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Original Article

Elevated IL-17A and IL-22 Regulate Expression of Inducible CD38 and Zap-70 in Chronic Lymphocytic Leukemia

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Background: In this study, we investigated the role and expression of interleukin (IL)−17A and IL-22 in chronic lymphocytic leukemia.

Methods: We evaluated the expression of markers above on CLL by ELISA, qRT-PCR, flow cytometric analysis and nonparametric Kruskal–Wallis test.

Results: Quantitative RT-PCR revealed that the mRNA levels of IL-17A and IL-22 in PBMCs of CLL patients were upregulated compared with those from healthy subjects (mean ± SD: 1.96 ± 0.232 vs.0.72 ± 0.15, $P < 0.001$ and mean ± SD: 2.45 ± 0.534 vs.0.81 ± 0.26, $P < 0.001$, respectivily). In addition, findings showed that the IL-17A and IL-22 plasma level was significantly elevated than that from healthy control group ($P < 0.001$). The median IL-17A and IL-22 in CLL patients and healthy control group were 48.28 ± 17.2 pg mL^{-1}; 20.01 ± 11.16 pg mL^{-1} and 58.68 ± 23.4 pg mL^{-1};16.47 ± 10.31 $P < 0.001$, respectively. The levels of IL-17A and IL-22 was not significantly associated with the different stages of disease (Rai stages; Kruskal–Wallis test $P > 0.05$).No significant relationship was found between expression of CD38 and higher median serum levels of IL-17A in patients, but patients with negative expression of ZAP-70 showed a significant association with higher median serum levels of IL-17A compared with healthy subjects. (57.84 pg mL^{-1} vs. 31.67 pg mL^{-1}; $P = 0.016$).

Conclusion: IL-22 is elevated and associated with CD38 and Zap-70 expression in patients with CLL. No significant correlation was found between expression of CD38 and increased levels of IL-17A, negative expression of ZAP-70 showed a significant association with increased levels of IL-17A.

Key terms: chronic lymphocytic leukemia; interleukins; QRT-PCR; ELISA; plasma

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Chronic lymphocytic leukemia (CLL), is one of the most common leukemia in adults and in the elderly worldwide (1), with an incidence of 22–30 per 100,000 in Western countries (2). It is characterized to be a heterogeneous disease with variable clinical pattern and

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evolution (3). Survival times from initial diagnosis have been indicated to be between 2 and 20 years (4). Different prognostic markers have been previously evaluated and these studies focused on evaluation of new prognostic markers that may be valuable in subgroups with favorable and poor clinical outcome in early CLL (4–7). These markers may be beneficial in early stage, progressive form, where patients can be treated before progression occurs (8). Many of these markers are mostly used clinical practice such as heavy-chain variable region (IgVH), CD38 levels, ZAP-70 expression, interphase fluorescence in situ hybridization (iFISH) abnormalities (4–9). It has been indicated that multifunctional cytokines are involved in the progression of malignancies via bidirectional regulation of inflammatory responses. Cytokines has been indicated to be an associated factor with survival in B-CLL cells and may be correlated with disease progression by upregulating antiapoptotic proteins and by furnishing prosurvival signals (10). Recent investigations have indicated that Th17 and Th22 cells and their effector cytokines can be involved in the pathogenesis of various autoimmune diseases and human tumors, such as Crohn's disease, lung and gastric cancer (11). Benham et al. found that increased IL-17 and IL-22 producing $CD4^+$ T cells can be considered as a feature of psoriatic arthritis (12). The role of Th22 and IL-22 in the pathogenesis of many kinds of solid tumors and also hematological malignancies has been shown including ALL, MDS and AML (13–15). The impact of Th17 in autoimmune diseases and against extracellular bacteria, various, fungi, and viruses is shown, but in cancer is controversial (16,17). However, its role has been described in a limited number of hematological malignancies such as AML, non-Hodgkin lymphoma and CLL (13,18,19).

This study was aimed to investigate the potential role of Th17 and IL-22 in chronic lymphocytic leukemia in association with clinical factors.

MATERIAL AND METHODS

Patients and Samples

Blood samples from 78 patients with CLL were obtained in the Clinics of Hematology–Oncology between 2013 and 2015, Tehran, Iran. All samples were collected before the start of any anticancer therapy including chemotherapy or anti-inflammatory drugs. Twenty-eight healthy volunteers were also included as controls (13 sex-matched and age-matched men and also 15 women; age range, 34–75 years, and median age: 62). It is worth noting that none of the CLL patients or control individual had kidney failure, active infections, and inflammatory diseases.

