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Exploring Potential Mutations in Human P53 Lymphocytes: Identification and Diagnostic Approaches

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ABSTRACT

This study investigated potential mutations in the P-53 gene in B lymphocytes from patients diagnosed with chronic lymphocytic leukemia (CLL). The expression of the p-53 protein was analyzed using the ELISA technique in twenty CLL patients across stages II-IV. In 17 out of 20 cases, the mean p-53 protein levels were 16.76 µg/dl, with a statistical probability of P = 0.034 and a coefficient of variation of 0.5%. The results showed that as CLL progresses, the amount of p-53 positive isoform proteins increases, with 15 ± 2% positivity in stages 1-2 and 100% in stages 3-4. Previous research has shown that the p-53 protein is involved in the inhibition of proteins such as protein kinase B, AMPK, and mTOR, which are involved in autophagy. This activity suggests that p-53 could be crucial for the development of novel therapies targeting autophagy in cancer cells. The ELISA technique has proven to be an effective prognostic tool, especially for customizing treatments when patients exhibit resistance to initial treatments.

Keywords: Chronic lymphocytic leukemia, CD-5 receptor, P-53 gene, Apoptosis, ELISA

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Introduction

Chronic B lymphocytic leukemia (CLL-B) is the most common type of leukemia seen in adults, particularly in older populations. The disease can present in various ways, depending on the patient's age and sex. Key players in the development and regulation of CLL-B include phosphoproteins such as p-53 and p-21, which are critical in controlling apoptosis and the disease's progression. In recent years, several paraclinical methods have been established to predict how CLL-B will evolve [1]. A significant finding is that the synthesis of mutant p-53 proteins in B lymphocytes, which become more stable, is involved in initiating the carcinogenesis process. This discovery has led to the use of multiple techniques like sandwich ELISA, single-stranded peptide microarrays (SSPMa), polymerase chain reaction (PCR), and next-generation sequencing (NGS) to detect and measure p-53 protein levels [2].

In addition to p-53 mutations, abnormalities in certain chromosomes—such as changes in the expressions of BCL-2, TCL1, and ZAP-70 genes, as well as micro-RNA variations—have been identified as useful markers for both diagnosis and prognosis of CLL-B. These molecular alterations suggest that patients with CLL-B should be carefully assessed for targeted treatments [3].

Li Fraumeni syndrome, a hereditary condition linked to an increased risk of cancer, arises from mutations in a single copy of the TP-53 gene. These mutations are often found in various cancers and can affect the transcriptional activity of the p-53 protein, leading to the generation of different protein isoforms that may influence cancer development [4-7].
This study investigates potential mutations in the P-53 gene in B lymphocytes from patients diagnosed with chronic lymphocytic leukemia (CLL).

Materials and Methods

To explore the correlation between p-53 protein expression at different stages of chronic B lymphocytic leukemia (CLL-B) and its potential impact on patient survival, we employed the ELISA technique to assess the frequency of p-53 protein expression in 20 CLL-B patients [8]. The ELISA kit used was a 96-well coated strip plate with components including a lyophilized assay, HRP-Streptavidin conjugate, biotinylated detection antibody, wash buffer, TMB substrate, and stop solution (sulfuric acid). The monoclonal p-53 antibody (PAb 240) used in this method is capable of distinguishing between mutant and wild-type p-53 by recognizing exposed epitopes after the protein undergoes denaturation or mutation, which alters its terminal structure. The ELISA kit is designed to analyze serum, plasma, or tissue homogenates but not other sample types.
For lymphocyte separation, we utilized a Ficoll-based protocol:

- Four 10 ml tubes were prepared by combining 24 parts Ficoll solution with 10 parts heparinized blood (100 units of heparin per mL) and 3 ml of IC-65 medium, resulting in a separation medium.
- After carefully layering 3 to 5 ml of whole blood onto the Ficoll-based medium, the tubes were centrifuged at 2000 rpm for 20 minutes.
- The resulting sediment containing macrophages, dead cells, and polynuclear cells was separated from the plasma and lymphocyte medium ring, which was extracted using a Pasteur pipette and placed in 25 ml cuvettes.
- The lymphocytes were washed twice at 1500 rpm and once at 1000 rpm, and then resuspended in a medium composed of 2% calf serum and IC 65 medium. This suspension was incubated in glass Petri plates at 36 °C.
- To test for macrophage adherence, the samples were incubated at 30 °C for 30 minutes, followed by another washing cycle to ensure removal of non-adherent cells.
- The lymphocytes were then resuspended in phosphate-buffered saline (PBS) after three additional washes and ultrasonic cell lysis from the CLL patients' EDTA blood samples.

