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Surface Evaluating *Galectin-9, CTLA-4,* and *ABCB1* Gene Expression as Diagnostic and Prognostic Markers in Acute Lymphoblastic Leukemia

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ABSTRACT

Acute Lymphoblastic Leukemia (ALL) remains a significant challenge in clinical hematology due to its complex mechanisms of immune evasion and resistance to treatment. The identification of reliable biomarkers is critical for improving diagnosis, prognosis, and therapeutic strategies. This study investigates the diagnostic and prognostic value of three biomarkers—*Galectin-9* (*Gal-9*), Cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*), and ATP-binding cassette subfamily B member 1 (*ABCB1*) gene expression—in ALL patients. A total of 40 ALL patients (25 treated and 15 in the first diagnosis group) and 25 healthy controls were enrolled. Total RNA extracted from peripheral blood. Complementarydeoxyribonucleic acid (cDNA) synthesized with reverse transcriptase. The synthesized cDNA is amplified by using quantitative real-time polymerase chain reaction (RT-qPCR).The findings were normalized to the Beta-2-Microglobulin (*β2M*) reference gene. One-way ANOVA was performed to analyze gene expression between groups, while correlation and receiver operating characteristic (ROC) curve analysis assessed clinical parameters and diagnostic potential. *Gal-9* levels were elevated in untreated patients ($p < 0.05$), whereas *CTLA-4* expression was reduced in untreated patients ($p < 0.01$). *ABCB1* levels were high in both groups ($p < 0.05$). Significant correlations were observed between *CTLA-4* and *ABCB1* ($r = 0.81$, $p < 0.0001$), and moderate correlations were found with *Gal-9* ($r = 0.35$, $p = 0.029$) and *ABCB1* ($r = 0.38$, $p = 0.016$). These findings underscore the potential of *Gal-9*, *CTLA-4*, and *ABCB1* as valuable biomarkers in ALL, with *CTLA-4* showing the highest diagnostic potential and *ABCB1*confirms its role in chemoresistance.

1.Introduction

Acute Lymphoblastic Leukemia (ALL) clones are a kind of hematologic malignancy that develops from lymphoid progenitors. Although this leukemia does occur in adults, it'sless frequent than in children (Malouf and Ottersbach, 2018, Pagliaro et al., 2024). Treatment options for ALL, including immunotherapy and chemotherapy, have improved, but patients still face a high chance of recurrence and a dismal prognosis. This is especially true for populations at high risk(Craddock et al., 2019). Early diagnosis, relapse prediction, and overall prognosis evaluation using accurate biomarkers are critical for improving clinical outcomes and tailoring therapies. An emerging biomarker in several malignancies, including ALL, is *Galectin-9* (*Gal-9*)(Noh et al., 2020, Yıldırım, 2024). Immunological modulation, immune response control, cell migration, and cell death are all impacted by *Gal-9*(Hsu et al., 2015). Overexpression of *Gal-9* is one mechanism by which ALL evades the immune system; this may reduce anti-tumor immunity and add time to the survival of leukemic cells(Zhang et al., 2024). According to Yildirimresearch, increased *Gal-9* expression is associated with a bad prognosis in ALL, which makes it a valuable biomarker for tracking the illness and predicting its destiny(Yıldırım, 2024).The homodimeric glycoprotein known as cytotoxic T-lymphocyte-associated antigen-4 (*CTLA-4*) was first detected on the surfaces of activated T cells in both humans and mice. It is a member of the superfamily of immunoglobulin (Ig) genes found in humans(Van Coillie et al., 2020). A number of malignancies, including ALL, have been associated to *CTLA-4* overexpression in recent investigations(Mansour et al., 2014, Pastorczak et al., 2021). An appealing immunotherapy target is *CTLA-4*, a receptor on the immune system that regulates T cell activation; its overexpression in ALL contributes to this appeal(Mu et al., 2023).As *CTLA-4* binds to its ligands, CD80 (B7.1) and CD86 (B7.2), present on antigen-presenting cells (APCs), it inhibits the production of Interleukin-2 (IL-2), Interferon-gamma (IFN-γ), Interleukin-4 (IL-4) cytokines, IL-2 receptor expression, and cell cycle progression(Simone et al., 2012). Some of the proposed mechanisms by which *CTLA-4* acts include blocking the production of cyclin-dependent kinases (CDK4/CDK6), competing with the positive T-cell costimulatory CD28 protein for ligands, and interfering with TCR signaling(Deng et al., 2018). Another mechanism by which *CTLA-4* may improve peripheral tolerance and downregulate immunological responses is its possible participation in suppressor CD4*CD25* T cell regulation, which has garnered considerable interest(Hossen et al., 2023).

