



Original article

RUNX1 mutation and elevated FLT3 gene expression cooperates to induce inferior prognosis in cytogenetically normal acute myeloid leukemia patients



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ABSTRACT

Background: Acute myeloid leukemia (AML) is a bone marrow malignancy having multiple molecular pathways driving its progress. In recent years, the main causes of AML considered all over the world are genetic variations in cancerous cells. The RUNX1 and FLT3 genes are necessary for the normal hematopoiesis and differentiation process of hematopoietic stem cells into mature blood cells, therefore they are the most common targets for point mutations resulting in AML.

Methods: We screened 32 CN-AML patients for FLT3-ITD (by Allele-specific PCR) and RUNX1 mutations (by Sanger sequencing). The FLT3 mRNA expression was assessed in all AML patients and its subgroups.

Results: Eight patients (25%) carried RUNX1 mutation (K83E) while three patients (9.37%) were found to have internal tandem duplications in FLT3 gene. The RUNX1 mutation data were correlated with clinical parameters and FLT3 gene expression profile. The RUNX1 mutations were observed to be significantly prevalent in older males. Moreover, RUNX1 and FLT3-mutated patients had lower complete remission rate, event-free survival rate, and lower overall survival rate than patients with wild-type RUNX1 and FLT3 gene. The RUNX1 and FLT3 mutant patients with up-regulated FLT3 gene expression showed even worse prognosis. Bradford Assay showed that protein concentration was down-regulated in RUNX1 and FLT3 mutants in comparison to RUNX1 and FLT3 wild-type groups.

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Conclusion: This study constitutes the first report from Pakistan reporting significant molecular mutation analysis of RUNX1 and FLT3 genes including FLT3 expression evaluation with follow-up. This provides an insight that aforementioned mutations are markers of poor prognosis but the study with a large AML cohort will be useful to further investigate their role in disease biology of AML.

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1. Introduction

Acute myeloid leukemia (AML) is a malignant neoplasm of the bone marrow characterized by the clonal expansion of abnormal myeloid progenitor cells having reduced ability to differentiate into mature cells (Shallis et al., 2019). Genetic screening plays a crucial role in defining treatment strategies for acute myeloid leukemia patients by categorize them into different prognostic groups (Medinger and Passweg, 2017). In recent times, the main causes of AML that have been admitted all over the world are genetic variations in cancerous cells. These chromosomal variations are related to good and bad responses to different treatments and are being used to determine the survival rate of AML patients (Döhner et al., 2015).

The RUNX1 gene is a Runt-related transcription factor that has multiple names i.e. core-binding factor subunit α -2 (CBFA2) or PEB-P2 α B) and AML1 protein. The AML1 protein unites to a core sequence TGTGGNNN (NNN can be either TTT or TCA) to form a heterodimeric complex with CBF β , this complex helps in the binding of RUNX1 protein with DNA to enhance transcription of other genes. The RUNX1 gene consists of three major domains i.e. 1st is the Runt homology domain (RHD), 2nd is the transcription activation domain (TAD) and 3rd is the suppression domain. The RHD domain is vital for heterodimerization with the CBF β subunit and the DNA binding. While the TAD domain is important for interaction with transcription coactivators. Recently, the function of the RUNX1 gene has been analyzed which showed its contribution as a positive regulator of other genes that help in cell adhesion and migration. The main causes of impaired hematopoietic differentiation are mutations in transcription factors or coactivators (Döhner et al., 2015).

The RUNX1 is associated indirectly with FMS like tyrosine kinase gene (FLT3), belongs to class III tyrosine kinase receptor family, which encodes a protein having 993 amino acids in humans. This protein is comprised of a Juxtamembrane (JM) dimerization domain, an extracellular ligand-binding domain, a transmembrane domain as well as a cytoplasmic domain which has a split tyrosine kinase motif (Markovic et al., 2005). The FLT3 protein is a type of receptor tyrosine kinase which showed its expression on early hematopoietic precursor cells and contributes to the survival and differentiation of stem cells (Murphy et al., 2003). The internal tandem duplication (ITD) in the FLT3 gene is the major type of FLT3 mutation which helps in FLT3 protein's constitutive phosphorylation thus disturbing the normal process of hematopoiesis leading to leukemogenesis (Choudhary et al., 2005). The internal tandem duplication arises due to the duplication of the Juxtamembrane domain's fragment which is a coding region encoded by FLT3 exons 14 and 15. The Juxtamembrane domain played a crucial role in the autoinhibition of the kinase (Schlenk et al., 2008; Mrózek et al., 2007). Accordingly, the current study was intended to assess the prognosis of RUNX1 gene mutations in AML patients and to assess the FLT3 gene expression and internal tandem duplication (ITD) mutation in acute myeloid leukemia (AML) patients and its clinical implications.

