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Differential regulatory effects of chemotherapeutic protocol on CCL3_CCL4_CCL5/CCR5 axes in acute myeloid leukemia patients with monocytic lineage

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ABSTRACT

Aims: AML (Acute myeloid leukemia) is characterized as a heterogeneous cancer. Chemokines play fundamental roles in the onset, progression cellular, migration, survival and improvement of AML therapy outcomes. The CCR5 receptors together with their ligands have indirect effects on the progression of cancer. In the present study, we have decided to investigate the impact of chemotherapy on the expression of CCR5 and its related ligands (CCL5, CCL4 and CCL3).

Main methods: In this study, peripheral blood and bone marrow specimens were collected prior and post the first stage of (7 + 3) chemotherapy from 25 AML-M4/M5 patients. The expression of CCR by Lymphocytes in peripheral blood was examined by flow cytometry and QRT-PCR. The serum levels of chemokines were measured by ELISA.

Key findings: There was not observed leukemic blast cells in peripheral blood smear at post first stage of chemotherapy. We found that the expression of CCR5 was attenuated in patients post the first stage of chemotherapy and the healthy control subjects. We have also observed that the serum levels of chemokines were elevated in AML patients prior to chemotherapy. Although in post-chemotherapy stage, only CCL3 was found to reach to the baseline level, CCL5 and CCL4 have not returned to the basal level and were significantly higher than healthy control subjects.

Significance: The current chemotherapy protocol was not able to completely inhibit CCL5 and CCL4. In conclusion, our findings in harmony with previous studies suggest that inhibition of chemokines along with chemotherapy in AML patients may aid therapy.

1. Introduction

Acute myeloid leukemia (AML) was initially defined as an aggressive and heterogeneous bone marrow (BM) malignancy. Therapy of AML includes intensive chemotherapy, either alone or in combination with allogeneic stem cell transplantation [1–3]. Chemokines alongside with their cognate receptors are pivotally involved in the pathogenesis of AML [4,5]. It has also been well evidenced that leukemic blasts represent abnormal degrees of responsiveness to cytokine stimulation, however, they are most often able to produce cytokines [6].

Likewise, other chemokine receptors, the CC chemokine receptor 5 (CCR5) which is now known as CD195 fits within the trimeric guanine nucleotide-binding protein-coupled, seven transmembrane receptors superfamily. The CC chemokine ligands CCL5, CCL4 and CCL3 bind to the CCR5 for their functional activity. All of these mediate oriented migration of cancer cells in parallel with contributing in processes of proliferation and survival of these cell types [5,7,8]. Additionally, it has been documented that CCR5 contributes to the events of tumor progression in several hematological cancers, including AML, chronic myeloid leukemia (CML) and multiple myeloma (MM) [9]. The native

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human AML cells have the tendency to generate CCL5 and expresses CCR5 and the CCL5/CCR5 interaction axis modulates metabolic events within the tumor microenvironment while tumor onset is going on to promote tumorgenesis [10,11].

Increased levels of CCL4 and CCL3 were observed in the bone marrow of patients who were suffering from CML (chronic myeloid leukemia) [12]. In more recent attempts, was demonstrated that the mouse tumor-infiltrated granulocytic and monocytic/myeloid-derived suppressor cells are able to produce high levels of CCR5 chemokine ligands including, CCL5, CCL4, and CCL3, all of which are responsible for the recruitment of Tregs (T regulatory cells). Therefore, a novel immune modulatory strategy could probably be achieved for cancer therapy by blocking of CCL5/CCR5 interaction axis [8]. Regardingly, modulation of cytokine network could be considered as a path to overcome therapy resistance. Further most, to improve the selectivity of AML treatment, for controlling the overall therapy-associated toxicity as well as ameliorate outcomes of AML treatment by approaching the modulation of cytokine networks in all of AML age groups [4]. Moreover, it has also been well documented that CCL5/CCR5 axis was able to effectively affect the development of tumor cells, and hence the axis has the capacity to be considered as a promising tool for the establishment of anticancer compounds [13,14]. In more recent studies, the CCL5/CCR5 axis was only examined in leukemia cells, nonetheless, CCR5 is predominantly expressed by several other cell types, including T regulatory cells, macrophages, dendritic cells and eosinophil's as well [4,8]. According to the aforementioned introductory comments, modulation of cytokine-dependent processes in AML could be considered as an appropriate therapeutic approach. Therefore, we sought to evaluate the effects of (7 + 3) therapy protocol which is currently applied as a chemotherapy regimen (for treatment of AML, worldwide) on CCL3-CCL4-CCL5/CCR5 functional axes. We have also examined the expression of CCR5 on Lymphocytes population in AML patients along with its ligands prior and post the first stage of chemotherapy in these patients.

