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Journal of Hematology and Allied Sciences

Original Research

Utility of mutation-specific immunohistochemical markers for predicting post-induction remission among acute myeloid leukemia patients – A prognostic test accuracy study

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Received: 30 May 2023
Accepted: 12 June 2023
EPub Ahead of Print: 25 July 2023
Published: 07 February 2024

DOI
10.25259/JHAS_19_2023

Quick Response Code:

ABSTRACT

Objectives: Various prognostically important genetic mutations are associated with acute myeloid leukemia (AML). Studies have found correlation between these mutations and expression of certain abnormal proteins in the tumor cells by immunohistochemistry (IHC). Common genetic mutations are nucleophosmin 1 (NPM1) and FMS like tyrosine kinase 3 (FLT3). This study aimed at studying the prognostic utility of surrogate IHC for these mutations-NPM1 IHC for NPM1 mutation, whereas C-X-C Chemokine Receptor type 4 (CXCR4) and Cluster of Differentiation 123 (CD123) IHC for FLT3 mutation in AML patients.

Material and Methods: This was a prognostic test accuracy study done in a tertiary care centre over a period of two years (2018–2020) under two subgroups: who attained remission (remission group) and who failed to achieve remission (not in remission group) after induction therapy. Prognostic IHC markers were performed on the diagnostic bone marrow biopsy.

Results: There were 70 cases in remission and 49 cases not in remission with median age of 32 and 31 years, respectively. Median total leucocyte count was significantly more in remission group ($P = 0.02$). AML subtype and cytogenetics wise, remission group, had significantly more M3 and M4 subtypes and translocations, while not in remission group had more M2 and M1 and more of normal and complex cytogenetics ($P = 0.01$ and 0.03, respectively). NPM1 and FLT3 mutation did not show significant association with remission status. IHC for NPM1, CXCR4, and CD123 was performed in the diagnostic bone marrow biopsy. Loss of nuclear localization of NPM1 and CXCR4 positivity by IHC was more in remission than not in remission (34.3% vs. 28.6% and 54.3% vs. 44.9%, respectively) which was not statistically significant. The expression of NPM1, CXCR4, and CD123 IHC had low sensitivity (34%, 54%, and 4.3%, respectively) to predict remission status. NPM1 IHC was highly significantly associated with NPM1 mutation and had high sensitivity (89%) and specificity (86%) to predict NPM1 mutation whereas CXCR4 and CD123 had low sensitivity, specificity to predict FLT3 mutation.

Conclusion: NPM1 IHC can be used as a surrogate to predict NPM1 mutation whereas CXCR4 and CD123 are not effective surrogates to predict FLT3 mutation.

Keywords: Acute myeloid leukemia, Bone marrow, Cluster of differentiation 123, C-X-C Chemokine receptor type 4, FMS like tyrosine kinase 3, Mutation-specific immunohistochemistry, NPM1, surrogate immunohistochemistry markers

INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous group of neoplastic disorders characterized by clonal expansion of myeloid progenitors (blasts) in the bone marrow (BM) and peripheral blood.

AML is associated with varied cytogenetics and genetic mutations. Within the past few years, many novel genetic mutations have been identified in essentially all types of AML, such as CCAAT enhancer binding protein alpha (CEBPA) and nucleophosmin 1 (NPM1), involving transcription factors, FMS like tyrosine kinase 3 (FLT3), Neuroblastoma rat sarcoma (NRAS), and Kirsten rat sarcoma (KRAS), which affect signal transduction and mutations in epigenetic regulators, including TET2, IDH1, IDH2, ASXL1, and DNMT3A.[1]

It has been observed that these mutations have an effect on patient outcome and treatment response, thereby making mutational analysis important in prognostication.[2] In addition, there are targeted therapies under trial for some of these mutations, which can be included in the treatment regimen for better response. Hence, evaluation of these prognostic mutations becomes an integral part in workup of AML patients. However, these mutations are assessed by conventional molecular methods which are expensive and not all resource limited settings can afford such mutational analysis. Studies have found correlation between these prognostic mutations and expression of certain abnormal proteins in the tumor cells. This can be estimated using antibodies directed against them by immunohistochemical methods.[3-6] The expression of these immunohistochemistry (IHC) markers when correlated to treatment response can be used as cost effective prognostic markers. This study aims to determine the predictive capacity of the surrogate mutation-specific immunohistochemical markers in determining treatment response.

Common genetic mutations in AML are NPM1 and FLT3; hence, this study aimed at studying the prognostic utility of surrogate IHC for these two mutations. In our study, NPM1 IHC has been used as a surrogate for NPM1 mutation, whereas C-X-C Chemokine Receptor type 4 (CXCR4) and Cluster of Differentiation 123 (CD123) IHC have been used for FLT3 mutation.[5,7,8] The objective of the study was to describe the pattern of expression of these mutation-specific IHC markers in AML and to assess their prognostic accuracy for post-induction remission outcome.

MATERIAL AND METHODS

Study design and settings

The study was carried out in a tertiary health care center in southern India over 2-year period (2018–2020) and was approved by the Institute Ethics Committee. The study design was a prognostic test accuracy, to determine the prognostic accuracy of mutation-specific IHC markers in AML patients under the two subgroups, AML patients who attained remission after induction treatment (remission group), and AML patients who failed to achieve remission after induction treatment (not in remission group).

