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EXPRESSION ANALYSIS OF CASPASE3 (CASP3) GENE IN LEUKEMIA PATIENTS

Original Research

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ABSTRACT

Background: Caspase 3 (CASP3) is a central executioner in the apoptotic pathway, regulating essential processes including cell homeostasis, hematopoietic development, and immune regulation. Dysregulation of CASP3 has been linked to tumorigenesis in several cancers such as breast, colorectal, and uterine malignancies. In leukemia, where defective apoptosis contributes to malignant cell survival, studies assessing CASP3 expression remain scarce. Understanding its expression profile in leukemia subtypes may provide insight into disease mechanisms and potential therapeutic targets.

Objective: To evaluate CASP3 gene expression in leukemia patients compared to healthy controls and to determine its association with clinical and demographic factors.

Methods: A case–control study was conducted involving 112 patients diagnosed with Acute Myeloid Leukemia (AML), Acute Lymphoblastic Leukemia (ALL), Chronic Myeloid Leukemia (CML), or Chronic Lymphocytic Leukemia (CLL), alongside age- and gender-matched healthy controls. Peripheral blood samples were collected in EDTA vacutainers, and total RNA was extracted using the Trizol method. RNA integrity was confirmed via gel electrophoresis before synthesizing complementary DNA using a High-Capacity Reverse Transcription Kit. Relative CASP3 expression was quantified using SYBR Green-based qPCR with β-actin as an internal control. Data were analyzed using the 2^-ΔΔCT method, with t-tests and ANOVA applied for statistical significance ($p<0.05$).

Results: CASP3 expression was significantly downregulated in leukemia patients compared to controls ($p=0.0012$). Patients receiving chemotherapy alone demonstrated higher CASP3 levels than those undergoing combined chemoradiotherapy, although this difference did not reach statistical significance ($p=0.0953$). No significant associations were found with age ($p=0.4616$), gender ($p=0.9425$), or white blood cell count ($p=0.4616$).

Conclusion: The marked reduction of CASP3 expression in leukemia supports its potential role in leukemogenesis through impaired apoptosis. These findings highlight CASP3 as a promising diagnostic biomarker and potential therapeutic target, warranting further molecular and functional investigations to explore its clinical applicability.

Keywords: Apoptosis, CASP3 protein, Caspase 3, Chemotherapy, Gene Expression, Leukemia, Quantitative PCR.

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INTRODUCTION

Cancer remains one of the foremost global health challenges, characterized by uncontrolled and abnormal cell proliferation that can affect virtually any organ or tissue, with its onset and progression influenced by both environmental and genetic factors (1). Hematological malignancies, particularly leukemia, hold a distinctive place within this spectrum due to their complex pathophysiology and profound impact on morbidity and mortality worldwide. Leukemia is a malignancy of the blood and bone marrow, marked by the excessive accumulation of immature or abnormal white blood cells, which disrupts normal hematopoiesis and impairs immune competence (2). Globally, leukemia accounts for approximately 8% of all cancer cases, with an estimated 437,033 new diagnoses and 309,006 related deaths reported in 2018 (3). The disease is broadly categorized into acute and chronic forms, each further subdivided according to the lineage of the affected white blood cells. Acute leukemias, such as Acute Lymphoblastic Leukemia (ALL) and Acute Myeloid Leukemia (AML), progress rapidly and are associated with poor outcomes in the absence of prompt intervention, whereas chronic types, including Chronic Lymphocytic Leukemia (CLL) and Chronic Myeloid Leukemia (CML), evolve more slowly but present unique therapeutic and clinical management challenges (4). Notably, ALL accounts for nearly 30% of all pediatric cancers, underscoring the significant burden of leukemia in children (5). Despite substantial therapeutic advances, leukemia persists as a leading cause of cancer-related mortality, highlighting the urgent need for a deeper understanding of its molecular underpinnings. A central hallmark of cancer is the evasion of apoptosis, the tightly regulated process of programmed cell death that maintains tissue homeostasis and prevents the propagation of damaged or malignant cells (1).