2. Material and methods

2.1. Study subjects and specimen collection

This case-control study was approved by the ethical committee of the Kerman University of Medical Sciences with the approval code IR.KMU.REC.1395.598. Informed written consent was also obtained from each participant.

Specimens were collected from 25 patients suffering from AML (M4 and M5) from 2017 to 2018 in the Shahid Bahonar University hospital of Kerman, Iran. AML patients were classified according to the FAB (French–American–British) classification. FAB subtype was further confirmed by immune phenotypic profiling (CD13, CD14, CD33, CD34, CD64, CD117 and HLA-DR). All of the studied patients received similar (7 + 3) current chemotherapy regimen and patients who had received a different chemotherapy protocol were excluded from the study. Peripheral blood smear (PBS) and bone marrow smear were both prepared from patients at the time of diagnosis and post the first stage of chemotherapy (following the fourth week of complete chemotherapy when complete blood count (CBC) was approximately normal) and percentage of blast cells was calculated.

A volume of 5 mL blood sample was taken from each patient prior initiation of chemotherapy and after the first stage of chemotherapy (following the fourth week of complete chemotherapy when complete blood count (CBC) was approximately normal) also serum samples were isolated and stored at −80 °C until being used in experiments. Participants of the healthy control group were selected from Kerman population and were then matched with AML patients with regard to demography, including age and sex status.

2.2. Total RNA isolation and cDNA production

Total RNA was isolated from the buffy coat of harvested samples using Trizol Reagent (Invitrogen, USA) according to the instructions of the manufacturer. Both, the quality and purity of isolated RNA samples were evaluated by agarose gel electrophoresis and measurement of optical density (A260/A280 ratio) by 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 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).

2.3. Performing the quantitative real-time PCR (QRT-PCR)

QRT-PCR was performed by adding 5 μL of Real Q Plus 2× Master Mix Green (Ampliqon, DK), 1 μL of the cDNA product, 0.5 μL of forward and reverse primers (0.5 μL equal to 10 pmol), and reaction mixture solution reached to 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 min and followed by 40 cycles including a denaturation step at 95 °C for 15 s and a combined annealing/elongation step at 60 °C for 60 s. The reaction was performed in the Rotor-Gene Q, Real-time PCR System (Qiagen, USA). A melting curve analysis was performed for verification of the specificity of the products. The fold induction or repression was measured relative to the control and calculated further adjusting with GAPDH as the housekeeping reference gene using the comparative Ct ($2^{-\Delta\Delta CT}$) method. The sequences for primers used in QRT-PCR are (all in 5_ to 3_direction) as follows: GAPDH Forward primer, GAAGGTGAAGGTCGGAGTC; GAPDH Reverse primer, GAAGATGGTGATGGGATTTC; CCR5 Forward primer, ATGTG AAGCAAATCGCAGCC; CCR5 Reverse primer, GCCAGGTTGAGCAGGT AGAT.

2.4. Flow cytometry analysis

In order to detect the expression of CCR5 on the membrane of peripheral blood Lymphocytes in AML patients and healthy controls, peripheral blood samples were treated with the indicated monoclonal antibodies and their isotype-matched negative control according to the manufacturer's instructions. Briefly, 5 μL of PE-conjugated anti-CD195 (BD, USA) was added to 50 μL peripheral blood samples, and after 30-minute incubation, red blood cells lysis solution (BD, USA) was used for RBC lysis. Cells (1×10^4) were analyzed by Partec system model PAS. Lymphocytes population was gated by forward scatter and side scatter. Percentage of CCR5 on evaluated cells was obtained using the provided software (Flow Max) in the Partec system model PAS.

