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Article

Expression Analysis of Caspase-3 (*CASP3*) Gene in Leukemia Patients Using Quantitative Polymerase Chain Reaction (qPCR) and Western Blot Techniques

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Abstract

CASP3 belongs to the caspases family and accounts for several critical activities, including apoptosis and other pathological and physiological processes. *CASP3* also influences the multiple functions in cellular development such as homeostasis, hematopoietic growth and embryonic growth. It has significant involvement in the initiation and development of cancers including breast, colon and uterine cancers. However, molecular studies on the *CASP3* gene in leukemia have not been widely conducted. The present study was designed to analyse the expression level of the *CASP3* gene in leukemia patients. The results were further compared between different parameters such as types of leukemia, age, gender, occupation, WBC count and location-based. About 112 leukemia blood samples along adjacent controls were collected to analyse the *CASP3* expression in this study cohort. The mRNA of each patient and control samples were isolated, quantified and converted to complementary DNA (cDNA). Relative expression of the *CASP3* gene was measured using the quantitative PCR (qPCR) and Western blot analyses. Results showed that the relative expression of *CASP3* was found to be significantly downregulated at both mRNA and protein levels in leukemia patients compared to normal controls. Further analysis showed that expression of *CASP3* was downregulated in patients receiving the combination of chemotherapy as well as radiotherapy compared with patients receiving chemotherapy. These findings from our research showed the lower expression of *CASP3* is associated with an increased risk of development and proliferation of leukemia. It can, therefore, be concluded that lower expression of the *CASP3* gene may contribute to the initiation and development of leukemia.

1. Introduction

Caspase 3 (*CASP3*) is a member of the cysteine-aspartic acid protease (caspase) family and a crucial protein involved in the apoptosis process. It has been associated with key processes including the execution of apoptosis, breaking proteins responsible for maintaining cellular integrity and functions, and activating other caspases for apoptotic signal [1]. Several functions in the cell progression including apoptosis, homeostasis, and embryonic and hematopoietic growth are associated with *CASP3*. *CASP3* failure contributes to increased proliferation and reduced differentiation of bone marrow cells in the hematopoietic cells [2]. *CASP3* is a main executioner caspase and is regulated by initiator caspase including caspase 8 and caspase 9. Two subunits (p12 and p17) of *CASP3* are produced from active *CASP3* enzyme by splitting the caspase 3 at the aspartate site [3]. Activated *CASP3* breaks down several cellular proteins during apoptosis and induces morphological alterations and DNA fragmentation in cells. Caspase facilitates certain types of programmed neuronal cell death based on *CASP3* and *caspase 1* including apoptosis and pyroptosis [4]. The role of *CASP3* has been discussed with multiple types of cancer. Different alleles of *CASP3* have been identified in the squamous cell carcinoma of the head and neck (SCCHN) and elevated the chance of SCCHN. Different alleles of *CASP3* are also associated with the chance of uterine cancer, NHL and multiple myeloma [5]. The lower expression of *CASP3* has been found associated with various types of cancer such as gastric cancer, hepatocellular carcinoma, leukemia, and prostate carcinoma [6-8]. Higher *CASP3* activity has been associated with a malignant form which amplified the apoptosis and poor prognosis. The alteration in the expression of *CASP3* has been associated with carcinogenesis and the progression of many tumors such as colorectal cancer [9]. The overexpression of caspase 3 has been seen in patients with acute leukemia including acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML) [10].

Leukemia is a type of blood cancer that arises in blood and bone marrow. It has a high mortality rate and the incidence varies globally with increased prevalence in East Asian countries. The majority of patients suffering from leukemia are diagnosed at later stages when the disease becomes chronic [11]. Apoptosis plays an important role in maintaining normal homeostasis of the

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cells in the body and makes biochemical and structural changes in cells so that macrophages can detect and engulf them. The decline in the rate of apoptosis can lead to carcinogenesis. Caspases are an important player and executioner in the apoptosis process, which has been reported to influence and control the rate of apoptosis, particularly *CASP3*. Expression variations of caspases have been reported in multiple cancers [12]. However, no study has been done to determine the expression level of *Caspase 3* in leukemia in the Pakistani population. This study was designed to determine the expression variation of *Caspase 3* in leukemia. Furthermore, the expression level of *Caspase-3* will be correlated with the demographic/pathological parameters of leukemia.

