The N-terminal CEBPA mutant in acute myeloid leukemia impairs CXCR4 expression

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Running head: C/EBPα regulates CXCR4 transcription
Abstract

CXC chemokine receptor 4 (CXCR4) is an essential regulator for homing, and maintenance of hematopoietic stem cells within the bone marrow niches. In the analyses of clinical implications of bone marrow CXCR4 expression in patients with acute myeloid leukemia, we not only found higher CXCR4 expression was an independent poor prognostic factor, irrespective of age, white blood cell counts, cytogenetics, and mutation status of NPM1/FLT3-ITD and CEBPA, but also showed CXCR4 expression was inversely associated with mutations of CEBPA, a gene encoding transcription factor C/EBPα. Patients with wild-type CEBPA had significantly higher CXCR4 expression than those with mutated CEBPA. We hypothesized that CEBPA might influence the expression of CXCR4. To test the hypothesis, we first examined endogenous CXCR4 expression in 293T and K562 cells overexpressing wild-type C/EBPα p42 and demonstrated that CXCR4 levels were increased in these cells, whilst the expression of the N-terminal mutant, C/EBPα p30, diminished CXCR4 transcription. We further showed p42 was bound to the CXCR4 promoter by the chromatin immunoprecipitation assays. Induction of p42 in the inducible K562-C/EBPα cell lines increased the chemotactic migration. Moreover, decreased expression of C/EBPα by RNA interference decreased levels of CXCR4 protein expression in U937 cells, thereby abrogating CXCR4-mediated chemotaxis. Our results provide, for the first time, evidences that C/EBPα indeed regulates the activation of CXCR4, which is critical for the homing and engraftment of acute myeloid leukemia cells, while p30 mutant impairs CXCR4 expression.
Introduction

CXCR4 is a rhodopsin-like G protein-coupled receptor and selectively binds the CXC chemokine CXCL12 [Stromal Cell-Derived Factor 1 (SDF-1)]. CXCR4 is expressed on multiple cell types including hematopoietic stem cells, lymphocytes, endothelial and epithelial cells. The SDF-1/CXCR4 pathway is involved in tumor progression, angiogenesis, metastasis, and cell survival. The expression of CXCR4 on leukemic cells as well as on malignant epithelial cells plays a crucial role in directing the metastasis of tumor cells to organs that express SDF-1. Several CXCR4 positive cancers were proved to metastasize to the bones and lymph nodes in a SDF-1-dependent manner in which the bone marrow (BM) in particular provided a protective environment for tumor cells. Furthermore, in mouse models, acute myeloid leukemia (AML) cells were shown to express functional CXCR4 that induced chemotaxis and migration of leukemia cells beneath BM stromal cells. Expression of CXCR4 in leukemic cells was associated with cell cycle arrest and reduced numbers of cell divisions, providing a potential mechanism for leukemia cells to evade the cell-killing effect of chemotherapy. Higher expression of CXCR4, measured by either immunohistochemical method or flow cytometry, has been identified as a poor prognostic marker for AML and may be an attractive target for the development of novel therapeutic approaches. However, whether the prognostic implication of higher CXCR4 expression on survival is independent from other prognostic factors, such as recently found genetic markers FLT3-ITD, NPM1 and CEBPA mutations, remains unclear.

AML is a heterogeneous hematologic malignancy characterized by proliferation but impaired differentiation of myeloid progenitors. The leukemogenesis is a
multi-step process involving accumulated genetic abnormalities and epigenetic
deregulations that perturb gene expression and disrupt cell differentiation.\textsuperscript{10} 
Transcription factors are main targets of mutations in AML. CCAAT enhancer binding
protein alpha (C/EBP\(\alpha\)) is a 42-kDa transcription factor that contains two
transactivation domains (TAD, TAD1 and TAD2) in the amino terminus and a basic
leucine zipper domain (bZIP) at its carboxy terminus for DNA binding.\textsuperscript{11,12} Mutations
in one or both alleles of \textit{CEBPA} are reported in about 7\% to 15\% of patients with
AML.\textsuperscript{13,14} These mutations can be divided into two major categories: one comprises
C-terminal mutations that disrupt the bZIP region and the other comprises N-terminal
mutations that disrupt the reading frame, resulting in translation of a 30-kDa C/EBP\(\alpha\)
p30 isoform. The N-terminal truncated mutant was shown to have a dominant-negative
effect.\textsuperscript{11,12} Though AML patients with C/EBP\(\alpha\) mutant have favorable prognosis,\textsuperscript{13,15}
the molecular mechanisms how \textit{CEBPA} mutations contribute to better treatment
response and improved outcomes are not yet fully understood.

In the present study, we analyzed the BM \textit{CXCR4} expression by quantitative
real-time polymerase chain reaction (RT-QPCR) in a cohort of 220 adult patients with
\textit{de novo} AML and found higher \textit{CXCR4} expression was an independent poor
prognostic factor. Moreover, \textit{CXCR4} expression was inversely associated with \textit{CEBPA}
mutation. In the study of the mechanism of this relationship between \textit{CXCR4}
expression and \textit{CEBPA} mutation, we found that C/EBP\(\alpha\) activates \textit{CXCR4}
transcription through direct binding to its promoter. We further demonstrated that the
overexpression of C/EBP\(\alpha\) increased SDF1-mediated directional migration of
leukemic cells, while the depletion of C/EBP\(\alpha\) diminished cell migration. These
results indicate a role of C/EBP\(\alpha\) in transcriptional control of \textit{CXCR4} gene expression
and emphasize the importance of this transcription factor in the regulation of
chemotactic SDF-1/CXCR4 axis in AML cells.

Methods

Patients and samples

Totally, 220 adult patients who were newly diagnosed as having de novo AML at the National Taiwan University Hospital (NTUH), had enough cryopreserved cells for molecular analyses, and had complete clinical and laboratory data were recruited for this study. Thirty healthy BM transplantation (BMT) donors were also enrolled as normal controls. Among them, one hundred and fifty-one (68.6%) patients received standard chemotherapy and were included for survival analysis.16,17 This study was approved by the Institutional Review Board of the NTUH; and written informed consent was obtained from all participants in accordance with the Declaration of Helsinki.

