

Original Article

Running Title: The Chemotherapeutic Effect on the CXCL12/CXCR4 Axis in AML

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Does the Chemotherapy Protocol Affect CXCL12/CXCR4 Axes in Acute Myeloid Leukemia Patients with Monocytic Differentiation?

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Abstract

Background: The CXCR4 receptor along with CXCL12 is believed to have an effect on the onset, progression, migration, and treatment complications and improve AML (acute myeloid leukemia) treatment outcomes. In this study, we investigated the impact of (7+3) chemotherapy protocol on the expression of CXCR4 and its related ligand CXCL12.

Method: In this case-control study, specimens were collected before and after the first cycle of chemotherapy of AML-M4 and AML-M5 patients. RT-PCR and flow cytometry techniques tested the CXCR4 expression. ELISA was used for measuring the serum level of CXCL12. Two samples, t-Test and Paired t-Test, were utilized for data analysis.

Results: We found that CXCR4 expression by Lymphocyte cells after chemotherapy was approximately similar to the CXCR4 expression in the healthy subjects. Moreover, CXCR4 expression was high prior to chemotherapy. The serum level of CXCL12 considerably increased in the patients before chemotherapy. However, after chemotherapy, CXCL12 was found to reach the baseline level in comparison to the healthy control group.

Conclusion: The (7+3) current chemotherapy inhibited CXCL12. Therefore, controlling chemokines along with chemotherapy in AML patients might be conducive to the treatment process or even prevent the relapse of the disease.

Keywords: Acute myeloid leukemia, Chemotherapy, Chemokine, CXCL12, CXCR4

Introduction

An aggressive and heterogeneous bone marrow (BM) malignancy is defined as acute myeloid leukemia (AML). The therapy of AML includes either intensive chemotherapy, alone or in combination with allogeneic stem cell transplantation.¹ Despite the sensitivity to chemotherapy, the long-term disease-free survival in AML sufferers has sustainably remained low and it was thus reported that the majority of the patients most often relapse from minimal residual disease (MRD).² The main location of MRD is BM, AML cells adhere to the BM components that may protect the chemotherapy reagents.³ Chemokines alongside their cognate receptors are extremely involved in the pathogenesis of AML.⁴ It has been also well evidenced that cytokine/receptor axes play crucial parts in leukemogenesis, AML cell persistence, and its treatment outcomes.⁵ Evidence has shown that leukemia cells respond differently to the stimulation of cytokines and leukemic blasts most often produce cytokines.⁶

The CXC chemokine ligand 12 (CXCL12) has been studied in various aspects not only in physiological, but also in clinical states. Attachment of CXCL12 to its receptor, which is called CXCR4 on peripheral immune cells, creates pleiotropic activities. CXCL12 attracts lymphocytes and monocytes and participates actively in neo-vascularization, metastasis, tumor growth, and inflammation, which all pivotally participate in cancer development and metastasis.⁷ Stromal cells produce CXCL12; this chemokine attracts both hematopoietic and non-hematopoietic tumor cells that express CXCR4.^{7, 8} The CXCR4 chemokine receptor is expressed by lymphocytes and has been observed to play pivotal parts in these cell traffic in homeostatic circumstances.⁹ This chemokine receptor (CXCR4) also

possesses a relative tendency to stimulate tumor progression, which is crucially required for metastatic spread towards organs where CXCL12 presents within the tissues/organs; these tissues/organs favor tumor cell survival and proliferation. CXCL12 also serves as a supportive mediator for forming new blood vessels which have been long known as neovascularization via recruiting endothelial cells towards the tumor microenvironment.⁷ Consequently, up-regulated expression of CXCR4 on AML blasts is well defined as a risk factor for relapse and overall poor prognosis.¹⁰ The CXCL12/CXCR4 interaction axis facilitates the retention and infiltration of AML cells in the skin tissues.¹⁰ Thus, the CXCL12/CXCR4 signaling axis may be promising targets for the development of antineoplastic agents.¹¹

In the recent studies, the CXCL12/CXCR4 axis has only been investigated in the leukemia cells of leukemic patients, yet not in Lymphocyte cells.