2. Material and Methods

Blood samples from 32 patients with cytogenetically normal acute myeloid leukemia (CN-AML) and 10 individuals from the control group were selected for this research work after obtaining written informed consent to take part in the study. Patients with acute myeloid leukemia were evaluated with the help of consultant hematologists at Jinnah and Mayo Hospitals, Lahore according to the standard clinical factors. Blood samples were collected in vacutainers coated with EDTA and stored at -20°C . Patients with AML (male = 19, female = 13) were aged 16–70 years. Fifty-nine percent (59%) of AML patients were men and 41% were women. The majority of patients (68.2%) were 13 to 40 years old, and only 3.1% of patients were ≥ 70 years old. Patients were divided into 2 groups: 1st group comprised of adults (19–75 years old) $n = 29$ while 2nd group was pediatrics (≤ 18 years old) $n = 3$ (Table 1). Variables in our study included patients' age, gender, clinical features, bone marrow blasts percentage, and FAB classification were the variables that were studied. Overall survival (OS) and event-free survival (EFS) were assessed using Kaplan-Meier estimates.

2.1. Protocol of treatment

Patients with < 60 years of age were given the standard '3 + 7' induction chemotherapy i.e. doxorubicin was given for three days and its dosage was $30\text{ mg/m}^2/\text{day}$ while cytarabine was given for seven days intravenously for 24 h and its dosage was $100\text{ mg/m}^2/\text{day}$. Patients with age > 60 years having performance status ranging from 0 to 2 and minimal co-morbidity were given the same induction therapy while patients with performance status > 2 were received low dose Ara-c $20\text{ mg/m}^2/12\text{ h}$ for ten days during every month. Patients having M3 FAB-subtype were treated with All-trans retinoic acid and anthracycline. After initiation of chemotherapy, bone marrow aspiration of patients was done during 21–28 days. During the consolidation phase, three to four courses of cytosine arabinoside were given to the patients in high dosage i.e. 3 g/m^2 for every 12 h on 1st, 3rd, and 5th days; total, 18 g/m^2 . Following this, there was a follow up of all patients once during every three months and their clinical parameters were recorded.

2.2. Molecular analysis of RUNX1 exon 3

All samples (both control and patients) were analyzed for mutation of the RUNX1 gene using the polymerase chain reaction (PCR) method. The DNA was extracted from all blood samples by using a commercially available Thermo Scientific Genomic DNA Purification Kit. The Nanodrop UV/VIS Spectrophotometer was used to check the integrity of isolated DNA, then exon 3 of RUNX1 gene was amplified by using the previously reported primers; F: 5'-GA GCTGCTGCTGAAGATCC-3' and R: 5'-CAAGCTAGGAAGACC GACCC-3' (Gaidzik et al., 2011). Thermal cycling conditions were: 95°C for 4 min and 35 cycles of 2 min at 95°C , 1 min at 60°C 1 min at 72°C with a final extension for 7 min at 72°C .

Table 1

Acute Myeloid Leukemia (AML) patients' characteristics included in the present cohort.

Patients' characteristics		Patient n = 32(%)
Sex	Males	19 (59)
	Females	13 (41)
Age wise categories	Paediatrics (≤ 18 years)	3 (9)
	Adults (19–75)	29 (91)
Average age	30 years (16–70)	