Reverse transcription-quantitative polymerase chain reaction (RT-QPCR) analysis of patient samples

BM mononuclear cells from 220 patients before chemotherapy and 30 healthy BMT donors were isolated and cryopreserved until use. Total RNA was extracted and reverse transcribed. The gene expression level was quantified utilizing TaqMan technology on the Applied Biosystem 7500 Fast Real-Time PCR System as described previously.18 Gene-specific primers and probe of CXCR4 were available as TaqMan Gene Expression Assay (Assay ID,Hs02330069_s1, Applied Biosystems). Each sample was tested at least twice independently. The amount of the target gene was normalized to that of the housekeeping gene RPLP0. The copies of target gene were quantified only after successful amplification of the internal control, using the standard curves derived from cloned plasmids. All data were presented as log ratio of the target
gene/RPLP0.

**Mutation analysis**

Mutation analyses of 17 relevant molecular alterations, including Class I mutations, such as FLT3/ITD and FLT3/TKD, NRAS, KRAS, JAK2, KIT and PTPN11 mutations and Class II mutations, such as MLL/PTD, CEBPA and RUNX1 mutations, as well as mutations in NPM1, WT1 and those genes related to epigenetic modification, such as ASXL1, IDH1, IDH2, TET2 and DNMT3A, were performed as previously described. Abnormal sequencing results were confirmed by at least 2 repeated analyses.

**Statistical analysis**

The discrete variables of patients with lower and higher BM CXCR4 expression were compared using the Chi-square tests or Fisher’s exact test. We used Mann–Whitney U-test to compare continuous variables and medians of distributions. Whole patient population was included for analyses of the correlation between CXCR4 expression and clinical characteristics, however, only those receiving conventional standard chemotherapy, as mentioned above, were included in analyses of survivals. Overall survival (OS) was measured from the date of first diagnosis to death from any cause or the last follow-up, whereas relapse was defined as a reappearance of at least 5% leukemic blasts in a BM aspirate or new extramedullary leukemia in patients with a previously documented remission. We adopted Kaplan–Meier estimation to plot survival curves, and used log-rank tests to examine the difference between groups. Relative risk (RR) and 95% confidence interval (CI) were estimated by Cox proportional hazards regression models to determine independent risk factors associated with survival in multivariate analyses. Two-sided P-values less than 0.05 were considered statistically significant. All statistical analyses were accomplished
Results

Correlation of BM CXCR expression with clinical and biological features and treatment outcome

The median value of BM CXCR4 expression in AML patients was used as the cut-off point to define lower- and higher-expression groups. Patients with higher BM CXCR4 expression were male predominant, older and had lower serum lactate dehydrogenase levels than those with lower expression (Table 1). Higher BM CXCR4 expression was more frequently found in patients with unfavorable-risk cytogenetics than in those with favorable-risk or intermediate-risk cytogenetics (P=0.0013) (Table 1). Patients with higher BM CXCR4 expression had a significantly higher incidences of NPM1 mutations (33.6% vs. 13.6%, P=0.0008), but a lower incidence of CEBPA mutations, either single or biallelic mutations (CEBPA\textsuperscript{double-mut}), than those with lower CXCR4 expression (6.4% vs. 18.2%, P=0.0125) (Table 2). In other words, patients with CEBPA mutation had significantly lower CXCR4 expression than those without the mutation (P=0.0019) (Supplementary Figure 1). Of the 151 AML patients undergoing conventional intensive induction chemotherapy, 111 (73.5%) patients achieved a complete remission (CR). The patients with higher BM CXCR4 expression had a trend of lower CR rate than those with lower expression (67.9% vs. 80.6%, P=0.0956, Table 1). With a median follow-up duration of 32 months (range 0.1 to 160), patients with higher BM CXCR4 expression had a significant shorter OS than those with lower expression (median 20 months vs. not reached, P=0.030) (Supplementary Figure 2). The same was also true among the patients with non-M3 AML (P=0.039).
Further, higher BM CXCR4 expression was an independently poor prognostic factor (RR 1.849, 95% CI 1.018-3.357, P=0.043) irrespective of age, white blood cell (WBC) counts, cytogenetics and other genetic markers NPM1/ FLT3-ITD and CEBPA mutations (Table 3).

**Enforced expression of wild-type C/EBPα activated CXCR4 expression**

Because patients with CEBPA mutations had lower CXCR4 expression than those without the mutation, we hypothesized that C/EBPα might regulate the expression of CXCR4, while its mutants lost the ability. To test this hypothesis, we first examined the relationship between CXCR4 and CEBPA expression using publicly available microarray data from the German AML Cooperative Group (AMLCG). Gene expression profiling data from forty-six patients with wild-type CEBPA were selected for the Pearson's correlation test. A significant linear correlation was observed between CEBPA and CXCR4 expression in these cases (R=0.46, P=0.003, Supplementary Figure 3). We then analyzed the sequence of CXCR4 and found that the CXCR4 promoter contains several conserved C/EBPα binding motifs, suggesting that C/EBPα may participate directly in the regulation of CXCR4 expression. To address the question, we generated wild-type C/EBPα expression vector pCMV-Flag-p42, the N-terminal truncated mutant pCMV-Flag-p30 and the C-terminal mutant pCMV-Flag-CTM (Figure 1A). We then examined the expression of CXCR4 protein and mRNA by Western blot analysis and RT-QPCR, respectively in HEK293T cells transfected with these C/EBPα expression vectors. As shown in Figure 1, overexpression of wild-type C/EBPα in HEK293T cells increased endogenous CXCR4 protein and mRNA level. In contrast, the expression of p30 diminished CXCR4 transcription (Figure 1B and 1C). To understand whether the enforced
expression of C/EBPα can directly activate the CXCR4 promoter activity, we prepared four reporter plasmids in which 2.3 kb, 0.8 kb, 0.3 kb and 0.2 kb of the human CXCR4 promoter were linked to the luciferase gene (Figure 2A). Similarly, co-transfection of pCMV-Flag-p42 expression construct increased the relative luciferase activity of the CXCR4 promoter (Figure 2B). However, p42 no longer activated CXCR4 promoter when all its C/EBPα binding sites were deleted (Figure 2B), indicating that the activation of CXCR4 transcription by C/EBPα p42 might be through these consensus motifs. On the other hand, the expression of p30 did not increase the CXCR4 promoter activity (Figure 2C), consistent with the data obtained from RT-PCR and Western blot analysis shown above (Figure 1B, 1C). The expression level of each Flag-tagged C/EBPα isoforms from one representative experiment was shown in Figure 2D. These data clearly show C/EBPα p42 as an activator of the CXCR4 promoter.

