

**ORIGINAL ARTICLE**

# Identification and Diagnosis of Possible Mutations in Human P-53 Lymphocytes

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## Résumé

In this study, some diagnosed cases of chronic lymphocytic leukemia (CLL) with possible mutations in the P-53 gene in the **P53 gene deletion in human lymphoma genome** human B lymphocyte genome were investigated. The ELISA technique was used to determine the frequency of p-53 protein expression in 20 patients diagnosed with CLL being analyzed in stages II-III / IV.

The average concentrations of p-53 proteins in 17 of the 20 cases were found to be 16.76 µg / dl, with CV = 0.5% and the probability index  $p = 0.034$ . It was found that the percentage of p-53 positive isoform proteins increased above normal with disease progression:  $15\% \pm 2$  in stages 1-2, compared to 100% in stages 3-4. In many studies in recent years, p-53 protein has been shown to mediate autophagy-inducing genes and stimulate autophagy by inhibiting protein kinase B, AMPK protein, and the mTOR complex. The role of p-53 protein in the autophagy of cancer cells could be used to develop a new anti-cancer therapeutic approach. ELISA method has proven to be a useful prognostic tool for CLL for the application of personalized treatment in cases diagnosed with CLL resistance to the specific treatment applied by the first line.

**Keywords:** Type B chronic lymphocytic leukaemia; P-53 gene; intrinsic apoptosis; CD-5 receptor; ELISA

## Introduction

The most common type of leukemia found in adults and the elderly is chronic B lymphocytic leukemia (CLL-B). It is characterized by different clinical expressions depending on the age or sex of the patient. Among the factors that control and regulate the apoptosis process, progression of the disease in the patients, p-53 protein and p-21 protein are considered to be of major importance. Over the last decade, several paraclinical investigation methods have been identified to predict the progression of the disease (1). It was discovered in the last few years that the production of some percentage of mutant p-53 proteins, with the increased stability in type B lymphocytes, leads to the carcinogenesis process. This discovery led to the identification and quantification of the p-53 protein by different methods such as immunohistochemistry (IHC),

polymerase chain reaction (PCR), single-stranded peptide microarray, (SSPMa), next-generation sequencing (NGS), and the sandwich enzyme-linked immunosorbent assay (sandwich ELISA) (2).

Specific chromosomal abnormalities, such as changes in micro-RNA forms and in the expressions of BCL-2, TCL1 and ZAP-70 genes, can serve as diagnostic and prognostic indicators for CCL-B disease progression and survival. In this scientific context, new therapeutic agents should be tested in the presence of these molecular lesions in CLL-B patients (3).

TP-53 gene mutation is a very common event in human neoplasia and a single allele is responsible for hereditary cancer susceptibility syndrome (Li Fraumeni). This variant encodes distinct isoforms

of the p-53 protein, which may disrupt its transcriptional activity (4-6).

### Materials and Methods

Using the ELISA technique, the frequency of p-53 protein expression in 20 representative patients diagnosed with CLL-B was analyzed (10), in order to investigate the relationship of the p-53 protein at the different stages of the disease and the impact on patient survival. ELISA Kit Component: Coated 96-well, Strip Plate 1, Standard (Lyophilized) 2 vials Assay, Diluent (5x) 1 vial x 15 ml, Biotinylated Detection Antibody 2 vials, HRP-Streptavidin Conjugate (800x) 1 vial x 200  $\mu$ l, Wash Buffer (20x) 1 vial x 25 ml, TMB Substrate 1 vial x 12 ml, Stop Solution sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) 1 vial x 8 ml, Plate Sealers 4.

The monoclonal p-53 antibody, PAb 240, used in the ELISA method recognizes both mutant and wild-type p-53 under denaturing conditions. Species reactivity is for human or rhesus monkey in conformity with the prospect. The monoclonal antibody PAb 240 recognizes an epitope that is structurally hidden in the wild-type conformation of p-53 and becomes exposed by denaturing the p-53 protein or the mutant conformations of p-53, where point mutations in the P-53 gene alter the terminal structure of the p-53 protein. This ELISA kit is recommended for use in serum, plasma or tissue homogenates. Using other types of sample is not supported. The sample collection protocols below were adapted from the references.

