

VLA-4 and CXCR4 expression levels show contrasting prognostic impact (favorable and unfavorable, respectively) in acute myeloid leukemia

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Abstract Very late antigen-4 (VLA-4) and CXC chemokine receptor 4 (CXCR4) perform critical roles in the adhesion of hematopoietic and leukemic stem cells to marrow stromal cells. This mechanism is associated with chemoresistance in patients with acute myeloid leukemia (AML). Here, we measured VLA-4 and CXCR4 expressions in leukemic myeloblasts to determine their prognostic implications. Using multicolor flow cytometry, positive VLA-4 and CXCR4 expressions were measured in leukemic myeloblasts in bone marrow aspirates that were obtained from newly diagnosed adult AML patients ($n=98$). VLA-4 expression was higher in patients at favorable or intermediate cytogenetic risk than in patients at poor risk ($p<0.001$ and $p=0.002$, respectively), but CXCR4 expression was not significantly different. Among the 72 non-

promyelocytic leukemia patients analyzed who received cytarabine + anthracycline-based induction chemotherapy, high VLA-4 expression was independently associated with a high probability of complete remission ($p=0.019$) and superior relapse-free survival (RFS) ($p<0.001$). However, high CXCR4 expression independently increased the probability of relapse ($p=0.002$) and was associated with a shorter RFS ($p=0.006$). When categorizing patients into three groups according to VLA-4 and CXCR4 expression levels, the group of high VLA-4 and low CXCR4 showed longer RFS ($p=0.001$) and overall survival (OS) ($p=0.011$) than the group of low VLA-4 or high CXCR4.

Keywords Acute myeloid leukemia · Adhesion molecule · VLA-4 · CXCR4

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Introduction

The adhesive and migratory properties of leukemic myeloblasts affect the survival, proliferation, and retention in the bone marrow (BM) and influence chemosensitivity [1]. Adhesion molecules, particularly very late antigen-4 (VLA-4) integrin and CXC chemokine receptor 4 (CXCR4), are generally expressed on acute myeloid leukemia (AML) blasts at varying levels and play key roles in directional migration and adhesion to stromal cells [2]. VLA-4 integrins interact with BM ligands, vascular cell adhesion molecule-1 (VCAM-1), and fibronectin. CXCR4 interacts with stromal cell-derived factor-1 (SDF-1; CXCL12), which is constitutively secreted by BM stromal cells. Because VLA-4 may mediate antiapoptotic signals [1] and CXCR4 may induce chemoresistance by regulating the cell cycle [3], therapeutic

agents that target VLA-4 and CXCR4 are currently under investigation [4, 5].

Several previous studies reported that low CXCR4 expression improved prognosis in AML patients [6–8], but it remains controversial whether VLA-4 is a marker of clinical outcomes. An initial study that included 25 non-promyelocytic AML patients and a mouse model has suggested that high VLA-4 expression was associated with poor chemosensitivity and short survival because of interactions between VLA-4 and fibronectin on stromal cells [1]. A follow-up study on 36 AML patients supported this finding [7]. However, a recent retrospective study of 175 cryopreserved AML specimens concluded that high functional VLA-4 activity was correlated with superior overall survival (OS) [9]. A subsequent prospective study on 216 pediatric AML patients also indicated that high VLA-4 expression was an independent marker of favorable prognosis [10]. However, there have been no earlier reports from prospective studies that evaluated VLA-4 expression as a prognostic marker in a large number of adult AML patients. Hence, in our current study, we have analyzed VLA-4 and CXCR4 expressions on leukemic blasts in 98 adult AML patients at the time of initial diagnosis and analyzed their prognostic impact in 72 patients.

Materials and methods

Patients

A total of 98 consecutive adult patients newly diagnosed with AML at Asan Medical Center (Seoul, Korea) between July 4, 2011, and May 25, 2012, were included in this study. AML diagnosis was defined according to the 2008 World Health Organization criteria. Our study was approved by the institutional review board of the Asan Medical Center, and all participants gave written informed consent (2013-0986).

All of the patients were tested with conventional karyotyping and molecular studies using HemaVision kit (DNA Diagnostic, Risskov, Denmark) for detecting 28 recurrent fusion transcripts. Patient characteristics are provided in Table 1.