**C/EBPα activates the CXCR4 transcription through direct binding to its promoter**

To examine the functional contribution of these putative C/EBPα binding sites to the CXCR4 promoter activity, we introduced mutation at those sites in the pGL2-CXCR4-0.8Kb reporter construct and measured the promoter activity mediated by C/EBPα p42 using luciferase activity assay. As shown in Figure 3A, the mutation at S1 or S2 site did not abolish the C/EBPα p42-mediated CXCR4 promoter activity. In contrast, the promoter activity decreased significantly when the S3 (-231/-246) site of the CXCR4 promoter was mutated, indicating the exclusive role of S3 site for C/EBPα p42-mediated transcriptional activation of CXCR4.

We then determined whether C/EBPα p42 could bind at the S3 site by gel-shift assays using the biotin-labeled double-stranded oligonucleotide covering S3 sequence
of the *CXCR4* promoter. Incubation of this probe with nuclear extract of HEK293T cells ectopically expressing C/EBPα p42 gave rise to the formation of a DNA-protein complex. This complex was shifted to higher molecular weight sites by the addition of the Flag peptides specific M2 or C/EBPα antibody, indicating the presence of C/EBPα and thus the bound antibody in the complex, but not by the GAPDH antibody (Figure 3B, lane 3-5, 11-12). The C-terminal mutant (CTM) also formed a complex with the probe (Figure 3B, lane 6). In contrast, p30 did not bind to the S3 probe (Figure 3B, lane 7). In addition, co-incubation of nuclear lysate containing p30 diminished the binding of p42 or CTM to the S3 probe (Figure 3B, lanes 9 and 10).

Next, we performed ChIP to test whether C/EBPα is directly involved in regulating the *CXCR4* promoter in HEK293T cells expressing Flag-p42 or Flag-p30. Primers specific to the *CXCR4* promoter were used for the PCR reaction. The result showed that wild-type C/EBPα p42 was associated with the *CXCR4* promoter, whereas the mutant p30 was not (Figure 3D). Similar results were observed when ChIP assays were performed using primers specific for the *Albumin* promoter in the PCR, consistent with the previous report that C/EBPα binds to the *Albumin* promoter.32 PCR using β-actin promoter primers was performed as the negative control. Based on these data, we conclude that C/EBPα is a direct activator of the *CXCR4* promoter.

**Ectopic expression of C/EBPα p42 increased the chemotactic migration in K562 cells in response to SDF-1 treatment**

To test whether ectopic expression of C/EBPα p42 or p30 in AML cells affects leukemic cell migration, the chemotaxis assays were performed. The inducible K562-C/EBPα cells stably expressed wild type or mutated C/EBPα were established. The pTripz-C/EBPα−p42 or p30 plasmids were packaged using HEK293T cells and
transduced into K562 cells as described.\textsuperscript{33} We used K562 cells because they do not express endogenous C/EBP\textalpha.\textsuperscript{34} Expression of C/EBP\textalpha p42 and C/EBP\textalpha p30 after the cells were exposed to doxycycline was successfully confirmed by Western blot analysis (Figure 4A).

To test whether the p42-mediated transcriptional activation of the \textit{CXCR4} gene can also be detected in the K562-C/EBP\textalpha p42 stable cell line, Western blot and RT-qPCR analysis were performed in these cells with or without doxycycline treatment. As shown in Figure 4A-B, CXCR4 protein and mRNA level were increased in K562-C/EBP\textalpha p42 cells after doxycycline treatment for 24 h. In contrast, expression of \textit{CXCR4} was not activated when p30 was induced. Protein and \textit{CXCR4} mRNA levels were not affected by doxycycline treatment in K562 parental cells. CXCR4 surface protein expression was also assessed using flow cytometry. As expected, surface CXCR4 expression was significantly increased in K562-C/EBP\textalpha p42 cells, but not K562- C/EBP\textalpha p30 cells, after doxycycline treatment. U937 cells were used as the positive control for the assay (Figure 4C).

To further assess the \textit{in vitro} functional responses of K562-C/EBP\textalpha stable cell lines, the migration of the cells in response to the SDF-1 treatment after C/EBP\textalpha was induced was examined. Consistent with increase of CXCR4 expression, induction of p42 in K562 cells significantly increased the migration index of these cells towards SDF-1. This chemotactic migration could be abolished by the treatment of AMD3100, a specific inhibitor for CXCR4. In contrast, induction of p30 did not affect the chemotactic migration toward SDF1 in K562 cells (Figure 4D).