2.3. Analysis of ITDs and expression of FLT3 gene employing Allele-specific polymerase chain reaction (ASPCR)

Total mRNAs were extracted from 32 AML patients with the help of TRIzol reagent. The Nanodrop UV/VIS Spectrophotometer was used to check the integrity of isolated mRNA, then the cDNA Synthesis kit was used to reverse-transcribe RNA to cDNA. The allele-specific polymerase chain reaction (ASPCR) was performed for the detection of ITD in the FLT3 gene with the help of previously reported primers i.e. F-14:5'-CTAACTGACTCATCATTCATCTCTGA-3' and R-155'-AAACATTTGGCACATTCATTC-3' (Burnatt et al., 2017). The GAPDH gene was used to make sure the equal loading of the sample by using the previously reported primers F: 5'-ACCA CAGTCCATGCCATCA-3' and R: 5'-TCCACCACCTGTGCTGTA-3' (Asad et al., 2012). Allele-specific Polymerase Chain Reaction (ASPCR) was used to identify internal tandem repeats in the FLT3 gene. Thermocycling conditions were: 95 °C for 4 min and 35 cycles at 95 °C for 2 min, 58 °C for 1 min and 72 °C for 1 min with a final extension for 7 min at 72 °C. Double bands have appeared on the gel for samples that were positive with FLT3-ITD while a single band was noticed for samples that were negative for this. To find the expression of the FLT3 gene in all AML patients (both mutants and non-mutants), the gel was further analyzed using different software i.e. ImageJ and GraphPad Prism 5.

2.4. Serology and protein quantification

The Control (10) and AML blood samples (32) were centrifuged at 5000 rpm for 7 min to separate the serum from the samples. Serum protein concentration in control and AML samples was estimated by Bradford assay as described previously (Bradford, 1976).

2.5. Statistical analysis

GraphPad Prism 5 and 9 were used for statistical analysis. For the comparison of different variables of the mutant and non-mutant groups, Fisher's exact test was performed. Kaplan-Meier survival analysis was used to calculate OS and EFS and compared

with the use of the 2-sided log-rank test. The OS was defined as the time duration from the time of AML diagnosis to the last follow-up/death. The EFS was the period from the time of AML diagnosis to failure of treatment/relapse/last follow-up/death. The level of significance was established at $P < 0.05$.

3. Results

3.1. Analysis of RUNX1 gene mutation

Sanger sequencing results of the RUNX1 gene (NM_001001890.3) exon 3, were aligned with the reference sequence to identify mutations in a particular exon. Results were analyzed with the help of BioEdit 7.2 software. In the present study, eight (25%) AML patients were observed to harbor the same heterozygous mutation K83E (rs121912498) at 247 nucleotide which changes Lysine amino acid into Glutamic acid. All samples of the control group had wild-type alleles (Fig. 1).

3.1.1. Correlation of RUNX1 mutation with clinical characteristics

The RUNX1 mutation was found to be associated with the male gender ($P = 0.034$) as compared to wild-type RUNX1. The RUNX1 mutants had higher median age (68.3 years) in comparison to the non-mutant group (54 years) ($P = 0.009$). A strong association of RUNX1 mutation was observed with high age ($P = 0.009$), elevated level of lactate dehydrogenase (LDH) ($P = 0.012$), and non-significant lower white blood cell count ($P = 0.09$) as compared to the non-mutant group. Patients harboring RUNX1 mutation had higher bone marrow blasts in contrast to non-mutants (68.6% vs 55.7%; $P = 0.036$) (Table 2).

3.1.2. Percentage of RUNX1 mutations in different FAB subtypes

The RUNX1 mutations were found in three AML patients having M0 FAB subtype (3 of 32; 9.4%) followed by M1 and M2 (2 of 32; 6.3% for both types) and M4 FAB subtype (1 of 32; 3.1%).

3.1.3. Effect of RUNX1 mutation on the prognosis of AML patients

The Kaplan-Meier analysis showed RUNX1 mutation to be a negative predictor of EFS ($P = 0.00322$) and OS ($P = 0.0367$). The EFS and OS rates were 34.3% and 58.3% respectively for RUNX1 mutant patients while it was 30.3% (EFS) and 65.1% (OS) for non-mutant patients (Fig. 2).

