IJHOSCR

Original Article

International Journal of Hematology-Oncology and Stem Cell Research

NPM1 and *FLT3-ITD/TKD* Gene Mutations in Acute Myeloid Leukemia

Shano Naseem[1], Jogeshwar Binota[1], Neelam Varma[1], Harpreet Virk[2], Subhash Varma[3], Pankaj Malhotra[3]

[1]Department of Hematology, Postgraduate Institute of Medical Education and Research, Chandigarh, India
[2]Department of Pathology, Postgraduate Institute of Medical Education and Research, Chandigarh, India
[3]Department of Internal Medicine, Postgraduate Institute of Medical Education and Research, Chandigarh, India

Corresponding Author: Shano Naseem, Department of Hematology, Postgraduate Institute of Medical Education and Research, Chandigarh, India
Tel: +91-172-2755131
Email:shanonaseem@yahoo.co.in

Received: 27, Sep, 2019
Accepted: 06, Dec, 2020

ABSTRACT

Background: A number of mutations have been reported to occur in patients with acute myeloid leukemia (AML), of which *NPM1* and *FLT3* genes mutations are the commonest and have important diagnostic and therapeutic implications.

Material and Methods: Molecular testing for *NPM1* and *FLT3* genes was performed in 92 de-novo AML patients. The frequency and characteristics of *NPM1* and *FLT3* mutations were analyzed.

Results: *Nucleophosmin 1(NPM1)* and *fms-like tyrosine kinase 3 (FLT3)* mutations were seen in 22.8% and 16.3% of patients, respectively. Amongst *FLT3* mutations, *FLT3*-ITD mutation was seen in 8.7% cases, *FLT3*-TKD in 5.4%, and *FLT3*-ITD+TKD in 2.2% cases. Certain associations between the gene mutations and clinical characteristics were found, including in *NPM1* mutated group- female preponderance, higher incidence in M4/M5 categories and decreased expression of CD34 and HLA-DR; and in *FLT3*-ITD mutated group- higher age of presentation, higher total leucocyte count and blast percentage.

Conclusion- AML patients with *NPM1* and *FLT3* mutations have differences in clinical and hematological features, which might represent their different molecular mechanism in leukemogenesis. The frequency of *NPM1* and *FLT3* mutations in this study was comparable to reports from Asian countries but lower than that reported from western countries. However, as the number of patients in the study was less, a larger number of patients need to be studied to corroborate these findings.

Keywords: Acute myeloid leukemia; Nucleophosmin 1(NPM1) mutation; fms-like tyrosine kinase 3 (FLT3) mutation

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogenous disease clinically, morphologically and genetically. Classification of AML has evolved from French-American-British (FAB) classification (1982) which was based on morphology alone to World Health Organization (WHO) classification that incorporates morphology, immunophenotype, cytogenetic and molecular genetic features in the classification schema and defines entities that are biologically and clinically homogenous. In the current 2017 WHO classification of AML, the category of recurrent genetic abnormalities, includes, in addition to 8 specific AML associated chromosomal translocations, category of AML with gene mutations for mutated nucleophosmin (*NPM1*), CCAAT/enhancer binding protein-α (*CEBPA*) and runt-related transcription factor (*RUNX1)* genes.[1]

About 30- 40% of AML cases have a recurrent chromosomal translocation and remaining 60-70%

cases show a normal karyotype. The molecular basis of AML in patients with normal karyotype is still poorly understood and this has led to research and identification of specific gene mutations. It has been shown that 85% of AML cases with normal karyotype have gene mutations, with *FLT3, NPM1, RUNX1* and *CEBPA* mutations being the commonest. *NPM1* mutations are seen in 45-60% of adult AML patients[2], *CEBPA* in 4-9% of AML cases[3], *RUNX1* in 4-16%[4], and fms-like tyrosine kinase-3 (*FLT3*) in 25-30% AML cases[5].

Mutations for *NPM1, RUNX1* and *CEBPA* gene have been included as a separate category in AML classification, based on the fact that patients harboring these mutations have consistent clinical and laboratory features and a favorable response to induction therapy is seen in patients with *NPM1* and biallelic *CEBPA* mutations; and adverse response is seen in patients with *RUNX1* mutations. *FLT3* mutations, however, have not been included as a separate category in WHO classification because *FLT3* mutation occurs across multiple AML subtypes[1].

