







Evaluation of cytokine levels as possible predicting elements in patients with chronic lymphocytic leukemia

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SUMMARY

OBJECTIVE: This study aimed to assess the patterns of serum cytokines in chronic lymphocytic leukemia patients at baseline and post-chemotherapy and investigate their association with response to treatment and chronic lymphocytic leukemia prognosis.

METHODS: Blood samples were taken from 32 subjects at their first medical visit after being diagnosed with chronic lymphocytic leukemia and 1 year after chemotherapy. Then, levels of cytokines and blood parameters in peripheral blood were measured. Correlation analysis was used to assess the indexes before and after chemotherapy as well as at different disease stages.

RESULTS: Most of the patients (45.80%) had stages I and III before initiation of treatment and after treatment, respectively. There were significant differences between levels of interleukin (IL)-6 ($p=0.006$) and IL-10 ($p=0.009$) before and after treatment. Notably, the difference in IL-10 levels before and after treatment was significantly higher in the advanced stages compared to that in the non-advanced stages ($p=0.007$). IL-6 and IL-10 were also higher in the expired patients compared to the survived cases.

CONCLUSIONS: Cytokines such as IL-6 and IL-10 may be considered predicting factors for chronic lymphocytic leukemia prognosis.

KEYWORDS: Chemotherapy. Cytokines. Lymphomas. Prognosis.

INTRODUCTION

Chronic lymphocytic leukemia (CLL) is considered the most prevalent adult leukemia¹. CLL is accompanied by dysregulation of the cytokine profile and immune response¹. Evidence from *in vitro* experiments indicated gene upregulation of inflammatory cytokines in the presence of CLL cells². As recently demonstrated, chronic inflammation contributes to cancer progression and even plays a role in cancer predisposition¹. An inflammatory microenvironment of CLL cells was suggested to be involved in the prolonged survival of the malignant cells². Thus, inflammatory conditions have been suggested to be important in the pathogenesis of CLL^{1,2}. The molecular interactions of adaptive immune cells (B and T cells) and innate immune cells (macrophages, dendritic, and nurse-like cells) with CLL cells contribute to the formation of a tumor-supportive microenvironment¹.

Immunologic abnormalities in CLL, such as the release of inflammatory (such as tumor necrosis factor [TNF] and interleukin [IL]-6, IL-8, IL-17) and anti-inflammatory cytokines (such as IL-10, IL-13, IL-4, and transforming growth factor

β), have been linked to type 1 and type 2 diabetes, as well as complications such as diabetic ketoacidosis and nephropathy^{1,3}. T-cell abnormalities, monocyte activation, and inflammatory markers were assumed to play a role in the loss of insulin secretory function by islet cells in this context^{3,4}. Interestingly, recent studies have proposed an association between insulin resistance and microvascular complications with levels of specific cytokines such as omentin and neuregulin-4⁵⁻⁷.

Aberrant secretion of multiple inflammatory and immunosuppressive cytokines, such as IL-10, TNF- α , and chemokine (C-X-C motif) ligand 9, has been linked to worsening CLL conditions¹. Secreted inflammatory cytokines such as IL-6 and IL-8 are thought to affect the CLL microenvironment and thus facilitate CLL development⁸. T-helper cells (Th), monocytes, and other immune cells produce IL-10, an anti-inflammatory and immunoregulatory cytokine that has strong immunosuppressive effects by suppressing the synthesis of Th1 cytokines such as TNF- α . IL-6 is released by a variety of cell types such as monocytes, normal hematopoietic cells, and lymphocytes¹. Serum cytokine

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patterns were suggested to reveal the underlying immune and inflammatory mechanisms involved in the survival and migration of CLL cells⁹. Hence, this study was designed to determine the role of cytokine status as a biomarker of treatment response and to investigate its association with CLL prognosis.

METHODS

Study design

This study was conducted in the Hematology and Oncology Department, Imam Reza Hospital, Mashhad, Iran, and enrolled 32 patients (14 females and 18 males) with a definite CLL diagnosis. Informed consent was obtained from the patients, and the study was approved by the ethics committee of Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1396.600). All patients received either chlorambucil-based or RCHOP treatment regimens. The patients were followed up every 3 months for 1 year. A medical interview and a complete physical examination were performed as part of the follow-up examinations to classify the disease stages.

Blood samples

Peripheral venous blood samples (4 mL) were taken from CLL patients at the initial medical visit following the diagnosis of CLL and again 1 year later.

Determination of serum levels of cytokines (IL-6 and IL-10) in CLL patients

The IL-6 and IL-10 serum levels were measured using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Inc., Minneapolis, MN, USA, and ENDOGEN Inc., Cambridge, MA, USA). For this purpose, the serum samples were dispensed (in triplicates) into the 96-well plate pre-coated with monoclonal anti-IL-6 and anti-IL-10 antibodies. To remove the unbound subjects, the plate was washed, followed by the addition of a conjugate solution containing polyclonal anti-IL-6 and IL-10 antibodies conjugated to horseradish peroxidase. Afterward, a substrate solution containing stabilized hydrogen peroxide and stabilized chromogen was added. Finally, the stop solution was added to terminate the reactions, and the absorbance was read using a microplate reader (BioTek, USA) at a wavelength of 450 nm. Finally, the cytokine levels were determined using the standard curves and presented as pg/mL.

