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Prognostically Significant Fusion Oncogenes and Gene Mutations in Pakistani AML Patients

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Abstract

Background: The onset and progression of leukemia is associated with many genetic abnormalities including gene mutations and production of fusion oncogenes. Molecular studies on fusion oncogenes and mutations in different populations have been done. However, not much research on correlation of the fusion oncogenes with acute myeloid leukemia have been done in Pakistan.

Methods: Genetic analysis of 105 AML patients was done to investigate *AML1-ETO* and *CBFB-MYH11* fusion oncogenes and mutations in *NPM1* and *NRAS* genes. The genomic DNA and cDNA were subjected to amplification, electrophoresis, and Sanger sequencing.

Results: The frequency of *AML1-ETO* was 26% in AML patients and 34.2% in AML-M2 patients. *CBFB-MYH11* was present in 11.4% AML patients. A total of six mutations in 4 regions of *NPM1* gene and 2 regions of *NRAS* gene were detected. 3'UTR of *NPM1* gene had three variants; g.1128C>T (57.1%), g.1185-/T insertion (80.95%), and g.1163A>T (57.14%) while c.867_871subGTGGA>CAAGTTTGC (2.86%) was present in exon 12. *NRAS* gene had two mutations c.12C>T (51.4%) and c.33A>T (11.43%) in exon 2. c.867_871subGTGGA>CAAGTTTGC, and g.1163A>T in *NPM1* gene and c.33A>T in *NRAS* gene were the novel findings in this population.

Conclusion: This genetic analysis may help to modulate the treatment strategies and improve survival of patients.



Introduction

Acute myeloid leukemia (AML) is a form of white blood cell cancer which is characterized by penetration of myeloid, blood, or other tissues by multiplication, aberrant differentiation or badly altered cells [1-3]. Among leukemia patients, AML is the most prevalent in adults with an average 68 years of diagnostic age. While outcomes in the younger age group have increased remarkably, the prognosis in older patients appears to be very poor [4]. AML is responsible for 15-20% of leukemia cases. As has been reported, AML in older patients is more likely to be complemented by a myelodysplastic process. It has unfavorable cytogenetic mechanisms which may also demonstrate multidrug resistance [4,5].

Being an exceedingly complex stem cells disease, AML can be associated with various cytogenetic defects and molecular mutations. Many pathogenetic processes involving replication, apoptosis, mutation, and DNA repair could have been involved in these anomalies. It is not triggered by a single genetic factor, but rather the result of multiple genetic transformations [6,7]. The genetic abnormalities may disrupt functions of hematopoietic transcription factors encoding genes or give rise to fusion oncogenes that are strongly correlated and differ significantly with respect to disease severity and treatment outcomes [8-10].

During several years of studies, numerous structural and functional effects of various types of translocations have been studied such as the ones causing chronic myeloid leukemia [3]. Similar chromosomal translocations facilitate the production of proteins in AML that are chimeric fusions. Fusion genes like *BCR-ABL*, *MLL-AF4*, *SIL-TAL1*, *TEL-AML1*, *E2A-PBX1*, *CBFB-MYH11* and *AML1-ETO* are associated with various types of leukemias including AML [11,13,14]. The fusion oncogenes *BCR-ABL*, *MLL-AF4*, *ETV6-RUNX11*, *E2A-PBX1* and *SIL-TAL1* genes have been investigated by Sabir et al. [11] which demonstrated the role of these fusion oncogenes among Pakistani population in the onset of AML. Iqbal et al. [12] reported that Pakistani leukemia patients substantially differ from western populations with respect to the frequencies of different classical fusion oncogenes.