NPM1 mutations are one of the most common genetic lesions seen in AML. They occur in 2-8% of childhood and 27-35% of adult AML cases. Taking only AML with normal karyotype, they are seen in 45-64% cases[6,7]. *NPM1* mutations are rather AML specific, since other human neoplasms consistently show nucleus-restricted *NPM1* expression. Also, *NPM1* and other recurrent genetic abnormalities are mutually exclusive. *NPM1* mutations are typically heterozygous with the leukemic cells retaining a wild-type allele. They usually occur at exon 12 of the *NPM1* gene[1,2].

NPM1 mutations have been associated with specific pathologic and clinical features- these are more frequent in M4 and M5 AML FAB categories, and in AML with prominent nuclear invaginations ("cuplike" nuclei). More than 95% of AML with *NPM1* are CD34 negative. Multilineage involvement (myeloid, monocytic, erythroid, and megakaryocytic but not lymphoid), higher blast and platelet counts, higher frequency of extra-medullary involvement in form of gingival hyperplasia and lymphadenopathy, female preponderance are other characteristic features of *NPM1* mutated AML[8,9]. Analysis of *NPM1* has important clinical implications, with normal karyotype AML patients carrying *NPM1* mutations, showing a higher complete remission rate than those without *NPM1* mutations after induction therapy[10].

FLT3 encodes a tyrosine kinase receptor that is involved in hematopoietic stem cell differentiation and proliferation. FLT3 is expressed on progenitor cells as well as on blast cells in most cases of AML. *FLT3* mutations are primarily of 2 types, internal tandem duplication (*FLT3*-ITD) within the juxtamembrane domain (75-80%) (involving exon 14 and sometimes part of exon 15), and mutations affecting codons 835 or 836 of the second tyrosine kinase domain (*FLT3*-TKD) (25-30%) (involving exon 20). The presence of *FLT3* has important prognostic and therapeutic implications. *FLT3*-ITD is associated with an adverse outcome, *FLT3*-TKD is also associated with poor prognosis; however, more data is needed[5].

Therapeutically, there are U.S. Food and Drug Administration (FDA) approved *FLT3* inhibitors, which have shown improved clinical response in AML patients, both as a single agent or in combination with chemotherapy[11-13]. The European Leukemia Network (ELN) therefore recommends that the results for *FLT3* mutational screening should be available within 72 hours in all AML patients, so that treatment decisions can be based on the mutation status of the patient[14].

As amongst the single gene mutations *NPM1* and *FLT3* are commonest and have significant prognostic and therapeutic implications, in this study, we evaluated the frequency and features of *NPM1* and *FLT3* mutations in our cohort of AML patients.

MATERIALS AND METHODS

A total of 92 cases with a diagnosis of de-novo AML, confirmed on the basis of hemogram findings, bone marrow examination and immunophenotyping were recruited in the study. All patients satisfied the diagnostic criteria of AML, i.e. all patients had more than 20% blasts in the peripheral blood or bone marrow and showed presence of myeloid lineage either by cytochemistry and/or immunophenotyping[1].

Immunophenotyping

The immunophenotyping analysis was carried out on peripheral blood or bone marrow aspirate sample. The panel of antibodies included CD13, CD33, CD117, CD10, CD19, CD79a, CD22, CD3, CD4, CD8, HLA-DR, CD34, TdT and anti-MPO labeled with either FITC, PE, PerCP, PE-Cy7, PerCP-Cy5.5, APC-H7 or APC fluorochromes. Additional panel of antibodies, including CD14, CD64, CD11c; or CD235a, CD71 or CD41, CD61 were put if monocytic, erythroid or megakaryocytic lineages were suspected on morphology or based on results of the initial panel of antibodies.

Staining was done using the lyse-wash technique. For cytoplasmic markers, permeabilization was done before adding the antibodies.

The cells in blast window (visualized as dim CD45 and low to intermediate side scatter) showing the presence of myeloid markers were taken as positive for confirmation of diagnosis of AML by immunophenotyping.

Polymerase chain reaction

Sample: In each case, 2-3 ml peripheral blood or bone marrow aspirate sample was collected for polymerase chain reaction (PCR).

RNA extraction: Total RNA was extracted from the sample using the commercial kit - QiAmp RNA blood mini kit (Qiagen, Germany) according to the manufacturer's instructions. The concentration and quality of extracted RNA was evaluated by measuring the absorbance of eluted RNA at 260nm in Nanodrop and calculating the absorbance ratio of A260/A280 nm or running a 1% formaldehyde gel electrophoresis for 18s and 28s RNA bands.

cDNA synthesis: Reverse transcriptase reaction was performed using the commercial cDNA synthesis kit - Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. The quality of cDNA was analyzed using the primers for β-actin housekeeping gene.

