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Relationship between CXC chemokine receptor 4 expression and prognostic significance in acute myeloid leukemia

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Abstract

CXC chemokine receptor 4 (CXCR4) expression on acute myeloid leukemia (AML) cells correlated with stromal cell derived factor-1α (SDF-1α) and retained hematopoietic progenitors and leukemia cells within the bone marrow microenvironment. Here, we examined CXCR4 expression in 134 de novo AML and 21 controls by flow cytometry, evaluated the relationship between CXCR4 expression and clinical characteristics, and elucidated the prognostic significance of CXCR4 expression in AML prospectively. We found that the CXCR4 expression was significantly higher in AML patients than controls ($P = .000$). One hundred thirty four cases of de novo AML patients were divided into 2 groups according to the median of CXCR4 relative fluorescence intensity (RFI). CXCR4 high group (RFI >4.23) had markedly shorter overall survival (OS) and disease-free survival (DFS) than CXCR4 low group (RFI ≤ 4.23) in 106 AML patients who received chemotherapy ($P = .002$; .026, respectively). Furthermore, in the 87 non-M3 patients who received induction therapy, there was a significant decrease for OS but not for DFS in the CXCR4 high group ($P = .047$ and .178, respectively). Moreover, high levels of CXCR4 expression independently increased the risk of relapse in both all AML and non-M3 patients who achieved complete remission (CR) after chemotherapy (odds ratio = 1.090, $P = .010$; odds ratio = 1.068, $P = .048$, respectively). Collectively, our data suggest that CXCR4 overexpression was an independent prognostic factor for disease relapse and poorer OS in both all AML and non-M3 patients. CXCR4 expression levels can be determined at disease presentation by the flow rapidly and easily. As such, CXCR4 could be used as a potential therapeutic target in AML patients with poor prognosis.

Abbreviations: AML= acute myeloid leukemia, APL= acute promyelocytic leukemia, ATAR = all-trans-retinoic acid, CR = complete remission, CXCR4 = CXC chemokine receptor 4, CXCL12 = Chemokine ligand 12, DFS = disease-free survival, DNR = daunorubicin, FLT3-ITD = FMS-like receptor tyrosine kinase 3, FLAG = combination of Fludarabine, Cytarabine and G-CSF (granulocyte colony-stimulating factor), HSCs = hematopoietic stem cells, IDA = Idarubicin, OS = overall survival, RFI = relative fluorescence intensity, SDF-1α = stromal cell derived factor-1α.

Keywords: acute myeloid leukemia, disease-free survival, overall survival, prognosis, relative fluorescence intensity of chemokine Receptor 4, relapse

1. Introduction

Acute myeloid leukemia (AML) is a hematopoietic clonal malignancy that is characterized by uncontrolled proliferation of hematopoietic stem cells (HSCs) and progenitors without the capacity to differentiate into mature cells.[1] The treatment and prognosis of AML patients depend on accurate cytogenetic and genetic examinations.[2] Despite the fact that various novel reagents have demonstrated clinical activities,[3] long-term outcomes of AML patients remain poor, which is mainly due to resistance to chemotherapy and disease relapse post-chemotherapy.[4–6]

In recent years, it has been reported that the interactions between leukemia cells and the bone marrow (BM) microenvironment were the major cause of resistance to chemotherapy and disease relapse in leukemia. The CXC chemokine receptor 4/stromal cell derived factor-1α (CXCR4/SDF-1α) axis played an important role in the crosstalk between AML leukemia cells and bone marrow microenvironment.[7–10] SDF-1α is produced by the bone marrow stromal cells, also known as chemokine ligand 12 (CXCL12), SDF-1α regulates leukemia cell trafficking by binding its cognate receptor CXCR4 on leukemia cells. Leukemia cells are recruited into and reside in the bone marrow, thereby acquire anti-apoptosis signals and favorable conditions for survival and growth.[11–15] However, there have been limited prospective studies evaluating the correlation between CXCR4 expression on leukemic cells and the clinical outcomes in AML

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TC and YY authors have contributed equally to this work.

Compliance with ethical standards: The study protocol was reviewed and approved by the Ethics Committee of West China Hospital of Sichuan University. All biological samples were obtained from patients and controls that had provided written informed consent in accordance with the tenets of the Declaration of Helsinki. Written informed consents for minor participants (<16 years old) were obtained from their parents or legal guardians.

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patients. Hence, in this study, we have analyzed CXCR4 expressions on leukemic cells in 134 newly diagnosed AML patients, evaluated the relationship between CXCR4 expression levels and clinical characteristics, and elucidated the prognostic implications of CXCR4 expression in AML prospectively.

2. Materials and methods

2.1. Patients and controls

A total of 134 de novo AML patients before treatment were recruited from the West China Hospital of Sichuan University from February 2012 to August 2015. Patients included in this study had an unequivocal diagnosis of AML based on the French–American–British (FAB)[16] and World Health Organization (WHO)[17] criteria in combination of clinical, morphological, immunophenotypic, and genetic features. Karyotype and cytogenetic risk analysis of AML patients were based on the United Kingdom medical research council (MRC) trials.[18] All patients who received induction chemotherapy were tested for internal tandem duplication of FMS-like receptor tyrosine kinase 3 (FLT3-ITD), NPM1(Nucleophosmin) mutations, CCAAT/enhancer binding protein α mutations, and receptor tyrosine kinase (KIT) mutations. A total of 21 healthy individuals who were diagnosed of non-hematological diseases without bone marrow infiltration were also recruited as controls.

