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ASSOCIATION OF RAS GENE EXPRESSION WITH NPM1 MUTATION IN ACUTE MYELOID LEUKEMIC PAKISTANI PATIENTS

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ABSTRACT:
Acute myeloid leukemia (AML) is one of the first tumor types sequenced at the whole genome level, in which mutations in nucleophosmin NPM1 and RAS are the most frequently acquired molecular abnormalities. In a current study, human blood samples of AML patients were collected from Jinnah hospital, Mayo hospital and Institute of Nuclear Medicine and Oncology (INMOL), Lahore. Clinical and haematological characteristic of AML patients of different age groups and genders according to AML1-ETO and CBFB-MYH11 transcripts, followed by the analysis of Neuroblastoma RAS (NRAS) coding sequence mutation, Nucleophosmin1 (*NPM1*) gene coding sequence mutation and *NPM1* 3'UTR Variant mutation through DNA and RNA isolation, Primer designing and sequence analysis through RT-PCR, which led to the detection of frequencies of FAB sub-types and frequency distribution of N-RAS and *NPM1* gene mutations in AML Pakistani patients. Statistically analysed results indicated that prevalence of this disease is common in adults (85.71%) as compared to paediatric (14.28%). AML fusion oncogene (AML1-ETO) was detected in 26.6% AML patients, with higher frequency in AML-M2 patients (34.2%). The percentage of AML patients with error was 2.86% with the frequency of 0.02. SNP5 and was not deviated from SNP1 and SNP2 because it also showed similar results, except for gender based (both) data ($p > 0.001$) and it shows significant correlation of gender with SNP5 while all other parameters gave non-significant results ($p < 0.001$). However, mutation in NRAS gene was detected in total 51.4% of AML patients. In conclusion, the role of different types of *NPM1* mutations, either individually or in the presence of RAS mutations may be essential for AML prognosis.

Keywords: Acute myeloid leukemia (AML), AML1-ETO, CBFB-MYH11, NRAS, *NPM1*, SNPs

INTRODUCTION

AML is an imitating disorder of primarily stem cells of bone marrow. It initiates via genetic mutations which results in the disturbance of normal regulation of differentiation, expansion & self-destruction of hematopoietic cells. This deregulation is responsible for the acceleration of more dissociable, but irregular, functionally undeveloped myeloid cell in blood & bone marrow (Abelson et al.,2018). These cells correspond to light microscopy myoblasts and are therefore called

(leukemic) blasts designated. The consequence of this cell expansion is an increasing displacement normal hematopoiesis. As with other neoplasms, cause genetic changes in the AML, stimulation of cancer-causing genes or the loss of functionality of anti-onco genes; however, is in hematological neoplasms, in difference from solid tumors, often just a single chromosomal NPM (Nucleic acid Protein B23, NO38 or numatrin) is a copious protein which is pervasive and extremely preserved. Located on chromosome 5q35, this gene has twelve axons (Welch et al., 2018 and Colombo *et al.*, 2011). NPM1 (ribonucleoprotein, made of 294 amino acids, molecular mass of 37KDa) has 3 well defined constructed and functional domains; a domain of C-terminal nucleic acid binding, a domain of amino terminal oligomerization and histone binding middle domain (Pronier et al., 2018). NPM mutation can be found in around 33% of AML cases, while few have been characterized, all being frame shift transformations by the inclusion of four bases. In tumors with multiple histological causes, including gastric (Manneli *et al.*, 2017), colon (Liu *et al.*, 2019), ovary (Zhu *et al.*, 2019), and prostate (Patnik *et al.*, 2019), NMP has shown to be upregulated (Kottaridis et al., 2001). In addition, the NPM1 gene is one of the main replicated targets of chromosomal translocations in hemopoietic malignancies. NPM1 is translocated with (2;5) encoding for the fusion of the protein NPM-ALK to produce the chimeric gene in ALCL (Port et al., 2014). Most translocation associates include t (3;5) in myelodysplasia/AML in severe acute cases of promyelocytic leukemia arising in an NPM-RAR fusion protein resulting in the signal peptide NPM-MLF142 and t (5;17).

In numerous signaling pathways, the RAS protein is an essential protein and administers normal cell growth and malignant transformation. RAS was found to be an activated cancer genes in human tumors about 40 years after its discovery. RAS oncogenes play important parts in tumor genesis and expansion by encoding a family of G-protein, so they are viewed as an important therapeutic target (Dohner *et al.*, 2017). Among three small GTP proteins in the RAS family (HRAS, NRAS, and KRAS), NRAS mutations occur more regularly than KRAS mutations, with rarely occurring HRAS mutations (< 1%). RAS mutations in compact tumors and blood related malignancies, including AML, have characteristically been considered as oncogenic events and are believed to be consistent with prognosis (Stein *et al.*, 2017). In AML, with an incidence of 15-40%, RAS mutations are one of the most frequent genetic alterations. The analytical function of mutations in RAS in AML still is unconvincing. Whereas few studies have shown little survival in RAS mutation patients, others have drawn a different conclusion (Daver *et al.*, 2015). Moreover, RAS mutations also play a strong role in the overall survival of adults and infants with AML (Liu *et al.*, 2019). Overall, approximately 10-25 % AML cases are accounted for by RAS mutation and are dominated in cases with inv (16) (p13q22)/t(16;16) (p13;q22) or inv(3)(q21q26)/t(3;3)(q21;q26) (Gunawardane *et al.*, 2013). The group of constitutively active mutated RAS gene results in severe consequences, including cancers and other diseases. Enabled RAS anchors on the membrane of the cell and activates RAS pathways for cell cycle regulation, differentiation, and signal transduction (Altman et al., 2018).

In Pakistan, no significant work has been performed yet to find out frequencies of fusion oncogenes in AML patients and possible mutated sites in NPM1 and NRAS gene, their correlation with patient characteristics and clinical response outcomes. So, present study was designed with an aim to investigate frequencies of common fusion oncogenes and gene mutations in Pakistani patients of AML.