Multiplex RT-PCR: It was carried for fusion genes *RUNX-RUNX1*, *CBFB-MYH11* and *PML-RARA* using the primers and PCR conditions as described previously by Pakakasama et al.[15]

PCR for *NPM1* mutation: It was carried using the primers and PCR conditions described by Noguera et al. The primers described target the region, including nucleotide 959 (Gene Bank accession number NM_002520) to the 3' end of the locus. This region of *NPM1* and its seven pseudogenes are highly homologous. Therefore, to rule out the amplification of pseudogenes, a C/A and C/G mutation had been introduced in forward and reverse primers, respectively.[16]

The PCR conditions were modified in-house and visualization of PCR products was carried out using 8% polyacrylamide gel electrophoresis (PAGE). Table 1 outlines the primers and PCR conditions for the *NPM1* assay.

Table 1: Details of PCR for *NPM1*

Primers- were used for exon 12 of *NPM1* gene Bold letters depict mismatch introduced (C to G and C to A mutation)		**Position Accession number NM_002520**	**Product size**
NM-F2	5' – ATC AAT TAT GTG AAG AAT TGC TT**A** C-3'	901–925	349 bp
NPM-Rev6	5' – ACC ATT TCC ATG TCT GA**G** CAC C- 3'	1249–1228	

PCR reaction- was performed in a 25 µL reaction volume comprising of:

Reagent	**Quantity**
Taq Buffer	2.5 µl
dNTP (0.2 mM)	0.4 µl
Primers (10 pmol)	forward = 0.2 µl reverse = 0.2 µl
Taq polymerase	0.4 µl
$MgCl_2$ (2 mM)	0.1 µl
Autoclaved distilled water	19.2 µl
cDNA	2 µl

PCR conditions

Step	**Temperature - time and number of cycles**
Pre-Denaturation	95°C - 5 min x one cycle
Amplification -Denaturation -Annealing -Extension	 95°C - 30 sec 56°C - 45 sec x 35 cycles 72°C - 30 sec
Final Extension	72ºC - 7 minutes
Hold at 4ºC	

PCR products were then run on 8% PAGE and visualized on gel-documentation system (InGenius 3, Syngene, USA).

In cases with no mutation, a band was observed at 349 bp, and in cases with *NPM1* mutation, an additional band was seen at 353 bp (corresponding to 4 bp insertion) (Figure 1).

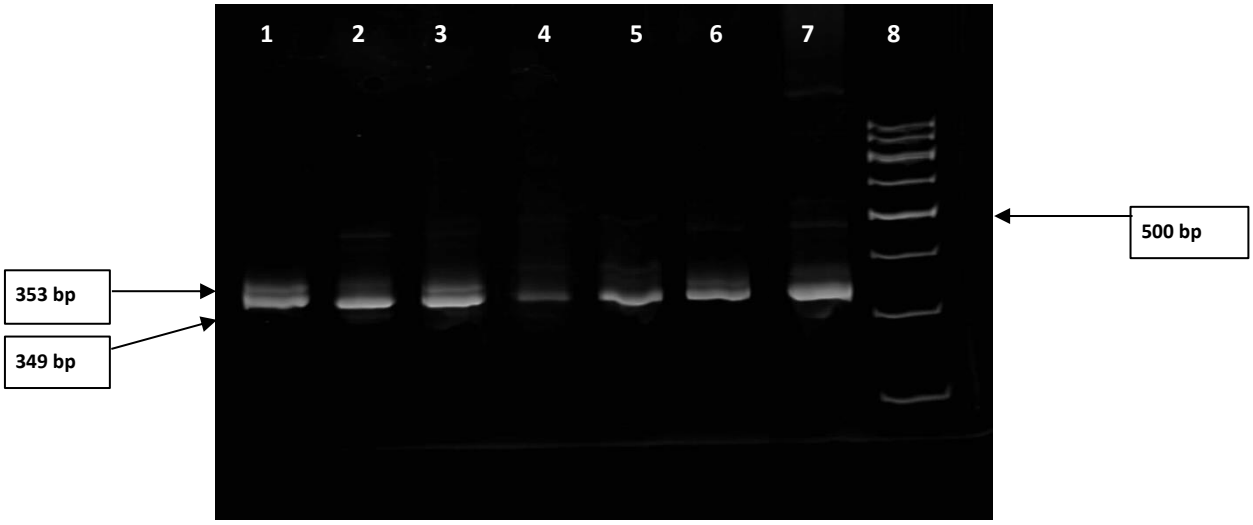

Figure 1: Polyacrylamide gel electrophoresis gel showing PCR products for *NPM1* mutation
***NPM1* mutation**: Lane 1, 3 and 6- showing an additional band at 353 bp (corresponding to 4 bp insertion) with normal band at 349 bp.
No mutation: Lane 2, 4, 5 and 7- showing a single band at 349 bp.
Lane 8= showing 100 bp ladder