Disease staging was classified based on the Rai classification system (20). Our research was performed according the Helsinki declaration and patients have signed an informed consent. Samples were centrifuged at 4000 rpm at 4°C for 20 min and plasma samples stored at −80°C until use.

Enzyme-linked Immunosorbent Assay (ELISA)

Plasma concentrations were measured using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Human IL-17A, IL-22 Immunoassay; R&D Systems). Elisa method was performed according to the manufacturer's recommendations.

Extraction of RNA and qRT-PCR

Quantitative RT-PCR was performed to measure the mRNA levels of IL-17A and IL-22 in peripheral blood mononuclear cell (PBMC). For this purpose, total RNA was extracted using miRNeasy Serum/Plasma Kit (Qiagen), according to the manufacturer's instructions. Transcriptor High Fidelity cDNA Synthesis Kit (Roche) was used to synthesize cDNA according to the manufacturer's protocol.

QRT-PCR was performed on Applied Biosystems 7500 real-time PCR system with TaqMan TaqMan reagents specific for human IL-17A and IL-22. GAPDH was served as a control and relative expression levels of mRNAs was counted with the threshold cycle (CT) method-fold change ($2\text{-}\Delta\Delta CT$).

Flow Cytometric Analysis of ZAP-70 and CD38

All procedures were performed as described previously (21), and a cutoff point of 20% (positive cells) was determined for ZAP-7 and CD38.

IGVH Gene Mutational Status

IgVH genes sequencing was performed (11), and obtained sequence were then aligned using GenBank databases.

Statistical Analysis

Nonparametric Kruskal–Wallis test followed by a Dunn multiple comparisons test were performed to analyze differences in cytokine levels between groups. All the statistical analyses were performed with Prism Version 5.00 (GraphPad Software) and SAS 9.2 (SAS Institute). Statistical analysis was considered to be statistically significant $P < 0.05$.

RESULTS

IL-17A and IL-22 Plasma Levels and Their Association with Clinical Factors

Our finding showed that the IL-17A plasma level was significantly elevated than that from healthy control group ($P < 0.001$). The median IL-17A level in CLL patients was 48.28 ± 17.2 pg mL^{-1}, and it was 20.01 ± 11.16 pg mL^{-1} in healthy control group. Furthermore, the plasma level of IL-22 was strongly higher as compared to healthy control Group (58.68 ± 23.4 pg mL^{-1};16.47 ± 10.31, $P < 0.001$). The levels of IL-17A and IL-22 was not significantly associated with the different stages of disease (Rai stages; Kruskal–Wallis test $P > 0.05$) (Fig. 1).

No significant relationship was found between expression of CD38 and higher median serum levels of IL-17A in patients, but patients with negative expression of

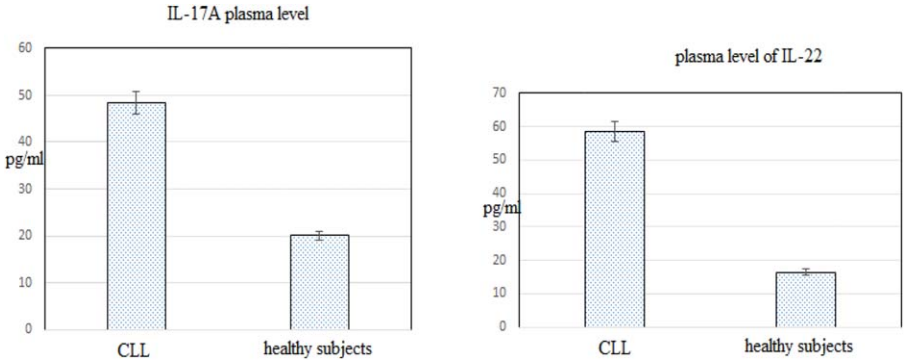

FIG. 1. IL-17A and IL-22 plasma levels in patents with CLL compared with normal control group. [Color figure can be viewed at wileyonlinelibrary.com]

ZAP-70 showed a significant association with higher median serum levels of IL-17A compared with healthy subjects. (57.84 pg mL^{-1} vs.31.67 pg mL^{-1}; $P = 0.016$). On the other hand, the high plasma level of IL-22 in CLL patients was linked to the patients with positive CD38 and ZAP-70 compared with healthy subjects (All $P < 0.001$).

