Published in final edited form as:

Clin Lymphoma Myeloma Leuk. 2013 December ; 13(6): 686–692. doi:10.1016/j.clml.2013.05.013.

CXCR4 Expression, CXCR4 Activation, and Wild Type *NPM1* Are Independently Associated with Unfavorable Prognosis in Patients with Acute Myeloid Leukemia

Sergej Konoplev, MD, PhD¹, Pei Lin, MD¹, C. Cameron Yin, MD, PhD¹, E. Lin, BS², Graciela M. Nogueras González, MPH², Hagop M. Kantarjian, MD³, Michael Andreeff, MD, PhD³, L. Jeffrey Medeiros, MD¹, and Marina Konopleva, MD, PhD³

¹Department of Hematopathology, The University of Texas MD Anderson Cancer Center, Houston, Texas

²Department of Biostatistics, The University of Texas MD Anderson Cancer Center, Houston, Texas

³Department of Leukemia, The University of Texas MD Anderson Cancer Center, Houston, Texas

Abstract

BACKGROUND—CXC chemokine receptor 4 (CXCR4) is activated by phosphorylation and is essential for migration of hematopoietic precursors to bone marrow. CXCR4 overexpression predicts unfavorable prognosis in patients with acute myeloid leukemia (AML). *Nucleophosmin* (*NPM1*) mutation is the most frequent genetic abnormality in AML patients and predicts a favorable prognosis. *In vitro* studies have suggested that mutant NPM decreases CXCR4-mediated chemotaxis by downregulating CXCR4, thereby linking the NPM and CXCR4 pathways.

PATIENTS AND METHODS—In a group of 117 untreated adults with AML we used immunohistochemistry to assess bone marrow specimens for CXCR4 and phosphorylated (p) CXCR4 (pCXCR4) expression. All cases were also analyzed for *NPM1* mutations using PCR-based methods.

RESULTS—CXCR4 expression was detected in 75 (64%) and pCXCR4 expression was detected in 31 (26%) patients. *NPM1* mutations were detected in 63 (54%) patients. *NPM1* mutations did not correlate with CXCR4 ($p = 0.212$) or pCXCR4 ($p = 0.355$) expression. The median 5-year overall survival was 27% (95% CI: 19-36%), with a median follow-up of 8 months (95%CI: 6-15). In a multivariate Cox proportional hazards model, reduced overall and progression-free survival rates were associated with a history of antecedent hematological disorder, failure to achieve

© 2013 Elsevier Inc. All rights reserved.

Corresponding author: Sergej Konoplev, MD, PhD, Department of Hematopathology, Unit 72, The University of Texas MD Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030; Telephone: (713) 792-4117. Fax: (713) 792-4821; skonople@mdanderson.org.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

CONFLICT OF INTEREST DISCLOSURES

All authors have no conflicts of interest.

complete remission, thrombocytopenia, unfavorable cytogenetics, CXCR4 expression, and wild type *NPM1*. pCXCR4 expression was independently associated with shorter progression-free survival.

CONCLUSION—There is no correlation between *NPM1* mutations and CXCR4 or phosphorylated CXCR4 expression suggesting that the CXCR4 and NPM pathways act independently in adult AML.

Keywords

Acute myeloid leukemia; AML; CXCR4; CXCR4 phosphorylation; CXCR4 activation; *NPM1*; prognosis

INTRODUCTION

Acute myeloid leukemia (AML) is estimated to affect 14,590 individuals in the United States in 2013.¹ Despite improvements in therapy over the past decade, 71% of these patients are expected to die of disease.¹ Therefore, biomarkers that predict and refine prognosis for AML patients are needed to improve risk-adapted therapy.

CXCR4 is a 352 amino acid rhodopsin-like G protein coupled receptor with no intrinsic kinase activity that selectively binds CXC chemokine stromal cell-derived factor 1 alpha (SDF-1 α), also known as CXCL12.^{2, 3} The CXCR4/ SDF-1 α interaction is essential for hematopoiesis as well as for general development, organogenesis, and vascularization.⁴⁻⁹ CXCR4 expression is tightly controlled at several levels. At the transcriptional level, a number of signaling molecules can either increase or attenuate CXCR4 expression.¹⁰⁻¹⁷ At the post-translational level, phosphorylation is a key mechanism of CXCR4 regulation. SDF-1 α -promoted tyrosine phosphorylation activates the JAK/ STAT pathway,^{18, 19} whereas cytokine-induced tyrosine phosphorylation promotes ligand-independent internalization of CXCR4.¹³ CXCR4 is phosphorylated in response to ligand binding in a G-protein-coupled receptor kinase 2-dependent fashion.²⁰⁻²² Receptor phosphorylation stimulates the interaction of β -arrestin with the carboxy terminus.^{21, 23} This interaction terminates CXCR4-mediated activation of G α i but promotes dynamin-dependent, clathrin-mediated receptor endocytosis and enhances CXCR4-RAF-dependent signaling.^{20, 23, 24} Phosphorylation of CXCR4 also can occur in response to the activation of other receptors and involves additional kinases, such as protein kinase C²⁰⁻²² or tyrosine kinases.^{13, 25}

CXCR4 expression has been detected in 23 cancers of various origins²⁶ and is the most common chemokine receptor expressed by cancer cells.²⁷ In the neoplastic setting, a number of other factors also can enhance CXCR4 expression, such as vascular endothelial growth factor,²⁸ activation of nuclear factor kappa B,²⁹ PAX3-FKHR,^{30, 31} and RET/PTC.³² Ubiquitination of CXCR4 is another mechanism of posttranslational regulation of CXCR4 expression.^{33, 34}

Nucleophosmin, encoded by the *nucleophosmin* (*NPM1*) gene on chromosome 5q35, is ubiquitously expressed in human tissues.^{35, 36} *NPM1* mutations occur in 25-35% of all AML patients and 45-64% of AML cases with a normal karyotype.^{37, 38} In AML patients, *NPM1*

mutations are associated with a favorable prognosis in the absence of *fms-related tyrosine kinase 3 gene (FLT3)* internal tandem duplications (ITD).³⁹ In two retrospective studies of patients with *FLT3* wild type AML, patients with *NPM1*-mutation did not benefit from allogeneic stem cell transplantation, in contrast with patients with wild type *NPM1*.^{39, 40} These data have led others to propose that AML patients with a normal karyotype should be tested routinely for *FLT3-ITD* and *NPM1* mutations thereby facilitating risk-adapted therapy.