TheATP-binding cassette subfamily B member 1 (*ABCB1)* transmembrane protein, sometimes called P-glycoprotein, is involved in the progression of multidrug resistance (MDR). In malignant cells, it actively facilitates the efflux of chemotherapy medications(Katayama et al., 2014, Engle and Kumar, 2022). Overexpression of *ABCB1*is a significant component of treatment resistance in ALL, particularly with regard to vincristine and daunorubicin(Aberuyi and Rahgozar, 2022, Álvarez-Carrasco et al., 2025). In individuals with ALL, this resistance is a common cause of treatment failure and recurrence(Buchmann et al., 2022). Consequently, knowing the relationship between *ABCB1* expression and treatment management and prognosis is crucial. Analyzing the complex molecular mechanisms of ALL might be achieved by integrating the *Gal-9*,*CTLA-4*, and *ABCB1* biomarkers. Most occurrences of chemotherapeutic resistance are mediated by *ABCB1*, *CTLA-4* may indicate immunological checkpoint resistance, and *Gal-9* can be utilized to evaluate immune evasion(Khan and Malik, 2024). Although there is growing evidence suggesting the activities of these indicators, their clinical significance in ALL has not been well studied. This project aims to find out how *Gal-9*, *CTLA-4*, and *ABCB1* contribute to ALL diagnoses, outcomes, and resistance to treatment. The possible diagnostic and prognostic utility of these biomarkers is the focus of this study. More tailored and efficient treatment plans for patients with ALL can be created by studying the association between expression levels and clinical outcomes, such as

disease progression, relapse, and patient survival.

2.Materials and Methods

2.1Patient and Control Samples

A total of 40 patients with ALL, including 25 (age 13-62 years) treated and 15 (age 9-43 years) newly diagnosed, were recruited into this case-control and cross-sectional study. Flow cytometry, blood smear examination, and bone marrow aspiration were performed to confirm the diagnosis of ALL, as shown in Table 1. A total of 25 healthypersons (age 18-32), who constituted the control group, were also included. The data and samples for this study were obtained from Nanakali Hospital, Erbil Governorate, Kurdistan, during the period of 15 September 2024 to 4 March 2025. Written informed consent was obtained from all subjects in accordance with institutional ethics. The study proposal was approved by an ethics review boardof Erbil Polytechnic University (Approval No. 25/0072 HRE).

Table 1: Baseline characteristics of ALL patients

characteristics	Value (n)
Total ALL patients	40
Treated	25 (62.5%)
Un-treated	15 (37.5%)
Male	28 (70%)
Female	12 (30%)
Flow cytometry confirmation	40
Bone marrow aspiration test	15 (newlydiagnosed patients)

2.2Sample Collection,Inclusion,and Exclusion Criteria

Peripheral blood samples (approximately 3 mL) were collected from both ALL patients and healthy controls using EDTA-treated tubes and bone marrow aspirate from new (untreated) cases. Both samples were immediately transported to the laboratory for processing. Inclusion criteria include the confirmed diagnosis of ALL by cytomorphology and immunophenotyping. Pre-treatment and on-treatment samples were available. Additionally, both the patient and their legal guardian are required to sign an informed consent document. Other hematologic malignancies or secondary tumors are considered exclusion criteria.Patients who have not yet started treatment or are on investigational therapies. Incomplete medical records or consent are also excluded.

2.3Flow Cytometry (Immunophenotyping)

Flow cytometry was employed to confirm the diagnosis of Acute Lymphoblastic Leukemia (ALL) in all patients using a standard panel of monoclonal antibodies for immunophenotyping. The method allows for the rapid analysis of cell surface and intracellular markers to identify leukemic cell populations. In this study, peripheral blood and bone marrow aspirates were processed and stained with a combination of antibodies targeting markers such as CD34, CD19, CD10, CD20, and other lineage-specific markers. The samples were then analyzed on a (BD FACSCanto™II) flow cytometer (BD Biosciences of Becton Dickinson Company), with results analyzed using FlowJo software. For the treated group, flow cytometry data were retrospectively collected from hospital records, while for the newly diagnosed (untreated) group, the flow cytometry analysis was performed prospectively in the lab during the diagnosis of new cases. Positive expression of specific markers consistent with the immunophenotypic profile of ALL was used for confirmation of diagnosis and classification of the leukemia subtype.

2.4Hematologic and Biochemical Tests

To back up the clinical diagnosis and evaluate the patient's general condition, hematologic and biochemical tests were performed. To investigate the morphology of the blood cells, a blood film was made using the peripheral blood sample and stained with prepared Leishman stain. Lymphoblasts, aberrant cell shapes, and other hematologic abnormalities indicative of ALL were examined on the blood film by using a microscope (Olympus of Japanese Multinational

Company) at 100-X with oil immersion. A Complete Blood Count (CBC) by hematologic analyzer (Medonic Coulter, an American product) was also done to evaluate the levels of platelets, hemoglobin (Hb), white blood cells (WBC), and red blood cells (RBC). Glucose levels, C-reactive protein (CRP) titers, lactate dehydrogenase (LDH), aspartate aminotransferase (AST), and creatinine were among the biochemical tests that were conducted using a biochemical analyzer (Biolis 50i, developed by Roche Diagnostics). Important information about the disease's course and treatment response was connected with the results of these tests, which measured kidney function, liver enzymes, inflammatory condition, and metabolic health generally.

2.5Bone Marrow Aspiration

The infiltration of leukemic cells was evaluated, and the diagnosis of ALL was confirmed by bone marrow aspiration. The posterior iliac crest of the hip bone was used to collect a bone marrow sample in accordance with established aseptic protocols. For microscopic analysis, the marrow aspirate was spread onto slides and then stained with Wright-Giemsastain or Leishman stain. The bone marrow aspirate was also subjected to molecular and cytogenetic testing.The percentage of blast cells in the marrow was quantified, and abnormal morphology indicative of leukemic infiltration was recorded. For the duration of the trial, bone marrow aspiration was crucial for determining the level of disease involvement and tracking therapy response.