3.2. Analysis of Allele-specific polymerase chain reaction (ASPCR) and expression of FLT3 gene

To detect the presence of FLT3-ITD, Allele-Specific Polymerase Chain Reaction (ASPCR) technique was used. The FLT3-ITD was found in three samples (A19, A27, A30) (9.37%) having FAB-M2

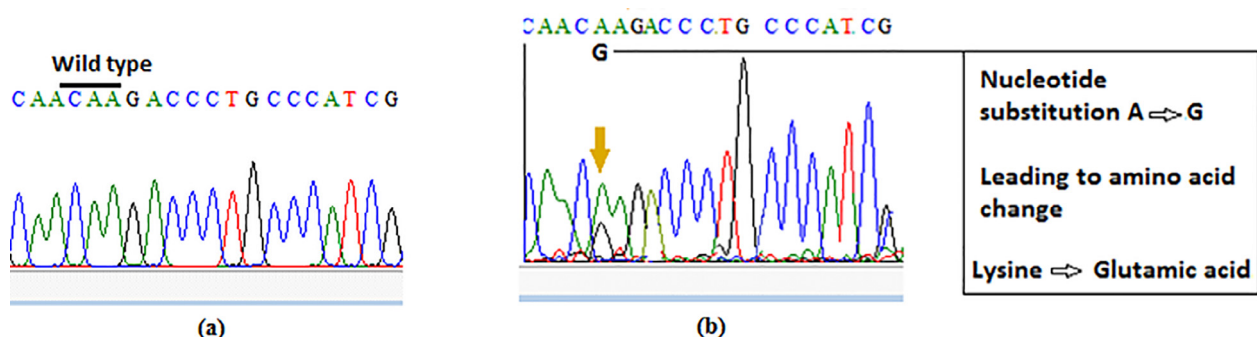


Fig. 1. Electropherogram showing (a) Wildtype sequence of RUNX1 and (b) mutant sequence showing a heterozygous mutation A > G that results into K83E protein change in eight Acute Myeloid Leukaemia patients. A: Adenine, G: Guanine.

Table 2

Clinical parameters of Acute Myeloid Leukemia patients included in present study (n = 32) (*=P < 0.05, **= P < 0.01).

Parameters	Patients with wild-type RUNX1	Patients with RUNX1 mutation	P-value
No. of patients	24	8	–
Mean age, years (range)	52 (25–82)	65 (52–79)	0.009*
Male gender, n.(%)	19 (59%)	7 (87.5%)	0.034*
Hemoglobin (g/dl), mean (range)	8.9 (5.3–14)	8.5 (4.7–11.5)	0.87
Platelets ($\times 10^3/\mu\text{l}$), mean (range)	48 (1–700)	37.6(8–1053)	0.93
TLC ($\times 10^3/\mu\text{l}$), mean (range)	35.4 (1.9 to 420)	12.5 (0.3 to 201)	0.09
LDH (U/L), mean (range)	456 (176–890)	620 (150–2987)	0.012**

g/dl = gram/decilitre, μl -microliter, U/L = units per liter, p-value = level of significance.

subtype and observed to be associated with high leucocyte count ($>400 \times 10^3/\mu\text{l}$) and high blast cells ($>70\%$) count (Fig. 3).

The gene expression analysis showed highly significant up-regulation in FLT3 of all AML samples [FLT3-wildtype (0.316 ± 0.05 folds) and FLT3-mutants (0.368 ± 0.03 folds)] in contrast of control group ($P = 0.0001$). The rate of complete remission (CR) was found to be inferior in FLT3-ITD positive AML patients than patients without this mutation ($P = 0.013$). The AML patients with wild-type FLT3 had lower CR rate (28.5%) as they were observed to

have higher FLT3 expression while the CR rate was 85% in patients showing low FLT3 expression. Follow-up showed a decreased level of FLT3 in AML patients when they achieve complete remission (Fig. 3).

The FLT3 gene expression analysis showed significant elevation in RUNX1 mutants as compared to non-mutant patients [RUNX1-wild-type (0.659 ± 0.03 folds) and RUNX1-mutants (0.825 ± 0.02 folds)]. The complete remission (CR) rate was worse in RUNX1 mutant patients having higher FLT3 expression than in RUNX1 mutant patients with comparatively low levels of FLT3 gene expression ($P = 0.043$). Detection of FLT3-ITD and quantification of FLT3 mRNA expression level in AML patients may serve as an indicator to assess the efficiency of therapy, predict treatment outcome, and examine minimal residual disease (Fig. 3).