Apoptosis is orchestrated by caspases, a family of cysteine-dependent proteases that degrade key cellular components during cell death (6). Among these, Caspase 3 (CASP3) is regarded as the principal executioner, cleaving structural and regulatory proteins to irreversibly commit the cell to apoptosis (7). In addition to its pivotal role in cell death, CASP3 participates in hematopoiesis and immune regulation, functions directly relevant to hematological malignancies (8). Alterations in CASP3 activity have been documented in various solid tumors, such as breast, colorectal, and lung cancers, where its downregulation is linked to chemoresistance and unfavorable prognosis (9,10). In malignant cells, reduced CASP3 activity may facilitate survival by impairing the apoptotic machinery that would otherwise eliminate them. Although these findings are well-established in solid cancers, the expression dynamics and clinical significance of CASP3 in leukemia remain comparatively underexplored. Emerging evidence suggests that CASP3 plays a critical role in the regulation of hematopoietic cell differentiation and apoptosis, both processes being profoundly disrupted in leukemia (11). Preliminary studies have indicated that aberrant CASP3 expression may influence treatment responsiveness, positioning it as a potential therapeutic target (12,13). Nevertheless, molecular investigations into CASP3 in leukemia are scarce, and its relationship to clinical parameters—such as leukemia subtype, age, gender, treatment regimens, and white blood cell counts—has yet to be systematically characterized. Given the centrality of apoptosis in cancer biology, clarifying the role of CASP3 in leukemia could open new avenues for therapeutic intervention, particularly strategies aimed at restoring apoptotic pathways in malignant hematopoietic cells. Grounded in this context, the present study seeks to evaluate CASP3 gene expression in patients with leukemia and to investigate its association with relevant clinical and demographic variables. It is hypothesized that alterations in CASP3 expression correlate with disease subtype and patient characteristics, and that such changes may hold diagnostic and prognostic value. By elucidating these molecular patterns, this research aims to contribute to the understanding of leukemia biology, support the development of apoptosis-targeted therapies, and ultimately improve clinical outcomes for affected patients.

METHODS

Study Design

The blood samples of 112 leukemia patients and their respective control groups were collected. The leukemia patients were diagnosed with one of the following types: Acute Myeloid Leukemia (AML), Acute Lymphoblastic Leukemia (ALL), Chronic Myeloid Leukemia (CML), and Chronic Lymphocytic Leukemia (CLL). Age- and gender-matched healthy controls were selected from the same geographic area. Blood samples were collected in EDTA vacutainers and stored at 4°C until further analysis. Detailed information, such as age,

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gender, occupation, and lifestyle factors (such as smoking), was recorded for each patient using a questionnaire, and consent was obtained for all subjects (14,15).

Ethical Considerations

All participants provided written informed consent before participating in the study. The study adhered to ethical guidelines and was approved by the Ethical Committee of Universitas Negeri Yogyakarta, Indonesia and Cholistan University of Veterinary and Animal Sciences, Bahawalpur (CUVAS). The research complied with all ethical regulations concerning human subjects, ensuring patient confidentiality and voluntary participation. Ethical approval was obtained in compliance with the Declaration of Helsinki (26).

Sample Collection

The blood samples of 112 leukemia patients and their respective control groups were collected. The leukemia patients were diagnosed with one of the following types: AML, ALL, CML, and CLL. Age- and gender-matched healthy controls were selected from the same geographic area. Blood samples were collected in EDTA vacutainers and stored at 4°C until further analysis. Detailed information, such as age, gender, occupation, and lifestyle factors (such as smoking), was recorded for each patient using a questionnaire, and consent was obtained for all subjects.

Inclusion Criteria

The inclusion criteria for leukemia patients required a confirmed diagnosis of one of the four types of leukemia, regardless of age, gender, or ethnicity. The control group consisted of healthy individuals with no history of blood cancer, matched in terms of age and gender (14).