The IgVH genes mutation was detected in 51 cases and unmutated genes were found in 27 cases. CD38 expression was >20% in 33 cases, and ZAP-70 expression was >20% in 28 cases (Table 1). A significant associating was found between the plasma levels of IL-22 and IgVH mutational status, but it was not significant in high level of IL-17A.

Moreover, other clinical parameters were not correlated with plasma levels of IL-17A and IL-22 including sex, age, Hb levels, platelet or lymphocyte count, and B2m levels.

Table 1
Clinical and Laboratory Features at CLL Diagnosis (Number of Patients= 78)

Characteristics	No. of patients	(%)
Age (years)	78	Median: 59; Range 33–81
Gender		
Male	43	55.12
Female	35	44.87
RAI stage		
0	31	39.74
1	16	20.51
2	9	11.53
3	10	12.82
4	12	15.38
IGHV mutation status		
Mutated (≥2%)	51	65.38
Unmutated (<2%)	27	34.61
CD 38		
Positive	33	37.17
Negative	45	62.82
Zap 70		
Positive	28	30.76
Negative	38	53.84
Unknown	12	15.38
β2-microglobulin, mg L^{-1}		
<2	65	83.33
>2	13	16.66
Hemoglobin: g dL^{-1}		
<10	15	19.23
>10	63	80.76
Platelet count: ($\times 10^9$/l)	78	Median 161, range between 3–646
WBC count ($\times 10^9$/l)	78	Median 48.54, range between 1.5–455

MRNA Levels of IL-17 and L-22

Quantitative RT-PCR was applied to evaluate the mRNA levels of IL-17A and IL-22 in PBMCs.

Our result showed that the mRNA levels of IL-17A in PBMCs of CLL patients was upregulated compared with those from healthy subjects (mean ± SD: 1.96 ± 0.232 vs.0.72 ± 0.15, $P < 0.001$). In addition, overexpression of IL-22 mRNA levels were presented in CLL patients as competed to healthy subjects (mean ± SD: 2.45 ± 0.534 vs. 0.81 ± 0.26, $P < 0.001$) (Fig. 2).

DISCUSSION

The role of Th17 in autoimmune diseases and against extracellular bacteria, various, fungi, and viruses has been previously described, but in cancer remain unclear (16,17).

Our results indicated that the IL-17A plasma level was significantly elevated than that from healthy control subjects. Also, mRNA levels of IL-17A in PBMCs of CLL patients was upregulated compared with those from healthy subjects No significant association was found between expression of CD38 and higher median serum levels of IL-17A in patients. However, patients with negative expression of ZAP-70 showed a significant correlation with increased median serum levels of IL-17A than in healthy individuals. Th17 cells distribution has been detected to show different pattern in solid tumors or hematological diseases. Elevated Serum Level of IL-17 found as a prognostic biomarker in multiple myeloma (22). Increased levels of circulating Th17 subset were detected in tumor tissues in uterine cervical cancer (23). Previous studies indicated that Th17 cells decreased in chronic myeloid leukemia (24,25). On the other hand, the elevated level of Th17 cells has been detected in multiple myeloma and acute myeloid leukemia. It has been suggested that the increased numbers of Th17 cells is association with favorable prognosis in patients

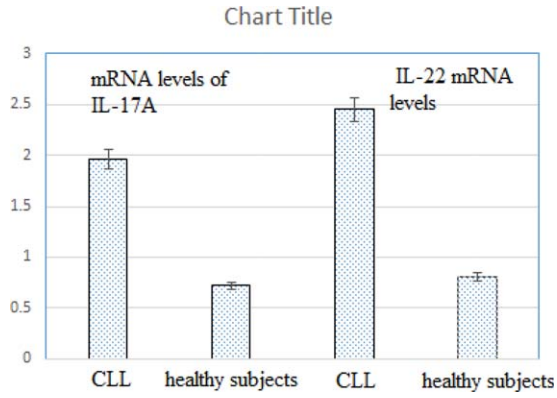

FIG. 2. MRNA level of IL-17A and IL-22 in patents with CLL compared with normal control. [Color figure can be viewed at wileyonlinelibrary.com]