3.2.1. Effect of FLT3-ITD mutation on the prognosis of AML patients

Kaplan-Meier estimates were calculated to show the poor prognosis of FLT3-ITDs on overall survival of AML patients which showed FL-ITD as a strong negative predictor of EFS ($P < 0.0001$) and OS ($P < 0.03$). The 2-year EFS and OS rates for FLT3 mutated patients were 33.3%, and 66.7% respectively while it was 31.1% (EFS) and 72.5% (OS) for patients with wild-type FLT3 (Fig. 4).

3.3. Serum protein quantification through Bradford assay

All AML samples were observed to have a very low concentration of protein as compared to healthy individuals ($P = 0.0004$).

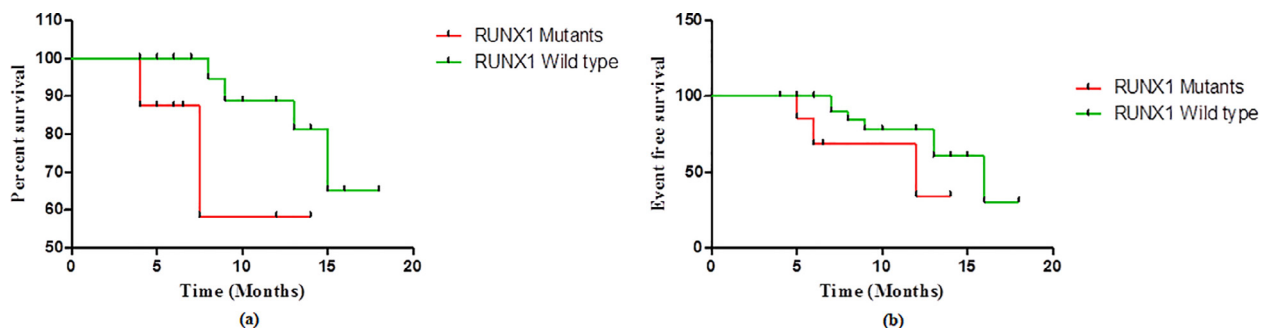


Fig. 2. Effect of RUNX1 mutation on prognosis of AML patients. Kaplan-Meier estimates showed low (a) Overall survival, (b) Event free survival in RUNX1 mutants as compared to non-mutants.

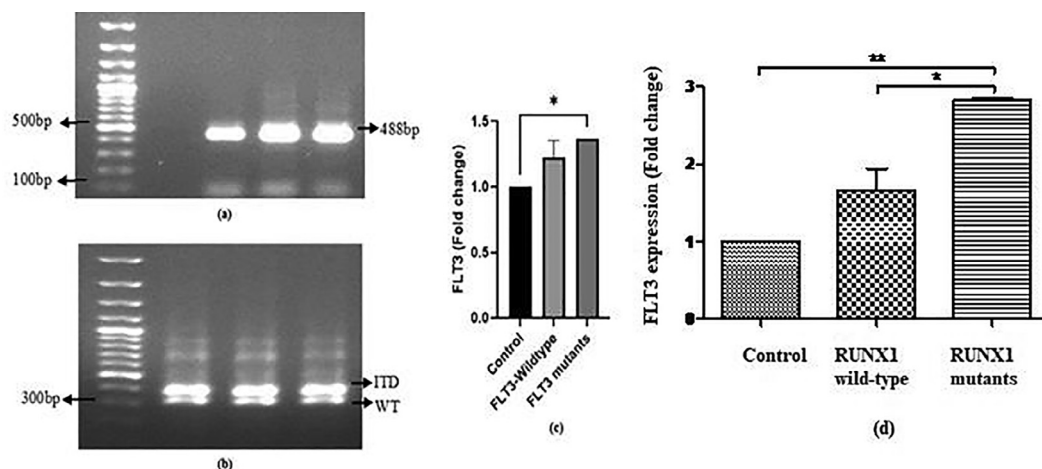


Fig. 3. (a) Image showing densitometric analysis showing gene expression of GAPDH in Control and Acute Myeloid Leukemia samples, (b) Representative diagram of densitometric analysis showing FLT3 gene expression and FLT3-ITD mutation detection in acute myeloid leukemia patients on 1.2% agarose gel (85 V:45 min). ITD: Internal tandem duplication, WT: wildtype, (c) Result showing significant (*=P < 0.05) upregulation in gene expression of FLT3 in AML samples in contrast of control group and (d) Result showing highly significant (*=P < 0.05, **=P < 0.01) elevation in expression of FLT3 mRNA level in RUNX1 mutants as compared to wild-type RUNX1 group.