Sample size

Initial sample size calculated was 73 cases in each group achieving post-induction remission and no remission, which was calculated taking sensitivity and specificity as 95%, confidence intervals as 95% and precision as 5%. However, the number of patients who failed to achieve remission was less than the initial estimate taken to calculate the sample size, possibly due to more effective induction regimens in recent years. Moreover, due to COVID-19 pandemic, there were reduced number of cases referred to the institute in the study year. Hence, finally 70 cases in remission group and 49 cases in not in remission group could be enrolled.

Enrolment of patients and Inclusion and exclusion criteria

All eligible patients whose remission status was known, and initial diagnostic BM biopsy was available were enrolled consecutively both in a prospective and a retrospective manner between January 2017 and October 2020. Patients with unavailable blocks, insufficient tissue to perform IHC, or where the primary diagnosis was made by flowcytometry without BM biopsy were excluded from the study. Peripheral blood findings such as hemoglobin, total leukocyte count (TLC), platelet count, and blast percentage and diagnostic BM findings such as cellularity, blast percentage marrow fibrosis, myelonecrosis, and subtype were recorded from the pathology records. The treatment, cytogenetic and mutation status, and survival data were recorded from medical oncology records [Figure 1].

Immunohistochemical markers used and their interpretation

Markers studied were NPM1, CXCR4, and CD123. All IHC staining for standardization and for individual cases was performed by manual staining method.

a. NPM1, Clone FC 61991 from Thermo Fischer at 1:100 dilution (standardized in wild type AML BM biopsy). NPM is localized to nucleus/nucleolus. Hence, this pattern of staining is taken as wild type or negative for NPM1 mutation. Cytoplasmic wispy staining or no staining at all is interpreted as positive for NPM1 mutation [Figure 2a-d, upper panel].
b. CXCR4, Clone 124824 from Abcam at 1:100 dilution (standardized in tonsil tissue). Cases with cytoplasmic or cytoplasmic and nuclear staining in >10% blasts were taken as positive. Positive IHC stain was taken as an indicator of FLT3 mutated status. Cases with <10% staining, smudgy staining, or no staining with or without internal control were taken as negative. This is taken as indicator of wild type FLT3 [Figure 2e-h, lower panel].
c. CD123/interleukin 3 receptor, clone 6H6 from stem cell at 1:100 dilution (standardized in Kikuchi lymphadenitis). Cases with cytoplasmic staining in

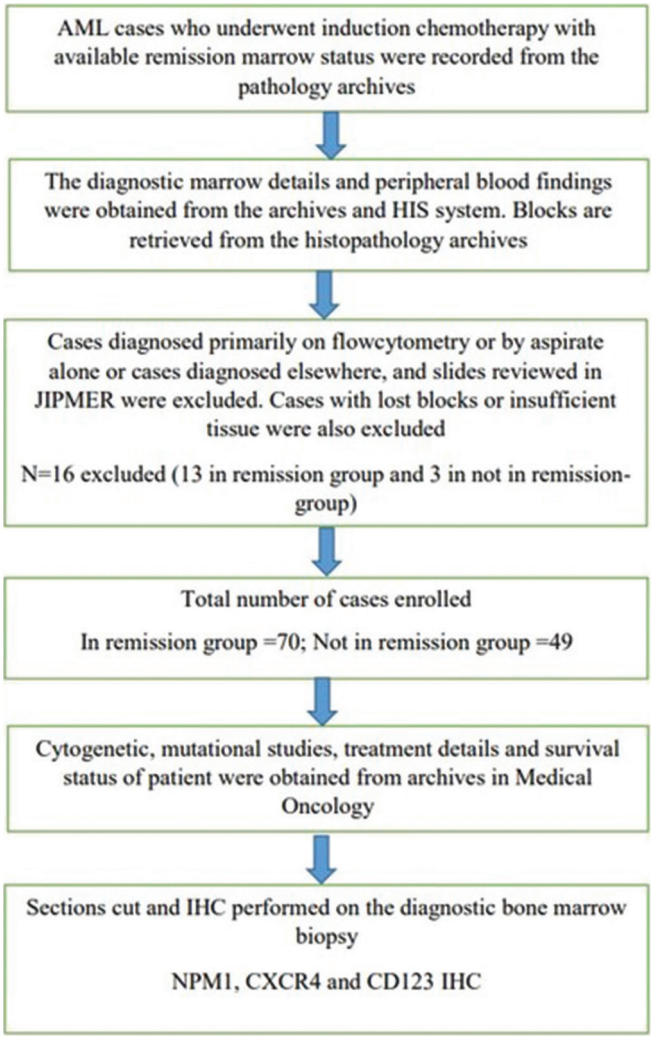

Figure 1: Flow chart depicting the study methodology.

>20% of blasts were taken as positive. Positive IHC stain was taken as indicator of the FLT3 mutated status. Cases with <20% staining or negative staining were taken as negative. This was taken as wild type FLT3 [Figure 3a-c, upper panel]. An illustrative case with all the three IHC markers done is given [Figure 3d-g, lower panel].

Statistical analysis

All the data collected were analyzed using SPSS software IBM PASW statistics (ver 20.0). The distribution of categorical variables was expressed as frequency and percentages. The continuous data were expressed as mean with standard deviation or median with range based on the normality of data. Normality of data was tested by Kolmogrov–Smirnoff test. Association of AML subtype, cytogenetics, expression of molecular mutations, and prognostic IHC to the remission groups was done using chi-square test or Fischer exact test. Association of continuous variables with remission status was done using independent Student *t*-test/Mann–Whitney U-test based on the normality of data. Prognostic test accuracy of the prognostic IHC markers to predict remission was expressed in sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). All the statistical analysis was carried out at 5% level of significance and $P < 0.05$ was considered as significant.