Genome sequencing studies of AML patients show it to be an intricate and diverse disease characterized by the existence of numerous leukemic genes, some repeatedly and other uncommonly mutated [13]. Different subtypes of mutations have been recognized which have broadly been classified into two groups; Class I mutations include activated signal-transduction pathways, enhancing the expansion or existence of hematopoietic precursor cells which include the mutations in *FLT3* and *RAS* gene family, while class II mutations affect transcription factors and impede

differentiation which include mutations in *CEBPA*, *MLL*, and *NPM1* [15-18]. Clinical characteristics and genetic analysis of about 45% of all AML cases among a wide subgroup of AML patients having a normal karyotype has been carried out. In the case of AML, translocation t(8;21) leads to the generation of *AML1-ETO* fusion oncogene which has been shown to influence differentiation, proliferation and apoptosis in both in-vitro and in-vivo models having association with a favorable prognosis [19]. Moreover, inv(16)(p13q22) rearrangement resulting in *CBFB-MYH11* fusion oncogene is found in approximately 10% of cases with de novo AML [20].

RAS genes regulate cellular proliferation by binding of a ligand to a variety of receptors and regulate cellular proliferation by cycling between inactive state i.e. (GDP) and active state (GTP). The *RAS* gene activates phosphorylation of activated mitogen protein kinase (MAPK) or (RAF) leading to cellular proliferation. So, if the *RAS* is mutated there will eventually be enhanced cellular proliferation [21, 49]. These pathogenetic mechanisms suggest that the presence of *NRAS* may have a prognostic significance. Patients harboring *NRAS* acquired two mutations in the coding sequence in exon 2 only. Activation of *NRAS* mutations plays a decisive role in oncogenesis in a large percentage of AMLs. *NRAS* mutations are the most common, occurring in 10–20% of AML patients [22].

NPM1 is a major stress-induced regulator of *p53* which functions because of cytotoxic drugs, hypoxia, and UV irradiation [23, 24]. Mutations in *NPM1* (exon 12) gene on chromosome 5q35 lead to frame shift and production of an elongated protein, which remains in the cytoplasm [35]. *NPM1* mutations relate to higher leukocyte counts [25-27, 35]. Mostly, *NPM1* mutations occur almost exclusively in de novo AML cases. Mutations in *NPM1* exon 12 are found in numerous patients with AML mostly having normal karyotype [35].

This study is designed to carry out genetic analysis of AML patients to figure out the genetic factors contributing to its development in Pakistani population. The nature and frequencies of classical fusion oncogenes, *AML1-ETO* and *CBFB-MYH11* in Pakistani AML patients, their comparison with other ethnic groups, presence of mutations in *NPM1* and *NRAS* genes and the correlation of the mutation data with clinical features must be investigated. This may help in understanding the disease outcomes which may be efficient and cost effective in terms of treatment and diagnostic protocols.

Methods

To conduct this study, the approval was taken from the ethical committee at the Institute of Zoology, University of the Punjab, Lahore. Patient recruitment proforma was formulated, patients were recruited, and informed consents signed from the participants. Blood samples were collected from 105 AML patients from August 2013 to December 2014 from Jinnah hospital, Mayo hospital and Institute of Nuclear medicine and oncology (INMOL) Lahore. The experimental work was completed in parasitology lab and central lab, Institute of Zoology, University of the Punjab, Quaid-e-Azam campus Lahore, Pakistan.

Blood was drawn from each AML patient and transferred to properly labelled EDTA coated vacutainer tubes. Screening tests were done to exclude patients having other infectious diseases. Genomic DNA and mRNA were extracted within 24 hours of blood collection and stored at -40 °C. All equipment was sterilized properly, the reagents were made using double distilled water and autoclaved. Light sensitive reagents were covered with aluminum foil and kept properly.

Nucleic Acids Extractions

Genomic DNA was isolated from whole white blood cells by using both the manual DNA isolation protocol using TRIzol reagent as well as kit-based isolation protocol (Promega Wizard Genomic DNA purification kit (Cat#A1120) according to the manufacturer's instructions depending upon the volume of the blood obtained from each AML patient. Both methods were properly optimized. Manual method of DNA extraction DNazol [29] was used for blood more than 3 mL to obtain high quality yield while kit-based method was used for DNA extraction from blood less than 3 mL to obtain maximum yield.

