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

Yuan-Yeh Kuo,[1] Hsin-An Hou,[2,3] Yin-Kai Chen,[3] Li-Yu Li,[1] Po-Hsuen Chen,[1] Mei-Hsuan Tseng,[3] Chi-Fei Huang,[3] Fen-Yu Lee,[4] Ming-Chih Liu,[4] Chia-Wen Liu,[4] Wen-Chien Chou,[3,5] Chieh-Yu Liu,[6] Jih-Luh Tang,[3] Ming Yao,[3] and Hwei-Fang Tien[3]

[1]Graduate Institute of Oncology, College of Medicine, National Taiwan University, Taipei; [2]Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taipei; [3]Division of Hematology, Department of Internal Medicine, National Taiwan University Hospital, Taipei; [4]Department of Pathology, National Taiwan University Hospital, Taipei; [5]Department of Laboratory Medicine, National Taiwan University Hospital, Taipei; and [6]Biostatistics Consulting Laboratory, Department of Nursing, National Taipei College of Nursing, Taiwan

ABSTRACT

CXC chemokine receptor 4 (CXCR4) is an essential regulator for homing and maintenance of hematopoietic stem cells within the bone marrow niches. Analysis of clinical implications of bone marrow *CXCR4* expression in patients with acute myeloid leukemia showed not only 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 this hypothesis, we first examined endogenous CXCR4 expression in 293T and K562 cells over-expressing 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, evidence 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)].[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.[1] 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.[1,2] 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.[3,4] 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.[5,6] Expression of CXCR4 in leukemic cells was associated with cell cycle arrest and reduced numbers of cell division, providing a potential mechanism for leukemia cells to evade the cell-killing effect of chemotherapy.[2] Higher expression of CXCR4, measured by either an immunohistochemical method or flow cytometry, has been identified as a poor prognostic marker for AML[7,8] and may be an attractive target for the development of novel therapeutic approaches.[9] 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.

Acute myeloid leukemia 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.[10] Transcription factors are main targets of mutations in AML. CCAAT enhancer binding protein alpha (C/EBPα) 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.[11,12] Mutations in one or both alleles of *CEBPA* are reported in approximately 7%-15% of patients with AML.[13,14] These mutations can be divided into two major categories: 1) comprising C-terminal mutations that disrupt the bZIP region; and 2) comprising N-terminal mutations that disrupt the reading frame, resulting in translation of a 30-kDa C/EBPα p30 isoform. The N-terminal truncated mutant was shown to have a dominant-negative effect.[11,12] Although AML patients with C/EBPα mutant have a favorable prognosis,[13,15] the molecular mechanisms by which *CEBPA* mutations contribute to better treatment response and improved outcomes are not yet fully understood.

In the present study, we analyzed BM CXCR4 expression

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The online version of this article has a Supplementary Appendix.
Manuscript received on March 25, 2014. Manuscript accepted on August 29, 2014.
Correspondence: yykuo@ntu.edu.tw

by quantitative real-time polymerase chain reaction (RT-QPCR) in a cohort of 220 adult patients with *de novo* AML and found higher *CXCR4* expression was an independent poor prognostic factor. Moreover, *CXCR4* expression was inversely associated with *CEBPA* mutation. Study of the mechanism of the relationship between *CXCR4* expression and *CEBPA* mutation revealed that C/EBPα activates CXCR4 transcription through direct binding to its promoter. We further demonstrated that the overexpression of C/EBPα increased SDF1-mediated directional migration of leukemic cells, while the depletion of C/EBPα diminished cell migration. These results indicate a role for C/EBPα in transcriptional control of *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

A total of 220 adult patients at the National Taiwan University Hospital (NTUH), with newly diagnosed *de novo* AML, enough cryopreserved cells for molecular analyses, and 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] The 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