3 Materials and Methods

3.1 Patients and Blood Samples Collection

Blood samples were collected from 105 acute myeloid leukaemia (AML) patients from Jinnah hospital, Mayo hospital and institute of nuclear medicine and oncology (INMOL), Lahore, Punjab, Pakistan from August 2013 to December 2014, after the Declaration of Helsinki was considered for all protocols, followed by the approval from ethical committee of Institute of Zoology, University of the Punjab, Lahore, Punjab, Pakistan. 3.0 ml blood was drawn from each AML patient, immediately transferred to pre-labelled (with sample number, patient name, age, time, and date of collection) EDTA coated vacutainers and centrifuged as per manufacturer ‘s instructions to avoid any clot

formation and to ensure less degradation of mRNA, processing for analysis of blood samples were started within 24 hours of blood collection. All samples were stored at -40°C for further use.

Clinical History and Patient's Data

Patient data forms and consent forms were designed to attain complete clinical history available since the time of AML diagnosis and consent was signed by the patients. Later, their follow up was done on monthly basis to record hematologic and cytogenetic response. The experimental work has completed in parasitology lab and central lab, Department of Zoology, University of the Punjab Quaid-e-Azam campus Lahore, Pakistan.

3.3 Molecular Analysis

Genomic DNA was isolated from whole white blood cells; by using manual protocol, kit (Promega Wizard Genomic DNA purification kit (Cat#A1120) protocol and both methods were optimized, which was depended upon the volume of blood obtained from AML patients. For instance, the samples contained volume above 3ml, manual method of DNA extraction was used to get high quality yield as well as due to cost efficacy.

1.3.1 DNA extraction

A bench/ area was reserved to isolate the DNA and sterilized by 75% ethanol before extraction was performed. The procedure was carried out by using nucleic acid extraction kit through DNAZOL method (Chomczynski *et al*., 1997).

3.3.2 Total RNA Isolation

For RNA isolation from the whole blood, PBMC pellet was isolated first. The RNA extraction procedure was adopted from previous published studies (Lowenberg et al., 1999; Slovak*et al*., 2000).

3.3.3 Primer Designing

Sequences of selected human genes were retrieved from NCBI (National Center for Biotechnology Information). Sequence was then BLAT (Basic Local Alignment Tool) in the bioinformatics tool (www.genome.ucsc.edu), in which whole sequence intron exon appeared. Primer pairs were designed after preliminary analysis of specific genes of humans through bioinformatics tool. (www.expasy.ch). To avoid genomic DNA contamination, only the sequence from exons was used for primer designing. The sequence from two exons was placed into other tool i.e. (www.primer3) for primer designing.

3.3.4 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

After the RNA isolation, the first strand cDNA was prepared using Fermentas kit (k1622). RT-reaction protocol and thermal conditions were optimised with slight variations (Byrd *et al*., 2002).

3.3.5 Sequencing analysis of PCR amplicons

The amplified PCR products were subjected to direct sequencing analysis for detection of any possible mutations present in NRAS and NMP1genes of AML patients. The facility for direct sequencing was provided by Centre of applied molecular biology (CAMB), University of the Punjab, Lahore. Nested RT-PCR products of processed samples were sequenced by using Sanger method. The forward primer (3306F) 5′-TGGTTCATCATCATTCAACGG-3′ and reverse primer (4000R) 5′-GGACATGCCATAGGTAGCA-3′ were used for sequencing reaction (Willis *et al*., 2005) as mentioned in table 3. Standard procedure for Big Dye® Terminator v3.1Cycle Sequencing Kit from Applied Biosystems, USA was used as per manufacturer 's instructions.

3.3.6 Analysis of Sequence Data

The sequenced templates were analysed by using sequencing analysis software GeneiousR7. The DNA sequences of NMP1 and NRA genes of healthy individuals were aligned against the reference sequence (NCBI GenBank accession number M14772.1). This was performed to find out any polymorphic variations in our population to avoid artefact findings with point mutations while analysing 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. Candidate genes for current study has been shown in Figure 1.

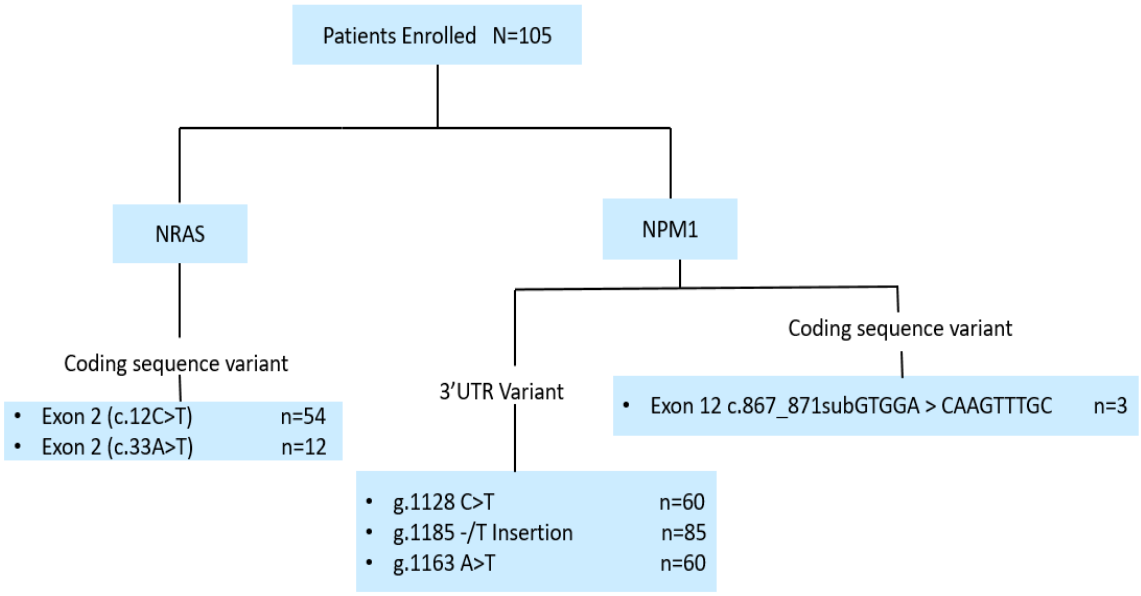


Figure 1 Flow chart of selected candidate genes for the AML study.