Materials and Chemicals Used

The following materials and chemicals were employed in experimental procedures for RNA extraction, cDNA synthesis, and quantitative PCR. Trizol reagent (Invitrogen, USA) for RNA extraction as described by (16). Chloroform (Merck, Germany) for phase separation during RNA extraction. Isopropanol (Merck, Germany) for RNA precipitation. Ethanol (Sigma-Aldrich, USA) for RNA washing. SYBR Green qPCR Master Mix (Affymetrix, USA) for quantitative PCR analysis. Primers for CASP3 and β-actin genes designed by Integrated DNA Technologies (USA). EDTA vacutainers (BD Biosciences, USA) for blood collection.

RNA was extracted using the Trizol method as described by (16), which enables the isolation of high-quality RNA from blood samples. Following RNA extraction, cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Invitrogen, USA) as per the manufacturer's protocol. For quantitative PCR (qPCR), the SYBR Green qPCR Master Mix was used, and the reaction conditions were optimized following standard protocols (17).

Table 1: Chemicals Used in this Study

Reagent	Source
RNA Isolation Reagents	
Trizol	Invitrogen
Chloroform	Merck
Isopropanol	Merck
Ethanol	Sigma
Gel Electrophoresis	
Ethidium Bromide	Sigma
Bromophenol Blue	Scharlau
Agarose	Invitrogen
cDNA Synthesis Reagents	
High-Capacity cDNA Reverse Transcription Kit	Invitrogen
Real-Time PCR Reagents	
SYBR Green qPCR master mix	Affymetrix
Primers	Integrated DNA Technologies

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RNA Extraction

Total RNA was extracted from blood samples using the Trizol method, as defined by (16), which allows for the simultaneous extraction of RNA, DNA, and proteins. In this study, 250 µL of blood was mixed with 500 µL of Trizol reagent. The mixture was vortexed and incubated at -20°C for 5 minutes. After the incubation, 200 µL of chloroform was added, and the sample was vortexed again. Following centrifugation at 12,000 rpm for 5 minutes at 4°C, three distinct layers formed: the bottom layer containing proteins, the middle layer containing DNA, and the top layer containing RNA. The RNA layer was carefully transferred to a new tube, and 500 µL of isopropanol was added for precipitation. After incubating the mixture at -20°C for 15 minutes, the RNA was pelleted by centrifugation at 13,000 rpm for 15 minutes. The pellet was washed with 75% ethanol, air-dried, and re-suspended in DEPC-treated water. The RNA was stored at -80°C for further analysis.

RNA Quantification and Quality Assessment

The quality of RNA was assessed using gel electrophoresis. A 1% agarose gel was prepared, and RNA samples were loaded for electrophoresis at 120V for 30 minutes. The gel was analyzed using the BioDoc Analyzer (BioRad, USA) to ensure RNA integrity before proceeding to cDNA synthesis. Only samples with distinct, non-degraded bands (indicative of high-quality RNA) were used for cDNA synthesis. This method is widely used for RNA integrity verification (18).

cDNA Synthesis

The High-Capacity cDNA Reverse Transcription Kit (Invitrogen) was used for synthesizing complementary DNA (cDNA) from the extracted RNA. The reaction mixture contained RNA, random hexamer primers, reverse transcriptase, DNTPs, and a reaction buffer. The reaction was carried out under the following conditions: 25°C for 5 minutes, 42°C for 60 minutes, and 70°C for 5 minutes. The resulting cDNA was used for quantitative PCR analysis (27).

Table 2: cDNA Synthesis Sample Mixture Composition

Component	Volume/Concentration
RNA	1 µL
Random Hexamer Primer	1 µL
5x Reaction Buffer	4 µL
RNA Inhibitor	1 µL
DNTPs	2 µL
Nuclease-Free Water	10 µL
RevertAid RT	1 µL

Table 3: Temperature and Time Interval for cDNA Synthesis Reaction

Step	Temperature (°C)	Time (minutes)
Step 1 (Hold 1)	25	5
Step 2 (Hold 2)	42	60
Step 3 (Hold 3)	70	5

The resulting cDNA was either used immediately for PCR amplification or stored at -20°C for later use.

