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# Clinical significance of FOXP3 mRNA expression in patients with B chronic lymphocytic leukemia: A preliminary study

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## Abstract

Chronic lymphocytic leukemia (CLL) is due accumulation of monoclonal B- cell lymphocytes in different organs in the body as the bone marrow. There is a positive relationship between T regs cells and the occurrence of CLL. The main objective of this study was to investigate the role of FOXP3 expression in peripheral blood in B- cell of CLL. This cross-sectional descriptive study included 30 newly diagnosed chronic lymphocytic patients and 30 normal controls. FOXP3 gene expression was assessed. CLL patients showed higher FOXP3 gene expression as compared to that identified in normal controls (3.5 ± 1.5 and 1 ± 0.5, respectively). In conclusion, FOXP3 gene expression was higher in CLL patients when compared with normal controls. The indication of such finding is discussed in this report.

**Keywords:** Chronic lymphocytic leukemia, FOXP3.

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## Introduction

The chronic lymphocytic leukemia (CLL) is due accumulation of monoclonal B lymphocytes in different area of the body as the bone marrow, the peripheral blood, and the different lymphoid organs. It accounts one of the most common leukemia in the developing countries.[1] It was reported that T cell dysfunction play an important role in the etiology, pathogenesis, and the progression of this disease.[2-3]

Several research studies were conducted in this field and revealed a positive relationship between regulatory T cells (Tregs) and the occurrence of CLL.[4-7] As it suppresses the immune response either by direct effect or by the release of immune suppressive cytokines which maintain tolerance and immune homeostasis.[8] Also, Tregs cells may modulate host T cell activity against tumor-associated antigens, this would lead to that tumor cells escape immune mechanisms.[9]

Treg cells are CD4+CD25 (IL-2 receptor) FOXP3 very low to no CD127 (IL-7 receptor) on their surface.[10-11] Moreover, the relationship

between Tregs and cancer is contradictory. Tregs may play a prognostic factor in CLL.[12] It has been suggested by many researchers that cancer progression through dampened antitumor responses and immunosuppression was due to elevated number of Treg cells.[13-15]

FOXP3 play an important role in the development and the function of Treg cells through the transcription factor forkhead box P3.[16] For the role of FOXP3+ Tregs and tumor, Sakaguchi and his colleagues, 2016[17] found that these cells are essential suppressors of antitumor responses so it can be used as prognostic factor. In order to prove this point recent research studies were conducted, showed that increased level of FOXP3+ Tregs was correlated with decreased metastasis and improved tumor progression.[18-25] Consequently, the aim of this study was to investigate the role of FOXP3 expression in peripheral blood B cell of CLL patients (B-CLL).

## Materials and Methods

*Ethical consideration*

The protocol of the study was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Suez Canal University (no. 4589, dated 26, July 2021). An informed consent was taken from each participant, who was assured about the confidentiality of his/her information.

This study included 30 newly diagnosed B-CLL patients obtained from oncology patients seeking Suez Canal University Hospital in Ismailia and 30 normal controls during the period from July 2021 till October 2021. The Rai clinical staging system was used to describe CLL stages.[1] Patients were divided into two groups, group (I): stages I and II CLL, included 20 cases, group (II): stages III and IV CLL, included 10 cases with an age range from 45 years to 66 years.

All patients were subjected to full clinical examination, complete blood count (CBC), Beta 2 microglobulin and Lactate Dehydrogenase (LDH) levels, bone marrow aspiration, immunophenotyping using routine panel of different chronic lymphoproliferative disorders (CD19, CD5, CD10, CD20, CD22, CD23, sIgM, CD38, CD79b, FMC7, CD103, CD123, kappa and lambda light chains) to confirm the diagnosis of B-CLL. Our inclusion criteria were according to the World Health Organization (WHO) principles for lymphoma classification,[26] and we excluded those who suffer from chronic diseases, malignancies, and those refused to participate in the study.