2.2. Chemotherapy regimens

A total of 106 patients enrolled in this study received the standardized chemotherapy. According to the clinical practice in our medical center, induction therapy for non-M3 patients was administered according to the standard "3+7" course (cytarabine 100–200 mg m^{-2} on days 1–7; idarubicin [IDA] 8–12 mg m^{-2} on days 1–3, or daunorubicin [DNR] 60–75 mg m^{-2} on days 1–3), and the dosage of IDA and DNR was adjusted according to the patient's condition. After the first induction cycle, patients with persistent disease (i.e., >5% BM blasts in the bone marrow at hematopoietic recovery) received a second induction therapy (original regimen, or FLAG regimen). The choice of post-remission treatments depended on both the physicians' options and the patients' willingness, including medium-high dose cytarabine, standard dose anthracycline plus cytarabine, or allo-hematopoietic stem cell transplantation. For patients with acute promyelocytic leukemia (APL, AML-M3), induction therapy included the combination of all-trans-retinoic acid (ATAR) and arsenic trioxide, with or without anthracycline. The post-remission therapy consisted of one or more of the drugs including ATRA, arsenic trioxide, anthracycline, and cytarabine. None of the patients did hematopoietic stem cell transplantation.

2.3. CXCR4 expression on objective cells detected by flow cytometry

We detected the expression of CXCR4 through flow cytometry (BD FACS Canto II, Becton Dickinson, NJ) on fresh BM leukemic cells within 8 hours after sample withdrawal. Briefly, the concentration of nucleated cells in BM sample suspensions was adjusted to 1×106/mL by phosphate buffer saline (PBS). Then, 20 μL APC-conjugated mouse anti-human anti-CXCR4 (CD184-APC, BD pharmingen, NJ) was used in combination with anti-CD34–FITC, anti-CD117–PE, and anti-CD45–PerCP (Becton Dickinson, NJ) to stain for the experimental tube panel. Twenty microliter isotype APC-conjugated mouse anti-human IgG2, κ (Becton Dickinson, NJ) was used in combination with anti-CD34–FITC, anti-CD117–PE, and anti-CD45–PerCP (Becton Dickinson, NJ) as isotype control tube panel. After incubation for 30 minutes in dark, 2 mL of lysing solution was added and sit for 5 minutes. The tubes were centrifugated for $300 \times g$ 5 minutes under 4 °C to discard the supernatant, and the cells were washed once with PBS. The cell suspensions were analyzed on a flow cytometer, which data were analyzed using the FACSDiva software (BD Bioscience, NJ).

2.4. Definition of variables

The expression of CXCR4 on the surface of leukemic cells was evaluated by flow cytometry, and the results were represented by the relative fluorescence intensity (RFI). CD45+/CD34+/CD117+cells (a few cells were CD45+/CD34–/CD117+ or CD45+/CD34+/CD117–) were the objective cells (leukemic cells) in AML group, CD45+/CD34+cells were the objective cells in control group. The expression of CXCR4 (CD184) was analyzed through single parameter histogram after gating on the objective cells. The APC (mouse IgG2, κ) isotype was used as the negative control (P1 region, Fig. 1A), and positive CXCR4 expression was defined when the objective cell tube labeled with anti-CXCR4 (CD184)-APC located in the right-side region of the isotype control (P2 region, Fig. 1B). The RFI of CXCR4 expression on the objective cell surfaces was calculated as dividing the anti-CXCR4-APC expression fluorescence intensity by isotype-APC expression fluorescence intensity.

2.5. Statistical analysis

Statistical analysis was performed using the SPSS Statistics 24.0 software (Chicago, IL). Comparison of continuous variables in 2 groups was carried out using the Mann–Whitney *U* test. Pearson chi-squared analysis or Fisher exact test was applied to compare the difference of categorical variables. Univariable and multivariable binary logistic regression analysis were used to predict the probabilities of complete remission (CR) or relapse. Disease-free survival (DFS) and overall survival (OS) were analyzed by the Kaplan–Meier method. Univariate and multivariate Cox proportional hazard models were fitted to assess the effect of patient characteristics on DFS and OS. Time of DFS was calculated from the date of CR to relapse or last follow-up examination. Time of OS was calculated from the date of first diagnosis to the date of death or last observation. All statistical tests were 2-sided, P values $<.05$ was considered statistically significant. GraphPad Prism version 6.0 (GraphPad Software, San Diego, CA) was used as the drawing tool.

3. Results

3.1. Clinical and laboratory characteristics of the patients with de novo AML

A total of 134 patients presented with de novo AML were recruited consecutively from West China Hospital of Sichuan University during February 2012 to August 2015, of which, 72 were men and 62 women. The age of the patients ranged from 15 to 83 years old, with a median of 47 years at diagnosis. According to FAB classification criteria, the AML patients consisted of 26 cases of M0/1, 44 cases of M2, 20 cases of M3, 19 cases of M4, 19 cases of M5, 4 cases of M6, and 2 cases of unclassification.