In 2007, Zhang and colleagues published data suggesting that the CXCR4-CXCL12 axis and NPM pathway are linked.⁴¹ These authors showed that CXCR4 forms complexes with NPM, and that overexpression of mutant NPM decreased CXCR4-mediated chemotaxis by downregulating CXCR4 expression.⁴¹ If relevant in vivo, *NPM1* mutation theoretically could result in less protection of leukemic cells by the bone marrow microenvironment suggesting that the favorable prognosis of *NPM1* mutation in AML patients could be related to negative regulation of CXCR4 expression and function by mutant NPM protein.

Recently, Chou and colleagues introduced a knock-in mouse model of *NPM1* mutation inserting TCTG after nucleotide c.857.⁴² and demonstrated that the expression of CXCR4/CXCL12-related genes was significantly suppressed in mutant myeloid precursors compared to myeloid precursors with wild type *NPM1*; similarly, suppression of CXCL12 and CXCR4 pathway signatures was detected by genome-wide expression microarray analysis on BM mononuclear cells in *NPM1*-mutated AML patients compared to *NPM1*-wild AML.⁴²

We and others have shown that CXCR4 expression correlates with inferior prognosis in adult AML patients;⁴³⁻⁴⁵ however, there are little data regarding pCXCR4 expression in AML. In addition, there are no studies analyzing the potential interaction of the CXCR4 and pCXCR4 pathways, and the NPM pathway in AML patients. Therefore, the goals of this study were to assess for CXCR4 and pCXCR4 expression as well as *NPM1* mutations and correlate with prognosis in adult patients with AML.

PATIENTS AND METHODS

Patient Identification

After approval of the protocol by the Institutional Review Board, the files of the Department of Hematopathology from January 1, 2003, to July 31, 2008 were searched for cases of AML with available banked bone marrow aspirate material and fixed, paraffin-embedded bone marrow core biopsy specimens. Patients who did not receive treatment at our institution were excluded. The diagnosis of AML was established based on the results of bone marrow (BM) aspiration and biopsy according to the current World Health Organization criteria. None of the patients received prior therapy. Available Wright-Giemsa stained BM aspirate smears and hematoxylin-eosin stained BM aspirate clot and trephine biopsy specimens were reviewed. Patient clinical information was obtained by review of medical records.

Morphologic, Cytochemical, and Immunophenotypic Analysis of Blasts

Bone marrow aspirate smears were assessed via cytochemical analysis for myeloperoxidase and alpha-naphthyl butyrate esterase using methods reported previously.⁴⁶ Flow cytometry immunophenotypic analysis was performed on BM aspirate specimens using a four-color FACScalibur cytometer (Becton Dickinson) and analyzed using the CellQuest software package (Becton Dickinson) as has been described.⁴⁷ Antibodies specific for the following antigens were used: CD3, CD7, CD10, CD13, CD19, CD20, CD33, CD34, CD45, CD56, CD64, CD117; HLA-DR, myeloperoxidase, and terminal deoxynucleotidyl transferase. All antibodies were obtained from Becton-Dickinson. Blasts were gated for analysis using CD45 expression and light side-scatter characteristics. Blasts were considered positive for an antigen based on an arbitrary cutoff level of at least 20% blasts that expressed the antigen compared with an isotype control.

Conventional Cytogenetic Analysis

Conventional cytogenetic analysis of BM aspirate specimens was performed using standard Giemsa trypsin G-banding procedures as described previously.⁴⁷ The results of cytogenetic studies were categorized according to a classification system proposed by Haferlach et al.⁴⁸ Briefly, the favorable risk category included AML cases associated with t(8;21), t(15;17), inv(16), and t(16;16), and the unfavorable risk AML category included cases with -5/5q-, -7/7q-, inv(3), t(3;3), 11q23 abnormalities, 17p abnormalities, and a complex aberrant karyotype (three or more abnormalities). All AML cases with other abnormalities or a normal karyotype formed an intermediate risk category.⁴⁸

Detection of *FLT3* and *NPM1* Mutations

Genomic DNA was extracted from BM aspirate specimens and *FLT3* was assessed for internal tandem duplication (ITD) in the activation loop of the tyrosine kinase domain using polymerase chain reaction (PCR) methods followed by capillary electrophoresis.⁴⁶ To detect *NPM1* mutation, exon 12 was amplified by PCR using the following primers: 5'-GATGTTGAACTATGCAAAGAGACA-3' (forward) and 5'-AACCAAGCAAAGGGTGGAGTT-3' (reverse). The PCR products were purified using a MinElute PCR purification Kit (Qiagen, Valencia, CA) and directly sequenced using a 5'-GGCATTGTTGGACAACACA-3' (reverse) primer, fluorescence dye chain-terminator chemistry (Sanger sequencing), and an ABI Prism 3100 or 3130 genetic analyzer (Applied Biosystems, Foster City, CA). The ABI GeneMapper software program (Applied Biosystems) was used to analyze the raw data.