Bone marrow 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 (TaqMan Gene Expression Assay; Assay ID, Hs02330069_s1, Applied Biosystems). Each sample was tested independently at least twice. 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 were performed as previously described. These included Class I mutations, such as FLT3/ITD and *FLT3*/TKD,[19] *NRAS*,[20] *KRAS*,[20] *JAK2*,[20] *KIT*[21] and *PTPN11*[22] mutations, and Class II mutations, such as *MLL*/PTD,[23] *CEBPA*[14] and *RUNX1*[24] mutations, as well as mutations in *NPM1*,[25] *WT1*,[26] and those genes related to epigenetic modification, such as *ASXL1*,[27] *IDH1*,[28] *IDH2*,[29] *TET2*[30] and *DNMT3A*.[17] 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 χ^2 tests or Fisher's exact test. We used Mann-Whitney U-test to compare continuous variables and medians of distributions. The entire 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 survival analysis. 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 5% or more 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 $P<0.05$ was considered statistically significant. All statistical analyses were made with SPSS 17 (SPSS Inc., Chicago, IL, USA) and Statsdirect (Cheshire, England, UK) software.

Results

Correlation of bone marrow CXCR4 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 predominantly male, 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 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*$^{double\text{-}mut}$), 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) (*Online Supplementary Figure S1*). 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 0.1-160 months), patients with higher BM *CXCR4* expression had a significantly shorter OS than those with lower expression (median 20 months *vs.* not reached; P=0.030) (*Online Supplementary Figure S2*). The same was also true among the patients with non-M3 AML (P=0.039). Furthermore, 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 this 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

Table 1. Comparison of clinical manifestations, treatment response and cytogenetic changes between AML patients with lower and higher BM *CXCR4* expression.*

Variables	Total (n=220)	Lower *CXCR4* expression (110, 50%)	Higher *CXCR4* expression (110, 50%)	*P* 0.0426
Gender†				0.0426
Male	104	44 (42.3)	60 (57.7)	
Female	116	66 (56.9)	50 (43.1)	
Age (year)‡	54.5 (15-90)	50.5 (15-86)	60 (15-90)	0.0131
Laboratory data‡				
WBC (×10⁹/L)	23.3 (0.4-42.3)	24.6 (0.4-175.9)	22.2 (0.9-423)	0.5087
Hemoglobin (g/dL)	7.9 (3.5-16.2)	8.0 (3.5-13.1)	7.8 (3.3-16.2)	0.6943
Platelet (×10⁹/L)	41.5 (2-455)	39.5 (2-331)	43 (6-455)	0.4105
Blast (×10⁹/L)	10.3 (0-369.1)	12.6 (0-162.8)	6.6 (0-369.1)	0.1646
LDH (U/L)	859 (271-13130)	963 (298-5559)	785 (271-13130)	0.019
Induction response†Δ	151	67	84	
CR	111	54 (80.6)	57 (67.9)	0.0956
PR/refractory	29	11 (16.4)	18 (21.4)	0.5342
Induction death	11	2 (3)	9 (10.7)	0.11277
Relapse	53	25 (46.3)	28 (49.1)	0.8499
Karyotypeξ				0.0013
Favorable	44	30	14	0.0106
Intermediate	146	72	74	0.6544
Unfavorable	20	4	16	0.0045
Unknown	10	4	6	

**The median value of CXCR4 expression in the total population was used as the cut-off point to define lower- and higher-expression groups. ΔOnly the 151 patients who received conventional intensive induction chemotherapy and then consolidation chemotherapy if CR was achieved were included in the analysis. ξ210 patients (104 higher CXCR4 expression and 106 lower CXCR4 expression patients) had chromosome data at diagnosis. There was no difference in 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. †Number (%) of patients. ‡Median (range). CR: complete remission; PR: partial remission.*

Group (AMLCG).[32] Gene expression profiling data from 46 patients with wild-type *CEBPA* were selected and the Pearson's correlation test was applied. A significant linear correlation was observed between *CEBPA* and *CXCR4* expression in these cases (R=0.46; *P*=0.003) (*Online Supplementary Figure S3*). 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 this 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 C). 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); this is consistent with the data obtained from RT-PCR and Western blot analysis described above (Figure 1B and C). The expression level of each Flag-tagged C/EBPα isoform from one representative experiment is 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 site S1 or S2 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, lanes 3-5 and 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