Researchers herein hypothesized that modulation of chemo/cytokine dependent pathways in AML is amongst approaches that could be standard chemotherapeutic regimens for improving the overall treatment outcome. Thus, we aimed to evaluate the effects of current chemotherapy (7+3) on the situation at the CXCL12/CXCR4 axis in AML patients after the first cycle of chemotherapy alongside healthy subjects.

Material and Methods

Study subjects and specimen collection

The Ethical Committee of the Kerman University of Medical Sciences approved the present case-control work under the approval code IR.KMU.REC.1395.598. The informed written consent was also obtained from each participant.

Specimens were collected from 25 patients suffering from AML (M4 and M5) in parallel with 25 healthy subjects between 2017 to 2018 in Shahid Bahonar University hospital, Kerman, Iran. The AML patients were classified and diagnosed based on FAB classification. The AML subtypes were defined according to the FAB standard criteria. The AML patients' FAB subtype was further confirmed with immune phenotypic profiling (CD117, CD64, CD34, CD33, CD14, CD13, and HLA-DR). The participants received the same (7+3) current chemotherapy regimen and those who were treated via a different chemotherapy protocol were excluded from the study. Peripheral blood smear (PBS) and BM smear were obtained from the patients at the time of diagnosis and following the first cycle of chemotherapy. Subsequently, we calculated the percentage of blast cells in BM and PBS.

A volume of 5 mL blood sample was taken from each patient prior to and following chemotherapy (first cycle of chemotherapy). The serum samples were stored at -80°C until used in the experiments. The healthy control subjects, who were not different with AML patients concerning age and sex status, were selected from the population of Kerman.

Total RNA isolation and cDNA production

Total RNA was isolated from the buffy coat of the harvested samples using Trizol Reagent (Invitrogen, USA) based on the instructions of the manufacturer. The quality and purity of the isolated RNA samples were evaluated with electrophoresing on agarose gels and measurement of optical density (A260/A280 ratio) applying a NanoDrop 1000 Spectrophotometer (Wilmington, DE, USA), respectively. To eliminate the genomic DNA from RNA preparations, DNase I, RNase-free kit from Thermo (Thermo Scientific, USA), was employed according to the manufacturer's

instructions. The reverse transcription (RT) reaction was conducted using the Revert Aid First Strand complementary DNA (cDNA) Synthesis kit purchased from Thermo (Thermo Scientific, USA).

Quantitative real-time PCR (QRT-PCR)

The QRT-PCR was performed by addition of 5 μL of Real Q Plus 2x Master Mix Green (Ampliqon, DK), 1 μL of the cDNA product, 0.5 μL of each forward and reverse primer (0.5 μL equal to 10 pmol). Reaction mixture solution reached a volume of 10 μL by adding 3 μL of nuclease-free water. The reaction mixtures were further incubated for an initial activation step at 95°C for 15 minutes followed by 40 cycles, including a denaturation step at 95°C for 15 seconds and a combined annealing/ elongation step at 60°C for 60 seconds. The reaction was performed in the Rotor-Gene Q, Real-time PCR System (Qiagen, USA). A melting curve analysis was produced for verification of the specificity of the products. The fold induction or repression was measured and compared to the control. We calculated further adjustment with GAPDH as the housekeeping reference gene using the comparative Ct ($2^{-\Delta\text{CT}}$) method. Table 1 represents the sequences for primers used in QRT-PCR.

Flow cytometry analysis

To detect the expression of CXCR4 (CD184) chemokine receptor on the membrane of peripheral blood lymphocyte cells in AML patients and healthy subjects, peripheral blood samples were treated with the indicated monoclonal antibodies and their iso-type-matched negative control according to the manufacturer's guidelines. Briefly, 5 μL of PE (phyco-erythrin) - anti-CD184 (BD, USA) was added to 50 μL of the suspension of the peripheral blood samples. Following 30 minutes of incubation, red blood cells lysis solution (BD, USA) was used for RBC lysis. 1×10^4 cells were analyzed with the Partec system

model PAS. The percentage of CXCR4 on the evaluated cells was obtained employing the provided software (Flow Max) in the Partec system model PAS.