Immunohistochemical Analysis

Expression of CXCR4 and pCXCR4 by blasts was analyzed using immunohistochemical methods as described previously.⁴⁹ Briefly, we used formalin-fixed, formic acid-decalcified, paraffin-embedded BM biopsy specimens. Total CXCR4 expression was assessed using a polyclonal rabbit antibody specific for CXCR4 (clone 2074; Abcam). A formalin-fixed, paraffin-embedded cell block of the Jurkat cell line, known to be positive for CXCR4,⁵⁰ was used as a positive control. Phosphorylated (p) CXCR4 was assessed using a rabbit polyclonal pCXCR4-specific antibody (kindly provided by Dr. Joshua Rubin, Washington

University, St. Louis, MO).⁵¹ A formalin-fixed, paraffin-embedded glioblastoma multiforme tissue array (kindly provided by Dr. Greg Fuller, The University of Texas MD Anderson Cancer Center, Houston, TX) was used as a positive control for pCXCR4 expression. CXCR4 and pCXCR4 expression by blasts was assessed by counting 5 random high-power microscopic fields (x400) and calculating the mean percentage of positive blasts (positive blasts/all blasts). A BM specimen was considered positive if at least 10% of blasts exhibited a cytoplasmic pattern of expression. Immunohistochemistry slides were reviewed independently by two authors (SK and PL) and discrepant interpretations were reviewed together using a multiheaded microscope to reach a consensus.

Statistical Analysis

The Pearson chi-square test (or Fisher exact two-tailed test) was used to assess associations among categorical variables. Kaplan-Meier methods were used to estimate overall survival (OS) and relapse-free survival (RFS), the latter computed starting from the date of diagnosis. Relapse or death was censored at the date of last follow-up if relapse or death was not observed. Univariate and multivariate Cox proportional hazards models were used to determine the association between tumor characteristics and survival (OS and PFS) according to age, sex, race, presence of antecedent hematological disorder,⁵² achievement of complete remission (CR), and laboratory data at the time of diagnosis. Specific laboratory data assessed included leukocyte and platelet counts; hemoglobin level; percentage of blasts in peripheral blood and BM; serum bilirubin, creatinine, and albumin levels; cytogenetic results; presence or absence of *FLT3* and *NPM1* mutations; and presence or absence of expression of CXCR4 and pCXCR4. In the multivariate Cox proportional hazards model, all potential covariates were included in the full multivariate Cox proportional hazards model. A backward elimination method was used to determine the final model. Statistical analysis was performed using the STATA/SE software program (version 11.2; StataCorp LP).

RESULTS

The study group included 117 AML patients, 62 (53%) men and 55 (47%) women, with median age of 61 years (range, 18-88). Twenty-six (22%) patients had an antecedent hematological disorder. All patients received intensive chemotherapy according to institutional protocols. Fifty-nine (50%) patients achieved CR, of whom 18 patients relapsed; 58 (50%) patients failed to achieve CR. Thirty-five (30%) patients were alive and 82 (70%) patients were dead at last follow-up. The median follow-up was 8 months ranging from less than a month for patients with early death up to 117 months. The 3-year OS rate was 32% (95% CI: 24-41%) and the 5-year OS rate was 27% (95% CI: 19-36%).

The laboratory data are summarized in Table 1. We detected CXCR4 expression in 75 (64%) patients and pCXCR4 expression in 31 (26%) patients, hence CXCR4 was found to be phosphorylated in 41% of cases. All p-CXCR4 positive were also positive for CXCR4, and all CXCR4-negative patients were also negative for pCXCR4. We detected *NPM1* mutations in 63 of 116 (54%) patients assessed. We did not identify *NPM1* mutations in patients with AML associated with t(8;21) or inv(16). In patients with *NPM1* mutations, 40 (63%) expressed CXCR4 and 15 (24%) expressed pCXCR4. In the patients with wild type

NPM1, 25 (47%) and 15 (28%) expressed CXCR4 and pCXCR4, respectively. *NPM1* mutations were not associated with CXCR4 ($p = 0.212$) or pCXCR4 ($p = 0.355$) expression. *FLT3-ITD* mutations were detected in 22 of 114 (19%) patients tested. In 21 patients with *FLT3-ITD* mutations assessed, 13 (62%) had CXCR4 expression and 7 (33%) had pCXCR4 expression. In a group of 77 patients with wild type *FLT3*, 50 (65%) had CXCR4 expression and 22 (29%) had pCXCR4 expression. *NPM1* mutations were significantly associated with *FLT3/ITD* mutations ($p = 0.040$). *FLT3-ITD* did not correlate with CXCR4 ($p = 0.797$) or pCXCR4 ($p = 0.672$) expression. There was no statistically significant correlation between therapeutic modalities and CXCR4 expression, pCXCR4 expression, *NPM1* mutations, or *FLT-3 ITD*.

In the univariate Cox proportional hazards model, shorter OS was significantly associated with older age (hazard ratio [HR]: 1.07; $p < 0.001$), history of antecedent hematologic disorder (HR: 2.21; $p = 0.006$), thrombocytopenia (HR: 1.01; $p = 0.002$), increased serum creatinine level (HR: 2.14; $p < 0.001$), decreased serum albumin level (HR: 1.38; $p = 0.031$), failure to achieve CR (HR: 5.26; $p < 0.001$), CXCR4 expression (HR: 1.75; $p = 0.042$), wild type *NPM1* (HR: 1.74; $p = 0.023$), and unfavorable cytogenetics (HR: 7.16; $p < 0.001$). We did not find a statistically significant association between OS and sex, leukocyte or platelet count, hemoglobin level, serum bilirubin level, *FLT3/ITD*, or pCXCR4 expression. The Kaplan-Meier curves for CXCR4 expression and *NPM1* status are presented in figures 1A and 1B, respectively. Of interest, when we analyzed the impact of CXCR4 expression separately in patients with wild type *NPM1* or mutated *NPM1*, CXCR4 expression had impact on OS only in patients with wild type *NPM1* ($p = 0.028$, figure 1C). CXCR4 expression in patients with *NPM1* mutations appeared to have worse OS but the difference did not reach the statistical significance ($p = 0.139$, figure 1D).