2. Methodology

2.1 Sample Collection of Leukemia Patients

The study was conducted at the Department of Biosciences, COMSATS University Islamabad from February 2020 to January 2021. All 112 blood samples of leukemia patients and 112 controls were collected in the EDTA vacutainer from the hospital named Pakistan Institute of Medical Sciences in Islamabad, Pakistan and placed in the refrigerator for further examination at 4 °C. Healthy individuals in the local area were selected as normal controls. The consent form was signed by involved patients and normal individuals and completed with all essential factors such as age, gender, addiction of each patient and ethnic background identified. The entire procedure was carried out after acceptance by the ethical committee of Comsat's University Islamabad (CUI, Ref # 7364).

2.2 Inclusion and Exclusion Criteria

This study included individuals who had a confirmed diagnosis of AML, ALL, CML, and CLL. We included newly diagnosed patients from the local population. Only those capable of understanding the study and providing informed consent were included, and we ensured that the blood samples collected were of adequate quality for RNA and protein extraction and subsequent analysis.

Patients with a history of other malignancies or hematologic disorders were excluded to maintain a clear focus on leukemia. Those with significant co-morbidities, such as uncontrolled diabetes, severe cardiovascular disease, or active infections, were also excluded, as these conditions could influence the study's outcomes. Patients who had undergone intensive chemotherapy, radiotherapy, or stem cell transplantation within the four weeks prior to sample collection were not included, to avoid potential alterations in gene expression levels that could confound our findings.

2.3 RNA Extraction from Blood Samples

Extraction of RNA was done by the Trizol method defined by [13] and the materials used were TRIzol™ Reagent (Thermo Fisher Scientific, Catalog number: 15596026), Isopropanol (Thermo Fisher Scientific, Catalog number: A416-500), Ethanol (Thermo Fisher Scientific, Catalog number: BP2818-500), Chloroform (Thermo Fisher Scientific, Catalog number: C298-500), and DEPC-treated Water (Thermo Fisher Scientific, Catalog number: AM9922).

2.4 RNA Extraction Procedure

250 µL of blood sample stored at -4 centigrade was put in the centrifuge tube of 1.5 mL. Trizol agent (500 µL) was added to the sample-containing tube and centrifuged for 30 seconds. After that tube was placed at -20 centigrade for 5 minutes. Then 200 µL of chloroform was added to the centrifuge tube and vortexed for 30 seconds. After centrifugation, the tube was kept at -20 centigrade for 3 minutes. Then tubes were removed from incubation and centrifuged at 12000 rpm for 5 minutes by refrigerator centrifuge machine. Three layers were formed after centrifugation, in which the bottom layer contained protein and debris, the middle layer consisted of DNA while upper layer contained RNA.

The upper layer which is RNA-containing, was transferred into another tube carefully. Then 500 µL chilled isopropanol was added into an RNA-containing tube and gradually shaken for RNA precipitation. After that tube was placed at -20 centigrade for 15 minutes. After incubation, the tube was centrifuged at 13000 rpm for 15 minutes and it caused the formation of RNA pellet. The supernatant was discarded and the pellet was washed with 1mL of 75 % ethanol. The resulting pellet was centrifuged for 3 minutes at 1000 rpm and then dried the RNA pellet for 20-25 minutes. After that RNA dissolved in the 30-50 µL of DPEC water and stored at -80 centigrade.

2.5 RNA Quantification

Gel electrophoresis was used to confirm the quality of extracted RNA. Gel was prepared by adding 1g agarose in 100 µL buffer solution of Tris Acetate EDTA (TAE) buffer (Thermo Fisher Scientific, Catalog number: B49). The solution was boiled and then allowed to cool down. The casting tray was fixed and an agarose-containing solution was poured and allowed for gel solidification, combs were removed from the gel. The extracted RNA and loading dye were loaded into the well in equal amounts. The reaction was set at 120V for 30 minutes.