Patients' risk status was classified as favorable, intermediate, or poor according to the 2011 National Comprehensive Cancer Network guidelines. All patients were tested for internal tandem duplication of FMS-like receptor tyrosine kinase 3 (*FLT3*-ITD). Patients with normal karyotype were evaluated for *NPM1* and *CEBPA* mutations, and patients with t(8;21) or inv(16) or t(16;16) aberrations were tested for *KIT* mutations. (Supplementary Table 1).

Of the 98 AML patients, we performed baseline study and analyzed clinical outcomes with 72 non-promyelocytic AML patients who received cytarabine + anthracycline-based induction therapy (Table 2).

Table 1 Clinical characteristics and risk status of patients with acute myeloid leukemia according to the 2008 WHO classification

Characteristics	Value
No. of patients	98
Sex	
Male	52
Female	46
Median age (range) (years)	54 (17–90)
Mean follow-up (range) (months)	23 (16–27)
Median WBC count (range) ($10^9/L$)	8 (0.3–487.5)
WHO classification	
AML with t(8;21)(q22;q22)	10
AML with inv(16)(p13.1q22) or t(16;16)	3
AML with t(15;17)(q22;q12)	12
AML with t(9;11)(p22;q23)	3
AML with <i>MLL</i> gene rearrangement	4
AML with t(6;9)(p23;q34)	1
AML with t(3;3)(q21;q26.2)	1
AML with myelodysplasia-related changes	29
AML, not otherwise specified	35

Flow cytometry

BM aspirates were obtained from 98 AML patients at the time of diagnosis. VLA-4 and CXCR4 expressions were assessed

Table 2 Treatment responses of 72 non-promyelocytic acute myeloid leukemia patients who received cytarabine + anthracycline-based induction therapy

Characteristics	No. of patient
Risk status	
Favorable	21
Intermediate	27
Poor	24
Induction regimen	
Cytarabine + daunorubicin	45
Cytarabine + idarubicin	27
Responses to induction chemotherapy	
CR after initial induction	39
CR after reinduction	6
Hypocellular marrow	4
Persistence	21
Death	2
Hematopoietic stem cell transplantation	
Allogeneic HSCT	24
Autologous HSCT	3
Total relapse	23
Total death	42

CR complete remission, HSCT hematopoietic stem cell transplantation

within 24 h after sample collection. Samples were labeled with specific antibodies, and erythrocytes were lysed using lysing solution. Peridinin-chlorophyll protein (PerCP)-conjugated anti-CD45 antibody, fluorescein isothiocyanate-conjugated anti-CD49d (VLA-4) antibody, and phycoerythrin-conjugated anti-CD184 (CXCR4) antibody were also simultaneously used to label samples. All antibodies (except CD45-PerCP [BD Bioscience, San Jose, CA, USA]) were obtained from Beckman Coulter, Brea, CA, USA. Isotypic controls were used as the negative control in separate tubes. Three-color flow cytometry was performed using the FACSCanto II flow cytometry system (BD Bioscience) and analyzed using FACSDiva software (BD Bioscience). Twenty thousand nucleated cells were acquired per tube, and leukemic blasts were isolated using CD45/side scatter gating. The expression levels (%) of CD49d (VLA-4) and CD184 (CXCR4) on the leukemic blasts were measured.

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (version 18.0; Chicago, IL, USA). Student *t* test or ANOVA was used for between-group analysis, and the Tukey method was used for the post hoc analysis. Univariable and multivariable binary logistic regression analysis was used to predict the probabilities of complete remission (CR) or relapse. The Kaplan-Meier method and log-rank test were used to evaluate OS (time from study entry until death or the last follow-up examination) and RFS (time from CR to relapse or last follow-up examination). For survival curves, patients were categorized into four groups according to the quartiles of the expression levels of each adhesion molecule, and patients were also assigned to combination groups by simple grouping according to the results of the survival analysis. The hazard ratios of RFS and OS were estimated using univariate and multivariate Cox regression modeling. For all analyses, tests were two-sided and *p* values <0.05 were considered statistically significant.

Results

Correlation between VLA-4 and CXCR4 expressions and risk status

Among the 98 AML patients, VLA-4 positivity ranged between 5.8 and 99.8 %, and the median value was 91.3 %. CXCR4 expression ranged between 0.2 and 85.1 %, and the median value was 13.9 % (Supplementary Fig. 1). Expression levels of VLA-4 and CXCR4 were not associated with patient's age, peripheral blood WBC count, platelets, and blast count in blood and BM (data are not shown). VLA-4 expression was higher in patients at favorable and intermediate risk compared with patients at poor risk ($p < 0.001$ and $p = 0.002$, respectively) (Supplementary Table 2). However, CXCR4 expression did not follow this trend ($p = 0.655$ and 0.759 , respectively).