In the multivariate Cox proportional hazards model (Table 3), shorter OS was associated with antecedent hematological disorder (HR: 2.63; $p = 0.005$), failure to achieve CR (HR: 5.88; $p < 0.001$), thrombocytopenia (HR: 1.02; $p < 0.001$), unfavorable cytogenetics (HR: 7.16; $p < 0.001$), CXCR4 expression (HR: 2.31; $p = 0.008$), and wild type *NPM1* (HR: 1.82; $p = 0.030$). In the univariate Cox proportional hazards model, shorter RFS was associated with failure to achieve CR (HR: 4.55; $p < 0.001$), older age (HR: 1.03; $p < 0.001$), antecedent hematological disorder (HR: 1.78; $p = 0.035$), increased serum creatinine level (HR: 1.97; $p < 0.001$), decreased serum albumin level (HR: 1.33; $p = 0.030$), CXCR4 expression (HR: 1.59; $p = 0.060$), pCXCR4 expression (HR: 1.02; $p = 0.0934$), wild type *NPM1* (HR: 0.62 $p = 0.028$), and unfavorable cytogenetics (HR: 9.76; $p < 0.001$). We did not observe a statistically significant association between RFS and sex, leukocyte or platelet count, hemoglobin level, serum bilirubin level, or *FLT3* mutation. In the multivariate Cox proportional hazards model (Table 4), shorter RFS was associated with antecedent hematological disorder (HR: 2.18; $p = 0.043$), failure to achieve CR (HR: 2.94; $p < 0.001$), thrombocytopenia (HR: 1.01; $p = 0.009$), unfavorable cytogenetics (HR: 5.94; $p = 0.002$), CXCR4 expression (HR: 3.59; $p = 0.001$), pCXCR4 expression (HR: 2.88; $p = 0.002$), and wild type *NPM1* (HR: 3.33; $p < 0.001$).

DISCUSSION

Based on data published by Zhang and colleagues⁴¹ which suggested that the CXCR4-CXCL12 axis and NPM pathways are linked, we hypothesized that there would be a correlation or possibly synergistic or antagonistic effects between CXCR4 and NPM in AML. The data we present, however, show no correlation between *NPM1* mutational status and frequency of CXCR4 expression. These data suggest that NPM and CXCR4 predict prognosis independently; a finding indirectly supported by the fact that the prognostic impact of *NPM1* mutations and CXCR4 expression was evident in multivariate analysis. Therefore, the NPM and CXCR4 pathways likely affect leukemic cell survival via separate mechanisms. Even if an interaction between *NPM1* and CXCR4 does occur in AML, it seems unlikely to have an important effect on patient prognosis. This being stated, it is worth noting that the number of patients in this study is relatively small, 117 patients, and it is possible that larger studies might detect overlap between *NPM1* and CXCR4 effects on prognosis. The potential group of interest could be a group of young patients with normal karyotype, who are known to have high frequency of *NPM1* mutation^{37, 38}. Alternatively, more precise and more quantitative detection of CXCR4 and pCXCR4 expression not achievable by immunohistochemistry, might uncover a relationship between the NPM and CXCR4 pathways. For example, flow cytometric assays provide quantitative evaluation of CXCR4 receptor density expression per cell including AML progenitor cells which might convey important functional consequences in CXCR4-expressing patients. In fact, recent studies by Chou et al. utilizing both murine knock-in model of AML and human AML specimens demonstrated remarkable suppression of CXCR4/CXCL12-related gene signatures, attesting to the role of bone marrow niche defect in *NPM1*-mutant leukemias.⁴² Interestingly, CXCR4 is not listed as one of the differentially expressed genes in their study, suggesting that differences in CXCL12 ligand expression could possibly account for the observed functional differences. Importantly, two recent papers in *Nature* confirmed the critical importance of CXCL12 produced by specialized stromal and endothelial cells within the bone marrow microenvironment for the support of normal HSC.^{53, 54} Further studies including quantitative assessment of CXCR4 and CXCL12 levels, bone marrow architecture and respective contributions of endothelial vs osteoblastic niches^{55, 56} are clearly warranted to delineate the role of perturbed microenvironment in *NPM1*-mutant AML including improved outcomes with cytarabine-containing regimens.

The SDF-1 α (CXCL12)-CXCR4 axis is known to play a key role in normal and leukemic hematopoiesis, and investigators are interested in this axis as a potential therapeutic target for AML patients. Tavor and colleagues have shown that inhibition of the SDF-1 α -CXCR4 axis by AMD3100 reduced the proliferation and survival of AML cell lines and primary AML cells and that many genes involved in regulation of cell proliferation are differentially expressed in AML cells treated with AMD3100 compared with control cells.⁵⁷ Tavor and colleagues also showed that a neutralizing treatment targeting CXCR4 led to inhibition of the repopulation and survival of human AML cells after engraftment in mice.^{57, 58} Importantly, this effect was considerably more significant in the leukemic stem cell compartment than in the normal human stem cell compartment, suggesting a therapeutic potential for CXCR4 neutralization in AML.⁵⁹

Recently, Uy and colleagues reported that plerixafor, a bicyclam small molecule antagonist of CXCR4 binding to CXCL12, combined with mitoxantrone, etoposide, and cytarabine yielded a promising CR rate of 39% in patients with relapsed or refractory AML.⁶⁰ These data support the hypothesis that inhibition of the CXCR4/CXCL12 axis can disrupt the interaction of neoplastic cells with the bone marrow microenvironment increasing the sensitivity of neoplastic cells to chemotherapy.