For RNA isolation from the whole blood, PBMC pellet was isolated first. The RNA extraction procedure was adopted from previous published studies [30,31]. For this purpose, blood with anticoagulant was aliquoted in Eppendorf tubes and cell lysis solution (Gentra USA) was added. The tubes were vortexed, left at room temperature, and centrifuged. The process was repeated for the complete lysis of RBCs and appearance of a pellet. The PBMC pellet was dissolved in the lysis solution which was followed by the RNA extraction procedure using TRIzol reagent.

Primer Designing

Sequences of selected human genes were retrieved from NCBI which were then BLAT (genome.ucsc.edu) and whole gene intronic and exonic sequences were obtained. Exon primer pairs were designed after preliminary analysis of specific human genes through bioinformatics tool (www.expasy.ch) for oncogenes and (<https://primer3.org/>) for selected *NPM1* and *NRAS*

(table 1,2). The primers were 20-22 nucleotides long with ~50% GC content and minimal complementarities to avoid dimer formations.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

cDNA was prepared using Fermentas kit (K1622) using the manufacturer's instructions. The RT-reaction protocol and thermal conditions were optimized. The cDNA was amplified by the nested PCR with optimized conditions.

Agarose Gel Electrophoresis

Genomic DNA and PCR products were visualized by agarose gel electrophoresis. 1% agarose was used to visualize genomic DNA and 2% for PCR products. The gel was observed under UV light to analyze the DNA. DNA was observed as fluorescent band and photograph was obtained by Fisher Scientific UVP PhotoDoc-It™ Imaging System.

DNA/RNA Quantitative and Qualitative Assessment

Quality of DNA/RNA was assessed by native agarose gel electrophoresis. The gel was observed under UV light. DNA was observed as fluorescent band and photograph was obtained by UVP PhotoDoc-It™ Imaging System. For the quantification of extracted genomic DNA NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) was used. The quantity was estimated, and DNA was diluted accordingly.

Sequencing Analysis of PCR Amplicons

The facility for direct sequencing was provided by Centre of applied molecular biology (CAMB), University of the Punjab, Lahore. The Nested RT-PCR products of fusion oncogenes and amplified PCR products of genomic DNA were subjected to direct sequencing analysis for detection of any possible mutations present in *NRAS* and *NMP1* genes of AML patients. Standard procedure for BigDye® Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems, USA was used as per manufacturer's instructions.

Analysis of Sequence Data

The sequenced templates were analysed using sequencing analysis software Geneious R7 [55]. The DNA sequences of *NMP1* and *NRAS* genes of healthy individuals were aligned against the reference sequence (NCBI GenBank accession number M14772.1). Polymorphic variations in our population were analysed to avoid artefact findings with point mutations within AML patient's sequences. Moreover, the gene sequences representing heterozygous mutations and those with noise in data were repeated for the whole procedure, to ensure the results are accurate and reproducible.

Gene	Mutation Type	Exon No. (Nucleotide change)	Genomic position	Known ID	No. of cases (%) n=105	Frequency
<i>N-RAS</i>	Coding Sequence variant (Synonymous Substitution)	Exon 2 (c.12C>T)	1:114716148	COSMIC_MUTATION COSM6981755 (Known CA>TT)	54 (51.4%)	0.514
<i>N-RAS</i>	Coding Sequence variant (Synonymous Substitution)	Exon 2 (c.33A>T)	1:114716128	Novel	12(11.43%)	0.114
<i>NPM1</i>	3'UTR Variant	g.1128C>T	5:171410673	rs1052205641	60 (57.1%)	0.571
<i>NPM1</i>	3'UTR Variant	g.1185-/T Insertion	5:171410729_171410730insT	rs776882863	85(80.95%)	0.809
<i>NPM1</i>	Coding Sequence variant (Substitution-Frameshift)	Exon 12 c.867_871subGTGGA > CAAGTTTGC	5:171410546	Novel COSM20859 at same location (c.867_868insTCCA (Insertion))	3(2.86%)	0.028
<i>NPM1</i>	3'UTR Variant	g.1163A>T	5:171410708	Novel rs1031458634 (Known g.1163AsubC)	60(57.14%)	0.571

Table 1: Frequency distribution in various mutations detected in *NRAS* and *NPM1* genes in Pakistani AML patients.