Statistical Analysis

The IBM SPSS statistics, version 22 and Graph pad Prism was used for statistical data analysis. The population was tested for Hardy-Weinberg equilibrium. Allelic and genotypic frequencies were calculated, and results were compared between cases and controls using an x2-test. The demographic characteristics of patients were also 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 chi square tests, respectively. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp. Source: https://www.ibm.com/analytics/spss-statistics-software)

4 RESULTS AND DISCUSSION

4.1 Gender and age wise distribution of AML patients

Incidence of AML was more in males (54.2%) than females (45.2%) (Figure 2 B). Majority of patients were adults (85.71%) while patients less than 18 years of age were only 14.28%. Median age of Pakistani AML patients was 33.5 years with a specific range (13-72 years), mean (33.5) and standard deviation (13.73) (Figure 2 A and 2C).

4.2 Clinical features at diagnosis

The clinical characteristics of patients presented at the time of diagnosis revealed that high grade fever was the most frequent event and reported in 85.71% AML patients. Weight loss (78.09%), organomegaly (76.19%), fatigue (66.66%) and muscle pains (57.14%) were other important diagnostic features presented by the patients (Figure 2 D). In addition, total leukocyte count (median 7.4±2.34) was observed in 78.09% of AML patients within normal range (4-11 x 10^9/L), however

21.90% individuals exhibited rise in TLC (>11 x 10^9/L). Platelet count (median 390.5±214.80) within the normal range (100-450 x 10^9/L) was observed in 97.14% individuals, whereas 2.85% patients were observed with elevated number of platelets (>450x 10^9/L) in peripheral blood. Mean corpuscular hemoglobin (MCH) (median 30.0± 22.80) within the normal range (26-32 PG) was observed in 96.19% in AML patients, while elevated level of MCH was found in only 3.80% of AML patients. Decreased level of hemoglobin in blood was presented by 90.47% individuals associated with this disease (Figure 2 E). Current data indicates that prevalence of this disease is common in adults (85.71%) as compared to paediatrics (14.28%) (Figure 2 B and C). There is no significance elevated level of TLC count and platelets count observed, however, decrease in Hb level observed in AML patients (Figure 2 D and E).

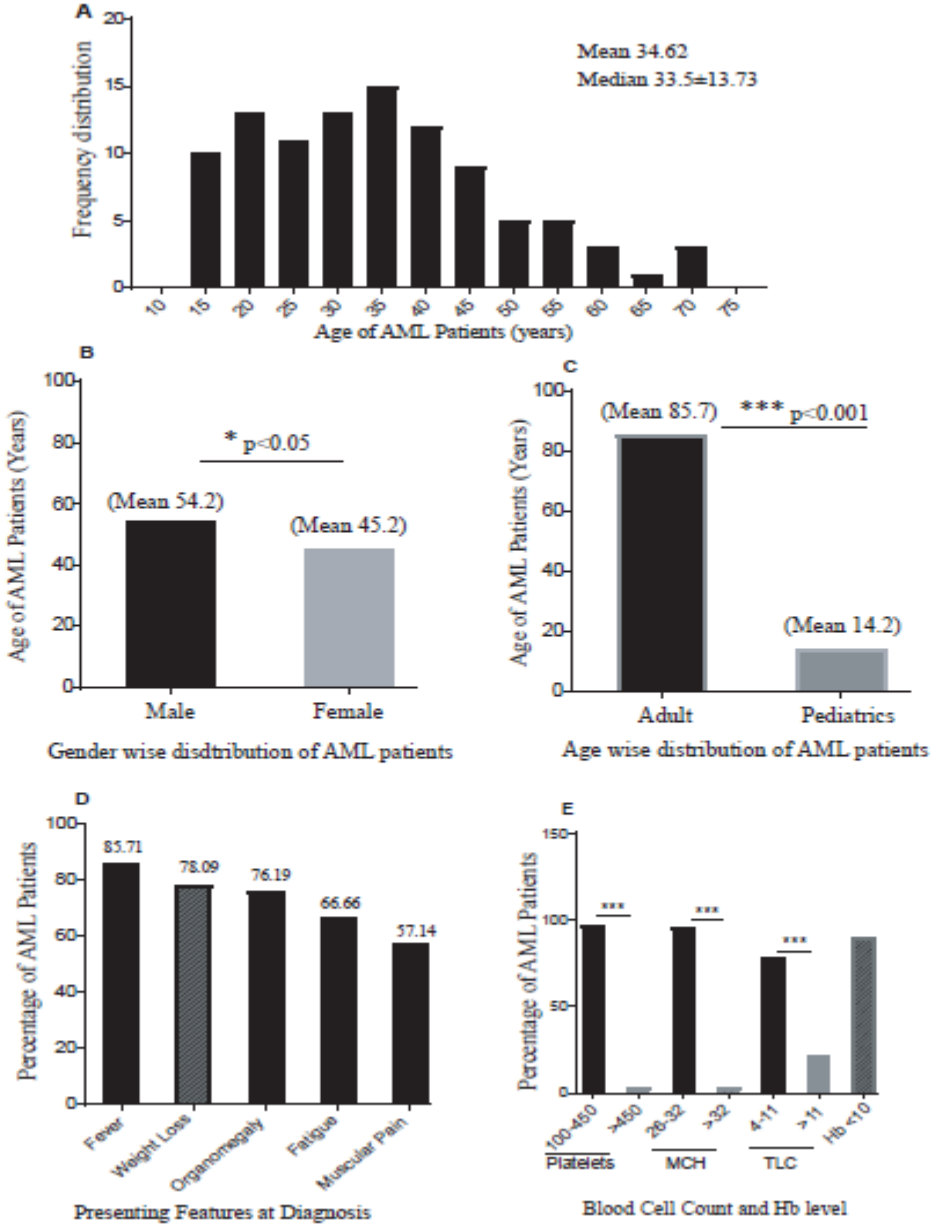


Figure 2 Age and gender wise frequency distribution of Pakistani AML patients (n=105)