RESULTS

In remission group, the median age with interquartile range (IQR) was 32 (21–44) years. Out of the 70 patients who attained remission that 48% belonged to the age group of 19–40, 16% were children and 5% elderly (above 60 years of age). Thirty-seven out of 70 (53%) patients were female and 33 out of 70 (47%) male patients with a female-to-male preponderance of 1.1:1. In not in remission group the median age with IQR was 31 (18–45) years. Out of the 49 patients who failed to achieve remission that 45% belonged to the age group of 19–40, 22% were children and 6% elderly (above 60 years of age). Twenty-seven out of 49 (55%) patients were male and 22 out of 49 (45%) female patients with a slight male-to-female preponderance of 1.2:1. Age and gender distribution between the two groups was not statistically significant.

Peripheral blood parameters at the time of diagnosis

At diagnosis, the mean hemoglobin in remission group was 6.6 (Standard Deviation [SD] 2.05) g/dL and in not in remission group was 6.9 (SD 1.84) g/dL. Median with IQR of platelet count in remission group was 32,000 (21,500–54,500) cells/cu.mm and in not in remission group was 39,000 (25,000–73,000) cells/cu.mm. Median with IQR of peripheral blood blast in remission group was 76 (38–89%) and in not in remission group 60 (13–86%). There was no significant difference in hemoglobin, platelet count, or peripheral blood blast percentage of the two groups ($P > 0.05$). The median TLC in remission group was 18,400 (6005–59,480) cells/cu.mm and in not in remission group was 9370 (2530–28,080) cells/cu.mm, and it was significantly higher in remission group as compared to the not in remission group ($P = 0.02$). The demographic and peripheral blood parameters at diagnosis in the two groups are given in Table 1.

BM aspiration-AML subtype

Among the remission subgroup M3 acute promyelocytic leukemia (APML) constituted the highest number of cases with 25.7% ($n = 18$), followed by M2 (22.8%, $n = 16$), M1 (21.4%, $n = 15$), and M4 (20.0%, $n = 14$). Among not in remission subgroups, M1 formed the majority with 32.6% ($n = 16$), followed by M2 (26.5%, $n = 13$) and M4 (14.3%, $n = 07$). This variation in subtypes between the two remission groups was found to be statistically significant with $P = 0.001$.