Statistical analysis

The collected data were statistically analyzed using the SPSS software version 16 (17.0.1, SPSS Inc., Chicago, IL, USA).

Kolmogorov-Smirnov tests were performed to assess data normality. To compare the differences in median and mean of serum IL-6 and IL-10 before and after treatment, Wilcoxon signed-ranks and paired samples tests were applied. Furthermore, the independent samples test and Mann-Whitney U-test were used to compare the mean and median of cytokines among disease stages. The results were presented as mean±SD. A p-value less than 0.05 was considered significant.

RESULTS

This study was performed on 32 CLL patients including 58.3% males and 41.7% females, with a mean age of 68.25±1.2 years (Table 1). B-symptoms (including severe

Table 1. Demographic and clinical features of chronic lymphocytic leukemia patients.

Parameters	Amount
Age, years, n (mean±SD)	68.25±1.2
Sex	
Female, n (%)	14 (41.7)
Male, n (%)	18 (58.3)
Rai stage of disease (before treatment), n (%)	
0	2 (8.3)
I	11 (45.8)
II	3 (12.5)
III	4 (16.7)
IV	4 (16.7)
Rai stage of disease (after treatment), n (%)	
0	0 (0)
I	6 (25)
II	2 (8.3)
III	10 (41.7)
IV	6 (25)
B symptoms	
Yes, n (%)	18 (75)
NO, n (%)	6 (25)
Treatment regimen	
Chlorambucil, n (%)	15 (62.5)
RCHOP, n (%)	2 (8.3)
NO change n (%)	7 (29.2)
Changed in the middle of treatment, n (%)	7 (29.2)
Changed at the beginning of treatment, n (%)	10 (41.7)
Recurrence, n (%)	7 (29.2)

RCHOP: rituximab, cyclophosphamide, doxorubicin hydrochloride, vincristine (Oncovin), prednisolone.

fatigue, weight, fevers $\geq 38.0^{\circ}\text{C}$, and night sweats) were observed in 25% of the patients.

As presented in Table 1, most of the patients (45.80%) had stage I of the disease before initiation of treatment. However, most of the patients experienced stage III of the disease during the follow-up (45.80) and after treatment (41.70).

The median values for the IL-6 serum levels before the initiation of treatment and after treatment were 0.045 pg/mL (range of 0.01–166.20 pg/mL) and 1.90 pg/mL (range of 0.08–106 pg/mL), respectively. In the case of IL-10 serum levels, the median values before the initiation of treatment and after treatment were 1.25 pg/mL (range of 0.00–38.10 pg/mL) and 2.85 pg/mL (range of 0.03–43 pg/mL), respectively. Before the initiation of treatment, serum levels of IL-6 and IL-10 were significantly lower than those in post-chemotherapy ($p=0.006$ and $p=0.009$, respectively).

Table 2 shows IL-6 and IL-10 serum levels in CLL patients with advanced (III, IV) and early stages (0, I, II). Cytokine levels in patients with different disease stages were evaluated to see if they could be used to identify patients based on disease stage and severity. There was no significant difference in IL-6 and IL-10 serum levels between patients with advanced stages and those with early stages before treatment starts. After treatment, IL-6 and IL-10 serum levels were found to be higher in patients with advanced stages than in patients with early stages, although the differences were not statistically significant. The median value for the difference between IL-10 before and after treatment was significantly higher in the advanced stages compared to that in the nonadvanced stages ($p=0.007$). However,

the median value for the difference between IL-6 before and after treatment did not show any significant difference in the advanced stages compared to that in the early stages ($p=0.06$).

As shown in Table 3, the median IL-6 level in non-survivors was significantly higher than that in survivors following the chemotherapy (2.95 [166.20–0.06] vs. 0.03 [6.20–0.01], $p=0.006$). Similarly, the mean IL-10 level in the dead patients was higher than in the alive patients (23.83 ± 17.55 vs. 2.67 ± 6.7 , $p=0.008$) before treatment initiation.

DISCUSSION

In this study, we determined the serum levels of IL-6 and IL-10 to investigate their association with response to treatment and CLL prognosis. Our findings revealed an increasing pattern of IL-6 and IL-10 levels after chemotherapy. These results were in line with the previous studies^{10,11}. Zhu et al. reported higher levels of IL-6 in the plasma of CLL patients who received chemotherapy compared to untreated controls in this regard¹⁰. Another recent study also showed that an increased production of autocrine IL-6 was associated with a worse clinical course of CLL¹¹.