2.6RNA Extraction

Total RNA was extracted from peripheral whole blood samples using the GeneAll Hybrid-R Blood RNA Kit (a product developed by a Korean biotechnology company), following the manufacturer's instructions. In short, the spin-column approach was used to extract total RNA from the blood samples after processing them to isolate the peripheral blood mononuclear cells (PBMCs). Gel electrophoresis was used to evaluate the integrity of the isolated RNA, and a NanoDrop spectrophotometer was used for quantification and qualification.

2.7Gel Electrophoresis of RNA

To assess the integrity of the RNA, an aliquot of the sample was subjected to 1% agarose gel electrophoresis. RNA was visualized under UV light after staining with ethidium bromide. The presence of distinct ribosomal RNA bands (28S and 18S rRNA) indicated high-quality RNA, while the absence of degradation was confirmed by the absence of smearing.

2.8cDNA Synthesis

The template for cDNA synthesis in reverse transcription was 1 µg of total RNA from every sample. The manufacturer's procedure was followed to carry out the reverse transcription using the (AddScript RT Master First Strand cDNA Synthesis Kit, a product of Bioneer Corporation, a South Korean biotechnology company).The reaction mixture was incubated at 50°C for 60 minutes and contained randomhexamer primers, deoxyribonucleotide triphosphates, and reverse transcriptase. The cDNA was kept at -20°C until it could be analyzed later.

2.9Real-Time qPCR Amplification

The cDNA samples were used as templates for quantitative real-time PCR (qPCR) analysis. Gene expression levels of *Gal-9*, *CTLA-4*, and *ABCB1* were measured using specific primers designed for each target gene listed in Table 2. The BIO-RAD CFX96 Real-Time System was employed to carry out the qPCR reactions. Each reaction was conducted in a final volume of 20 µL, which included 10 µLof SYBR Green PCR Master Mix, 1 µL of cDNA template, and 0.5 µM of forward and reverse primers for each gene of interest, and the full volume is then filled with nuclease-free distilled water. The amplification circumstances were as follows: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of 95°C for 30 seconds, 60°C for 30 seconds annealing, and 72°C for 30 seconds extension. Melting curve analysis was performed at the end of each run to confirm the specificity of the amplification.

Table 2: Primer sequences

Genes	Primer Sequence (Forward)	Primer Sequence (Reverse)	References
Gal-9	5'-GGACGGACTTCAGATCACTGT-3'	5'-CCATCTTCAAACCGAGGGTTG-3'	(Wang et al., 2021)
CTLA-4	5'-ACGGGACTCTACATCTGCAAGG-3'	5'-CCCCGAACTAACTGCTGCAA-3'	(Al-Harbi et al., 2023)
ABCB1	5'-GTCTACAGTTCGTAATGCTGACGT-3'	5'-TGTGATCCACGGACACTCCTAC-3'	(Pravdic et al., 2023)
β2m (reference gene)	5'-GTGGAGCATTCAGACTTGTCTTT-3'	5'-TGCTTACATGTCTCGATCCCAC-3'	(Ahmadi et al., 2018)

2.10Normalization and Data Analysis

The β2m housekeeping gene was used to normalize the expression levels of target genes *Gal-9*, *CTLA-4*, and *ABCB1*. This gene was selected for its steady expression across all samples. The $2^{-\Delta\Delta Ct}$approach was used to determine relative gene expression levels. Here, ΔCt represents the distance between the target gene's Ct values and the reference gene, and ΔΔCt signifies the difference between the experimental and control groups.

2.11Statistical Analysis

Statistical analysis was performed using (GraphPad Prism 9.5.1). The expression data of *Gal-9*, *CTLA-4*, and *ABCB1*in treated and untreated B-ALL patients were compared to the control group using ANOVA (one-way) accompanied with post-hoc analysis (Tukey test).The correlation matrix was used to correlate between biomarkers. The correlation between gene expression and clinical parameters such as treatment response, disease progression, and relapse was evaluated. A p-value < 0.05 was considered statistically significant.

3.Results

This study comprised 40 patients diagnosed with acute lymphoblastic leukemia (ALL), including 25 treated patients aged 13 to 62 years and 15 newly diagnosed, untreated patients aged 9 to 43 years. Among the 40 cases, 28 were male and 12 were female. The diagnosis of acute lymphoblastic leukemia (ALL) was validated with flow cytometry, blood film examination, and bone marrow aspiration. Moreover, 25 healthy controls were incorporated as a comparative group, with ages spanning from 18 to 32 years.

3.1Immunophenotyping

The expression of CD markers in ALL patients revealed varied distribution patterns. For Human Leukocyte Antigen-DR (HLA-DR), 77.5% were negative, 17.5% moderate, and 5% dim. WhileCD10 was expressed as 22.5% negative, 20% dim, and 57.5% moderate. CD19 showed 15% negative, 4% dim, and 77.5% moderate, while CD20 was mostly negative (87.5%) and moderately expressed in 2.5%. CD34 expression was 20% negative, 22.5% dim, and 57.5% moderate. CD45 had 27.5% negative, 65% moderate, and 7.5% dim expression. For CD79a, 19.5% were negative, 15.5% dim, and 65% moderate. CD22 showed 82.5% negative, 15% dim, and 2.5% moderate expression. CD99 was 67.5% negative, 10.5% dim, and 22% moderate, and TdT showed 36.5% negative, 45% dim, and 18.5% moderate expression, as shown in Table 3. These variations reflect the immunophenotypic characteristics of ALL across the patient cohort.