Study subjects were included in the following assessments:

1. Full detailed history and clinical examination including, age, sex, enlargement of superficial lymph nodes, liver, and spleen due to the infiltration of secondary lymphoid organs and systemic symptoms such as loss of weight, fever without infections, night sweats, fatigue, and repeated infections.

2. Laboratory investigation:

-Peripheral blood (PB) samples (5 ml) were collected on EDTA, under complete aseptic conditions, and divided into 3 tubes for CBC and morphological studies, immunophenotyping and the polymerase chain reaction (PCR) assay.

-CBC was performed using cell counter (ABX Micros 60, France), according to the manufacturer's instructions. The CLL diagnosis was primarily based on the morphological characteristics of the lymphoid cells by PB films stained with Leishman stain and examined by experienced pathologist.

-Immunophenotyping was performed by flowcytometry (Becton Dickinson FACS Caliber flow cytometer, Germany) by using a panel of monoclonal antibodies (MoAbs) against B-lymphoid – associated antigens: (CD2, CD5, CD19, CD22, CD23, CD37, CD79b, FMC7, anti-human kappa light chain and anti-human lambda light chain), and the common prognostic marker CD38.

-Total RNA extraction, cDNA, and real time PCR reaction for FOXP3 gene expression analysis[27] in CLL patients:

Total RNA was isolated from 2 ml PB by using the RNeasy Mini Kit (QIAampmini kit, Qiagen, USA) according to the manufacturer's instructions. cDNA was synthesized by

incubating 10µl total RNA (contain up to1 µg RNA) in a 20 µl reaction mixture, consisted of reverse transcriptase (RT) buffer, and RT primer mixture. The entire reaction was performed at 42°C and then inactivated at 95°C using QuantiTectR RT kit (QIAGEN, USA), according to the manufacturer's instructions.

The real-time PCR was performed on the StepOne Applied biosystem (Real-Time Thermocycler, Chicago, USA) using SYBR Green QuantiTectR Primer Assay (PCR Master Mix, Qiagen, USA). Each 25 µl PCR reaction contained 5 µl of cDNA (up to 100 ng), 12.5µl SYBR Green PCR Master Mix, 2.5µl PCR primers specific for human FOXP3 {FOXP3_1_SG QuantiTect Primer Assay (Qiagen, USA)} and 5µl RNase-free water. For each sample another reaction set was made for the internal control gene, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH), by using human GAPDH primer instead of human FOXP3 primer. The real-time PCR reaction consisted of an initial denaturation step at 95°C for 5 min followed by 35 cycles (denaturation at 95°C for 5s then combined annealing and extension at 60°C for 10 s). PCR reaction with human GAPDH primer was used as the internal control. Relative gene expression presented the data of the gene of interest (FOXP3) relative to internal control gene. The threshold cycles (CT, at which an increase in reporter fluorescence above the baseline signal was first detected), were determined. The GAPDH was used as endogenous control and for normalization (ΔCT) of the mRNA levels for the gene of interest. The endogenous control was subtracted from respective gene giving the ΔCT as a reflection of the relative mRNA expression. The calculations of relative gene expression were done using the ΔΔCT method. Because the amount of product doubles in each cycle the relative gene expression was calculated using the formula 2-ΔΔCT, as given in the manufacturer's instructions.

*Statistical analysis*

Statistical analysis was performed by SPSS for Windows software version 16.0 (SPSS, Chicago, IL, USA). data of target gene expression levels were highly skewed, non-parametric statistical method was used. We used Kruskal–Wallis H-test and Mann–Whitney U-test to compare gene expression levels between groups and clinical parameters. All probabilities were two-tailed. A *p*-value of <0.05 was considered statistically significant.

