *VLA-4* expression was significantly higher in leukemia cells from patients who were younger at the time of diagnosis of relapse (*P*=0.018) and from those with very early/early relapse (*P*=0.011). Accordingly, leukemia cells from patients stratified into the high-risk groups S3/S4 showed higher *VLA-4* expression compared to those in the intermediate risk group S2 (*P*=0.002, *Online Supplementary Figure S2C*). Furthermore, we compared the *VLA-4* expression levels between four different risk stratification subgroups (S2-, MRD low; S2+, MRD high; S3 and S4). The *VLA-4* expression levels differed significantly between these groups (Kruskal Wallis test: *P*=0.012, Figure 1G). Within the intermediate risk group S2, *VLA-4* expression was higher in the group with a high MRD load after induction therapy (Table 1). The median *VLA-4* expression in leukemia cells from patients in first relapse who did not respond to therapy (non-responders, median *VLA-4*

Table 1. Clinical and biological characteristics of the studied BCP-ALL patients and *VLA-4* mRNA expression in correlation with these parameters.

Parameter	Representativeness[1]					VLA-4 expression[2]		N. of patients with low VLA-4[3]		N. of patients with high VLA-4[3]		
	Total n. of patients		Study cohort									
	N	%	N	%	*P*	Median (Min/Max)	*P*	N	%	N	%	*P*
Total	100	100	56	100		8.3 (1.0 / 148.1)		25	100	26	100	
Gender					0.606		0.959					
male	61	61	37	66		8.6 (1.0 / 148.1)		17	68	17	65	1.0
female	39	39	19	34		7.4 (1.9 / 100.4)		8	32	9	36	
Age at relapse					0.900		0.018					0.027
< 5 years	19	19	9	16		20.3 (4.6 / 109.9)		1	4	7	27	
≥ 5 and < 10 years	45	45	26	46		9.0 (1.0 / 33.1)		11	44	13	50	
≥ 10 years	36	36	21	38		6.3 (2.5 / 148.1)		13	52	6	23	
Time point					0.294		0.011					0.096
very early	21	21	18	32		22.7 (2.8 / 148.1)		5	20	11	42	
early	27	27	12	21		11.1 (2.5 / 33.1)		5	20	7	27	
late	52	52	26	46		6.0 (1.0 / 100.4)		15	60	8	31	
Site					0.411		0.441					0.291
BM isolated	78	78	47	84		9.5 (1.0 / 109.9)		19	76	23	89	
BM combined	22	22	9	16		6.2 (2.9 / 148.1)		6	24	3	11	
Risk group					1.0		0.002					0.025
high risk (S3/S4)	38	38	27	48		13.7 (2.5 / 148.1)		8	32	17	65	
intermediate risk (S2)	62	62	29	52		5.7 (1.0 / 100.4)		17	68	9	35	
- MRD status (S2 only)					0.102		0.294					0.017
MRD low	20	39	16	59		4.1 (1.1 / 28.8)		12	75	2	36	
MRD high	31	61	11	41		9.5 (1.0 / 100.4)		4	25	7	64	
no data	11	(18)	2	(7)				1	(6)	-	-	
Peripheral blood count					0.207		0.112					0.423
< 1/μL	14	16	3	6		4.4 (3.4 / 8.6)		2	8	1	5	
≥ 1 and < 10000/μL	63	70	38	75		6.9 (1.1 / 100.4)		19	79	15	68	
≥ 10000/μL	13	14	10	20		18.8 (1.0 / 148.1)		3	13	6	27	
no data	10	(10)	5	(9)		13.7 (2.5 / 24.3)		1	(4)	4	(15)	
Immunophenotype					0.863		0.025					0.122
pro-B ALL	10	11	7	13		19.1 (7.7 / 48.5)		1	4	5	20	
common ALL	55	58	31	56		5.7 (1.1 / 100.4)		17	68	11	44	
pre-B ALL	29	31	17	31		9.5 (1.0 / 109.9)		7	28	9	36	
bilineage	1	1	-	-		-		-	-	-	-	
no data	5	(5)	1	(2)		148		-	-	1	(4)	
BCR/ABL					1.0		-					-
negative	85	98	55	98		8.6 (1.0 / 148.1)		24	96	26	100	
positive	2	2	1	2		3.7		1	4	-	-	
no data	13	(13)	-	-		-		-	-	-	-	
TEL/AML1					0.807		0.070					0.099
negative	75	85	49	87		9.5 (1.0 / 148.1)		20	80	25	96	
positive	13	15	7	13		5.7 (1.1 / 12.1)		5	20	1	4	
no data	12	(12)	-	-		-		-	-	-	-	
MLL/AF4					1.0		0.005					0.235
negative	82	92	52	93		7.5 (1.0 / 109.9)		25	100	23	89	
positive	7	8	4	7		43.4 (19.2 / 148.1)		-	-	3	11	
no data	11	(11)	-	-		-		-	-	-	-	
Events					0.637		0.014					0.052
continuous complete remission	28	32	12	23		3.7 (1.1 / 20.3)		10	40	2	8	
subsequent relapse	37	43	26	51		9.0 (1.0 / 100.4)		12	48	14	54	
non-response	11	13	8	16		29.0 (1.3 / 109.9)		3	12	5	19	
death in CR[4]	8	9	2	4		21.3 (9.5 / 33.1)		-	-	2	8	
induction death[5]	2	2	2	4		98.3 (48.5 / 148.1)		-	-	2	8	
secondary malignancy	1	1	1	2		28.8		-	-	1	4	
non-protocol	13	-	5	-		-		-	-	-	-	
Frontline protocol					0.851		0.181					0.297
ALL-BFM 86/90/95I	32	32	18	32		4.5 (1.0 / 100.4)		11	44	5	19	
ALL-BFM 2000I	40	40	23	41		9.5 (2.1 / 109.9)		10	40	11	42	
COALL	25	25	14	25		12.1 (1.3 / 148.1)		4	16	9	35	
others	3	3	1	2		24.3		-	-	1	4	

