Original Article
Middle East Journal of Cancer; October 2022; 13(4): 573-580

Evaluation of the Expression Levels of Notch Signaling Pathway-Related Genes; *JAG1, CXCR4*, and *MIB1* in Acute Myeloid Leukemia Patients

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Please cite this article as: Ghafelehbashi SH, Sadeghizadeh D, Rohollah F, Pahlavanneshan S, Sadeghizadeh M. Evaluation of the expression levels of Notch signaling pathway-related genes; *JAG1, CXCR4*, and *MIB1* in acute myeloid leukemia patients. Middle East J Cancer. 2022;13(4):573-80. doi: 10.30476/mejc.2021.89042.1504.

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Abstract

Background: Acute myeloid leukemia (AML) is a complex disease characterized by clonal expansion of undifferentiated myeloid precursors, resulting in impaired hematopoiesis and bone marrow failure. Different genetic and environmental factors are believed to be involved in the pathogenesis of AML. Notch signaling with a tumor suppressing plays a role in myeloproliferative disorder and is a negative regulator of myeloid progenitor commitment. Crosstalk between Notch signaling and CXCR4 axis is a matter of debate in AML.

Method: In the current case-control study, we evaluated the expression level of *CXCR4, JAG1*, and *MIB1*, which are all involved or related to Notch signaling in adult AML patients. Blood samples were obtained from 25 AML and 17 healthy individuals and the expression level of the selected genes was evaluated via the real-time polymerase chain reaction.

Results: Our results revealed the increased expression of *JAG1*, but decreased expression of *CXCR4* in AML patients in Iranian population of AML patients. Moreover, some gender-associated effects on the expression of *JAG1* and *CXCR4* were detected, which may be related to sex hormones. The expression level of *MIB1* did not change significantly. The correlation analysis showed no correlations between the age of the patients and the expression levels of the genes.

Conclusion: Herein, for the first time, we suggested some new evidence regarding the complex role of Notch signaling-related genes (*CXCR4* and *JAG1*) in the pathogenesis of AML in Iranian patients.

Keywords: Notch, *JAG1, CXCR4, MIB1*, Acute myeloid leukemia

Received: December 30, 2020; Accepted: August 28, 2021

Introduction

Acute myeloid leukemia (AML) is defined as a group of hematopoietic cell disorders that lead to the uncontrolled proliferation of undifferentiated myeloid progenitors.[1] Despite the improvement in its treatment, five-year survival rate has been reported to be about 35%-40% and the disease is still incurable in most patients.[2] Several factors could increase the susceptibility to AML, including environmental factors (such as Benzene and high-level ionizing radiation), and genetic and epigenetic dysregulations.[3,4] These abnormalities lead to the disruption of the normal mechanisms of growth, proliferation, and differentiation of blood precursors.[5] Since AML is highly heterogeneous amongst patients and different ethnic groups, precision medicine and personalized treatment are considered to be challenging.[6] Hence, unraveling molecular mechanisms underlying AML progression could be conducive to tackling the disease effectively.

Gene expression profiling, genome-wide association studies, as well as protein profiling, have shed further light on the molecular mechanisms controlling AML initiation, progression, and relapse.[7,8] For note, Notch signaling is a causative signaling in myeloproliferative disorders and a negative regulator of myeloid progenitor commitment.[9] The Notch signaling pathway, including Notch receptors and ligands, such as DLL1 and JAG1, contribute to regulating gene expression in nucleus.[10] Activation of this signaling pathway has emerged as a prognostic marker in patients with acute lymphoblastic leukemia (ALL), yet it is matter of debate in AML.[11,12] Previous studies have shown that Notch activation in AML is suppressed and its reactivation induced cell cycle arrest, differentiation, and apoptosis in AML-initiating cells.[13,14] Activation of Notch signaling leads to increased number of hematopoietic stem cell (HSC) and blood progenitor cells.[15,16]

JAG1 is expressed on the surface of osteoblasts and is attached to Notch receptor on the surface of HSC cells.[17] One of the other molecules involved in the activation of the Notch signaling pathway as a ubiquitin ligase is *MIB1*; certain studies have shown that *MIB1* mutants result in reduced Notch1 activity.[18]

One of the main pathways related to Notch signaling and hematopoiesis is *CXCR4/CXCL12* axis, which is identified as the regulator of lymphocytes orientation to bone marrow.[19] *CXCR4* receptor is a G-protein coupled receptor