Multiplex PCR for *FLT3*-ITD and TKD mutation: It was carried using the primers and PCR conditions described by Bianchini et al, which covers exon 14-15 for ITD and exon 20 for TKD mutations.[17] For TKD, restriction enzyme digestion was carried out using EcoRV restriction enzyme. The visualization of PCR products was carried out using 3% gel electrophoresis. Table 2 outlines the primers and PCR conditions for the *FLT3* assay.

Thereafter, digestion of PCR products was done with EcoRV at 37°C x 1 hour

[8 µl PCR product + 14 µl autoclaved distilled water + 2 µl 10X buffer + 0.5 µl EcoRV]

Table 2: Details of Multiplex PCR for *FLT3- ITD and TKD*

Primers- used for *FLT3*-ITD & TKD gene		**Accession number**	**Product size**
14F	5'- TGT CGA GCA GTA CTC TAA ACA-3'	NM_004119	366 bp
15R	5'- ATC CTA GTA CCT TCC CAA ACT C-3'	NM_004119	
20F	5' – CCG CCA GGA ACG TGC TTG- 3'	NM_004119	114 bp
20R	5' – GCA GAC GGG CAT TGC CCC- 3'	NM_004119	

PCR reaction- was performed in a 25 µL reaction volume comprising of-	
Reagent	**Quantity**
Taq Buffer	2.5 µl
dNTP (0.2 mM)	0.4 µl
Primers (10 pmol)	0.3 µl of 14F and 15R and 0.4 µl of 20R and 20F
Taq polymerase	0.5 µl
$MgCl_2$ (1.5 mM)	0.1 µl
Autoclaved distilled water	19.7 µl
cDNA	1.5 µl

PCR conditions		
Step	**Temperature - time and number of cycles**	
Pre-Denaturation	95°C -10 min –one cycle	
Amplification		
-Denaturation	95°C - 30 sec	
-Annealing	56°C - 45 sec	x 40 cycles
-Extension	72°C - 30 sec	
Final Extension	72ºC - 10 minutes	
Hold at 4ºC		

Products were then run on 3% agarose gel and visualized using gel-documentation system.

FLT3- ITD- In absence of mutation- a single band at 366 bp was seen; and in presence of ITD mutation- an additional band of larger size was seen.

FLT3- TKD- In absence of mutation- complete degradation of 114 bp product was seen- with two smaller bands of 68 bp and 46 bp; and in presence of mutation- undigested 114 bp band along with two smaller bands of 68 bp and 46 bp was seen; indicating the removal of EcoRV recognition site due to TKD mutation, as depicted in Figure 2.