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Relapse protocol					0.434			0.007					0.350
ALL-REZ BFM 02 Pilot	73	73	46	82		7.4	(1.0 / 100.4)		24	96	22	85	
ALL-REZ BFM 2002	14	14	5	9		51.6	(8.0 / 148.1)		1	4	4	15	
non-protocol	13	13	5	9			-		-	-	-	-	
Stem cell transplantation					0.967			0.338					0.996
none	57	59	34	63		8.3	(1.1 / 148.1)		15	60	15	65	
matched sibling donor	11	12	5	9		4.6	(1.7 / 13.7)		3	12	3	8	
haploidentical donor	4	4	2	4		3.4	(2.5 / 4.3)		1	4	1	-	
unrelated donor	24	25	13	24		10.6	(1.0 / 42.2)		6	24	7	27	
no data	4	(4)	2	(4)			32.0		-	-	-	-	

[1]Frequencies of clinical, diagnostic, therapeutic parameters and events of the VLA-4 study patient cohort compared with the total ALL-REZ BFM 2002 group of patients. [2]VLA-4 mRNA expression in correlation to clinical, diagnostic, and therapeutic parameters of the total VLA-4 study cohort (n=56). [3]VLA-4 mRNA expression in correlation to clinical, diagnostic, and therapeutic parameters of the two groups with VLA-4 expression below and equal to/above the median (Only patients who were treated according to ALL-REZ BFM were included; n=51). [4]Both patients of the VLA-4 study cohort showed poor response to therapy, measured by MRD and morphological analysis (see Online Supplementary Table S1) [5]One of two patients of the VLA-4 study cohort showed poor response to therapy as measured by morphological analysis (see Online Supplementary Table S1). BCP, B-cell precursor; ALL, acute lymphoblastic leukemia; BM: bone marrow; CR: complete remission. Time point of relapse: very early (within 18 months after first diagnosis of ALL); early (18 months after first diagnosis of ALL until 6 months after cessation of frontline therapy); or late (more than 6 months after cessation of frontline therapy). Site of relapse: isolated BM (more than 25% leukemia cells in the BM, no evidence of extramedullary disease); combined BM (more than 5% leukemia cells in the BM combined with CNS, testis or other extramedullary disease). High-risk: early (isolated) or very early (isolated or combined BM) relapse of a BCP-ALL. Intermediate risk: late (isolated or combined BM), or early combined relapse of a BCP-ALL; pEFS after chemotherapy was estimated at 35-40% for this group. MRD: low level, less than 10^{-3} leukemia cells in BM aspiration after the second induction course (F2, week 5); MRD high level: equal or more than 10^{-3} leukemia cells. Probability of EFS can be estimated at above 60% for MRD low and at below 30% for MRD high intermediate-risk patients.