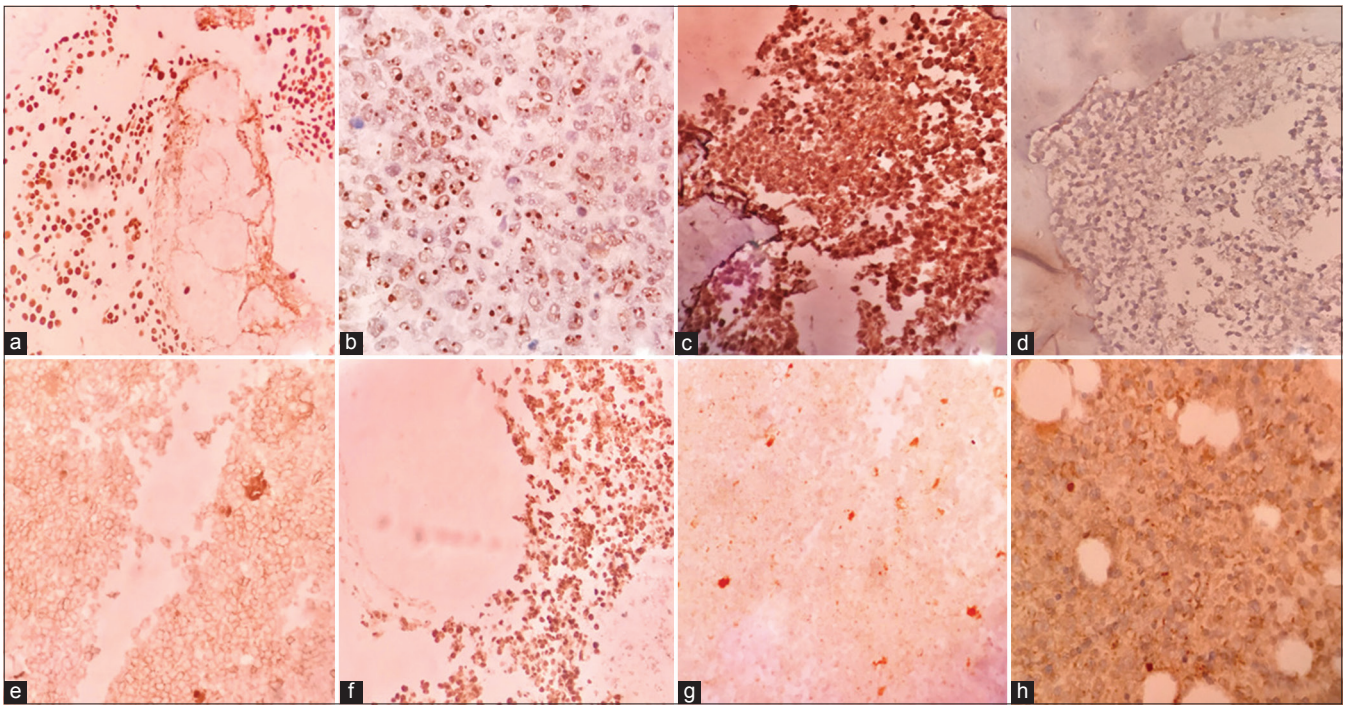

Figure 2: Upper panel shows patterns of staining of NPM1 (a) nuclear, (b) nucleolar, (c) cytoplasmic, and (d) nil staining interpreted as wild type for patterns (a and b) and mutant type for patterns (c and d), respectively (IHC ×400); lower panel shows patterns of staining of CXCR4 (e) cytoplasmic, (f) cytoplasmic and nuclear, (g) negative (internal control megakaryocytes positive), and (h) smudgy staining interpreted as positive (surrogate for FLT3 mutation) for patterns (e and f) and negative for patterns (g and h), respectively (IHC ×400). NPM1: Nucleophosmin 1, IHC: Immunohistochemistry, CXCR4: C-X-C chemokine receptor type 4, FLT3: FMS like tyrosine kinase 3.

Table 1: Demographic and peripheral blood parameters at time of diagnosis.

Parameter	Remission group (*n*=70)	Not in remission group (*n*=49)	*P*-value
Age			
Median with IQR	32 (21–44)	31 (18–45)	0.59
0–18	11 (16%)	12 (24%)	
19–40	34 (48%)	22 (45%)	
40–60	22 (31%)	12 (24%)	
>60	3 (5%)	3 (6%)	
Gender			
Male	33 (47%)	27 (55%)	0.39
Female	37 (53%)	22 (45%)	
Hemoglobin			
Mean (SD) g/dL	6.6 (2.05)	6.9 (1.84)	0.3
TLC			
Median (IQR) Cells/cu.mm	18,400 (6005–59,480)	9370 (2530–28,080)	**0.02**
Platelet			
Median (IQR) Cells/cu.mm	32,000 (21,500–54,500)	39,000 (25,000–73,000)	0.19
Blast count			
Median (IQR) percentage	76 (38–89)	60 (13–86)	0.07

IQR: Interquartile range, SD: Standard deviation, TLC: Total leukocyte count, Bold value: Statistically significant

Hence, M3 and M4 were seen significantly more in remission group as compared to the not in remission group. Overall (n = 119), M1 was the most common subtype with 26.0% (n = 31), followed by M2 with 24.4% (n = 29), M4 with 17.6% (n = 21), and M3 with 15.9% (n = 19).

BM biopsy

Cellularity of BM biopsy, presence of myelonecrosis, and myelofibrosis was noted from the diagnostic BM biopsy. Among remission patients, 97.2% had hypercellular marrow and 2.8% had normocellular biopsy. None of the cases in remission group had hypocellular marrow. Among not in remission cases, 89.8% had hypercellular marrow, 6.1% had hypocellular marrow, and 4.1% had normocellular marrow. However, this was not statistically significant with P = 0.09. None of the remission cases had myelonecrosis, one out of 49 cases (2.0%) in not in remission group had 90% of core showing myelonecrosis. Four cases each in remission group (5.7%) and not in remission group (8.2%) had myelofibrosis at diagnosis. Degrees of fibrosis along with other BM findings are listed in Table 2.

Cytogenetics

Cytogenetic details were available for 59 patients in remission and 26 patients in not in remission group. In remission group, translocations were the most common cytogenetic