Characteristics	Mutations in <i>NPM1</i> and <i>NRAS</i>					
	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6
Positive (N)	54	12	60	85	3	60
Percentage	51.4	11.43	57.1	80.95	2.86	57.1
p-value (sig=0.05)						
Platelets (Mean)	0.998	0.058	0.748	0.847	0.458	0.289
Hb Levels (Mean)	0.870	0.324	0.659	0.441	0.265	0.933
Total Leukocytes (Mean)	0.755	0.285	0.726	0.891	0.474	0.317
Fever (°F) (mean values)	0.307	0.224	0.132	0.80	0.340	0.502
Age	0.952	<0.001	0.727	0.854	0.376	0.206
Gender	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

Table 2: Correlation of *NPM1* and *NRAS* gene mutations with various clinical parameters.

Statistical Analysis

The IBM SPSS statistics, version 22 and Graph pad Prism were used for statistical analysis. The demographic characteristics of patients were analyzed statistically. For all analyses, a p-value of less than 0.05 was considered statistically significant. The continuous and categorical data was analyzed by independent sample t-test and correlation tests.

Results

The Clinical data of 105 AML patients under investigation was arranged according to clinical characteristics and response outcomes. Incidence of AML was more in males (54.8%) than females (45.2%). Majority of patients in the present study were adults. Median age of Pakistani AML patients was 33.5 years with a range (13-72 years). High grade fever was the most frequent clinical characteristic. Weight loss, organomegaly, fatigue, and muscle pains were other important diagnostic features. Complete blood count revealed that 78.09% AML patients had normal total leukocyte count, but others had elevated TLC count. 97.14% individuals had normal platelet count. Mean corpuscular hemoglobin (MCH) within the normal range (26-32 pg) was observed in 96.19% in AML patients. Decreased level of haemoglobin in blood was presented by 90.47% individuals. 54.7% individuals were in chronic phase, 28.7% in accelerated phase and

16.6% in blast phase of disease. Mutational analysis indicated the presence of fusion oncogenes and mutated *NRAS*, and *NPM1* genes were found in various samples. Colocalization of several mutations was found in some samples.

Patient characteristics and detection of *AML1-ETO* transcripts

AML1-ETO transcripts were detected as 395 bp in the first and 260 bp in the second PCR product, which was found in 26.6% individuals. *AML1-ETO* transcripts in M2 were detected in 34.2%. *GAPDH* was used as an internal control (Fig 1 & 2). A comparison of clinical parameters such as age, gender, hepatomegaly, splenomegaly, and extramedullary tumefaction between *AML1-ETO* positive groups in AML patients was made but no significant difference was found.

Patient characteristics and detection of *CBFB-MYH11* transcripts

CBFB-MYH11 transcripts were detected as 180 bp in the first and 280bp in the second PCR product, which was found in 11.4%. *GAPDH* was used as internal control. 4.7% AML- M2 patients were present with gender ratio being 3:2 (M:F) and the median age 18 years. *CBFB-MYH11* transcripts in M2 were not detected (figure 3).

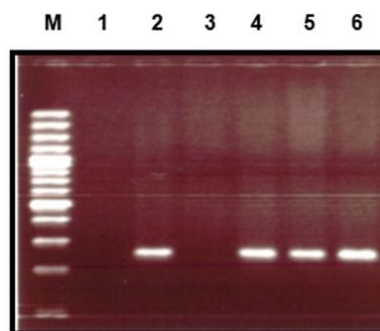


Figure 1: *AML1-ETO* transcript of 395 bp detected by RT-PCR by using 1Kb DNA ladder.