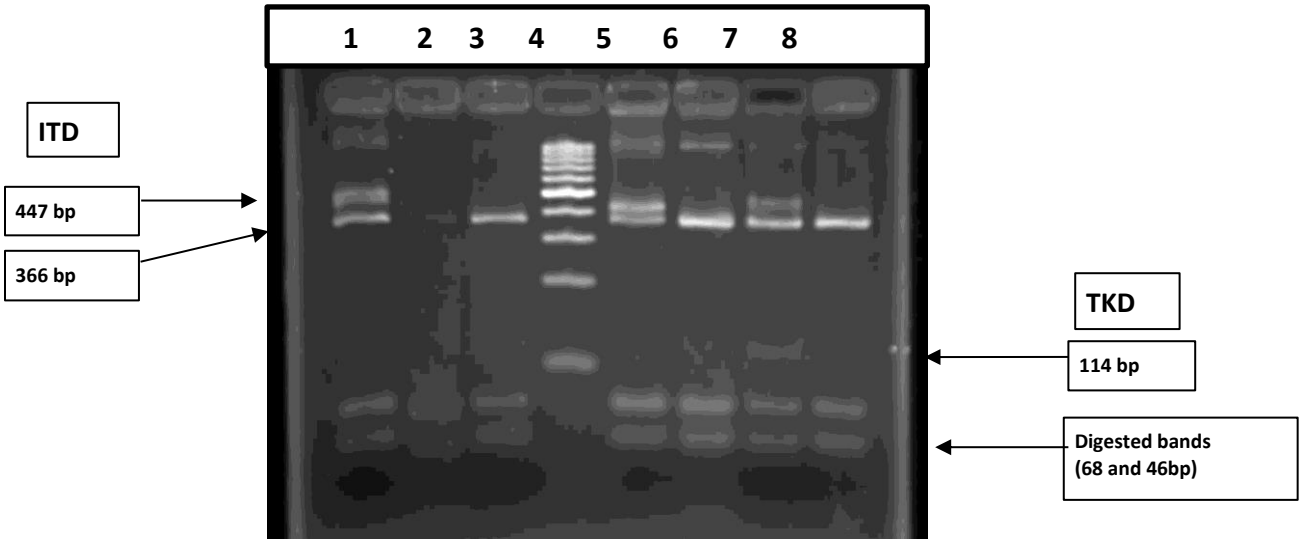

Figure 2: Agarose gel showing PCR products for *FLT3*- ITD and *FLT3*-TKD mutation.
ITD mutation: Lane 1, 5 and 7- An additional larger band is seen- in presence of ITD mutation.
Lane 3, 6 and 8- Single band at 366 bp is seen – in absence of ITD mutation.
TKD mutation: Lane 7- Presence of undigested 114 bp band indicates TKD mutations.
Lane 1,3,5,6 and 8- Complete digestion of 114-bp product indicates the absence of TKD mutations.
Lane 4= showing 100 bp ladder

RESULTS

A total of 92 patients were included in the study, of which 60 were males and 32 females (male:female ratio=1.9:1). The median age of patients was 35 years (range= 6 months – 89 years).

***NPM1 and FLT3-ITD/TKD* mutations**

Of the 92 cases studied, *NPM1* mutation was seen in 22.8% cases (21/92 cases) and *FLT3* mutations in 16.3% (15/92 cases) - [ITD in 8.7% (8/92 cases), TKD in 5.4% (5/92 cases) and ITD+TKD in 2.2% (2/92 cases)]. *NPM1* and *FLT3* mutations were not seen together in any case.

For analysis the cases were grouped as-

- *NPM1* and *FLT3* (ITD and TKD)- Negative (n=56, 60.9%)
- *NPM1* positive and *FLT3* (ITD and TKD) negative (n=21, 22.8%)
- *NPM1* negative and *FLT3*-ITD positive (n=8, 8.7%)
- *NPM1* negative and *FLT3*-TKD positive (n=5, 5.4%)
- *NPM1* negative and *FLT3*-ITD + TKD positive (n=2, 2.2%)

The details of the groups are summarized in Table 3.

Table 3: Characteristics of AML cases- with and without *NPM1*, *FLT3*-ITD and *FLT3*-TKD mutations