\textbf{Depletion of \textit{CEBPA} reduced chemotactic migration toward SDF-1 in U937 cells}

Finally, we tested whether depletion of \textit{CEBPA} in U937 cells reduce chemotactic
migration toward SDF-1. We used U937 cells because they express high levels of endogenous C/EBPα.\textsuperscript{34} Inducible U937 cell lines stably transfected with \textit{shCEBPA} (U937-\textit{shCEBPA}) or scramble control plasmid were established. Cells treated with doxycycline were subjected to Western blot analysis and \textit{in vitro} migration assay. As shown in Figure 4E, C/EBPα protein level reduced with a concurrent decrease of CXCR4 expression in U937-\textit{shCEBPA} cells after doxycycline treatment. Furthermore, the depletion of C/EBPα significantly decreased the migration index in response to SDF-1 (Figure 4F). These data suggest that wild-type C/EBPα induces CXCR4 expression and in turn increases the SDF-1-mediated directional migration of leukemic cells.

**Discussion**

In this study, we found \textit{CXCR4} expression was negatively associated with \textit{CEBPA} mutation. Moreover, to the best of our knowledge, this study was the first to demonstrate a role of C/EBPα in transcriptional control of \textit{CXCR4} gene expression and emphasize the importance of this transcription factor in the regulation of chemotactic SDF-1/CXCR4 axis in AML cells.

In addition to genetic and epigenetic aberrations of hematopoietic progenitors, impaired BM microenvironment may also contribute to the development of AML.\textsuperscript{35} BM microenvironment provides support for self-renewal, homing, engraftment and proliferative potential of hematopoietic stem cells, in which \textit{SDF-1/CXCR4} axis plays an essential role. Emerging data also show that adhesion to the BM stromal cells affects survival and proliferation of AML cells.\textsuperscript{36} So, it is prudent to investigate the clinical implications of CXCR4 in AML. In this study, we identified AML patients with higher BM \textit{CXCR4} expression had distinct clinical and laboratory characteristics.
and poorer outcome. In addition, higher BM CXCR4 expression was a poor prognostic factor independent of age, cytogenetic and gene mutations. Furthermore, we demonstrated for the first time an inverse relationship between CEBPA mutations and expression of CXCR4. The finding that CXCR4 mRNA levels dropped in patients with mutant CEBPA, compared to those with wild-type CEBPA, suggests that C/EBPα may participate in regulating CXCR4 gene expression.

In the current study, we provided several evidences to demonstrate that wild-type C/EBPα truly regulates the expression of CXCR4, while mutant p30 loses this action. Firstly, enforced expression of wild type C/EBPα-p42 increased CXCR4 protein and mRNA levels in 293T and K562 cells (Figures 1B and 1C and 4A and 4B) and it also activated the promoter activity of CXCR4 gene in 293T cells, while mutant p30 did not (Figure 2). Secondly, ChIP assay indicated the binding of C/EBPα-p42, but not p30, in the CXCR4 promoter region (Figure 3D). Thirdly, we located the functional C/EBPα binding site at -231/-246 within the promoter region of CXCR4 gene (Figure 3B) and observed that mutation at the S3 (-231/-246) site caused a reduction of C/EBPα-p42-mediated activation of CXCR4 promoter (Figure 3A). Taken together, our results demonstrated that C/EBPα-p42 is a direct activator of CXCR4 transcription through direct binding to the CXCR4 promoter region, whereas the C/EBPα-p30 isoform cannot bind to the same region in vitro and in vivo. Furthermore, p30 diminished the binding of p42 or CTM to the S3 probe in the gel-shift assays (Figure 3B, lanes 9 and 10) suggests that p30 acts as a dominant-negative isoform in the regulation of CXCR4 transcription. Similarly, Cleaves et al found the relative affinity of C/EBPα-p30 for C/EBP-binding sites on the GR and PU.1 genes were reduced compared with C/EBPα-p42. In the NOD/SCID-leukemia mouse model, CXCR4 was reported to participate in the migration, repopulation, and development of AML.
cells in the BM by regulating their anchorage to the stromal microenvironment and cell survival.\textsuperscript{6} How does the effect of \textit{CEBPA} mutant on CXCR4 expression contribute to the leukemogenesis awaits further study.

We and others reported that AML patients with \textit{CEBPA} mutation had favorable survival.\textsuperscript{13,14,38} The better prognosis in AML with \textit{CEBPA} mutation may be partially explained by the lower \textit{CXCR4} expression in these patients. \textit{CXCR4}-mediated contact between leukemia cells and stromal cells has been shown to result in cell cycle arrest and reduced numbers of cell divisions, providing a potential mechanism for leukemia cells to escape chemotherapy effect.\textsuperscript{2} In mouse models, \textit{CXCR4} antagonists were demonstrated to induce the mobilization of AML cells from the protective stromal microenvironments into the circulation and enhance the sensitivity of the tumor cells to chemotherapy.\textsuperscript{4,39} Moreover, in a phase 1/2 study, the addition of Plerixator, a \textit{CXCR4} antagonist, to cytotoxic chemotherapy increased the rate of remission.\textsuperscript{40} Our in vitro data that blockage of C/\textit{EBPα}-p42 induced \textit{CXCR4} expression indeed reduces directional migration of leukemic cells in response to the SDF-1 treatment also support the hypothesis. Compatible with these findings, AML patients with \textit{CEBPA} mutation, in whom most (20 among 27 patients, Table 2) show lower \textit{CXCR4} expression, have a higher CR rate (86.3\% vs. 71.3\%) and longer overall survival (median, not reached vs. 22 months) than other patients (data not shown). However, there must be additional reasons to explain the better clinical outcome in \textit{CEBPA}-mutated patients since both \textit{CEBPA} mutation and \textit{CXCR4} expression are independent prognostic factors in this study. Further studies are needed to answer the question. It also remains to be determined whether patients with \textit{CEBPA} mutation, who already have lower \textit{CXCR4} expression, will not get further improvement in survival from the treatment of \textit{CXCR4} antagonists.
It is interesting to note that a subset of patients harboring wild-type \textit{CEBPA} showed low CXCR4 expression. We suggest that other mechanisms might affect the activity of C/EBP\textalpha in these cases, such as phosphorylation on serine 21 or SUMOylation on lysine 161 of C/EBP\textalpha.\textsuperscript{41} In addition to dysfunction of C/EBP\textalpha, recent report demonstrated that CXCR4-Serine 339 phosphorylation is a prognostic marker in AML patients and a critical regulator of migration, homing and retention of leukemic cells.\textsuperscript{42} Therefore, dysfunction of CXCR4-Serine 339 phosphorylation might be the alternative way of CXCR4 deregulation in AML.

