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Original Article

Measurements of p53 Isoform Protein Concentration and Energetic Levels in Cells of CLL Using Enzymatic Methods

Aurelian Udriştioiu *[1], Alexandru Giubelan [1], Ana Velcea [1], Liviu Martin [1], Manole Cojocaru [2], Ioan-Ovidiu Gheorghe [3]

[1]Faculty of Medicine, General Medical Care, Titu Maiorescu University of Bucharest, Târgu Jiu, Romania.
[2]Faculty of Medicine, Romanian Academy of Scientist, Titu Maiorescu University of Bucharest, Romania.
[3]Faculty of Medical Science and Behaviour, Constantin Brâncuși University, Târgu Jiu, Romania.

*Corresponding Author: Aurelian Udriştioiu; *aurelianu2007@yahoo.com*

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Abstract

Background/Objectives: Aim of this works of researches proposed the detection of the isoform p53 protein in the patients with Chronic Lymphocytic Leukemia of type B, (CLL-B), performed using the quantitative sandwich Enzyme linked immunoassay ELISA for the direct detection of the p53 isoform protein, a product of the mutant P53 gene. ***Methods:*** Additionally, the cellular energy levels, particularly in CLL-B, were measured using a bioluminescence method. The study employed a quantitative sandwich ELISA method for the direct detection of the p53 isoform protein, a product of the mutant P53 gene Adenosine triphosphate, (ATP), was measured using the standard bioluminescence principle on an automatic LKB analyzer with an ATP monitoring reagent. ***Results:*** The presence of isoform p53 protein in the studied group was calculated at 17%. Unfavorable evolution and transformation of CLL into Diffuse Large B-Cell Lymphoma (DLBCL) were observed in 3.5% of the cases. In such as malignant diseases, increased ATP values were observed in Chronic Lymphatic Leukemia in values of 4.30-4.55 µM ATP, (Normal energy in B Cells: $\bar{x}$ = 0.35 µM ATP, (SD = 0.42). ***Conclusion:*** Maturation state of the clonal tumor and the prognosis of patients with CLL depend on anaerobic metabolism and the expression of the p53 protein isoform.

Keywords: ***chronic lymphocytic leukemia; P53 gene, isoform p53 protein, CD-5 receptor, Diffuse Large Lymphoma, adenosine triphosphate.***

1.1. Introduction

Chronic lymphocytic leukemia (also known as "chronic lymphoid leukemia" or CLL) is a type of cancer affecting white blood cells, specifically lymphocytes. These cells accumulate mainly in the bone marrow and blood. Morphologically, the neoplastic cells resemble mature lymphocytes found in peripheral blood. In the majority of CLL patients, the malignant cells are clonal B cells arrested in the differentiation pathway between pre-B cells and mature B cells. Only 2-5% of patients with chronic CLL exhibit a T-cell phenotype.

In CLL-B, the DNA of B cells is mutated, impairing their ability to fight infection while allowing them to proliferate uncontrollably and crowd out healthy blood cells. This study included patients with CLL who had an absolute lymphocyte count of over 5,000 cells, as determined by cytological examination of a peripheral blood smear stained with May-Grunwald Giemsa, and with less than 10% prolymphocytes present in the peripheral blood smear, [**Figure 1**].

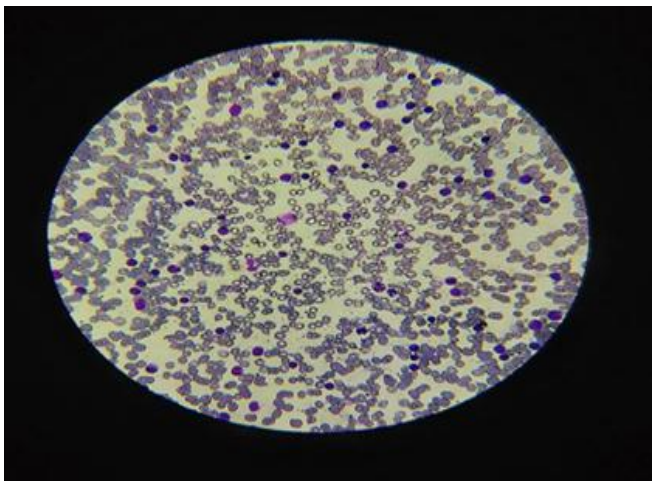


Figure 1: Lymphocytes with Gumprecht nuclear shadows, in CLL on peripheral smear, colored with May-Grunwald Giemsa