2.5. Chemokines assay

The serum levels of CCL5 (RANTE), CCL4 (MIP-1β) and CCL3 (MIP-1α) were measured by ELISA using kits purchased from R&D systems, UK, in patients and healthy subjects, immediately further collection and serum isolation. All of assays were conducted according to manufacturer's guidelines. The detection limit for each kit was as follow: 6.6 pg/mL for CCL5, 11 pg/mL for CCL4, 10 pg/mL for CCL3.

2.6. Statistical analysis

Data were analyzed using SPSS software version 22 (SPSS Inc., Chicago, IL, USA).

Quantitative data were presented as Mean ± SEM. For comparison of the underlying factors, two sample *t*-Test and Paired *t*-Test were used. The differences have been considered, only if $P < 0.05$.

Table 1
Clinical and demographic characteristics of AML patients and healthy controls.

	Age (MEAN ± SEM)	Gender	M4/M5	% Blast cells in BM[a] (mean ± SEM)	% Blast cells in PB[a] (mean ± SEM)	WBC count in PB[b] (mean ± SEM)	Extramedullary involvement
Patient	41.45 ± 4.7	12 M/13 F	16/9	47 ± 5.8	46.45 ± 8.6	8360 ± 1158[c]	No
Healthy control	40 ± 3.2	10 M/15 F	–	–	–	8050 ± 963	–

AML = Acute myeloid leukemia.
BM = bone marrow.
PB = peripheral blood.
SEM = Standard Error of Mean.
[a] At the time of diagnosis.
[b] White blood cell (WBC) count in peripheral blood.
[c] Post first stage of chemotherapy.

3. Results

3.1. The response of AML patients to the chemotherapy

We have observed that AML patients have attained a partial response to chemotherapy that was seen (7.9 ± 1.2%) of blast cells in BM post first stage of chemotherapy also was not observed blast cells in PBS at post first stage of chemotherapy. Clinical and demographic characteristics of AML patients and healthy control group are shown in Table 1.

3.2. Expression of CCR5 by Lymphocytes population

We sought to find whether if CCR5 receptor was expressed by Lymphocytes population in AML patients post the first stage of chemotherapy in comparison with healthy control group. We have initially detected the mRNA expression of CCR5 by QRT-PCR. We found that the expression of CCR5 was low in all of AML patients at post first stage of chemotherapy and all members of the healthy control group. The comparative analysis of CCR5 expression indicated that patients had an approximate similarity with the healthy control group, however, the expression of CCR5 was not significantly differed in studied group ($P > 0.05$) (Fig. 1). We have then verified the expression of CCR5 by flow cytometry and we found similar results with the QRT-PCR results (Figs. 2, 3).

3.3. Analysis of the serum levels of chemokines

When we have analyzed, the serum level of CCL5 was found that it was considerably increased in AML patients prior chemotherapy and post the first stage of chemotherapy, however, the serum level of CCL5 has significantly decreased post first stage of chemotherapy. Although, the serum level of CCL5 has remarkably been decreased further chemotherapy in AML patients however, it has not backed to its basal level (the CCL5 level in healthy controls) (Table 2 and Fig. 4A). We have also observed that as similar as CCL5, the serum level of CCL4 was increased in AML patients prior to chemotherapy and the further first stage of chemotherapy. The serum level of CCL4 has significantly decreased post first stage of chemotherapy. Again, CCL4 was remarkably reduced in AML patients post-chemotherapy but it has not reached to the level which was observed in healthy control subjects (the basal level) (Table 2 and Fig. 4B). In the present study, we have found that prior chemotherapy AML patients had a very high level of CCL3 while post first stage of chemotherapy CCL3 has significantly decreased to the baseline level as similar as the level which was observed in the healthy control group. There was not a significant difference between AML patients post first stage of chemotherapy and healthy control group with regard to CCL3 serum level (Table 2 and Fig. 4C). Our results indicated that the current chemotherapy regimen (7 + 3) in patients suffering from AML with monocytic differentiation in the first stage of chemotherapy has not affected the expression of CCR5 by Lymphocytes population, however, it has significantly down-tuned the serum measures of CCL5, CCL4, and CCL3 chemokines. However, only CCL3 was found to reach the baseline level compared to the healthy control group, but CCL5 and CCL4 have not backed to their basal level. Therefore, this chemotherapy protocol has not completely inhibited CCL5 and CCL4 (Fig. 4).