(A) Age (years) wise frequency distribution of AML patients. (B) Percentage of gender wise distribution of AML patients (black bar =male, mean 54.2 and grey bar = female, mean 45.2). (C) Percentage of adult (black bar) and paediatric AML patients (grey bar) (D) Clinical manifestation in AML patients (E) Percentage of laboratory test values of AML patients. The data compared and evaluated by t-test was found statistically significant at *p<0.05, ***p<0.001 level.

4.3 Frequencies of FAB sub-types in AML patients in Pakistan

Out of total of 105 patients of AML, the frequency by FAB classification appeared as M0, M1, M2, M3, M4, M5, M6 and M7 subtypes, among which frequency of FAB subtype M1 is higher (31.42%), whereas, the frequency of FAB subtype M2 was 28.57%. The frequencies of other subtypes including M0, M3, M4, M5, M6 and M7 were 16.1%, 6%, 10%, 8%, 1% and 0% respectively (Figure 3).

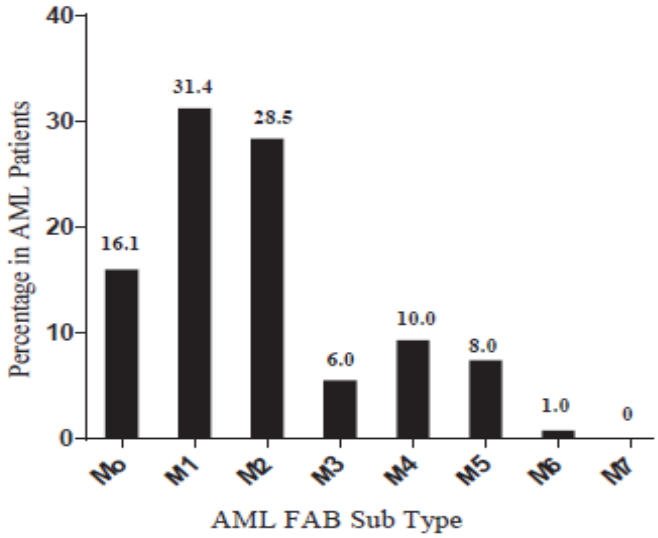


Figure 3 Frequency of AML FAB subtype in Pakistani AML patients (n=105)

4.4 Patient characteristics and detection of AML1-ETO transcripts

AML1-ETO translocation t (8;21) were detected in 6-8% of all AML cases and in 20-30% of FAB M2 AML via routine karyotyping (Grimwade et al., 1998). Despite the immense majority (>90%) of t (8;21) were described as M2 AML, this aberration has also been reported, at lower frequencies, in M1 and M4 AML, and in rare cases of myelodysplastic and myeloproliferative syndromes (Français de, 1990). In a current study, AML1-ETO transcripts were detected as 395bp in the first PCR product and 260bp in the second PCR product (26.6%) and in internal control (26.6%), with positive GAPDH results (Figure 4 and Figure 6). AML1-ETO transcripts in M2 were detected in 34.2% by using GAPDH as an internal control (Figure 5).

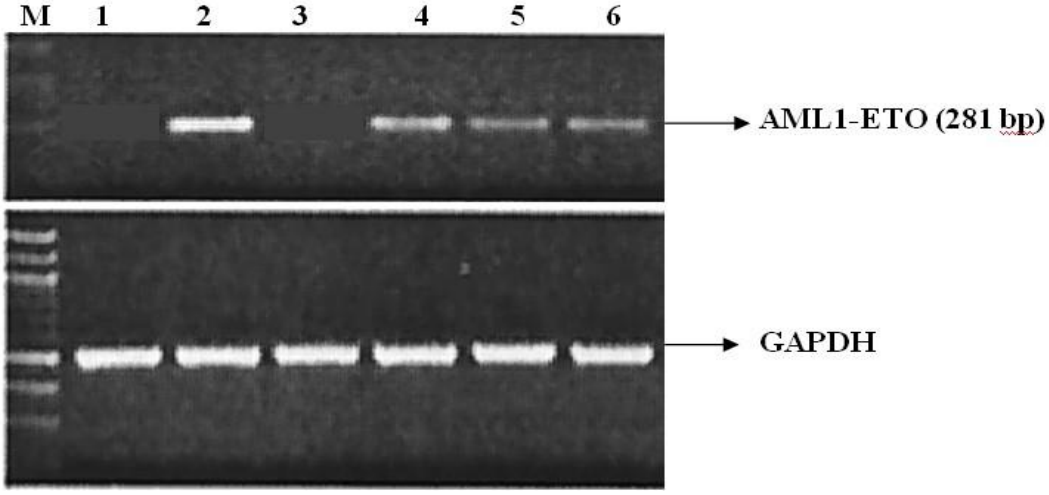


Figure 4 Detection of AML1-ETO transcript by RT-PCR in AML patients.
M= Marker Lane, Lane 2, 4, 5, 6: positive patients, lane 1 & 3 negative patients