1.2. Aim

The aim of this paper is to highlight the stages of Chronic Lymphocytic Leukemia of type B (CLL-B) that do not meet the standard treatment criteria for malignant hematological diseases due

to mutations in the p53 gene, with progression to Diffuse Large Lymphoma. Additionally, this work aims to emphasize the measurement of cellular energy levels in various malignant diseases especially in CLL-B, resulting from gene dysregulation, using a bioluminescence method on an LKB bioluminescence analyzer.

1.3. Immunophenotyping of CLL-B Cells

The diagnosis of CLL-B was confirmed by immunophenotyping. All samples included in the study consisted of lymphocytes expressing positive $CD19^{+}$, $CD20^{+-}$, (with variable intensity), $CD5^{+}$, and $CD23^{+}$ cell receptors. The $CD38^{+}$ receptor was considered positive if the lymphocytes exhibited a higher staining intensity than the granulocytes in the same sample. This positivity was associated with the presence of the ZAP-70 protein, which has prognostic significance for treatment in CLL.

1.4. Participant Type

Patients diagnosed with CLL-B and hospitalized in the Hematology Departments of the Oncology Institute between November 2016 and September 2019 were included, Age Group: Seniors, Gender: Both, Target Number of Participants: 20, Recruitment Start Date: 01/11/2016, Recruitment End Date: 01/09/2019, location, Romania

1.5. Materials and Methods Used on the ELISA Line

The study employed a quantitative sandwich ELISA method for the direct detection of the p53 isoform protein, a product of the p53 gene. The used kit had the, specificity: Human p53 protein (aa20-25) and format: purified product using a monoclonal antibody (clone DO-1, isotype IgG2a). Techniques Applicable was the immune-enzymatic method, Enzyme Linked Immune Assay, ELISA. The PAb 240 antibody was used for its specific binding to denatured p53 protein (Wang PL et al., 2001). Compatible Sample Types was applied in cells of plasma and serum on Solid Support with 96-well microplates.

1.6. Principle of the ELISA method and technique

This analysis is based on the sandwich ELISA principle. Each well of the microtiter plate is pre-coated with a specific capture antibody. When standards or samples are added, the target antigen in this case, the p53 protein binds to the capture antibody.

ELISA Protocol from prospect:

- Prepare all reagents, samples, and standards as instructed in the guide manual.
- Add 100 µl of standard or sample to each well.
- Incubate for 2.5 hours at room temperature (RT) or overnight at 4°C.
- Add 100 µl of the prepared biotin antibody to each well.
- Incubate for 1 hour at RT.
- Add 100 µl of the prepared streptavidin solution to each well.
- Incubate for 45 minutes at RT.
- Add 100 µl of TMB One-Step Substrate Reagent to each well.
- Incubate for 30 minutes at RT.
- Add 50 µl of stop solution to each well.
- Read the plate at 450 nm immediately on the ELISA analyzer.

1.7. Results obtained by the ELISA Method

After analyzing 85 CLL samples at various stages (from stage 0, "watch and wait," to stage IV), 20 patients were selected for further investigation. These patients were evaluated for the detection of p53 protein isoforms associated with resistance to oncological treatments, including Rituximab, Cyclophosphamide, Doxorubicin hydrochloride, Vincristine sulfate (Oncovin), and Prednisone (R-CHOP) after two cycles of relapse. This selected group comprised 16 men and 4 women, aged 39-85 years.

Male Results: Protein Concentration in p53, µg/dL): 20, 15, 18, 40, 10, 12, 14, 60, 30, 10, 13, 15, 5, 10, 15, 12; Female Result: 140, 30, 13, 10. Normal values for cell lines on the ELISA equipment were: 0.25-0.5 µg/dL (or 2.5-5 ng/mL).