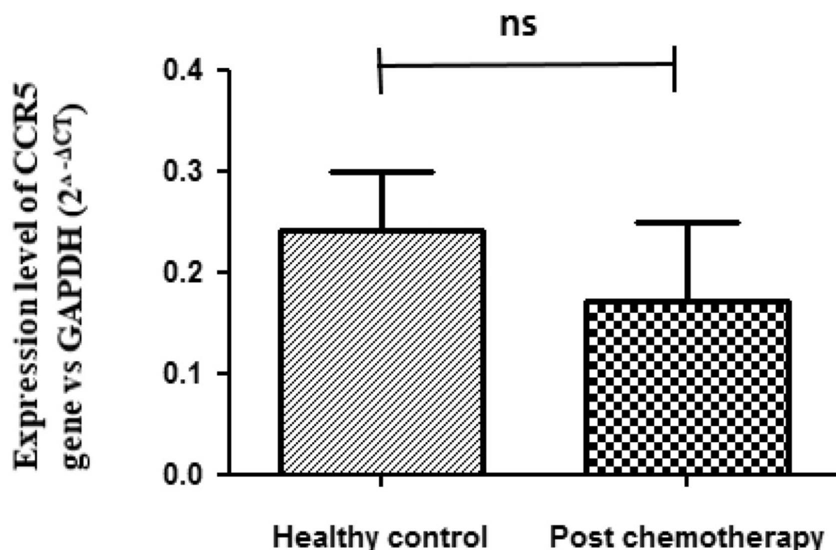

Fig. 1. Demonstrates the mRNA expression of CCR5 in AML patients post chemotherapy (n = 9) and healthy controls (n = 11). The quantitative real-time PCR using specific primers for CCR5 and GAPDH was performed. All values were normalized against GAPDH. There was no statistically significant difference (P = 0.5025). Bar represent Mean ± SEM of Healthy control = 0.2422 ± 0.057 and Mean ± SEM of Post chemotherapy = 0.1713 ± 0.078.

