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Discovery of Isoform *p-53* Protein in Failed Cases of Chronic Lymphocytic Leukemia by Elisa Method

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Abstract

Introduction: Some diagnosed cases of chronic lymphocytic leukaemia (CLL) with possible mutations of the *p-53* gene in the human B lymphocyte genome were investigated in this study.

Method: Using the ELISA technique, the frequency of *p-53* protein expression in 20 patients diagnosed with CLL, was analyzed, including the relationship of this protein to the disease status, in the stages II-III/IV.

Results: The frequency of increased expression of the isoform *p-53* protein in type B-CLL was found to be 15% per cent. The mean concentrations of the *p-53* proteins in 17 cases out of the 20, was found to be 16.76 μg/dl, with CV=0.5% and the probability index p=0.034. The percentage of the *p-53* positive isoform proteins increased above the normal values with disease progression: 15% ± 2 in stages 1-2, compared to 100% in stages 3-4.

Conclusion: The ELISA method has proved a useful prognostic tool of CLL because was able to identify the patients with *p-53* isoform proteins and can be considered a screening method for the applying of personalized treatment in the cases diagnosticated with resistance CLL to the specific treatment applied of the first line.

Keywords: Lymphocytic leukemia • *p-53* protein • Apoptosis • ZAP-70 • CD38 receptor

Key Highlights

i. *P-53* Gene mutations are the most common genetic abnormalities of cancer. They have been extensively studied in various mature B cell malignancies, including Chronic Lymphocytic Leukemia, (CLL).

ii. In recent years, more attention has been paid to the importance of the p53 expressed protein in CLL, and a combination with low survival and non-response to classical conventional chemotherapy.

iii. Identifying different *P-53* gene mutations is very important because these mutations have an impact on patients' clinical course in CLL with the *p-53* protein mutant isoform.

Introduction

Type B chronic lymphocytic leukemia (CLL-B) is the most common type of leukemia in adults and the elderly. It is characterized by different clinical expressions depending on the patient's age or gender. 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 immune sorbent 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 inthe 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 iso forms 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, 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 × 15 ml, Biotinylated Detection Antibody 2 vials, HRP-Streptavidin Conjugate (800x) 1 vial × 200 µl, Wash Buffer (20x) 1 vial × 25 ml, TMB Substrate 1 vial × 12 ml, Stop Solution H_2SO_4 1 vial × 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

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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 ml 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 Oditson 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. Thelymphocytes 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-Oditson medium from the Turk chamber). The washed lymphocytes were resuspended in 10 ml medium IC 65 + 0.2 ml 2% calf serum. 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°Cfor 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 (1×10, 3×10^3 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 × g for 10 minutes at a temperature within the range 2°C-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 micro titer plate wells were covered with 100 µl of the appropriate antibody (capture antibody) 'at a rate of'1 µg/ml-10 µg/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 µl 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 µl 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 µl 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 µl 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.

The optical density (OD) was measured spectro photo metrically at a wavelength of 450 nm ± 2 nm. The OD value 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 (Figure 1).

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).

Laboratory hematological diagnosis of CLL type B was confirmed with the Immunopheno typing using monoclonal antibodies, (Flow Cytometry), analyzing the monoclonal antibodies in CD5+, CD19+, CD20+, CD23+, CD28+ 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-

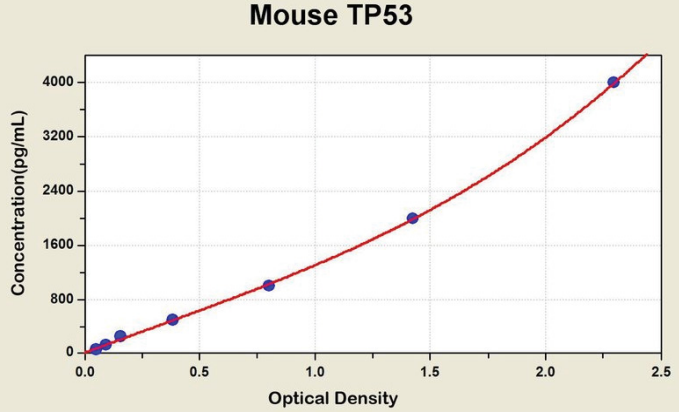

Figure 1. Standard curve from serial dilution data with the x-axis (logarithmic scale) *vs.* Y-axis (linear) absorption.