2.6 cDNA Synthesis

The reverse transcription kit (Invitrogen, Catalog number: 4368814) is used to synthesize the cDNA from RNA. The process for cDNA synthesis are:

The mixture of reagents used for cDNA synthesis is shown in Table 1. The variable concentration of each regent was used, but the overall concentration should not exceed 20 µL.

Table 1. 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
Revert Aid RT	1 µL

The specific temperature and time interval applied in every step are given in Table 2.

Table 2. Temperature and time interval for cDNA synthesis reaction

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

The cDNA was either used immediately or kept at -20°C for later use.

Human *beta-actin*, a housekeeping gene acts as an endogenous control to check the quality of cDNA products. PCR is used for amplification to check the quality of cDNA after primer designing of *beta-actin*. The products of PCR were checked by 2% agarose gel. Before RT-PCR scrambling, the specific primers of the target gene were designed and optimized.

2.7 Real-Time Polymerase Chain Reaction

To verify the expression of the selected gene, a Real-Time PCR (RT-PCR) machine was used (Bio-Rad, Catalog number: CFX Connect). The procedure is described below.

2.7.1 Primer Designing and Optimization for RT

The sequence of the target gene was recruited through the Ensemble Genome browser and was also proved through the NCBI database. The primer quest tool of Integrated DNA Technology (IDT) was used for the primer design. Primer blast\USCS In silico PCR is also used for the verification of primers. The same procedure was used for designating *beta-actin* gene primers which was used as internal control. To achieve the final concentration of 10mM, each primer was diluted in ddH_2O and stored at -20°C for further investigation.

The details of the primers are given in Table 3.

Table 3. Sequence for the CASP3 and ß-actin genes primers for Real-Time PCR

Gene	Primer	Sequence	Annealing Temperature	Product length
ß-actin	Forward	5-TTCTCTGACCTGAGTCTCCTT-3	55	116
ß-actin	Reverse	5-ACACCCACAACACTGTCTTAG-3	55	116
CASP3	Forward	5-ACCTCAGGGAAACATTCAGAAA-3	56	143
CASP3	Reverse	5-GCTCAGAAGCACACAAACAAA-3	56	143

2.7.2 qPCR Amplification

The amplification process in qPCR for the cDNA of each of the samples was carried out using the reagent mixture are shown in Table 4. All reactions were carried out in triplicates.

Table 4. 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 Syber Green	3 µL
Total volume	**10 µL**

2.8 Data Analysis

The relative expression of mRNA of *CASP3* and *ß-actin* was examined by $2^{-\Delta\Delta CT}$ method.

2.9 Western Blot Analysis

This study conducted Western blot analysis to evaluate the expression of *CASP3* protein in 2 normal blood samples and 6 leukemia samples. From the blood samples, the peripheral blood mononuclear cells (PBMCs) were extracted using Ficoll-Paque Plus (GE Healthcare, Cat. No. 17-1440-02). The isolated PBMCs were then lysed using RIPA buffer (ThermoFisher Scientific, Cat. No. 89900) containing a protease inhibitor cocktail (ThermoFisher Scientific, Cat. No. 78429) to extract total protein. The lysates were incubated on ice for 30 minutes with intermittent vortexing and then centrifuged at 14,000 × g for 15 minutes at 4°C. The supernatant, which contained the extracted protein, was carefully collected and stored at -80°C until further analysis.