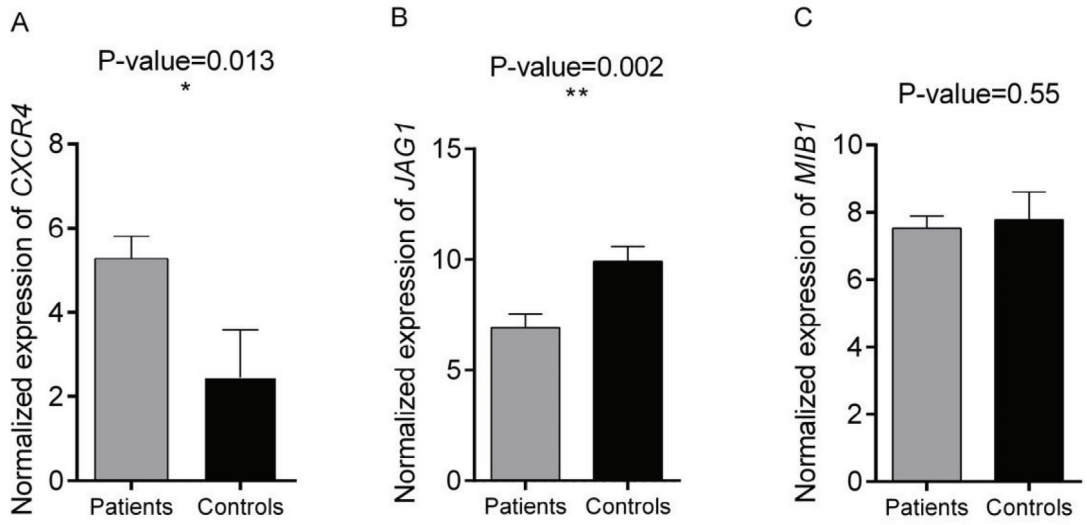

Figure 1. *CXCR4, JAG1,* and *MIB1* expression level in PBMCs of AML patients. Using independent t-test analysis, the expression of *JAG1* significantly increased while the expression of *CXCR4* significantly decreased in AML patients compared to the healthy controls (A and B, respectively). *MIB1* expression level did not significantly change among the patients (C). The expression values of each sample were normalized against GAPDH mRNA expression. *$P < 0.05$, ** $P < 0.005$
AML: Acute myeloid leukemia

(GPCR) located on the surface of HSC and attached to *CXCL12* chemokine ligand on HSCs.[20] By directing the leukemic cells in the bone marrow niche, *CXCL12* regulates their engraftment and survival.[21] Elevated expression of *CXCR4* has been indicated to correlate with shortened survival of the patients. The Notch signaling pathway regulates the *CXCR4* expression in several cell types and is essential for controlled proliferation and apoptosis.[22,23]

Based on the specific genetic background of the Iranian population, we conducted the present work to evaluate the expression of *CXCR4, JAG1,* and *MIB1* genes as Notch signaling pathway-related genes in the PBMCs of adult patients with AML.

Materials and Methods

Samples preparation

In this case-control study, 5 milliliters of blood sample was collected from 25 AML patients clinically diagnosed by an expert oncologist in the Imam Khomeini Hospital (Tehran, Iran). Additionally, 17 age-matched healthy controls were selected from the individuals without any personal or familial cancer history. All the procedures were under ethical standards and international agreements (Helsinki Declaration of 1964, revised 2013). The study was approved by the Ethics Committee of Tarbiat Modares University (ethics code: 1203429). Informed consent was obtained from all the participants.

Extraction of RNA and elimination of DNA

RNA was isolated using the RNATM-plus (CinnaGen Inc., Iran) after PBMC isolation via Ficoll-Paque solution gradient. The quality and integrity of the extracted RNAs were verified through spectrophotometry and 1% agarose gel electrophoresis. Moreover, to eliminate any probable genomic DNA contamination, the