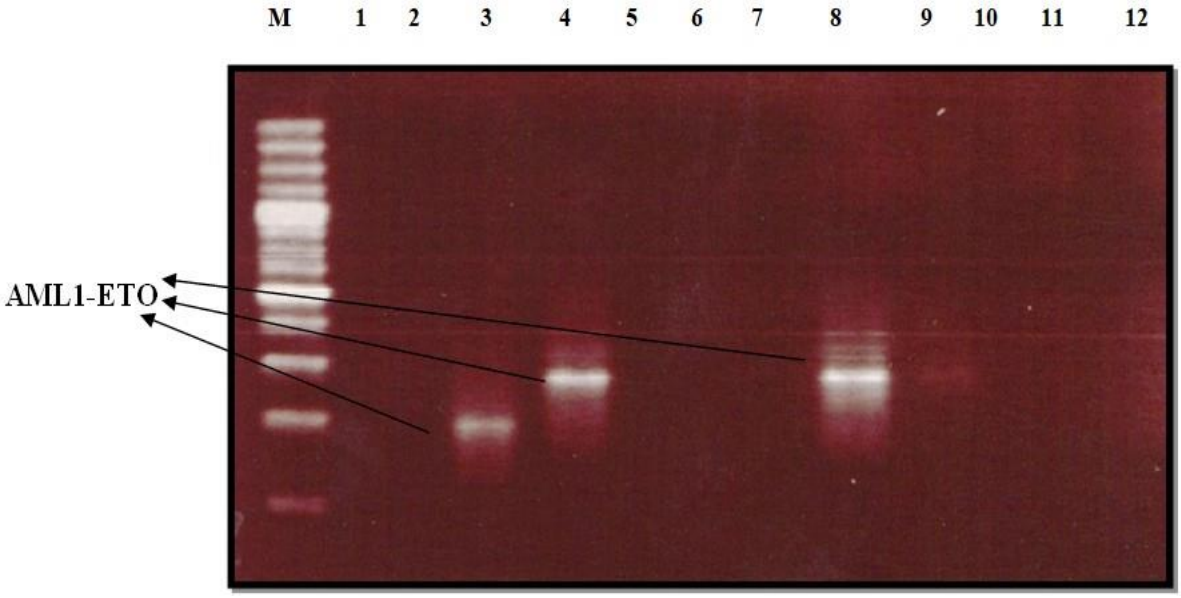


Figure 5 Detection of AML1-ETO transcript by RT-PCR in AML-M2 patients.

Lane M: Marker, **Lane 1**: Primer control (no RNA), **Lane 2, 5, 6, 7, 9, 10, 11 & 12:** Negative patients, **Lane 3, 4 & 8:** Positive patients.

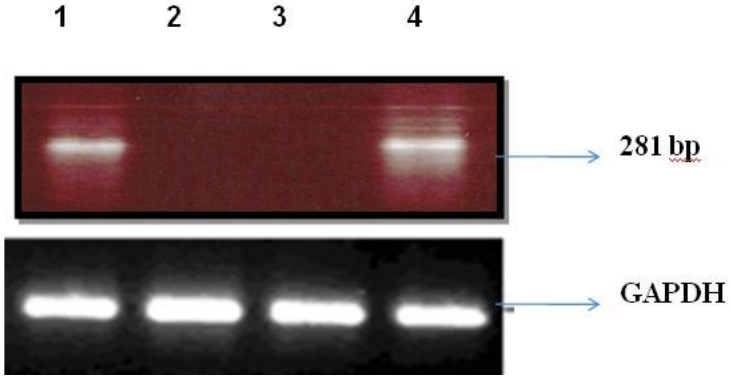


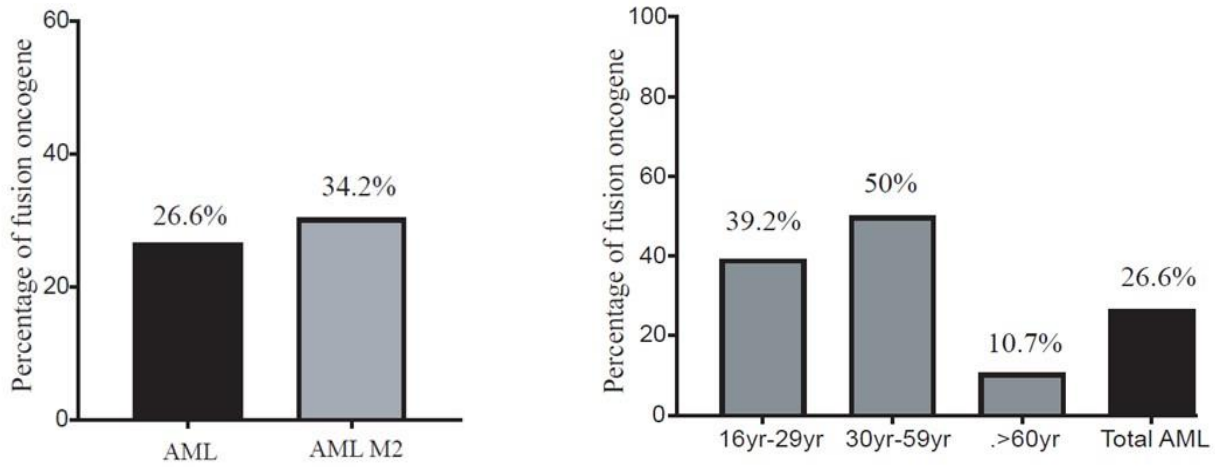
Figure 6 Detection of AML1-ETO transcript by RT-PCR in AML-M2 patients.
Lane 1 & 4: Positive patients, **Lane 2 & 3:** Negative patients.

4.5 Clinical and hematological characteristic according to AML1-ETO

A comparison of clinical parameters such as age, gender, hepatomegaly, splenomegaly and extramedullary tumefaction between AML1-ETO positive AML patients and AML1-ETO positive AML-M2 patients demonstrated that frequency of AML fusion oncogene (AML1-ETO) was detected in 26.6% in AML patients. However, frequency of this fusion oncogene was higher in AML-M2 patients 34.2%) (Figure 7 a). In addition, no significant difference had been observed in clinical and laboratory findings (Table 1). Moreover, frequency of AML1-ETO fusion oncogene was higher in age between 30 to 59 years (50%), followed by age between 16- 29 years (29%) (Table 2 and Figure 7 b).