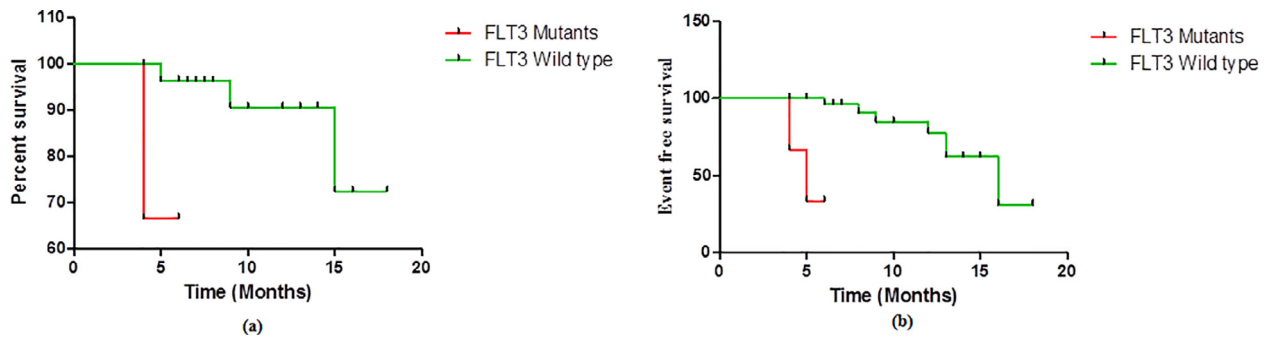


Fig. 4. Effect of FLT-ITD mutation on prognosis of AML patients. Kaplan-Meier estimates showed low (a) Overall survival, (b) Event free survival in FLT3 mutants as compared to non-mutants.

The RUNX1 and FLT3 mutant and non-mutant groups had very low protein concentration in contrast to the healthy individual's group ($P < 0.0001$ and $P = 0.0092$ respectively). Protein concentration was

down-regulated in RUNX1 and FLT3 mutants in comparison of RUNX1 and FLT3 wild-type groups but this decrease was not up significant level (Fig. 5 & Table 3).