VLA-4 and CXCR4 expressions as predictor of CR or relapse

Of 72 non-promyelocytic leukemia patients who received cytarabine + anthracycline-based induction chemotherapy, 45 patients achieved CR after initial or reinduction therapy. According to the univariable binary logistic regression model, old age was associated with low CR probability ($p = 0.036$) and favorable risk status indicated higher probability of CR than intermediate or poor risk status ($p = 0.016$ and 0.004 , respectively; Table 3). High VLA-4 expression significantly increased CR probability ($p = 0.008$), but CXCR4 was not associated with the rate of CR ($p = 0.686$). According to the multivariable analysis, VLA-4 expression, age, and risk status were significantly associated with CR probability ($p = 0.019$, 0.014 , and 0.049 , respectively; Table 3).

Risk of relapse was analyzed with 50 patients, including 45 patients who achieved CR after initial or reinduction therapy and 5 patients who achieved CR after salvage chemotherapy. High VLA-4 expression was found to be associated with low relapse probability

Table 3 Probability of complete remission in 72 non-promyelocytic acute myeloid leukemia patients who received cytarabine + anthracycline-based induction therapy

Prognostic marker	Univariable			Multivariable		
	OR	95 % CI	<i>p</i>	OR	95 % CI	<i>p</i>
Age	0.963	0.931–0.998	0.036	0.948	0.908–0.989	0.014
Risk status			0.016			0.049
Favorable vs poor	11.227	2.127–59.262	0.004	8.437	1.370–51.952	0.021
Favorable vs intermediate	7.600	1.470–39.293	0.016	7.638	1.333–43.759	0.022
Intermediate vs poor	1.477	0.489–4.460	0.489	1.105	0.303–4.023	0.880
VLA-4 (%)	1.046	1.012–1.081	0.008	1.044	1.007–1.083	0.019
CXCR4 (%)	1.005	0.981–1.029	0.686	–	–	–

OR odds ratio, CI confidence interval

Table 4 Probability of relapse in 50 acute myeloid leukemia patients who achieved complete remission (CR) after chemotherapy

Prognostic marker	Univariable			Multivariable		
	OR	95 % CI	<i>p</i>	OR	95 % CI	<i>p</i>
Risk status			0.014			0.012
Favorable vs poor	0.115	0.023–0.567	0.008	0.082	0.013–0.512	0.008
Favorable vs intermediate	1.015	0.242–4.255	0.983	0.968	0.181–5.173	0.970
Intermediate vs poor	0.114	0.022–0.591	0.010	0.084	0.013–0.539	0.009
VLA-4 (%)	0.919	0.861–0.982	0.012	–	–	–
CXCR4 (%)	1.052	1.016–1.089	0.005	1.059	1.018–1.101	0.004

OR odds ratio, CI confidence interval

($p=0.012$; Table 4), and high CXCR4 expression was correlated with high relapse probability ($p=0.005$; Table 4). In multivariable analysis, high CXCR4 expression level increased relapse risk independent to risk status ($p=0.004$; Table 4) and high level of VLA-4 decreased relapse risk independent to risk status ($p=0.050$; Supplementary Table 3).

Survival according to VLA-4 and CXCR4 expressions

In the Kaplan-Meier survival curve analysis, we assigned patients to quartile groups according to expression levels of VLA-4 and CXCR4, respectively. The level of statistical significance was determined by log-rank test. The