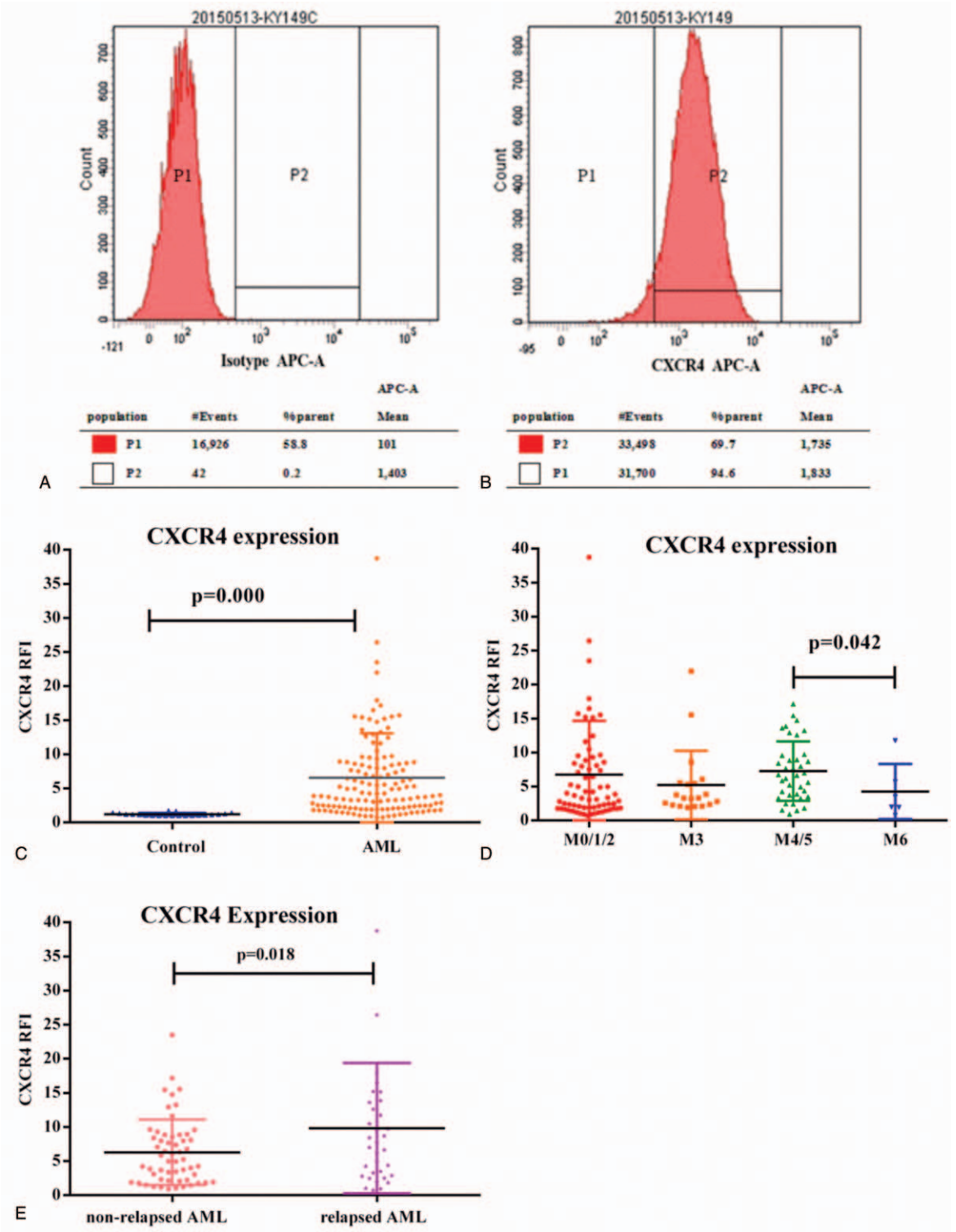

Figure 1. The relative fluorescence intensity (RFI) of CXCR4 expression. A: The histogram plots refer to isotype control. B: The objective cells located on the right side of the isotype control door (p2 region) were judged to be positive for CXCR4 expression. C: The comparison of the RFI of CXCR4 expression between the AML patients group and controls group. D: The comparison of the CXCR4 RFI among the FAB subtypes [FAB M0/M1/M2, FAB M3, FAB M4/M5, FAB M6] of AML patients. E: The comparison of the CXCR4 RFI between the relapsed patients and the patients without relapse in AML group who achieved CR after chemotherapy. NOTE: The distributions of the RFI of CXCR4 expression in controls and AML patients were presented with scatter plots. The median level of the RFI of CXCR4 expression in each group was shown with horizontal line. AML = acute myeloid leukemia, CR = complete remission, CXCR4 = CXC chemokine receptor 4, FAB = French–American–British.

Based on European Leukemia Net group cytogenetic risk criteria,[2] 48 patients were classified as a favorable risk group, 42 patients were intermediate risk group, 30 patients were adverse risk group, and 14 patients cannot be classified. Of the 134 de novo AML patients, 106 cases (87 cases of non-M3) had received the induction therapy. The baseline characteristics of the AML participants were detailed in Table 1.

3.2. Comparison of CXCR4 expression between the AML patient group and control group

By comparing the RFI of CXCR4 expression between the AML patient group and control group, we found that CXCR4 expression was significantly up-regulated in AML patients (0.74–40.17; median = 4.23) compared with the controls (1.01–1.74; median = 1.44) ($P = .000$, Fig. 1C). We also analyzed the differences among AML subtypes categorized by the FAB classification, that is, myelocytic subtypes (FAB M0/M1/M2), promyelocytic subtypes (FAB M3), myelomonocytic/monocytic subtypes (FAB M4/M5), and erythroleukemic subtypes (FAB M6). There was a tendency of high CXCR4 expression in M4/5 subtypes, and the level of CXCR4 expression was significantly higher in M4/M5 FAB subtypes (0.93–17.17: median = 7.27) than M6 FAB subtypes (0.74–5.68: median = 2.58) ($P = .042$, Fig. 1D). However, no difference of CXCR4 expression of in the other FAB subtypes was observed.

