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THE DYSREGULATED EXPRESSION OF THE P53 GENE IS ASSOCIATED WITH POOR PROGNOSIS IN CEBPA MUTANT ACUTE MYELOID LEUKEMIA PATIENTS

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Abstract

The main objective of the current investigation is to examine the influence of altered p53 gene expression on the prognosis in CEBPA mutant acute myeloid leukemia (AML) patients. AML is a heterogeneous disorder that arises due to acquired genetic anomalies such as CEBPA and TP53. The CEBPA transcription factor plays a critical role in the differentiation of specific blood cells, while the p53 gene functions as a protector of the genome, preventing the development of tumors and regulating various genes involved in cell cycle arrest, DNA repair and programmed cell death. Nevertheless, there is a paucity of comprehensive data concerning the evaluation of p53 expression, which is the resultant of the p53 gene, particularly in hematologic malignancies. In this research investigation, a cohort of thirty individuals diagnosed with AML and twenty-five healthy control participants, who granted informed consent, were enrolled. The evaluation of p53 expression levels was carried out through quantitative Real-time PCR (qRT-PCR). Statistical assessments were performed utilizing one-way ANOVA and Tukey test, facilitated by GraphPad Prism software version 9.0.0. The $p53^{wt}/CEBPA^{mut}$ group (0.20 ± 0.032 SEM) and $p53^{mut}/CEBPA^{mut}$ group (0.06 ± 0.011 SEM) demonstrated a significant decrease in p53 expression compared to the control group (1.00 ± 0.010 SEM) with statistical significance ($p<0.0001$) for both comparisons respectively. Furthermore, the $p53^{mut}/CEBPA^{mut}$ group exhibited a significantly lower level of p53 expression ($p<0.01$) when contrasted with the $p53^{wt}/CEBPA^{mut}$ group. In relation to both the overall survival and event-free survival results, a univariate analysis indicated a statistically significant variance in overall survival ($p=0.0299$, HR = 3.401) and event-free survival ($p=0.0128$, HR = 3.002) between individuals with $p53^{wt}/CEBPA^{mut}$ and $p53^{mut}/CEBPA^{mut}$.

Keywords: Acute myeloid leukemia, p53 gene, gene expression, prognosis.

INTRODUCTION

Acute myeloid leukemia is a rapidly developing form of blood cancer that often exhibits resistance to therapeutic interventions, as documented in various studies (Arber et al., 2016; Döhner et al., 2017; Vago and Gojo, 2020; Dozzo et al., 2022; Stubbins et al., 2022;). The condition is characterized by the abnormal proliferation of primitive hematopoietic cell lines or precursor cells, leading to an imbalance between immature malignant cells and mature blood cells due to impaired myeloid cell differentiation. While individuals of any age can be affected, the prevalence of this disorder is significantly higher among individuals aged 60 and above (Seo et al., 2022; Tislevoll et al., 2023).

AML accounts for 15 to 20% of cases in pediatric populations, while it represents 80% of cases in older individuals (Beaton et al., 2020; Cacace et al., 2022; Chianese et al., 2023). The incidence of AML is higher in males compared to females (Yi et al., 2020). Epidemiological investigations conducted in various countries, such as the United States, United Kingdom, Australia, Canada, Algeria, and Denmark, have indicated that men have a 1.2-1.6 times higher lifetime risk of developing AML (Shallis et al., 2019; Alshemmari et al., 2022). According to the cancer statistics for 2023, it is anticipated that there will be 20,380 new cases of AML in the United States, leading to an estimated 11,310 deaths (Siegel et al., 2023). The incidence of AML is on the rise in both adult and pediatric populations in Pakistan; however, due to the absence of a cancer registry, the precise number of AML cases in the country remains unknown (Sultan et al., 2018; Ghafoor et al., 2020). In the year 2020, an approximate 8,305 new instances of leukemia were reported in Pakistan, resulting in an expected 6,261 fatalities. However, there is a lack of precise information regarding cases of Acute Myeloid Leukemia (AML) as indicated by Ali et al. (2022).While survival rates have notably improved in younger demographics, the prognosis for elderly AML patients remains unfavorable (Récher et al., 2022).