Table 3: CD markers expression (Immunophenotyping) (n=40)

Marker	Negative (%)	Dim (%)	Dim to Moderate (%)	Moderate (%)	Bright (%)	Heterogeneous (%)
HLADR	77.5	-	5	17.5	-	-
CD 10	22.5	20	-	57.5	-	-
CD 19	15	4	-	77.5	-	3.5
CD 20	87.5	2.5	-	2.5	-	7.5
CD 34	20	22.5	-	57.5	-	-
CD 45	27.5	65	-	7.5	-	-
CD 79a	19.5	15.5	-	65	-	-
CD 22	82.5	15	-	2.5	-	-
CD 99	67.5	10.5	-	22	-	-
TdT	36.5	45	-	18.5	-	-

3.2Gene Expression Alterations in ALL: Response to Treatment and Implications for Drug Resistance and Immune Modulation

In the untreated group, *GAL-9* and *ABCB1*have comparable expression levels, with fold increases slightly above 1.3; however, *CTLA-4* expression is significantly reduced, with a fold change below 0.3. *GAL-9* expression exhibits a slightly decrease post-treatment, whereas *CTLA-4* expression experiences a modest rise; however, it remains the lowest of the three genes. In the treated group, *ABCB1*expression is the greatest, with a small rise relative to the untreated group, approaching 1.4, as shown Figure 1.The data indicate that therapy preserves elevated *ABCB1*expression and modestly increases *CTLA-4* expression, while slightly reducing *GAL-9* expression, reflecting gene-specific responses to treatment.

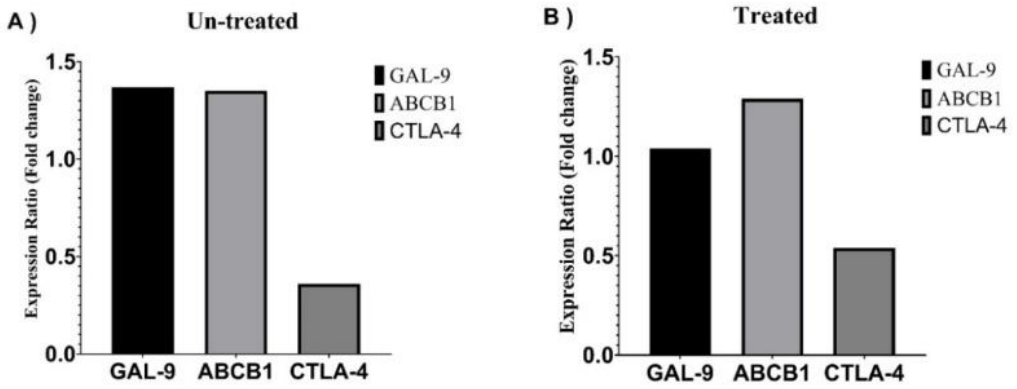

Figure 1: Relative expression changes of three genes—*GAL-9*, *CTLA-4*, and *ABCB1*—in both the untreated (Panel A) and treated (Panel B) of ALL patients.

3.3mRNA expression ofGal-9,CTLA-4, and ABCB1 in ALL: Statistical Analysis and Comparisons

One-way ANOVA with subsequent post hoc comparisons indicated substantial disparities in expression patterns. The untreated group (Mean; 4.59, 95% CI: 3.65-5.54) exhibited a substantially elevated expression of *GAL-9*in comparison to both the treated (Mean; 5.73, 95% CI; 5.26- 6.20) and control groups (Mean; 5.66, 95% CI; 5.31-6.00) ($p < 0.05$); however, no significant difference was noted between the treated and control groups.*CTLA-4* expression wasobviously reduced in the untreated group (Mean; 8.24, 95% CI; 7.13-9.35) compared to the control group

(Mean; 6.42, 95% CI; 6.07-6.76)($p < 0.01$) but did not vary substantially from the treated group.Compared to the control group, the untreated group had significantly higher *ABCB1* expression (Mean; 2.78, 95% CI; 1.22-4.34) ($p<0.05$). However, there were no significant differences observed between the treated group and the other groups. These data demonstrate that therapy differentially influences gene expression across several genes, with *GAL-9* demonstrating the highest reaction,as shown in Figure **2:** .