3.3. Comparison of the clinical and laboratory characteristics between AML patients with low and high CXCR4 expression

Median CXCR4 RFI on leukemia cells was 4.23 (0.74–40.17), by which, we categorized patients into $CXCR4^{low}$ and $CXCR4^{high}$ expression group. The comparison of clinical and laboratory characteristics between the $CXCR4^{low}$ AML group and the $CXCR4^{high}$ AML group was shown in Table 1. There were no significant differences in age, sex, hemoglobin concentration, platelet count, peripheral white blood cell count, peripheral blood leukemia-cell (PBLC) count, and bone marrow blast percentage ($P > .05$, Table 1) between the $CXCR4^{low}$ AML group and the $CXCR4^{high}$ AML group. However, the $CXCR4^{high}$ AML group showed a significantly higher incidence of M4/M5 subtypes ($P = .002$), higher frequency of NPM1 mutation ($P = .021$) and FLT3-ITD mutation ($P = .016$) than the $CXCR4^{low}$ AML group. On the contrary, the $CXCR4^{low}$ AML group showed an increased incidence of C-kit mutation ($P = .030$) than the $CXCR4^{high}$ AML group. Although CXCR4 expression on AML cells showed no impact on CR rate neither after first nor salvage therapy (first induction chemotherapy CR 51% in $CXCR4^{low}$ AML group and 43% in $CXCR4^{high}$ AML group, $P = .495$; overall CR 73% in $CXCR4^{low}$ AML group and 67% in $CXCR4^{high}$ AML group, $P = .421$), the $CXCR4^{high}$ AML group had a marginally higher relapse rate (RR) ($P = .056$) than the $CXCR4^{low}$ AML group. Details were seen in Table 1.

3.4. The impact of CXCR4 expression on survival in AML patients

To determine the prognostic impact of CXCR4 expression on leukemia cells, we evaluated the overall survival (OS) (n = 106), disease-free survival (DFS) (n = 106) in AML patients who received induction therapy. Considering the special prognostic features of acute promyelocytic leukemia (APL), we conducted a separate analysis of the impact of CXCR4 expression on OS and DFS in the non-M3 patients (n = 87).

The median follow-up time was 23 (range, 1–61) months. There were 106 patients (87 cases of non-M3) treated with standardized chemotherapy regimens (as described above) and analyzed for clinical outcomes, of which 74 cases (58 cases of non-M3) achieved CR (overall CR 69.8%, non-M3 CR 66.7%) after their overall induction therapy. However, 36 (32 case of non-M3) of them experienced a disease relapse or progression. Overall, 44 cases of the AML patients survived while 62 died. The median DFS was 16.1 months (95% confidence interval [CI]: 6.5–25.6) and the median OS was 23.3 months (95% CI: 14.6–31.8). The 1-year DFS and OS were 51% and 68%, the 2-year DFS and OS were 38% and 48%, and the 3-year DFS and OS were 5% and 19%, respectively.

According to the univariable binary logistic regression model, the favorable risk status indicated a higher probability of CR than intermediate or adverse risk status in the all AML patients ($P = .001$ and .020, respectively, Table 2). However, only the favorable risk status indicated a significantly higher probability of CR than intermediate risk status in the non-M3 patients (OR = 3.943, 95% CI = 1.145–13.582, $P = 0.030$, Table 2). But the levels of CXCR4 expression was not associated with the CR rate in both all AML and non-M3 patients ($P = .421$ and .644, respectively).

The Kaplan–Meier method was used to estimate DFS and OS for all AML patients, non-M3 patients, and all the relapsed patients (Fig. 2). In the 106 AML patients who received induction therapy, there was a significant decrease both for OS and DFS in the $CXCR4^{high}$ group ($P = .002$ and .026, respectively, details seen in Fig. 2A and B). In the 87 cases of non-M3 patients who received induction therapy, there was a significant decrease for OS but not for DFS in the $CXCR4^{high}$ group ($P = .047$ for OS, $P = .178$ for DFS, details seen in Fig. 2C and D).

To define the prognostic significance of CXCR4 expression as well as other parameters, including age, WBC count, and cytogenetic risk, univariate Cox proportional hazard models for OS and DFS were performed, after which factors associated with at least borderline significance ($P < .10$) were further subjected to multivariate analysis. For all AML patients, univariate analyses indicated that CXCR4 high expression (RFI >4.23) and high WBC count ($>20 \times 10^9$/L) were significant predictive factors resulting in a reduced OS and DFS. For non-M3 patients, univariate analyses indicated that CXCR4 high expression (RFI >4.23) was significant predictive factors only for a reduced OS ($P = .047$) but not for DFS ($P = .178$), only high WBC count ($>20 \times 10^9$/L) was significant predictive factors for a reduced OS and DFS in non-M3 patients ($P = .006$ and .025). In multivariate Cox analysis, CXCR4 high expression (RFI >4.23) and high WBC count ($>20 \times 10^9$/L) were independent markers of poor OS both in all AML patients and in non-M3 patients, and cytogenetic risk (favorable) were independent markers of good OS both in all AML patients and in non-M3 patients (Tables 3 and 4). But the CXCR4 high expression (RFI >4.23) has no prognostic significance for DFS neither in all AML nor in non-M3 patients ($P > .05$, respectively).

3.5. The impact of CXCR4 expression on relapse in AML patients

There were total 36 (32 cases of non-M3) patients experienced disease relapse in 74 (58 cases of non-M3) AML patients who achieved complete remission (CR) after chemotherapy. As

Table 1

Comparison of clinical and laboratory characteristics between low and high CXCR4 expression groups of the AML patients.