Separation of lymphocytes from total blood using Ficoll gradient was done by follow steps:

a. C 65 separation medium was prepared from 9% Ficoll in distilled water and 34% the Odiston solution in distilled water, and then 24 parts of the Ficoll solution were mixed with 10 parts of blood which were harvested on heparin, at 100 units of H / ml blood + 3 ml of IC-65 medium in 4 tubes of 10 ml.

b. 3 to 5 ml of the total blood were carefully inserted in the sample tube on the separation medium and centrifuged for 20 minutes at 2000 rpm.

c. In the centrifuge tubes we obtained plasma, with the medium ring of lymphocyte, below which was the Ficoll and Odiston media; in the base tub we obtained the sediment with

macrophages and polynuclear and dead cells. With a Pasteur pipette the lymphocyte ring was carefully removed into 25-ml cuvettes.

d. The lymphocytes were washed on 3 occasions, once every 10 minutes at 1500 rpm, and on 2 occasions, once every 10 minutes at 1000 rpm (the first wash removed the Ficoll-Odiston medium from the Turk chamber). The washed lymphocytes were resuspended in 10 ml medium IC 65 + 0.2 ml 2% calf serums. After resuspension, the suspension was introduced into Jena glass Petri dishes and stored at 36 °C.

e. For macrophage adhesion in the glass Petri dishes, the samples were kept in a thermostat at 30 °C for 30 minutes. The lymphocytes were later counted in the Turk chamber and then washed thrice again in 3 ml of IC 65 medium in 25 ml cuvettes. The required lymphocyte count was placed on the IC 65 medium per ml (1 x 10, 3 x 10, 3 cells / ml).

The lymphocytes were collected from the EDTA samples of CLL patients by centrifugation and the supernatants removed. The cells were washed 3 times and then resuspended in tampon phosphate (PBS). Lysis of cells by ultrasound was performed 4 times. The cells were centrifuged at 1500 x g for 10 minutes at a temperature within the range 2-8 °C to remove other cellular debris. Alternatively, the cells could have been frozen at -20° C and warmed to room temperature for 3 hours.

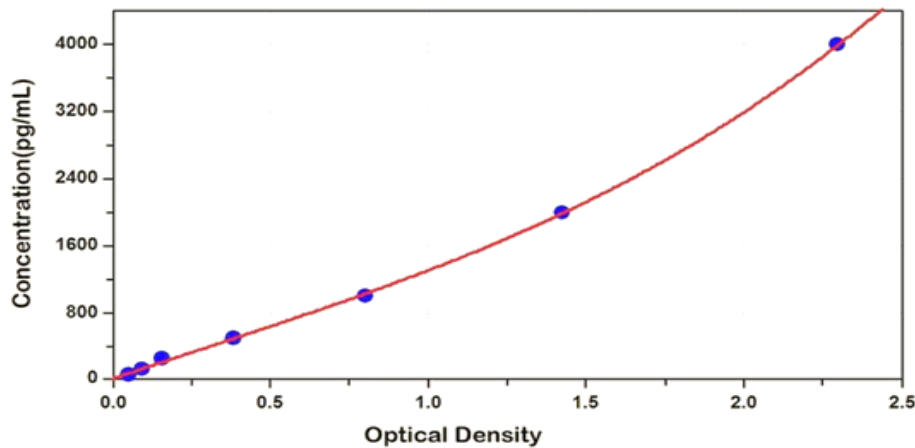
The microtiter plate wells were covered with 100  $\mu$ l of the appropriate antibody (capture antibody) 'at a rate of' 1-10  $\mu$ g / mL in the coating buffer. The plate was covered and incubated overnight at 4 °C, and then washed 3 times in ELISA Wash Buffer. To each well 150  $\mu$ L of blocking solution was added and incubated for 60 minutes at 37 °C. The mixture was then washed 4 times in wash buffer. The samples were diluted with wash buffer (ELISA), and 100  $\mu$ L of target antigen and appropriately diluted standards were added into the relevant wells. The mixture was then incubated for 90 minutes at 37 °C and then carefully washed 3 times in wash buffer.

Next, 100  $\mu$ L of the conjugate detection antibody (Streptavidin HPR Complex) was added to each well and incubated for 1 hour at 37 °C. The resulting mixture was washed 3 times in wash buffer and 100  $\mu$ L of an appropriate substrate solution (TMB) added to each well. Incubation

was done at room temperature (and in darkness), for 30 minutes or until a change in the color of the well contents as achieved. Lastly,  $H_2SO_4$  stop solution was added and a series of dilutions of the positive control standard carried in duplicates or triplicates, the last well of each series being the negative control mark.