Table 1. Comparison of clinical and laboratory findings between AML1-ETO positive group

Patients	**AML**	**AML-M2**
Age (median	Positive=105	Positive= 30
Sex (M:F)	(57:48)	(18:12)
WBC (*10^3/L)	20.2	19.6
Hemoglobin(g/dl)	6.2	6.0
Platelet (*10^3/L)	45.3	50.0



(a) **(b)**

Figure 7 Frequency distribution of AML1-ETO fusion oncogene in (a) AML and AML M2 Pakistani Patients (b) Adult AML Pakistani Patients in Different Age Groups.

Black bar = frequency of AML1-ETO fusion oncogene in total AML patients, while gray bar = frequency of AML1-ETO fusion oncogene in sub-type AML M2.

Table 2 Fusion oncogenes (%) in AML patients of various age groups

Fusion Oncogene	Chromosomal aberrations	M: F	16-29 Y	30-59 Y	> 60 Y	Total
AML1-ETO	t(8;21)	1.5:1	39.2%	50%	10.7%	26.6%
CBFB-MYH11	inv(16)(p13q22)	1.4:1	41.6%	50%	8.3%	11.4%

M= Male F= Female Y= Years, >= Greater than

4.6 Patient characteristics and detection of CBFB-MYH11 transcripts

CBFB-MYH11 transcripts were detected as 180 bp and 280bp in the 1st and 2nd PCR products respectively in 11.4% patients (Table 4 and Figure 8) by keeping GAPDH as internal control. Blood samples of all AML- M2 patients, with gender ratio of 3:2 (M:F) and the median age of 18 years, were examined for the detection of CBFB-MYH11 fusion oncogene, but CBFB-MYH11 transcripts were not detected. Moreover, frequency of CBFB-MYH11 fusion oncogene was higher (50%) in age group between 30 -59 years and 41.6% in age group between 16 -29 years (Table 2 and Figure 9).

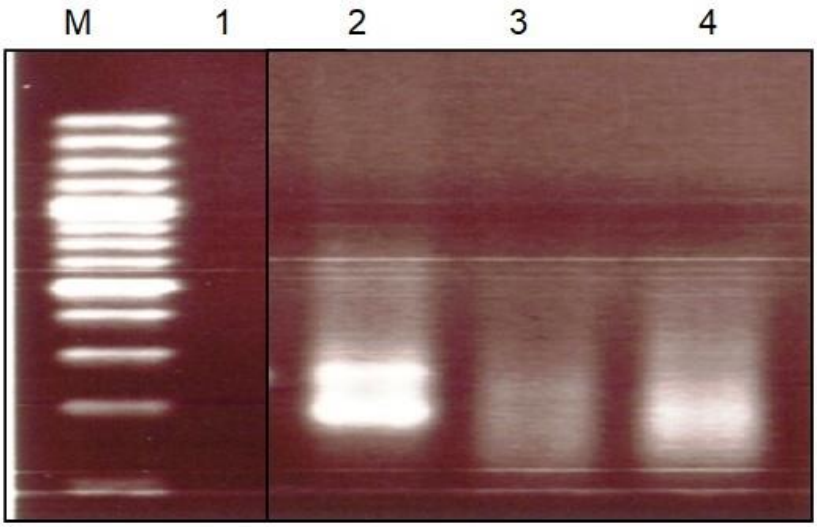


Figure 8 Detection of CBFB-MYH11 transcript by RT-PCR in AML patients.
Lane M: Marker, **Lane 1:** Primer control (no RNA), **Lane 2, 3 & 4:** Positive patients

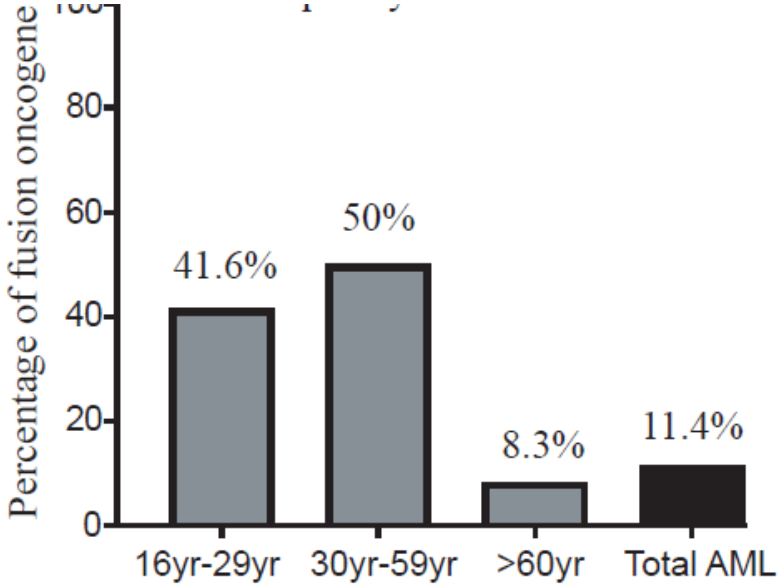


Figure 9 Frequency distribution of CBFB-MYH11 fusion oncogenes in adult AML Pakistani patients in different age groups.