Characteristic	*NPM1* negative/ *FLT3* negative	*NPM1* positive/ *FLT3* negative	*NPM1* negative/ *FLT3* -ITD positive	*NPM1* negative/ *FLT3* -TKD positive	*NPM1* negative/*FLT3* -ITD & TKD positive
Number of patients	56 (60.9%)	21 (22.8%)	8 (8.7%)	5 (5.4%)	2 (2.2%)
Age in years, Mean, (range)	35 (1-75)	28.4 (0.6-76)	49.6 (11-89)	25 (11-58)	53 (44-61)
Male:female	39:17 (2.3:1)	7:14 (1:2)	5:3 (1.7:1)	2:3 (1:1.5)	0:2 (0:2)
Clinical features					
-Fever	25 (44.6%)	11 (52.4%)	4 (50%)	2 (40%)	2 (100%)
-Weakness	12 (21.4 %)	6 (28.6%)	1 (12.5%)	0	1 (50%)
-Breathlessness	5 (8.9%)	1 (4.8%)	0	0	0
-Bone pains	2 (3.6%)	3 (14.3%)	4 (50%)	1 (20%)	1 (50%)
-Bleeding	13 (23.2%)	5 (23.8%)	1 (12.5%)	1 (20%)	0
-Pallor	28 (32.1%)	13 (61.9%)	5 (62.5%)	3 (60%)	2 (100%)
Organomegaly					
-Spleen	9 (16.1%)	10 (47.6%)	2 (25%)	3 (60%)	1 (50%)
-Liver	13 (23.2%)	11 (52.4%)	1 (12.5%)	1 (20%)	0
-Lymphadenopathy	7 (12.5%)	5 (23.8%)	2 (25%)	2 (40%)	1 (50%)
Hemoglobin, g/L, mean, range	7.5 (3.8-12.4)	7.4 (3.9-11)	8.3 (6-10.6)	9.7 (8-12.6)	6 (3.6-8.3)
Reticulocyte, %, mean, range	1.6 (0.1-9.4)	1.8 (0.6-9.7)	2.3 (0.3-8.2)	1.1 (0.7-2)	1 (0.8-1.2)
Platelet count, $x10^9$/L, mean, range	49.8 (2.7-258)	57.6 (2.9-277)	59.4 (16-279)	113 (10-207)	22 (9-35)
TLC, $x10^9$/L, mean, range	18.3 (1.1-149.4)	57.4 (3.3-397.6)	121.9 (4.8-408.2)	24 (1-88.5)	52.4 (26.2-78.6)
Peripheral Blood blast%, mean, range	30 (0-97)	35 (0-86)	69 (8-95)	36 (0-96)	26 (21-30)
Bone Marrow blast%, mean, range	38 (0-97)	51 (0-83)	63 (0-97)	44 (3-88)	61 (60-62)
Bone Marrow hypercellularity, % cases	98	95	88	100	100
FAB-					
-M0	8 (14.3%)	0	0	1 (20%)	0
-M1	12 (21.4%)	2 (9.5%)	2 (25%)	0	0
-M2	18 (32.1%)	2 (9.5%)	2 (25%)	1 (20%)	0
-M3	12 (21.4%)	0	2 (25%)	2 (40%)	0
-M4	4 (7.1%)	11 (52.4%)	0	1 (20%)	1 (50%)
-M5	2 (3.6%)	4 (19%)	2 (25%)	0	0
-M6	0	2 (9.5%)	0	0	1 (50%)
-M7	0	0	0	0	0
PCR-% cases positive					
-PML-RARA	12 (21.4%)	0	2 (25%)	2 (40%)	0
-RUNX-RUNX1	2 (3.6%)	0	0	0	0
-CBFB-MYH11	0	0	0	1 (20%)	0
FCM-% cases positive					
-CD34	42 (75%)	10 (47.6%)	3 (37.5%)	3 (60%)	1 (50%)
-HLA-DR	40 (71.4%)	13 (62%)	4 (50%)	3 (60%)	1 (50%)
-B-lymphoid markers	7 (12.5%)	5 (23.8%)	0	1 (20%)	0
-T-lymphoid markers	6 (10.7%)	1 (4.8%)	2 (25%)	0	0

Briefly, patients with *FLT3*-ITD and *FLT3*-ITD+TKD mutations had higher age of presentation and this was statistically significant ($p < 0.05$). Male predominance was seen in *NPM1/FLT3* unmutated and patients with *FLT3*-ITD mutation. However, female predominance was seen in patients with *NPM1, FLT3*-TKD and *FLT3*-ITD +TKD mutations.

Fever was the most common presentation for all group of patients. Splenomegaly, hepatomegaly and lymphadenopathy were seen in 12- 60% patients and no statistical difference was seen in the different groups.

The hemoglobin and reticulocyte count did not show statistical difference in the different groups. However, patients with *FLT3*-TKD had higher platelet counts and with *FLT3*-ITD had higher total leucocyte count (TLC) and blast percentage in peripheral blood, and this was statistically significant ($p < 0.05$).

In the *NPM1/FLT3* unmutated group, FAB subtype M2 was the commonest, in *NPM1* mutated group M4/M5 was the commonest, whereas in the *FLT3*-ITD mutated group, FAB subtype M2, M3 and M4 were seen equally and in *FLT3*-TKD mutated group, M3 was the commonest.

No statistically significant difference was observed for bone marrow cellularity, myeloid to erythroid ratio or nature of erythropoiesis, presence of Auer rods, cytoplasmic granulations or MPO positivity, between the unmutated and *NPM1* mutated and *FLT3* mutated groups.

On flow cytometry immunophenotyping, myeloid antigens- CD13, CD33, CD117 and MPO were expressed in most of the cases with no significant difference between the groups.

B-lymphoid markers- CD19 and CD20 were seen in up to 20% cases in *NPM/FLT3* unmutated group, *NPM1* mutated and FLT3-*TKD* mutated group, however were not seen in *FLT3*-ITD mutated group.