Characteristic	Case N (%)	$CXCR4^{low}$group (RFI ≤4.23)N (%)	$CXCR4^{high}$group (RFI >4.23)N (%)	*P* value
No. of patient	134	67	67	
Age, y, median (range)	47 (15–83)	47 (16–83)	47 (15–82)	.975
Gender				.083
No. of males	72 (53.7)	41	31	
No. of female	62 (46.3)	26	36	
CBC (median, range)				
WBC ($\times 10^9$/L)	40.6 (0.5–368)	44 (0.6–368)	37 (0.53–234)	.558
PBLC (×109/L)	30 (0–335)	30 (0–335)	27 (1–192)	.766
Platelets ($\times 10^9$/L)	64.79 (3–465)	58 (6–428)	79 (3–465)	.124
Hemoglobin, g/L	79.1 (8–148)	79 (8–148)	80 (44–123)	.790
BM blast cells (%)	64.7 (10.0–97.5)	65 (14–97)	65 (10–98)	.980
FAB classification				.075
M0/1	26 (19.4%)	14 (21%)	12 (18%)	
M2	44 (32.8%)	25 (37%)	19 (28%)	
M3	20 (14.9%)	13 (19%)	7 (10%)	
M4	19 (14.2%)	7 (11%)	12 (18%)	
M5	19 (14.2%)	4 (6%)	15 (22%)	
M6	4 (3.0%)	3 (5%)	1 (1%)	
Unclassifiable	2 (1.5%)	1 (1%)	1 (1%)	
FAB M4/5	38 (28.4%)	11 (29%)	27 (71%)	.002*
FAB non-M3	114 (85.1%)	54 (47%)	60 (53%)	.089
WHO classification (2008)				.045*
AML with *t* (8;21)	15	10 (15%)	5 (7%)	
AML with inv (16)/*t* (16;16)	5	3 (5%)	2 (3%)	
APL with *t* (15;17)	20	12 (18%)	8 (12%)	
AML with *t* (9;11)	1	1 (1.5%)	0	
AML with *t* (6;9)	1	1 (1.5%)	0	
AML with inv (3) or *t* (3;3)	1	1 (1.5%)	0	
AML with mutated NPM1	20	5 (7%)	15 (22%)	
AML with mutated CEBPA	9	6 (9%)	3 (5%)	
AML not otherwise specified	60	27 (40%)	33 (49%)	
Therapy-related	2	1 (1.5%)	1 (2%)	
Karyotype classifications				.105
Favorable	48 (35.8%)	23 (49%)	25 (51%)	.719
Intermediate	42 (31.3%)	23 (55%)	19 (45%)	.349
Adverse	30 (22.4%)	11 (37%)	19 (63%)	.097
No data	14 (10.4%)	10 (71%)	4 (29%)	
Karyotypes#		67	67	.105
t (8;21)	15 (11%)	10 (67%)	5 (33%)	.060
t (15;17)	20 (15%)	12 (60%)	8 (40%)	.101
Inv (16)/*t* (16;16) (p13;q22)	5 (4%)	3 (60%)	2 (40%)	.190
Complex	6 (5%)	1 (17%)	5 (83%)	.070
Others	10 (7%)	3 (30%)	7 (70%)	.120
Normal	64 (48%)	28 (44%)	36 (56%)	.162
No data	14 (10%)	10 (71%)	4 (29%)	.189
Gene mutations#				
Double CEBPA (+/–)	9/110 (7.6%)	6/50 (11%)	3/60 (5%)	.107
NPM1 (+/–)	20/96 (17.2%)	5/50 (9%)	15/46 (25%)	.021*
FLT3-ITD (+/–)	18/101 (15.1%)	4/52 (7%)	14/49 (23%)	.016*
c-KIT (+/–)	5/117 (4.1%)	5/53 (9%)	0/64 (1%)	.030*
First induction CR (+/–)#	49/106 (46.2%)	23/22 (51%)	26/35 (43%)	.495
Overall CR (+/–)#	74/106 (69.8%)	33/12 (73%)	41/20 (67%)	.421
Overall RR (+/–)#	36/106 (34.0%)	11/34 (24%)	25/36 (41%)	.056
Non-M3 CR (+/–)#	58/87 (66.7%)	25/11 (69.4%)	33/18 (64.7%)	.644
Non-M3 RR (+/–)#	32/87 (36.8%)	11/25 (30.6%)	21/30 (41.2%)	.312

AML = acute myeloid leukemia; BM blast = bone marrow blast; CBC = complete blood cell count; CEBPA = CCAAT/enhancer binding protein α; CR = complete remission; FAB = French–American–British classification; PBLC = peripheral blood leukemia-cell count; RR = relapse rate; WBC = white blood cells; WHO = World Health Organization.

Percentage was equal to the number of happened incidence or positive patients divided by total cases in each group.

* $P < .05$, with statistical significance.

Table 2

Univariable analyses of prognostic marker for complete remission in AML patients who received induction therapy.

	All AML (n = 106)			Non-M3 (n = 87)		
Prognostic marker	**OR**	**95%CI**	***P***	**OR**	**95%CI**	***P***
CXCR4RFI low vs high	0.706	0.302–1.650	.421	1.012	0.949–1.080	.644
Cytogenetic risk			**.006***			.088
Favorable vs intermediate	6.907	1.890–21.680	**.001***	3.943	1.145–13.582	**.030***
Favorable vs adverse	3.289	1.241–11.162	**.020***	1.849	0.512–7.004	.338
Intermediate vs adverse	2.013	0.645–6.283	.189	2.082	0.645–6.708	.219
Age	0.975	0.928–1.027	.070	0.973	0.943–1.003	.080

Univariable binary logistic regression analysis was used.

AML = acute myeloid leukemia, CI = confidence interval, CXCR4 RFI = relative fluorescence intensity of chemokine receptor 4, OR = odds ratio.