Figure 2: AML1-ETO transcript of 260 bp detected by RT-PCR by using 1kb DNA ladder.

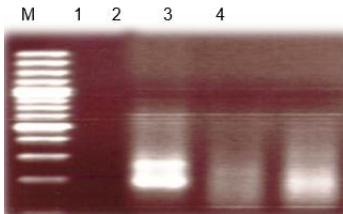


Figure 3: CBFB-MYH11 transcript detected by RT-PCR by using 1kb DNA ladder.

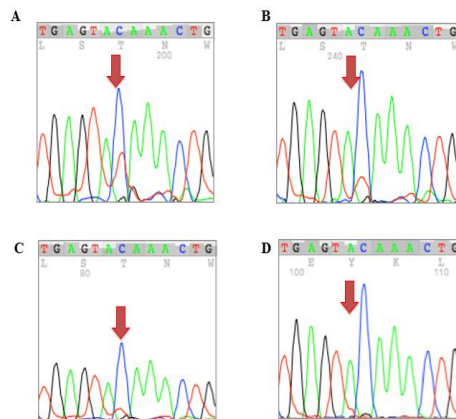


Figure 4: Mutation c.12C>T in Exon 2 at 12 nucleotide base pair in A,B,C chromatogram as compared to wild type D chromatogram.

Neuroblastoma *RAS* (*NRAS*)

Two alternations were observed in the *NRAS* gene, a substitution of nucleotide C with T (c.12C>T) in Exon 2 at nucleotide 12 in 54 (51.4%) AML patients which was a known mutation while the other was the replacement of A with T (c.33A>T) in Exon 2 at nucleotide 33 in 12 (11.43%) AML patients (Fig. 2). The *NRAS* mutations had a higher prevalence of adults (n=53) predominantly males (83.33%) as compared to few affected females (14.81%). Significant correlation of SNP1 was found with male and female ratio with 0.001 p-value. Other factors including platelet count, Hb levels, TLC count, fever, and age were not significantly correlated. SNP2 had no significant correlation with biochemical parameters. However, it was significantly correlated (p=0.001<0.05) with gender and age.

Nucleophosmin1 (*NPM1*) 3'UTR Variant mutation

Three types of mutations in 3'UTRs of *NPM1* gene were observed in the patients. Replacement of nucleotide C with T (g.1128C>T) at genomic position 5:171410673 was found in 60 (57.1%) AML patients. The p-values for

platelet count, Hb levels, total leukocytes, fever, and age were non-significant for SNP3 whereas gender ratio had significant correlation with the positive AML patients with more males being affected as compared to females.

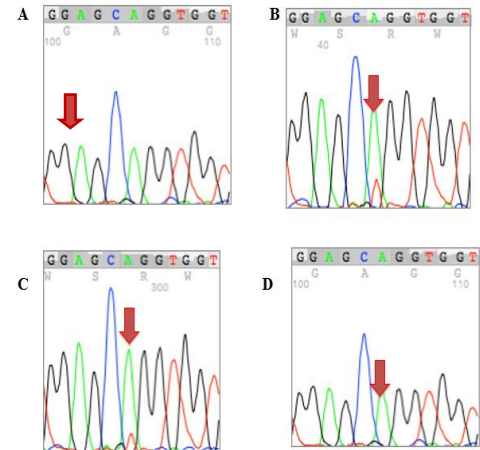


Figure 5: Mutation c.33A>T in Exon 2 at 33 nucleotide base pair in A,B,C chromatogram as compared to wild type D chromatogram.

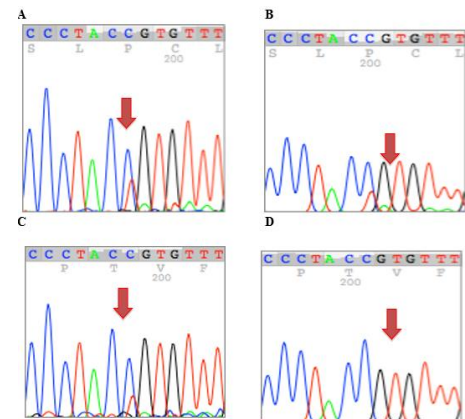


Figure 6: Translocation mutation in 3'UTR of *NPM1* g.1128C>T resulting in homozygous mutant in A chromatogram, heterozygous mutant in B,C chromatogram and wild type in D chromatogram.