After centrifugation, the supernatant was removed, and the residual cell debris was eliminated through four more rounds of centrifugation at 1500 x g for 10 minutes, at temperatures ranging between 2–8 °C. The cells could also be frozen at -20 °C and thawed after 3 hours for further processing.
For ELISA, 100 µl of the capture antibody (1–10 µg/mL) was added to each well of the microtiter plate, followed by incubation overnight at 4 °C. After three washes with the ELISA wash buffer, each well received 150 µL of blocking solution and was incubated at 37 °C for 60 minutes. The plate was then washed again, and 100 µL of diluted target antigen and standard solutions were added to the wells. After 90 minutes of incubation at 37 °C, the wells were washed three times, and 100 µL of the conjugate detection antibody (Streptavidin HPR Complex) was added, followed by another hour of incubation at 37 °C.
After the final washes, 100 µL of TMB substrate solution was added to each well, and the plate was incubated at room temperature in the dark for 30 minutes or until a color change was observed. The reaction was stopped by adding an H2SO4 stop solution. A standard curve was generated using the absorbance data (OD 450 nm) and serial dilutions of the positive control. The p-53 protein concentration in the samples was determined by comparing their OD values to the standard curve, with duplicate or triplicate measurements for each sample **(Figure 1)** [9].

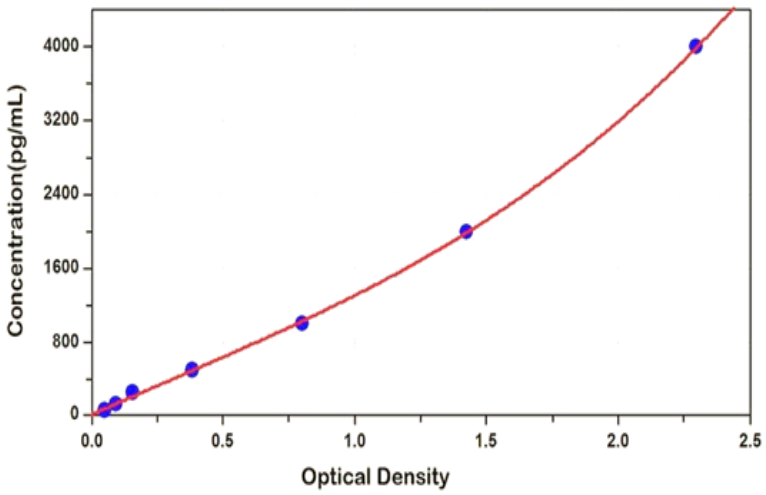

Figure 1. Standard curve from serial dilution data with the x-axis (logarithmic scale) vs. Y-axis (linear) absorption

Patients who presented with symptoms such as coughing, night sweats, and chest discomfort underwent a detailed medical evaluation. Ultrasound and clinical examination revealed signs of adenopathy and/or splenomegaly, with the spleen measuring 3 cm larger than its typical size.

A hemogram was performed on each patient using a 5-diff hematology analyzer, and May-Grunwald-Giemsa staining was applied to blood smears for cytological examination of both peripheral blood and bone marrow. Leukemic cells were identified in peripheral blood smears based on their distinctive microscopic features, which included small nuclei, lymphocytes with no nucleoli, and fully or partially condensed chromatin **(Figure 2)**.