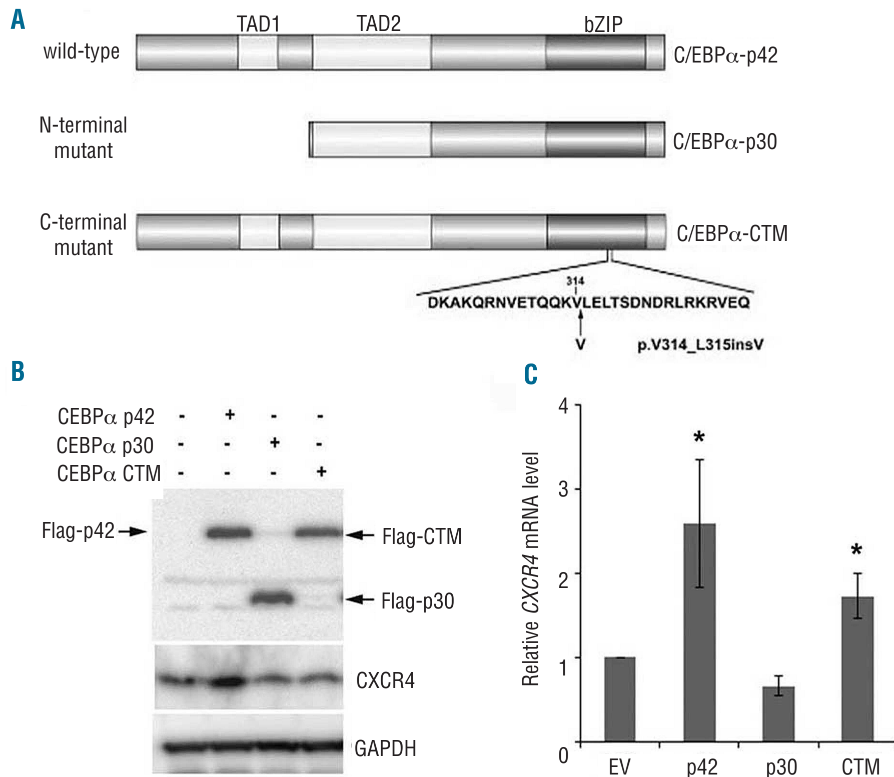

Figure 1. Ectopic expression of C/EBPα activates the *CXCR4* gene expression. (A) Schematic diagram of C/EBPα expression constructs. The N-terminal mutation is represented by the p30 isoform (C/EBPα-p30). C-terminal mutation (C/EBPα-CTM) contains an amino acid insertion p.V314_L315insV. Diagrams were constructed using Domain Graph (DOG) v.2.0.[31] (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+SD (n=3); *P<0.05; **P<0.01.

Table 2. Comparison of genetic alterations between AML patients with lower and higher BM *CXCR4* expression.

Variables	Number (%) of patients with the gene mutation			
	Whole cohort (n=220)	Lower *CXCR4* expression (110, 50%)	Higher *CXCR4* expression (110, 50%)	P
FLT3/ITD	55 (25)	32 (29.1)	22 (20)	0.1582
FLT3/TKD	17 (7.7)	6 (5.5)	11 (10)	0.3127
NRAS	26 (11.8)	11 (10)	15 (13.6)	0.5317
KRAS	7 (3.2)	3 (2.7)	4 (3.6)	>0.9999
PTPN11	14 (6.4)	5 (4.5)	9 (8.2)	0.4084
KIT	9 (4.1)	6 (5.5)	3 (2.7)	0.4986
JAK2	1 (0.5)	0	1 (0.5)	>0.9999
WT1	13 (5.9)	7 (6.4)	6 (5.5)	>0.9999
NPM1	52 (23.6)	15 (13.6)	37 (33.6)	0.0008
CEBPA	27 (12.3)	20 (18.2)	7 (6.4)	0.0125
CEBPA$^{double-mut}$	18 (8.2)	15 (13.6)	3 (2.7)	0.0054
RUNX1	29 (13.2)	15 (13.6)	14 (12.7)	>0.9999
MLL/PTD	13 (5.9)	8 (7.3)	5 (4.5)	0.5692
IDH1	13 (5.9)	5 (4.5)	8 (7.3)	0.5692
IDH2	31 (14.1)	11 (10)	20 (18.2)	0.1201
ASXL1	23 (10.5)	9 (8.2)	14 (12.7)	0.3785
TET2	32 (14.5)	16 (14.5)	16 (14.5)	>0.9999
DNMT3A	36 (16.4)	14 (12.7)	22 (20)	0.2016