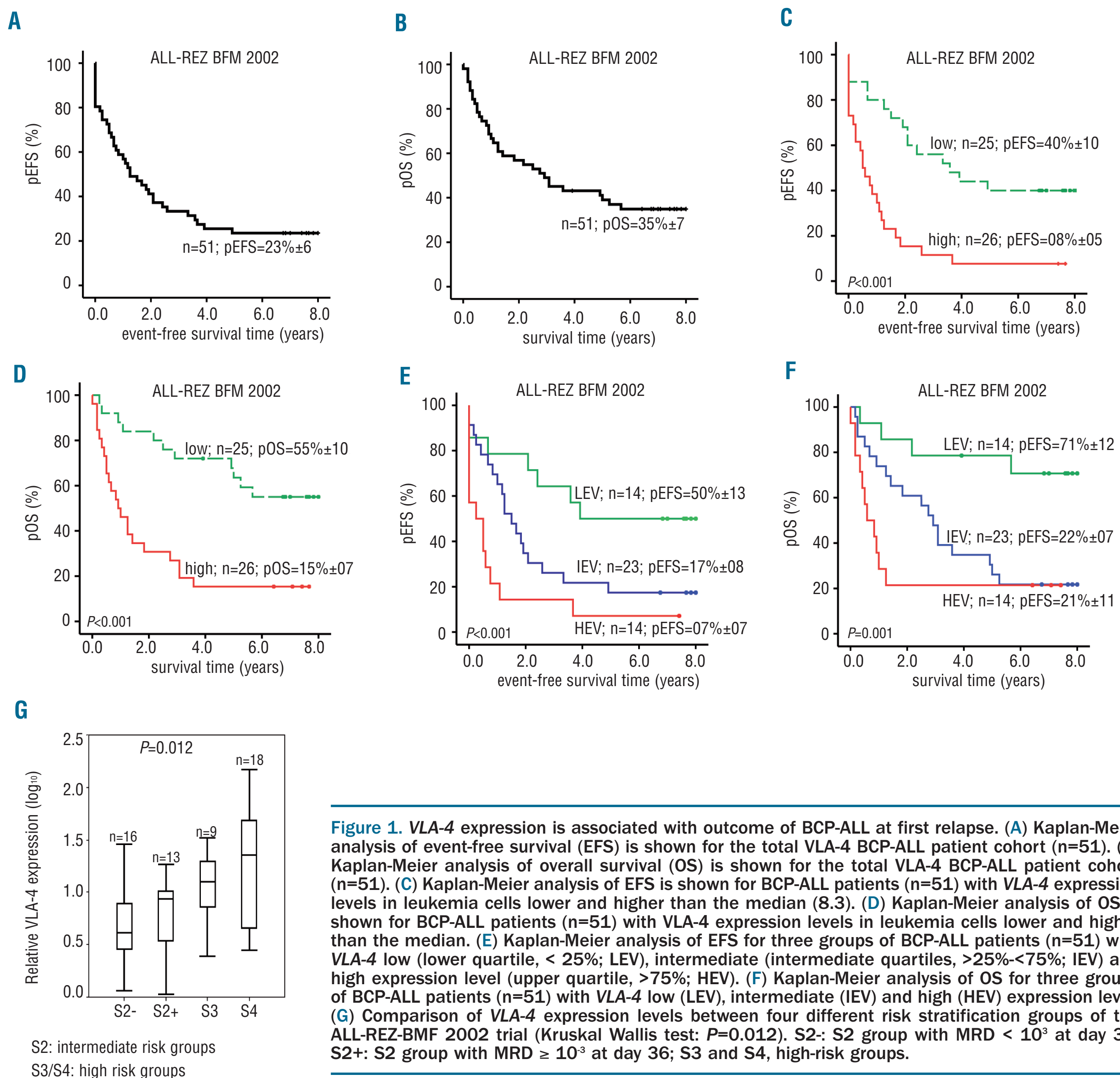

Figure 1. ***VLA-4*** **expression is associated with outcome of BCP-ALL at first relapse. (A) Kaplan-Meier analysis of event-free survival (EFS) is shown for the total VLA-4 BCP-ALL patient cohort (n=51). (B) Kaplan-Meier analysis of overall survival (OS) is shown for the total VLA-4 BCP-ALL patient cohort (n=51). (C) Kaplan-Meier analysis of EFS is shown for BCP-ALL patients (n=51) with *VLA-4* expression levels in leukemia cells lower and higher than the median (8.3). (D) Kaplan-Meier analysis of OS is shown for BCP-ALL patients (n=51) with VLA-4 expression levels in leukemia cells lower and higher than the median. (E) Kaplan-Meier analysis of EFS for three groups of BCP-ALL patients (n=51) with *VLA-4* low (lower quartile, < 25%; LEV), intermediate (intermediate quartiles, >25%-<75%; IEV) and high expression level (upper quartile, >75%; HEV). (F) Kaplan-Meier analysis of OS for three groups of BCP-ALL patients (n=51) with *VLA-4* low (LEV), intermediate (IEV) and high (HEV) expression level. (G) Comparison of *VLA-4* expression levels between four different risk stratification groups of the ALL-REZ-BMF 2002 trial (Kruskal Wallis test: *P*=0.012). S2-: S2 group with MRD < 10^{3} at day 36; S2+: S2 group with MRD ≥ 10^{-3} at day 36; S3 and S4, high-risk groups.**