The TP53 gene, which encodes the tumor protein p53, serves dual roles as a transcription factor and a tumor suppressor. TP53, located on chromosome 17p13, is the most commonly mutated gene in various types of cancers (George et al., 2021; Grob et al., 2022). The collective activation of multiple genes that serve to impede the onset and progression of cancer is triggered by the interaction of the wild-type p53 protein with specific DNA response elements. Under normal circumstances, the p53 signaling pathway is activated in reaction to a range of stressors, enabling different transcriptional activities within cells such as halting the cell cycle, repairing DNA, inducing senescence, and ultimately triggering apoptosis. This process serves to inhibit the formation and progression of tumors (Marei et al., 2021).

Approximately 5-15% of cases of acute myeloid leukemia (AML) exhibit TP53 mutations, which encompass five distinct domains: N-terminal transactivation, proline-rich, DNA-binding, oligomerization, and C-terminus regulation (Granowicz and Jonas, 2022). The DNA-binding region of the p53 gene is a common site for alterations in cancer cells, with missense mutations being the most prevalent type of modification. These mutations result in a protein with diminished ability to bind to a specific DNA sequence, thereby affecting the p53 transcriptional pathway (Baugh et al., 2018).

The utilization of real-time polymerase chain reaction (PCR) in clinical diagnostics holds considerable importance due to its capability to identify subtle variations such as point mutations, changes in gene expression levels, and modifications in gene copy numbers. Furthermore, it is instrumental in the analysis of cancer biomarkers (Mitas et al., 2001; Sadia et al., 2020). This methodology is distinguished by its capacity to analyze numerous samples concurrently, facilitating

the accurate detection and quantification of gene expression levels with high sensitivity (Bernard and Wittwer, 2002; Sadia et al., 2020).

The presence of mutated p53 in cells results in genetic instability, as it loses its ability to regulate cell proliferation and effectively repair DNA damage. This continuous genomic damage can potentially lead to the development of neoplasia (Al-Joudi et al., 2001; Sadia et al., 2020). The immunohistochemical assessment of p53 expression is a widely employed technique in the examination of ductal carcinoma in situ (DCIS), whereas molecular biology screening approaches are not as commonly employed. There is a scarcity of studies that have employed molecular biology screening methods, such as Constant density gel electrophoresis (CDGE) or single-strand conformational polymorphism (SSCP), to explore p53 sequence variations in DCIS cohorts. These investigations subsequently confirmed the observed altered mobility patterns in SSCP/CDGE through DNA sequence analysis (Livak and Schmittgen, 2001; Sadia et al., 2020).

Despite the significant influence of p53 mutations on the onset and advancement of different malignancies, along with their association with poor overall survival rates in AML patients, the consequences of altered p53 expression in individuals with AML have not been extensively explored (Bossi et al., 2006; Hajian, 2017; Davoodi et al., 2018; Pei et al., 2023). This study presents findings indicating a notable dysregulation in p53 expression among AML patients harboring CEBPA mutations.

MATERIALS AND METHODS

Blood Sample Collection

In this study, a cohort of thirty individuals recently diagnosed with AML and twenty-five healthy volunteers were selected from Jinnah and Mayo hospitals in Lahore. Prior to their participation, all individuals provided written consent. The AML diagnosis was verified by haematologists according to the 2016 World Health Organization (WHO) classification criteria. The patients were observed over a period of 3 to 6 years for survival analysis. The samples had previously been analyzed for potential alterations in the CEBPA gene. A comprehensive patient data form documented clinical, hematological, and biochemical parameters for each participant. Blood samples containing CEBPA mutations were stored in Ethylene Diamine Tetra-acetic acid (EDTA) tubes at a temperature of -20°C.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA was isolated from blood samples stored at -20°C using a homogenizer, centrifuge, vortex, and Eppendorf tubes, along with chemicals such as TRIzol, chloroform, 75% ethanol, and nuclease-free water. Following this, the generation of complementary DNA (cDNA) was carried out using the RevertAid First Strand cDNA Synthesis Kit. Subsequently, the Real Time Expression analysis of the target genes was conducted using the SYBR Green quantitative polymerase chain reaction (qPCR) mix (Zokeyo, Wuhan, China), with cDNA serving as the template and relevant primers in triplicate. The primers employed in this study were previously reported by Goudarzipour et al. (2017) for the p53 gene, with the forward primer sequence ACCGGCGCACAGAGGAAGAGAA and the reverse primer sequence TGGGGAGAGGAGCTGGTGTTGT. The GAPDH gene, also referred to as glyceraldehyde 3 phosphate dehydrogenase gene, was utilized as a reference gene for normalization in accordance with the primer sequences provided by Kwon et al. (2021): TTGGCTACAGCAACAGGGTG (forward primer) and GGGGAGATTCAGTGTGGTGG (reverse primer). The qRT-PCR procedure initiated with an initial denaturation phase at 95ºC for 2 minutes, followed by denaturation at 95ºC for 15 seconds, annealing at 62ºC for 30 seconds, and extension at 72ºC for 30 seconds. These denaturation, annealing, and extension steps were iterated over 40 cycles, as depicted in figure 1. The respective Ct values of the samples were compared against a housekeeping gene. The quantification of the target gene expression was conducted utilizing the 2-