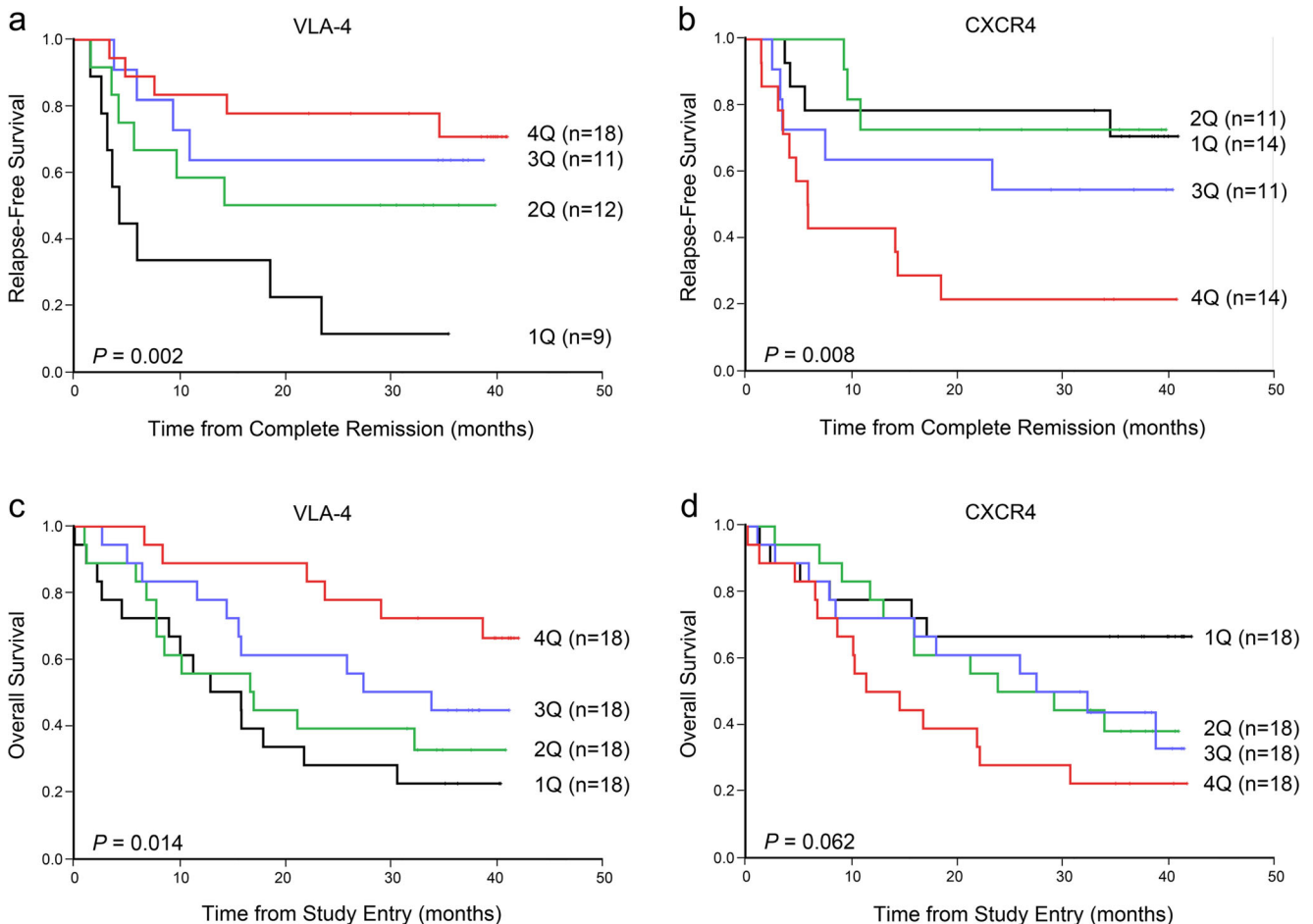


Fig. 1 Kaplan-Meier estimates of relapse-free survival (RFS) and overall survival (OS) in 72 non-promyelocytic acute myeloid leukemia patients that were divided according to VLA-4 and CXCR4 expression quartiles. Patients with expression levels in the lower quartile were assigned to group 1Q, and patients with expression levels in the upper quartile were

assigned to group 4Q. Groups 2Q and 3Q were divided according to the median values. RFS (**a, b**) and OS (**c, d**) association with VLA-4 and CXCR4 expression is shown. The *p* values were determined using the log-rank test

Table 5 Relapse-free survival in 72 non-promyelocytic acute myeloid leukemia patients

Prognostic marker	Univariate			Multivariate		
	HR	95 % CI	<i>p</i>	HR	95 % CI	<i>p</i>
Age	1.005	0.978–1.033	0.725	1.024	0.994–1.055	0.115
Risk status			0.001			0.002
Favorable vs poor	0.194	0.071–0.531	0.001	0.181	0.063–0.519	0.001
Favorable vs intermediate	0.749	0.217–2.590	0.648	0.751	0.225–2.508	0.641
Intermediate vs poor	0.213	0.073–0.622	0.005	0.241	0.080–0.728	0.012
VLA-4 (%)	0.962	0.944–0.981	<0.001	0.956	0.934–0.979	<0.001
CXCR4 (%)	1.025	1.009–1.041	0.002	–	–	–