In summary, the current study shows a close association of \textit{CEBPA} mutation with lower CXCR4 expression and provides novel evidences that C/EBP\textalpha regulates the activation of \textit{CXCR4}, while C/EBP\textalpha mutant p30 loses the ability. Since CXCR4 blockage in AML cells may disrupt their interaction with the BM niche and sensitizes them to chemotherapy, patients with higher CXCR4 expression may get benefits from the treatment of CXCR4 antagonists, but whether patients with \textit{CEBPA} mutation, who already have lower CXCR4 expression, need this kind of therapy remains to be determined.

\textbf{Acknowledgements}

We thank Dr. Wilhelm Krek for providing \textit{CXCR4} promoter reporter constructs. This work was partially sponsored by grants NSC 100-2314-B-002-112-MY3 and 100-2628-B-002-003-MY3 from the National Science Council (Taiwan), MOHW103-TD-B-111-04 from the Department of Health (Taiwan) and NTUH 102P06, UN101-014 and 102-015 from the Department of Medical Research, National Taiwan University Hospital and the Scholarship from the Taiwan Society of Hematology.
Authorship and Disclosures

Y.-Y. K. designed the study, performed the experiments, analyzed and interpreted data, and wrote the manuscript. H.-A.H. designed and planned the study, contributed data management, statistical analysis and wrote the manuscript. Y.-K.C. planned the study, contributed data management, and wrote the manuscript. C.-Y.L was responsible for statistical analysis and interpretation of the statistical findings. L.-Y.L., P.-H.C., M.-H.T., C.-F.H., F.-Y.L., M.-C.L. and C.-W.L. performed the experiments and interpreted data. W.-C.C., J.-L.T and M.Y. participated in data collection and provision of patients. H.-F.T. designed and planned the study, wrote the manuscript and coordinated the study over the entire period. The authors declare no competing financial interests.
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Table 1. Comparison of clinical manifestations, treatment response and cytogenetic changes between AML patients with lower and higher BM CXCR4 expression*

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total (n=220)</th>
<th>Lower CXCR4 expression (110, 50%)</th>
<th>Higher CXCR4 expression (110, 50%)</th>
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<td>Age (year):‡</td>
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<td>50.5 (15-86)</td>
<td>60 (15-90)</td>
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<td>Laboratory data:‡</td>
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<td>WBC (/μL)</td>
<td>23275 (380-42300)</td>
<td>24620 (380-175900)</td>
<td>22160 (890-423000)</td>
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<td>Hemoglobin (g/dL)</td>
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<td>7.8 (3.3-16.2)</td>
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<td>Platelet (×1,000 /μL)</td>
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<td>39.5 (2-331)</td>
<td>43 (6-455)</td>
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<td>Blast (/μL)</td>
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<td>12620 (0-162750)</td>
<td>6636 (0-369070)</td>
<td>0.1646</td>
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<td>LDH (U/L)</td>
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<td>963 (298-5559)</td>
<td>785 (271-13130)</td>
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<td>Induction response†△</td>
<td>151</td>
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<td>84</td>
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<td>CR</td>
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<td>Relapse</td>
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<td>25 (46.3)</td>
<td>28 (49.1)</td>
<td>0.8499</td>
</tr>
<tr>
<td>Karyotypeζ</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Favorable</td>
<td>44</td>
<td>30</td>
<td>14</td>
<td>0.0106</td>
</tr>
<tr>
<td>Intermediate</td>
<td>146</td>
<td>72</td>
<td>74</td>
<td>0.6544</td>
</tr>
<tr>
<td>Unfavorable</td>
<td>20</td>
<td>4</td>
<td>16</td>
<td>0.0045</td>
</tr>
<tr>
<td>Unknown</td>
<td>10</td>
<td>4</td>
<td>6</td>
<td></td>
</tr>
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</table>

*The median value of CXCR4 expression in total population was used as the cut-off point to define lower- and higher-expression groups.
†Number (%) of patients
‡Median (range)
△Only the 151 patients who received conventional intensive induction chemotherapy and then consolidation chemotherapy if CR was achieved, were included in the analysis.
ζTwo hundred and ten patients, including 104 higher CXCR4 and 106 lower CXCR4 patients, had chromosome data at diagnosis. There was no difference of cytogenetic changes such as simple, complex, t(8;21), t(15;17), inv(16), t(11q23), t(7;11), -5/-5q- and -7/-7q- between patients with higher and lower CXCR4 expression.

Abbreviation: CR, complete remission; PR, partial remission.
Table 2. Comparison of genetic alterations between AML patients with lower and higher BM CXCR4 expression