CXCR4 activation is thought to be primarily ligand dependent, supported by the anti-tumor efficacy of the agent AMD 3100, an antagonist of CXCL12 binding.⁶¹⁻⁶⁶ CXCR4 is phosphorylated in response to ligand binding in a G-protein-coupled receptor kinase 2–dependent fashion.²⁰⁻²² Receptor phosphorylation stimulates the interaction of β -arrestin with the carboxy terminus ceasing CXCR4-mediated activation of $G\alpha_i$, promoting dynamin-dependent, clathrin-mediated receptor endocytosis, and enhancing CXCR4-RAF-dependent signaling.^{20, 21, 23, 24} Regulation of phosphorylation and internalization has significant effects on CXCR4-mediated cellular responses, and for this reason some authors believe that the descriptions of total CXCR4 expression alone are inadequate. In this study, we demonstrated that CXCR4 is phosphorylated in 41% of AML patients, and that phosphorylated (activated) CXCR4 expression independently predicts poorer RFS but not for OS in adults with AML. This result is similar to observations in adult patients with acute lymphoblastic leukemia,⁴⁹ but the precise mechanism of this phenomenon and the upstream kinases involved remains to be elucidated. Notably, PIM1 kinase which is frequently activated in *FLT3/ITD*-mutant AML was reported to cause phosphorylation of serine 339 in the CXCR4 intracellular domain,⁶⁷ however, we did not observe higher frequency of CXCR4 phosphorylation in *NPM1*-mutant AML which additionally carried *FLT3-ITD* mutations.

CONCLUSIONS

In summary, we could not demonstrate any correlation between *NPM1* mutations and CXCR4 or phosphorylated CXCR4 expression suggesting that in adult AML patients. Nevertheless, both *NPM1* mutations and CXCR4 expression predict prognosis. These data suggest that the CXCR4 and NPM pathways act independently. Our findings support the notion that targeting CXCR4 may improve outcomes in AML, and implicate routine testing CXCR4 expression as an additional prognostic marker in AML.

Acknowledgments

We thank Dr. Joshua Rubin for providing a rabbit polyclonal phosphorylated CXCR4-specific antibody, Dr. Greg Fuller for providing a glioblastoma multiforme tissue array, Geneva Williams for manuscript preparation, and Don Norwood for editing the manuscript.

Funding sources: this work was supported in part by National Institutes of Health grants CA016672, P30 CA016672, 2P50 CA100632-06

REFERENCES

1. Siegel R, Naishadham D, Jemal A. Cancer statistics, 2013. *CA Cancer J Clin.* 2013; 63:11–30. [PubMed: 23335087]

2. Murphy PM, Baggiolini M, Charo IF, et al. International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol Rev.* 2000; 52:145–76. [PubMed: 10699158]
3. Fredriksson R, Lagerstrom MC, Lundin LG, Schiöth HB. The G-protein-coupled receptors in the human genome form five main families. Phylogenetic analysis, paralogon groups, and fingerprints. *Mol Pharmacol.* 2003; 63:1256–72. [PubMed: 12761335]
4. Tachibana K, Hirota S, Iizasa H, et al. The chemokine receptor CXCR4 is essential for vascularization of the gastrointestinal tract. *Nature.* 1998; 393:591–4. [PubMed: 9634237]
5. Nagasawa T, Tachibana K, Kishimoto T. A novel CXC chemokine PBSF/SDF-1 and its receptor CXCR4: their functions in development, hematopoiesis and HIV infection. *Semin Immunol.* 1998; 10:179–85. [PubMed: 9653044]
6. McGrath KE, Koniski AD, Maltby KM, McGann JK, Palis J. Embryonic expression and function of the chemokine SDF-1 and its receptor, CXCR4. *Dev Biol.* 1999; 213:442–56. [PubMed: 10479460]
7. Ma Q, Jones D, Borghesani PR, et al. Impaired B-lymphopoiesis, myelopoiesis, and derailed cerebellar neuron migration in CXCR4- and SDF-1-deficient mice. *Proc Natl Acad Sci U S A.* 1998; 95:9448–53. [PubMed: 9689100]
8. Zou YR, Kottmann AH, Kuroda M, Taniuchi I, Littman DR. Function of the chemokine receptor CXCR4 in haematopoiesis and in cerebellar development. *Nature.* 1998; 393:595–9. [PubMed: 9634238]
9. Nagasawa T, Hirota S, Tachibana K, et al. Defects of B-cell lymphopoiesis and bone-marrow myelopoiesis in mice lacking the CXC chemokine PBSF/SDF-1. *Nature.* 1996; 382:635–8. [PubMed: 8757135]
10. Moriuchi M, Moriuchi H, Turner W, Fauci AS. Cloning and analysis of the promoter region of CXCR4, a coreceptor for HIV-1 entry. *J Immunol.* 1997; 159:4322–9. [PubMed: 9379028]
11. Cristillo AD, Highbarger HC, Dewar RL, Dimitrov DS, Golding H, Bierer BE. Up-regulation of HIV coreceptor CXCR4 expression in human T lymphocytes is mediated in part by a cAMP-responsive element. *FASEB J.* 2002; 16:354–64. [PubMed: 11874984]
12. Jourdan P, Vendrell JP, Huguet MF, et al. Cytokines and cell surface molecules independently induce CXCR4 expression on CD4+ CCR7+ human memory T cells. *J Immunol.* 2000; 165:716–24. [PubMed: 10878344]
13. Wang J, Guan E, Roderiquez G, Calvert V, Alvarez R, Norcross MA. Role of tyrosine phosphorylation in ligand-independent sequestration of CXCR4 in human primary monocytes/macrophages. *J Biol Chem.* 2001; 276:49236–43. [PubMed: 11668182]
14. Feil C, Augustin HG. Endothelial cells differentially express functional CXC-chemokine receptor-4 (CXCR-4/fusin) under the control of autocrine activity and exogenous cytokines. *Biochem Biophys Res Commun.* 1998; 247:38–45. [PubMed: 9636650]
15. Salcedo R, Wasserman K, Young HA, et al. Vascular endothelial growth factor and basic fibroblast growth factor induce expression of CXCR4 on human endothelial cells: In vivo neovascularization induced by stromal-derived factor-1alpha. *Am J Pathol.* 1999; 154:1125–35. [PubMed: 10233851]
16. Phillips RJ, Mestas J, Gharaee-Kermani M, et al. Epidermal growth factor and hypoxia-induced expression of CXC chemokine receptor 4 on non-small cell lung cancer cells is regulated by the phosphatidylinositol 3-kinase/PTEN/AKT/mammalian target of rapamycin signaling pathway and activation of hypoxia inducible factor-1alpha. *J Biol Chem.* 2005; 280:22473–81. [PubMed: 15802268]
17. Gupta SK, Lysko PG, Pillarisetti K, Ohlstein E, Stadel JM. Chemokine receptors in human endothelial cells. Functional expression of CXCR4 and its transcriptional regulation by inflammatory cytokines. *J Biol Chem.* 1998; 273:4282–7. [PubMed: 9461627]
18. Vila-Coro AJ, Rodriguez-Frade JM, Martin De Ana A, Moreno-Ortiz MC, Martinez AC, Mellado M. The chemokine SDF-1alpha triggers CXCR4 receptor dimerization and activates the JAK/STAT pathway. *FASEB J.* 1999; 13:1699–710. [PubMed: 10506573]
19. Zhang XF, Wang JF, Matczak E, Proper JA, Groopman JE. Janus kinase 2 is involved in stromal cell-derived factor-1alpha-induced tyrosine phosphorylation of focal adhesion proteins and migration of hematopoietic progenitor cells. *Blood.* 2001; 97:3342–8. [PubMed: 11369622]