PCR and Primer Optimization

PCR amplification was performed on β-actin (housekeeping gene) to confirm the integrity of the cDNA. The PCR product was visualized using 2% agarose gel electrophoresis. The successful amplification of β-actin ensured the quality of cDNA for downstream quantitative PCR analysis (19). The primers for both CASP3 and β-actin were designed using the PrimerQuest Tool from Integrated DNA Technologies (IDT, USA) to ensure optimal primer sequences and reaction conditions.

Quantitative PCR (qPCR)

Quantitative PCR (qPCR) was used to assess the relative expression of the CASP3 gene in the leukemia and control samples. Specific primers for CASP3 and β-actin were designed using the PrimerQuest Tool (Integrated DNA Technologies). The qPCR reactions were carried out on a StepOne Plus Real-Time PCR System (Applied Biosystems). The cycling conditions used were as follows: an initial denaturation at 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute.

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Table 4: Sequence for the CASP3 and β-actin Gene Primers for Real-Time PCR

Gene	Primer	Sequence	Annealing Temperature (°C)	Product Length (bp)
β-actin	Forward	5'-TTCTCTGACCTGAGTCTCCTT-3'	55	116
	Reverse	5'-ACACCCACAACACTGTCTTAG-3'	55	116
CASP3	Forward	5'-ACCTCAGGGAAACATTCAGAAA-3'	56	143
	Reverse	5'-GCTCAGAAGCACACAAACAAA-3'	56	143

Table 5: Reagent Mixtures Used for the qPCR Reaction

Reagents	Volume per 10 µL Reaction
cDNA	1 µL
Forward Primer	1 µL
Reverse Primer	1 µL
RNase-Free Water	4 µL
2X SYBR Green Master Mix	3 µL
Total Volume	10 µL

DATA ANALYSIS

The relative expression of CASP3 was calculated using the 2-ΔΔCT method, with β-actin as the internal control (18). All data were analyzed using GraphPad Prism software (GraphPad, USA). Statistical significance between leukemia patients and controls was determined using ANOVA for multi-group comparisons and Student's t-test for pairwise comparisons. A p-value of < 0.05 was considered statistically significant.

RESULTS

The study analyzed CASP3 gene expression in a cohort of 112 leukemia patients compared with age- and gender-matched healthy controls. The mean age distribution indicated that 35% of patients were aged ≤25 years and 65% were aged >25 years, mirroring the control group proportions. The patient group comprised 38% females and 62% males, while the control group included 36% females and 64% males. Among leukemia cases, the distribution of subtypes was as follows: Acute Lymphoblastic Leukemia (ALL) 43%, Acute Myeloid Leukemia (AML) 22%, Chronic Lymphocytic Leukemia (CLL) 2%, and Chronic Myeloid Leukemia (CML) 33%. Occupational categorization revealed that 46% were housewives, 35% were students, and 19% were laborers. In terms of residence, 60% were from urban areas and 40% from rural areas. Most patients (81%) were undergoing chemotherapy alone, whereas 19% received combined chemotherapy and radiotherapy. White blood cell (WBC) counts were >10,000/µL in 33% and <10,000/µL in 67% of cases. Quantitative real-time PCR analysis demonstrated that CASP3 gene expression was significantly downregulated in leukemia patients compared to healthy controls (p=0.0012 as shown in Figure 1). When stratified by age, expression levels were higher in patients aged >25 years compared to those ≤25 years; however, the difference was statistically non-significant (p=0.4616) as shown in Figure 2. Gender-based analysis revealed negligible variation in CASP3 expression between males and females (p=0.9425) as shown in Figure 3.