Enzyme-linked immune sorbent assay (ELISA)

Following specimen collection, the separated serum sample was immediately frozen and stored at -80°C until further use. The serum levels of CXCL12 were measured with the relative ELISA kit (R&D system, Minneapolis, USA). All of the assays were performed according to the manufacturer's guidelines. The sensitivity of the kits was 2 pg/mL.

Statistical analysis

The data were statistically analyzed utilizing SPSS software version 22 (SPSS Inc., Chicago, IL, USA).

Mean \pm SEM was used for quantitative data presentation. Paired t-Test and two sample t-Test were used for the comparison of the studied factors. The differences were considered to be significant if only $P < 0.05$.

Results

The response of AML patients to treatment

We observed that the patients attained partial response to chemotherapy; they showed ($7.9 \pm 1.2\%$) of blast cells in BM after the first cycle of chemotherapy. We did not observe blast cells in PBS following the first cycle of chemotherapy. None of the patients had extramedullary involvement. Table 2 depicts the demographic and clinical characteristics of the healthy control group and the patient group.

CXCR4 expression by Lymphocyte cells and leukemic cells

We sought to find whether the CXCR4 chemokine receptor was expressed by Lymphocyte cells in the patients after the first cycle of chemotherapy in comparison to the healthy control subjects, in whom CXCR4 expression by leukemic cells was also evaluated. We detected the expression

of CXCR4 via the QRT-PCR and flow cytometry.

Herein, a comparative analysis of CXCR4 chemokine receptor expression indicated that almost all of the patients had approximately a similar fashion of expression to the healthy control subjects after chemotherapy and expression of CXCR4 was not significantly different ($P > 0.05$). In addition, there was a significant difference between the patients before chemotherapy and the healthy controls and between the patients before and after chemotherapy ($P < 0.05$). (Figures 1-3).

The serum levels of chemokines analysis

In the present study, we observed an increased level of CXCL12 in the patients before chemotherapy while the serum levels of CXCL12 significantly decreased to the baseline level similar to the level observed in the healthy control subjects. No significant differences were observed between the patients after the first cycle of chemotherapy and the healthy control subjects ($P > 0.05$) (Figure 4).

Our results also indicated that (7+3) current chemotherapy regimen in the patients suffering from AML with monocytic differentiation in the first cycle of chemotherapy affected the condition of the CXCL12/CXCR4 axis; accordingly, this therapeutic regimen significantly down-tuned the serum measures of CXCL12.

Discussion

A large body of evidence indicated that after being cancerous, cells start change in their chemokine expression profile. The altered chemo/cytokine network can disrupt activation of relative signaling pathways and thus overcome resistance to treatment, thereby increasing AML treatment capacity, reducing treatment complications, and improving AML treatment outcomes.⁵ It has been well addressed that AML cells can highly express CXCR4 and thus be recruited

into the BM in response to CXCL12, through which pro-survival and anti-apoptosis signals are provided. The BM microenvironment is also able to protect leukemia cells from cytotoxic chemotherapeutic agents via the CXCL12/CXCR4 signaling axis, which is an important mechanistic path for the chemotherapeutic resistance and further leukemia relapse.¹² It has been well established that expression of CXCR4 may be associated with poor prognosis in AML patients.^{13, 14}

According to the below literature, the level of CXCL12 increased in the AML patients and this increase may have a poor prognosis in patients. Furthermore, several studies have indicated that the high level of expression of CXCR4 is a poor prognostic factor in AML patients.¹⁵ We also observed an increased CXCL12 in the AML patients in our study, yet there are no investigations on the effect of chemotherapy on the CXCL12 and on the CXCR4 expression by Lymphocyte cells in peripheral blood, which are changed or not after chemotherapy. This work is the first to focus on this matter; therefore, we aimed to evaluate possible effects of current chemotherapy (7+3) regimen on the situation of the CXCL12/CXCR4 axis in AML patients with monocytic differentiation.