For the detection used a standard curve with the serial dilution data on the x-axis (logarithmic scale) and the (linear) absorption data on the y-axis was plotted. Information on antigen concentrations in the different types of sample

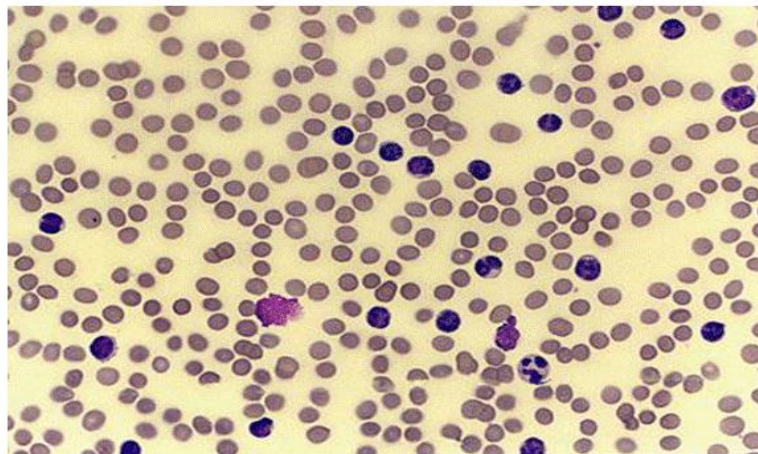
may be obtained from the published literature. However, it is often necessary to carry out a series of dilutions for each sample type. A colored product is formed in proportion to the concentration of human p53 protein present in the samples and the optical density (OD) value of 450 nm  $\pm$  2 nm, on ELISA line, was proportional to the concentration of p-53 protein. The calculation of the concentration of p-53 protein in the samples was done by comparing the OD of the samples to the standard curve. Samples were also measured in duplicates or triplicates (7) Figure 1.



**Figure 1. Standard curve from serial dilution data with the x-axis (logarithmic scale) vs. Y-axis (linear) absorption**

In the full physical examination of patients diagnosed with CLL were encompassed all patients which have been admitted to hospital with symptoms and clinical features of CLL, such as cough, night sweating, and retrosternal pain. Clinical examination and ultrasounds revealed adenopathy and/or splenomegaly, with spleen enlargement of 3 cm above the normal diameter. For each of the cases, a 5 Diff Hematology

Analyzer was used to perform a hemogram, and blood smear cytology exams on peripheral blood and medullary bone marrow were carried out by May-Grunwald-Giemsa staining. The leukemia cells found in the peripheral blood smear had characteristic microscopic morphology, with the small nuclei having mature lymphocytes with full or partially aggregated chromatin and lacking nucleoli, Figure 2.



**Figure 2. The appearance of microscopic smear in Chronic Lymphocytic Leukemia (CLL). Numerous small lymphocytes with an incised nucleus disposed of peripheral blood**

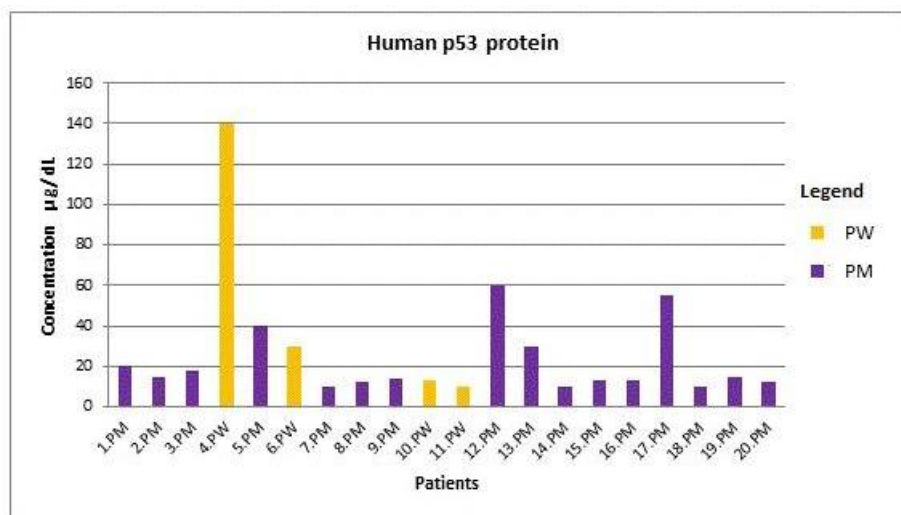
Laboratory hematological diagnosis of CLL type B was confirmed with the Immunophenotyping using monoclonal antibodies, (Flow Cytometry), analyzing the monoclonal antibodies in CD5<sup>+</sup>, CD19<sup>+</sup>, CD20<sup>+</sup>, CD23<sup>+</sup>, CD28<sup>+</sup> receptors, and with B lymphocytes expressing IgM or IgG heavy chains with kappa or lambda light chains. Diagnosis of patients with chronic lymphocytic leukemia type B (CLL-B), the clinical stages of the disease, and the patient responses to chemotherapy were based on criteria recommended by the International CLL Workshop (6). The studied cases with CCL diseases were chosen in function of the absolute number of lymphocytes (> 5000) in the Hemogram with differential count, presented in last 3 months, the aspect of blood film on the microscopic slides, with less 10% prolymphocytes, executed from peripheral blood films, colored with the hematological solution May-Grunwald Giemsa. In the study were enrolled the cases with CD19<sup>+</sup>, CD20<sup>+</sup>, CD5<sup>+</sup> and CD23<sup>+</sup>, positive by flow cytometry technique.