<table>
<thead>
<tr>
<th>Variables</th>
<th>Number (%) of patients with the gene mutation</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Whole cohort (n=220)</td>
<td>Lower CXCR4 expression (110, 50%)</td>
<td>Higher CXCR4 expression (110, 50%)</td>
</tr>
<tr>
<td>FLT3/ITD</td>
<td>55 (25)</td>
<td>32 (29.1)</td>
<td>22 (20)</td>
</tr>
<tr>
<td>FLT3/TKD</td>
<td>17 (7.7)</td>
<td>6 (5.5)</td>
<td>11 (10)</td>
</tr>
<tr>
<td>NRAS</td>
<td>26 (11.8)</td>
<td>11 (10)</td>
<td>15 (13.6)</td>
</tr>
<tr>
<td>KRAS</td>
<td>7 (3.2)</td>
<td>3 (2.7)</td>
<td>4 (3.6)</td>
</tr>
<tr>
<td>PTPN11</td>
<td>14 (6.4)</td>
<td>5 (4.5)</td>
<td>9 (8.2)</td>
</tr>
<tr>
<td>KIT</td>
<td>9 (4.1)</td>
<td>6 (5.5)</td>
<td>3 (2.7)</td>
</tr>
<tr>
<td>JAK2</td>
<td>1 (0.5)</td>
<td>0</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>WTI</td>
<td>13 (5.9)</td>
<td>7 (6.4)</td>
<td>6 (5.5)</td>
</tr>
<tr>
<td>NPM1</td>
<td>52 (23.6)</td>
<td>15 (13.6)</td>
<td>37 (33.6)</td>
</tr>
<tr>
<td>CEBPA</td>
<td>27 (12.3)</td>
<td>20 (18.2)</td>
<td>7 (6.4)</td>
</tr>
<tr>
<td>CEBPA&lt;sup&gt;double-mut&lt;/sup&gt;</td>
<td>18 (8.2)</td>
<td>15 (13.6)</td>
<td>3 (2.7)</td>
</tr>
<tr>
<td>RUNX1</td>
<td>29 (13.2)</td>
<td>15 (13.6)</td>
<td>14 (12.7)</td>
</tr>
<tr>
<td>MLL/PTD</td>
<td>13 (5.9)</td>
<td>8 (7.3)</td>
<td>5 (4.5)</td>
</tr>
<tr>
<td>IDH1</td>
<td>13 (5.9)</td>
<td>5 (4.5)</td>
<td>8 (7.3)</td>
</tr>
<tr>
<td>IDH2</td>
<td>31 (14.1)</td>
<td>11 (10)</td>
<td>20 (18.2)</td>
</tr>
<tr>
<td>ASXL1</td>
<td>23 (10.5)</td>
<td>9 (8.2)</td>
<td>14 (12.7)</td>
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<tr>
<td>TET2</td>
<td>32 (14.5)</td>
<td>16 (14.5)</td>
<td>16 (14.5)</td>
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<tr>
<td>DNMT3A</td>
<td>36 (16.4)</td>
<td>14 (12.7)</td>
<td>22 (20)</td>
</tr>
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### Table 3. Multivariate analysis (Cox regression) on the overall survival in AML patients

<table>
<thead>
<tr>
<th>Variables</th>
<th>Hazard ratio</th>
<th>95% CI</th>
<th>P value</th>
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<tr>
<td>Age*</td>
<td>3.490</td>
<td>1.945-6.264</td>
<td>&lt;0.001</td>
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<tr>
<td>WBC†</td>
<td>2.379</td>
<td>1.337-4.232</td>
<td>0.003</td>
</tr>
<tr>
<td>Karyotype‡</td>
<td>3.402</td>
<td>1.001-11.562</td>
<td>0.049</td>
</tr>
<tr>
<td>CXCR4∆</td>
<td>1.849</td>
<td>1.018-3.357</td>
<td>0.043</td>
</tr>
<tr>
<td>NPM1/FLT3-ITD®</td>
<td>0.468</td>
<td>0.179-1.222</td>
<td>0.121</td>
</tr>
<tr>
<td>CEBPAπ</td>
<td>0.227</td>
<td>0.054-0.957</td>
<td>0.043</td>
</tr>
</tbody>
</table>

*Age older than 50 years relative to age 50 years or younger.
†WBC greater than 50,000/μL vs. less than or equal to 50,000/μL.
‡Unfavorable-risk cytogenetics vs. others.
∆Higher BM CXCR4 expression vs. lower CXCR4 expression.
®NPM1+/FLT3-ITD− vs. other subtypes.
πCEBPA double-mut vs. other subtypes.
Figure Legends

**Figure 1. Ectopic expression of C/EBPα activates the CXCR4 gene expression.** (A) Schematic diagram of C/EBPα expression constructs. The N-terminal mutation is represented by the p30 isoform (C/EBPα-p30). C-terminal mutation (C/EBPα-CTM) contains an amino acid insertion p.V314_L315insV. Diagrams were constructed using Domain Graph (DOG), version 2.0.43. (B) 293T cells were transfected with pCMV-tag2B (control), pCMV-Flag-p42, pCMV-Flag-p30 or pCMV-Flag-CTM. After 48 h, cells were then harvested and analyzed by Western blotting with anti-M2, CXCR4, or GAPDH antibody. (C) A parallel sets of cells were harvested for RNA preparation followed by RT-QPCR analysis using primers specific for human CXCR4 and GAPDH. Data represent mean ± s.d. (n=3), * P < 0.05, ** P < 0.01.

**Figure 2. Wild-type C/EBPα, but not that with deletion of putative C/EBPα binding sites, activates CXCR4 promoter.** (A) Schematic representation of the human CXCR4 promoter reporter constructs. Putative C/EBPα binding sites are shown as boxes. (B) 293T cells were co-transfected with the human CXCR4 promoter reporter constructs (full length or that with deletion of putative C/EBPα binding sites) and pRL-TK in the presence of pCMV-tag2B empty vector (EV), as control, or pCMV-Flag-p42 as indicated. The luciferase activities normalized by Renilla luciferase activities were calculated relative to that in the cells transfected with
full-length **CXCR4** promoter reporter construct, pGL2-**CXCR4-2.6K**, and the control vector, which was set arbitrarily at 1. Values are the averages of three independent determinations. Data represent mean ± s.d. (n=3), * P < 0.05, ** P < 0.01. (C) 293T cells were co-transfected with pGL2-2.6K constructs in the presence of C/EBPα expression plasmids (N-terminal or C-terminal mutants, wild-type C/EBPα, or empty vector). The luciferase activities were determined as described in panel B. (D) Western blot data showing the expression of Flag-tagged C/EBPα isoforms in those cells described in panel C.