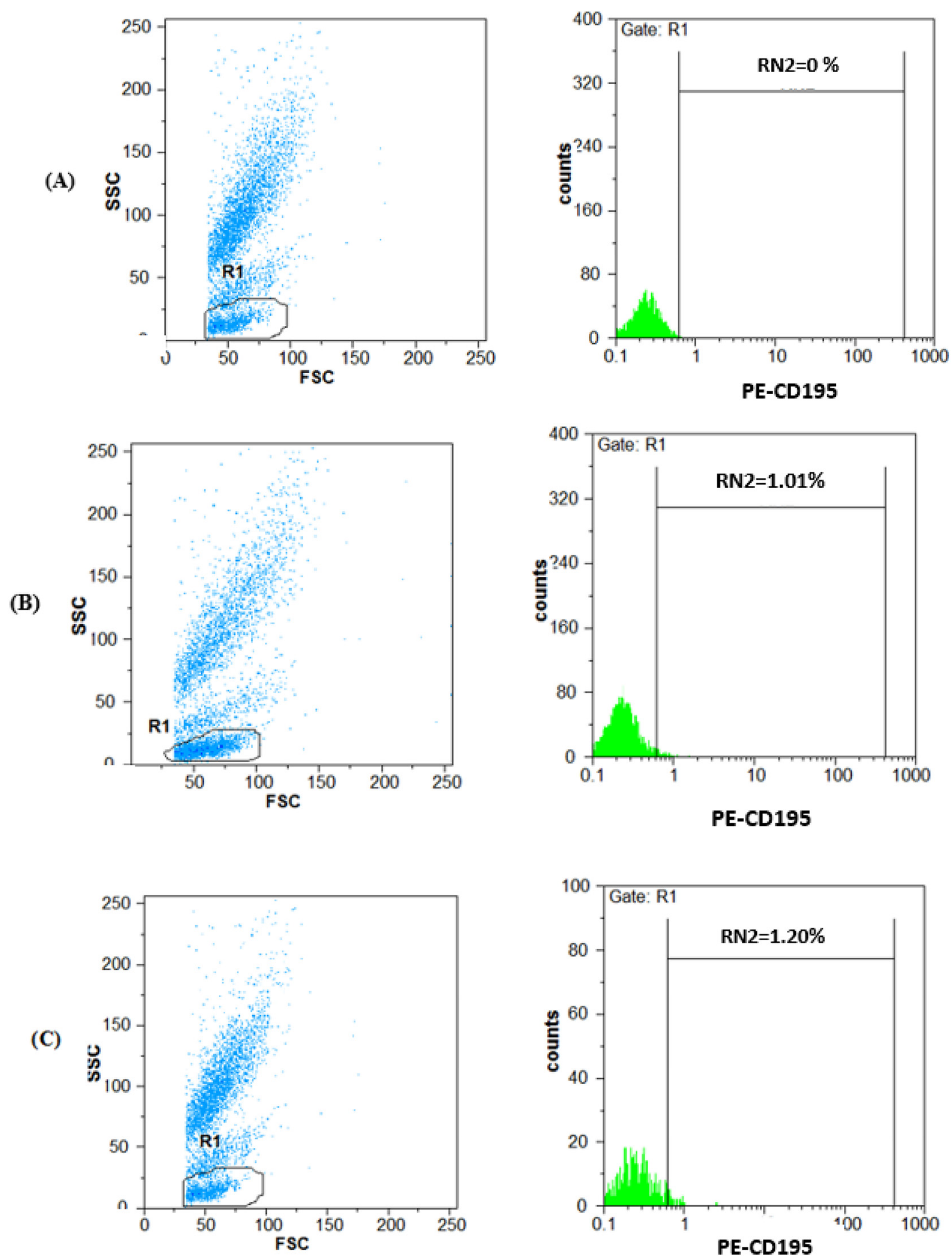

Fig. 2. Demonstrates the expression of chemokine receptor in AML patients post chemotherapy and healthy subjects. Cells were stained with PE_conjugated MAb CD195 and analyzed by flow cytometry. Cells were gated based on side light and forward light scatter. Histograms represent the PE fluorescence obtained using isotype-matched murine MAb as negative control (A), MAb against CD195 in a patient post chemotherapy (B) and Healthy control (C). R1 gates Lymphocyte cells and RN2 indicates the cells that express CD195.

4. Discussion

Evidences are in favor of the concept that cancer cells are able to establish changes in the chemokine system. Consequently, altered cyto/chemokine network can disrupt activation of signaling pathways [4]. Depending on the tumor type, the stage and composition of immunity, inhibition of chemokines or their receptors may have positive or destructive effects on the development of the disease [13].

The current study was aimed to examine the effects of frequent chemotherapeutic regimen (7 + 3) on CCL3-CCL4-CCL5/CCR5 axes in AML patients with monocytic differentiation.

Scientists reported that adaptive immune cells have undergone changes after chemotherapy in AML patients [15]. Therefore, we have evaluated CCR5 expression by Lymphocyte cells post first stage of chemotherapy and healthy control group. We have also examined CCL5, CCL4 and CCL3 prior and post the first stage of chemotherapy and in healthy control group.

Regardingly, we found that the expression of CCR5 down-regulated in Lymphocytes population of AML patients following the first stage of chemotherapy as well as members of the healthy control group,