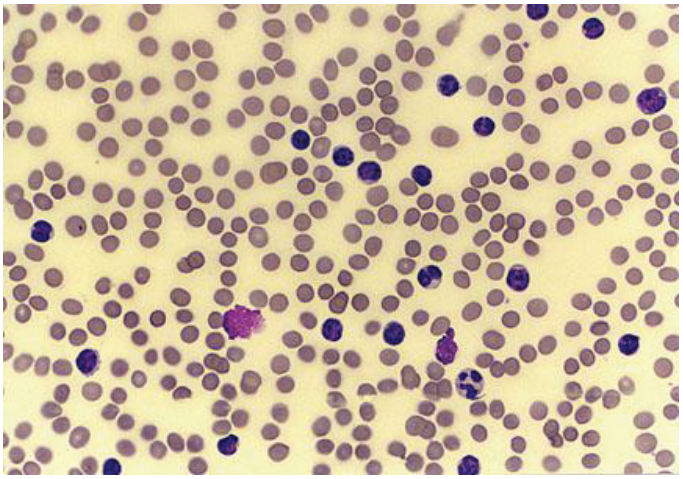

Figure 2. The appearance of microscopic smear in Chronic Lymphocytic Leukemia (CLL). Numerous small lymphocytes with an incised nucleus disposed of peripheral blood.

Grunwald Giemsa. In the study were enrolled the cases with CD19+, CD20+, CD5+ and CD23+, 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 blood samples were collected at the Hematology Departments of the Hospital University of Bucharest, from patients admitted from November 2015 to September 2019. All patients were subjected to evaluation.

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, (µg/dl) and women's results of protein concentration *p-53* were 140, 30, 13, 10 (µg/dl) (Figures 3 and 4).

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 µg/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 µg/dl-40 µg/dl, (m=24.5 µg/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).

For the statistical interpretations was used: Method Sensitivity (MS)=number of cases with abnormal values of the tumor marker (*p-53*>10 µg/dl)/total number of cases with positive *p-53* protein × 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) × 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) × 100=3/3=100% (CI 95%); Sample Student's t-test with the null hypothesis that the population mean is equal to a specified value μ_0, one uses the "t" student. 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 α=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, where "x" is the sample mean, "s" is the sample standard deviation, and "n" is the sample size {Standard table factors Student}.

$$t = \frac{\overline{x} - \mu}{s / \sqrt{n}}$$

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.

Discussion

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.

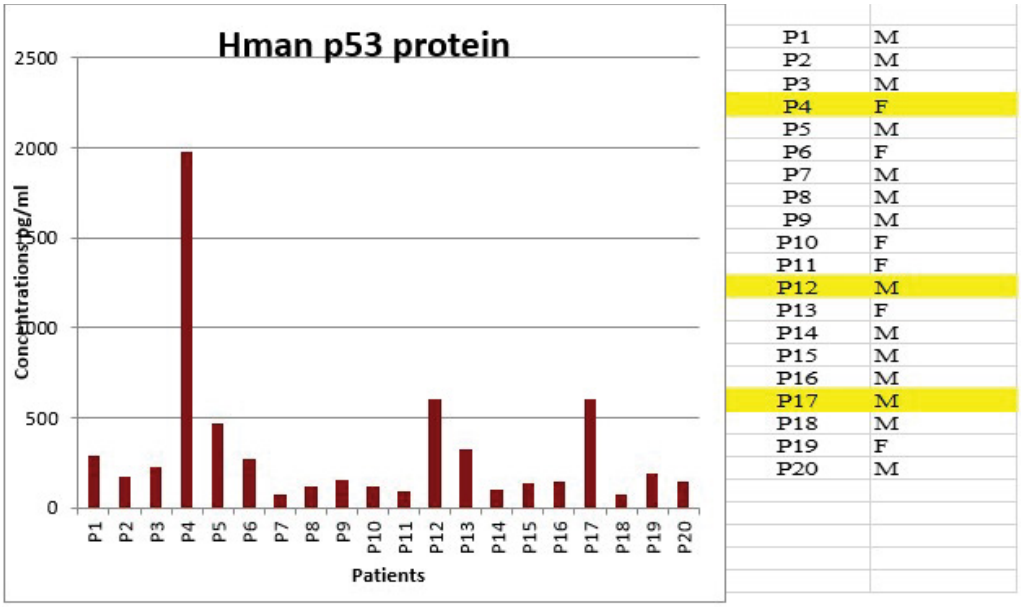

Figure 3. Values of p-53 protein co-concentration, assays performed on the ELISA line. Research on the ELISA system was performed in the Research Laboratory of the Immunology Department of the Stefan Nicolau Institute in Bucharest, in a retrospective study 2019.