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.[33] 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, we performed chemotaxis assays. 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.[34] We used K562 cells because they do not express endogenous C/EBPα.[35] Expression of C/EBPα p42 and C/EBPα p30 after the cells were exposed

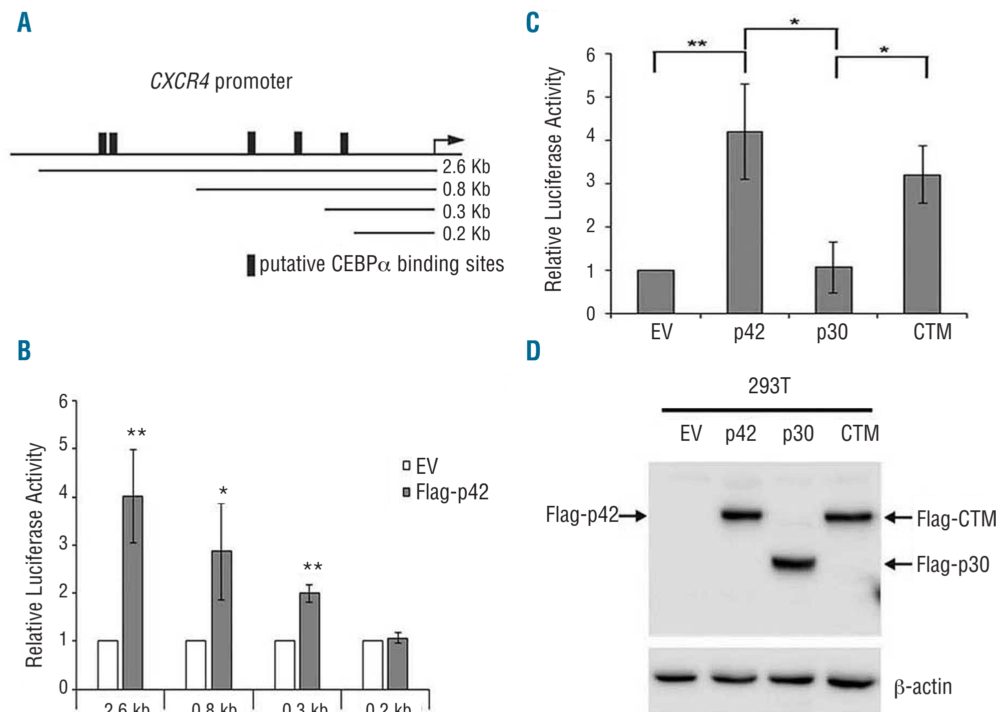

Figure 2. Wild-type C/EBPα, but not that with deletion of putative C/EBPα binding sites, activates *CXCR4* promoter. (A) Schematic representation of the human *CXCR4* promoter reporter constructs. Putative C/EBPα binding sites are shown as boxes. (B) 293T cells were co-transfected with the human *CXCR4* 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 3 independent determinations. Data represent mean+SD (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 (B). (D) Western blot data showing the expression of Flag-tagged C/EBPα isoforms in those cells described in (C).

Table 3. Multivariate Cox regression analysis on the overall survival in AML patients.