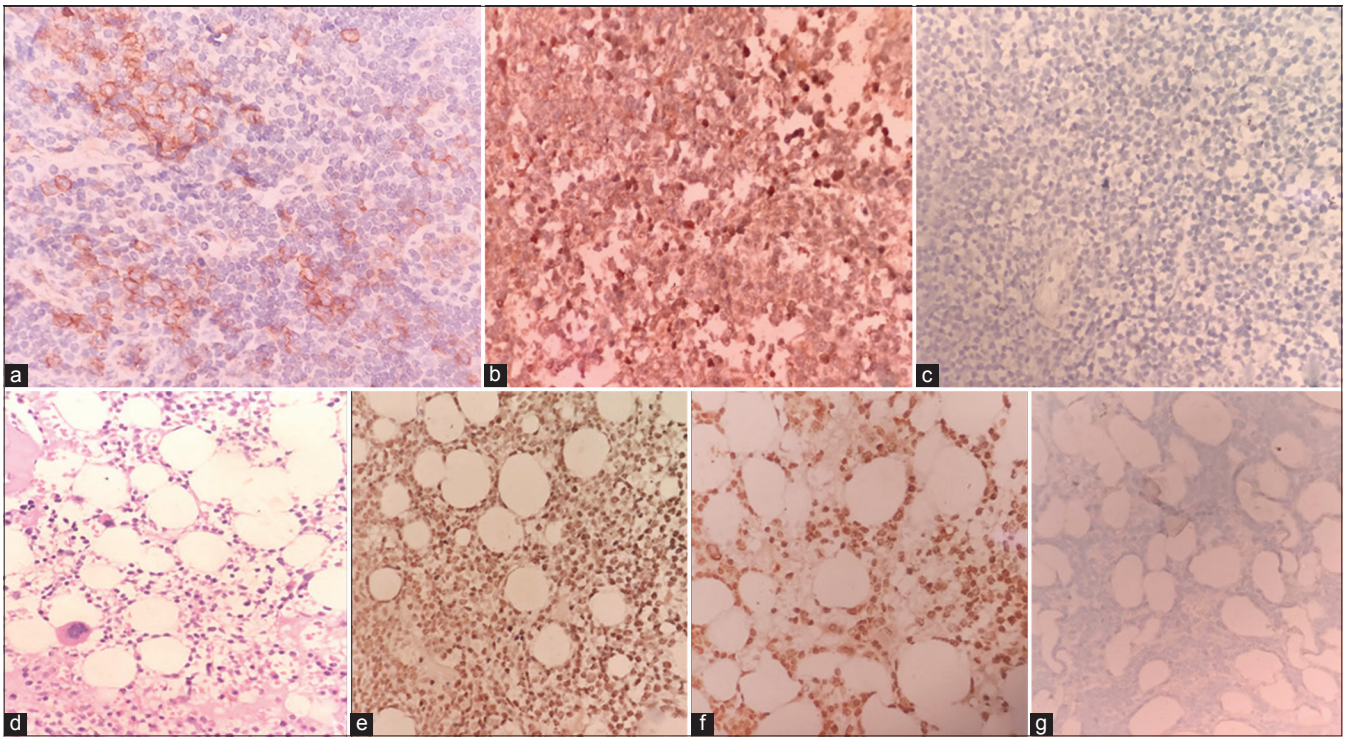

Figure 3: Upper panel shows patterns of staining of CD123 showing (a) positivity in plasmacytic dendritic cells of kikuchi lymphadenitis as part of standardization, (b) cytoplasmic, and (c) negative staining interpreted as positive (surrogate for FLT3 mutation) for pattern (b) and negative for pattern (c) (IHC ×400); lower panel shows a case of hypocellular AML showing (d) bone marrow biopsy morphology (H&E, ×400), (e) nuclear pattern of NPM1 IHC, (f) cytoplasmic positivity for CXCR4 IHC, and (g) negative staining for CD123 in the blasts (IHC ×400). CD123: Cluster of differentiation 123, FLT3: FMS like tyrosine kinase 3, IHC: Immunohistochemistry, NPM1: Nucleophosmin 1, CXCR4: C-X-C chemokine receptor type 4, AML: Acute myeloid leukemia.

abnormality constituting 45.8% (n = 27), followed by normal cytogenetics in 37.2% (n = 22) of cases and trisomy in 6.8% (n = 4) cases. Among translocations, most common was t(15,17) followed by t(8,21). In not in remission group, normal cytogenetics formed the majority with 42.3% (n = 11) cases, followed by complex cytogenetics and translocations with each 23.1% cases (n = 6). Remission group had more translocations as compared to not in remission group, while complex cytogenetics and normal cytogenetics were observed with increasing frequency in not in remission group. This was statistically significant with P = 0.03. The cytogenetic profile among the two groups is given in Table 3.

Mutational status of molecular markers

Out of 119 cases, NPM1 mutation status was known in 63 cases (52.9%) of which 38 were in remission and 25 not in remission and FLT3 mutation status was known in 65 cases (54.6%) of which 38 were in remission and 27 not in remission.

NPM mutation was seen in 19 cases out of 63 (30%) and the rest 44 cases (70%) were wild type NPM. Out of 38 cases in remission whose NPM 1 status was known, 24 (63%) were wild type and 14 (37%) mutated. Out of 25 cases of not in remission with known NPM1 status, 20 (80%) were wild type and 05 (20%) mutated. FLT3 mutation was seen in 16 cases out of 65 (24.6%) and rest 49 cases (75.4%) were wild type for FLT3. Out of 38 cases in remission whose FLT3 status was known, 26 were wild type and 12 mutated. Out of 27 cases of not in remission with known FLT3 status, 23 were wild type and 4 mutated. Association of NPM1 and FLT3 mutation status with remission groups was not statistically significant (P = 0.1). Hence, in our study, NPM1 and FLT3 mutation had no significant association with remission status, as summarized in Table 4.

Expression pattern of prognostic immunohistochemical markers and its association with remission status

The various patterns of expression of these IHC markers are given in Table 5.

NPM1 IHC patterns observed in our study were nuclear, nuclear and nucleolar, cytoplasmic wispy, and negative staining (external control positive). Nuclear and nuclear with nucleolar staining pattern was considered as retained NPM IHC staining which is a marker of wild type NPM1.

Table 2: AML subtypes and bone marrow biopsy findings between the remission groups.

Subtype	Remission (N=70) *n* (%)	Not in remission (N=49) *n* (%)	*P*-value
Subtype			
M0	01 (1.4)	05 (10.2)	**0.001**
M1	15 (21.4)	16 (32.6)	
M2	16 (22.8)	13 (26.5)	
M3	18 (25.7)	01 (2.0)	
M4	14 (20.0)	07 (14.3)	
M5	01 (1.4)	01 (2.0)	
M6	0	01 (2.0)	
AML-MDS	02 (2.8)	02 (4.1)	
APMF	0	01 (2.0)	
Not typed	03 (4.2)	02 (4.1)	
Bone marrow cellularity			
Hypercellular	68 (97.2)	44 (89.8)	0.09
Normocellular	02 (2.8)	02 (4.1)	
Hypocellular	00	03 (6.1)	
Myelofibrosis			
Absent	66 (94.3)	45 (91.8)	0.7
Present	04 (5.7)	04 (8.2)	
Grade 1	02 (2.9)	03 (6.1)	
Grade 2	01 (1.4)	01 (2.0)	
Grade 3	01 (1.4)	00	

AML: Acute myeloid leukemia, AML-MDS: Acute myeloid leukemia-Myelodysplastic syndrome, APMF: Acute panmyelosis with myelofibrosis, Bold value: Statistically significant

Table 3: Cytogenetic profile in remission groups.