* $P < .05$, with statistical significance. According to the univariable binary logistic regression model, the favorable risk status indicated a higher probability of CR than intermediate or adverse risk status in the all AML patients ($P = .001$ and $P = .020$, respectively). The favorable risk status indicated a significantly higher probability of CR than intermediate risk status in the non-M3 patients ($P = .030$).

described above, the $CXCR4^{high}$ group (RFI >4.23) showed a tendency of higher relapse rate (RR) than the $CXCR4^{low}$ group (RFI ≤4.23) (42% vs 24%; $P = .056$). Also, in the subgroup analysis, the median CXCR4 RFI 10.07 in relapsed patients (n = 36) was significantly higher than the median CXCR4 RFI 5.90 in patients without relapse (n = 38) ($P = .018$, Fig. 1E). To further investigate the clinical significance of CXCR4 expression on disease relapse, we divided the 36 cases of relapsed patients into 2 groups according to their median RFI value (RFI = 7) of CXCR4 expression. Notably, the relapsed patients with high CXCR4 expression (RFI >7) showed significantly shorter OS than those with low CXCR4 expression (RFI ≤7) ($P = .014$; HR = 2.238 [1.27–5.509], Fig. 2E). Furthermore, we analyzed the prognostic markers of relapse in all AML (n = 74) and non-M3 (n = 58) CR patients, including CXCR4 expression levels, the incidence of M4/M5 subtypes, cytogenetic risk, and age. In all AML CR patients, univariable analysis indicated that increased CXCR4 expression levels and higher incidence of M4/M5 subtypes were significantly correlated with high relapse probability ($P = .010$ and .007, respectively, Table 5). In the non-M3 CR patients, univariable analysis indicated that increased CXCR4 expression showed a tendency of higher relapse probability ($P = .052$, Table 5), meanwhile, higher incidence of M4/M5 subtypes was significantly correlated with high relapse probability ($P = .035$, Table 5). By multivariable analysis, we demonstrated that high CXCR4 expression levels independently increased relapse risk both in all AML and non-M3 patients who achieved complete remission after chemotherapy (OR = 1.090, $P = .010$; OR = 1.068, $P = .048$, respectively), which further suggest the feasibility of using CXCR4 expression as an independent risk factor for relapse both in all the AML and non-M3 patients. These results listed in Table 5.

4. Discussion

It has been reported that acute myeloid leukemia cells highly expressing CXCR4 are recruited into the bone marrow by SDF-1α, through which pro-survival and anti-apoptosis signals are provided.[19] The bone marrow microenvironment can protect leukemia cells from cytotoxic chemotherapeutic agents through the SDF-1α/CXCR4 signal pathway, which is an important mechanism of chemotherapeutic resistance and relapse of leukemia.[6,7,11,12,14,15] Recent studies showed that CXCR4 had significant prognostic significance in AML.[20,21] However, the extent of the impact remains elusive. In the present study, we demonstrate that CXCR4 expression was significantly higher in AML group than control group. Our study also showed that M4/M5 FAB subtypes with myelomonocytic/monocytic feature had a tendency of high CXCR4 RFI expression among all FAB subtypes. Especially, it was worth to be noted that the incidence of AML-M4/M5 subtypes was increased in $CXCR4^{high}$ expression group. Such data are consistent with the result from a previous study showing that the incidence of M4/M5 subtypes was significantly higher in $CXCR4^{high}$ group, yet they did not mention the relative levels of CXCR4 expression in M4/M5 subtypes.[21] CXCR4 overexpression has been confirmed to be associated with extramedullary infiltration in many hematological malignancies.[22,23] Therefore, we speculate that in M4/M5 subtypes, the specific clinical features of monocytic leukemia such as prone to extramedullary infiltration and relapse may be correlated with its CXCR4 overexpression.

CXCR4 expression has been extensively studied in a variety of human hematological malignancies and many other neoplastic diseases, such as multiple myeloma (MM), myelodysplastic syndrome (MDS), chronic lymphocytic leukemia (CLL), pediatric AML, epithelial ovarian cancer, breast tumor, and non-small cell lung cancer, in which it has been associated with poor clinical outcomes, metastasis, invasion, and chemotherapy resistance.[24–30] It has been reported that CXCR4 expression may be associated with poor prognosis in patients with AML,[20,21,31,32] but none of these studies included an accurate cut-off value for prognosis evaluation. In this study, we used the median RFI of CXCR4 expression level 4.23 to differentiate the patients into $CXCR4^{high}$ and $CXCR4^{low}$ group for the first time. Our results demonstrate that the $CXCR4^{high}$ group (RFI >4.23) had remarkably reduced OS and DFS in all AML patients, significantly decreased for OS but not for DFS in 87 non-M3 patients. In addition, CXCR4 high expression (RFI >4.23) was an independent poor prognostic factor for OS in multivariate Cox analysis not only in all AML patients but also in non-M3 AML patients. These ensuing results based on the cut-off value of 4.23 strongly suggest the significant prognostic impacts of CXCR4 overexpression in AML.

The present study also indicated that CXCR4 high expression was an independent relapse factor of AML patients. Firstly, we found that $CXCR4^{high}$ group (RFI >4.23) had a tendency of higher relapse rate, compared with $CXCR4^{low}$ group (RFI ≤4.23) ($P = .056$). Secondly, the RFI of CXCR4 expression in relapsed patients was significantly higher than that in patients without relapse ($P = .018$) in 74 cases of patients who achieved CR after chemotherapy Furthermore, high CXCR4 expression level independently increased relapse risk in both all AML and non-M3 patients who achieved CR after chemotherapy ($P = .010$ and $P = .048$, respectively) in multivariable binary logistic