To quantify the protein concentration, we used the Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific, Cat. No. 23225). For Western blotting, equal amounts of protein (30 µg) from each sample were mixed with Laemmli sample buffer (ThermoFisher Scientific, Cat. No. 39001) containing 2-mercaptoethanol and heated at 95°C for 5 minutes. The proteins were separated by electrophoresis on a 10% SDS-PAGE gel at 120V for 90 minutes and then transferred onto a nitrocellulose membrane (ThermoFisher Scientific, Cat. No. 88018) using the iBlot 2 Dry Blotting System (ThermoFisher Scientific, Cat. No. IB21001). The membrane was blocked for 1 hour at room temperature with 5% non-fat dry milk in TBS-T (Tris-buffered saline with 0.1% Tween 20) and then incubated overnight at

4°C with a primary antibody against *CASP3* (Caspase-3 (8G10) Rabbit mAb, Cell Signaling Technology, Cat. No. 9665) diluted 1:1000 in 5% non-fat dry milk in TBS-T. The following day, the membrane was washed with TBS-T and incubated for 1 hour at room temperature with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (ThermoFisher Scientific, Cat. No. 31460) diluted 1:5000 in TBS-T.

For detection, the membrane was treated with the Pierce™ ECL Western Blotting Substrate (ThermoFisher Scientific, Cat. No. 32106) for 5 minutes, and the protein bands were visualized using the iBright FL1500 Imaging System (ThermoFisher Scientific, Cat. No. A44115). The intensity of the *CASP3* protein bands was quantified using iBright Analysis Software (ThermoFisher Scientific). The relative expression levels of *CASP3* were normalized against a loading control, *β-actin* (Mouse Anti-β-Actin Monoclonal Antibody, ThermoFisher Scientific, Cat. No. MA1-91399), to ensure accurate comparison across all samples.

2.10 Statistical Analysis

Graph Pad Prism software (Version 10.3.0) was used for the statistical analysis of the data. ANOVA and Student's t-tests were used to analyze statistical differences among different groups. These tests were selected for analyzing gene expression variation due to their specific capabilities. ANOVA was used to compare gene expression across multiple groups, such as different cancer stages or age categories, to identify significant differences among them. Student's t-test was employed for pairwise comparisons between two groups, such as gender differences, to assess if there were significant differences in gene expression between these two categories. The statistical significance was established at $p<0.05$.

3. Results

3.1 Gel Electrophoresis

The integrity of the RNA was confirmed using gel electrophoresis, followed by analysis with a Gel Doc Analyzer (Bio-Rad, Cat. No. 1708195), as presented in Figure 1.

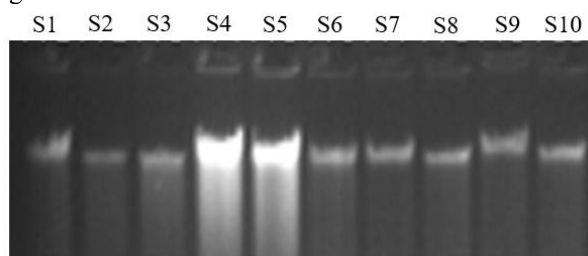

Figure 1. Results of RNA gel electrophoresis exhibit RNA was intact (A representative gel electrophoresis image of RNA extraction and analysis).

3.2 Real-time Polymerase Chain Reaction

Firstly, Real-time polymerase chain reaction (qPCR) analysis was used to analyse the expression of the *CASP3* gene in leukemia patients as well as control samples. An overall summary of the different parameters for the demographic characterization of the cohorts used in this study is shown in Table 5.

Table 5. Demographic characterisation of the 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)	-
Previous Therapy	Chemotherapy	91(81)	-
	Chemotherapy and Radiotherapy	21(19)	-
WBC	>10000	37(33)	-
	<10000	75(67)	-

This age threshold was chosen based on previous literature and epidemiological data indicating that the biological and clinical behavior of leukemia can differ significantly between younger and older populations.

In the present research, the expression level of the *CASP3* gene was examined in leukemia patients. Quantitative real-time PCR analysis was used to evaluate the relative expression of the *CASP3* gene in leukemia patients and associated control samples. Significant downregulation of the *CASP3* gene in leukemia patients was observed relative to normal control as shown in Figure 2 (a).

Relative expression of the *CASP3* gene was identified between two age groups of the patients. The relative expression of the gene was found to be higher in patients aged >25 years compared to relative expression in patients aged <25 years and results indicated statistically non-significant as shown in Figure 2 (b) and statistically non-significant relative expression was seen between two gender groups such as males and females as shown in Figure 2 (c).