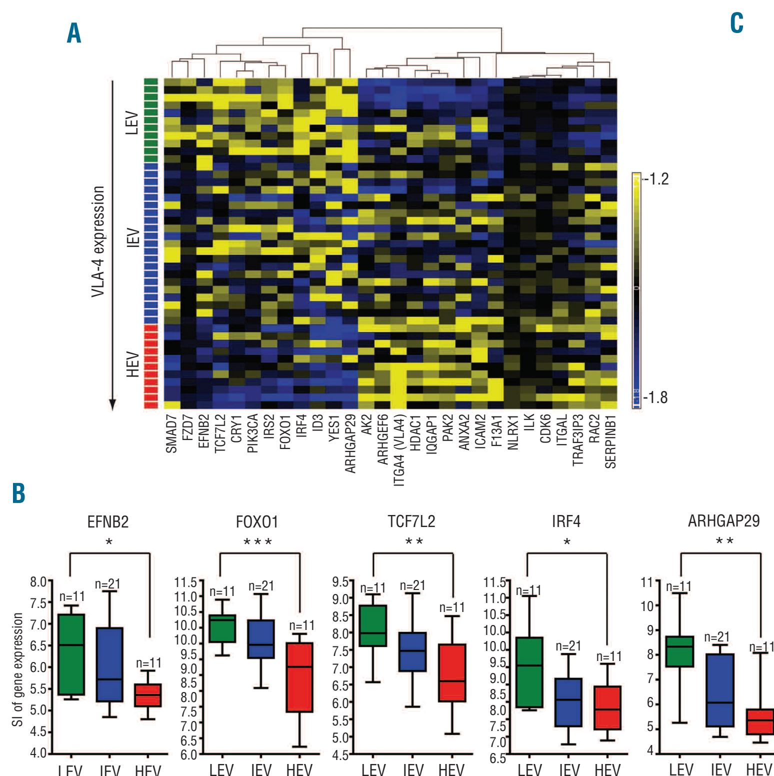

Figure 2. ***VLA-4*** **expression is associated with distinctive gene expression changes. (A) Expression levels of 27 genes identified by comparison of relapsed BCP-ALL patients with low (lower quartile, < 25%; LEV, green), intermediate (intermediate quartiles, >25%-<75%; IEV blue), and high (upper quartile, >75%; HEV, red) expression of *VLA-4*. Expression levels are shown as differences with respect to the mean over all patients (n=43). Patients were ordered according to *VLA-4* expression levels. Genes are clustered according to down- and up-regulation in HEV- *versus* LEV-groups. (B) Box plots of down-regulated genes in HEV- *versus* LEV-groups obtained by chip analysis in the three groups (LEV: green; IEV: blue; HEV: red). Signal intensities (SI) of expression of five representative genes: *EFNB2*, *FOXO1*, *TCF7L2*, *IRF4*, and *ARHGAP29* are shown. (C) Box plots of up-regulated genes in the HEV- *versus* LEV-groups, obtained by chip analysis in the three groups (LEV: green; IEV: blue; HEV: red). Signal intensities (SI) of expression of two representative genes are shown: *ICAM2* and *F13A1*. Statistical analysis was done by ANOVA (*P<0.05; **P<0.001; ***P<0.0001).**

expression=29) and who suffered a subsequent relapse (median=9.0) was significantly higher than that in leukemia cells from the patients remaining in continuous complete remission (median=3.7) (Table 1). In particular, of the four patients with high *VLA-4* levels who died during induction therapy or of a therapy-related cause (Table 1), three had a poor response to therapy (*Online Supplementary Table S1*), and died of sepsis. High *VLA-4* expression was also associated with a more immature pro-B-cell immunophenotype (P=0.025). *VLA-4* expression was higher in leukemia cells from four patients with *MLL/AF4* fusion genes. There was no correlation between the presence of *TEL/AML1* fusion genes and *VLA-4* expression.

To study the impact of *VLA-4* expression on treatment outcome, we divided the patients into groups with high and low *VLA-4* expression, defined as those with an expression level above or below the median *VLA-4* expression (median=8.3), respectively (Table 1). The probabilities of event-free survival (23% ± 6) and overall survival (35%±7) of the total study cohort are shown in Figure 1A-B. Kaplan-Meier estimates revealed that patients with *VLA-4* levels higher than median in leukemia cells had a significantly lower probability of event-free survival (8%±5 *versus* 40%±10; P<0.001; Figure 1C) and overall survival (15%±7 *versus* 55%±10; P<0.001; Figure 1D) than those with *VLA-4* levels lower than the median at a follow-up of 8 years. To exclude biases related to genetic alterations in leukemia cells, separate Kaplan-Meier analyses were conducted excluding *MLL/AF4*$^+$ (n=3, high *VLA-4* expression) and *TEL/AML1*$^+$ patients (n=6). As expected, the exclusion of either group did not diminish the prognostic significance of *VLA-4* expression (*MLL/AF4*$^-$: event-free survival: 08%±05 *versus* 43.5%±.10; overall survival: 20 ±08 *versus* 55%±10; *Online Supplementary Figure S3A-B*; *TEL/AML1*$^-$: event-free survival: 08% ± 06 *versus* 33%±.10; overall survival: 16%±08 *versus* 46%±11; *Online Supplementary Figure S3C-D*).