ΔΔCT method, with the p53 gene expression normalized relative to the GAPDH gene serving as an internal control.

Table 1: Components with final concentrations for preparing the qRT-PCR reaction mixture.

Components	The volume of the final concentration.
Syber Green	**5 ul**
NFW	**2 ul**
F Primer	**1 ul**
R Primer	**1 ul**
Template	**1 ul (total rec volume 10 ul)**

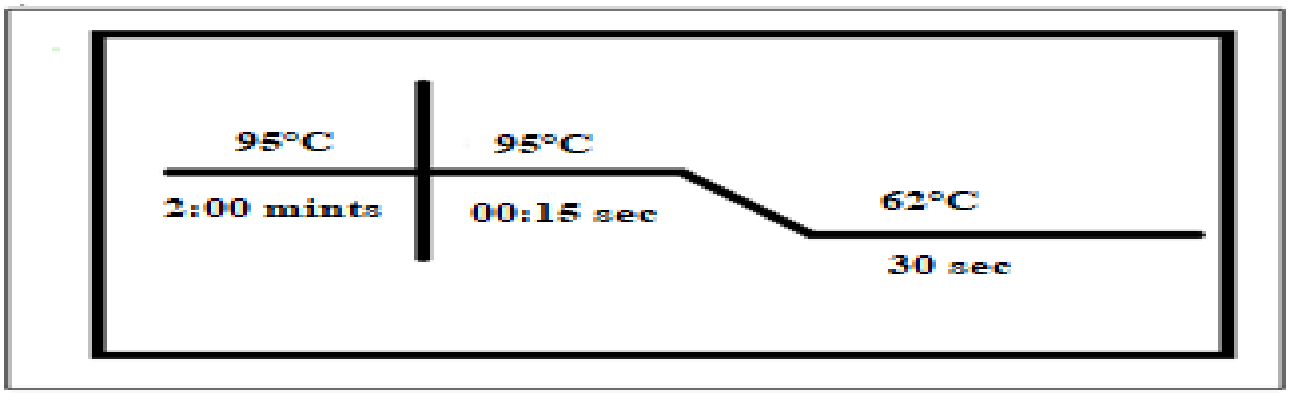


Figure 1: Quantitaive real time polymerase chain reaction conditions for the amplification of target template

STATISTICAL ANALYSIS

The Fisher exact test was utilized to compare various variables between groups with and without mutations. Gene expression levels were evaluated using the 2-ΔΔCT method described by Livak and Schmittgen (2001). Statistical analysis was conducted with GraphPad Prism software (version: 9.0.0), with a significance level of $p<0.05$. One-way ANOVA was used to compare gene expression differences between ^{p53wt}/CEBPA^{mut}, p53^{mut}/CEBPA^{mut} patients, and the control cohort. Additionally, the Tukey test was utilized to explore relationships among various subgroups. Kaplan-Meier survival curves were constructed to evaluate overall survival (OS) and event-free survival (EFS), with the log rank test employed to compare these curves.