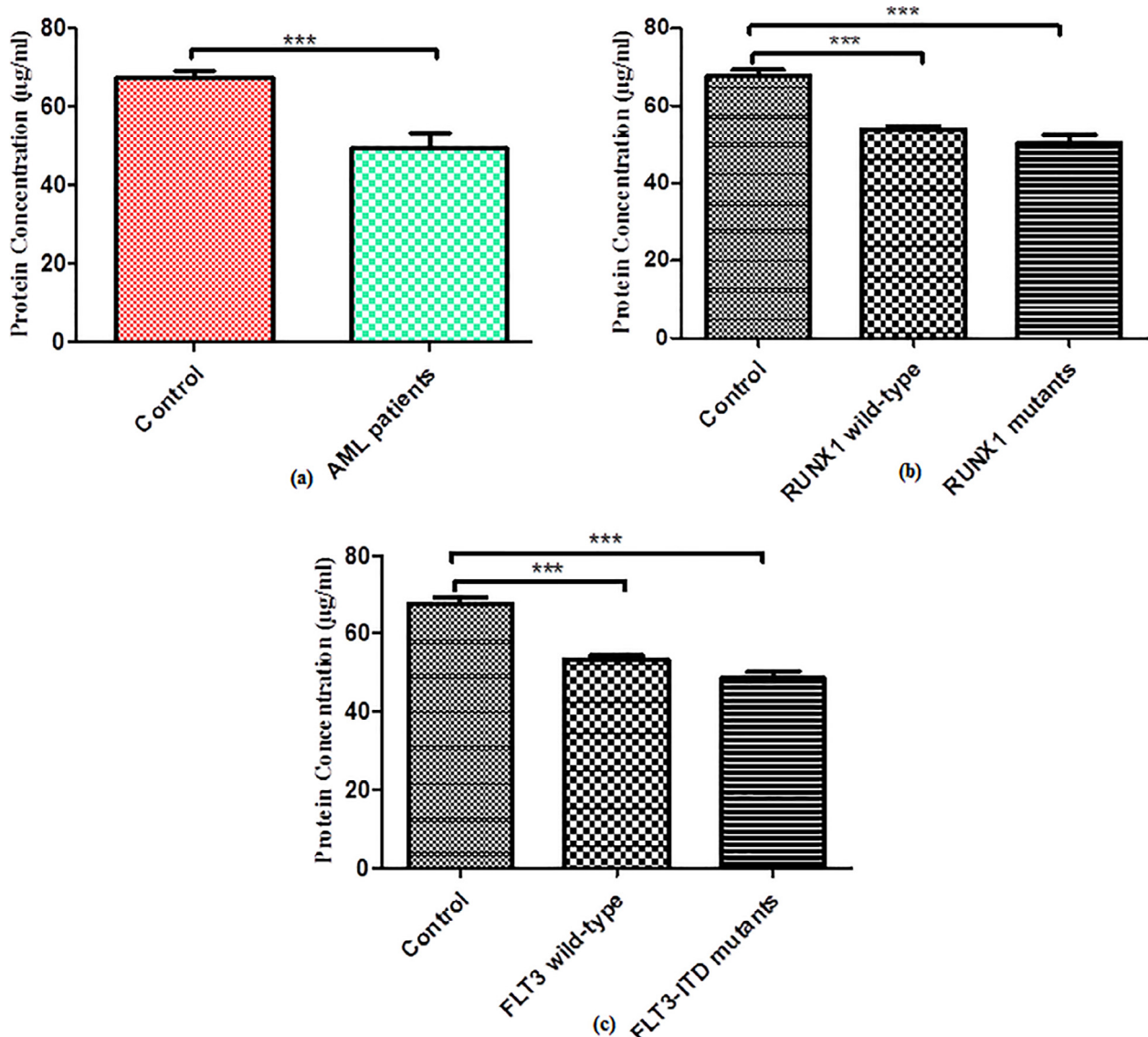


Fig. 5. (a) Highly significant down-regulation of Serum protein conc. (µg/ml) in Acute Myeloid Leukemia patients in comparison of control group (*= $P < 0.05$, **= $P < 0.01$ and ***= $P < 0.001$), (b) Highly significant down-regulation of serum protein conc. (µg/ml) in RUNX1 wild-type and RUNX1 mutant patients in comparison of control group (*= $P < 0.05$, **= $P < 0.01$ and ***= $P < 0.001$) and (c) Highly significant down-regulation of serum protein conc. (µg/ml) in FLT3 wild-type and FLT3 mutant patients in comparison of control group (*= $P < 0.05$, **= $P < 0.01$ and ***= $P < 0.001$).

Table 3

Spectrophotometric determination of serum protein concentration in different groups of Acute Myeloid Leukemia Patients included in present cohort (* = $P < 0.05$, ** = $P < 0.01$ and *** = $P < 0.001$).

Concentration of protein ($\mu\text{g/ml}$)				
Genes	Control (Mean $\pm$ S.E.M)	Mutants (Mean $\pm$ S.E.M)	Wildtype (Mean $\pm$ S.E.M)	P-value
RUNX1	67.70 $\pm$ 1.564	53.88 $\pm$ 0.859	50.35 $\pm$ 2.173	<0.0001***
FLT3	67.70 $\pm$ 1.564	63.46 $\pm$ 0.903	48.49 $\pm$ 1.739	0.0092**

$\mu\text{g/ml}$ = -microgram per milliliter, S.E.M = standard error of mean, P-value = level of significance.

4. Discussion

In our study including homogeneous and uniformly treated CN-AML patients, RUNX1 mutation was found in 25% of patients higher than the frequency reported previously (Gaidzik et al., 2011; Tang et al., 2009) who only studied patients below the age of 60 years. The RUNX1 mutations were found to be associated with the male gender which is in the harmony with prior studies indicating its highest ratio in male AML patients having the FAB-M0 subtype (Mendler et al., 2012). Three patients (9.37%) were found to harbor ITDs in the FLT3 gene having the FAB-M2 subtype. Among FAB subtypes, FLT3 mutations were more commonly reported within the M2, M3 subtypes (Schnittger et al., 2000). The overall frequency of FLT3 mutations in our study (9.37%) was less than the previous studies (Levis and Small, 2003; Stirewalt and Radich 2003) which might be attributed to the small sample size of our study. In our cohort, FLT3-ITD and RUNX1 mutations were mutually exclusive as two FLT3-ITD positive patients were also RUNX1 mutants (Gaidzik et al. 2011; Schnittger et al., 2011; Tang et al., 2009). One AML patient who did not harbor FLT3-ITD at diagnosis has been found to acquire it at the time of relapse raises the significance of evaluating ITDs in the FLT3 gene early.