20. Haribabu B, Richardson RM, Fisher I, et al. Regulation of human chemokine receptors CXCR4. Role of phosphorylation in desensitization and internalization. *J Biol Chem.* 1997; 272:28726–31. [PubMed: 9353342]
21. Orsini MJ, Parent JL, Mundell SJ, Marchese A, Benovic JL. Trafficking of the HIV coreceptor CXCR4: role of arrestins and identification of residues in the C-terminal tail that mediate receptor internalization. *J Biol Chem.* 2000; 275:25876.
22. Signoret N, Oldridge J, Pelchen-Matthews A, et al. Phorbol esters and SDF-1 induce rapid endocytosis and down modulation of the chemokine receptor CXCR4. *J Cell Biol.* 1997; 139:651–64. [PubMed: 9348282]
23. Cheng ZJ, Zhao J, Sun Y, et al. beta-arrestin differentially regulates the chemokine receptor CXCR4-mediated signaling and receptor internalization, and this implicates multiple interaction sites between beta-arrestin and CXCR4. *J Biol Chem.* 2000; 275:2479–85. [PubMed: 10644702]
24. Sun Y, Cheng Z, Ma L, Pei G. Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. *J Biol Chem.* 2002; 277:49212–9. [PubMed: 12370187]
25. Su SB, Gong W, Grimm M, et al. Inhibition of tyrosine kinase activation blocks the down-regulation of CXC chemokine receptor 4 by HIV-1 gp120 in CD4+ T cells. *J Immunol.* 1999; 162:7128–32. [PubMed: 10358157]
26. Balkwill F. The significance of cancer cell expression of the chemokine receptor CXCR4. *Semin Cancer Biol.* 2004; 14:171–9. [PubMed: 15246052]
27. Zlotnik A. Chemokines and cancer. *Int J Cancer.* 2006; 119:2026–9. [PubMed: 16671092]
28. Bachelder RE, Wendt MA, Mercurio AM. Vascular endothelial growth factor promotes breast carcinoma invasion in an autocrine manner by regulating the chemokine receptor CXCR4. *Cancer Res.* 2002; 62:7203–6. [PubMed: 12499259]
29. Helbig G, Christopherson KW 2nd, Bhat-Nakshatri P, et al. NF-kappaB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. *J Biol Chem.* 2003; 278:21631–8. [PubMed: 12690099]
30. Tomescu O, Xia SJ, Strezlecki D, et al. Inducible short-term and stable long-term cell culture systems reveal that the PAX3-FKHR fusion oncoprotein regulates CXCR4, PAX3, and PAX7 expression. *Lab Invest.* 2004; 84:1060–70. [PubMed: 15184910]
31. Libura J, Drukala J, Majka M, et al. CXCR4-SDF-1 signaling is active in rhabdomyosarcoma cells and regulates locomotion, chemotaxis, and adhesion. *Blood.* 2002; 100:2597–606. [PubMed: 12239174]
32. Castellone MD, Guarino V, De Falco V, et al. Functional expression of the CXCR4 chemokine receptor is induced by RET/PTC oncogenes and is a common event in human papillary thyroid carcinomas. *Oncogene.* 2004; 23:5958–67. [PubMed: 15184868]
33. Marchese A, Benovic JL. Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting. *J Biol Chem.* 2001; 276:45509–12. [PubMed: 11641392]
34. Marchese A, Raiborg C, Santini F, Keen JH, Stenmark H, Benovic JL. The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4. *Dev Cell.* 2003; 5:709–22. [PubMed: 14602072]
35. Cordell JL, Pulford KA, Bigerna B, et al. Detection of normal and chimeric nucleophosmin in human cells. *Blood.* 1999; 93:632–42. [PubMed: 9885226]
36. Chang JH, Olson MO. Structure of the gene for rat nucleolar protein B23. *J Biol Chem.* 1990; 265:18227–33. [PubMed: 2211699]
37. Schnittger S, Schoch C, Kern W, et al. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. *Blood.* 2005; 106:3733–9. [PubMed: 16076867]
38. Boissel N, Renneville A, Biggio V, et al. Prevalence, clinical profile, and prognosis of NPM mutations in AML with normal karyotype. *Blood.* 2005; 106:3618–20. [PubMed: 16046528]
39. Dohner K, Schlenk RF, Habdank M, et al. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. *Blood.* 2005; 106:3740–6. [PubMed: 16051734]