Cytogenetics	Remission (N=59) *n* (%)	Not in remission (N=26) *n* (%)
Translocation	27 (45.8)	06 (22.1)
t (15,17)	16	01
t (8,21)	10	04
t (16,16)	01	0
t (6,9)	0	01
Normal	22 (37.2)	11 (42.3)
Trisomy	04 (6.8)	02 (7.7)
+4	02	0
+8	01	02
+21	01	0
Complex	02 (3.4)	06 (23.1)
Deletions	02 (3.4)	01 (3.8)
11q23	01	0
5q	01	0
7	0	01
Inversion	01 (1.7)	0
Hypoploidy	01 (1.7)	0

P=0.03

Cytoplasmic and negative staining was taken as loss of nuclear NPM1 IHC, which is a marker of mutated NPM1. In remission group, 65.7% showed retained staining, while, in not in remission group, 71.4% showed retained staining.

The CXCR4 IHC patterns observed in our study were cytoplasmic, cytoplasmic and nuclear, negative staining with or without internal control, and smudgy staining pattern. Cytoplasmic, cytoplasmic and nuclear staining of 10%, or more blasts were considered positive staining while negative or smudgy staining pattern was taken as negative. In remission group, 54.3% showed positive staining pattern and 45.7% showed negative staining pattern. In not in remission group, 44.9% showed positive staining pattern and 55.1% showed negative staining.

CD123 staining patterns observed were cytoplasmic or negative staining pattern. Cytoplasmic staining in 20% or more of blasts was considered as positive staining. About 4.3% cases in remission and 4.1% cases in not in remission showed positive CD123 staining.

Overall, loss of nuclear localization of NPM1 IHC was seen in 38 (31.9%) cases, CXCR4 positivity was seen in 60 (50.4%) cases, and CD123 positivity in 5 (4.2%) cases. Loss of nuclear localization of NPM1 and CXCR4 positivity by IHC was more in remission than not in remission (34.3% vs. 28.6% and 54.3% vs. 44.9%, respectively). The patterns of IHC staining, that is, loss of nuclear localization of NPM1 IHC (mutated form), CXCR4 IHC positivity, and CD123 IHC positivity did not show any significant association with remission status, as shown in Table 6.

Prognostic test accuracy of IHC markers to estimate remission status

Sensitivity of NPM1 IHC to predict remission was 34% with a specificity of 71%. PPV was 63% and NPV 43%. Sensitivity of CXCR4 IHC to predict remission was 54% with a specificity of 55%. PPV was 63% and NPV 46%. Sensitivity of CD123 IHC to predict remission was 4.3% with a specificity of 96%. PPV was 60% and NPV 41% [Table 7].

Hence, from the above, NPM1, CXCR4, and CD123 cannot act as a standalone prognostic test to determine remission status.

IHC markers as surrogates for genetic mutations

Loss of nuclear localization of NPM1 IHC was highly significantly associated with NPM1 mutation. NPM1 IHC had a sensitivity of 89% and specificity of 86% to predict NPM1 mutation with a PPV of 74% and NPV of 95%. Hence, NPM1 IHC can be used as immunohistochemical surrogate for NPM1 mutation. CXCR1 IHC had a sensitivity of 56%, specificity of 45% to predict FLT3 mutation with a positivity predictive value of 25%, and NPV of 76%. Meanwhile none of the cases with CD123 IHC positivity had FLT3 mutation;

Table 4: Association of NPM1 and FLT 3 mutation with remission groups.

Mutation	Remission (%)	Not in remission (%)	Total	Statistical significance
NPM1 (*n*=63)				
Wild	24 (63)	20 (80)	44	Chi-square=2.03
Mutated	14 (37)	05 (20)	19	*P*=0.15
FLT3 (*n*=65)				
Wild	26 (68.4)	23 (85.2)	49	Chi-square=2.39
Mutated	12 (31.6)	04 (14.8)	16	*P*=0.12

NPM1: Nucleophosmin 1, FLT3: FMS like tyrosine kinase 3

Table 5: Pattern of expression of NPM1, CXCR4, and CD123 IHC.

IHC pattern	Remission group (N=70) *n* (%)	Not in remission group (N=49) *n* (%)
NPM1		
Nuclear	41 (58.6)	33 (67.3)
Nuclear and nucleolar	05 (7.1)	02 (4.1)
Cytoplasmic	20 (28.6)	14 (28.6)
Negative staining	04 (5.7)	0
CXCR4		
Cytoplasmic	37 (52.9)	22 (44.9)
Cytoplasmic and nuclear	01 (1.4)	0
Negative	15 (21.4)	15 (30.6)
Smudgy	17 (24.3)	12 (24.5)
CD123		
Cytoplasmic	03 (4.3)	02 (4.1)
Negative	67 (95.7)	47 (95.9)

NPM1: Nucleophosmin 1, IHC: Immunohistochemistry, CXCR4: C-X-C chemokine receptor type 4, CD123: Cluster of differentiation 123

hence, sensitivity and PPV cannot be estimated. CD123 had a specificity of 90% and a NPV of 73% to predict FLT3 mutation. Hence, both CXCR4 and CD123 IHC are not surrogates for predicting FLT3 mutation status [Table 8].

DISCUSSION

AML is the most common type of leukemia in adults. AML is a heterogenous neoplastic disorder characterized by clonal expansion of myeloid progenitors (blasts) in the BM and peripheral blood. AML patients undergo induction chemotherapy, response to which is assessed by BM examination and achievement of remission status. There are many prognostic mutations in AML, most common of which are NPM1 and FLT3. This study aimed to correlate surrogate IHC for the above mutations and other clinicohematological parameters with remission status.