CXCR4 receptor was initially discovered on lymphocytes in inflammatory tissues. Multiple organs, such as liver, lymph nodes, and BM, widely express CXCL12 that is a chemotactic factor for lymphocyte cells in the normal circumstances. Lymphocytes take part in the metastasis because of their association with CXCR4.¹⁶

Denkert et al. indicated the favorable prognostic of lymphocytic infiltration in patients who received anthracycline/taxane chemotherapy. These patients attained a good outcome after the treatment compared to the patients without dense lymphocytic

infiltration in the tumors. Moreover, Loi et al. showed the prognostic significance of lymphocytic infiltration and indicated the potential favorable role of immunogenic treatments in breast cancer.¹⁶

There is a reverse correlation between the decrease in CXCR4 and CD8+ TILs at the intratumoral site of breast cancer.¹⁶

Certain studies have shown that chemotherapy alerts adaptive immune cells in AML patients.¹⁷ The secretion of cytokine by chemotherapy potentially affects host tissues and resultantly, the treatment response and prognosis. In a study, chemotherapy for ALL, due to B.M damage, functionally deregulated stromal cells of BM and reduced CCL3.¹⁸ In other words, chemotherapy caused secretion of various inflammatory cytokines, including CCL5, and triggered CCL3 depletion.¹⁹

In one study similar to this work, scientists observed that the CCR5 expression was attenuated in patients after the first cycle of chemotherapy and the healthy control group. They also found that the serum levels of CCL3, CCL4, and CCL5 chemokines were elevated in AML patients prior to chemotherapy. They observed that only CCL3 reached the baseline level in the after-chemotherapy cycle while CCL5 and CCL4 did not return to the basal level and were high in patients compared to the healthy control group. In fact, chemotherapy inhibited CCL3, but that could not inhibit CCL4 and CCL5. According to the findings of other studies and the current paper, these chemokines have a poor prognosis in AML patients.²⁰

In the present study, we observed that the expression of CXCR4 was significantly higher in the AML patients than that in the age and gender-matched control group. Our data are consistent with the results obtained in a previous study showing that the incidence of M4/M5 subtypes was significantly higher in CXCR4.²¹ In the

present research, we evaluated the expression of CXCR4 on Lymphocyte cells in peripheral blood of AML patients after chemotherapy.

We found that no differences between the expression of CXCR4 on Lymphocyte cells in peripheral blood in the patients after the first cycle of chemotherapy and the healthy control subjects.

Faaij CM et al. reported that the CXCL12/CXCR4 axis participates in EAML (extra medullary-acute myeloid leukemia) of the skin in 15 pediatric AML patients.¹⁰

The CXCL12, which was secreted by MSCs (Mesenchyme Stem cells), participate in hematopoietic cell proliferation and survival.²² Similar mechanisms could describe the interaction between MSCs and AML cells, which impairs the cytotoxic effects of chemotherapy regimens.²³

Regulatory T cells (Tregs), tumor-associated macrophages (TAMs), and myeloid-derived suppressor cells (MDSCs) are immunosuppressive cells that play a role in tumor immune evasion. Tregs and MDSCs mediate their suppressive effects by inhibiting T-cell. Additionally, they provide a microenvironment in which cancer cells can expand and evade host immunosurveillance. CXCL12 is involved in the chemotaxis of MDSCs and Tregs. MDSCs and Tregs recruitment are conducted by CXCR4.^{24, 25} Hence, we could say that the reduced level of CXCL12 after the first cycle of chemotherapy is useful for patients.

Cytokine secretion undergoes certain changes by chemotherapy; thus, it affects the prognosis and response to treatment.¹⁹ Therefore, we examined this signal on Lymphocyte cells in peripheral blood to see whether it changes after chemotherapy.