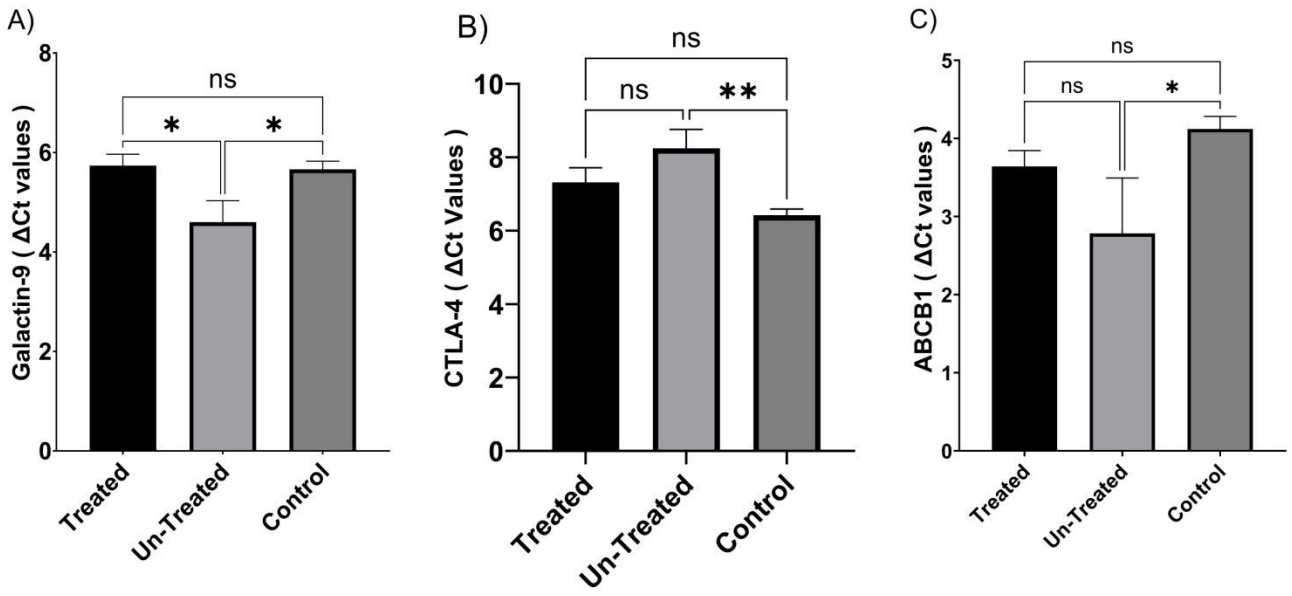

Figure 2: Gene expression levels of *Galectin-9*, *CTLA-4*, and *ABCB1* across treated, untreated, and control groups. Panel (A) shows *Galectin-9* expression (ΔCt values) in the treated, untreated, and control groups, with significant differences observed between untreated group with both groups (*$p < 0.05$).Panel (B) illustrates *CTLA-4* expression, with a significant reduction in the untreated group compared to the control group (**$p < 0.01$) and non-significant change with treated group.Panel (C) displays *ABCB1* expression, where the untreated group shows significantly higher expression compared to the control group (*$p < 0.05$) and no significant difference with treated group. Note that the ΔCt values are inversely proportional with gene expression levels.

3.4Correlations between Gal-9, CTLA-4, and ABCB1 Expression: Insights into Immune Modulation and Drug Resistance

Correlation studies revealed substantial positive associations among the expression levels of *Gal-9*, *CTLA-4*, and *ABCB1*. Both scatter plots and the correlation matrix indicate that *Gal-9* expression has a moderate connection with *CTLA-4* (r = 0.35) and *ABCB1*(r = 0.38), accompanied by statistically significant p-values (p = 0.029 and p = 0.016, respectively). According to the results, *Gal-9* could be involved in controlling the expression of drug transporters and immunological checkpoints. A significant positive correlation was identified between *CTLA-4* and *ABCB1*(r = 0.81, p < 0.0001), demonstrating a strong link between immune modulation and multidrug resistance, as shown inFigure 3: .

The hematologic, cellular, and biochemical characteristics of untreated (n=15) and treated (n=25) ALL patients were evaluated and are listed in Table 4. In the untreated group, 93% had high bone marrow blasts and 67% had high whole blood blasts, while treatment led to improvements in these parameters, with 53% of

treated patients showing normal WBC counts and 60% having normal lymphocytes. Red blood cell counts and hemoglobin were lower in untreated patients, but treatment helped normalize these levels. Platelet counts were low in 67% of untreated patients but improved in 48% of treated patients. Biochemically, treated patients showed improvements in kidney and liver function, with 80% having normal urea and 84% with normal AST. CRP, a marker of inflammation, was elevated in untreated patients but decreased in treated patients. Elevated LDH and glucose levels were more common in untreated patients, with treatment reducing these markers. Overall, treatment led to improvements in most parameters, though some abnormalities persisted.