40. Schlenk RF, Dohner K, Krauter J, et al. Mutations and treatment outcome in cytogenetically normal acute myeloid leukemia. *N Engl J Med.* 2008; 358:1909–18. [PubMed: 18450602]
41. Zhang W, Navenot JM, Frilot NM, Fujii N, Peiper SC. Association of nucleophosmin negatively regulates CXCR4-mediated G protein activation and chemotaxis. *Mol Pharmacol.* 2007; 72:1310–21. [PubMed: 17715399]
42. Chou SH, Ko BS, Chiou JS, Hsu YC, Tsai MH, Chiu YC, Yu IS, Lin SW, Hou HA, Kuo YY, Lin HM, Wu MF, Chou WC, Tien HF. A knock-in Npm1 mutation in mice results in myeloproliferation and implies a perturbation in hematopoietic microenvironment. *PLoS One.* 2012; 7:e49769. [PubMed: 23226219]
43. Rombouts EJ, Pavic B, Lowenberg B, Ploemacher RE. Relation between CXCR-4 expression, Flt3 mutations, and unfavorable prognosis of adult acute myeloid leukemia. *Blood.* 2004; 104:550–7. [PubMed: 15054042]
44. Spoo AC, Lubbert M, Wierda WG, Burger JA. CXCR4 is a prognostic marker in acute myelogenous leukemia. *Blood.* 2007; 109:786–91. [PubMed: 16888090]
45. Konoplev S, Rassidakis GZ, Estey E, et al. Overexpression of CXCR4 predicts adverse overall and event-free survival in patients with unmutated FLT3 acute myeloid leukemia with normal karyotype. *Cancer.* 2007; 109:1152–6. [PubMed: 17315232]
46. Oyarzo MP, Lin P, Glassman A, Bueso-Ramos CE, Luthra R, Medeiros LJ. Acute myeloid leukemia with t(6;9)(p23;q34) is associated with dysplasia and a high frequency of flt3 gene mutations. *Am J Clin Pathol.* 2004; 122:348–58. [PubMed: 15362364]
47. Hao S, Sanger W, Onciu M, Lai R, Schlette EJ, Medeiros LJ. Mantle cell lymphoma with 8q24 chromosomal abnormalities: a report of 5 cases with blastoid features. *Mod Pathol.* 2002; 15:1266–72. [PubMed: 12481006]
48. Haferlach T, Schoch C, Löffler H, et al. Morphologic dysplasia in de novo acute myeloid leukemia (AML) is related to unfavorable cytogenetics but has no independent prognostic relevance under the conditions of intensive induction therapy: results of a multiparameter analysis from the German AML Cooperative Group studies. *J Clin Oncol.* 2003; 21:256–65. [PubMed: 12525517]
49. Konoplev S, Jorgensen JL, Thomas DA, et al. Phosphorylated CXCR4 is associated with poor survival in adults with B-acute lymphoblastic leukemia. *Cancer.* 2011; 117:4689–95. [PubMed: 21456010]
50. Hesselgesser J, Liang M, Hoxie J, et al. Identification and characterization of the CXCR4 chemokine receptor in human T cell lines: ligand binding, biological activity, and HIV-1 infectivity. *J Immunol.* 1998; 160:877–83. [PubMed: 9551924]
51. Woerner BM, Warrington NM, Kung AL, Perry A, Rubin JB. Widespread CXCR4 activation in astrocytomas revealed by phospho-CXCR4-specific antibodies. *Cancer Res.* 2005; 65:11392–9. [PubMed: 16357147]
52. Estey EH, Pierce S, Keating MJ. Identification of a group of AML/MDS patients with a relatively favorable prognosis who have chromosome 5 and/or 7 abnormalities. *Haematologica.* 2000; 85:246–249.
53. Ding L, Morrison SJ. Haematopoietic stem cells and early lymphoid progenitors occupy distinct bone marrow niches. *Nature.* 2013; 495:231–5. [PubMed: 23434755]
54. Greenbaum A, Hsu YM, Day RB, Schuettpeiz LG, Christopher MJ, Borgerding JN, et al. CXCL12 in early mesenchymal progenitors is required for haematopoietic stem-cell maintenance. *Nature.* 2013; 495:227–30. [PubMed: 23434756]
55. Ding L, Saunders TL, Enikolopov G, Morrison SJ. Endothelial and perivascular cells maintain haematopoietic stem cells. *Nature.* 2001; 481:457–62. [PubMed: 22281595]
56. Spradling A, Drummond-Barbosa D, Kai T. Stem cells find their niche. *Nature.* 2001; 414:98–104. [PubMed: 11689954]
57. Tavor S, Eisenbach M, Jacob-Hirsch J, et al. The CXCR4 antagonist AMD3100 impairs survival of human AML cells and induces their differentiation. *Leukemia.* 2008; 22:2151–5158. [PubMed: 18769446]
58. Zeng Z, Shi YX, Samudio IJ, et al. Targeting the leukemia microenvironment by CXCR4 inhibition overcomes resistance to kinase inhibitors and chemotherapy in AML. *Blood.* 2009; 113:6215–24. [PubMed: 18955566]