To assess whether a gradual division by *VLA-4* expression would further strengthen discrimination of prognostic groups, we split the patients' samples into three groups according to whether they had low expression of *VLA-4* (LEV; lower quartile, <25%), intermediate expression (IEV; interquartile range, >25%-<75%) or high expression (HEV; upper quartile, >75%). Patients with LEV had a significantly better probability of event-free survival (Figure 2E) and overall survival (Figure 2F) than those with IEV and HEV at a follow-up of 8 years (event-free survival: P<0.01; overall survival: P=0.001), indicating a clear correlation between *VLA-4* expression levels and outcome in these groups. In addition, the median event-free survival was significantly (P=0.002) shorter in the HEV-group (0.37 years) than in the IEV (1.5 years) and LEV groups (5.3 years). Thus, through analysis of *VLA-4* expression we identified prognostic groups with high/low event-free and overall survival probabilities and early/late subsequent events. The multivariate Cox regression analysis (Table 2), including *VLA-4* expression, time point, age, immunophenotype and *MLL/AF4* fusion gene, revealed an independent prognostic significance of *VLA-4* expression (hazard ratio, HR=3.43, P=0.001) next to time point of relapse (HR= 3.81, P=0.006). For patients stratified into the intermediate risk group S2, MRD was additionally included in the multivariate Cox regression model but only *VLA-4* expression was found to be of independent prognostic significance (P=0.014) since this group comprises only

patients with time point early/late and MRD was already used for risk assessment (Table 2).

VLA-4 *expression is associated with distinctive gene expression changes*

To identify pathways associated with *VLA-4* expression at ALL relapse, we studied gene expression profiling data available for a subset of 43 patients with relapsed ALL from the total cohort of 56 patients. The event-free and overall survival probabilities of these patients are shown in *Online Supplementary Figure S4A-B*. Gene expression analysis confirmed our *VLA-4* level results obtained with QRT-PCR in the defined groups (IEV, LEV, HEV; P<0.001, Figure 2A). We identified 414 genes differentially expressed in correlation with *VLA-4* expression between the three groups with a P-value of 0.01 and a minimal relative fold change of 1.3 between the HEV and the LEV groups (*Online Supplementary Table S3*: 142 down-regulated genes; *Online Supplementary Table S4*: 272 up-regulated genes). According to the fold change and P-value, we selected 27 genes related to known pathways (Figure 2A). The differential expression, fold change and statistical analysis are summarized in Table 3. When comparing the most extreme groups, HEV *versus* LEV, 12 of the genes were down-regulated by up to 6-fold (e.g. *EFNB2*, *FOXO1*, *TCF7L2*, *IRF4*, *ARHGAP29*; Figure 2B) and 16 were up-regulated up to 2.8-fold (e.g. *ICAM2*, *F13A1*, *PIK3CA*; Figure 2C). Most of these genes are involved in PI3K/Akt, Pten, Ephrin (*PIK3CA*, *ILK*, *FOXO1*, *ICAM2*, *RAC2*, *IRS-2*, *ITGAL*, *PAK2*, *F13A1*, *EFNB2*), Wnt/beta-catenin (*TC7FL2*, *HDAC1*), and Rho GTPase signaling pathways (*Rac2*, *ARHGAP29*, *ARHGEF6*).

Blocking of VLA-4 signaling overcomes the supportive effect of stromal cells

Having shown that *VLA-4* expression is associated with prognosis and distinctive gene expression changes, we next evaluated the effect of VLA-4 signaling on BCP-ALL cell adhesion, survival and drug resistance using the BCP-ALL cell line REH which expresses high amounts of VLA-4 (*Online Supplementary Figure S1A,B*) as a model system. In co-culture with stromal cells (L87/4) the adhesion of REH cells was reduced after treatment with VLA-4 blocking antibodies (Figure 3A). This effect was not observed in the absence of stromal cells. In addition, the stromal cell-induced proliferation of REH cells was less pronounced after treatment with VLA-4 blocking antibodies (Figure 3B). To assess whether blocking of VLA-4 signaling overcomes the protection of leukemia cells from cytarabine-induced cytotoxicity by stromal cells,[21] we treated REH cells in mono-culture and in co-culture with cytarabine (Ara-C) in the presence or absence of anti-VLA-4 antibodies. Blocking of VLA-4 in leukemia cells significantly abolished the cytoprotective effect of stromal cells (Figure 3B). Consistent with these findings, expression of the anti-apoptotic protein BCL-2, was also decreased in leukemia cells (Figure 3C), revealing that VLA-4 signaling has the ability to regulate the sensitivity of leukemia cells to chemotherapy.