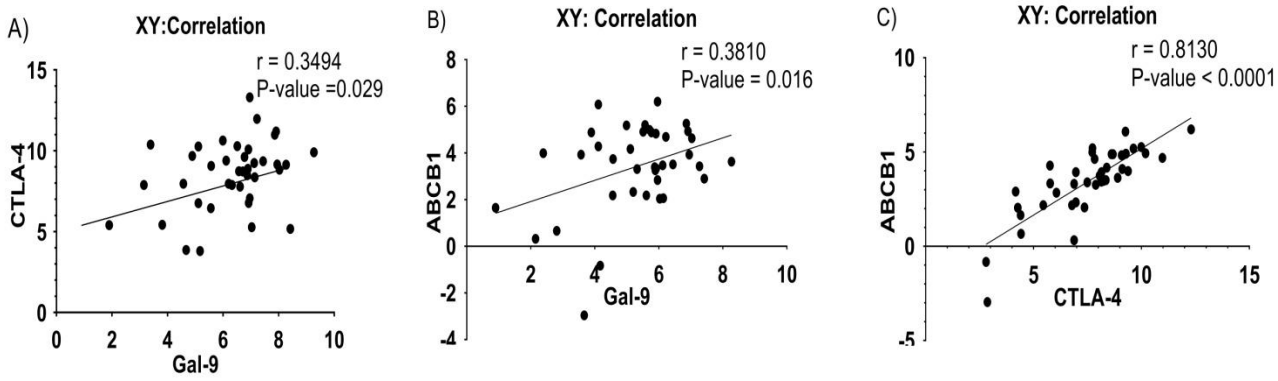

Figure 3: Correlation analysis between gene expressions. Panel (A) shows a moderate positive correlation between *CTLA-4* and *Galectin-9*. Panel (B) demonstrates a weak positive correlation between *ABCB1* and *Galectin-9*.Panel (C) reveals a strong positive correlation between *ABCB1* and *CTLA-4*.

Table 4: Hematologic, cellular and biochemical characteristics of ALL patients.

Parameter	Untreated (n=15)			Treated (n=25)		
	Low (%)	Normal (%)	High (%)	Low (%)	Normal (%)	High (%)
Bone marrow blasts	0	7	93	-	-	-
Whole blood blasts	0	33	67	-	-	-
WBC	14	33	53	44	48	8
Lymphocyte	0	40	60	8	88	4
RBC	53	47	0	48	52	0
Hb.	73	27	0	54	46	0
Platelet	67	20	13	36	48	16
Urea	13	74	13	16	80	4
Creatinine	33	60	7	20	80	0
GOT(AST)	0	73	27	0	84	16
GPT(ALT)	0	93	7	0	60	40
CRP	0	40	60	0	76	24
LDH	0	20	80	12	64	24
Glucose	7	73	20	4	44	52

3.5Implications for Hematologic and Metabolic Homeostasis: Correlations between Gene Expression and Clinical Parameters

Correlation heatmaps were created to investigate the correlations between gene expression and clinical parameters by comparing ΔCt values of *Gal-9*, *CTLA-4*, and *ABCB1* with other hematological and biochemical markers shown in Figure 4:.In Panel A, ΔCt*Gal-9,CTLA-4, and ABCB1*exhibited moderate to strong positive associations among themselves, aligning with prior findings (r = 0.43–0.89). Significantly,

ΔCt*CTLA-4* and ΔCt*ABCB1* exhibited a positive correlation with red blood cell (RBC) count, hemoglobin (Hb), and hematocrit (HCT) (r = 0.30–0.38), while demonstrating a negative correlation with red cell distribution width (RDW) (r = -0.32 to -0.41). This finding indicates a possible correlation between the expression of immune/drug resistance genes and the erythropoietic state. In Panel B, ΔCt*ABCB1*and *CTLA-4* had a robust positive connection (r = 0.89) and were inversely correlated with lymphocyte-to-monocyte (L:M) and neutrophil-to-lymphocyte (N:L) ratios (r = -0.55 to -0.87), signifying a negative association with inflammatory or immune response indicators. Panel C demonstrated negative correlations between ΔCt*Gal-9* and various metabolic markers, including LDH, uric acid, and total serum bilirubin (TSB) (r = -0.28 to -0.56). In contrast, ΔCt*ABCB1* and *CTLA-4* exhibited positive associations with TSB (r = 0.64 and 0.77, respectively), creatinine (r = 0.52–0.56), and GOT (r = 0.48–0.51). These data suggest a potential relationship between compromised renal and hepatic function and the expression of *CTLA-4* and *ABCB1*.