Eighteen (18) patients in stage A (0) (diagnosed patients without treatment), being the first year of CLL diagnosis, remained in medical observation; and 22 patients in Stage B (patients with the stable disease), who responded to rituximab plus

cyclophosphamide, doxorubicin, vincristine, and prednisone (R-CHOP) first-line treatment and had undergone 12 months of treatment with normal hemogram values, were not considered eligible for this study. The CCL stage classification referred to is according to Binet. In accordance with the B/C Binet disease phase, 20 patients who had, after one year of R-CHOP treatment resistance, been defined as “failure to complete” or “partial remission” were included in this study. All the 20 patients were treated at the time of this study and all had leukemic B lymphocytes in over 80% of their peripheral blood. CLL-B (10) blood samples were collected at the Hematology Departments of the Hospital University of Bucharest, from patients admitted from November 2015 to September 2019.

### Results

Of the 20 patients studied, 14 men have aged 55-85 years and 6 women aged 39-85 years. Patients were treated at the time of these investigations with cytostatic and immunotherapy specific for CLL. For the men the results of protein concentration p-53 detection were 20, 15, 18, 40, 10, 12, 14, 60, 30, 10, 13, 13, 5, 10, 15, 12, ( $\mu\text{g} / \text{dl}$ ) and women's results of protein concentration p-53 were 140, 30, 13, 10, ( $\mu\text{g} / \text{dl}$ ), Figure 3.



**Figure 3.** Values of p-53 protein co-concentration, assays performed on the ELISA line.

Statistical interpretations: the concentration of p-53 protein, in the 17 cases with p-53 protein expression, after excluding the 3 out-line cases present in the study, was calculated at the average value of  $16.76\mu\text{g} / \text{dl}$ , with  $STDEV = 8.35$ ,  $CV =$

0.5% and the probability index (NORMDIST) p, was calculated in the value of  $p = 0.034$ . The reference interval was established between the values  $10-40\mu\text{g} / \text{dl}$ , ( $m = 24.5\mu\text{g} / \text{dl}$ ).

The pathological values in the 3 cases of highly

elevated p-53, reflecting the concentration of p-53 protein mutant, were calculated in 2 men in the amount of 60 µg / dl, respectively in 50 µg / dl, and in the female case, it was calculated in the amount of 140 µg / dl, the frequency of chronic lymphocytic leukemia being higher in men than in women, in ratio 2/1, in accordance with the data

from the specialized literature. The overall frequency of positivity of the p53 protein, in the increased number of CLL cases studied, was 15% (3 out of 20 cases). The expression of the high-concentration p53 proteins in stage 2/3 of the disease was associated with a significantly weaker response to chemotherapy ( $p = 0.034$ ) Table 1.