In our study, the median age at presentation for AML was 32 years in the remission group and 31 years in the not in remission group. Many studies from western population have shown that AML is a disease of elderly and occurs at a median age of 70.[9-11] However, the previous studies from India, Pakistan, and Malaysia have shown median age at presentation of 32–39 years,[12-14] similar to our study. Sultan *et al.*[12] have attributed this difference to genetic and geographical differences between western and Asian populations. Studies have shown a male preponderance in AML.[12,13,15] In our study, the remission group had a slight female preponderance 1.1:1, while the not in remission group had a male preponderance 1.2:1. In a study performed by Kulsoom *et al.*,[16] 8.5% of patients were children <15 years; in our study, 16 % of remission cases and 22% of not in remission cases were under 18 years of age. Hence, our study shows a relatively higher incidence of childhood AML.

Mean hemoglobin from the previous studies of AML in Asian countries range between 8.1 g/dL and 8.4 g/dL.[12,16] Our study shows a lower mean hemoglobin of 6.6–6.9 g/dL. Reasons for this variation are not known, possibly can be due to delayed presentation and hence extensive marrow replacement or a pre-existing nutritional deficiency anemia. Contrary to the previous studies showing association of increased TLC to adverse prognosis,[17-19] our study showed that the remission group had increased median total leucocyte count as compared to the not in remission group. Extensive literature search did not yield any similar studies with significant increase in TLC in the remission group. Possible explanation to this variation can be that this study compares TLC with respect to remission status only, which is part of treatment response. The other disease outcomes and survival analysis are not studied. Studying TLC with respect to other disease outcomes like early mortality, relapse, and reduced disease-free survival may depict an association with increased TLC and adverse outcome.

Median platelet count in the remission group was 32,000 cells/cu.mm while in not in remission group was 39,000 cells/cu.mm. This was similar to the findings in previous study by Kulsoom *et al.*,[16] with a median platelet count of 36,000/cu.mm. Median blast percentage in the peripheral blood was 60% and 76% in the not in remission and the remission group, respectively. Literature shows median values of 48–56%. Hence, our study had a slightly higher peripheral blasts as compared to the previous studies.

Table 6: Association of NPM1, CXCR4, and CD123 IHC with remission groups.

IHC marker	Pattern	Total (119) *n* (%)	Remission (70) *n* (%)	Not in remission (49) *n* (%)	Chi-square statistic	*P*-value
NPM1	Loss	38 (31.9)	24 (34.3)	14 (28.6)	0.44	0.5
	Retained	81 (68.1)	46 (65.7)	35 (71.4)		
CXCR4	Positive	60 (50.4)	38 (54.3)	22 (44.9)	1.02	0.3
	Negative	59 (49.6)	32 (45.7)	27 (55.1)		
CD123	Positive	5 (4.2)	03 (4.3)	02 (4.1)	0.003	0.96
	Negative	114 (95.8)	67 (95.7)	47 (95.9)		

NPM1: Nucleophosmin 1, IHC: Immunohistochemistry, CXCR4: C-X-C chemokine receptor type 4, CD123: Cluster of differentiation 123

Table 7: Prognostic accuracy of immunohistochemical markers to predict remission status.

IHC marker	Status	Remission	Not in remission	Sensitivity	Specificity	PPV	NPV
NPM1	Loss	24	14	34%	71%	63%	43%
	Retained	46	35				
CXCR4	Positive	38	22	54%	55%	63%	46%
	Negative	32	27				
CD123	Positive	03	02	4.3%	96%	60%	41%
	Negative	67	47				

NPM1: Nucleophosmin 1, IHC: Immunohistochemistry, CXCR4: C-X-C chemokine receptor type 4, CD123: Cluster of differentiation 123

Table 8: Association and diagnostic accuracy of immunohistochemical markers as surrogates for genetic mutations.

NPM1 IHC and NPM1 mutation (*n*=63)							
NPM 1 IHC	**NPM1 mutated**	**NPM1 wild type**	**Statistical significance**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
Loss of nuclear expression	17	06	Chi-square=32.9	89%	86%	74%	95%
Retained NPM1	02	38	*P*<0.001				
CXCR4 IHC and FLT3 Mutation (*n*=65)							
CXCR4 IHC	**FLT3 mutated**	**FLT3 wild type**	**Statistical significance**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
Positive	09	27	Chi-square=0.0064	56%	45%	25%	76%
Negative	07	22	*P*=0.93				
CD123 IHC and FLT3 Mutation (*n*=65)							
CXCR4 IHC	**FLT3 mutated**	**FLT3 wild type**	**Statistical significance**	**Sensitivity**	**Specificity**	**PPV**	**NPV**
Positive	00	05	-	NA	90%	NA	73%
Negative	16	44					

NPM1: Nucleophosmin 1, IHC: Immunohistochemistry, CXCR4: C-X-C chemokine receptor type 4, CD123: Cluster of differentiation 123, FLT3: FMS like tyrosine kinase 3, PPV: Positive predictive value, NPV: Negative predictive value

In the remission group, M3 constituted the highest number of cases followed by M2, M1, and M4. In the not in remission cases, M1 had the highest number of cases, followed by M2 and M4. This significant increase in M3 cases in the remission group can be explained by the fact that APML cases have favorable outcome with good response to ATRA-ATO therapy and most patients achieve complete remission. Overall, most common subtype in our study was M1 followed by M2, M4, and M3. Similar to our study, the previous studies have also shown that M2, M1, M3, and M4 are the most common subgroups of AML.[12,16] Our study showed an overall incidence of AML with myelofibrosis of 6.7% at presentation. None of the remission cases had hypocellularity, while 6.1% of not in remission cases had hypocellular AML. This finding is similar to previous studies showing that hypocellular AML accounts for 5–12% of overall AML with poorer patient outcome.[20]