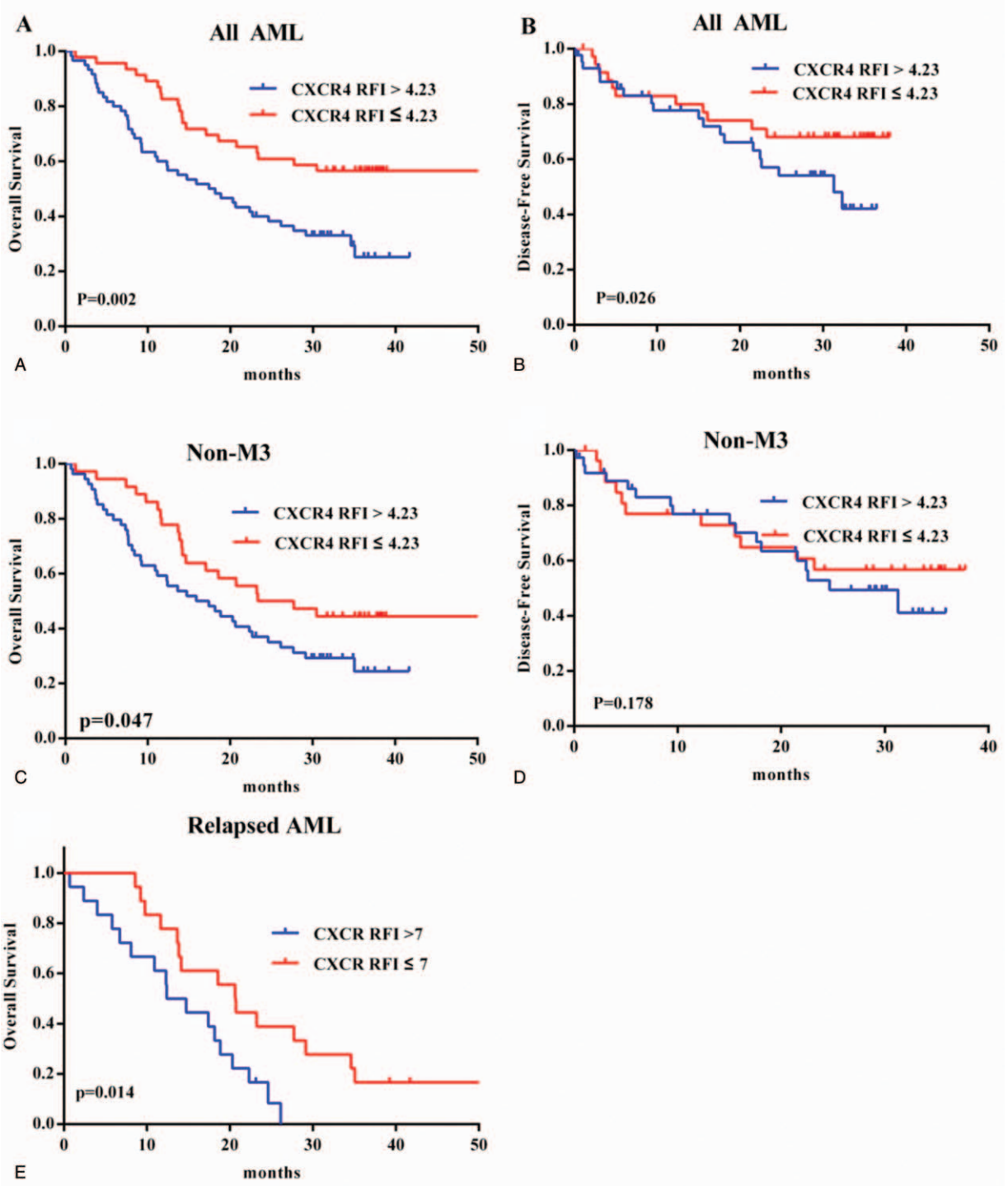

Figure 2. The prognostic impact of the relative fluorescence intensity (RFI) of CXCR4 expression in AML patients. A: overall survival (OS) in all AML patients. B: Disease-free survival (DFS) in all AML patients. C: OS in non-M3-AML patients. D: DFS in non-M3-AML patients. E: OS in all relapsed AML patients. The *P* values were determined using the log-rank test. Survival analyses were performed by Kaplan–Meier methods. CXCR4 = CXC chemokine receptor 4.

regression analysis. Interestingly, recent studies also provided evidence that targeting to disrupt the SDF-1α/CXCR4 signal pathway by using CXCR4 inhibitors could improve the chemosensitivity of AML cells in vitro, in vivo, and in clinical trials,[33–35] indicating that CXCR4 is critically related to chemotherapeutic efficiency, and consequently, the prognosis and relapse. Consistent with such result, the present study suggested that CXCR4 expression could be used to predict the relapse and poor prognostic of AML patients. While minimal residual disease (MRD) may be the main cause of relapse in AML patients, there were limited studies on the relationship between CXCR4 expression and MRD in AML. Of these, it has been reported that leukemia cells were

Table 3

Multivariate analyses of prognostic markers for OS and DFS in 106 AML patients who received induction therapy.

	DFS		OS	
Prognostic markers	**Hazard ratio (95% CI)**	***P***	**Hazard ratio (95% CI)**	***P***
CXCR4 RFI >4.23	1.807 (0.792–4.964)	.160	2.400 (1.229–4.688)	**.010***
Age >50	–	–	–	–
WBC >20	2.318 (1.082–4.964)	.031*	2.109 (1.167–3.815)	**.014***
Cytogenetic risk		.724		.130
Favorable vs intermediate	0.737 (0.266–2.404)	.557	0.478 (0.231–0.988)	**.046***
Intermediate vs adverse	0.813 (0.291–2.266)	.692	0.960 (0.638–2.492)	.506
Favorable vs adverse	0.907 (0.368–2.390)	.133	0.602 (0.293–1.238)	.168

Multivariate binary logistic regression analysis was used.