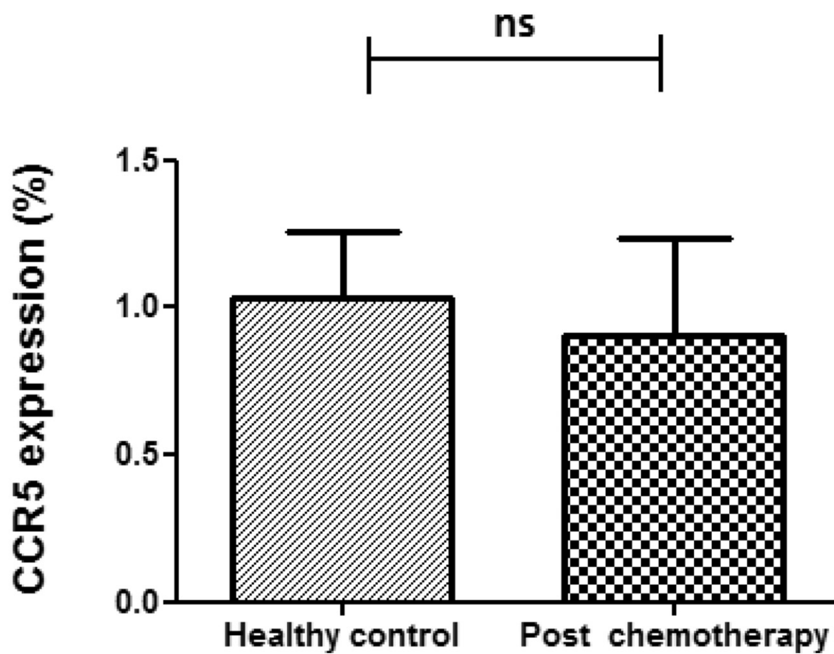

Fig. 3. Demonstrates the expression of CCR5 by Lymphocytes population in patients post chemotherapy (n = 25) and healthy control (n = 25). There was no statistically significant difference (P = 0.74). The results are from 1×10^4 cells flow cytometry analysis. Bar represent Mean ± SEM of Healthy control = 1.030 ± 0.2301 and Mean ± SEM of Post chemotherapy = 0.9018 ± 0.3348.

Table 2
Serum levels of CCL5, CCL4 and CCL3 chemokines in AML patients and healthy control.

Chemokine	Serum levels of chemokines (pg/mL)[a] mean ± SEM					
	AML patients		Healthy control subjects	p-Values[b]		
	Prior-chemotherapy	Post-chemotherapy		Healthy control VS prior chemotherapy	Healthy control VS post-chemotherapy	Prior chemotherapy VS post-chemotherapy
CCL5	1312 ± 100.5	442.2 ± 114.0	125.9 ± 3.680	P < 0.0001	0.0198	0.0002
CCL4	1414 ± 108.2	310.6 ± 18.37	130.8 ± 15.48	P < 0.0001	P < 0.0001	P < 0.0001
CCL3	888.4 ± 138.7	146.0 ± 6.279	151.5 ± 15.18	P < 0.0001	0.7886	0.0003

[a] pg/mL as measured by ELISA.
[b] The differences have been considered, only if P < 0.05.

chemotherapy has not significantly affected the expression of CCR5 by Lymphocytes population in peripheral blood of AML patients. Our results indicated that the serum levels of CCL5, CCL4, and CCL3 have significantly increased in AML patients with monocytic differentiation compared to healthy subjects that proposing an inflammatory state associated with the suppression of T cell-related immune response. This is inconsistent with a recently published article that proposed a double-faced cell mediated immunity in some disorders and malignancies, including AML, which means that T cells exhibit stimulated phenotype, while their activity has been suppressed [16]. In line with our study, Astrid Marta Olsnes and colleagues evaluated the expression of CCL3 and CCL5 by AML blast cells and observed that these cell types produce both of chemokines [17,18]. Additionally, Yang Wang et al. reported that the serum level of CCL3 was increased in AML patients [19].

Our results, which indicated an elevation in CCL5, CCL4 and CCL3 in patients, were consistent with the previous studies.

CCL5 is considered as a molecular biomarker by which the presence of tumor-dependent macrophages (TAM) could be confirmed in tissue, this chemokine has also the ability to enhance the expression of angiogenesis factors, including VEGF (Vascular endothelial growth factor) [20]. Immune Suppressive Myeloid Cells (ISMCs) are described as a negative immune regulator against the tumor. The CCL5/CCR5 axis also pushes precursor cells into the bone marrow toward granular and monocytic granulocyte group in tumorigenesis. These ISMCs themselves increase regulatory T cells, and subsequently the regulatory T cells, however, they reduce TCD8+ cells that play a potential role against tumor cells [14].

Furthermore, another ligand of CCR5 is CCL3 which has a direct growth inhibitory effect against normal hematopoietic stem/progenitor cells (HSPCs) [21]. It also induces the proliferation of leukemia cells [4,22]. In a study, investigators demonstrated that CCL3/CCR5 interaction axis is pivotally involved in the storage of AML blast cells in the skin [18].

Koichi Takahashi et al. reported that a high concentration of CCL4 and CCL3 was associated with poor prognosis in patients with diffuse large B cell lymphoma (DLBCL) [23].