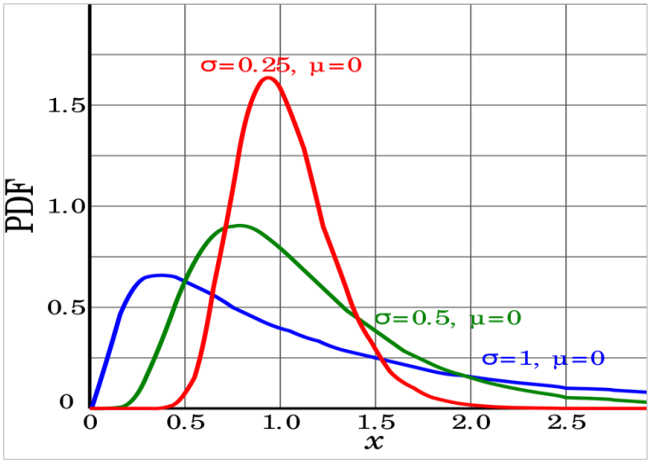
1.8. Statistical Interpretations

After excluding 3 outlier cases, the concentration of the p53 isoform protein (representing the p53 mutant gene) was calculated in 17 cases. The mean value was 14.8 µg/dL, with a standard deviation (STDEV) of 6.46 and a coefficient of variation (CV) of 0.4%. The probability index (NORMDIST, "p") was calculated at $p = 0.079$. Multivariate analyses (MA) for overall survival (OS) were performed on variables with $p < 0.20$ at univariate analysis, with gradual elimination of insignificant variables.

The presence of p53 protein isoforms in the studied group was calculated at 17%. Unfavorable evolution and transformation of Chronic Lymphocytic Leukemia into Diffuse Large B-Cell Lymphoma (DLBCL) were observed in 3.5% of the cases. According to meta-analyses in the specialized literature, Diffuse Large Lymphoma is considered a rare disease. Comparisons between categorical and numerical variables were performed using appropriate statistical methods using Fisher test.

1.9. Fisher test

Number of patients in the study group = 85; Eligible 20 a + b = 3 + 17; Ineligible 65. c + d = 5 + 60. Total a + c b + d a + b + c + d (= n) = 85; p = {a + b} \ {a} x {c + d} \ {c}: [{n} \ {a + c}] = 20 / 3x65 / 5 / 8,125 = 10.2 = 1.1 log in base 10, result that we indicate that all statements are true. {\ frac {(a + b)! (c + d)! (a + c)! (b + d)!} [a! b! c! d! n!], in the calculations made by the statistical software where, (n / k), is the binomial coefficient and the symbol "!" show that factorial, {**Graphic 1**}.



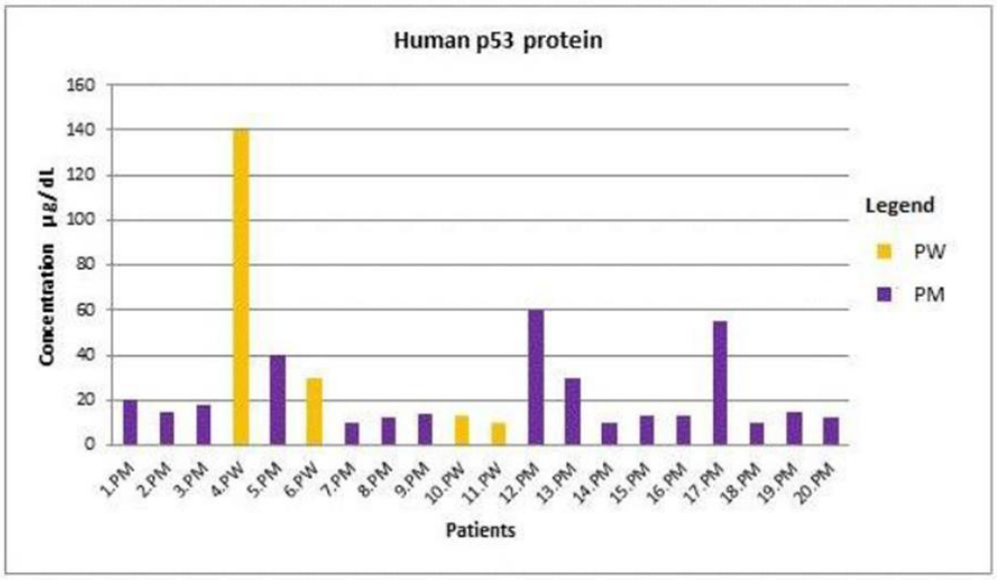
Graphic 1: All expressions under the sign of the logarithm are indicated as positive which is under the sign of the logarithm and as a basis

Frequency of p-53 isoform protein in cohort of study was evaluated with ANOVA test, Calculation of frequency of isomorph p-53 protein by ANOVA test positive, in value 1.1 and indicates a linear relationship between the 2 variables, [**Table 1**].