RESULTS

Clinical features of CEBPA mutant AML patients

The research study involved the analysis of 30 samples obtained from individuals diagnosed with AML of various genders and ages. Additionally, 25 healthy volunteers were included as control subjects, with their participation being voluntary and based on informed consent. In the group of patients with AML, 63% were men (n=19) and 37% were women (n=11), with an average age of 37.0 ± 17.5 years. The control group included 25 healthy participants, with 15 males and 10 females, having an average age of 41.2 years and a median age of 35.5 years (ranging from 25 to 65 years). Symptoms reported by the AML patients included fever (43%), cough (16.7%), body pain (20%), weight loss (23.3%), anorexia (13.3%), bleeding (16.7%), blurred vision (10%), edema (6.7%), dizziness (20%), and hepatomegaly (3.3%). The French-American-British (FAB) classification revealed M1 in 13.3%, M2 in 26.7%, M4 in 46.7%, M5 in 6.7%, M6 in 3.3%, and M7 in 3.3% of the cases, with no instances of M0 or M3 observed. Based on cytogenetic analysis, 26.7% of patients had abnormal findings while 73.3% had normal results. The distribution of patients across induction, consolidation, remission, and relapse chemotherapy cycles was 36.7%, 30%, 20%, and 13.3%, respectively as depicted in table 2.

Table 2: The clinical features of AML patients (n=30) included in the present study.

clinical Measures	Features	Patients n= 30 (%)	
Gender	Men	19	(63.3%)
	Women	11	(36.7%)
Age	<20	3	(10%)
	>20	27	(90%)
Median age	37		
Signs at diagnosis	Fever	13	(43%)
	Cough	5	(16.7%)
	Body pain	6	(20%)
	Weight loss	7	(23.3%)
	Anorexia	4	(13.3%)
	Bleeding	5	(16.7%)
	Blurred vision	3	(10%)
	Oedema	2	(6.7%)
	Dizziness	6	(20%)
	Hepatomegaly	1	(3.3%)
FAB Type	M0	----	
	M1	3	(10%)
	M2	9	(30%)
	M3	----	
	M4	14	(46.7%)
	M5	2	(6.7%)
	M6	1	(3.3%)
	M7	1	(3.3%)
Cytogenetics	Abnormal	8	(26.7%)
	Normal	22	(73.3%)
Chemo cycle	Induction	11	(36.7%)
	Consolidation	9	(30%)
	Remission	6	(20%)
	Relapse	4	(13.3%)

Hematological Features of CEBPA Mutant AML Patients

The hemoglobin levels in individuals with CEBPA mutant AML ranged from 3.8 to 11.4 g/dl, with an average of 7.6 g/dl and a standard error of the mean (S.E.M.) of 0.39 g/dl. In this study, the hemoglobin levels of patients were notably lower than typical values, indicating a high prevalence of anemia within this patient cohort. Platelet counts in the research exhibited a range of values from 11 to 869×103/µl, with a mean of 46.3×103/µl and a standard error of the mean (S.E.M.) of ± 9.1. The total leukocyte count varied between 3.7 and 125.1×103/µl, with an average of 25.1×103/µl and an S.E.M. of ± 6.5.

Leukocytosis was observed in 12 patients (40%), leukocytopenia in 9 patients (30%), normal leukocyte count in 4 patients (13.3%), and hyper-leukocytosis in 5 patients (16.7%). The percentage of blast cells was 55.13% for p53 wild type and 65.28% for p53 mutant in CEBPA mutant AML patients. Table 3 illustrates the distribution of TLC count percentages.

Table 3. The clinical characteristics of the 30 patients with CEBPA mutant AML (p< 0.05)

Parameters	$p53^{wt}$ /$CEBPA^{mut}$	$p53^{mut}$ /$CEBPA^{mut}$	P-value
Number of patients (%)	11 (36.7%)	19 (63.3%)	__
Average hemoglobin levels (g/dl),	8.4 (5.3-12.8)	7.6 (3.8-11.4)	0.512
Average platelet counts ($\times10^3$/ul),	51.4 (3-785)	46.3 (11-869)	0.218
Range of total leukocyte counts ($\times10^3$/µl),	29.9 (1.5 to 136.2)	25.1 (3.7 to 125.1)	0.636
percentage of blast cells	55.13%	65.28%	0.301

P53 expression among $p53^{wt}$/$CEBPA^{mut}$ and $p53^{mut}$/$CEBPA^{mut}$ AML patients and healthy volunteers:

The mean Ct values (± standard deviation) of p53 in individuals with $p53^{wt}$/$CEBPA^{mut}$, $p53^{mut}$/$CEBPA^{mut}$ AML, and healthy controls were found to be 23.685 ± 3.45, 25.515 ± 3.90, and 21.476 ± 1.91, respectively. Subsequently, the relative p53 expression levels in $p53^{wt}$/$CEBPA^{mut}$ AML samples were compared to those in standard blood samples, resulting in a 1-fold change in the control samples. An increase in fold change above 1 indicated up-regulation, while a decrease below 1 indicated down-regulation. The majority of blood samples from individuals with p53 wild type in CEBPA mutant AML cohort exhibited a significant (P<0.0001) four asterisks (****) (0.20 ± 0.032 SEM) down-regulation in p53 expression compared to the control group (1.00 ± 0.010 SD). In most CEBPAmut AML samples, the relative gene expression of p53mut showed a significant (P<0.0001) four asterisks (****) (0.06 ± 0.011 SEM) down-regulation compared to healthy controls (1.00 ± 0.010 SD). The relative fold change value of p53 mutant in CEBPA mutant AML samples demonstrated a significant (P<0.01) two asterisks (**) (0.20 ± 0.032 SEM) down-regulation compared to p53 wild type (0.06 ± 0.011 SEM). All specimens displayed aberrant gene expression profiles when compared to the control specimens, as illustrated in Figure 2.

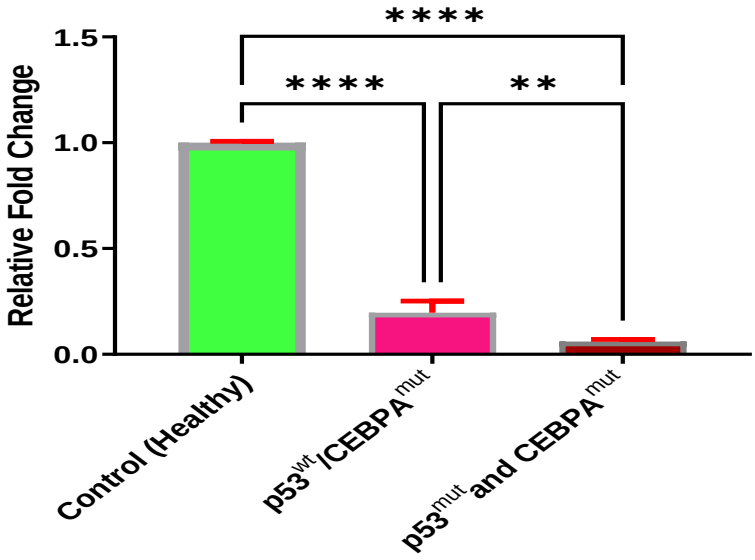
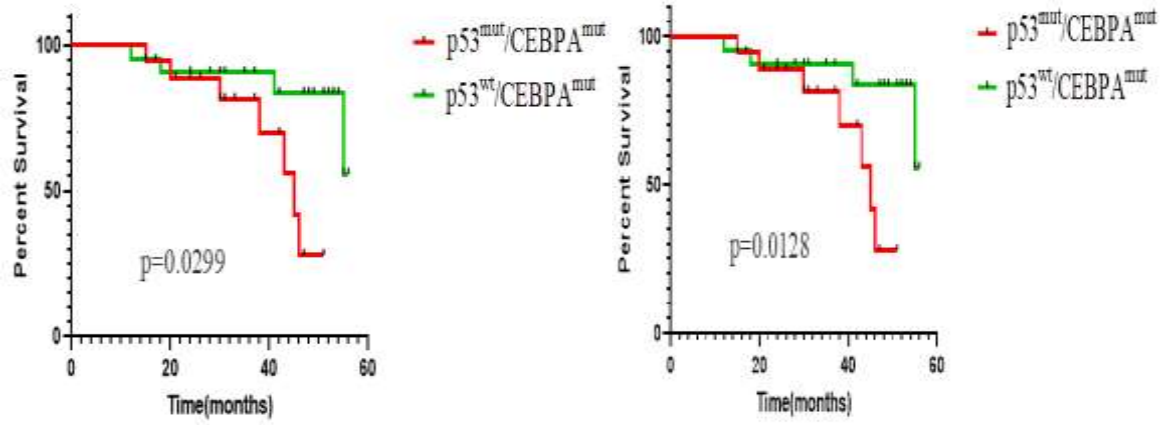


Figure 2. Relative expression of p53 in 30 AML patients and 25 healthy volunteers was measured from Ct values and normalized against a reference gene (GAPDH). A significant relative fold change difference (p<0.0001) four asterisks (****) between $p53^{wt}$ (0.20 ± 0.032 SEM) of CEBPA mutant AML patients in comparison to normal control group (1.00 ± 0.010 SEM) was identified. A significant relative fold change difference (p<0.0001) four asterisks (****) between $p53^{mut}$ (0.06 ± 0.011 SD) of CEBPA mutant AML patients and healthy groups was identified. A significant relative fold change difference (p<0.01) two asterisks (**) between $p53^{wt}$ (0.20 ± 0.032 SEM) and $p53^{mut}$ (0.06 ± 0.011 SEM) CEBPA mutant AML groups was identified.