59. Tavor S, Petit I, Porozov S, et al. CXCR4 regulates migration and development of human acute myelogenous leukemia stem cells in transplanted NOD/SCID mice. *Cancer Res.* 2004; 64:2817–24. [PubMed: 15087398]
60. Uy GL, Rettig MP, Motabi IH, et al. A phase 1/2 study of chemosensitization with the CXCR4 antagonist plerixafor in relapsed or refractory acute myeloid leukemia. *Blood.* 2012; 119:3917–24. [PubMed: 22308295]
61. Rubin JB, Kung AL, Klein RS, et al. A small-molecule antagonist of CXCR4 inhibits intracranial growth of primary brain tumors. *Proc Natl Acad Sci U S A.* 2003; 100:13513–8. [PubMed: 14595012]
62. Smith MC, Luker KE, Garbow JR, et al. CXCR4 regulates growth of both primary and metastatic breast cancer. *Cancer Res.* 2004; 64:8604–12. [PubMed: 15574767]
63. De Clercq E. The bicyclam AMD3100 story. *Nat Rev Drug Discov.* 2003; 2:581–7. [PubMed: 12815382]
64. Gerlach LO, Skerlj RT, Bridger GJ, Schwartz TW. Molecular interactions of cyclam and bicyclam non-peptide antagonists with the CXCR4 chemokine receptor. *J Biol Chem.* 2001; 276:14153–60. [PubMed: 11154697]
65. Hatse S, Princen K, Bridger G, De Clercq E, Schols D. Chemokine receptor inhibition by AMD3100 is strictly confined to CXCR4. *FEBS Lett.* 2002; 527:255–62. [PubMed: 12220670]
66. Hendrix CW, Flexner C, MacFarland RT, et al. Pharmacokinetics and safety of AMD-3100, a novel antagonist of the CXCR-4 chemokine receptor, in human volunteers. *Antimicrob Agents Chemother.* 2000; 44:1667–73. [PubMed: 10817726]
67. Grundler R, Brault L, Gasser C, et al. Dissection of PIM serine/threonine kinases in FLT3-ITD-induced leukemogenesis reveals PIM1 as regulator of CXCL12-CXCR4-mediated homing and migration. *J Exp Med.* 2009; 206:1957–70. [PubMed: 19687226]

CLINICAL PRACTICE POINTS

- CXCR4 expression and wild type *NPM1* gene are associated with shorter overall and progression free survival in adult patients with AML
- Phosphorylated CXCR4 expression is associated with shorter progression free survival in adult patients with AML
- There is no correlation between *NPM1* mutations and CXCR4 or phosphorylated CXCR4 expression in adult AML suggesting that CXCR4 and NPM pathways act independently.
- These data provide a rationale for independent targeting CXCR4 and NPM pathways in leukemic blasts to improve an outcome in AML patients.

Figure 1.

A) Adverse effect of CXCR4 expression on overall survival in the entire study group; B) Adverse effect of wild type *NPM1* on overall survival in the entire study group; C) Adverse effect of CXCR4 expression on overall survival in patients with wild type *NPM1*; D) Adverse effect of CXCR4 expression on overall survival in patients with mutated *NPM1*; all curves are drawn using Kaplan-Meier method.

Table 1

Summary of Laboratory Data

Variable	Median (range)
White blood cell count, $\times 10^9/L$	23.8 (0.3-200.5)
Platelet count, $\times 10^9/L$	51 (5-306)
Hemoglobin level, g/dL	9.2 (4.1-14.8)
Peripheral blood blasts count, %	44 (0-99)
Bone marrow blasts count, %	64 (20-96)
Albumin level, g/dL	3.6 (1.4-4.9)
Bilirubin level, mg/dL	0.5 (0.1-2.7)
Creatinine level, mg/dL	1.0 (0.5-4.4)
Cytogenetics	No. of Patients (%)
Favorable	19 (16)
Intermediate	70 (60)
Unfavorable	28 (24)
No analyzable metaphases	3 (3)
<i>NPM1</i>	
Mutated	63 (54)
Wild-type	53 (45)
Not done	1 (1)
<i>FLT3</i>	
<i>ITD</i>	22 (19)
Wild-type	92 (79)
Not done	3 (3)
CXCR4 expression	
Positive	75 (64)
Negative	40 (34)
Not done	2 (2)
pCXCR4 expression	
Positive	31 (26)
Negative	70 (60)
Not done	16 (14)

Table 2

Multivariate Cox Proportional Hazards Model for overall survival.

Variable	<i>P</i>	HR	95% CI
Antecedent hematological	0.005	2.63	1.34-5.16
Failure to achieve CR	<0.001	5.88	3.11-11.11
Thrombocytopenia	<.001	1.02	1.01-1.03
Unfavorable cytogenetics	<0.001	7.16	2.62-19.60
CXCR4 expression	0.008	2.31	1.25-4.29
Wild type <i>NPM1</i>	0.030	1.82	1.06-3.13

Table 3

Multivariate Cox Proportional Hazards Model for Relapse-Free Survival

Variable	<i>P</i>	HR	95% CI
Antecedent hematological disorder	0.043	2.18	1.03-4.64
Thrombocytopenia	0.009	1.01	1.00-1.01
Unfavorable cytogenetics	0.002	5.94	1.90-18.56
CXCR4 expression	0.001	3.59	1.73-7.46
pCXCR4 expression	0.002	2.88	1.49-5.56
Wild type <i>NPM1</i>	<0.001	3.33	1.81-6.25