Table 1: Calculation of frequency of isomorph p-53 protein by ANOVA test

The interval of concentration (i)	The middle classes (m)	Frequency of concentration (f)
30-32	31	1
27-29	28	0
24-26	25	0
21-23	22	0
18-20	19	2
15-17	16	2
12-14	13	8
9-11	10	4
6-8	7	0
3-5	4	1 17%

Very high pathological values in the 3 cases of p-53 were calculated in 2 men in the value of 60 µg / dL, respectively at 40 µg / dL, and in the case of females it calculated in the amount of 140 µg / dL, the frequency chronic lymphocytic leukemia with transformation into Diffuse Large Lymphoma, (DLL), [**Graphic 2**].



Graphic 2: Very high pathological values in the 3 cases of p-53 were calculated in 2 men with the value of 60 µg / dl, respectively at 40 µg / dl, and in the case of females it was calculated in the amount of 140 µg / dl, the frequency of Chronic Lymphocytic Leukemia with transformation into Diffuse Large Lymphoma, (DLL).

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 2**).

Table 2: 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 = 50µg / dL and 60µg / dL, respectively* *1 Female = 140µg / dL*	*p value 0.034*
Hematological parameters in peripheral blood	***Mean values of haemogram:*** *No. Leukocytes = 35-50 x 10³/µL* *Hb = 11.8g / dL;* *Platelet = 140 x 10³/µL* *Lymphocytes = 65-80%*	*No. Leucocytes = 250-500 x 10³/µL* *Hb = 8.6g / dL* *Thrombocytosis = 45x10³/µL* *Limphocytes = 85-90%*	*p value 0.05*

The pathological values in the three cases of highly elevated p53 (reflecting the concentration of mutant p53 protein) were as follows: in two male patients, the values were 60 µg/dL and 50 µg/dL, respectively; in one female patient, the value was 140 µg/dL. The frequency of chronic lymphocytic leukemia (CLL) was higher in men than in women (a 2:1 ratio), which is consistent with the specialized literature. Overall, p53 protein positivity was observed in 15% of the CLL cases studied (3 out of 20 cases). High-concentration p53 protein expression in stage II/III of the disease was associated with a significantly poorer response to chemotherapy (p = 0.034).

2. Energy Measurement and Bioluminescence Analysis

The main stimulus for malignant tumor progression is hypoxia resulting from predominant anaerobic metabolism. One aim of this work was to emphasize the measurement of cellular energy levels in various malignant diseases especially in CLL-B-due to gene dysregulation. ATP was measured using the standard bioluminescence principle on an automatic LKB analyzer with an ATP monitoring reagent, ATP Standard (10^6 mol ATP/mL), Tris-EDTA buffer, and TCA-EDTA lysis. All results were statistically analyzed using Microsoft Excel. Principle of the Reaction: ATP + luciferin + O_2 → oxyluciferin + AMP + PPi + CO_2 + Light.

Lymphocytes were obtained from the peripheral blood of healthy individual and from hospitalized patients with selected diseases from the departments of Oncology Plasma was collected from patient samples using vacutainers with EDTA or heparin as an anticoagulant by centrifuging the samples for 15 minutes at 4500 rpm (280 G). After the blood sample has been centrifuged and its plasma has been separated from red blood cells, the plasma is fractionated into four distinct fractions placed on a layer of white blood cells(lymphocytes). With a pipet, a quantity of 100µL is extracted from the lymphocyte ring.

2.1. Procedure

- A 100 µL drop is taken from the lymphocyte layer in 3 mL of Wash Buffer medium.
- Introduce the lymphocyte layer into 25 ml cuvettes with 3 ml Wash Buffer medium for washing the lymphocytes,
- Washing is performed three times: first at 1500 rpm for 10 minutes, then twice at 1000 rpm for 10 minutes each.
- Lysis of the washed lymphocytes is carried out using a device as Mini Wave Smart Laboratory microwave.
- Run Luminometer Analyzer by introduction cuvettes of Lymphocyte Layer Extraction of lymphocytes for processing and reaction catalyzed by luciferase from the ATP kit.