Critical number	Men Concentration of protein p-53 µg/dl		Women Concentration of protein p-53 µg/dl
1-PM	20		-
2-PM	15		-
3-PM	18		-
4-PF	-		140
5-PM	40		-
6-PF	-		30
7-PM	10		-
8-PM	12		-
9-PM	14		-
10-PF	-		13
11-PF	-		10
12-PM	60		-
13-PF	30		-
14-PM	10		-
15-PM	13		-
16-PM	13		-
17-PM	55		-
18-PM	10		-
19-PF	15		-
20-PM	12		-

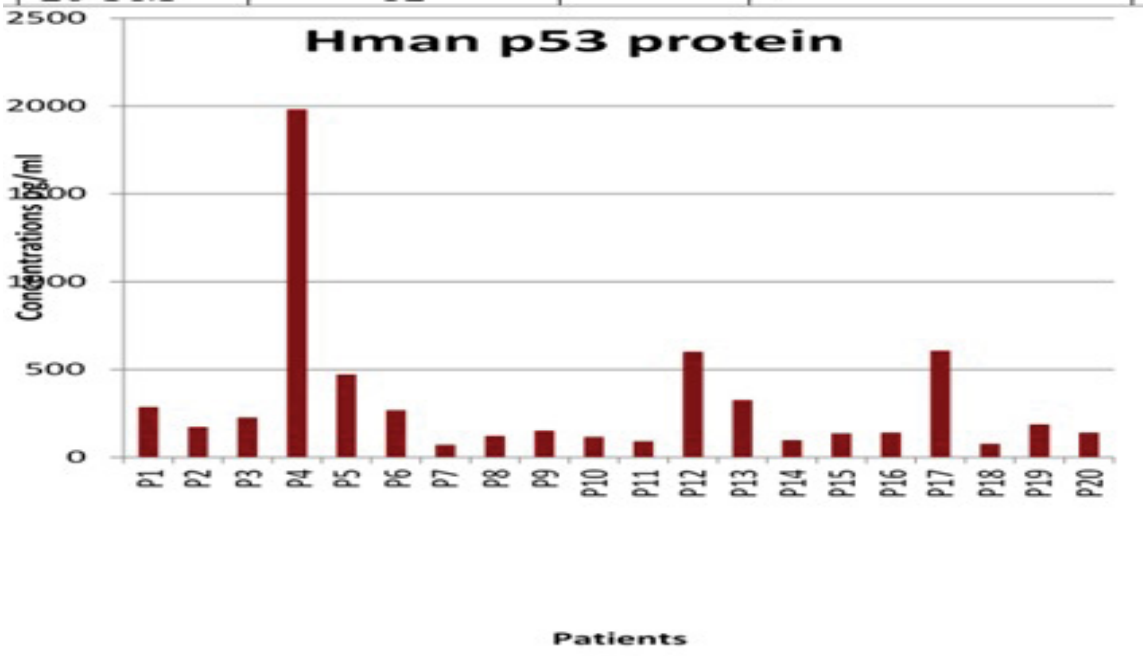

Figure 4. Quantification of p-53 isomorphic protein concentrations in CLL cases on the ELISA analyzer.

Table 1. Expression of hemogram parameters and p53 protein concentration in different stages of CLL-B.

CLL-Age patients	CLL stage I/II, (n=17 patients) P-53 protein concentration in reactive limfocytes B	CLL stage III/IV (n=3 patients] Percentage of p53 izoform proteins	p-value
The age of patients with LLC, ranging from 39 to 85 years.	The average p-53 protein concentration in CLL, 16.76 µg/dl	P-5 izofoorm proteins with elevated values was present in 15% (3 of 20 cases) 2 Men=50 µg/dl and 60 µg/dl, respectively 1 Female=140 µg/dl	0.034
Hematological parameters in peripheral blood	Mean values of haemogram: No. Leukocytes=35-50 × 10^3/dl; Hb=11.8g/dl; Platelet=140 × 10^3; Lymphocytes=65-80%	No. Leucocytes=250-500 × 10^3/dl; Hb=8.6g/dl; Thrombocytosis=45 × 10^3/dl; Limphocytes=85-90%	0.05