Variables	Hazard ratio	95% CI	P value
Age*	3.490	1.945-6.264	<0.001
WBC†	2.379	1.337-4.232	0.003
Karyotype‡	3.402	1.001-11.562	0.049
CXCR4Δ	1.849	1.018-3.357	0.043
NPM1/FLT3-ITD@	0.468	0.179-1.222	0.121
CEBPAπ	0.227	0.054-0.957	0.043

Age over 50 years compared to age 50 years or younger. †WBC greater than 50×10^9/L versus less than or equal to 50×10^9/L. ‡Unfavorable-risk cytogenetics versus others. ΔHigher BM CXCR4 expression versus lower CXCR4 expression. @NPM1+/FLT3-ITD- versus other subtypes. πCEBPAdouble-mut versus other subtypes.

to doxycycline was successfully confirmed by Western blot analysis (Figure 4A). To test whether the p42-mediated transcriptional activation of the *CXCR4* gene can also be detected in the K562-C/EBPα 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 and B, CXCR4 protein and mRNA level were increased in K562-C/EBPα p42 cells after doxycycline treatment for 24 h. In contrast, expression of *CXCR4* was not activated when p30 was induced. Protein and *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, after doxycycline treatment, surface CXCR4 expression was significantly increased in K562-C/EBPα p42 cells, but not K562-C/EBPα p30 cells. U937 cells were used as the positive control for the assay (Figure 4C). To further assess the *in vitro* functional responses of K562-C/EBPα stable cell lines, we examined the migration of the cells in response to the SDF-1 treatment after C/EBPα was induced. Consistent with an 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).

Depletion of CEBPA *reduced chemotactic migration toward SDF-1 in U937 cells*

Finally, we tested whether depletion of *CEBPA* in U937 cells reduce chemotactic migration toward SDF-1. We used U937 cells because they express high levels of endogenous C/EBPα.[35] Inducible U937 cell lines stably transfected with *shCEBPA* (U937-shCEBPA) or scramble control plasmid were established. Cells treated with doxycycline were subjected to Western blot analysis and *in vitro* migration assay. As shown in Figure 4E, C/EBPα protein level decreased with a concurrent decrease of CXCR4 expression in U937-*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 *CXCR4* expression was negatively associated with *CEBPA* mutation. To the best of our knowledge, this is the first study to demonstrate a role for C/EBPα in transcriptional control of *CXCR4* gene expression and to confirm 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.[36] The BM microenvironment provides support for self-renewal, homing, engraftment and the proliferative potential of hematopoietic stem cells, in which *SDF-1/CXCR4*