In acute leukemia subtypes, CASP3 expression was slightly higher in AML patients compared to ALL, with no statistically significant difference (p=0.8646) as shown in Figure 4. Among chronic leukemia subtypes, CML exhibited higher expression levels than CLL, again without statistical significance (p=0.7933) as shown in Figure 5. Occupational differences showed the highest expression in housewives, followed by laborers and then students, although the differences were not significant (p=0.3924) as shown in Figure 6. Patients residing in urban areas had higher CASP3 expression compared to those from rural areas, but the difference was not statistically significant (p=0.6073) as shown in Figure 7. Regarding treatment modality, CASP3 expression was higher in those receiving chemotherapy alone compared to patients receiving combined chemotherapy and radiotherapy; however, the p-value approached but did not reach statistical significance (p=0.0953) as shown in Figure 8. Analysis based on WBC count showed higher CASP3 expression

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in patients with WBC <10,000/µL compared to those with counts >10,000/µL, though this difference was non-significant (p=0.4616) as shown in Figure 9.

Table 6: Demographic characterization of study cohort used for the analysis in this study

Parameter		Total number of cancer patients (%)	Total number of controls (%)
Age	≤25	39(35)	39(35)
	>25	73(65)	73(65)
Gender	Female	43(38)	40(36)
	Male	69(62)	72(64)
Types of leukemia	ALL	48(43)	-
	AML	25(22)	-
	CLL	3(2)	-
	CML	36(33)	
Occupation	Labor	21(19)	-
	Housewives	51(46)	-
	Class Student	40(35)	-
Localization	Urban	67(60)	-
	Rural	45(40)	-
Therapy	Chemotherapy	91(81)	-
	Chemotherapy and Radiotherapy	21(19)	-
WBC	>10000	37(33)	-
	<10000	75(67)	-

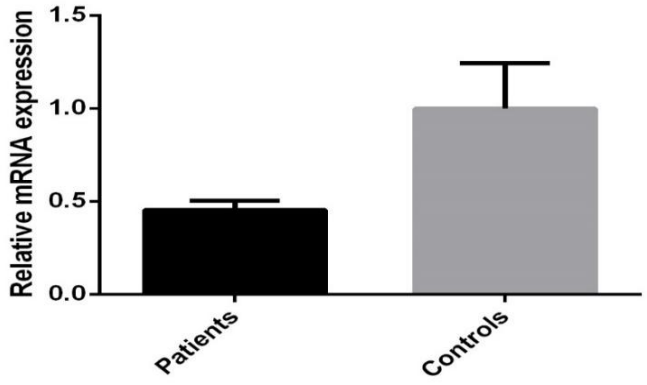

Figure 1: *Change in relative expression of CASP3 gene in leukemia patients and controls (p=0.0012)*

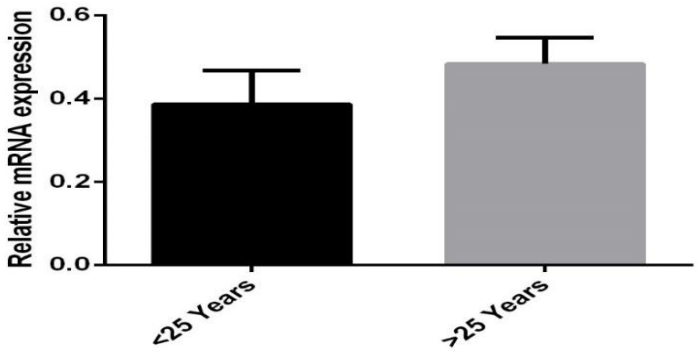

Figure 2: *Relative expression of CASP3 gene in two age groups of leukemia patients*

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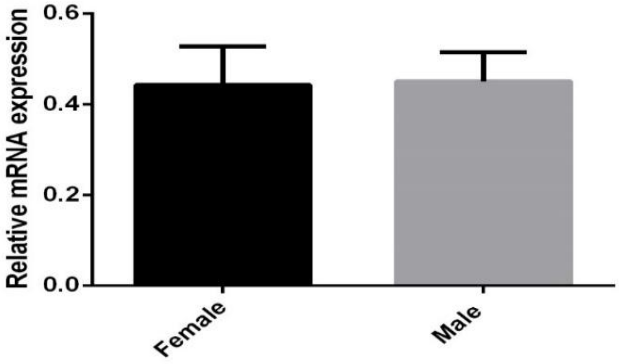