2.2 Results of ATP concentration on Luminometer Analyzer

ATP concentrations analysis with standard deviations, (SD), were determined as follows: normal T cells: $\bar{x}$ = 1.39 µM ATP, (SD = 0.41), normal B cells: $\bar{x}$ = 0.35 µM ATP, (SD = 0.42), T cells in malignant diseases: $\bar{x}$ = 0.17 µM ATP, (SD = 0.46), B cells in malignant diseases, $\bar{x}$ = 3.06 µM ATP, (SD = 0.45), B cells in leukemia:, $\bar{x}$ = 4.33 µM ATP, (SD = 1.5), T cells in leukemia:, $\bar{x}$ = 0.09 µM ATP, (SD = 1.7), [**Table 3**].

Table 3: Concentration of ATP (µM) in malignant diseases determined by bioluminescence

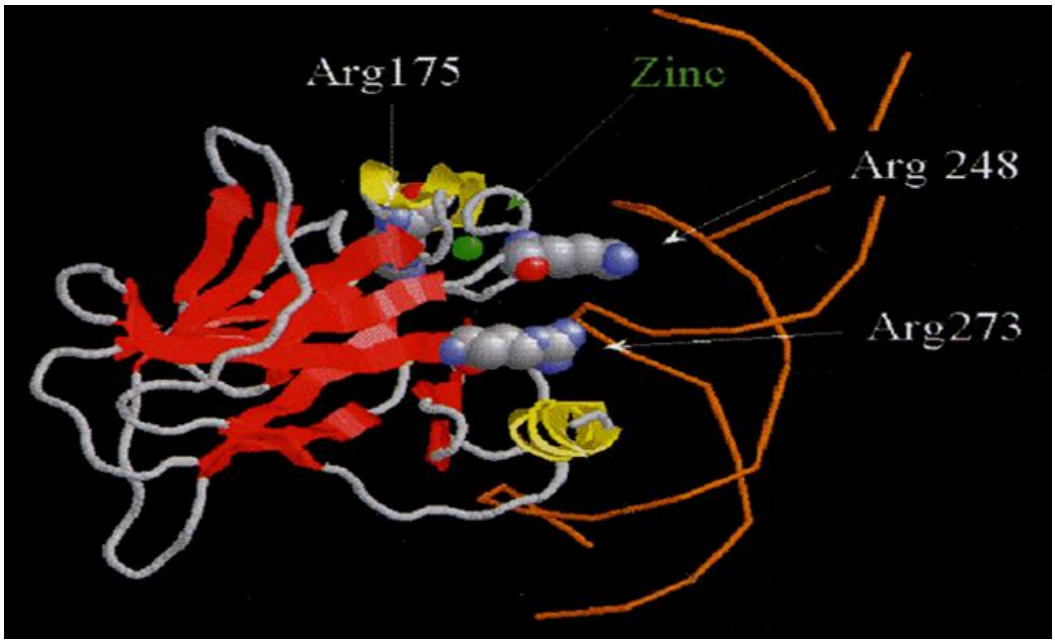
Normal conc. ATP in T Cells	Normal conc. ATP in B cells	Conc. ATP in T cells, malignant diseases	Conc. ATP in B cells, malignant disease	Conc. ATP in B cells, CLL	Conc. ATP in T cells, CLL
$\bar{x}$ = 1.39	$\bar{x}$ =0.35	$\bar{x}$ == 3.06	$\bar{x}$ = 0.17	$\bar{x}$ =4.33	$\bar{x}$ = 0.09
SD = 0.41	SD = 0.42	SD = 0.46	SD = 0.45	SD =1.5	SD =1.7

3. Discussions

Deletion in the short arm of chromosome 17 is associated with rapid disease progression, short remission periods, and decreased overall survival in chronic CLL. Specifically, deletions at 17p13 lead to loss of function of the tumor suppressor gene p53, while deletions at bands 11q22-q23 are linked to extensive lymph node involvement, aggressive disease, and shorter survival. These structural and biochemical changes in malignant cells influence bioenergetic processes, which in turn affect the expression of cellular oncogenes.