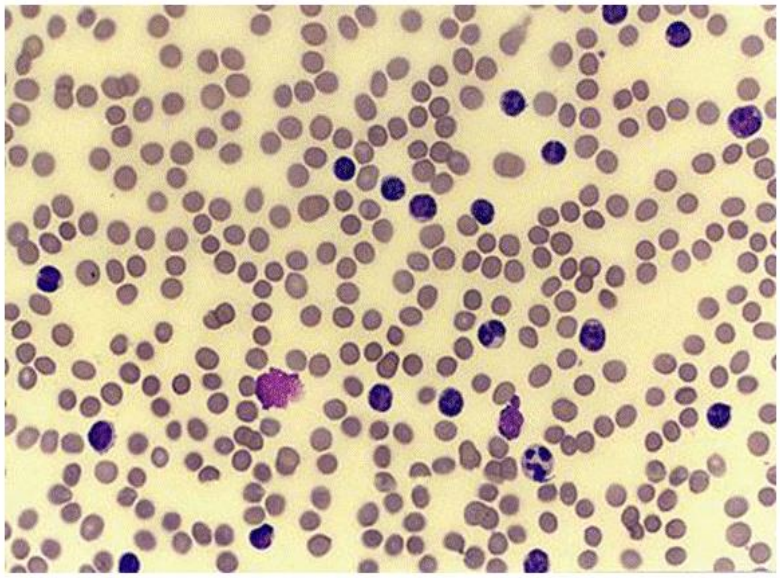

Figure 2. The appearance of microscopic smear in chronic lymphocytic leukemia (CLL); numerous small lymphocytes with an incised nucleus disposed of peripheral blood

The diagnosis of CLL type B was confirmed through a combination of hematological testing and immunophenotyping using monoclonal antibodies, with flow cytometry identifying B cells producing IgM or IgG heavy chains and kappa or lambda light chains. The expression of $CD5^+$, $CD19^+$, $CD20^+$, $CD23^+$, and $CD28^+$ receptors was examined using monoclonal antibodies. The classification of clinical stages and patient responses to treatment followed the international CLL workshop criteria [6]. Patients were selected based on blood films with fewer than 10% prolymphocytes and lymphocyte counts exceeding 5000 in their hemogram from the past three months. May-Grunwald Giemsa staining was used for blood smear analysis, and flow cytometry was employed to identify patients with specific markers of $CD19^+$, $CD20^-$, $CD5^+$, and $CD23^+$.

The study excluded 22 stage B patients who had completed one year of treatment with R-CHOP (rituximab, cyclophosphamide, doxorubicin, vincristine, and prednisone) and had stable disease with normal hemogram results. 18 stage A patients who were diagnosed but not yet treated were under continued observation. Binet staging was used to classify the patients. The research focused on 20 patients who were classified as "failure to complete" or "partial remission" after a year of resistance to R-CHOP treatment, corresponding to the B/C Binet disease phase. By the time of the study, all 20 patients were still undergoing treatment, with over 80% of their peripheral blood consisting of leukemic B cells. Blood samples were collected from patients admitted between November 2015 and September 2019 at the Hematology Departments of the University Hospital of Bucharest.

Results and Discussion

The study involved 20 patients, consisting of 6 women aged 39 to 85 and 14 men. At the time of the study, all patients were receiving cytostatic therapy and specific immunotherapy for CLL. Protein concentration levels of p-53 were measured, and the results are summarized in **Figure 3**. In the male patients, the p-53 concentrations ranged from 5 to 60 µg/dl, with individual values being 20, 15, 18, 40, 10, 12, 14, 60, 30, 10, 13, 13, 5, 10, 15, and 12 µg/dl. For the female patients, the p-53 protein levels were 140, 30, 13, and 10 µg/dl.