The most common cytogenetic abnormality in the remission group was translocation, predominantly t(15,17) and t(8,21), while in not in remission cases majority belonged to normal cytogenetics and complex cytogenetics. This is in concordance with the previous studies showing that t(15,17)

and t(8,21) have good risk and better treatment response and patient outcome, while normal cytogenetics form intermediate risk and complex cytogenetics form poor risk with adverse outcome.[21-23] In remission group, 37.2% had normal cytogenetics while in not in remission 42.3% had normal cytogenetics, this is similar to the previous reported data of normal cytogenetics in de novo AML 40–45%.[22,23]

In our study, NPM1 mutation was seen in 30% of cases and FLT3 mutation in 24.6% of cases. The previously reported studies[13,24-26] have shown NPM1 mutation in 12–21% of AML and FLT3 mutations in 9–25% of AML. Contrary to the previous studies stating that NPM1 mutation confers good prognosis and better treatment response and FLT3 adverse prognosis, our study failed to show any significant difference in response to induction chemotherapy. This again can be due various causes like studying association of mutation status with respect to remission status only and not with other patient outcomes, confounding factors such as age, adverse cytogenetics, and additional comorbidities may in addition contribute to the disease outcome.

In our study, loss of nuclear/nucleolar localisation and concomitant absence of cytoplasmic staining (in spite of repeating) was interpreted as negative staining pattern (external control positive) suggestive of NPM1 mutation. However, some of these cases were wild type NPM1. Possible explanation to this lack of staining could be due to loss of antigenicity due to excessive decalcification or reduction in antigenicity in old paraffin blocks stored for longer duration.[27-29] CXCR4 antibody produced smudgy and non-specific background staining which in spite of using blockers did not improve the staining pattern. This antibody was a novel research monoclonal antibody, with limited prior use in research. Hence, we could not compare this smudgy background staining with prior works to identify the cause and rectification of the same.

In our study, both loss of nuclear NPM1 expression and positive CXCR4 expression were seen more in remission patients as compared to not in remission patients. However, this was not statistically significant. This is in contrary to previous literature that NPM1 mutation and thereby loss of nuclear NPM1 was associated with better prognosis[6,25,30] and more chances of complete remission and increased CXCR4 expression[3,31] were associated with reduced chance of complete remission and poor prognosis. CD123 expression had no association with remission status, this is again in contrary to the previous studies stating CD123 expression which is associated with lower complete remission states and reduced overall survival.[32-34] Overall, these IHC markers had low sensitivity to predict remission status.

Interestingly, loss of nuclear localization of NPM1 IHC was highly significantly associated with NPM1 mutation. NPM1 IHC was seen to have a sensitivity of 89% and specificity of 86% to detect NPM1 mutation. This is similar to the previous studies[6,7,35,36] with sensitivity of 90–100% and specificity of 87–100%. Hence, NPM1 may be used as a cost effective surrogate immunohistochemical marker for NPM1 exon 12 mutation. CXCR4 expression did not have any association with FLT3 mutation in this study; however, literature search showed that FLT3 mutation increased the expression of CXCR4 on the blasts surface.[8,37] As seen in Table 8, none of the FLT3 positive cases in our study showed CD123 expression. This is at par with prior study stating that CD123 did not show any association with FLT3 mutation.[38]

Limitations of this study

Reduced cases enrolled in not in remission group due to better induction chemotherapy regimens in the recent past and reduced case load due to COVID-19 pandemic. Data on molecular and genetic studies were not available for all cases for comparison, as these studies were done by outsourcing and not all patients could afford the same. Smudgy staining pattern and background staining caused interference in interpreting CXCR4 IHC. This did not improve despite adding a blocker. Complete lack of nuclear/nucleolar/cytoplasmic staining in NPM1 IHC-interpreted as negative, which possibly was due to loss of antigenicity, interfered with the results.

CONCLUSION

Loss of nuclear localization of NPM1 IHC and CXCR4 IHC positivity were seen to be expressed more in remission cases as compared to not in remission cases although without statistical significance. CD123 IHC expression did not have any association with remission status. NPM1 IHC may be used as a cost-effective surrogate for NPM1 exon 12 mutations in a resource poor setting. CD123 and CXCR4 IHC did not correlate with remission status or with FLT3 mutation and hence cannot act as effective surrogate for the same.

Ethical approval

The research/study complied with the Helsinki Declaration of 1964.

Declaration of patient consent

The Institutional Review Board (IRB) permission obtained for the study.

Financial support and sponsorship

Institute intramural fund.

Conflicts of interest

There are no conflicts of interest.

Use of artificial intelligence (AI)-assisted technology for manuscript preparation

The authors confirm that there was no use of artificial intelligence (AI)-assisted technology for assisting in the writing or editing of the manuscript and no images were manipulated using AI.

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How to cite this article: Muralidaran M, Kayal S, Harichandrakumar KT, Kar R. Utility of mutation-specific immunohistochemical markers for predicting post-induction remission among acute myeloid leukemia patients – A prognostic test accuracy study. J Hematol Allied Sci. 2023;3:125-35. doi: 10.25259/JHAS_19_2023