The p53 protein is an oligomeric transcription factor composed of 393 amino acids and is organized into five structural and functional regions [1].

Restoration of p53 function can induce apoptosis. However, in many cancers, p53 activity is compromised, either due to mutations in the p53 gene or alterations in its regulatory state. In hereditary cancers, five p53 gene mutations have been identified; for example, mutation screening detected a carrier of the Arginine mutation (Arg-248) [Scheme 1].



Scheme 1: Three-dimensional x-ray structures of the p53 protein in isomorphic form.

Theoretical studies suggest that elevated ATP concentrations in malignant B lymphocytes from CLL can impair p53-mediated apoptosis. Apoptosis is regulated by a cascade of caspases, initially produced in an inactive pro-form. Once cleaved, caspases activate pathways leading to programmed cell death. Pro-apoptotic proteins (Bax, Bad, Bak, and Bid) trigger apoptosis within the mitochondria. Under hypoxic conditions, resistance to apoptosis occurs at both the mitochondrial and cytosolic levels. For instance, accumulation of Bax in the mitochondria promotes the release of cytochrome c into the cytosol a process that is markedly reduced under hypoxia [3].

In addition, apoptosis via the extrinsic pathway is initiated by death ligands, such as Fas ligand or tumor necrosis factor (TNF), leading to activation of caspases 8 and 3 and the apoptotic protease activating factor (APAF). Hematopoietic cells, particularly lymphoid cells, express Bcl-2 (primarily in the nucleus and endoplasmic reticulum), an oncogene that may act as a general suppressor of genes directly regulating apoptosis [4].

Each cell requires energy to maintain its structure, synthesize reserve substances, and perform various cellular functions (e.g., plasma movement, nuclear division, and cell synthesis). Energy-generating substances in normal cells are primarily carbohydrates, followed by fats and proteins. This phenomenon is particularly prevalent in aggressive malignancies, many of which are hypoxic. Hypoxia induces an imbalance between reduced mitochondrial species and available oxygen, resulting in increased reactive oxygen species (ROS). The toxicity of ROS can lead to apoptotic cell death. Schematic representation of hypoxia-induced bioenergetic alterations and ROS-mediated [5].

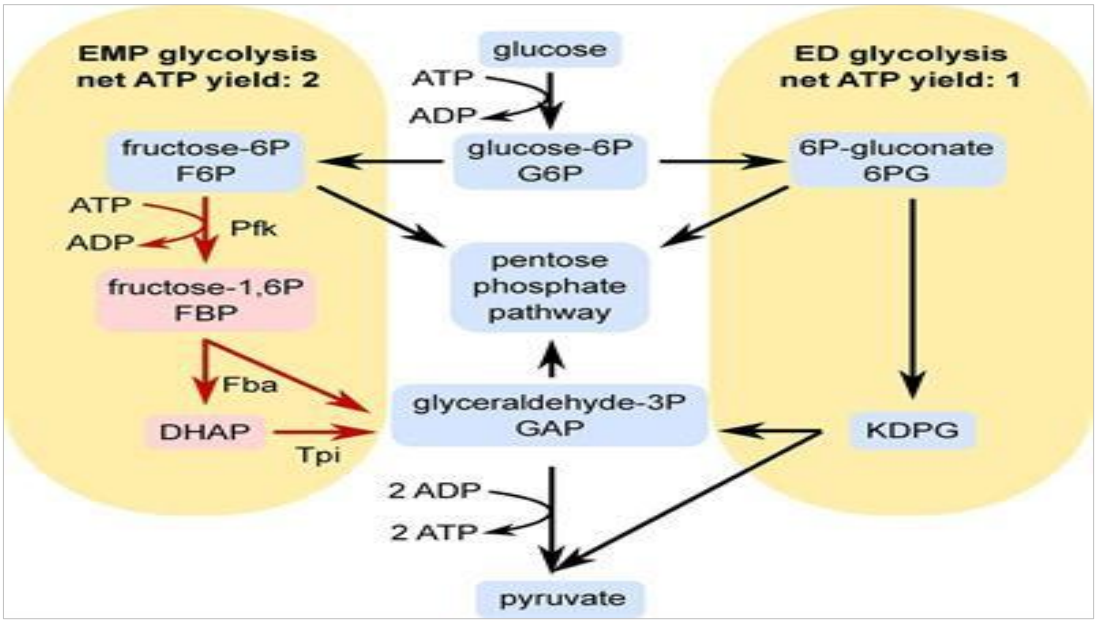
Alterations in the physiological processes of oxidative phosphorylation, a decrease in respiratory levels, and an increase in anaerobic glycolysis during malignancy are accompanied by an increase in the intensity of cellular bioluminescence. Thus, bioluminescence appears to be an intrinsic component of metabolism rather than the product of specialized photogenic organs.