Relative expression of the *CASP3* gene in leukemia patients was higher in AML than ALL although the differences observed were statistically non-significant as shown in Figure 2 (d). Relative expression of the *CASP3* gene in leukemia patients was upregulated in CML compared to CLL. The results observed were statistically non-significant as shown in Figure 2 (e).

Relative expression of the *CASP3* gene in leukemia patients was observed higher in housewives in contrast to labour-followed class students. However, the differences

in expression were observed as statistically non-significant as shown in Figure 1 (f). Relative expression of the *CASP3* gene in leukemia patients was seen higher in the individuals living in urban areas as compared to rural, with statistically non-significant as shown in Figure 2 (g).

Relative expression of the *CASP3* gene in leukemia patients was compared between therapies such as chemotherapy and radiotherapy and the expression was upregulated in patients with chemotherapy compared to chemotherapy & radiotherapy although the differences were statistically non-significant as shown in Figure 2 (h). Relative expression of the *CASP3* gene in leukemia patients was compared with different WBC counts of leukemia patients. The expression was observed higher in individuals having WBC <10000 than WBC >10000. However, the difference in expression was observed statistically non-significant as shown in Figure 2 (i).

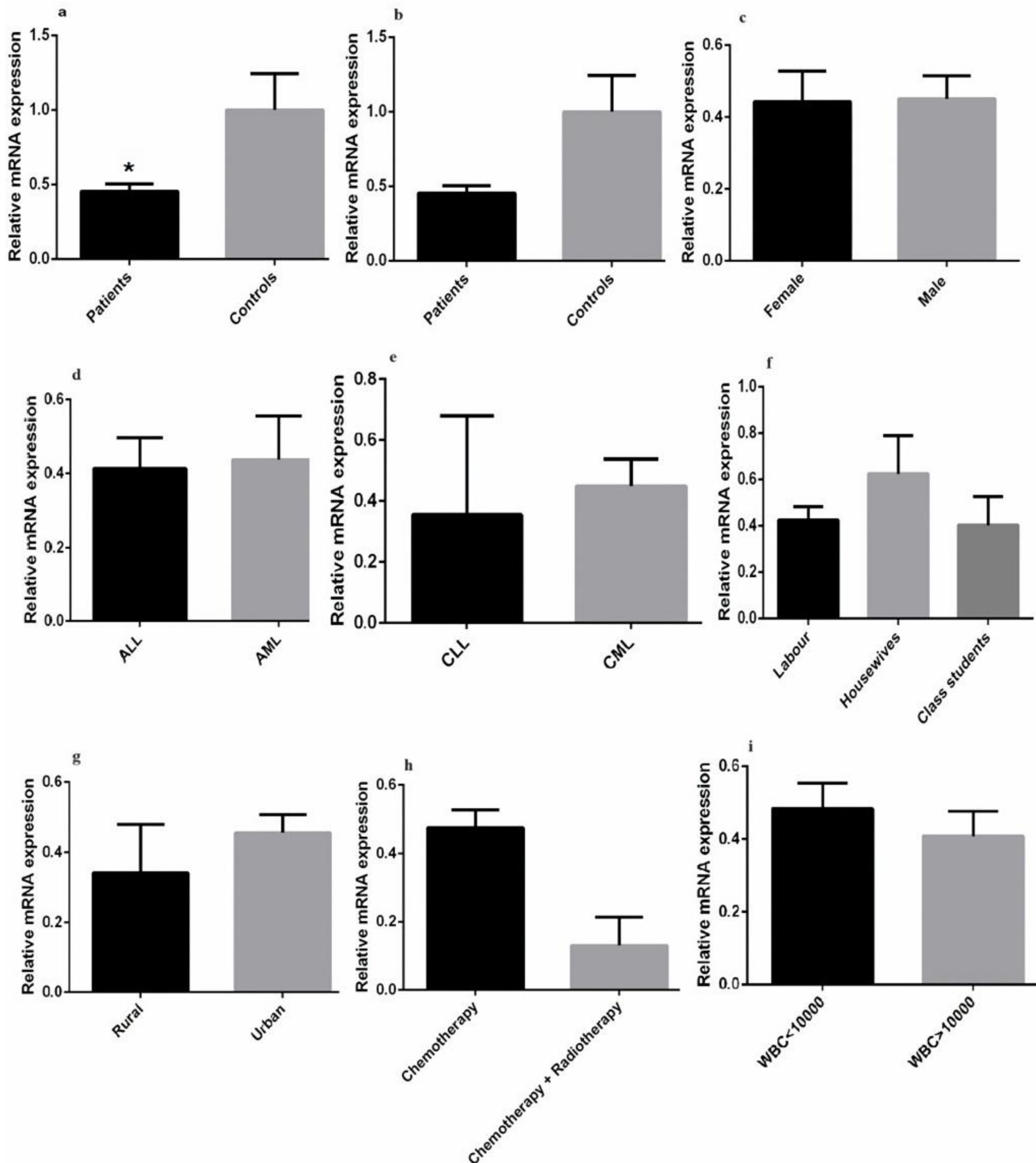