Figure 3: *Comparison of relative expression of CASP3 gene between males and female's patients of leukemia*

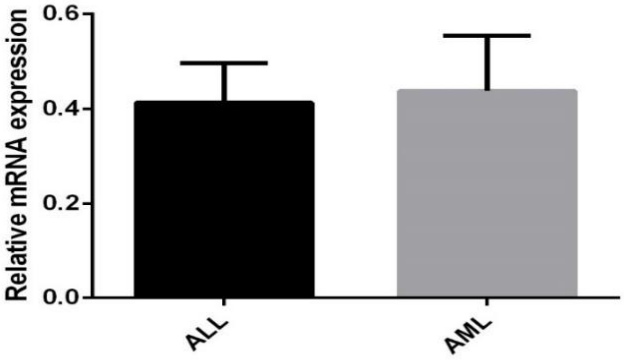

Figure 4: *Comparison of relative expression of CASP3 gene between ALL and AML*

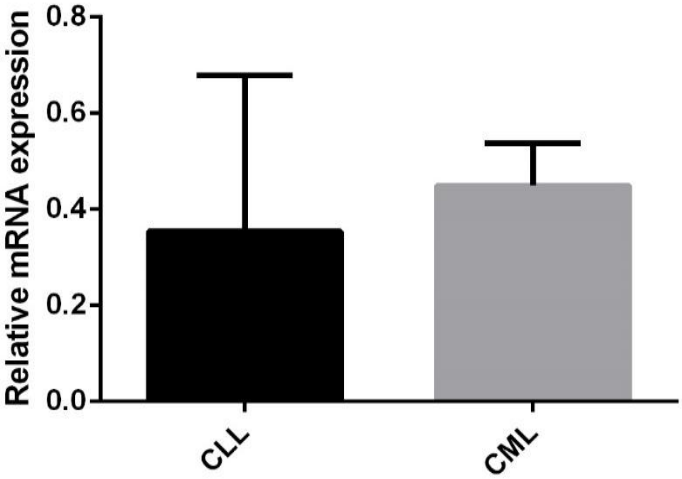

Figure 5: *Comparison of relative expression of CASP3 gene between CLL and CML*

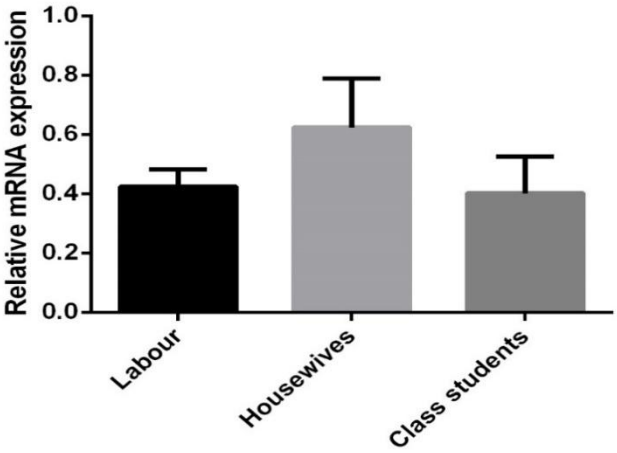

Figure 6: *Comparison of relative expression of CASP3 gene in association with occupation*

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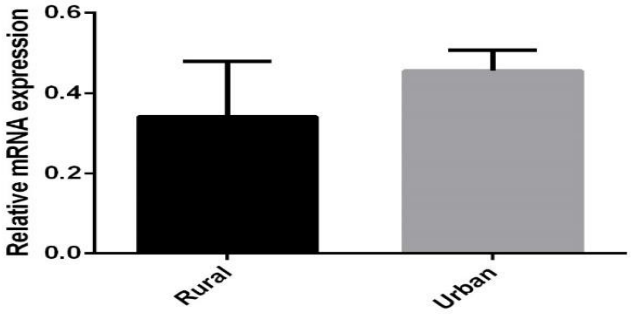