with CLL (26). Some studies have also indicated IL-17+ T cells are associated with tumor pathogenesis in a little number of malignancies such as ovarian and renal cell carcinoma (27). In consist with our study, Hus et al. (4) found that the IL-17A concentration of peripheral blood was significantly higher in CLL patients compared with healthy subjects. Moreover, they reported that the high plasma level of IL-17A inversely related to the stage of disease, but we didn't find significant association between the levels of IL-17A and the different stages of disease. Furthermore, the high level of Th17 was detected in peripheral blood mononuclear cells and bone marrow mononuclear cells in patients with acute myeloid leukemia. Also, it was indicated that high Th17 cell frequency is associated with poor prognosis (28). Th17 cells in involved in the pathogenesis of AML and may be a prognostic predictor (28). Th17/IL-17 axes may be associated with development of non-Hodgkin's lymphoma, and acute myeloid leukemia (18,29). On the other hand that the downregulation of IL-17-producing T cells and expansion of Treg cells, has been reported to be correlated with progression of CLL (19). Further comprehensive and functional studies are required for clarification of the role of IL-17 in CLL patients.

In the present study, the plasma level of IL-22 was strongly higher as compared to healthy control group. High IL-22 mRNA levels were presented in CLL patients as competed to healthy subjects. In addition, the high plasma level of IL-22 in CLL patients was linked to the patients with positive CD38 and ZAP-70 compared with healthy subjects. Regulatory role of IL-22 in some biological process has been indicated such as cell cycle control, cell growth and proliferation. In addition, IL-22 might be involved in tumor genesis. The role of Th subsets in the immune pathogenesis of CLL needed further clarification. It has been recently found that Th22 cells and IL-22 may participate in ALL, MDS and AML (13–15). Recent study indicated that Th22 frequency and level of IL-22 were increased in AML patients than in healthy subjects (30). Increased expression of peripheral Th22 was detected to be correlated with the late-stage MDS as compared to early-stage MDS (15). In this study, the levels of IL-22 were not significantly associated with the different stages of disease (Rai stages).

Liu et al. suggested that circulating Th22 cells as well as Th17 cells are significantly increased in the peripheral blood of gastric cancer patients with tumor progression. Yu et al. indicated that plasma levels of IL-22 and percentage of Th22 and Th17 subsets were markedly elevated in AML patients (13). Moreover, plasma levels of the cytokines IL-1β, IL-6, IL-17, IL-22, IL-23, and TGF-β1 were strongly elevated in AML patients (28). The frequencies of PB or BM Th22 cells were high in ND CML patients (24). High level of plasma IL-22 was detected in B-CLL patients compared with healthy individuals in chronic lymphocytic leukemia. Patients presenting with high CD38 expression had significantly higher plasma IL-22 levels compared with those with low CD38 (31). This finding is in accordance with our result that the high plasma level of IL-22 in CLL patients was linked to the patients with positive CD38 and ZAP-70 compared with healthy subjects. CD38 was described as a novel marker of poor prognosis. In the current study, No statistically significant difference was determined between plasma levels of IL-22 in different d stages.

A significant associating was found between the plasma levels of IL-22 and IgVH mutational status, but it was not significant in high level of IL-17A.

There was no significant association between IL-22 levels and other clinical parameters including age, sex, Hb levels, platelet or lymphocyte count, B2m levels. Our findings are more or less in agreement with Heiba et al. (31). CD38 is an unfavorable prognosis and is associated with activation and proliferation of CLL cells (32,33). The role CD38 has been described in signal transduction, aschemotaxis and homing more efficient, was found that this marker act as an integrator of proliferative and migratory signals.

It has been suggested that high plasma IL-22 levels, high expression of IL-22 and high expression of CD38 may be involved in synergy to activate genetic programs relevant for proliferative responses and inhibition of apoptosis. And can be associated with poor prognosis in B-CLL patients (31) as well as different interleukins has been reported as prognostic biomarker on cancers (34). However, functional studies are needed for more clarification. In conclusion, the present study suggested that IL-22 is elevated and its expression is associated with CD38 and Zap-70 expressions in patients with CLL. Negative expression of ZAP-70 showed a significant association with increased levels of IL-17A, while no significant correlation was found between expression of CD38 and increased levels of IL-17A.

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