Our study shows that RUNX1 and FLT3 mutations are a significant indicator of poor prognosis in both younger and older CN-AML patients. Our data are in harmony with previously published data as it showed that FLT3-ITD mutation is found to be associated with the high relapse rate, shorter OS, and EFS in CN-AML patients who harbor the aforementioned anomaly as compared to the patients having wild-type FLT3. The FLT3-ITD is found to be associated with high leukocyte count ($>400 \times 10^3/\mu\text{l}$) and high blast cell ($>70\%$) count as mentioned previously (Xu et al., 2012). The patients having RUNX1 mutation were observed to show poor response to intensive induction therapy and only three RUNX1 mutated patients (37.5%) were able to get CR but 2 RUNX1 mutated patients who achieved CR died within 7.5 months after getting relapse phase confirming other findings who report RUNX1 mutation to be associated with shorter EFS and OS in CN-AML patients (Tang et al., 2009).

The gene expression analysis showed highly significant up-regulation in the FLT3 level in all AML samples specifically in RUNX1 mutated patients. Previously, it had been shown that high FLT3 expression and FLT3-ITD is associated with poor outcome of AML patients exhibiting their essential function in the differentiation block (Radomska et al., 2006; Zheng and Small, 2005). But it has been confirmed by earlier studies that high expression of FLT3 is alone not sufficient to induce leukemogenesis (Reckzeh et al., 2012). However, the expression of RUNX1 protein is influenced by FLT3 expression and FLT-ITD by the FLT3 signaling pathway. It means elevated FLT3 level and disturbed RUNX1 protein level together synergize to cause AML. High FLT3 protein levels might further induce alteration in RUNX1 protein level by several different mechanisms. Previously, it has been shown that FLT3 signaling affects the RUNX1 protein level by the process of phosphorylation indicate that high expression of FLT3 elevates gene expression of RUNX1 directly (Cauchy et al., 2015).

The variations and mutations in amino acids are genetic causes of human diseases. Mutations can affect stability and protein folding (Alfalah et al., 2009; Ode et al., 2007; Koukouritaki et al., 2007), protein function (Yamada et al., 2006), and interactions of one protein with the others (Jones et al., 2007; Ung et al., 2006), as well as protein expression and subcellular localization (Krumbholz et al., 2006; Tiede et al., 2006). Mutations in proteins have an important role in the onset and development of cancer (Hanahan and Weinberg, 2000). By changing a gene's instructions to make a protein, a mutation can cause the protein to malfunction or to be missing entirely. The protein function can be affected by the direct impact of a mutation on a protein. These consist of a change in the interaction of the protein with other biomolecules, like other proteins or DNA or RNA or lipids, or change in the interaction with ligands i.e. enzyme substrates. Also, the variations in protein stability, e.g. destabilization leading to higher degradation rates changed protein concentration are the possible effects of gene mutations (Reva et al., 2011). We observed low serum protein concentration in our both mutant groups (RUNX1 and FLT3 mutant groups) in comparison with non-mutant AML subjects. This might indicate the mutational impact on overall serum protein concentration.

5. Conclusion

At present no sufficient data exist stating correlation of RUNX1 and FLT3 mutations with up-regulated FLT3 gene expression in Pakistani AML patients. Summarizing the finding of this investigation, we give further confirmation of the poor prognosis of RUNX1 and FLT3-ITD mutations in uniformly treated CN-AML patients. Our results will give insight in defining alternative treatment strategies for AML patients having RUNX1 mutations and up-regulated FLT3 gene expression. Our study also showed the significance of FLT3-ITD assessment in all relapsed AML patients as one patient who did not have FLT3 mutation at the time of diagnosis has been found to acquire it during relapse. Early identification of FLT3-ITD might thus be helpful for this group of patients to overcome poor prognosis.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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