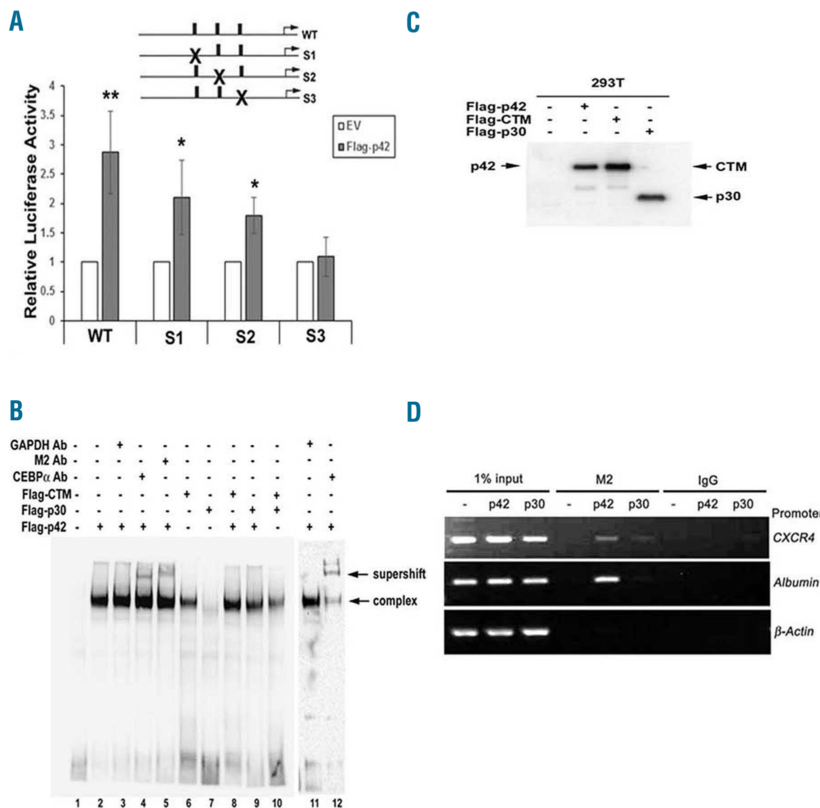

Figure 3. C/EBPα p42 activates *CXCR4* transcription through direct binding to the proximal C/EBPα binding site in the *CXCR4* promoter. (A) C/EBPα-dependent transactivation of the *CXCR4* promoter. Human *CXCR4* 0.8 kb promoter fragment containing wild-type C/EBPα binding sites (shown as boxes) or mutated sites (indicated by 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 3 independent determinations. Data represent mean+SD (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 (lanes 3-5) or 1 μg (lanes 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 (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.

axis plays an essential role. Emerging data also show that adhesion to the BM stromal cells affects survival and proliferation of AML cells.[37] The clinical implications of CXCR4 in AML should, therefore, be examined. In this study, we observed that AML patients with higher BM *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, cytogenetics 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 provide evidence 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 C, 4A and B) 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 demonstrate 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* or *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) suggesting that p30 acts as a dominant-negative isoform in the regulation of *CXCR4* transcription. Similarly, Cleaves et al. found the