**Table1. Expression of hemogram parameters and p53 protein concentration in different stages of CLL-B**

CLL Stage	No. patients	P-53 protein * Average values	P53 isoform proteins Elevated values	Hematological parameters**	P value
I/II	17	16.76 µg / dL	-	Leukocyte = 35-50 x 10 <sup>3</sup> / dL Hb = 11.8g / dL Platelets = 140 x 10 <sup>3</sup> /dL Lymphocytes = 65-80%	0.034
III/IV	3	-	15%	Leucocytes = 250-500 x 10 <sup>3</sup> /dL Hb = 8.6g / dL platelets = 45x10 <sup>3</sup> / dL Lymphocytes = 85-90%	0.05

\*concentration in reactive lymphocytes B

\*\*in peripheral blood

For the statistical interpretations was used: Method Sensitivity (MS) = number of cases with abnormal values of the tumor marker ( $p-53 > 10 \mu\text{g} / \text{dL}$ ) / total number of cases with positive p-53 protein  $\times 100 = 15/20 = 75\%$ , which means a good sensitivity (Normal values = 60-90%, CI 9%); Method Specificity (SP) = number of cases with normal p-53 protein values (range 10-40) / total number of CLL-B patients (20)  $\times 100 = 17/20 = 85\%$ , which means a good specificity (Normal values = (70-90%, CI 95%); Positive predictive value of the method (PPV) = number of cases with real abnormal values of p-53 ( $p-53$  mutant = 3) / total number of cases with abnormal values of p-53 ( $p-53$  with values higher than the cutoff)  $\times 100 = 3/3 = 100\%$  (CI 95%).

Using the formula for the “t” statistic, “t” was found to be equal 2. For a two-sided test at a common level of significance  $\alpha = 0.05$ , the critical values from the “t” distribution over 20 degrees of freedom are  $-2.01$  and  $2.01$ . The calculated “t” does not exceed these values hence the null hypothesis cannot be rejected with 95% per cent confidence. The Student’s t-test can be calculated using the given formula (1), where “x” is the sample mean, “s” is the sample standard

deviation, and “n” is the sample size {Standard table factors Student}.

$$t = (x_{\text{mean}} - \mu) / (s / \sqrt{n}) \quad (1)$$

In the equation feature for this formula,  $t = 2.01$ . The degree of freedom used in this test was  $n - 1$ . Although the patient population did not need to be normally distributed, the distribution of the population of the sample means was assumed to be normal.

### Discussions

In different studies, have been shown that in CLL, TP-53 gene mutations are commonly detected in two alleles of chromosome 17p, occurring in more than 15% of cases. Patients with such abnormalities of both alleles show increased resistance to treatment than patients with the deletion of only one of the 17p alleles (8,9).

In the presented work with CLL, in the stage I/II, ( $n=17$  patients) was registered in the average p-53 protein concentration in CLL, 16.76, ( $\mu\text{g}/\text{dl}$ ), with mean values of hemogram: hematological parameters in peripheral blood, Leukocytes numberer = 35-50 x 10<sup>3</sup> / dL, Hb = 11.8g /dL; Platelet = 140 x 10<sup>3</sup>/dL, Lymphocytes in

peripheral blood = 65-80% Table 1.

Statistical interpretations: the concentration of p-53 protein, in the 17 cases with p-53 protein expression, after excluding the 3 out-line cases present in the study, was calculated at the average value of 16.76 $\mu$ g / dL. In the CLL stage III/IV (n = 3 patients Table 1, the percentage of p53 isoform protein with elevated values was present in 15% (3 of 20 cases), such as 2 Men = 50 $\mu$ g / dL and 60 $\mu$ g / dl, respectively and 1 Female = 140 $\mu$ g /dL, p value 0.034; Hematological parameters in peripheral blood were changed with the Leucocytes leukocyte number = 250-500 x 10<sup>3</sup> /dL, Hb = 8.6g / dl, Thrombocytosis = 45x10<sup>3</sup> / dL, Lymphocytes in peripheral blood= 85-90%, ( p value = 0.05).

In some international studies, the immunological characteristics of patients with CLL having p-53 protein positive, were measured by

immunohistochemistry, (IHC). In the second part of the study CLL on the stage I/II, (n=47 patients), the P-53 protein isoform concentration in reactive Lymphocyte B, the average p-53 protein concentration in CLL was on average 47 U/m with 16.7% samples studied, (7 out of 42 cases), Hematological parameters in peripheral blood, Leucocyte number = 35x10<sup>3</sup> / dl, Hb = 12,2g / d, Thrombocyte = 140 x x10<sup>3</sup> / dL, Lymphocytes in peripheral blood = 75-80%. The average p-53 protein concentration in CLL was on average 47 U/m, in CLL on the stage III/IV (n = 140 patients] and percentage of p53 isoform varied from 7-32%.; Hematological parameters in peripheral blood where changed as Leukocytes number = 350 x10<sup>3</sup> / dL, Hb = 10,8g / dL, Thrombocytes = 80 x 10<sup>3</sup> / dL, Lymphocytes in peripheral blood = 80-90%; Percent p-53 positive was in percent of 15%<sup>10</sup> Table 2.