The above-mentioned studies showed that the elevated expression of CXCL12 alongside its relative receptor (CXCR4) is associated with poor prognosis in AML

patients. Our results indicated that the serum level of CXCL12 significantly increased in the AML patients with monocytic differentiation compared to the healthy subjects. This proposes an inflammatory state, but after the first cycle of chemotherapy, CXCL12 decreased significantly and reached back to the basal level in the healthy control group.

Overall, further investigation should be conducted to evaluate the roles and mechanism(s) of chemokines in the treatment of AML patients. It is also important to assess their upstream and down gene targets both before and after chemotherapy for a better understanding of the molecular basis. Additionally, further attention should be paid to the evaluation of the cytogenetic effects on chemokine expression.

Conclusion

Our results revealed that the expression of CXCL12 chemokine in the AML patients with monocytic differentiation was completely inhibited via chemotherapy. In conclusion, based our data and the findings of previous studies, chemokines expression inhibition along with chemotherapy in AML patients with monocytic differentiation may be conducive to the treatment by decreasing the treatment duration, changing the chemotherapy drug dose, and prevention of the recurrence disease in patients; accordingly, we should also evaluate the pattern of the expression of other chemokines in AML patients in parallel with their upstream signaling pathway targets by blocking the pathways to delineate their mechanisms of expression.

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Conflict of Interest

None declared.

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Table 1. Sequences of the used primers for quantitative real-time PCR

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Size (bp)
GAPDH	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	226
CXCR4	AATGGGCTCAGGGGACTATG	CTGTACTTGTCCGTCATGCT	186

GAPDH= Glyceraldehyde 3-phosphate dehydrogenase
bp = base pair

Table 2. Demographic and clinical characteristics of the healthy controls and AML patients

	Healthy Control	Patient
Gender	12 M 13 F	12 M 13 F
Age (Mean \pm SEM)	40 \pm 3.2	41.45 \pm 4.7
M4 M5	-	16 9
% Blast cells in BM ^{Δ} (Mean \pm SEM)	-	47 \pm 5.8
% Blast cells in PB ^{Δ} (Mean \pm SEM)	0	46.45 \pm 8.6
WBC count in PB* (Mean \pm SEM)	8050 \pm 963	8360 \pm 1158 [#]

SEM= Standard Error of Mean

BM= bone marrow

PB= peripheral blood

^{Δ} at the time of diagnosis

*White blood cell (WBC) count in peripheral blood.

[#] post first cycle of chemotherapy.

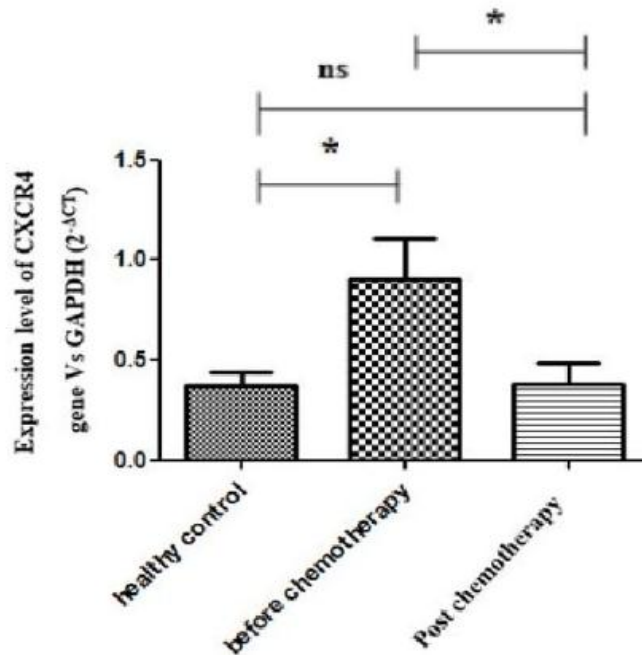


Figure 1. The mRNA expression of CXCR4 in the AML patients before/after chemotherapy and the healthy controls. The quantitative real-time PCR using specific primers for CXCR4 and GAPDH was performed. All the values were normalized against GAPDH. There was a significant difference between the patients before chemotherapy and the healthy controls and between the patients before and after chemotherapy ($*P<0.05$). In addition, no statistically significant differences were observed between the healthy controls and the patients after chemotherapy ($P>0.05$).