AML = acute myeloid leukemia, CI = confidence interval, CXCR4 RFI = relative fluorescence intensity of chemokine Receptor 4, DFS = disease-free survival, OS = overall survival, WBC = white blood cells.

* $P < .05$, with statistical significance. In all AML patients, through multivariate Cox analysis, CXCR4 high expression (RFI>4.23) and high WBC count ($>20 \times 10^9$/L) were independent markers of poor OS ($P = .010$ and $P = .014$, respectively), the cytogenetic risk (favorable) were independent markers of good OS ($P = .046$). Only high WBC count ($>20 \times 10^9$/L) were significant predictive factors resulting in a reduced DFS ($P = .031$).

Table 4

Multivariate analyses of prognostic markers for OS and DFS in 87 non-M3 patients who received induction therapy.

	DFS		OS	
Prognostic markers	**Hazard ratio (95% CI)**	***P***	**Hazard ratio (95% CI)**	***P***
CXCR4 RFI >4.23	–	–	1.968 (1.006–3.853)	.048*
Age >50	–	–	–	–
WBC >20	2.328 (0.943–4.764)	.063	2.109 (1.167–3.815)	.018*
Cytogenetic risk	–	–		.106
Favorable vs intermediate	–	–	0.441 (0.207–0.952)	.037*

Univariable binary logistic regression analysis was used.

CI = confidence interval, CXCR4 RFI = relative fluorescence intensity of chemokine Receptor 4, DFS = disease-free survival, OS = overall survival, WBC = white blood cells.

* $P < .05$, with statistical significance. In non-M3 patients, through multivariate Cox analysis, CXCR4high expression (RFI>4.23) and high WBC count ($>20 \times 10^9$/L) were independent markers of poor OS ($P = .048$ and $P = .018$, respectively), the cytogenetic risk (favorable) were independent markers of good OS ($P = .037$).

protected by the stromal cells from cytotoxic chemotherapeutics and represented a reservoir for minimal residual disease and relapse through SDF-1α/CXCR4 bio-axis. Thus, it will be intriguing to further investigate the potential correlation of CXCR4 and MRD in AML.

The current study also demonstrated that the $CXCR4^{high}$ expression group (RFI >4.23) showed higher NPM1 and FLT3-ITD mutation rate than the $CXCR4^{low}$ expression group (RFI ≤4.23). Also, patients harboring NPM1 and FLT3-ITD mutation had a tendency of high CXCR4 expression. It remains controversial whether CXCR4 expression is associated with NPM1 and FLT3-ITD mutation in AML patients. In a study of 142 AML patients, NPM1 mutation was significantly correlated with higher CXCR4 expression, indicating that NPM1-wild-type (wt) down-regulated CXCR4 expression.[21] In another study of 117 patients who were tested for NPM1 mutations by PCR, there was no correlation between CXCR4 expression and NPM1 mutation.[36] It has been reported that CXCR4 expression was significantly higher in FLT3-ITD AML than in FLT3/wt AML.[37] The possible mechanism was that FLT3-ITD may activate CXCR4 signaling, thereby further affecting the function of the SDF-1α/CXCR4 axis. Therefore, our data provide important

Table 5

Univariate and multivariate analyses prognostic markers for relapse patients who achieved complete remission (CR) after chemotherapy.

		Univariable			Multivariable		
Relapse (RP/CR NO)	**Prognostic marker**	**OR**	**95%CI**	***P***	**OR**	**95%CI**	***P***
AML (36/74)	CXCR4	1.095	1.021–1.173	.010*	1.090	1.021–1.166	.010*
	Risk status			.269	–	–	–
	Age	1.017	0.984–1.052	.314	–	–	–
	M4/5	3.900	1.332–11.423	.007*	3.240	1.324–7.932	.010*
Non-M3 (32/58)	CXCR4	1.069	0.999–1.144	.052	1.068	1.001–1.140	.048*
	Risk status			.385	–	–	–
	Age	1.000	0.972–1.029	.980	–	–	–
	M4/5	3.900	1.332–11.423	.035*	2.703	1.064–6.867	.037*

Univariable and multivariable binary logistic regression analysis were used.

AML = acute myeloid leukemia, CI = confidence interval, CXCR4 = chemokine Receptor 4, M4/5 = M4/M5 FAB subtypes, OR = odds ratio, risk status = (Favorable vs intermediate; Favorable vs adverse; Intermediate vs adverse); RP/CR NO = the number of relapsed and complete remission patients.

* $P < .05$, with statistical significance. Univariable analysis indicated that increased CXCR4 expression levels and higher incidence of M4/M5 subtypes were significantly correlated with high relapse probability ($P = .010$ and $P = .007$, respectively) in all AML CR patients, only higher incidence of M4/M5 subtypes was significantly correlated with high relapse probability in the non-M3 CR patients ($P = .035$). Multivariable analysis indicated that high CXCR4 expression levels independently increased relapse risk both in all AML and non-M3 CR patients ($P = .010$ and $P = .048$, respectively), so dose higher incidence of M4/M5 subtypes ($P = .010$ and $P = .037$, respectively).

information on utilizing CXCR4 inhibition as a targeted therapy in FLT3-ITD AML patients. It is worth mentioning that, due to the diagnosis time limit of the cases included in this study, the latest WHO stratified criteria for prognosis cannot be used. We hope to make up for this regret in future studies.

Collectively, our findings imply that CXCR4 overexpression (RFI >4.23) is an independent prognostic factor for disease relapse and poorer OS in both all AML and non-M3 patients. The levels of CXCR4 expression can be determined rapidly and easily at disease presentation by the flow. Therefore, CXCR4 could be used as a potential therapeutic target in AML patients with poor prognosis.

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