HR hazard ratio, CI confidence interval

lower and upper quartiles of VLA-4 were 76.1 and 95.5 %, and CXCR4 were 5.5 and 32.0 %, respectively. High VLA-4 expression was associated with longer RFS ($p=0.002$; Fig. 1a), and high CXCR4 expression was associated with shorter RFS ($p=0.008$; Fig. 1b). High VLA-4 expression was also associated with superior OS ($p=0.014$; Fig. 1c). High CXCR4 expression showed trend of shorter OS ($p=0.062$; Fig. 1d).

Cox regression modeling was used to analyze the expression levels of VLA-4 and CXCR4. Age and expression levels of VLA-4 and CXCR4 were considered continuous variables, and risk status was considered categorical variables. Patient age and risk status were used as covariates in multivariate analysis. The univariate analysis showed that high VLA-4 and low CXCR4 expressions were associated with a superior RFS ($p<0.001$ and $p=0.002$, respectively; Table 5). In multivariate analysis, VLA-4 and CXCR4 expressions were independent markers of RFS ($p<0.001$ and $p=0.002$, respectively; Table 5 and Supplementary Table 4). OS was increased along with VLA-4 expression level ($p=0.010$; Table 6) and decreased along with CXCR4 expression ($p=0.027$) in univariate analyses. Multivariate analysis did not indicate significant correlations between OS and VLA-4 expression ($p=0.100$; Table 6) and between OS and CXCR4 expression ($p=0.297$; data are not shown).

Table 6 Overall survival in 72 non-promyelocytic acute myeloid leukemia patients

Prognostic marker	Univariate			Multivariate		
	HR	95 % CI	<i>p</i>	HR	95 % CI	<i>p</i>
Age	1.043	1.019–1.068	<0.001	1.057	1.029–1.085	<0.001
Risk status			0.001			0.001
Favorable vs poor	0.153	0.057–0.412	<0.001	0.140	0.049–0.400	<0.001
Favorable vs intermediate	0.307	0.113–0.833	0.020	0.340	0.125–0.929	0.035
Intermediate vs poor	0.498	0.259–0.959	0.037	0.412	0.199–0.852	0.017
VLA-4 (%)	0.983	0.970–0.996	0.010	0.987	0.972–1.002	0.100
CXCR4 (%)	1.015	1.002–1.028	0.027	–	–	–

HR hazard ratio, CI confidence interval

Survival according to combination groups

Expression levels of two kinds of adhesion molecules were combined, and the patients were categorized into three groups. Patients with the high VLA-4 expression level over the median value (>91.3 %) and low CXCR4 expression level below the median value (<13.9 %) were assigned to group A ($n=20$). The patients with low VLA-4 level in the lower quartile (<76.1 %) or high CXCR4 level in the upper quartile (>32.0 %) were assigned to group C ($n=28$), and the remaining patients were assigned to group B ($n=24$). Kaplan-Meier survival analysis of the RFS (Fig. 2a) and OS (Fig. 2b) demonstrated significant survival differences between the groups ($p<0.001$ and $p=0.009$, respectively). Group A was associated with the best outcomes, and group C was associated with the worst outcomes in both the RFS and OS analyses. In multivariate Cox regression analysis, group A showed superior RFS and OS compared with group C ($p=0.001$ and 0.011 , respectively; Table 7).

Discussion

VLA-4 integrin and CXCR4 chemokine receptor activation plays similar roles in the adhesion of leukemic myeloblasts