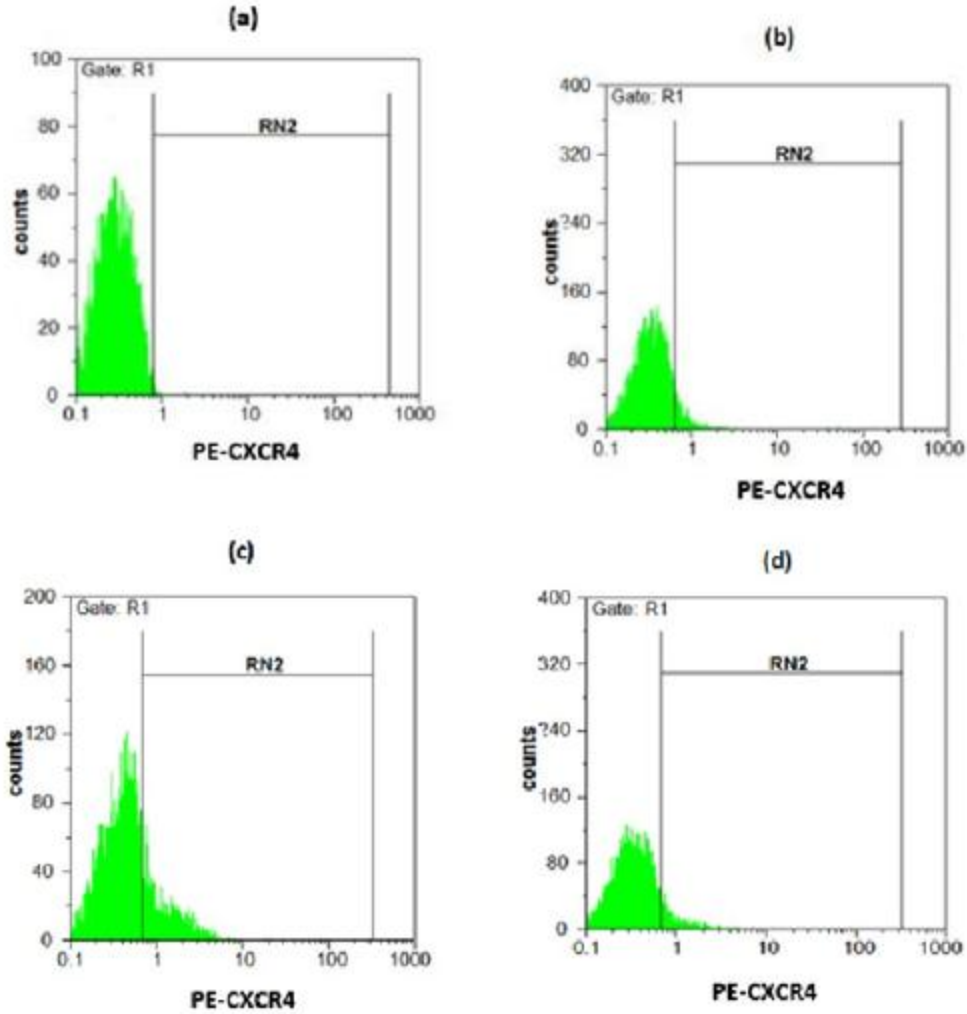


Figure 2. The expression of the CXCR4 chemokine receptor in the AML patients before/after chemotherapy and the healthy controls. The cells were stained with PE-conjugated MAb CD184 (CXCR4) and analyzed with flow cytometry. Histograms represent the obtained PE fluorescence using isotype-matched murine MAb as a negative control (a), MAb against CD184 in the healthy control subjects (b), a representative patient before chemotherapy (c), and a representative patient after chemotherapy (d). RN2 indicates the cells that express CXCR4.