Interestingly, our results showed that IL-10 levels were significantly higher in the end stages of the disease (Rai stage III or IV). Evidence from cellular experiments confirmed the secretion of IL-6 and IL-10 in the supernatant of cultured CLL^{8,12,13}. There are some reports suggesting that IL-6 in the CLL microenvironment inhibits proliferation but prolongs survival through suppressing apoptosis of CLL

Table 2. Comparison of cytokine levels in the chronic lymphocytic leukemia stages.

Cytokines	Status	Early stages (0, I, II), median (min–max)	Advanced stages (III, IV), median (min–max)	p-value+
IL-6	Before treatment	0.035 (0.01–166.20)	0.06 (6.20–0.01)	0.90
	After treatment	3.1 (106.00–0.6)	1.10 (3.50–0.08)	0.05
	Difference of before and after treatment ($\Delta\text{IL-6}$)	1.70 (-6.90–60.20)	0.73 (-2.03–2.70)	0.06
IL-10	Before treatment	1.55 (0.01–16.00)	1.15 (38.10–0.05)	0.64
	After treatment	1.35 (18.00–0.03)	3.65 (43.00–0.90)	0.06
	Difference of before and after treatment ($\Delta\text{IL-10}$)	0.065 (-3.25–5.60)	2.62 (-36.10–2.00)	0.007

IL: interleukin. +Mann-Whitney U-test.

Table 3. The comparison of the levels of IL-6 and IL-10 between chronic lymphocytic leukemia non-survivors and survivors.

Cytokine, Value	Dead	Alive	p-value
IL-6, median (min–max)	2.95 (166.20–0.06)	0.03 (6.20–0.01)	0.006+
IL-10, mean (mean \pm SD)	23.83 \pm 17.55	3.67 \pm 2.32	0.008#

IL: interleukin. +Mann-Whitney U-test. #Independent samples test.

cells^{11,14}. *In vitro* studies showed that secretion of specific inflammatory cytokines like IL-6 alters the biology of the CLL microenvironment, which correlates with progressive CLL⁸. el-Far et al. claimed prognostic values of IL-10 and IL-6 for non-Hodgkin's lymphoma¹⁵. These findings support the idea that inflammatory and regulatory cytokines and related immune molecules have been raised in the serum of CLL patients⁹. There is accumulating evidence suggesting that the secretion of certain autocrine cytokines, such as IL-6 and IL-10, may contribute to the severe course of CLL¹⁶. In this regard, Fayad et al. showed the correlation of IL-6 and IL-10 serum levels with the severity of the disease¹⁶. Furthermore, an increased percentage of monocytic myeloid-derived suppressor cells producing IL-10 in CLL patients was reported at Rai I/II and III/IV stages. IL-10, an immunoregulatory cytokine, inhibits the production of other cytokines and suppresses immune responses¹. It seems that IL-10 secretion in CLL patients may be associated with the suppression of the immune response against CLL¹³. Recent evidence indicates poor prognosis and outcome of the disease, accompanied by increased levels of IL-6 and IL-10 in the advanced stages of CLL¹⁷⁻¹⁹. Hence, in this study, aberrant secretion of IL-6 and IL-10 seems to be involved in worsening the CLL conditions.

In this study, we found that the expired patients had much greater levels of IL-6 and IL-10 than the survivors. Consistently, IL-6 or IL-10 serum levels in CLL patients were shown to be associated with adverse disease features and 3-year survival¹⁶.

Based on our data, the median IL-6 levels in survived and dead patients were 2.95 and 0.03 pg/mL, respectively. Similarly, another study proposed that survival chance was higher in patients with the IL-6 level less than 3 pg/mL¹⁸. Wang et al. also reported a higher mean value of IL-6 linked to shorter progression-free survival¹¹.

Regarding the IL-10 level, a higher value was observed in the expired patients compared to the survived patients. Consistent with our findings, according to Fayad et al., the 3-year survival rate of CLL patients with high levels of IL-10 was 45%, while it was 85% in those with low levels of IL-10. They proposed an IL-10 cutoff of greater than 10 pg/mL as a predictor of poor prognosis¹⁶. Our data support the previous findings^{9,15}. In this context, Yan et al. indicated a higher level of IL-10 with reduced survival⁹. el-Far et al. indicated higher levels of IL-6 and IL-10 with poor survival¹⁵. Therefore, IL-6 and IL-10 may predict the outcome of patients with CLL.

CONCLUSIONS

Our findings revealed that serum levels of IL-6 and IL-10 increased in parallel with the CLL progression, with high levels of the particular cytokines in non-survivors. Hence, more aggressive treatments are recommended in patients with higher levels of cytokines. However, more studies on the mechanisms that contribute to CLL resistance and strategies to overcome them are required.

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AUTHORS' CONTRIBUTIONS

MK: Data curation, Formal Analysis. **AR:** Data curation, Formal Analysis, Writing – original draft, Writing – review & editing. **AA:** Data curation, Formal Analysis, Writing – original draft, Writing – review & editing. **FLA:** Writing – original draft, Writing – review & editing. **NA:** Writing – original draft, Writing – review & editing. **AA:** Supervision.

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