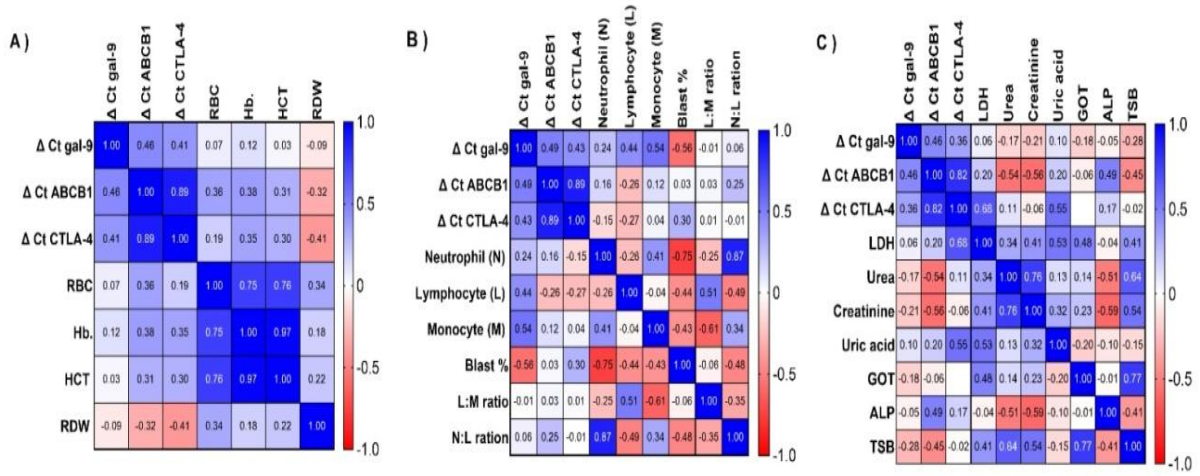

Figure 4:Network correlation matrix (Heatmaps) showing relationships between gene expression and various biological markers. Panel (A) displays correlations between gene expressions of *Galectin-9*, *CTLA-4*, *ABCB1*, and blood parameters, with moderate to strong correlations observed among certain genes. Panel (B) illustrates correlations between gene expressions and immune cell counts (neutrophils, lymphocytes, monocytes) as well as blast percentage and ratios, highlighting significant associations. Panel (C) shows correlations between gene expressions and biochemical parameters, including LDH, urea, creatinine, uric acid, GOT, ALP, and total serum bilirubin (TSB), revealing both positive and negative relationships. The color gradient represents the strength of the correlations, from blue (strong positive) to red (strong negative).

3.6Diagnostic Evaluation of Gal-9,CTLA-4, and ABCB1as Biomarkers in ALL: ROC Curve Analysis

The result of Figure 5: displays ROC curve studies for three biomarker genes, *GAL-9*, *CTLA-4*, and *ABCB1*, to evaluate their diagnostic efficacy. *GAL-9* has a low AUC of 0.55 and a non-significant P-value of 0.515, signifying inadequate and statistically irrelevant discriminating capacity.Among the three, *CTLA-4*'s AUC of 0.72 and highly significant P-value of 0.002 demonstrate its higher diagnostic performance, suggesting its acceptable and reliable potential as a diagnostic biomarker.The AUC for *ABCB1* is 0.66, and the P-value is 0.043, indicating that it has a limited diagnostic effectiveness.

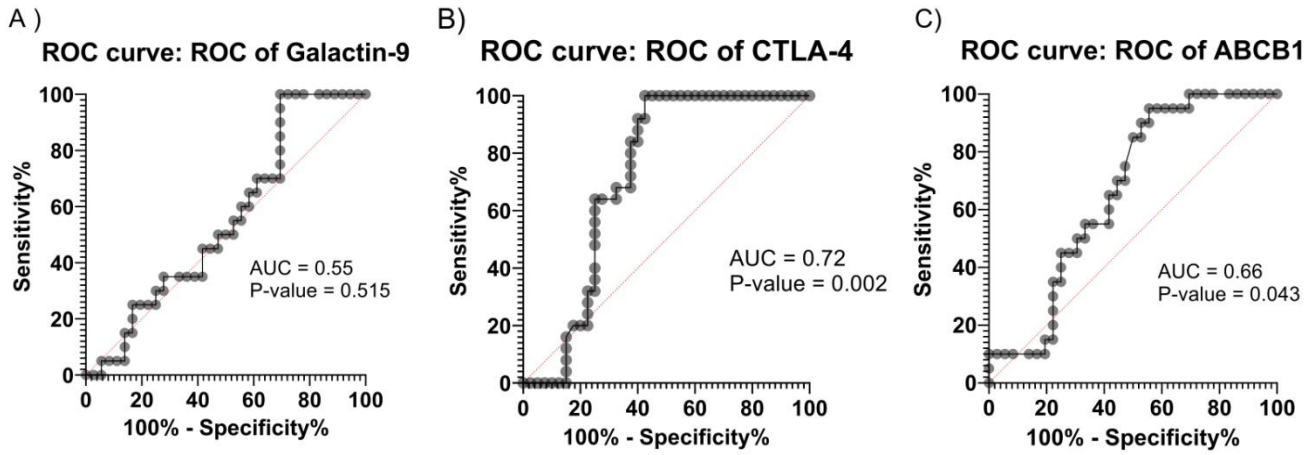

Figure 5: ROC curve analysis for the gene expressions of *Galectin-9*, *CTLA-4*, and *ABCB1*. Panel (A) shows the ROC curve for *Galectin-9*, with an AUC of 0.55 (p = 0.515).Panel (B) displays the ROC curve for *CTLA-4*, with an AUC of 0.72 (p = 0.002). Panel (C) presents the ROC curve for *ABCB1*, with an AUC of 0.66 (p = 0.043). *Galectin-9* indicating poor diagnostic ability, while others are significant.

4.Discussion

The main goal of this study was to identify the role of *Gal-9*,*CTLA-4*, and *ABCB1*in the development, progression, and resistance to therapy of acute lymphoblastic leukemia (ALL).It was shown by our research that distinct expression patterns of these biomarkers are present in both treated and untreated patients, suggesting that they might be useful diagnostic tools due to the strong associations found with clinical characteristics. It is suggested by these results that the regulation of these biomarkers is altered in response to treatment, which is consistent with a complicated interaction between immune modulation and drug resistance processes.