T-lymphoid markers- CD3 was uniformly negative in all groups, CD7 and CD4 expression were seen in *NPM/FLT3* unmutated, *NPM1* and *FLT3*-ITD mutated groups.

Both *NPM1* and *FLT3*-ITD mutated cases showed CD34 expression in less than 50% cases. However, no statistical difference was observed in the expression of markers.

DISCUSSION

AML is a clonal stem cell neoplasm that is characterized by accumulation of myeloid blast cells, principally in the marrow and impaired production of normal blood cells. Molecular genetic analysis of acute leukemia has been at the forefront of research into the pathogenesis of cancer. The remarkable molecular heterogeneity of AML has made a genetic-based classification essential for accurate diagnosis, prognostic stratification, monitoring of minimal residual disease and developing targeted therapies.

NPM1 gene mutations are the most common genetic lesion described in adult de-novo AML. Analysis of *NPM1* has important clinical implications, with patients showing a higher complete remission rate than AML-NK without *NPM1* mutations after induction therapy.[10] The favorable response to therapy has been postulated to the fact that nucleoplasmic and cytoplasmic recruitment of NPM wild type (NPMwt) mediated by the NPM mutants might interfere with its functions. NPM is a nucleocytoplasmic shuttling protein with prominent nucleolar localization, regulates the ARF-p53 tumor-suppressor pathway; translocations involving the *NPM* gene cause cytoplasmic dislocation of the NPM protein. NPMwt protects hematopoietic cells from p53-induced apoptosis in conditions of cellular stress; the level of genotoxic stress appears to regulate this effect, it is speculated that NPM mutants fail to protect cells and renders them more susceptible to chemotherapy-induced high-level genotoxic stress.[9]

FLT3 encodes a tyrosine kinase receptor that is involved in hematopoietic stem cell differentiation and proliferation. *FLT3* is expressed on progenitor cells as well as on blast cells in most cases of AML. Mutations in *FLT3* are common in AML, seen in approximately 25-30% of AML patients. *FLT3* mutations are primarily of 2 types, *FLT3*-ITD and *FLT3*-TKD, of which *FLT3*-ITD is more common and associated with an adverse outcome[18,19].

The frequency of *NPM1* mutation in this study was 22.8% and *FLT3* mutations was 16.3%. The *NPM1*

frequency was similar to previously reported study done by us on a separate cohort of 100 AML patients, where we had tested for *NPM1* mutations by immunohistochemistry[20].

In current study, among the *FLT3* mutations, ITD mutations were common than TKD mutations, as has been previously reported[5]. Also, we found 2.2% (2/92) cases with both *FLT3*- ITD and TKD mutations. This frequency is comparable to 1.7%, previously reported by Thiede et al, where they found both *FLT3*-ITD and TKD mutations together in 17 of the 979 patients tested by them.[21]

A large UK study on 1312 AML patients by Lazenby et al, reported *NPM1* mutations in 21% patients and *FLT3*-ITD mutations in 16% patients. The authors also studied the prognostic relevance of these mutations and found that the *FLT3* mutation was associated with an inferior survival, and *NPM1* mutation had a significantly higher remission rate irrespective of treatment approach[22].

Another large recent study by Juliusson et al. from Sweden on 1461 AML [non - acute promyelocytic leukemia (APL)] reported 30% and 25% patients with *NPM1* and *FLT3*-ITD mutations in their study cohort. Their findings on this group of patients also reported that the prognostic impact of *FLT3*-ITD and *NPM1* mutation in adult AML is age-dependent[23].

Previous study from India by Chauhan et al. on 161 AML patients showed *NPM1* mutations in 21% patients, *FLT3*-ITD in 22% and *FLT3*-TKD in 3% patients[24].

Boonthimat et al. from Thailand on 400 AML patients reported *NPM1* and *FLT3*-ITD mutations in 26% and 33% patients, respectively.[25]

Overall, the frequency of *NPM1* and *FLT3* mutations in this study was similar to reported studies from this part of the sub-continent, which have reported it from 14-30% and 11-25% for *NPM1* and *FLT3* mutations, respectively[24-28]. But, the frequency was lower compared to western studies which indicated 21-55% and 16-40% for *NPM1* and *FLT3* mutations, respectively[2,5,7,21-23,29,30].

However, as the number of patients in our study were less and included all AML cases, a larger prospective study on cytogenetically characterized AML cases will provide a more definitive data on this. Table 4 summarizes the details of *NPM1* and *FLT3* mutations in AML cases studied by various authors.