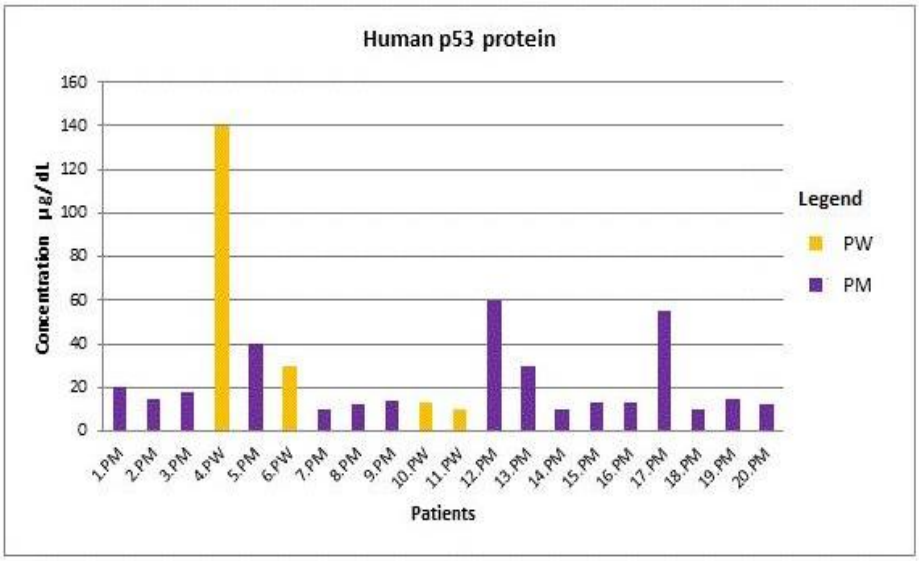

Figure 3. Values of p-53 protein co-concentration, assays performed on the ELISA line.

The statistical analysis revealed that in the 17 cases where p-53 protein expression was detected (excluding the 3 outliers), the average p-53 concentration was 16.76 µg/dl, with a probability index (NORMDIST) of 0.034, a standard deviation of 8.35, and a coefficient of variation (CV) of 0.5%. The reference range for p-53 protein levels was established at 24.5 µg/dl, with values ranging from 10 to 40 µg/dl.

Among the 20 patients, two males and one female showed significantly higher p-53 concentrations, measuring 60 µg/dl and 140 µg/dl, respectively, which were classified as pathogenic levels based on previous studies. It was noted that chronic lymphocytic leukemia (CLL) is more prevalent in men, with a 2:1 male-to-female ratio. In total, 3 cases in this study had a p-53 protein positivity rate of 15% in the progression of CLL.

A significant correlation (P = 0.034) was observed between elevated p-53 protein concentrations and poor treatment response in patients at stages 2 and 3 of the disease **(Table 1)**.

Table 1. 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 10^3 /dL Hb = 11.8g / dL Platelets = 140 x 10^3 /dL Lymphocytes = 65-80%	0.034

III/IV	3	-	15%	Leucocytes = 250-500 x 10 ^{3} /dL Hb = 8.6 g /dL platelets = 45x10 3 /dL Lymphocytes = 85-90%	0.05

*concentration in reactive lymphocytes B
**in peripheral blood

The following statistical analyses were conducted: The sensitivity (MS) of the method was calculated as the proportion of cases with abnormal p-53 values (greater than 10 µg/dL) out of the total number of p-53 positive cases, yielding 75% (15/20), which demonstrates good sensitivity (with a normal range of 60-90%, CI 9%). The specificity (SP) was determined by dividing the number of cases with normal p-53 levels (ranging from 10-40 µg/dL) by the total number of CLL-B patients (20), resulting in 85%, indicating good specificity (normal range 70-90%, CI 95%). The positive predictive value (PPV) was calculated by dividing the number of cases with true p-53 outliers (3 cases) by the total number of cases with abnormal p-53 levels, resulting in 100% (CI 95%).
For the "t" statistic, it was calculated that "t" = 2. The critical values for a two-sided test with 20 degrees of freedom at a significance level of $\alpha = 0.05$ were ± 2.01. Since the calculated "t" value did not exceed these critical values, the null hypothesis could not be rejected at the 95% confidence level. The student's t-test can be performed using the formula provided in Eq. 1, where "x" is the sample mean, "s" is the sample standard deviation, and "n" is the sample size **(Table 1)**.