Insertion of 'T' (g.1185-/T Insertion) at genomic position 5:171410729_171410730insT was the most frequently occurring mutation that was present in 85 (80.95%) AML patients. Platelet count, Hb levels, total leukocytes, fever, and age indicated no correlation between SNP4 found in AML patients whereas gender indicated significant correlation with a higher prevalence of affected males than females (fig 3). Replacement of A with T (g.1163A>T) in nucleotide 1163 at genomic position 5:171410708 was found in 60 (57.1%) AML patients. No significant correlation of SNP6 was found with platelet count, Hb levels, total leukocytes, fever, and age however, significant correlation was found with gender (p-value 0.001<0.05)

with equally affected individuals of different age groups but a higher ratio of affected males than females.

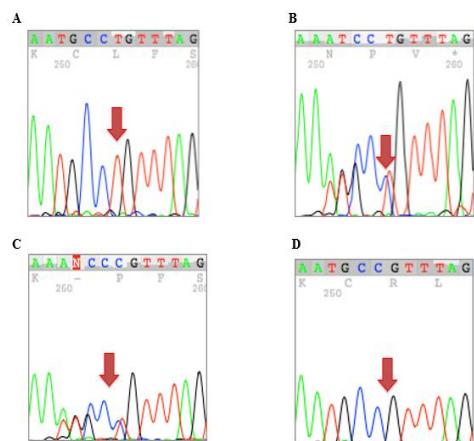


Figure 7: g.1185-/T Insertion mutation in 3'UTR of NPM1 represented by A,B,C chromatogram and wildtype by D chromatogram.

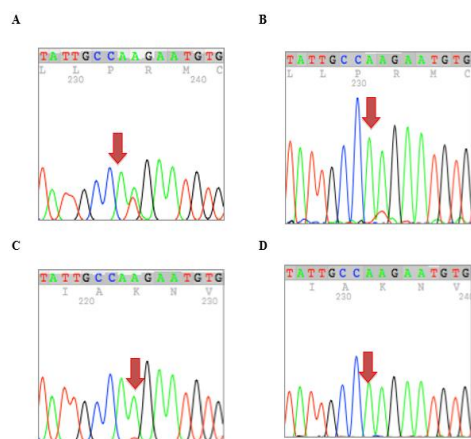


Figure 8: Substitution mutation in 3'UTR of NPM1 g.1163A>T in A,B,C chromatogram and wildtype in D chromatogram

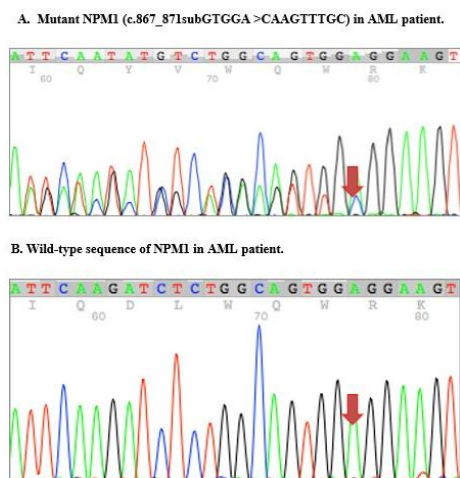


Figure 9: (A) c.867_871subGTGGA >CAAGTTTGC mutation in NPM1 gene of AML patients; (B) Wild type sequence of NPM1 in AML patients.