P53 expression in different FAB subtypes of AML.

The research investigated the modified p53 expression in samples from AML patients across various FAB subtypes utilizing the One Way ANOVA test. Although no statistically significant variance in p53 expression was detected among the distinct FAB subcategories, an evident distinction was observed. Specifically, reduced p53 expression was identified in the M1, M2, M6, and M5 subtypes. In comparison to the control group, these subcategories displayed a distinct reduction in p53 expression, with no discernible correlation among them. Noteworthy is the lowest expression level observed in the M1 subtype, while the highest expression level was noted in the M4 subtype.

Analysis of the survival rates of wild type and mutated p53 CEBPA mutant AML patients:
The GraphPad Prism software version 9.0.0 was utilized to evaluate the overall survival and event-free survival. Univariate analysis revealed a statistically significant difference in overall survival (p=0.0299, HR=3.401) and event-free survival (p=0.0128, HR=3.002) between patients with $p53^{wt}/CEBPA^{mut}$ and $p53^{mut}/CEBPA^{mut}$ (Table 4). The corresponding survival curves are depicted in Figure 3.



(A) Overall Survival **(B) Event-Free Survival**

Figure 3: Kaplan-Meier survival results for 30 patients with CEBPA mutant and p53 acute myeloid leukemia. (A) Shows the overall survival of CEBPA mutant AML patients with Wild-type p53 (green) and those with p53 mutation (red). (B) Represents event-free survival.

Table 4. Various factors utilized for conducting survival analysis of p53 mutated and wild type in CEBPA mutants among AML patients.

Graphs of survival	95% confidence interval.	Hazard Ratio	Survival Proportions		P-value
			$p53^{mut}/CEBPA^{mut}$	$p53^{wt}/CEBPA^{mut}$	
Overall survival	0.977-11.85	3.401	46.00	59.00	0.0299
Event-free survival	1.086-8.299	3.002	38.00	52.00	0.0128

DISCUSSION

The primary objective of this study is to investigate the expression profile of the p53 gene in different groups of CEBPA mutant AML patients using real-time PCR. This research is innovative as there is a lack of prior literature documenting similar results. Lukas et al. (2000) highlighted the underutilization of molecular biology screening methods in evaluating p53 expression, in contrast to the more commonly used immunohistochemical analysis in ductal carcinoma in situ (Sadia et al., 2020). The study focused on CEBPA mutant AML patients and revealed a decrease in p53 expression across various patient cohorts. These results are consistent with earlier research that has emphasized the significant role of the p53 gene in the development of hematologic malignancies (Ahmadzadeh et al., 2018; Pei et al., 2023).

The tumor suppressor protein p53 is frequently inactivated in various cancer types, either due to genetic mutations or dysregulation mechanisms. The dysregulation of the tumor suppressor protein p53 can occur due to increased levels of the p53 inhibitor MDM2 or disturbances in the movement of p53 within the cell from the nucleus to the cytoplasm. This disruption may be caused by abnormalities in the transporter proteins importin and exportin, or the overexpression of the cytoplasmic retention protein CUL9/PARC. While p53 mutations are rare in acute myeloid leukemia (AML) cells, impaired p53 function is a common occurrence in AML cases (Colombo et al., 2002; Seipel et al., 2016).

The disruption of elements within the p53 signaling pathway is a frequent occurrence in the onset and advancement of different types of cancers (Bossi et al., 2006; Green and Kroemer, 2009; Li et al., 2015; Shakweer and El-Sheshtawy, 2017; Hajian, 2017). Therefore, it is essential to assess the

p53 gene and its associated components in cancerous cells. This research observed a reduction in the overall expression levels of the p53 gene in the M1, M2, M6-M7, and M5 FAB subtypes among patients diagnosed with acute myeloid leukemia (AML) due to genetic mutations. While previous studies have identified mutations in the p53 gene in various human cancers, and mutations in p53 have been documented in a substantial number of human malignancies, the frequency of mutations was unexpectedly lower in AML patients compared to other cancer types (Peller and Rotter, 2003; Goudarzipour et al., 2017; Rahmé et al., 2023).