All living cells contain the macro-energetic molecule adenosine triphosphate (ATP), which is maintained at a fairly constant level for each cell type and is rapidly lost upon cell death. It has been observed that the electrical charge of the cell membrane remains relatively constant at approximately 0.85 eV under resting conditions. If the energy charge decreases below 0.85 eV, corresponding to ATP levels below 1.75 µM per cell [6].

ATP serves as the energy source for enzymes such as DNA polymerase and DNA ligase, which are critical for repairing damaged DNA nucleotides caused by intracellular free radicals or extracellular physical, chemical, and biological toxic factors. In addition, the p53 protein the product of the p53 gene has additional roles that may overlap with its tumor suppressive capacity. These roles include mediating the response to DNA damage, regulating aging metabolism, and influencing stem cell differentiation [7].

Based on this information, it can be inferred that certain malignant diseases arise due to the dysregulation of gene expression over time and under specific lifestyle conditions. Structural and biochemical changes in malignant cells influence the development of characteristic bioenergetic processes, which in turn modulate the expression of cellular oncogenes at the expense of anti-oncogenes that are normally expressed under aerobic conditions [8].

Ongoing research has highlighted that the direction of the synthesis reaction (ADP + Pi → ATP + H_2O) can be reproducibly controlled in vitro, depending on the concentration of system factors, pH, and ionic strength. This finding suggests that functional energy control may exist by forming weak bonds between the chemical complexes of the newly formed biologically active substances (see Scheme 5). Otto Warburg observed that many cancers lose their capacity for mitochondrial respiration, limiting ATP production to anaerobic glycolytic pathways [9], [Scheme 2].



Scheme 2: Pathways of ATP Production in Anaerobic Glycolytic Pathways

Hypoxia reduces a cell's ability to maintain its energy levels because ATP production from glycolysis is less efficient than that from oxidative phosphorylation. In response, cells adapt by activating the expression of glycolytic genes, sometimes harboring mutations. Severe hypoxia induces a high mutation rate, resulting in point mutations that may be due to reduced DNA mismatch repair activity [10].

The mechanism involves inhibition of glycolytic ATP production via a Randle-like cycle, while increased mitochondrial uncoupling renders cancer cells unable to produce compensatory ATP via oxidative phosphorylation, even when the tricarboxylic acid (TCA) cycle remains intact. One mitochondrial adaptation to elevated reactive oxygen species (ROS) is the overexpression of uncoupling protein 2 (UCP2), as reported in multiple human cancer

cell lines Increased UCP2 expression is also associated with reduced ATP production in malignant oxyphilic mouse leukemia and human lymphoma cell lines [11].

The most direct induction of apoptosis by hypoxia occurs via inhibition of the electron transport chain in the inner mitochondrial membrane. Lack of oxygen impairs proton transport, leading to a decrease in the mitochondrial membrane potential. Under conditions of mild hypoxia, cell survival is mediated by phosphoinositide-3 kinase (PI3K); however, in severe hypoxia or anoxia, cells initiate cascades leading to apoptosis [12].

Following DNA damage, the p53 protein a tumor suppressor mutated in over 60% of human tumors that normally suppresses cell division plays a critical role in regulating apoptosis. Genetic alterations can be identified using fluorescent probes via fluorescent in situ hybridization (FISH) [13]. Chromosomal evaluation by FISH can detect prognostically significant abnormalities in CLL, such as deletion of part of the short arm of chromosome 17 (del 17q), which targets the cell cycle regulator p53 and is particularly deleterious [14].