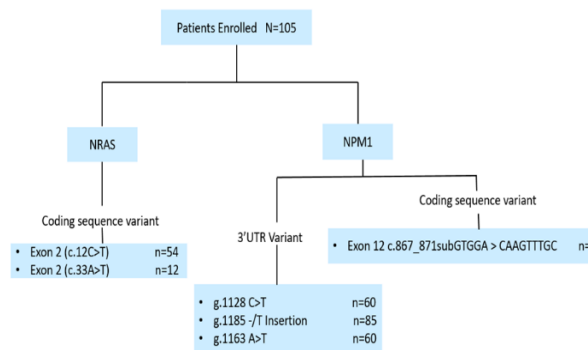


Figure 10: Different mutations detected in coding sequence and 3'UTR of NPM1 and NRAS genes in variable no. of individuals out of total 105 investigated AML patients.

Nucleophosmin1 (*NPM1*) Gene coding sequence mutation

Replacement of the sequence GTGGA with CAAGTTGC c.867_871subGTGGA >CAAGTTTGC in exon 12 of *NPM1* gene in 3 (2.86%) AML patients was a novel finding in this population. SNP5 showed significant correlation with gender ratio with greater number of males affected ($p\text{-value}=0.001<0.05$) while other parameters were not correlated.

Discussion

Genetic, molecular, and biochemical studies on the onset and progression of leukemia have indicated genetic abnormalities involving oncogene mutation and fusion oncogene production to be the major cause of leukemia. The prevalence of different fusion oncogenes and chromosomal abnormalities associated with leukemia vary in different ethnic groups [32,33]. These studies are aiding in devising strategies for differential diagnosis, prognosis, treatment, and management of leukemia. Pakistani leukemia patients differ from other populations with respect to the frequencies of different classical fusion oncogenes. However, data has not been provided on chromosomal changes and the clinical correlation of the fusion oncogenes [12].

AML is a malignant bone marrow disease wherein hematopoietic precursors are arrested in an early stage of progression. It differs from other related blood disorders by the occurrence of more than 20% blasts in the bone marrow [12,15]. Acute leukemias are characterized by recurring chromosomal aberrations and gene mutations. The pathogenesis of disease at molecular level is not fully understood and treatment grading is complex, especially for patients who have seemingly normal karyotype. Gene mutations have played an important role as biological and diagnostic markers in the treatment of AML. It is anticipated that molecular classification of AML not only allows accurate analytical predictions in numerous patients

but also opens future helpful treatment options for the patients [15].

Acute leukemia associated fusion protein *AML1-ETO* is a product of the chromosome translocation (8; 21) frequently occurring in AML [34]. This fusion oncoprotein blocks leukemia cell differentiation and induces growth arrest with increased sensitivity to apoptosis induction. The inv(16)(p13q22) rearrangement results in a *CBFB-MYH11* fusion gene [14]. In our study, the frequency of *AML1-ETO* fusion oncogene was 26.6% while frequency of *CBFB-MYH11* was 11.4% among the investigated AML patients. The frequency of *AML1-ETO* fusion oncogene in sub-type AML-M2 was 34.2%. The frequency of *AML1-ETO* and *CBFB-MYH11* fusion oncogenes were significantly high in age between 30–59 years.

Mutations in *NPM1* gene on chromosome 5q35 lead to frame shift and production of an elongated protein, which occurs in about one third of AML patients [35,36]. It is one of the 3 most common mutations in AML and relates to higher leukocyte counts especially in the presence of *FLT3-ITD* fusion oncogene [35]. *NPM1* mutations may occur in de novo AML cases or can be co-expressed with *RUNX1-RUNX1T1* transcripts [37,38]. Similar results of *NPM1* gene mutations on exon 12 were found in this study. The most frequently detected mutation in Pakistani AML patients was *NPM1* 3'UTR Variant (g.1185-/T Insertion), found in 80.95% of the investigated patients while the other 3'UTR variants were g.1128C>T and g.1163A>T with a prevalence rate of 57.1% each. g.1163A>T was a novel mutation in this ethnic region. c.867_871subGTGGA>CAAGTTTGC was another novel mutation in the exon 12 of Pakistani AML patients with a prevalence rate of 2.86%. The probable cause of the onset of AML due to mutations in the 3'UTR be the modification of gene expression or uncontrolled production of abnormal proteins.