The significance of the p53 pathway in maintaining cellular integrity highlights the necessity of confining p53 abnormalities to gene mutations. Nevertheless, it is crucial to acknowledge that deviations in gene expression can also produce comparable outcomes, a fact that has been disregarded. Additionally, studies have demonstrated that individuals harboring p53 mutations face adverse prognoses, treatment-resistant conditions, and restricted short-term survival (Haferlach et al., 2008; Rahmé et al., 2023).

Despite the significant impact of abnormalities in the p53 gene on the progression of cancer and its substantial prognostic relevance, there is a lack of comprehensive studies on p53 expression, particularly in hematologic malignancies. The results of the current study indicate a notable overall decrease in p53 gene expression in individuals with acute myeloid leukemia (AML). These findings suggest that irregularities in gene expression may be more significant than gene mutations, as evidenced by the reduced p53 expression in the majority of AML patients and across all AML subtypes except M4. Our investigation unveiled a general decrease in p53 expression in AML patients. Previous studies conducted on cell lines or non-clinical samples have shown inconsistent results. For example, Shikami et al. (2006) observed a decrease in p53 protein levels in AML cells with the t(8;21) translocation, without any changes in mRNA levels. While our research did not directly measure p53 protein levels, it is suggested that patients with reduced p53 mRNA levels may also exhibit lower p53 protein levels (Shikami et al., 2006). In contrast, our study found a general reduction in p53 levels in individuals with CEBPA mutant AML. Conversely, Fu et al. (2014) reported that the emergence of AML1-ETO fusion genes leads to the binding of the AML1-ETO fusion protein to the AML1 binding site, resulting in increased expression of early growth response gene 1 (EGR1), which subsequently activates p53 and PTEN expression (Fu et al., 2014).

Various studies have been carried out in different contexts or using diverse cell lines, which were distinct from our own investigation. For instance, Bellodi and colleagues (2006) suggested in a separate study that the PML/RARa fusion gene hinders the transcription of p53, leading to a decrease in the growth-inhibiting and apoptosis-inducing effects of p53. However, our current study revealed a significant reduction in the overall expression of p53 in individuals with acute myeloid leukemia (AML). Furthermore, it is plausible to suggest that the level of p53 expression may be associated with both overall survival and treatment response, considering the favorable prognosis associated with the PML-RARα mutation (Shakweer and El-Sheshtawy, 2017; Nikzad et al., 2017).
Recent studies have shown that patients diagnosed with AML have demonstrated poor overall survival and event-free survival rates. The use of Kaplan-Meier survival curves was employed to assess overall survival and event-free survival from the time of diagnosis to the last follow-up, comparing individuals with $p53^{wt}/CEBPA^{mut}$ and $p53^{mut}/CEBPA^{mut}$. The results of the research indicate a more positive prognosis in the $P53^{wt}/CEBPA^{mut}$ group. Current research has indicated that the presence of CEBPA mutation in the bZIP domain, whether categorized as $CEBPA^{sm}$ or $CEBPA^{dm}$, is associated with a favorable outcome in de novo AML (Wakita et al., 2022). Conversely, the existence of p53 mutations in patients with or without complex karyotypes has been linked to an unfavorable prognosis (Granowicz and Jonas, 2022; Kayser and Levis, 2023).
Hence, the expression of p53 may serve as a promising prognostic marker and a predictor of recurrence likelihood, presenting novel therapeutic possibilities. Nevertheless, evaluating it in a

more extensive patient group in a varied setting is imperative. This current investigation sought to analyze p53 expression in the Pakistani populace through real-time PCR; however, forthcoming studies should contemplate enlarging the sample size to achieve a more comprehensive understanding.

CONCLUSION

The results of the present investigation demonstrate a notable reduction in p53 expression within the CEBPA mutant AML group, implying a potential association with unfavorable prognosis and survival rates among afflicted individuals. Additional studies are necessary to clarify the specific impact of variations in p53 expression in AML patients as pivotal molecular mechanisms in cancerous cells.

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