4.7 Neuroblastoma RAS (NRAS) coding sequence mutation

The *RAS* oncogene family has three members: *KRAS*, *NRAS* and *HRAS*. These genes encode a family of highly homologous GTPases that are involved in various cellular activities, such as growth, proliferation and differentiation.RAS family mutations are often observed across cancer types. Molecular analyses have revealed that nucleotide substitutions and chromosomal translocations are the two major genetic alterations associated with leukemia. Previous studies demonstrated that 20 to 40% of patients of AML have N-RAS mutations**.**

In current study, two mutations were observed AML patients, and genome of 54 Pakistani AML patients had alternation of nucleotide C with T in Exon 2 at number 12 nucleotide base pair (Figure 10 A-D). Mutated patients among AML patients were 51.4% with the frequency of 0.514, with highest significance was found was in accordance with male and female ratio (p= 0.001). Platelet count, Hb levels, total leucocytes and, fever (°F) and age were not significant in correlation (p-values 0.998, 0.870, 0.755, 0.307 and, 0.952 respectively). Moreover, another mutation observed in NRAS gene was the replacement of A with T in Exon 2 at number 33 nucleotide base pair. 12 AML patients were found mutated in the whole sample (11.43 % and 0.114 frequency) (Figure 10 E-H). Evaluation of the correlation of SNP2 with biochemical parameters revealed no significant relation except higher significance in gender and age (p-value< 0.001). p-values of 0.058, 0.324, 0.285, 0.224 were obtained based on platelet count, Hb levels, total leukocyte count and fever respectively (Table 3).

4.8 Nucleophosmin1 (*NPM1*) Gene coding sequence mutation

NPM1 protein is involved in several cellular processes, centrosome duplication and cell proliferation. It has been studies that mutation in NPMI gene is responsible for causing AML. The associated pathways of *NPM1* are the development of hepatocyte growth factor signaling pathway and tyrosine kinases pathways that help in nucleic acid binding and protein homo dimerization activities. The WHO classification considers mutated NPMI in AML patients as provisional entity and it recommends diagnosis of NPM1 cytogenetically normal AML.

Current data reveals that among 105 AML patients, three patient's genomes were found mutated in coding sequence such as in Exon 12 of gene NPM1, a sequence GTGGA found mutated with the sequence CAAGTTGC, from nucleotide base number 867 to 871 (Figure 10 I-J). AML patients with error were 2.86% with the frequency of 0.02. SNP5 was not deviated from SNP1 and SNP2 because it also showed similar results with the exception of gender wide data (p> 0.001) which showed significant correlation of gender ratio with SNP5 while all other parameters gave non-significant p-

values (0.458, 0.458, 0.474, 0.340 and, 0.376 for platelet count, Hb levels, total leucocytes, fever (°F) and age respectively) (Table 3).

4.8 Nucleophosmin1 (NPM1) 3'UTR Variant mutation

Current data demonstrated that 3'UTR variant mutation was most frequent in AML patients. There were three different types of mutations which were observed in the 105 AML patients. Sixty AML patients with the percentage of 57.1 and frequency of 0.571 were mutated via translocation of nucleotide C with T in the gene 1185 in the 3'UTR of NPM1 (Figure 10 K-N).SNP3 evaluation gave us the results with the non-significant P values for platelet count, Hb levels, total leucocytes, fever (°F) and age as 0.748, 0.659, 0.726, 0.132, and 0.727 respectively while significant P-value was only obtained by the analysis of gender ratio which was 0.001 (Table 3). This significant value shows the correlation with the positive AML patients. The next mutation which was seen was even more frequent. Insertion of T in gene 1185 happened in 85 patients with the percentage of 80.95 and frequency of 0. 809.SNP4 also showed very similar results like other mutations. The P values for platelet count, Hb levels, total leucocytes, fever (°F) and age were 0.847, 0.441, 0.891, 0.80, and 0.854 respectively (Table 3). These were non-significant values and therefor showed no correlation between AML patients and other .001 significant values were only obtained from the data of gender analysis. Another mutation which was observed in 60 patients among 105 under examined patients, was the replacement of A with T in the gene.1163 (Figure 10 O-R and Figure 10 S-V). The percentage went on up to 57.14% and the recorded frequency was 0. 571.SNP6 was when analyzed gave us similar values to that of previous ones. The values for platelet count, Hb levels, total leucocytes and, fever (°F) and age were 0.289, 0.933, 0.317, 0.502, 0.206 respectively (Table 3). The value for gender analysis in SNP6 shows a significant value of 0.001 with significant correlation.

Table: 3 Characteristics of AML patients by SNP1 to SNP6 status.

SNPs	Patients/ Characteristics	Platelet count (x 10^9/L)	Hb levels	Total leucocytes	Fever (°F)	Age	Male	Female
SNP1 (% age /mean)	Positive	307.4	7.05	7.26	100.8	38.2	78.90%	16.70%
	Negative	307.4	7.02	7.13	11.5	38.3	21.10%	83.30%
	p-value	0.998	0.87	0.755	0.307	0.952	<0.001	
SNP2 (% age /mean)	Positive	250	6.7	6.6	101.1	22.5	78.90%	16.70%
	Negative	314.8	7	7.2	100.6	40.2	21.10%	83.30%
	p-value	0.058	0.324	0.285	0.224	<0.001	<0.001	
SNP3 (% age /mean)	Positive	304.3	7	7.1	100.8	38.7	78.90%	16.70%
	Negative	311.4	7	7.2	100.5	37.7	21.10%	83.30%
	p-value	0.748	0.659	0.726	0.132	0.727	<0.001	
SNP4 (% age /mean)	Positive	308.4	7.07	7.2	100.6	38.4	78.90%	16.70%
	Negative	303.1	6.88	7.1	101.1	37.7	21.10%	83.30%
	p-value	0.847	0.441	0.891	0.8	0.854	<0.001	
SNP5 (% age /mean)	Positive	260	7.7	6.33	101.3	30.7	78.90%	16.70%
	Negative	308.8	7	7.2	100.6	38.4	21.10%	83.30%
	p-value	0.458	0.265	0.474	0.34	0.376	<0.001	
SNP6 (% age /mean)	Positive	297.1	7.03	7	100.6	40	78.90%	16.70%
	Negative	320.5	7.04	7.4	100.7	36	21.10%	83.30%
	p-value	0.289	0.933	0.317	0.502	0.206	<0.001	