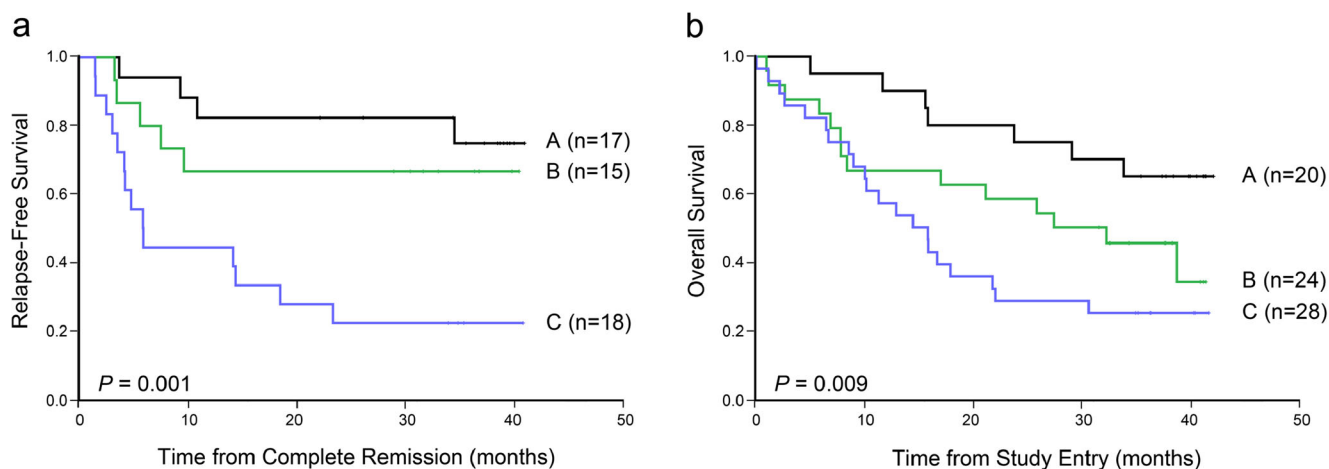


Fig. 2 Kaplan-Meier estimates of relapse-free survival and overall survival according to the combination groups. Group A: 20 patients with the high VLA-4 expression level over the median value (>91.3 %) and low CXCR4 expression level below the median value (<13.9 %).

Group B: 24 patients who do not belong to group A or C. Group C: 28 patients with low VLA-4 level in the lower quartile (<76.1 %) or high CXCR4 level in the upper quartile (>32.0 %). The *p* values were determined using the log-rank test

to BM stroma [2]. Their functions are executed via different mechanisms, whereby integrins are responsible for the initial attachment of leukemic cells to the stroma [11] and chemokine receptor activation is necessary to retain firm adhesion [12]. However, the results of our current study show that VLA-4 and CXCR4 expressions demonstrate contrasting results in terms of clinical outcomes in patients with non-promyelocytic AML.

Previous studies on the prognostic impact of VLA-4 expression on AML blasts report conflicting results. Initial studies suggested that high VLA-4 expression was associated with poor prognosis [1, 7], but studies on larger patient populations reported high VLA-4 expression in association with improved survival [9, 10]. When we referred to previous studies to define interactions of VLA-4 integrin to its ligands, several

features were noted. The binding of the VLA-4 integrin to its ligand depends on not only the expression level but also affinity level, which increases according to conformational changes [13]. VLA-4 integrin does not interact specifically to BM stroma cells. VCAM-1 and fibronectin are the major ligands of the VLA-4 integrin and are expressed on marrow stromal cells, but these ligands are also distributed on the surface of endothelial cells and are secreted as blood-soluble type [14, 15]. Soluble VCAM-1 is elevated to high levels in the plasma of leukemia patients [14], and it may be enough to overcome the VLA-4 expression. The adhesion of the hematopoietic progenitor to the BM microenvironment via the VLA-4 pathway is regulated by other agents, in particular the FLT3-ligand [16]. Accordingly, VLA-4 expression on leukemic blasts should not be considered a simple factor that

Table 7 Multivariate survival analysis with combination groups

Prognostic marker	Relapse-free survival			Overall survival		
	HR	95 % CI	<i>p</i>	HR	95 % CI	<i>p</i>
Age	0.997	0.968–1.027	0.844	1.053	1.024–1.082	<0.001
Risk status			<0.001			<0.001
Favorable vs poor	0.129	0.043–0.381	<0.001	0.115	0.042–0.318	<0.001
Favorable vs intermediate	0.805	0.242–2.681	0.724	0.325	0.119–0.886	0.028
Intermediate vs poor	0.160	0.051–0.504	0.002	0.355	0.176–0.718	0.004
Combination groups ^a			<0.001			0.040
Group A vs group C	0.145	0.046–0.457	0.001	0.325	0.137–0.775	0.011
Group A vs group B	0.844	0.212–3.362	0.809	0.465	0.186–1.165	0.102
Group B vs group C	0.172	0.053–0.554	0.003	0.699	0.342–1.430	0.327

HR hazard ratio, CI confidence interval

^a Group A: 20 patients with the high VLA-4 expression level over the median value (>91.3 %) and low CXCR4 expression level below the median value (<13.9 %). Group B: 24 patients who do not belong to group A or C. Group C: 28 patients with low VLA-4 level in the lower quartile (<76.1 %) or high CXCR4 level in the upper quartile (>32.0 %)

induces adhesion to BM stromal cells, thereby resulting in chemoresistance.