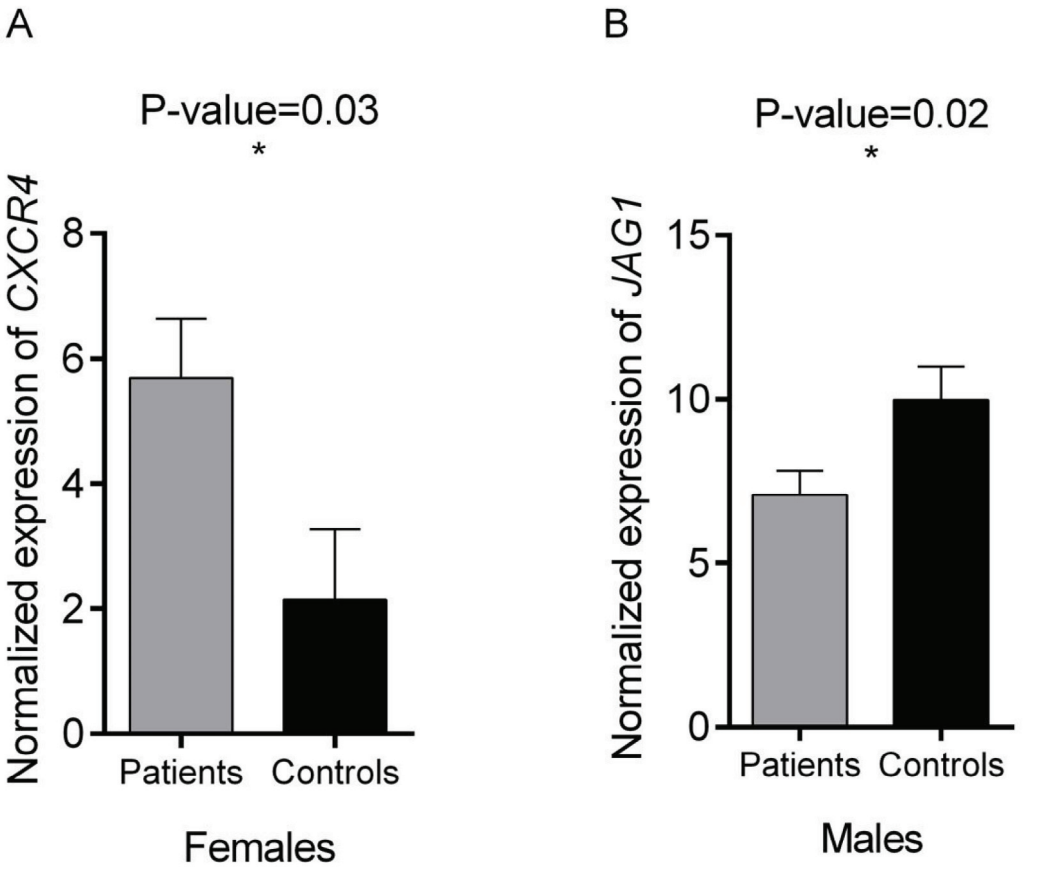

Figure 2. This figure shows the normalized expression of selected genes in the male and female subgroups. The mRNA expression level of *CXCR4* decreased significantly only in the AML female patients (A). Expression level of *JAG1* increased significantly only in the AML male patients (B).

* $P < 0.05$; AML: Acute myeloid leukemia

samples were treated with DNase I (Fermentas, Lithuania).

Complementary DNA (cDNA) synthesis and real-time polymerase chain reaction (RT-PCR) quantification

cDNA synthesis was performed employing RT-PCR kit (Takara Shuzo, Otsu, Japan). OligodT and Random Hexamer primers, in a total volume of 20µL reaction mixture, were used under the manufacturer's instruction. All the quantitative gene expression analyses were assessed as duplicates with EvaGreen I master mix (Salise Biodyne, Japan) via Step One Applied Biosystems (Applied Biosystems, USA). The mRNA expression levels of *JAG1* (Forward primer (FP): TACTGGCACCTGCAGTCACC, Reverse primer (RP): GAAGCAGAACACGGGCGTTG), *CXCR4* (FP: AGTGGCCGACCTCCTCTT, RP: TTGGCCTCTGACTGTTGGT), and *MIB1*(FP: AGCCAGAAACAGTGTAAGAGGG, RP: GTTTGGGGATTCATTGCTGCT) were normalized to *GAPDH*(FP: CCATGAGAAG-TATGACA, RP: GAGTCCTTCCACGAT) as the internal control. The relative expression of the selected genes was calculated using the $2^{-\Delta\Delta Ct}$ formula.

Statistical analysis

The normality of the data was checked using the Shapiro-Wilk test. Independent t-test and/or Mann-Whitney U-test were utilized to investigate the differences in the expression levels of the genes among the patients and controls. The receiver operating characteristic (ROC) curve analysis was retrieved to determine the optimal cut-off values of the expression levels of the selected genes as potential biomarkers. Finally, Spearman or Pearson correlation coefficient tests were employed to determine the potential correlations between the expression levels of the selected genes and age.

All the statistical analyses were performed via GraphPad Prism 6.01 (GraphPad Software, Inc., CA, USA) and *P*-value of ≤ 0.05 was considered to be significant.