## Results

The demographic findings of the studied population showed that the age range of the patients was from 45 to 66 years with 53.3% females. While in the control group the age ranged from 50 to 70 years with 46.6% males. For the CBC findings, the level of hemoglobin, blood lymphocytes, platelet count and bone marrow lymphocytes were 10g/dl, 30 $\times 10^9$/L, 120 $\times 10^9$/L and 55%, respectively for the patient group. For the control group their findings were 12g/dl, 1.5 $\times 10^9$/L and 180 $\times 10^9$/L, respectively. According to Rai clinical staging, 16 cases were in stage 0-I while 14 cases were in stage III and IV. For CD38 %, 10 cases were positive. And lastly for Beta 2 microglobulin and Lactate Dehydrogenase (LDH) levels, half of the cases were within normal range. For FOXP3 mRNA, the relative expression in the studied CLL patients and the control groups were 3.5 ± 1.5 and 1 ± 0.5, respectively (Table 1).

**Table 1.** Clinical characteristics of 30 CLL patients and 30 controls.

| Parameter | CLL patients | Control group |
|---|---|---|
| Age range | 45-66 | 50 -70 |
| Sex (male/female) | 14/16 | 16/14 |
| Hemoglobin g/dl | 10 (8.0 -13.5) | 12 (11- 14) |
| Blood lymphocytes $\times 10^9$/L | 30 (24 – 60) | 1.5 (2 -3) |
| Platelets count $\times 10^9$/L | 120 (50 -230) | 180 (200 – 230) |

**Table 1.** Continued.

| Parameter | CLL patients | Control group |
|---|---|---|
| Bone marrow lymphocytes $\times 10^9$/L | 55% (45 -90) | - |
| Rai clinical staging | | |
| Early (0 –II) | 16 | - |
| Late (III – IV) | 14 | |
| CD38 % (cutoff 20 %) | | |
| Negative | 20 | - |
| Positive | 10 | |
| Beta 2 microglobulin | | |
| Below 2.5 μg/dl | 16 | - |
| Above 2.5 μg/dl | 14 | |
| LDH | | |
| Normal 420 | 15 | 30 |
| Above 420 U/l | 15 | - |
| Foxp3 mRNA relative expression | 3.5 ± 1.5 | 1 ± 0.5 |

Lactate Dehydrogenase (LDH)

FOXP3 expression and some clinical characteristics in CLL cases are shown in Table 2. Of the 30 CLL cases, 16 patients (53.3%) showed low FOXP3 expression. The difference in age, hemoglobin level and lymphocyte and platelet counts, was statistically significant (Table 2).

**Table 2.** FOXP3 expression and clinical characteristics in CLL cases.

| | High FOXP3 expression n=14 | Low FOXP3 expression n=16 | *P* value |
|---|---|---|---|
| Age<br>Mean± Standard deviation (SD) | 60 ± 5 | 50± 6 | 0.04 |
| Sex<br>Male/female | 6/8 | 10/6 | NS |
| Lymphocytes % | 56 ± 10 | 30±12 | 0.02 |
| Hemoglobin g/dl | 8.4±1.2 | 10.5±2 | 0.025 |
| Platelets Count $\times 10^9$/L | 60±26 | 160 ± 85 | 0.01 |

$P \geq 0.05$ is not significant (NS).

For the relation between FOXP3 expression and other prognostic factors in CLL patients, the FOXP3 gene was expressed in higher level in patients above 60 years old and in advanced stages (II-III) according to the WHO performance, the Rai staging, and the International Prognostic Index (IPI) as presented in Table 3.

**Table3.** Relation between FOXP3 expression and other prognostic factors in the 30 CLL patients.

| | No. of patients | FOXP3 expression |
|---|---|---|
| Age | | |
| < 60 | 22 | Low |
| > 60 | 8 | high |
| WHO performance | | |
| 0-I | 20 | Low |
| II-IV | 10 | high |
| Rai stage | | |
| 0-II | 16 | Low |
| III-IV | 14 | high |
| IPI | | |
| L + L-I | 22 | Low |
| H-I + H | 8 | High |
| B symptoms | | |
| Absent | 12 | Low |
| Present | 18 | High |
| Serum LDH level | | |
| Normal | 18 | Low |
| >Normal | 12 | High |

International Prognostic Index (IPI).

Table 4 shows the multivariate analysis of FOXP3 expression, age, erythrocyte sedimentation rate (ESR), beta-2 microglobulin, WHO performance status and IPI score.