This abnormality is found in 10% of patients with CLL and is associated with a poor prognosis. Deletion of the long arm of chromosome 13 (del 13q) is the most common genetic abnormality in CLL, with approximately 50% of patients exhibiting this effect. These patients tend to have the best prognosis, with many living for many years without the need for therapy. DNA-damaging agents may increase the expression of p53 and its transactivation activity, suggesting that p53 plays a protective role against the accumulation of mutations and the subsequent progression to malignancy [15].

Furthermore, hypoxia can prevent apoptosis by inducing the expression of the anti-apoptotic protein IAP-2. A typical cellular response to hypoxia mediated by hypoxia-inducible factor 1α (HIF-1α) is the expression of insulin-independent GLUT transporters, ensuring maximum glucose uptake for glycolytic ATP generation [16, 17].

In its normal form, the p53 protein halts cell division when DNA damage is detected, thereby allowing time for DNA repair before errors are duplicated and passed on to daughter cells. Overexpression of normal p53 protein can result in either G1 arrest, mediated by the p21 protein, or in the induction of apoptosis [18].

The p53 protein has become an attractive therapeutic target for anticancer drug discovery. Three classes of p53-targeting compounds have been identified and characterized. The first class consists of compounds that activate or restore wild-type p53 function; for example, Nutlin-1-a cis-imidazole analog has been shown to be effective in human cancers. The second class includes agents such as PKI-587, a dual competitive ATP inhibitor that targets PI3K (specifically PI3K-α and PI3K-γ) and mTOR. This orally bioavailable inhibitor rescues the p53 protein isoform that carries a mutation in the p53 gene. The third class comprises heterocyclic compounds such as WYE-354, a bi-ATP-competitive kinase inhibitor that selectively blocks mTORC1/2 activity [19].

Antibodies to human p53 have been detected in patients with cancer. These antibodies are highly specific for malignant diseases and are rarely found in healthy donors or in patients with benign conditions. This immune response is correlated with the presence of a p53 gene mutation, which leads to the accumulation of a dysfunctional p53 protein in tumor cells [20,21].

4. Conclusions

Gene mutations, deletions, and translocations serve as biomarkers that reflect the individual proteomic and genomic profiles of various cancers. The structural and biochemical alterations in malignant cells influence the development of characteristic bioenergetic processes, which in turn condition the expression of cellular oncogenes at the expense of anti-oncogenes normally expressed under aerobic metabolism.

In malignant diseases, like in CLL-B, blocked apoptosis may be attributable to high ATP concentrations produced via anaerobic metabolism, which can suppress the expression of anti-oncogene proteins, particularly the isoform p53 protein. Further studies are necessary to identify patients with elevated ATP levels who harbor mutations, translocations, or deletions in the p53 gene, located on chromosome 17p13, Next Generation Sequencing, (NSG) and CRISPR technology.

Declarations

Acknowledgements

Dr. Cristiana Tanase, Titu Maiorescu University, Faculty of Medicine, Professor Molecular Biology, Clinical Biochemistry and Research Management, PhD theses coordinator, Academic titles MD, PhD, Dr. Habil., Professor, Senior Researcher I.

Conflict of Interest Statement

The author declares no conflict of interest. The author declares that have no known competing financial interests or personal relationships that could have influenced the work reported in this study.

Ethics approval and consent to participate approved

Aurelian Udristioiu, as prim author of this article, “Measurements of p53 Isoform Protein Concentration and Energetic Levels in Cells of CLL Using Enzymatic Methods”, I confirm that I have had personal full access to all aspects of the research and writing process, and took final responsibility for this paper and I made revision of article for intellectual content.

All authors contributed to the intellectual content of this paper and in this work of research have accomplished the following the follow requirements: [1]AU, Conceptualization, [1]AG, Formal analysis, [1]AV, Funding acquisition, [1]LV, Investigation, Visualization, [2]MC, Resources, Writing original draft, [3]IO-G, Writing - review & editing, Methodology, Software. Finaly, Aurelian Udristioiu[1]*, agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

All experiments were performed following relevant guidelines and regulations with the Declarations section: ANMCS: Accreditation. Also, all methods were carried out by relevant guidelines and regulations of the Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects.

Patient consent statement

Informed consent was obtained from subject of this study when he was admitted to hospital departments, in him declaration of acceptance for the necessary paraclinical investigations for diagnosis and treatment. The patient signed the individual accord to be supplementary investigated the blood samples.

Funding Statement

Not applicable

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