A previous retrospective study on soluble VCAM-1 studied cryopreserved specimens from adult AML patients has reported favorable prognostic implications of VLA-4 [9]. Despite some differences in the study conditions, the findings of our current prospective study using fresh specimens and the direct measurement of VLA-4 expression using flow cytometry agree with that earlier retrospective study: specifically, high VLA-4 expression improves survival in adult patients. Achievement of CR is one of the most important goals in AML treatment, as it demonstrates early and powerful prognostic impact on patient survival. Although the effects of VLA-4 expression on CR probability have not been identified to date both in the pediatric or adult AML patients, we here report the prognostic impact of VLA-4 expression in response to induction chemotherapy in adult AML patients and its usefulness as a prognostic marker. VLA-4 expression in adult AML patients can also be used as a favorable prognostic marker, as indicated by a previous study in pediatric patients [10].

A previous study of 90 AML patients indicated that high CXCR4 expression resulted in shorter RFS [8], and another study of 90 AML patients suggested that low CXCR4 expression was correlated with longer RFS and OS [6]. Recent study on 36 AML patients reported that lower expression of CXCR4 was associated with CR achievement and high expression of CXCR4 negatively influenced OS [7]. Our current study reveals the same results, namely that high CXCR4 expression is an independent prognostic factor for a poor RFS and correlates with a short OS. Unlike VLA-4, CXCR4 predominantly interacts with only SDF-1/CXCL12 [17], and CXCR4 and SDF-1/CXCL12 induce hematopoietic stem cells to remain in a quiescent state (G0) [3], thereby inducing chemoresistance. A CXCR4 antagonist (plerixafor) has been approved for mobilizing CD34+ hematopoietic stem cells in autologous HSCT during the treatment of non-Hodgkin's lymphoma and plasma cell myeloma [18]. According to a recent phase 1/2 study, plerixafor improves chemosensitivity in patients with relapsed or refractory AML [4]. Thus, measuring CXCR4 expression in leukemic myeloblasts can be a useful independent prognostic marker of RFS and be used to determine potential new drugs.

We consolidated the prognostic implications of VLA-4 and CXCR4 expressions into combination groups to integrate the interactions between VLA-4/VCAM-1 and CXCR4/SDF-1 pathways. The analyses resulted in simultaneous high VLA-4 expression (>91.3 %), and low CXCR4 expression (<13.9 %) was an independent marker for superior survival. It could be the result of incorporation of findings in the current study, high VLA-4 expression was associated with high CR achievement rate, while low CXCR4 expression showed low relapse rate. Considering that, this might be related to the different mechanisms of the chemotherapeutic drugs.

Anthracycline seems to more effectively eradicate VLA-4-expressing myeloblasts, but chemoresistance (particularly in association with cytarabine, an S phase-specific drug) could be encouraged by CXCR4 and cause myeloblasts to remain quiescent. However, it needs further investigations; controlling both pathways would provide more effective treatment strategy.

In conclusion, high VLA-4 expression in leukemic myeloblasts is an independent prognostic marker of a favorable outcome in terms of CR and RFS in adult AML patients, whereas high CXCR4 expression is a marker of poor prognosis, relapse, and short RFS in these populations. Combination of expression levels of VLA-4 and CXCR4 provides independent and robust prognostic value in adult AML patients.

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Authorship C-JP and K-HL designed and conducted the research. MHB collected, analyzed, and interpreted data; performed statistical analysis; and wrote the manuscript. S-HO collected, analyzed, and interpreted data. B-RL performed flow cytometric experiments. SHP performed morphologic analysis and molecular analysis. YJK, Y-UC, and SJ performed morphologic analysis. J-H Lee and K-HL managed patients, provided clinical data, and are involved in prognostic analysis. NK performed and analyzed flow cytometric data. J-H Lim and E-JS performed chromosomal analysis.

C-JP and K-HL contributed equally to this study, having full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

Conflict of interest The authors have no conflicts of interest to report.

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