Figure 2. Expression analysis of CASP3 in leukemia and control samples using qPCR. (a) Change in the relative expression of the *CASP3* gene in leukemia patients and controls (b) Relative expression of the *CASP3* gene in two age groups of leukemia patients (c) Comparison of relative expression of *CASP3* gene between male and female patients of leukemia (d) Comparison of relative expression of *CASP3* gene between ALL and AML (e) Comparison of relative expression of *CASP3* gene between CLL and CML (f) Comparison of relative expression of *CASP3* gene in association with occupation (g) Comparison of relative expression of *CASP3* gene in association with localization (h) Comparison of relative expression of *CASP3* gene in association with therapy (i) Comparison of relative expression of *CASP3* gene in association with WBC count. *p-value < 0.05.

3.3 Western Blot Analysis

Next, Western blot analysis of *CASP3* protein was performed in 6 leukemia and 1 normal control sample to quantity the relative protein level of *CASP3.* In Figure 3 (a), CASP3 expression is assessed in one control sample and six leukemia samples, with β-actin serving as a loading control to ensure equal protein loading across samples. The CASP3 bands are visible in all samples but display varying intensities, suggesting differences in expression levels. In Figure 3 (b), the relative *CASP3/β-actin* densities are quantified and presented as a bar graph, showing that the leukemia group has a significantly lower *CASP3* expression compared to the control group. Therefore, these findings suggest that *CASP3* was significantly down-regulated at both mRNA and protein levels in leukemia patients as compare to the controls.