Figure 7: *Comparison of relative expression of CASP3 gene in association with localization*

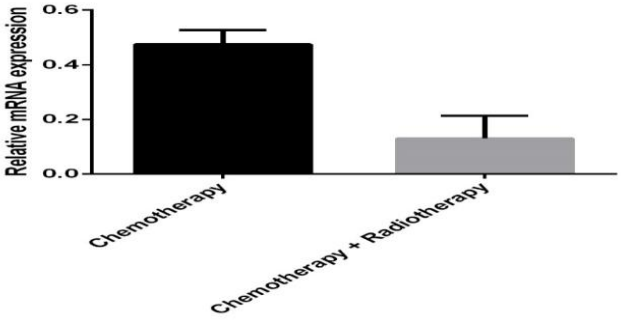

Figure 8: *Comparison of relative expression of CASP3 gene in association with therapy*

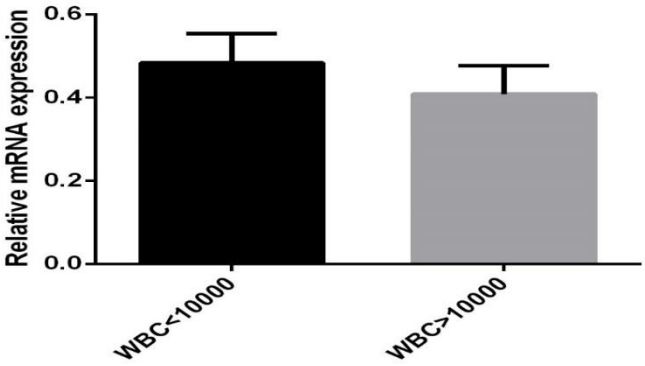

Figure 9: *Comparison of relative expression of CASP3 gene in association with WBC count*

DISCUSSION

The present study demonstrated that CASP3 expression was significantly downregulated in leukemia patients compared with healthy controls, suggesting an important role for this gene in the pathogenesis of leukemia. This finding aligns with earlier evidence indicating that CASP3, a critical executioner in the apoptotic cascade, is frequently dysregulated in cancers, leading to impaired apoptosis and uncontrolled cellular proliferation (19-21). In the context of leukemia, where defects in apoptotic regulation are well-recognized contributors to disease progression, reduced CASP3 expression may facilitate the survival and expansion of malignant hematopoietic cells. The observed statistical significance (p=0.0012) reinforces the likelihood of CASP3's involvement in disease biology and supports its potential relevance as a molecular marker. The absence of significant differences in CASP3 expression between age groups or genders suggests that, within this cohort, demographic variables may not exert a major influence on its regulation. Although these results differ from some reports that have indicated gender- or age-related modulation of apoptotic pathways, such discrepancies could be attributed to variations in sample size, genetic background, or disease characteristics across studies (22-24). Similarly, analyses across leukemia subtypes revealed no significant differences in CASP3 expression between acute or chronic forms, although small sample sizes—particularly in the CLL group—limit the interpretive power of these findings. This lack of subtype-specific significance may indicate a broader role for CASP3 across the leukemia spectrum; however, more extensive and balanced datasets are needed to confirm this hypothesis. No statistically significant associations were found between CASP3 expression and clinical parameters such as white blood cell count, occupation, or geographical location. The absence of correlation with WBC count suggests that CASP3 regulation may be

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independent of overall leukocyte burden and instead related to molecular events driving leukemogenesis. Environmental or occupational factors were not strongly linked to CASP3 levels, though heterogeneity within subgroups and the absence of detailed exposure data may have masked potential relationships. The analysis of treatment type revealed a non-significant trend towards higher CASP3 expression in patients receiving chemotherapy alone compared to combined chemoradiotherapy. While not conclusive, this trend raises the possibility that therapeutic modality could influence apoptotic regulation, a finding that merits further exploration in longitudinal settings.