Table 2. Univariate and multivariate analysis for survival after relapse.

	N. of patients	pEFS	HR	CI 95% lower	CI 95% upper	*P* value
Univariate analysis (all patients)						
VLA-4 median						<0.001
Low	25	0.40±0.10	1.00			
High	26	0.08±0.05	3.32	1.70	6.51	
Time point						0.004
Late	23	0.35±0.10	1.00			
Early	12	0.17±0.11	1.79	0.80	4.04	
Very early	16	0.13±0.08	3.79	1.77	8.12	
Immunophenotype						0.160
Pro-B ALL	6	0.00±0.00	1.00			
Common ALL	28	0.32±0.09	0.45	0.18	1.12	
Pre-B ALL	16	0.19±0.10	0.72	0.27	1.90	
(no data)	1					
MLL/AF4						0.005
Negative	48	0.25±0.06	1.00			
Positive	3	0.00±0.00	6.86	1.77	26.52	
Age at relapse						
< 5 years	8	0.13±0.12	1.00			0.202
≥ 5 and < 10 years	24	0.25±0.09	0.46	0.19	1.12	
≥ 10 years	19	0.26±0.10	0.42	0.17	1.05	
Multivariate analysis (all patients)						
VLA-4 median						0.001
Low	25		1.00			
High	26		3.43	1.68	7.02	
Time point						0.006
Late	23		1.00			
Early	12		1.35	0.59	3.08	
Very early	16		3.81	1.71	8.50	
Multivariate analysis (S2 patients only)						
VLA-4 median						0.014
Low	16		1.00			
High	9		4.04	1.35	12.11	

Univariate analysis of all clinical parameters significantly associated with VLA-4 expression. Model of multivariate analysis of all patients included parameters of univariate analyses. Multivariate analysis of S2 patients only additionally included MRD in the model. Best models are shown. HR: hazard ratios.

Discussion

In this study, we assessed the clinical and prognostic relevance of *VLA-4* expression in bone marrow-derived leukemia cells of pediatric patients with first relapse of BCP-ALL. Patients with higher *VLA-4* levels in their leukemia cells had significantly worse event-free and overall survival probabilities than those with lower expression. Accordingly, high levels of *VLA-4* expression were associated with clinical and biological high-risk parameters, i.e. early time point and high-risk stratification. In multivariate Cox regression analysis *VLA-4* expression was a significant independent prognostic determinant of event-free survival. ALL patients at first relapse with *VLA-4* levels higher than the median rarely remained in continuous complete remission (8%) compared to those with *VLA-4* levels lower than median (40%; Table 1). However, there was no significant difference in subsequent relapse rates between these groups (54% *versus* 48%), perhaps because of the presence of different minor subclones at first relapse that could not be analyzed for *VLA-4* expression. Addressing whether *VLA-4* expression levels vary between first to second relapse, we analyzed ten matched leukemia cell samples. Five patients showed similar *VLA-4* expression in second relapse, three had higher and two had lower *VLA-4* expression levels (*Online Supplementary Table S2*). Although in these two cases, *VLA-4* expression

Table 3. Differential expression, fold change and pathway analysis of 27 identified genes.

