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

Shano Naseem1, Jogeshwar Binota1, Neelam Varma1, Harpreet Virk2, Subhash Varma3, Pankaj Malhotra3

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

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\(^5\), CEBPA in 4-9% of AML cases\(^3\), RUNX1 in 4-16\(^{\circ}\)\(^{\circ}\), 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. \(^\text{15}\)

**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. \(^\text{16}\)

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- used for exon 12 of NPM1 gene | Position Accession number | Product size |
|----------------------------------------|---------------------------|--------------|
| NM-F2 5’ – ATC AAT TAT GTG AAG AAT TGC TTA C-3’ | NM_002520 901–925 | 349 bp |
| NPM-Rev6 5’ – ACC ATT TCC ATG TCT GAG 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   |
| MgCl₂ (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      | x 35 cycles                            |
| - Denaturation     | 95°C - 30 sec                          |
| - Annealing        | 56°C - 45 sec                          |
| - Extension        | 72°C - 30 sec                          |
| Final Extension    | 72°C - 7 minutes                       |
| Hold at 4°C        |                                        |

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. 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   |
| MgCl₂ (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                           |
| -Extension            | 72°C - 30 sec                           |
| Final Extension       | 72°C - 10 minutes                       |
| Hold at 4°C           |                                          |

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.
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 positive/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                        | 29 (52.1%)                  | 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 (8-10.6)                     | 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 (9-97) | 35 (9-96) | 69 (8-95) | 36 (9-96) | 26 (9-96) |
| Bone Marrow blast%, mean, range | 38 (9-97) | 51 (9-97) | 63 (3-88) | 44 (9-97) | 61 (9-97) |
| 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                                     |
| -RUNX2-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.\(^9\) 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. In current study, among the FLT3 mutations, ITD mutations were common than TKD mutations, as has been previously reported. 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. 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.

Another large recent study by Julisson 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.

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.

Boonthimat et al. from Thailand on 400 AML patients reported NPM1 and FLT3-ITD mutations in 26% and 33% patients, respectively. 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. But, the frequency was lower compared to western studies which indicated 21-55% and 16-40% for NPM1 and FLT3 mutations, respectively.

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  | 979 | - | 20%, (n=200) | 8%, (n=79) | 2%, (n=17) |
| Germany and USA/ 2005 | 300 (NK-AML) | 48%, (n=145) | 32%, (n=97) | 9%, (n=29) | - |
| USA/ 2007  | 295 (children only) | 8%, (n=23) | 19%, (n=52/270) | - | - |
| United Kingdom/ 2014 | 1312 | 21%, (n=252) | 16%, (n=199) | - | - |
| Spain/ 2013  | 303 | 53%, (n=161) | 31%, (n=94) | - | - |
| Sweden/ 2020  | 1461 (non-APL) | 30%, (n=488) | 25%, (n=356) | - | - |
| Thailand/ 2008  | 400 | 26%, (n=105) | 33%, (n=107) | - | - |
| China/ 2009  | 220 | 16%, (n=36) | 11%, (n=22) | - | - |
| Saudi Arabia/2014  | 97 | - | 14% (n=14) | 4% (n=4) | 0% (n=0) |
| India/ 2013 | 161 | 21%, (n=34) | 22%, (n=35) | 3%, (n=5) | 0% (n=0) |
| India/ 2017 | 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.12,21 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.11,32 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 findings need 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.