Table 4: Frequency of *NPM1*, *FLT3*-ITD and *FLT3*-TKD mutations in AML cases

Country/ Year	Number of patients studied	*NPM1* mutation %, (number of patients)	*FLT3-ITD* mutation %, (number of patients)	*FLT3* -TKD mutation %, (number of patients)	*FLT3-ITD+TKD* mutation %, (number of patients)
Germany/ 2002[21]	979	-	20%, (n=200)	8%, (n=75)	2%, (n=17)
Germany and USA/ 2005[29]	300 (NK-AML)	48%, (n=145)	32%, (n=97)	9%, (n=29)	-
USA/ 2007[6]	295 (children only)	8%, (n=23)	19%, (n=52/270)	-	-
United Kingdom/ 2014[22]	1312	21% (n=252)	16%, (n=199)	-	-
Spain/ 2013[30]	303	53%, (n=161)	31%, (n=94)	-	-
Sweden/ 2020[23]	1461 (non-APL)	30%, (n=488)	25%, (n=356)	-	-
Thailand/ 2008[25]	400	26%, (n=105)	33%, (n=107)	-	-
China/ 2009[26]	220	16%, (n=36)	11%, (n=22)	-	-
Saudi Arabia/2014[27]	97	-	14% (n=14)	4%, (n=4)	0% (n=0)
India/ 2013[24]	161	21%, (n=34)	22%, (n=35)	3%, (n=5)	0% (n=0)
India/ 2017[28]	111(non-APL)	14%, (n=16)	11%, (n=12)	-	-
Present study	92	22.8%, (n=21)	8.7%, (n=8)	5.4%, (n=5)	2.2%, (n=2)

NK-AML, Normal karyotype acute myeloid leukemia. APL, acute promyelocytic leukemia. -, Not available.

Among the clinical features, similar frequency of fever and lymphadenopathy were seen in *NPM1* mutated and *FLT3*-ITD mutated groups. The hemoglobin and platelet counts were lower than normal in all groups. However, cases with *FLT3*-ITD mutation had higher total leucocyte count and blast percentage in peripheral blood. This finding is in concordance to previous reports for *FLT3*-ITD mutated cases.[24]

In the *NPM1/FLT3* unmutated group, FAB subtype M2 was the commonest, in *NPM1* mutated group M4/M5 was the commonest, whereas in the *FLT3*-ITD mutated group, FAB subtype M1, M2, M3 and M5 were seen equally and in *FLT3*-TKD mutated group, M3 was the commonest. These findings are also in concordance to previous reports[1,2,21].

The expression of flow cytometric markers seen in the current study, including decreased expression of CD34 and HLA-DR in *NPM1* mutated cases has been reported in previous studies also[1,2]. Other markers were not found to be significantly associated with *NPM1* or *FLT3* mutation status.

None of the *NPM1* mutated cases showed co-presence of *RUNX-RUNX1*, *PML-RARA* and *CBFB-MYH11* fusion genes. This is in concordance with the fact that the *NPM1* mutations and the WHO categories of recurrent genetic abnormalities are mutually exclusive.[1]

FLT3 mutations have been reported to occur in 30-40% cases of APL[31,32]. In this study, we found *FLT3* mutations in 25% cases of APL. Cases with *FLT3* mutations are associated with a higher TLC, microgranular morphology, and involvement of the bcr3 breakpoint of PML.[1]

In the present study, *NPM1* and *FLT3* mutations were not seen in any patient together in contrast to previous studies, which reported the co-occurrence of these mutations in 13-30% cases[2,24]. This aspect also needs to be studied further in a larger cohort of patients.

CONCLUSION

In the present study, the frequency of *NPM1* and *FLT3* mutations was 22.8% and 16.3% in de novo AML patients. Certain associations between gene mutations and clinical characteristics were also found, including in *NPM1* mutated group- female preponderance, higher incidence in M4/M5 categories and decreased expression of CD34 and HLA-DR; and in *FLT3*-ITD mutated group- higher age of presentation, higher TLC and blast percentage.

None of the cases in our study were positive for both *NPM1* and *FLT3* mutations, which is in contrast to previous studies, as a concomitant *FLT3* mutation has been reported in 13-30% AML cases with mutated *NPM1*.

These finding needs to be evaluated in a larger cohort of AML patients.

ACKNOWLEDGEMENTS

This study was supported by Intramural Research grant (No.71/6-Edu/13/1502) from Postgraduate Institute of Medical Education and Research, Chandigarh, India.

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