**Figure 3. C/EBPα p42 activates CXCR4 transcription through direct binding to the proximal C/EBPα binding site in the CXCR4 promoter.** (A)

C/EBPα-dependent transactivation of the **CXCR4** promoter. Human **CXCR4** 0.8 kb promoter fragment containing wild-type C/EBPα binding sites (indicated as boxes) or mutated sites (indicated as X) were linked to firefly luciferase gene and were co-transfected with the C/EBPα-p42 expression or control vector in 293T cells. The luciferase activities normalized by Renilla luciferase activities were calculated relative to that in the cells transfected with wild-type pGL2-**CXCR4-0.8kb** and the control vector, which was set arbitrarily at 1. Values are the averages of three independent determinations. Data represent mean ± s.d. (n=3), * P < 0.05, ** P < 0.01.

(B) C/EBPα binds specifically to a site within the human **CXCR4** proximal promoter.
The biotin-labeled double-stranded oligonucleotide containing -231/-245 region of the CXCR4 promoter was mixed with 5 μg of nuclear protein extracted from 293T cells ectopically expressing Flag-p42, Flag-p30 or Flag-CTM. For the supershift assay, 0.2 μg (lane 3-5) or 1 μg (lane 11-12) of specific antibody was added to the reaction mixtures. (C) Western blot showing the protein level of Flag-tagged C/EBPα isoforms in those cells described in panel B with empty vector as control. (D) 293T cells transfected with pCMV-tag2B, pCMV-Flag-p42 or pCMV-Flag-p30 were treated with formaldehyde for ChIP assays using anti-M2 antibody or normal mouse IgG. DNA isolated from immunoprecipitate was amplified by PCR with primers specific for the CXCR4, Albumin or β-Actin promoter.

**Figure 4. C/EBPα regulates chemotactic migration.** K562 cells were stably transfected with doxycycline inducible C/EBPα-p42 or C/EBPα-p30 constructs. (A) Total cell lysate from indicated K562 stable lines were analyzed for the relative expression of C/EBPα, CXCR4 and GAPDH proteins with specific antibodies as indicated. (B) Total RNA was analyzed for the expression of CXCR4 mRNA in the presence or absence of doxycycline by RT-QPCR. The relative CXCR4 mRNA level in U937 cells was set arbitrarily at 1. (C) Surface CXCR4 expression on K562-p42 or K562-p30 cells detected by flow cytometry using monoclonal Allophycocyanin (APC)-conjugated 12G5 antibody. Values indicate the relative fluorescence of CXCR4.
detected. U937 cells were used as positive control. (D) Transwell migration of parental K562, K562-p42 or K562-p30 cell lines in response to recombinant SDF-1α (30 nM). Cells were pre-cultured with or without doxycycline for 1 day and then treated with or without AMD3100 (10 μM) for 1 h before carrying out the migration assay. The results reflect the mean of 3 independent experiments. The error bars represent SD of the mean (⁎P<0.05). (E) U937 cells were stably transfected with doxycycline inducible shCEBPA construct. Total cell lysate from indicated U937 stable lines were analyzed for the relative expression of C/EBPα, CXCR4 and GAPDH proteins with specific antibodies as indicated. (F) Transwell migration of U937-scramble or U937-shCEBPA cell lines in response to recombinant SDF-1α. Cells were pre-cultured with or without doxycycline for 3 days and then treated with or without AMD3100 (10 μM) for 1 h before carrying out the migration assay. The results reflect the mean of 3 independent experiments. The error bars represent SD of the mean (⁎P<0.05)
A

K562 parental p42 p30
Dox - + - + - +

- CEBPα p42
- CEBPα p30

CXCR4

GAPDH

B

Relative CXCR4 mRNA level (log2)

Dox - + - + - +
U937 K562 K562-p42 K562-p30

C

U937

Isotype CXCR4

Counts vs CXCR4 expression level

K562-p42 K562-p30

Isotype CXCR4

Counts vs CXCR4 expression level

D

Chemotaxis Index

- p42 p42 + AMD3100 p30

E

U937

scramble shCEBPA

Dox - + - +

CXCR4 CEBPα

GAPDH

F

Chemotaxis Index

AMD3100 - + - +
scramble shCEBPA
ONLINE SUPPLEMENTARY METHODS SECTION

Antibodies and reagents

Anti-C/EBPα, anti-GAPDH and anti-β-actin antibodies were purchased from Genetex Biotechnology. M2 antibodies and doxycycline were from Sigma-Aldrich.

Cell culture

Human embryonic kidney 293T cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% calf serum. Human myeloid U937 and erythroleukemic K562 cell lines were grown in RPMI 1640 medium (Invitrogen) containing 10% fetal bovine serum (FBS; HyClone). To establish the inducible K562-C/EBPα cells that stably express wild type or mutated C/EBPα, the pTripz-C/EBPα-p42 and p30 plasmids were packaged using HEK293T cells and transduced into K562 cells as described.1,2 Subclones resistant to puromycin (2 µg/ml) were obtained by limiting dilution.

RT-QPCR analysis of human cell lines

Total RNA was isolated from cells using Trizol reagent (Invitrogen). cDNA was synthesized using ImProm-II reverse transcriptase (Promega). RT-QPCR analysis was performed using 7500 Fast Real-Time PCR System (Applied Biosystems) and Fast SYBER Green Master Mix (Applied Biosystems). CXCR4 was normalized to 18S rRNA.
Transfection and luciferase assays

HEK293T cells were transfected using a DNA mixture containing ScreenFect in vitro DNA transfection reagent (InCella). After transfection for 24 h, cells were analyzed for reporter activity assays. Transfected cells were assayed for both firefly and Renilla luciferase activities using the dual luciferase assay system (Promega).