The TIM-3 ligand *Gal-9* is an important modulator of immunological suppression and T-cell exhaustion(Anderson, 2014). Expression of *Gal-9* was higher in the untreated group before treatment but dropped after therapy. Based on these findings, it appears that the leukemic condition has a greater impact on *Gal-9* expression than the treatment itself. Wiesma et al. (2019) reported that *Gal-9* is an immunomodulator that may do double duty, potentially inducing T-cell death while simultaneously enhancing regulatory T-cell activity. Elevated *Gal-9* expression in untreated ALL patients may impact tumor microenvironment immunosuppression, which in turn contributes to leukemia persistence (Wiersma et al., 2019). This lends credence to the idea that *Gal-9*, a key regulator of the immune response in leukemia, might be a therapeutic target.

Despite a significant decrease in the untreated group, *CTLA-4* expression did not seem to increase after treatment. An immunological rebound or compensatory reactivation of immune checkpoints following cytoreduction may explain why there is no rise, as opposed to prior studies on other blood cancers that have demonstrated an upregulation of *CTLA-4*(Salik et al., 2020). The immunosuppressive role of *CTLA-4* means that it can still be modulated to influence therapeutic responses and immune evasion.

Because ABCB1 is known to increase the efflux of chemotherapeutic medicines, it is confirmed that its action in multidrug resistance remains post-treatment.This persistence might be explained by the continuing expression of *ABCB1*, which is likely caused by cellular alterations induced by cytotoxic stress. Such persistent expression may contribute to the persistence of drug resistance after treatment.

Furthermore, our correlation analysis revealed that *Gal-9,CTLA-4*, and *ABCB1* are highly correlated.The strong positive connection between *CTLA-4* and *ABCB1* ($r = 0.81$, $p < 0.0001$) suggested that there could be a regulatory axis linking drug resistance pathways with immune checkpoint signaling much more precisely. It is still not known how exactly therapeutic pressure in cancer cells might co-select for immune evasion and drug resistance mechanisms, but previous research suggested that these two processes are related(Sharma et al., 2017). The modest correlations between *Gal-9* and both *ABCB1* and *CTLA-4*(Anderson, 2014, Tran, 2021) provide additional evidence.

Among the biomarkers studied, *CTLA-4* demonstrated the highest area under the curve (AUC) in our receiver operating characteristic (ROC) analysis (0.72, $p = 0.002$), underscoring its diagnostic value. Although this AUC falls within the range of modest diagnostic accuracy(Zhu and Wu, 2023, Sobhani et al., 2023), it indicates that *CTLA-4* could serve as a useful non-invasive biomarker for assessing ALL status. Similarly, while *ABCB1*also showed some discriminatory power, *Gal-9* did not reach statistical significance.

Further evidence supporting the relevance of these genes in disease pathogenesis comes from the correlation of their expression with various clinical and hematologic markers. A potential connection between immune checkpoint activation, medication resistance, and hematologic homeostasis was observed, as indicated by positive correlations with erythropoietic indices (RBC, Hb, HCT) and inverse correlations with inflammatory ratios (L\:M, N\:L).While a causative relationship between the overexpression of *Gal-9* and *ABCB1* and systemic organ stress or treatment-related toxicity cannot be definitively proven, positive correlations with renal and hepatic function markers (such as creatinine, GOT, and TSB) suggest a possible association.

A growing body of evidence indicates that *Gal-9* and *CTLA-4* have prognostic importance in leukemia. Because of its function in immune evasion, *Gal-9* suppresses anti-tumor immunity and is associated with T-cell exhaustion, both of which may add to the leukemic clone's survival(Yang et al., 2021). *CTLA-4* levels have been linked to a poorer prognosis in certain cancers, including acute lymphoblastic leukemia (ALL)(Sadeghi et al., 2022, Onishi et al., 2022). Therefore, these biomarkers not only depict the immunological terrain of ALL, but they also provide helpful predictive information about the course of the illness and its response to treatment.

Collectively, our findings demonstrated that *GAL-9*, *CTLA-4*, and *ABCB1* do not function independently but rather as components of a well-coordinated network that controls the immune system's interactions with malignancies and responses to therapy. Our ability to combat immune evasion and medication resistance could benefit from a better understanding of this connection. In order to understand if these markers' changes affect ALL prognoses or outcomes of therapy, future research should use functional investigations and longitudinal analysis to examine the molecular basis ofthis relationship.

One major limitation was the difficulty in obtaining samples from patients before the initiation of chemotherapy, which could have provided more accurate baseline data. Additionally, the small sample size of 40 ALL patients may also limit the generalizability of the findings, and larger studies are needed for validation.

5.Conclusion

The results of this study show that treatment interventions affect the variation in *Gal-9*, *CTLA-4*, and *ABCB1* expression in ALL. The most promising diagnostic tool was *CTLA-4*, but the fact that *ABCB1* levels were high even after therapy confirmed its role in drug resistance. Newly diagnosed (untreated) patients had a significantly higher level of *Gal-9*, suggesting a function for *Gal-9* in immune modulation prior to treatment. A possible co-regulatory axis linking immune evasion and chemoresistance was suggested by the significant connections revealed among the three genes. These results go counter to those of other studies that have focused on how different stages of disease and treatments affect gene expression.The incorporation of these biomarkers may improve

diagnostic precision and prognosis evaluation. Moreover, simultaneously targeting immunological checkpoints and efflux transporters may constitute a potential therapeutic approach. These results highlight the significance of integrated biomarker analysis in enhancing ALL treatment.

6.Conflict of interested

None

7.References

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