Gene symbol	Probe set ID	*P* value over all three groups	*P* value (HEV *vs.* LEV)	Fold-Change (HEV vs. LEV)	Pathway
ARHGAP29	203910_at	0.00006	0.00002	-6.0	Rho GTPase protein signal transduction
YES1	202932_at	0.002	0.0006	-3.8	Adherens / tight junction
ID3	207826_s_at	0.008	0.003	-3.2	TGF-β
FOXO1	202723_s_at	0.0003	0.0001	-3.1	Integrin, PI3K/ Akt
EFNB2	202668_at	0.03	0.009	-2.9	Integrin, MAP/ERK, ephrin
SMAD7	204790_at	0.009	0.003	-2.5	TGFβ
TCF7L2	212762_s_at	0.006	0.001	-2.5	Wnt / β-catenin
IRF4	204562_at	0.007	0.005	-2.2	Regulation of transcription
IRS2	209184_s_at	0.004	0.003	-2.1	Insulin signaling
CRY1	209674_at	0.01	0.004	-2.0	Circadian clock in mammals
PIK3CA	204369_at	0.02	0.006	-1.9	Integrin, PI3K/ Akt, Pten
FZD7	203706_s_at	0.01	0.008	-1.8	Wnt / β-catenin
ILK	201234_at	0.003	0.003	1.3	Integrin, PI3K/ Akt, Pten
NLRX1	219680_at	0.0001	0.00004	1.3	NFκ
ITGAL	213475_s_at	0.01	0.008	1.4	Integrin, PI3K/ Akt
CDK6	207143_at	0.005	0.001	1.4	P53 / cell cycle
PAK2	208877_at	0.008	0.002	2.1	MAPK / ERbB, Rho GTPase
HDAC1	201209_at	0.02	0.006	1.7	Wnt / β-catenin, TGFβ
RAC2	207419_s_at	0.003	0.009	1.8	PI3K/ Akt, Rho GTPase
IQGAP1	200791_s_at	0.002	0.0004	2.3	Adherens junction
ANXA2	213503_x_at	0.01	0.004	2.1	Angiogenesis / fibrinolysis
ARHGEF6	209539_at	0.004	0.001	2.2	Actin cytoskeleton / interferon
SERPINB1	213572_s_at	0.006	0.002	2.2	Serine protease inhibitor
AK2	212174_at	0.006	0.002	2.4	Metabolic
TRAF3IP3	213888_s_at	0.0002	0.00005	2.3	JNK
F13A1	203305_at	0.006	0.002	2.6	PI3K/ Akt
ICAM2	213620_s_at	0.001	0.0006	2.7	Integrin, PI3K/ Akt

Twenty-seven genes differentially expressed between the high expression VLA-4 *group (HEV), intermediate expression* VLA-4 *group (IEV), and low expression* VLA-4 *group (LEV) are shown.* P *value: two-way ANOVA statistical analysis, probeset ID: Affymetrix GeneChip® U133A.*

was lower at second relapse, the relative *VLA-4* expression was still high (HEV/IEV group). Nevertheless, the median *VLA-4* expression in leukemia cells from patients who suffered a subsequent relapse or who did not respond to therapy was higher than the median in patients in continuous complete remission, suggesting an involvement of VLA-4 in the persistence of leukemia cells during relapse therapy (Table 1). The detailed analysis of outcome revealed that the significant difference between the VLA-4 groups was largely accounted for by the disparity in response to therapy, next to subsequent relapse. In particular, also the patients experiencing death during induction therapy (2/2) or in continuous complete remission (1/2) showed a poor response to therapy (*Online Supplementary Table S1*), indicating that response to therapy is the main factor responsible for the divergence of these groups. In line with this, patients with higher MRD load showed higher *VLA-4* levels than those with low MRD burden.

In a few previous studies of adult and pediatric ALL, VLA-4 expression was associated with different pathophysiological and clinical features, such as peripheral blood cell count,[14,22-25] but significant correlations between VLA-4 expression levels and outcome could not be clearly established,[14,22-24] probably due to the consideration of heterogeneous, non-uniformly treated groups and small numbers of patients. In acute myeloid leukemia, more systematic studies have been published, with contradictory results, some finding that high VLA-4 expression was associated with adverse outcome[16,26] while others found that it was associated with improved outcome.[27,28] In our study of uniformly treated patients at first relapse, we observed that high VLA-4 expression was associated with an adverse outcome as well as with high-risk features, i.e. age,[29] immunophenotype and time of relapse.[4]

To identify molecular determinants underlying the prognostic relevance of *VLA-4* expression at ALL relapse, we studied gene expression profiles of leukemia cells from 43 ALL patients in our study cohort. Different *VLA-4* expression levels were characterized by distinctive gene expression changes. Most of the genes differentially expressed are involved in the PI3K/Akt, ephrin, Wnt and Rho GTPase signaling pathways. FOXO1, a member of the family of Forkhead transcription factors, is primarily regulated by the PI3K/Akt pathway and its inactivation/activation plays a crucial role in B-cell proliferation and tumor suppression by inducing apoptosis.[30-33] Down-regulation of