**Table 2. Immunological characteristics of patients with CLL having p-53 protein positive measured by IHC**

CLL Stage	No. Patients	P-53 * Average values	P 53 isoform Elevated values	Hematological parameters**	p53 <sup>+</sup>	p-53 Average values	P value
I/II	47	16.7%	-	Leukocytes =35x10 <sup>3</sup> /dL. Hb = 12.2g/dL Platelets = 140x10 <sup>3</sup> /dL Lymphocytes = 75-80%	4/30	47 U/mL	0.398
III/IV	140	-	7-32% positive	Leukocytes =350x10 <sup>3</sup> /dL. Hb = 10.8g/dL Platelets = 80x10 <sup>3</sup> /dL Lymph = 80-90%	3/12	15% positive	0.398

\*P-53 protein concentration in reactive Lymphocyte B

\*\* In peripheral blood

Recent studies have shown that the TP-53 gene is a tumor suppressor gene that is its activity stops the formation of tumors. In the tumor cell, the nuclear protein p-53 binds to the DNA, stimulating another gene, CDKN1A, to produce a protein called p-21, which interacts with a protein, CDKN2, to stop the cell division in case of DNA damage. It has been shown that the nuclear p-53 protein protects the cell from malignant processes, but the cytoplasmic p-53 protein, through its isoforms, may gain new functions to promote carcinogenesis processes (11,12). The modified activity of the p-53 protein isoform affects DNA

damage and the tumor cell phenotype. These findings suggest that the phosphorylation of the p-53 protein to Serine-15 amino acid is, therefore, an important focal point in p-53 activation. The replacement of serine with another amino acid, alanine causes the partial failure of p-53 to inhibit cell cycle progression. Also, the p-53 protein has been identified as an important regulator of glucose transport, and the transcriptional repression of both GLUT1 and GLUT4 receptors has been demonstrated. By contrast, the p-53 mutant does not affect GLUT1 and GLUT4 receptor activity in the malignant cell.

In the personal previous study, the intracellular ATP concentrations (i-ATP) have been found to be 10-fold higher in malignant tissues, in the range of 200 to more than 500 3M, than in normal tissues both *in vitro* and *in vivo*<sup>13</sup>. The internalized “I”-ATP promotes cell growth rate, increases cell survival, and, through the phosphor-ester connections which bind the p-53 isoform proteins, maintains the cell in an anti-apoptosis state. More recently, it was shown that the blocking analogues of ATP substantially reduced resistance to targeted chemotherapy in different cancers (13,14).

Another research has shown that a different mechanism which can trigger TP-53 dysfunction is the high expression of MDM2 protein (8,9). This protein suppresses TP-53 gene and miRNA genes, including miR-34a, a downstream effector of p-53 (15). Since micro-ARN, miR-34a is involved in the induction of apoptosis and cell cycle disruption, a more aggressive course of the disease can be correlated with the excessive expression of miR-34a and its effect associated with the increased intensity of CD20, FMC7, CD79b receptors on the B cell surface (16).

Also, the increased expression of CD38 receptors [16], ZAP-70 protein kinase, and unmutated IGHV chains have been reported in relation to the deletion of 17p chromosome, resulting in the poor prognosis of this group of ZAP70 patients in CLL [17, 18]. Furthermore, it was discovered that the tumors with autophagic defects were more susceptible to several anti-malignant agents (19, 22).

In many studies conducted in the past few years, it has been shown that p-53 protein mediates genes that induce autophagy and stimulate autophagy by inhibiting the protein kinase B, AMPK protein, and mTOR complex (20, 21). The role of p-53 protein in the autophagy of cancer cells could be used to develop a new anti-cancer therapeutic approach. (23, 24).

### Conclusions

The evolution of stages of Chronic Lymphocytic Leukemia of type B, which do not fall within the standard treatment criteria for malignant hematological diseases due to deletions or mutations of the P-53 gene with non-functional p-53 proteins, can be followed by Elisa technique as a screening method (25).

In the context of a heterogeneous malignant disease, such as CLL-B, this simple and inexpensive ELISA method, such as employed in this study, proves useful for identifying patients to be considered as candidates for personalized therapeutic strategies, based on the mutation of the TP- 53 gene and the presence of p-53 isoform protein.

### Conflict of Interests

There is no conflict of interest.

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