**Table 4.** Multivariate analysis of FOXP3 expression, age, erythrocyte sedimentation rate (ESR), beta-2 microglobulin, WHO performance status and IPI score.

| | OR | 95% CI for OR (Lower-upper) | *P* value |
|---|---|---|---|
| Age | 1.2 | 0.5 -2 | NS |
| FOXP3 mRNA Expression | 1.9 | 0.8 -0.95 | 0.02 |
| ESR | 0.5 | 0.3 -1.1 | NS |
| Beta2 microglobulin | 1.5 | 1.1-2.5 | 0.03 |
| IPI | 0.9 | 0.3 -2.5 | NS |
| WHO performance | 1.1 | 0.7 – 3.1 | NS |

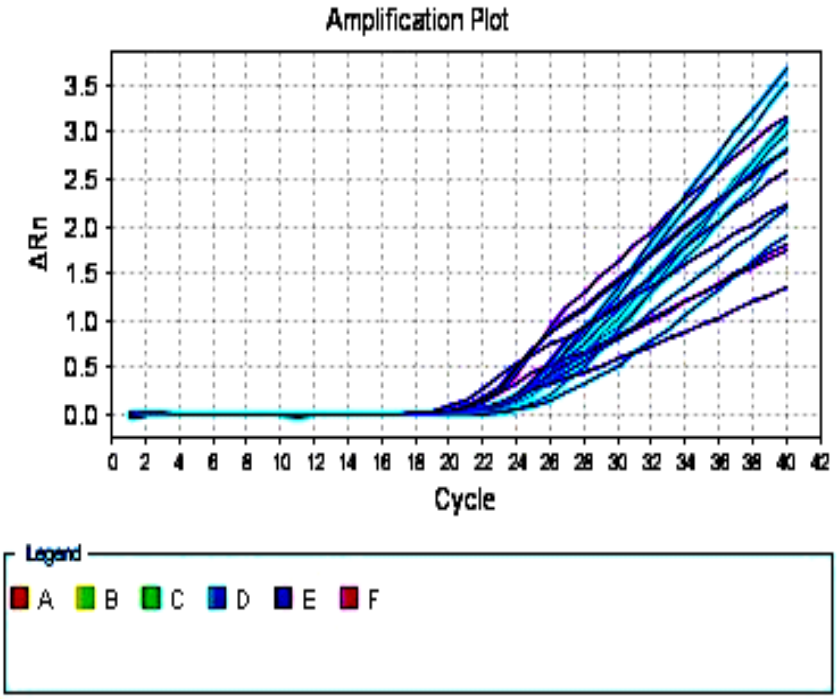
*P* ≥ 0.05 is not significant (NS). Erythrocyte Sedimentation Rate (ESR)

Table 5 shows that of the 30 CLL cases, high FOXP3 expression was observed in 4 cases with complete response and in 12 cases with relapse and such difference was statistically significant ($p$ =0.002 and 0.001, respectively).

**Table 5.** FOXP3 expression in 30 CLL patients and clinical outcome.

| | No. of high FOXP3 expression | No. of low FOXp3 expression | *P* value |
|---|---|---|---|
| Complete response (CR) | 4 | 24 | 0.002 |
| Relapse | 12 | 6 | 0.001 |

$p \leq 0.05$ is significant.




Purple color: CT cycle of FOXP3 gene Blue color: CT cycle of housekeeping gene

**Figure 1.** Amplification Plot of Real Time PCR Reaction for FOXP3 Gene Expression Analysis.

## Discussion

This is a cross sectional descriptive study to determine the FOXP3 gene expression. It included 30 newly diagnosed B-CLL patients chronic lymphocytic obtained from oncology patients to detect and 30 controls. In the present study the age of control and patient groups was from (45 to 66) and (50 to 70) years, respectively as shown in Table 1, whereas other studies showed comparable values of 46 and 64.[28]