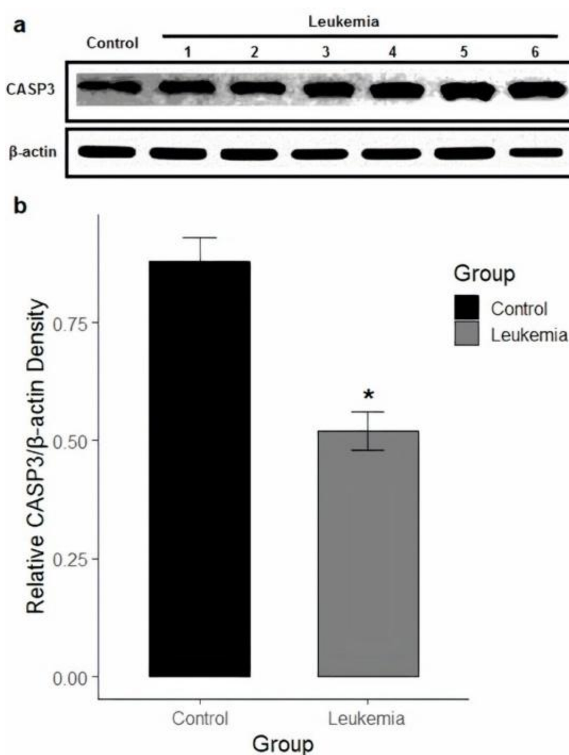

Figure 3. Downregulation of *CASP3* expression in leukemia cells compared to normal control: Western blot analysis and quantification. (a) This panel shows the Western blot analysis of *CASP3* and *β-actin* expression in a control sample and six leukemia samples. *CASP3* expression was detected across all samples, with β-actin serving as a loading control. (b) Quantification of *CASP3* expression relative to β-actin is presented as a bar graph, with black bars representing the control group and grey bars representing the leukemia group. *p-value < 0.05.

4. Discussion

The present was designed to explore and analyze the expression association of the *CASP3* gene to the development and proliferation of leukemia. Caspase 3 is a key executioner caspase that plays an important role in cell processes and apoptosis. It is a tumor suppressor gene because of its key role in apoptosis ultimately deregulation in its expression leads to progression and development and progression of cancer. This gene has been reported to show a significant role in the modulation and signaling pathways of apoptosis and the activation of the caspase cascade during the process of apoptosis as well [14]. The role of the *CASP3* gene has been identified in different cancers including gastric, breast, and colon cancers [15]. *CASP3* is also a main factor of apoptosis and it can inactivate several proteins that are associated with the normal structure and cell cycle. Downregulated expression of the *CASP3* gene causes the inhibition of apoptosis of tumor cells [16]. The lower expression of the *CASP3* gene has been observed in breast cancer patients [17] hepatocellular carcinoma as well [18] and human prostate cancer [19]. CASP3 also modulates the invasion, migration, and metastasis of colon cancer cells, thereby proving it a potential therapeutic target for colon cancer therapy [20]. In our study, the relative expression of the *CASP3* was upregulated in leukemia patients with AML leukemia type compared to CML, ALL and CLL. Svingen *et al.* discussed similar findings in cancer patients [6]. CASP3 plays a critical role in influencing cell death which is an important procedure for mediating the development and proliferation of cancerous cells in leukemia. Therefore, any dysregulation in the CASP3 could to the initiation and development of leukemia such as lower expression of CASP3 can hinder its functions [21]. The leukemia cells exhibit uncontrolled proliferation as they are resistant to apoptosis due to the dysregulated CASP3 [22].

In the present study, *CASP3* expression was evaluated in a cohort of leukemia patients undergoing various therapies. The expression of *CASP3* was found to be upregulated in leukemia patients receiving chemotherapy alone compared to those undergoing both chemotherapy and radiotherapy, aligning with similar findings reported by *Zhou et al.* (2018) in colorectal cancer patients (20). Additionally, higher *CASP3* expression was observed in leukemia patients older than 25 years compared to those younger than 25 years, consistent with results previously published by *Ke et al.* (2021) in breast cancer patients [8]. Finally, *CASP3* expression was elevated in leukemia patients from urban areas compared to those residing in rural areas, echoing the findings of *Zhang et al.* (2013) in breast cancer patients [23].

5. Conclusion

The findings of the current study suggested the downregulation of *CASP3* expression in leukemia development and proliferation. It indicated the future use of this gene as a diagnostic biomarker for leukemia patients. Further cell line-based studies could enhance the awareness of the actual mechanism of the *CASP3* gene and create an efficient target for the treatment of leukemia.

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Authors Contribution

Muhammad Aqib Riaz - Literature Review, Sampling, Experiment Design, Experimental Work, Result Analyses

Muhammad Abubakar - Sampling, Experiment Design, Methodology, Resources

Rooma Ayyoub - Primer Design, Experiment Design, Methodology, Resources

Ahmad Nawaz Khan - Methodology, Resources

Yasir Hameed - Experiment Design, Result Analyses

Conflict of Interest Statement

The author declares that they have no conflict of interest.

Ethical Approval and Informed Consent

This study was approved by the ethical committee of Comsat's University Islamabad (CUI, Ref # 7364). Moreover, written informed consent was obtained from all the patients and healthy individuals prior to sample collection.

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