Results

Blood samples were obtained from a total of 25 AML patients (18 males, 7 females) and 17 control individuals (10 males, 7 females). The mean age of the patients and controls were 30 and 28.3 years old, respectively. Based on the French-American-British (FAB) criteria, the most

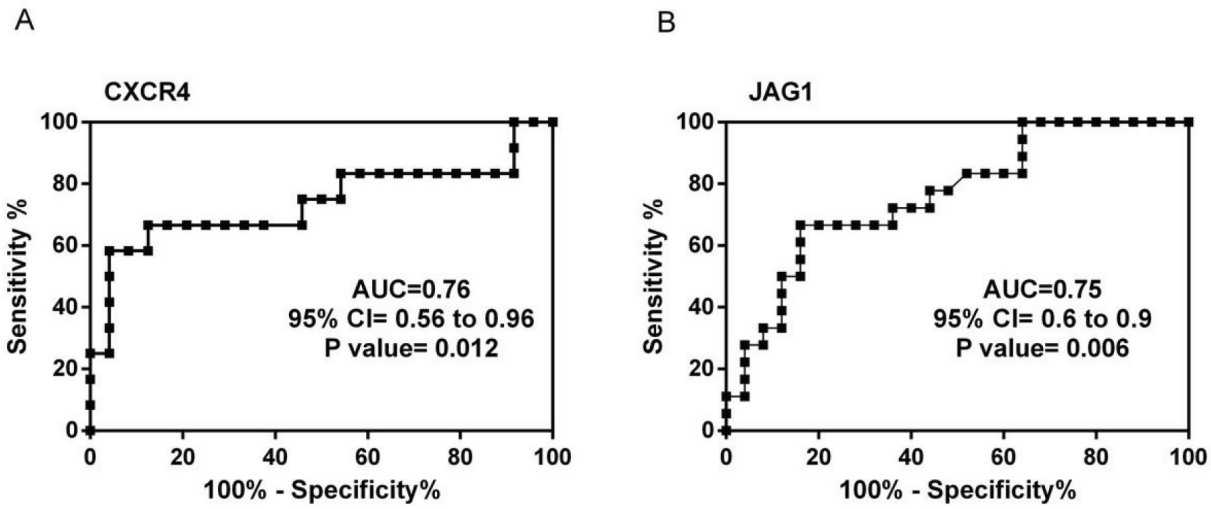

Figure 3. This figure shows the ROC curve analysis of *CXCR4* (A) and *JAG1* (B) gene expression for discriminating the AML patients from the healthy controls. ROC curve analysis was retrieved by plotting sensitivity versus specificity for each threshold value. The area under the curve showed an accuracy level of >0.7.
ROC: Receiver operating characteristic; AML: Acute myeloid leukemia

frequently observed AML subtypes were M3 and M4 in our subjects.

Following statistical analysis, our results implied that the expression level of *CXCR4* significantly decreased (P = 0.01, Figure 1A); whereas the expression of *JAG1* (P = 0.002, Figure 1B) significantly increased in AML patients compared with the healthy controls. However, we did not find any significant differences concerning the expression of *MIB1* between the patients and controls (P = 0.5, Figure 1C). In stratification for gender, *CXCR4* expression significantly decreased in the females compared with that in males (P = 0.03, Figure 2A). Moreover, there was a significant difference concerning *JAG1* expression between the male AML patients and controls (P = 0.02, Figure 2B).

We also performed ROC curve analysis in order to evaluate the sensitivity and specificity of *JAG1* and *CXCR4* expressions as potential biomarkers for clinical diagnosis of AML patients. The area under the curve (AUC) values for *CXCR4* and *JAG1* were 0.75 (95% confidence interval (CI) = 0.6 to 0.9, P = 0.006, sensitivity: 66%, and specificity: 81%) and 0.76 (95% CI = 0.56 to 0.96, P = 0.012, sensitivity: 58%, specificity: 100%), respectively (Figure 3A, and B). Furthermore, we could not find any statistically significant correlations between the expression level of the selected genes and the patients' age. In addition, there were no correlations among the expression levels of the selected genes (P > 0.05).

Discussion

Herein, we reported a significant increase in *JAG1* expression as Notch signaling player and a decrease in *CXCR4* expression in patients with AML. To the best of our knowledge, it is the first report in Iranian population with ethnic background, which highlights the crosstalk between Notch signaling and other chemokine signaling regulating AML progressions. Moreover, we observed a significant difference in *JAG1* expression between the male and female participants, which highlighted the potential role of sex hormones.