In our study, we observed a remarkable increase in the frequency of FOXP3$^{+}$T cells expression in patients with CLL. However, this increase was obviously clear only in the advanced stages of the disease. Also, this expression was correlated with LDH level, a surrogate marker of tumor burden. These findings agreed with different previous studies.[29-31]

Several studies were conducted *in vitro,* and mouse models suggested that Treg cells play an important role in suppression of the immune system in cancer patients.[25-29] It was found that there is a relationship between the increased number of these immune cells and the solid tumor.[30] Several researchers reported that there was an increase in the number of Treg cells in CLL while their role in disease progression is still under investigation.[30-32]

A study by Stephen & Peter, 2019[32] reported that there was a correlation between disease stage and impairment of Treg function. Also, in agreement with our findings, a study by Giannopoulos *et al*., 2021[34] observed that the drastic increase of CD4$^{+}$CD25$^{+}$FOXP3$^{+}$ Tregs in 80 CLL patients has a strong implication on the disease progression. Moreover, the study by D'Arena et al., 2018[36] found that CD4$^{+}$CD25$^{hi}$CD127$^{low}$ cells have strong correlation with the advanced clinical stage.

In the present study, we found that FOXP3 gene expression was higher in advanced CLL according to WHO performance and presence of B cell symptoms and those who suffer from relapse (12/30) as shown in Table 5. These finding agreed with several studies[36-39] but not in correlation with others.[39-42] And this difference could be explained due to the use of different Treg markers by flow cytometry and due to ethnic variation.[44]

For the Rai stage, LDH, beta 2 microglubulin levels and CD38%, it was found that there was a slight increase of FOXP3 gene expression when compared with normal controls. Such observation agreed with the results reported by D'Arena et al., 2018[35] who found that patients with high risk of CLL, Treg cells were increased in their peripheral blood. So, Treg cells can fight cancer as they play a role in immune evasion by tumor cells.[45]

The study by Boćko and his colleagues 2020[47] demonstrated that patients suffering from CLL exhibit a systemic T cell dysregulation, played an important role in the accumulation of $CD4^{+}FOXP3^{+}$ T cells which interact with malignant cells. This could be considered an innovative therapy to treat malignancy.

In the same context, an experimental study conducted by Jak et al., 2009[7] revealed that the accumulation of Tregs in CLL can be explained to its extensive proliferation induced by CD27/CD70 interaction in the lymph node proliferation centers and by consequence lead to decrease in the sensitivity to apoptosis. Several studies reported the strong collaboration between Treg number and the dysfunctional Vγ9Vδ2 T cells in untreated CLL patients could contribute to the functional role of Tregs in this setting.[47]

So, to treat CLL on immunotherapeutic role targeting Treg cells, it is highly important to investigate their biologic and regulatory functions.[45-48] Lad et al., 2018[14] focused on the role of Treg cells in the pathogenesis of CLL as we did in the current study.

For the prognostic effect of Treg cells and malignancy, several studies reported that Treg cells were correlated with tumor stage, size, subtypes, and any depletion of these cells may contribute to tumor progression.[41-43]

It is noteworthy that CLL patients with bad prognosis showed lower Treg cell levels. This outstands that Treg level correlated neither with the expression of Zeta-chain-associated protein kinase 70 (ZAP-70) nor with the expression of CD38.[44] Furthermore, the increased expression of FOXP3+ Treg in CLL patients was correlated with the stage of the disease. These findings underline the pivotal role of Treg in the restriction of T-cell responses in the peripheral blood from CLL patients.[49-54]

In conclusion, our findings indicated that FOXP3 gene expression was higher in CLL patients when compared with normal controls and thus could provide a future modality treatment in those patients.

## Author Contributions

NE, contributed to the study conception and design. GAI, contributed to study conception. HE, contributed to material preparation, data collection and analysis. RE, contributed to material preparation, data collection and analysis. All authors read and approved the final manuscript.

## Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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## Ethical approval

The protocol of the study was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine, Suez Canal University (no. 4589, dated 26, July 2021).

## Informed consent

An informed consent was taken from each participant, who was assured about the confidentiality of his/her information.

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