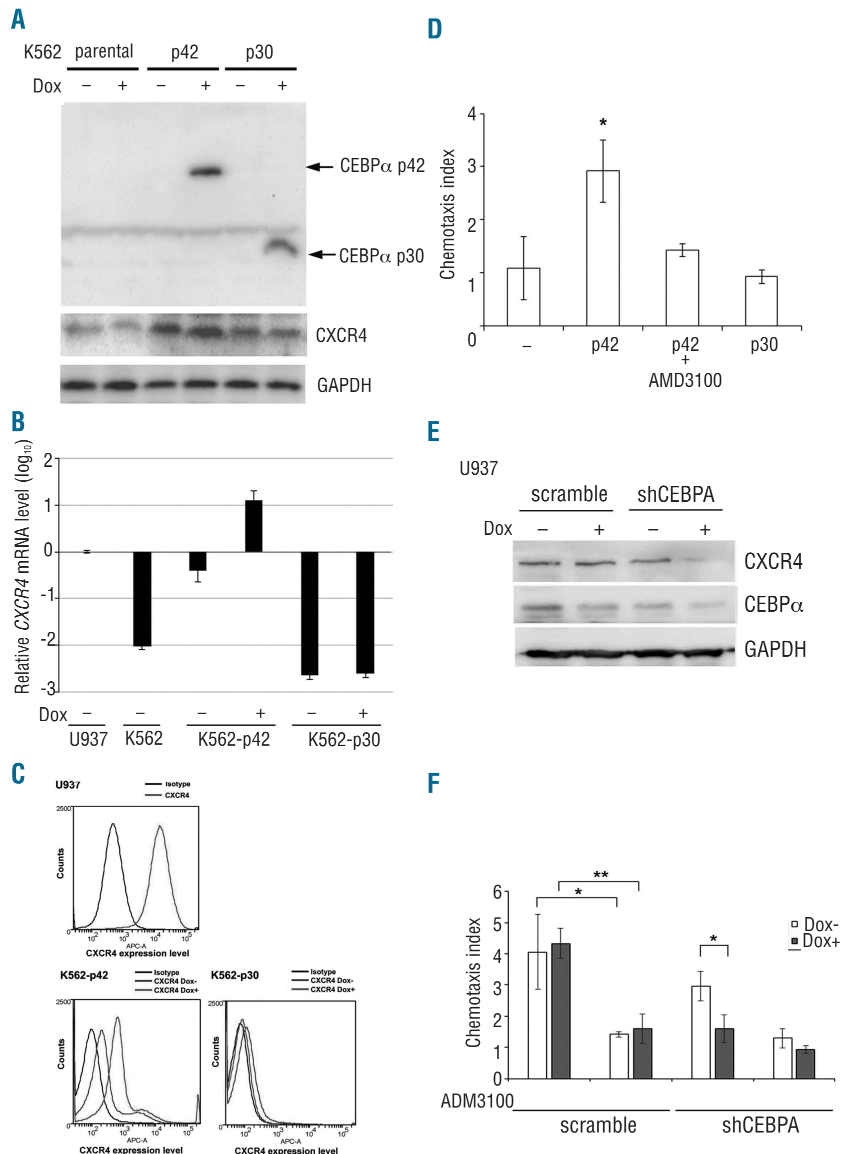

Figure 4. C/EBPα regulates chemotactic migration. K562 cells were stably transfected with doxycycline inducible C/EBPα-p42 or C/EBPα-p30 constructs. (A) Total cell lysate from indicated K562 stable lines were analyzed for the relative expression of C/EBPα, *CXCR4* and GAPDH proteins with specific antibodies 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 one 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 three 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).

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.[38] 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.[6] The way in which the effect of *CEBPA* mutant on *CXCR4* expression contributes to leukemogenesis awaits further study.

We and others have reported that AML patients with *CEBPA* mutation had a favorable survival.[13,14,39] The better prognosis in AML with *CEBPA* mutation may be partially explained by the lower *CXCR4* expression in these patients. CXCR4-mediated contact between leukemia cells and stromal cells has been shown to result in cell cycle arrest and a reduction in cell division, providing a potential mechanism for leukemia cells to escape chemotherapy effect.[2] In mouse models, 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.[4,40] Moreover, in a phase I/II study, the addition of plerixator, a CXCR4 antagonist, to cytotoxic chemotherapy increased the rate of remission.[41] Our *in vitro* data showing that blockage of C/EBPα -p42 induced *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 *CEBPA* mutation, in whom most patients (20 of 27) (Table 2) show lower *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 other reasons for the better clinical outcome in *CEBPA*-mutated patients since, in this study, both *CEBPA* mutation and *CXCR4* expression are independent prognostic factors. Further studies are needed to provide answers to these questions. It also remains to be determined whether patients with *CEBPA* mutation, who already have lower *CXCR4* expression, will not gain further improvement in survival from the treatment of *CXCR4* antagonists.

It is interesting to note that a subset of patients harboring wild-type *CEBPA* showed low *CXCR4* expression. We suggest that other mechanisms might affect the activity of C/EBPα in these cases, such as phosphorylation on serine 21 or SUMOylation on lysine 161 of C/EBPα.[42] In addition to dysfunction of C/EBPα, a 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.[43] Therefore, dysfunction of CXCR4-Serine 339 phosphorylation might be the alternative means of CXCR4 deregulation in AML.

In summary, the current study shows a close association of *CEBPA* mutation with lower *CXCR4* expression, and provides new evidence that C/EBPα regulates the activation of *CXCR4*, while C/EBPα mutant p30 loses this ability. Since CXCR4 blockage in AML cells may disrupt their interaction with the BM niche and sensitize them to chemotherapy, patients with higher CXCR4 expression may benefit from the treatment of CXCR4 antagonists. But it remains to be determined whether patients with *CEBPA* mutation, who already have lower CXCR4 expression, need this kind of therapy.

Acknowledgments

We thank Dr. Wilhelm Krek for providing CXCR4 promoter reporter constructs.

Funding

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

Information on authorship, contributions, and financial & other disclosures was provided by the authors and is available with the online version of this article at www.haematologica.org.

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