Some investigations have suggested that elevated levels of CCL5, CCL3 is associated with poor prognosis in AML also elevated level of CCL4 is associated with poor prognosis in some disorders. Therefore, controlling the expression chemokines in AML patients may aid treatment and thus prevent the relapse of the disease by this fashion. Yuting Ma and colleagues designed immunotherapy by a combination of a vaccine, chemotherapy in addition to a Toll_like receptor3 agonist (VCT) for non-treatable tumors such as melanoma and glioma. They treated mice with VCT that had already been treated with CCL5. Their results demonstrated that treatment was delayed because CCL5 had recruited and caused accumulation of CCR5 expressing immune cells, which are pivotally important for the development of the tumor. Thus, taking together these observations may in a way confirm that the inhibition of CCL5 may probably improve the therapeutic effects of VCT [13].

Secretion of cytokine by chemotherapy potently affects host tissues

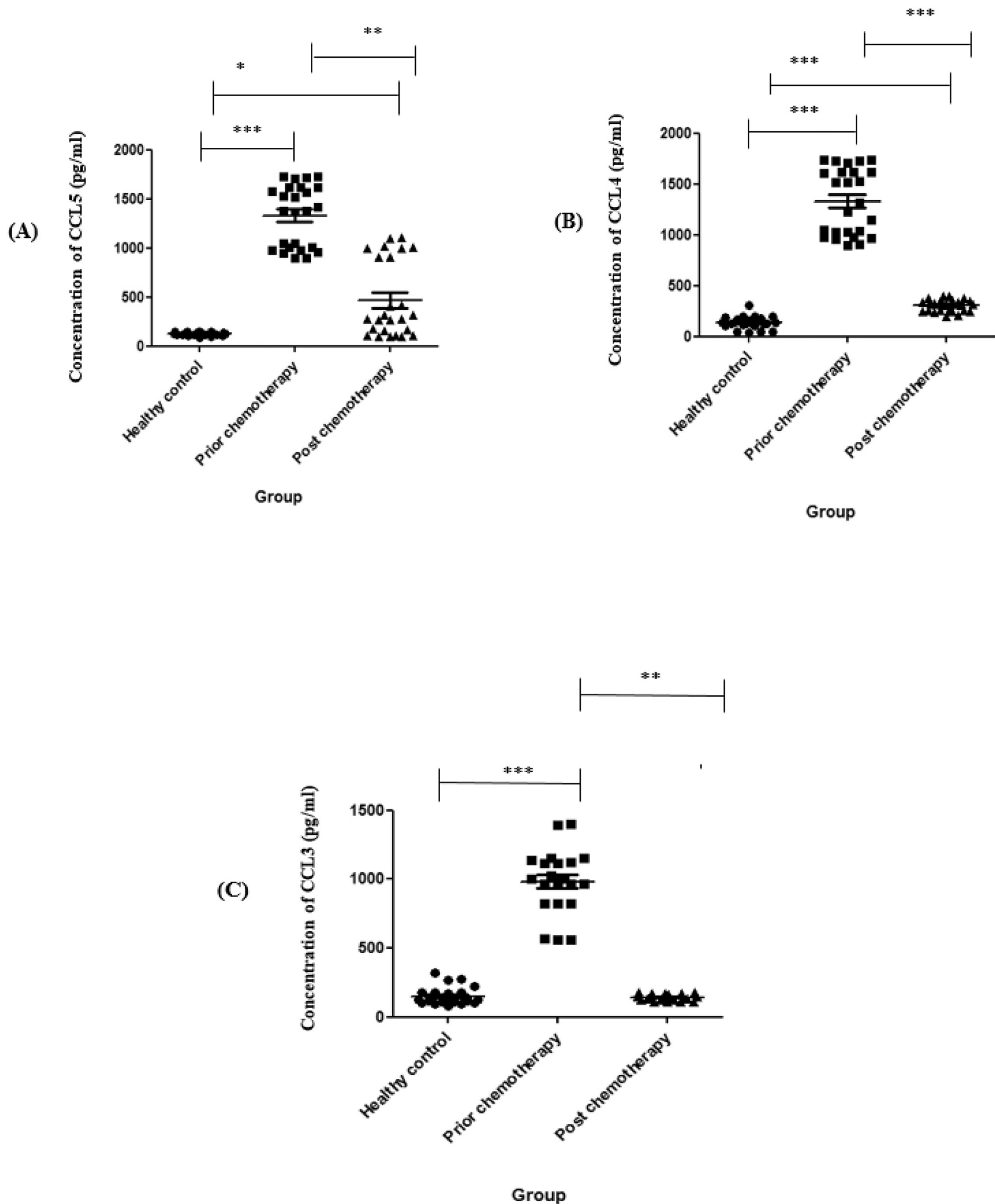

Fig. 4. Demonstrates the serum levels of CCL5, CCL4 and CCL3 in AML patients. The chemokines serum levels were analyzed by ELISA.
Profile A shows the serum levels of CCL5 in AML patients prior and post chemotherapy ($n = 25$) alongside with healthy controls ($n = 25$). There was significant difference in patients prior chemotherapy with healthy controls (***$P < 0.0001$) and post chemotherapy with healthy controls (*$P < 0.05$). Also there was significant difference in patients prior and post chemotherapy (**$P < 0.001$).
Profile B shows the serum levels of CCL4 in AML patients prior and post chemotherapy ($n = 25$) alongside with healthy controls ($n = 25$). There was significant difference in patients prior/post chemotherapy with healthy controls (***$P < 0.0001$). Also there was significant difference in patients prior and post chemotherapy (***$P < 0.0001$).
Profile C shows the serum levels of CCL3 in AML patients prior and post chemotherapy ($n = 25$) alongside with healthy controls ($n = 25$). There was significant difference in patients prior chemotherapy with healthy controls (***$P < 0.0001$). There was not significant difference in patients post chemotherapy with healthy controls ($P > 0.05$). Also there was significant difference in patients prior and post chemotherapy (**$P < 0.001$).

and resultantly the treatment response and prognosis. It has been found that cytokines including CCL5 are involved in resistance to cytotoxic agents through autocrine/paracrine signaling [24].

Considering the elevated levels of CCL5, CCL4, and CCL3 in AML patients with monocytic differentiation, this elevation is associated with poor prognosis. Hence we sought to carefully look if the first phase of chemotherapy is able to suppress these chemokines?