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, (µg/dl), with mean values of hemogram: hematological parameters in peripheral blood, Leukocytes number=35-50 × 10^3/dl, Hb=11.8 g/dl; Platelet=140 × 10^3/ µg/dl, Lymphocytes in peripheral blood=65%-80%. 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 µg/dl,

In the CLL stage III/IV (n=3 patients], the percentage of p53 isoform

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

CLL- Age patients	CLL stage I/II, (n=47 patients) P-53 protein concentration in reactive Lymphocyte B	CLL stage III/IV (n=140 patients) Percentage of p53 isoform proteins	p-value
The age of patients with LLC, ranging from 52 to 84 years, with average 62 years.	In the samples studied at CLL it was 16.7% (7 out of 42 cases).	The percentage of positive p53 cells varied from 7%-32%.	0.398
Hematological parameters in peripheral blood	No. Leukocyte=35 × 10^3/dl; Hb=12.2 g/dl; Thrombocyte=140 × 10^3/dl; Lymphocytes=75%-80%;	No. Leukocytes=350 × 10/dl; Hb=10.8g/dl; Thrombocytes=80 × 10^3/dl; Lymphocytes=80%-90%	0.398
p53 (+ve) n=7/42	4/30	3/12	0.398
The mean p-53 protein concentration: 27.9 U/ml in healthy people	The average p-53 protein concentration in CLL was on average 47U/m	Percent p-53 positive=15%.	0.398

protein with elevated values was present in 15% (3 of 20 cases), such as 2 Men=50 μg/dl and 60 μg/dl, respectively and 1 Female=140 μg/dl, p value 0.034; Hematological parameters in peripheral blood were changed with the Leucocytes leukocyte number=250-500 × 10^3/dl, Hb=8.6 g/dl, Thrombocytosis=45 × 10^3/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 measured by IHC, in the international studies, CLL stage I/II, (n=47 patients), the *P-53* protein isoform concentration in reactive Lymphocyte B, was in the samples studied, in the mean *p-53* protein concentration: 27.9 U/ml in healthy people. The average *p-53* protein concentration in CLL was on average 47 U/m with the percent *p-53* positive=15%. (p=0.398), (7 out of 42 cases, Hematological parameters in peripheral blood, Leucocyte number=35 × 10^3/dl, Hb=12,2g/dl, Thrombocyte=140 × 10^3/dl, Lymphocytes in peripheral blood=75%-80%. The average *p-53* protein concentration in CLL was on average 47 U/m, in CLL stage III/IV (n=140 patients] and percentage of p53 iso form varied from 7%-32%; Hematological parameters in peripheral blood where changed as Leukocytes number=350 × 10^3/dl, Hb=10,8 g/dl, Thrombocytes=80 × 10^3/dl, Lymphocytes in peripheral blood=80%-90%; Percent *p-53* positive was in percent of 15% [7] (Table 2).

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 [8]. It has been shown that the nuclear *p-53* protein protects the cell from malignant processes, but the cytoplasmic *p-53*protein, through its iso forms, may gain new functions to promote carcinogenesis processes [9].

The modified activity of the *p-53* protein iso form 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 μM, than in normal tissues both *in vitro* and *in vivo* [10]. 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 [11].

Another research has shown that a different mechanism which can trigger *TP-53* dysfunction is the high expression of MDM2 protein [12,13]. This protein suppresses *TP-53* gene and miRNA genes, including miR-34a, a downstream effector of *p-53* [14]. 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 [15].

Also, the increased expression of CD38 receptors, ZAP-70 protein kinase, and un mutated 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 [16,17]. Furthermore, it was discovered that the tumors with autophagic defects were more susceptible to several anti-malignant agents [18]. 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 [19]. The role of *p-53* protein in the autophagy of cancer cells could be used to develop a new anti-cancer therapeutic approach.

Conclusion

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. 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.

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Declaration of Interest Statement

This manuscript, "The Inhibitor Role of the Isoform *p-53* Protein in Failed Cases of Chronic Lymphocytic Leukemia", is an original research which has not been published and is not under consideration elsewhere. No authors declared any potential conflicts of interest.

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