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

For statistical analysis, the sensitivity (MS) of the method was calculated as the percentage of cases with abnormal p-53 values (> 10 µg/dL) among all p-53 positive cases. This resulted in 75% sensitivity, which is considered good (normal range: 60-90%, CI 9%) [10, 11]. The specificity (SP) was calculated by dividing the number of cases with normal p-53 levels (10-40 µg/dL) by the total number of CLL-B patients, yielding 85%, indicating good specificity (normal range: 70-90%, CI 95%) [10, 11]. The positive predictive value (PPV) was determined as 100% because all three outlier cases with elevated p-53 protein concentrations were true positive cases, representing 100% (CI 95%).
For the "t" statistic, a value of 2 was calculated, and the critical values for a two-sided test with 20 degrees of freedom at a significance level of $\alpha = 0.05$ were ± 2.01. Since the calculated "t" did not exceed these values, the null hypothesis could not be rejected at the 95% confidence level. The student's t-test can be calculated using the formula provided, where "x" is the sample mean, "s" is the sample standard deviation, and "n" is the sample size **(Table 1)** [12].
Several studies have shown that TP-53 gene mutations are commonly found in two alleles of chromosome 17p, affecting more than 15% of CLL patients. Those with mutations in both alleles show greater resistance to treatment compared to those with only one allele deleted [10, 11]. In the study with 17 patients, the average p-53 protein concentration was 16.76 µg/dL. The hematological parameters showed leukocyte counts of 35-50 x 10^3/dL, platelets at 140 x 10^3/dL, hemoglobin at 11.8 g/dL, and lymphocytes in peripheral blood between 65-80%.
The study also indicated that in stage III/IV CLL (3 patients), the p-53 protein isoform was present in 15% of the cases, with protein concentrations of 50-60 µg/dL in males and 140 µg/dL in a female (P-value = 0.034). Hematological parameters in these patients showed leukocyte counts between 250-500 x 10^3/dL, hemoglobin at 8.6 g/dL, thrombocytosis at 45 x 10^3/dL, and lymphocytes at 85-90%.
In other studies, immunohistochemistry (IHC) was used to assess the immune characteristics of CLL patients with p-53 protein expression. In one study, 47 patients in stages I and II were analyzed, with 16.7% (7 of 42 cases) showing p-53 isoform expression in reactive lymphocytes. Hematological parameters in these cases included leukocytes at 35 x 10^3/dL, hemoglobin at 12.2 g/dL, platelets at 140 x 10^3/dL, and lymphocytes at 75-80%. The p-53 isoform concentration ranged from 7-32%, with an average of 47 U/m for stage III/IV patients (140 cases).
Recent research indicates that the TP-53 gene acts as a tumor suppressor, preventing cancer growth. When p-53 binds to DNA in tumor cells, it activates the CDKN1A gene, which produces the p-21 protein to inhibit cell proliferation during DNA damage. Nuclear p-53 protects the cell from cancerous processes, while cytoplasmic p-53 isoforms may contribute to cancer development. Phosphorylation of p-53 at serine-15 is crucial for its

activation, and the substitution of serine with alanine reduces its ability to halt the cell cycle. Moreover, p-53 protein regulates glucose transport by inhibiting GLUT1 and GLUT4 receptors, which are not affected by mutations in p-53 [12, 13].

Studies have also shown that ATP concentrations in cancerous tissues are significantly higher than in normal tissues, accelerating cell growth and survival. ATP analog blockers have been shown to reduce cancer resistance to targeted treatments [13-17].

Excessive MDM2 protein production can contribute to TP-53 dysfunction, with overexpression of miR-34a inducing apoptosis and cell cycle disruption. This miRNA, along with others like TP-53 and miR-34a, is repressed by the p-53 protein. The ZAP-70 protein kinase, unmutated IGHV chains, and increased CD38 expression are associated with a poor prognosis in CLL patients with 17p chromosomal deletions. Studies also indicate that targeting tumors with autophagic defects may enhance the effectiveness of anti-cancer therapies [18-24].

Finally, the p-53 protein inhibits the AMPK protein, mTOR complex, and protein kinase B, regulating genes that promote autophagy. This suggests that manipulating the p-53 protein's role in cancer cell autophagy could offer new therapeutic strategies [25-27].

Conclusion

The ELISA method proves useful as a diagnostic tool for tracking the stages of type B chronic lymphocytic leukemia, particularly in cases where traditional criteria for treating hematological cancers are not applicable due to deletions or mutations in the P-53 gene, leading to dysfunctional p-53 proteins [28].

This cost-effective and straightforward ELISA technique, as demonstrated in the study, plays a key role in identifying patients who may benefit from individualized treatment approaches, especially in the context of the complex nature of CLL-B.

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Conflict of Interest: None

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Ethics Statement: None

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