AML is a highly aggressive hematological malignancy and the most prevalent type of acute leukemia in adults. Notch signaling is known to have a tumor suppressor role in AML; its reactivation was found to be a promising anti-AML therapy.[24] Significant efforts have been made to combat master molecular drivers of the disease and to understand the underlying signaling pathways which direct onset and progression of AML.[25,26] Notch signaling is one of the main mechanisms, through which different cell types are generated from precursor cells.[27] Dysregulation of Notch signaling and its related molecules in AML pathogenesis has been suggested by several previous papers;[7,10,13,28] for instance, it has been strongly suggested that NOTCH1 in AML represses the expression of oncogenes, such as FLT3, through the induction of the expression of HES1.[13,28]

Aberrant expression of Notch signaling ligands, such as *JAG1* and *DLL-1*, has been reported in ALL and postulated to be important in AML.[17,29,30] We observed a significant upregulation in *JAG1*, which is in accordance with the reports by other researchers.[29,30] It has been stated that *JAG1*, through an autocrine signaling, can activate its own expression in leukemic cells although it can activate Notch signaling through Notch1 receptor in ALL.[31,32] It is notable that *JAG1* can influence AML patients' response to chemotherapy potentially through its high expression in AML blasts, which directs increased proliferation.[29] Additionally, our observation of an Iranian population further confirmed that aberrant expression of *JAG1* may have prognostic effects on different populations with ethnic differences.

We observed different expressions of *JAG1* in males and females, which might be attributed to the impact of sex hormones. Despite the limitation in our sample size, particularly after the stratification by gender, sex hormones were found to affect the expression of *JAG1*, which can explain, at least in part, different expressions of *JAG1* in the males and females. For instance, 17beta-estradiol (E2) increased *JAG1* levels by limiting the repressive effects of miR-21 on *JAG1* 3′UTR.[33]

Crosstalk between Notch signaling and other

related chemokines, such as *CXCR4, SDF1, CCL19,* and *CCL25*, is correlated with the exacerbation and progression of T- and B-ALL.[12] High expression of *CXCR4*, as a direct transcriptional effect of Notch1, leads to B-cell infiltration to other organs, like spleen, liver, and lymph nodes; it may also cause T-ALL infiltration in bone marrow and other organs.[34] Although these observations suggested pharmacological roles of *CXCR4* in ALL, the role of this signaling is still controversial in AML. The *SDF1/CXCR4* signaling axis has been reported as a strong player for the crosstalk between stromal cells and leukemic cells in AML.[12] This axis is also known to contribute to migration of hematopoietic and lymphopoietic cells.[35] In vivo culture of leukemia cells supported this notion by the overexpressed level of *CXCR4*.[21] For note, several studies have shown amelioration of chemoresistance by inhibiting *CXCR4* in culture.[24,36] Moreover, high *CXCR4* expression has been reported to be correlated with increased autophagy in AML patients; however, our findings were on the contrary since we observed decreased expression of *CXCR4* in AML patients.[36] It is worth mentioning that decreased level of *CXCR4* have been also observed in AML CD34+ cells in comparison with normal progenitors.[37] Overall, it seems that expression of *CXCR4* is highly context-dependent and certain parameters, such as tumor grade, gender, cell type, and tumor microenvironment, could influence its expression. Our obtained results revealed that although the research was carried out on an Iranian population with specific genetic background, the study could be more beneficial and generalizable by increasing the number of participants.

MIB1 did not show any differences between the patients and controls, but Staber et al. reported increased level of *MIB1* protein in bone marrow of AML patients in comparison with their controls.[38]

The area under the ROC curve for both *CXCR4* and *JAG1* was > 0.7. Therefore, the expression levels of *CXCR4* and *JAG1* in the PBMCs may be predictive for AML patients. Further prospective studies are necessary to verify and test the usefulness of this cut-off value to distinguish AML patients and healthy controls. Small sample size was one of the main limitations in this study and a heterogeneous combination of cells in whole blood may have overshadowed the variation of our selected gene expression. We could also suggest that future research follow these genes in different AML tumor grades and protein levels.

Conclusion

The results in the present paper indicated the aberrant expression level of some important genes related to Notch signaling in Iranian AML patients. Although there is a growing number of genes and mutations involved in the pathogenesis of AML, population-based evidence, which might be important for personalized medicine therapy, is poorly understood.

Acknowledgment

The authors gratefully acknowledge the contribution of the patients and healthy individuals.

Funding

This work was financially supported by Tarbiat Modares University.

Availability of data and material

The data are available by the corresponding author upon request.

Conflict of Interest

None declared.

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