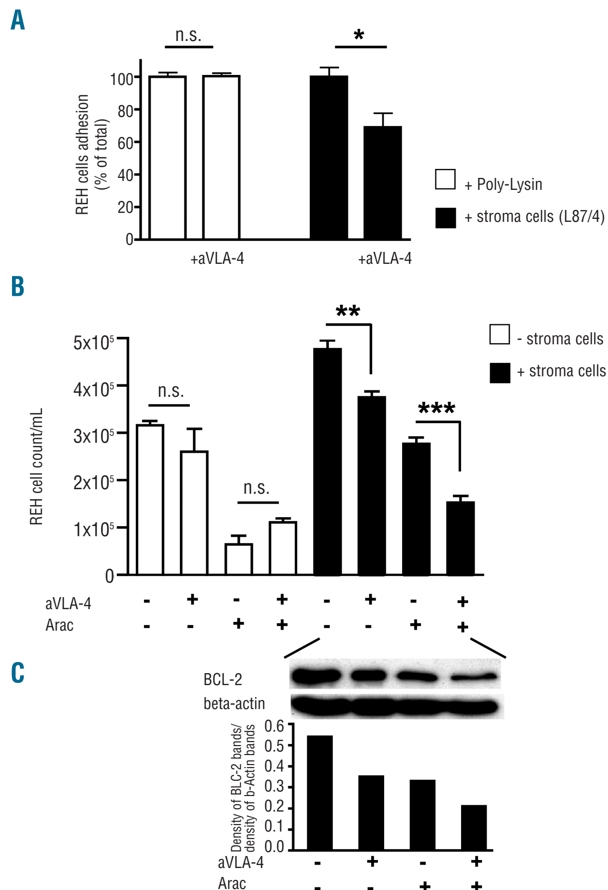

Figure 3. Blocking of VLA-4 signaling overcomes the supportive effect of stromal cells. (A) For cell adhesion assay, the BCP-ALL cells (REH) were fluorescence-labeled and seeded into 24-well plates coated with poly-lysine or the stromal cell line L87/4 in the absence or presence of the blocking anti-VLA-4 monoclonal antibody. The percentage of adherent cells measured by the fluorescence plate reader is shown relative to control samples without blocking antibodies. (B) REH cells were cultured either alone or in co-culture with L87/4 stromal cells with or without blocking anti VLA-4 monoclonal antibody and cytarabine (ARA-C, 1 μM). After 48 h, proliferation was measured by the MTS tetrazolium assay and cell numbers per microliter were calculated from appropriate cell dilution series. Columns, mean of at least three independent experiments; bars, SD (*P*<0.01; ****P*<0.001). (C) Western-blot analysis of the anti-apoptotic protein BCL-2. REH cells were grown in co-culture with L87/4 stromal cells and were treated with or without blocking anti VLA-4 monoclonal antibody and/or ARA-C for 48 h. β-actin served as the control for equal protein loading. The density of dots was measured. The ratios of BCL-2 density to β-actin density are shown.**

FOXO1 and TCF7L2 has been shown to correlate with metastasis and poor prognosis in renal cell carcinoma,[34] as observed in the HEV-group. Furthermore, the low expression level of *EFNB2* in the HEV-group is consistent with a recently published study showing that epigenetic silencing of *EPH/EPHRIN* family genes contributes to ALL pathogenesis.[35] Rho GTPases (Rac2, Cdc42),[36,37] ARHGEF6[38] and PAK2 (p21-activated kinases) transduce signals leading to migration, adhesion, and survival of many blood cells and are often over-expressed in multiple cancer types.[39] *Rac* genes have been shown to regulate leukemogenesis,[40,41] and B-cell development.[37] In line with these findings Rac2, PAK2 and ARHGEF6 were up-regulated in the HEV-group with worse event-free and overall survival probabilities. It has been recently shown that VLA-4 mediates survival in chronic lymphocytic leukemia through two different pathways: by binding to Vcam-1 and subsequent PI3K/Akt pathway activation or by interacting with MMP9/CD44 leading to STAT3 phosphorylation and MCL-1 expression.[42] We did not observe any differential expression of CD44 and MCL-1 in our data set (*data not shown*), suggesting that VLA-4 signaling probably contributes via the PI3K/Akt pathway to leukemia cell survival in relapsed BCP-ALL.

Many studies have revealed that VLA-4 regulates adhesion, migration and survival of leukocytes and hematopoietic stem cells.[12] Furthermore, blocking VLA-4 signaling increases chemotherapy-induced apoptosis in acute myeloid leukemia[16] and decreases the ability of leukemia cells to engraft in bone marrow in a xenograft mouse model.[43] Consistent with these findings, our co-culture experiments showed that inhibition of VLA-4 signaling in BCP-ALL overcomes the cytoprotective effect of stromal cells. Thus, targeting receptors/pathways involved in the VLA-4/PI3K pathway may be a promising therapeutic option in relapsed ALL, like the application of clinically approved antibodies against VLA-4[44,45] or PI3K inhibitors.[19]

In conclusion, our study reveals that high *VLA-4* expression is an indicator of poor prognosis and a potential therapeutic target in relapsed childhood BCP-ALL. Furthermore, our results show that cellular and biological effects related to VLA-4 signaling play a decisive role in survival and/or response to therapy of childhood BCP-leukemia cells.

Authorship and Disclosures

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