A study conducted by Bhattacharyya et al. [39] in India demonstrated 27.9% prevalence of *NPM1* in AML patients, while it was 27.5% in Germany [35], 15.4% in China [40], 26.3% in Thailand [41], 7% in South Africa [42], 21% in Egypt [43], and between 19.1%–20.3% in the AML patients in Bulgaria [38]. These statistics show varying prevalence of *NPM1* gene with varying geographic areas with prevalence rates often higher than 20% in Asia.

This study also showed a disturbance in leukocyte count in AML patients with *NPM1* mutation. Hb levels and platelet count were dysregulated with *NPM1* mutation in a study by Yi et al. [44] in the AML patients but no dysregulation was found in this study. According to a study, *NPM1* mutations were higher in male patients than females [45] which was contradicted by Kern et al., [46] stating higher *NPM1* mutations in

females than males (7:13). This study also indicated higher prevalence of *NPM1* in males than females.

NRAS gene mutation in the pathophysiology of AML patients was also investigated in this study. *RAS* gene activates and causes phosphorylation of activated mitogen protein kinase (*MAPK*) leading to cellular proliferation which gets disturbed in the presence of mutations [47]. *NRAS* mutations are the most common, occurring in 10–20% of AML patients [48]. In Indian population the mutations in *NRAS* gene in AML patients are about 5%, whereas it is 10% in French population, 11% in the UK and higher in the rest of Europe [49].

This study demonstrated the prevalence rates to be 12% (c.33A>T) and 54.1% (c.12C>T) in two coding regions of exon 2 of *NRAS* gene which is significantly greater than the prevalence rates around the world. Our study showed a significant correlation of *NRAS* mutation with reduced leukocyte counts as demonstrated by Bacher et al. [50]. It also showed significant correlation of age and gender with *NRAS* mutations. Dunna et al. [49] and also concluded higher prevalence of *NRAS* mutations in males than females.

AML may develop due to activating *NRAS* mutations simultaneously with *NPM1* mutations, occurring commonly in the favorable-risk population [51]. Akram et al. [52] showed disturbance in platelet count, Hb levels and leukocyte count of the AML patient with *NRAS* mutation. Our study also signified these results with the relation of *NRAS* mutation with platelet count, leukocyte count and Hb levels.

Although the extrapolative scale of *NRAS* mutations in AML patients is not decisive [53], yet several large cohort studies indicated that *NRAS* mutations in AML did not influence the prognosis of patients [54]. That is why, given the prevalence of *NRAS* mutations in AML patients, more research on clinical characteristics and possible prognostic factors should be done.

In Pakistan, no significant work has been performed to find out frequencies of fusion oncogenes in AML patients, possible mutations in *NPM1* and *NRAS* genes, and their correlation with patient characteristics and clinical response. This study has proven to be beneficial to determine the role of fusion oncogenes in Pakistani AML patients. It has given new insights into the mutational range and prognostic elements of *NRAS* and *NPM1* genes in AML patients. More detailed mutational analysis and large prospective studies are needed in the future for better prognostication of *NRAS* and *NPM1* in monitoring the development and proliferation of AML. It must be explored by parallel comparison with non *NRAS* and *NPM1* mutation associated AML. Our conclusion needs validation in a larger and prospective cohort.

Competing Interest

The authors declare that there is no conflict of interest.

Author Contributions

Muhammad Tayyab: Conception and design, Experimental Work, Analysis and interpretation of data, Rough Draft

Tanveer Akhtar: Supervision, Research Project Administration

Zahra Tariq: Analysis and Interpretation,

Mahwish Khan: Final Draft, writing and Revision

Muhammad Imran Qadeer: Final Draft and Revision

Tuba Fayyaz: Draft editing

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