Plasmid construction

The DNA fragment of CEBPA was obtained by PCR from K562 genomic DNA and cloned in-frame in the EcoRI and XhoI sites of pCMV-Tag2B plasmid. For generating tetracycline inducible plasmids, wild type CEBPA or p30 mutant cDNA were subcloned in the AgeI and XhoI sites of pTRIPZ plasmid. The human CEBPA shRNA were cloned into the lentiviral vector pTRIPZ plasmid containing a RFP reporter. The shRNA oligonucleotide sequence targeting CEBPA was as described.\textsuperscript{3}

The pGL2-CXCR4 plasmid containing human CXCR4 promoter region fused to the promoterless pGL2 firefly luciferase reporter was a generous gift of Wilhelm Krek.\textsuperscript{4}

The pGL2-CXCR4-0.8 kb was used to PCR amplify to generate a 0.2-kb fragment of the CXCR4 promoter region, which was subcloned to the pGL2 vector, producing pGL2-CXCR4-0.2 kb. Mutant versions of pCMV-tag2B- C/EBP\(\alpha\) (pCMV-tag2B-C/EBP\(\alpha\)-CTM) and pGL2-CXCR4-0.8 kb (pGL2-CXCR4-S1, pGL2-CXCR4-S2 and pGL2-CXCR4-S3) were generated using Quick-Change Site-directed Mutagenesis kit.
Chromatin immunoprecipitation assays (ChIP)

Chromatin immunoprecipitation was performed as described\(^2\). In brief, cells were treated with formaldehyde at a final concentration of 1% for 10 min at room temperature. Glycine was added at a final concentration of 125 \(\mu\)M to quench cross-linking. Cell lysates were sonicated to generate DNA fragments averaging <1 kb. After pre-clearance, cell extracts were incubated with 1 \(\mu\)g of antibody at 4°C overnight, followed by precipitation with protein A/G Sepharose beads. After washing, elution, deproteination, and heating, immunoprecipitated DNA was extracted and ethanol precipitated. DNA was resuspended in 50 \(\mu\)l of Tris-EDTA buffer. PCR was applied to the immunoprecipitated DNA with the following thermal cycling program: 30 s at 94 °C, 30 s at 55 °C and 1 min at 72 °C (30 cycles), followed by an extension time at 72 °C for 5 min. PCR products was analyzed on 2.0% agarose gel and visualized by ethidium bromide staining.

Preparation of nuclear extracts and gel-shift analysis

The preparation of nuclear extracts from HEK293T cells expressing C/EBP\(\alpha\) and gel-shift reactions were as described previously\(^2\). The gel-shift analysis was performed using gel-shift assay kits from Signosis (Sunnyvale, CA). For super-shift assays, antibodies specific for C/EBP\(\alpha\) or GAPDH were added to the nuclear extracts for 30 min on ice prior to the DNA binding reaction. After incubation at room
temperature for 30 min with biotin-labeled probes and nuclear extracts (5.0 μg protein), samples were analyzed by electrophoresis at 150 V for 1.5 h through non-denaturing 4% polyacrylamide gels. After separation by 4% non-denaturing polyacrylamide gel electrophoresis, the binding reaction mixtures were transferred onto a nylon membrane, and the membrane was UV-cross-linked. The biotin-labeled DNA was probed with streptavidin-HRP conjugate for chemiluminescence detection.

**Chemotaxis assay**

A total of 5x10^5 cells in a volume of 200 μl were added to the top chamber of Transwell culture inserts (Corning Costar, Lowell, MA) with a pore size of 5 μm. Inserts were placed in wells containing 800 μl of RPMI medium with or without SDF-1 (R&D Systems). Chemotaxis assays were performed at 37°C for 24 hours. Cells were then counted in triplicates using a hemocytometer.

**Flow cytometry**

K562 cells were washed with cold PBS, blocked with 5% FBS in PBS on ice for 30 min and then treated with APC-conjugate of mouse anti-human-CXCR4 monoclonal antibody (5 μg/ml, clone 12G5), or with an isotype control, on ice for 1 h. Cells were then washed and analyzed by BD-Canto flow-cytometer (BD Biosciences, San Jose, CA).

**ChIP and Quantitative RT-PCR oligonucleotides**
human CXCR4 promoter sense primer 5’-CGCGGGGGAATGGGCGGTTGGAAGC-3’, antisense primer 5’-GCCGGACAGGACCTCCCAGAGGCATTTCCTC-3’; human albumin promoter sense primer 5’-CGACGACCCCATTCAAAGTCG-3’, antisense primer 5’-CTCTCCGGAATCGAACCCTGA-3’; human β-actin promoter sense primer 5’-ACGCCAAAACGCTTCCTCCCTCCTC-3’, antisense primer 5’-CATAAAAAGCGACTTTCGGAACGGC-3’. human CXCR4 sense primer 5’-CTGAGAAGCATGACGGACAA-3’, antisense primer 5’-TGGAGTGTGACAGCTTGGAG-3’; human 18S rRNA sense primer 5’-CGACGACCCCATTCAAAGGTG-3’, antisense primer 5’-CTCTCCGGAATCGAACCCCTGA-3’

ONLINE SUPPLEMENTARY REFERENCES

Figure S1. CXCR4 expression is negatively associated with C/EBPα mutations.

There is negative association of BM CXCR4 mRNA expression and CEBPA mutation (P=0.0019). Intriguingly, there is significant differences in CXCR4 mRNA expression between patients with CEBPA double mutations (CEBPA\textsuperscript{double-mut}) and those with single mutation (CEBPA\textsuperscript{single-mut}, P=0.0075) or those without mutation (CEBPA\textsuperscript{wild}, P=0.0002).
Supplementary Figure 2

Figure S2. Kaplan–Meier survival curves for overall survival stratified by BM CXCR4 mRNA expression. A total of 151 patients with AML who received conventional intensive chemotherapy showed that patients with higher BM CXCR4 expression had shorter overall survival than those with lower expression (P=0.03).
Figure S3. Correlation of *CEBPA* and *CXCR4* mRNA levels in AML patients.

Scatter plot of log2-transformed intensities between *CEBPA* and *CXCR4* expression in the patients with wild-type *CEBPA* from the German AMLCG cohort reported by Dufour et. al.\textsuperscript{5} The Pearson correlation coefficient (R), level of statistical significance (P), and regression line (dash line) are shown.