Our data indicated a striking difference in the pattern of chemokines levels post the first stage of chemotherapy; (7 + 3) current chemotherapy regimen in patients suffering from AML with monocytic differentiation. To the best of our knowledge, this study is very novel because this is the first study which has addressed a fundamental role for CCL3-CCL4-CCL5/CCR5 axes in AML patients prior/post-chemotherapy. Thus, there exists a gap of information in this field. As we have demonstrated, only CCL3 has returned to its basal level when compared to healthy subjects and CCL5, CCL4 have been decreased but have not backed to the basal level and they are still high in the serum compared to the healthy control group. Thus, it probably could be concluded that chemotherapy has not been completely able to inhibit the expression of CCL5 and CCL4 chemokines (Fig. 4).

Thus, firstly, Chemotherapy caused secretion of various inflammatory cytokines including CCL5 by different tumor and immune cell lines [24].

Secondly, production of these chemokines is probably related to the regulatory effects of G-CSF (granulocytic- colony stimulating factor) which is consumed by the AML patients for recovery of their immune system post-chemotherapy and the stimulate cytokines production [25]. Also it can be due to changes in adaptive immune cells in AML patients post chemotherapy [15].

In one study, chemotherapy for ALL, due to B.M damage, functionally deregulates stromal cells of bone marrow and reduces CCL3 [26]. Thus, chemotherapy may be the cause of CCL3 depletion in our study. In other words, Chemotherapy caused secretion of various inflammatory cytokines including CCL5 and the cause of CCL3 depletion.

Overall, the role of chemokines in AML and its etiology, pathogenesis and treatment is a very complex topic, and more studies are deserved to at least, in part clarify the role of chemokine network in treatment of AML. It is also important to look at the expression of these chemokines at the mRNA level and also at their down and upstream gene targets both prior and post-chemotherapy to achieve a better understanding of the molecular basis of the AML and its response to treatment. More attention needs to also be paid to evaluate the role of patient's cytogenetic in chemokine expression.

5. Conclusion

Our data indicated that chemotherapy has not been completely able to inhibit CCL5 and CCL4 chemokines in AML patients with monocytic differentiation. In conclusion, taken together in an overall view, our findings in harmony with previous studies suggest that inhibition of chemokines expression along with chemotherapy in AML patients with monocytic differentiation may aid therapy by either reducing the duration of treatment, decreasing the dose of chemotherapy drugs, or even preventing the relapse of the disease in patients.

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Declaration of competing interest

None of the authors declared conflict of interest.

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