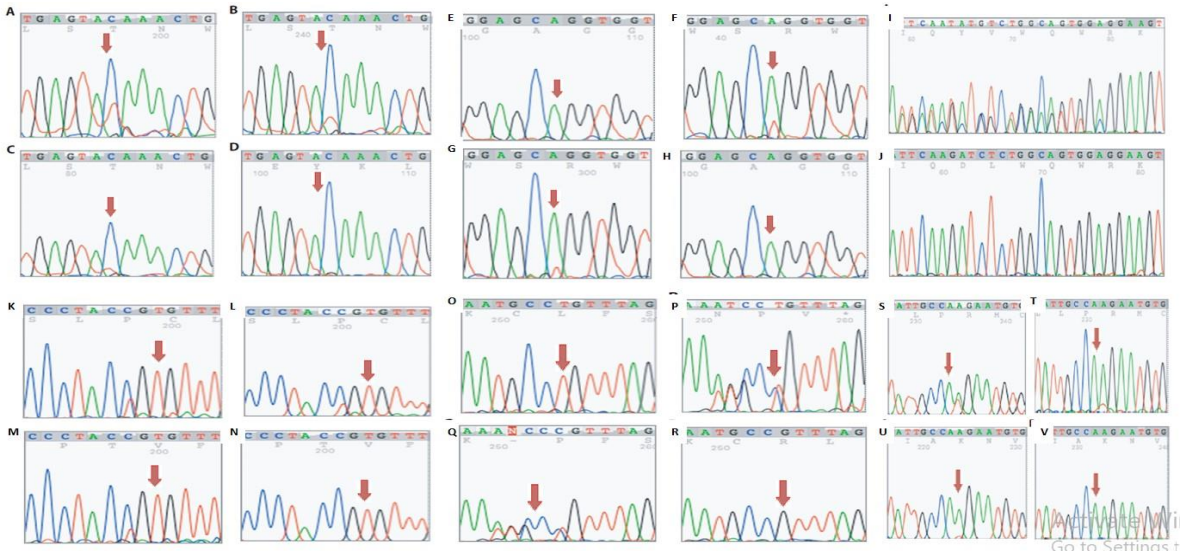


Figure 10 Detection of mutant N-RAS (c.12C>T and c.33A>T), NPMI, 3'UTR of NPM1 (g.1185insT, g.1163AsubT and g.1163AsubT) and wild type in exon 2 in AML Pakistani patients by Sanger sequencing.

(A, B, C) Mutant N-RAS (c.12C>T) in three different AML patient's sample, **(D, H)** Wild-type sequence N-RAS (exon 2) in AML patient**, (E, F, G)** Mutant N-RAS (c.33A>T) in three different AML patients, **(I and J)** Mutant NPM1 in exon 12 (c.867_871subGTGGA >CAAGTTTGC) and wild-type in AML patients, **(K)** Homozygous Mutant NPM1 3'UTR (g.1185insT) in AML patient, **(L, M)** Heterozygous Mutant NPM1 3'UTR (g.1185insT) in two different AML patients, **(N)** Wild-type sequence NPM1 3'UTR in AML patient, **(O, P, Q)** mutant NPM1 3'UTR (g.1163AsubT) in three different AML patients, **(R)** Wild-type sequence NPM1 3'UTR in AML patient, **(S, T, U)** mutant NPM1 3'UTR (g.1163AsubT) in three different AML patients, **(V)** Wild-type sequence NPM1 3'UTR in AML patient. Red arrow points toward base substitution in mutants with respect to normal sequence and is indicating nucleotide change in chromatograms.

4.9 Frequency distribution of N-RAS and NPM1 gene mutations in AML Patients

Data regarding frequency distribution of NPM1 and NRAS in 105 AML patients showed that most frequently detected mutation in Pakistani AML patients was NPM1 3'UTR variant (g.1185-/T Insertion) (80.95%) as compared to other mutations (Table 3). Most observed two mutations detected were in NPM1, g.1128C>T and g.1163A>T detected in NPM1 3'UTR Variant 60% and 57% respectively (Table 4). However, mutation in NRAS gene was detected in 51.4% patients (Table 3 and Figures 10).

Table 4 Frequency distribution of N-RAS and NPM1 gene mutations in 105 AML Patients

Gene	Mutation Type	Exon No. (Nucleotide change)	Genomic position	Known ID	No. of cases (%) n=105	Frequency
N-RAS	Coding Sequence variant (Synonymous Substitution)	Exon 2 (c.12C>T)	1:114716148	COSMIC_MUTATION (COSM6981755) (Known CA>TT)	54 (51.4%)	0.514
N-RAS	Coding Sequence variant (Synonymous Substitution)	Exon 2 (c.33A>T)	1:114716128	Novel	12(11.43%)	0.114
NPM1	3'UTR Variant	g.1128C>T	5:171410673	rs1052205641	60 (57.1%)	0.571
NPM1	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
NPM1	3'UTR Variant	g.1163A>T	5:171410708	Novel (rs1031458634) (Known g.1163AsubC)	60(57.14%)	0.571

CONCLUSION

A relatively consistent *NPM1* mutation rate was observed, but with variations in types of mutations. The role of different types of *NPM1* mutations, either individually or in the presence of other common gene mutations with RAS may be essential for AML prognosis. *NPM1* mutations are consistently present in approximately 10% of AML cases. However, the observation of a high variety of *NPM1* mutations merits further studies, to determine their individual contribution to the pathogenesis of AML and their comprehensible relation to RAS and AML prognosis.

DISCLAIMER

The data used for this research was taken absolutely without any conflict of interest between the authors and patients because we do not intend to use this data as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by any agency or company, rather it was funded by personal efforts of the authors.

ETHICAL APPROVAL

It is not applicable.

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