The study possesses several strengths, including the use of a well-defined case–control design, matched demographic characteristics between groups, and standardized laboratory protocols for RNA extraction and quantitative PCR. The inclusion of multiple demographic and clinical parameters allowed for a broad exploratory analysis of potential associations with CASP3 expression. However, important limitations must be acknowledged. The relatively modest sample size and uneven distribution among leukemia subtypes, particularly the very small CLL group, limit statistical power and may have obscured subtype-specific differences. The reliance solely on mRNA expression without accompanying protein-level validation leaves uncertainty regarding the functional impact of the observed downregulation, as transcriptional changes do not always translate to alterations in protein abundance or activity. Furthermore, the cross-sectional design precludes assessment of temporal changes in CASP3 expression during disease progression or treatment response. The study also did not incorporate genetic or epigenetic profiling, such as promoter methylation analysis, microRNA regulation, or single nucleotide polymorphism identification, which could elucidate the mechanisms underlying CASP3 dysregulation. Additionally, treatment-related variables such as drug type, dosage, and duration were not controlled, potentially introducing variability in CASP3 expression patterns. Future research should aim to address these limitations through larger, multi-center studies with balanced subtype representation and incorporation of both gene and protein expression analyses (24,25). Longitudinal designs would allow for the assessment of CASP3 dynamics throughout the course of therapy, including remission and relapse phases, to evaluate its potential as a predictive biomarker. Integration of molecular profiling—encompassing epigenetic, transcriptional, and translational regulation—would provide a more comprehensive understanding of CASP3's role in leukemia. Functional studies using in-vitro and in-vivo models could clarify whether restoring CASP3 expression enhances apoptosis and improves therapeutic outcomes. Additionally, the exploration of pharmacological agents capable of modulating CASP3 activity, in combination with standard chemotherapy, could open novel therapeutic avenues. Overall, the findings of this study strengthen the evidence that CASP3 plays a role in leukemia pathogenesis through its involvement in apoptosis regulation. Although the results do not support associations with most demographic or clinical variables, the consistent downregulation observed in leukemia patients underscores the importance of further investigation into CASP3 as both a biomarker and a potential therapeutic target.

CONCLUSION

This study concluded that CASP3 expression was markedly reduced in leukemia patients compared to healthy controls, supporting its potential role in leukemogenesis through the disruption of apoptotic pathways that normally regulate malignant cell survival. The observed expression patterns across different leukemia subtypes and treatment groups suggest that CASP3 may hold value as a molecular marker in understanding disease biology and could, with further validation, serve as a diagnostic or prognostic biomarker. While the findings provide meaningful insights into leukemia pathophysiology, their clinical translation requires confirmation through comprehensive molecular, genetic, and functional investigations. Such work will be essential to establish CASP3's utility not only in disease monitoring but also as a possible therapeutic target aimed at restoring apoptosis in malignant hematopoietic cells.

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AUTHOR CONTRIBUTION

Author	Contribution
Abeer Afzal	Substantial Contribution to study design, analysis, acquisition of Data Manuscript Writing Has given Final Approval of the version to be published
Rafia Anwer	Substantial Contribution to study design, acquisition and interpretation of Data Critical Review and Manuscript Writing Has given Final Approval of the version to be published
Areeba Musferah	Substantial Contribution to acquisition and interpretation of Data Has given Final Approval of the version to be published
Maria Mumtaz	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
Syeda Komal Abbas Naqvi	Contributed to Data Collection and Analysis Has given Final Approval of the version to be published
Sahrish Haji	Substantial Contribution to study design and Data Analysis Has given Final Approval of the version to be published
Areej Safdar	Contributed to study concept and Data collection Has given Final Approval of the version to be published
Samra Zafar	Writing - Review & Editing, Assistance with Data Curation
Safdar Ali	Writing - Review & Editing, Assistance with Data Curation
Shazia Aslam*	Writing - Review & Editing, Assistance with Data Curation

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