REFERENCES

1. Arber DA, Orazi A, Hasserjian RP, et al. Introduction and overview of the classification of the myeloid neoplasms. In: Swerdlow SH, Campo E, Harris NL, et al. WHO classification of tumours of haematopoietic and lymphoid tissues. 2017 (Revised 4th edition); IARC press: Lyon. pp. 15-28.
2. Falini B, Mecucci C, Tiacci E, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 2005;352(3):254-66.
3. Nerlov C. CEBPalpha mutations in acute myeloid leukemias. Nat Rev Cancer. 2004;4(5):394-400.
4. Mendler JH, Maharry K, Radmacher MD, et al. RUNX1 mutations are associated with poor outcome in younger and older patients with cytogenetically normal acute myeloid leukemia and with distinct gene and MicroRNA expression signatures. J Clin Oncol. 2012; 30(25):3109-18.
5. Patnaik MM. The importance of FLT3 mutational analysis in acute myeloid leukemia. Leuk Lymphoma. 2018;59(10):2273-2286.
6. Brown P, McIntyre E, Rau R, et al. The incidence and clinical significance of nucleophosmin mutations in childhood AML. Blood. 2007; 110(3): 979-85.
7. Thiede C, Kohl S, Creutzig E, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood. 2006; 107(10):4011-20.
8. Haferlach C, Mecucci C, Schnittger S, et al. AML with mutated NPM1 carrying a normal or aberrant karyotype show overlapping biologic, pathologic, immunophenotypic, and prognostic features. Blood. 2009; 114(14):3024-32.
9. Falini B, Bolli N, Liso A, et al. Altered nucleophosmin transport in acute myeloid leukaemia with mutated NPM1: molecular basis and clinical implications. Leukemia. 2009; 23(10):1731-43.
10. Boissel N, Renneville A, Biggio V, et al. Prevalence, clinical profile, and prognosis of NPM mutations in AML with normal karyotype. Blood. 2005; 106(10):3618-20.
11. Smith CC, Wang Q, Chin CS, et al. Validation of ITD mutations in FLT3 as a therapeutic target in human acute myeloid leukaemia. Nature. 2012; 485(7397):260-3.
12. Stone RM, Mandrekar SJ, Sanford BL, et al. Midostaurin plus chemotherapy for acute myeloid leukaemia with a FLT3 mutation. N Engl J Med. 2017; 377(5):454-64.
13. Zarrinkar PP, Gunawardane RN, Cramer MD, et al. AC220 is a uniquely potent and selective inhibitor of FLT3 for the treatment of acute myeloid leukaemia (AML). Blood. 2009; 114(4):2984-92.
14. Döhner H, Estey E, Grimwade D, et al. Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 2017; 129(4):424-447.
15. Pakakasama S, Kajanchumphol S, Kanjanapongkul S, et al. Simple multiplex RT-PCR for identifying common fusion transcripts in childhood acute leukemia. Int J Lab Hematol. 2008; 30(4):286-91.
16. Noguera NI, Ammatuna E, Zangrilli D, et al. Simultaneous detection of NPM1 and FLT3-ITD mutations by capillary electrophoresis in acute myeloid leukemia. Leukemia. 2005; 19(8): 1479-82.
17. Bianchini M, Ottaviani E, Grafone T, et al. Rapid detection of Flt3 mutations in acute myeloid leukemia patients by denaturing HPLC. Clin Chem. 2003; 49(10):1642-50.
18. Kottaridis PD, Gale RE, Frew ME, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML10 and 12 trials. Blood. 2001; 98(6):1752-9.
19. Bacher U, Haferlach C, Kern W, et al. Prognostic relevance of FLT3-ITD mutations in AML: the combination matters—an analysis of 3082 patients. Blood. 2008; 111(5): 2527-37.
20. Rastogi P, Naseem S, Varma N, et al. Nucleophosmin mutation in de-novo acute myeloid leukemia. Asia Pac J Clin Oncol. 2016; 12(1): 77-85.
21. Thiede C, Steudel C, Mohr B, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. Blood. 2002; 99(12):4326-35.
22. Lazenby M, Gilkes AF, Marrin C, et al. The prognostic relevance of FLT3 and NPM1 mutations on older patients treated intensively or non-intensively: a study of 1312 patients in the UK NCRI AML16 trial. Leukemia. 2014; 28(10): 1953-9.
23. Julliusson G, Jadersten M, Deneberg S, et al. The prognostic impact of FLT3-ITD and NPM1 mutation in adult AML is age-dependent in the population-based setting. Blood Adv. 2020; 4(6): 1094-1101.
24. Chauhan PS, Ihsan R, Singh LC, et al. Mutation of NPM1 and FLT3 genes in acute myeloid leukemia and their association with clinical and immunophenotypic features. Dis Markers. 2013; 35(5):581-8.
25. Boonthimat C, Thongnoppakhun W, Auewarakul CU. Nucleophosmin mutation in Southeast Asian acute myeloid leukemia: eight novel variants, FLT3 coexistence and prognostic impact of NPM1/FLT3 mutations. Haematologica. 2008; 93(10):1565–9.
26. Ruan GR, Li JL, Qin YZ, et al. Nucleophosmin mutations in Chinese adults with acute myelogenous leukemia. Ann Hematol. 2009; 88(2): 159–66.
27. Elyamany G, Awad M, Fadalla K, et al. Frequency and prognostic relevance of FLT3 mutations in Saudi acute myeloid leukemia patients. Adv Hematol. 2014; 2014:141360.
28. Sazawal S, Singh N, Jain S, et al. NPM1 and FLT3 mutations in acute myeloid leukemia with normal karyotype: Indian perspective. Indian J Pathol Microbiol. 2017; 60(3):355-359.
29. Döhner K, Schlenk RF, Habdank M, et al. Mutant nucleophosmin (NPM1) predicts favorable prognosis in younger adults with acute myeloid leukemia and normal cytogenetics: interaction with other gene mutations. Blood. 2005; 106(12):3740-6.
30. Pratcorona M, Brunet S, Nomdedeu J, et al. Favorable outcome of patients with acute myeloid leukemia harboring a low-allelic burden FLT3-ITD mutation and concomitant NPM1mutation: relevance to post-remission therapy. Blood. 2013;121: 2734-38.
31. Breccia M, Loglisci G, Loglisci MG, et al. FLT3-ITD confers poor prognosis in patients with acute promyelocytic leukemia treated with AIDA protocols: long-term follow-up analysis. Haematologica .2013; 98: e161-3.
32. Callens C, Chevret S, Cayuela JM, et al. Prognostic implication of FLT3 and Ras gene mutations in patients with acute promyelocytic leukemia (APL): a retrospective study from the European APL Group. Leukemia. 2005; 19: 1153-60.