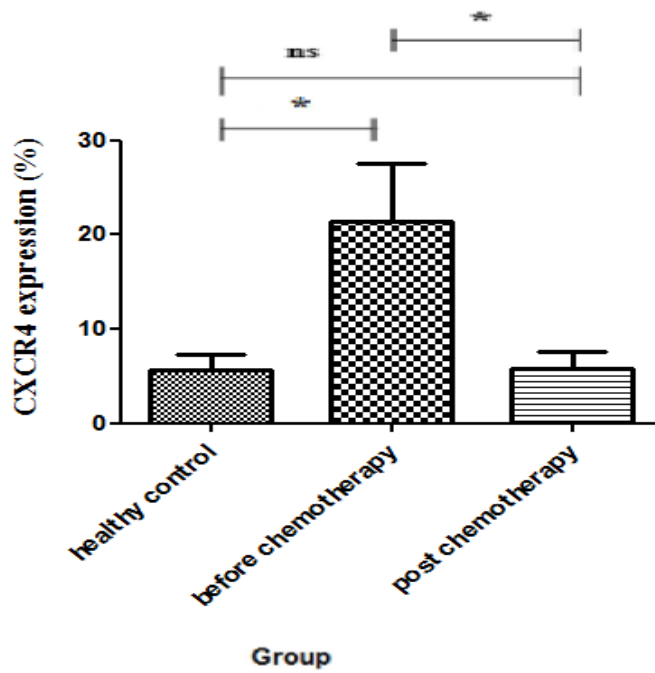


Figure 3. The expression of CXCR4 in the patients before/after chemotherapy and the healthy subjects. There was a significant difference between the patients before chemotherapy and the healthy controls and between the patients before and following chemotherapy ($*P<0.05$). In addition, no statistically significant differences were observed between the healthy control and the patients after chemotherapy ($P> 0.05$).

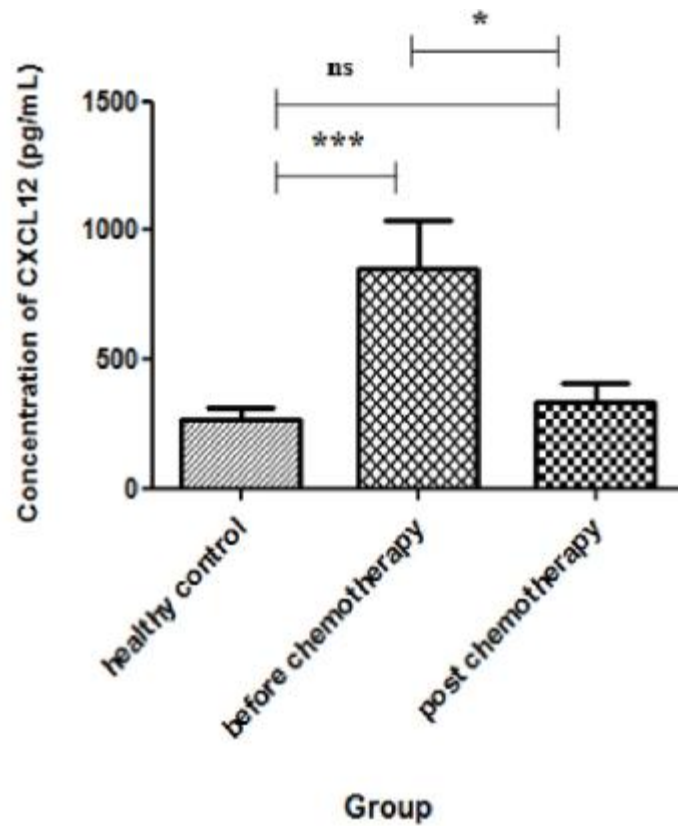


Figure 4. The serum level of CXCL12 in the AML patients. The chemokine serum level was analyzed utilizing ELISA. There was a significant difference between the patients before chemotherapy and the healthy controls ($***P<0.0001$). There were no significant differences between the patients after chemotherapy and the healthy controls ($P>0.05$). Additionally, there was a significant difference between the patients before and after chemotherapy ($*P<0.05$).