Prevalence and Clinical Significance of FLT3 and NPM1 Mutations in Acute Myeloid Leukaemia Patients of Assam, India

Jina Bhattacharyya¹ · Sukanta Nath² · Kandarpa Kumar Saikia² ·
Renu Saxena³ · Sudha Sazawal³ · Manash Pratim Barman⁴ · Dushyant Kumar²

Received: 23 September 2016 / Accepted: 25 April 2017 / Published online: 28 April 2017
© Indian Society of Haematology & Transfusion Medicine 2017

Abstract Acute Myeloid Leukaemia (AML) is one of the common forms of haematological malignancy in adults. We analysed the prevalence and clinical significance of FMS-like tyrosine kinase 3 (FLT3) and Nucleophosmin 1 (NPM1) mutations in AML patients of North East India. Co-prevalence and clinical significance of three recurrent chromosomal translocations namely t(15; 17), t(8; 21), t(16; 16) and expression of epidermal growth factor receptor (EGFR), flow markers were also documented and co-related with disease progress. We analysed bone marrow aspirates or peripheral blood samples from 165 newly diagnosed AML patients. All clinical samples were analysed by Real Time PCR and DNA sequencing based assays. NPM1 was the most frequently detected mutation in the study population (46/165 = 27.90%, 95% CI 20.75–35.05). FLT3 mutations were detected in 27/165 (16.40%, 95% CI 10.45–22.35) patients with internal tandem duplication (FLT3-ITD) in 24/165 (14.60%, 95% CI 8.91–20.29) and FLT3-D835 in 3/165 (1.80%, 95% CI 0–4.13) patients. NPM1 mutations were associated with a higher complete remission rate and longer overall survival

($P < 0.01$) compared to FLT3-ITD whereas FLT3-ITD showed adverse impact with poor survival rate ($P < 0.01$), leukocytosis ($P < 0.01$) and a packed bone marrow. EGFR expression was more in patients with NPM1 mutation compared to FLT3 mutation ($P = 0.09$). Patients with FLT3 and NPM1 mutations uniformly expressed CD13 and CD33 whereas CD34 was associated with poor prognosis ($P \leq 0.01$) in patients with NPM1 mutation. FLT3-ITD was associated with inferior overall survival. However the clinical significance of FLT3-D835 was not clear due to small number of samples. NPM1 mutation showed better prognosis with increased response to treatment in the absence of FLT3-ITD.

Keywords Acute Myeloid Leukaemia (AML) · FMS-like tyrosine kinase 3 (FLT3) · Nucleophosmin 1 (NPM1) · Prognosis

Abbreviations

FLT3 FMS-like tyrosine kinase 3
NPM1 Nucleophosmin 1

Jina Bhattacharyya and Sukanta Nath have contributed equally to this work.

✉ Kandarpa Kumar Saikia
kksaikia@gmail.com

¹ Department of Clinical Haematology, Gauhati Medical College and Hospital, Guwahati, Assam, India

² Department of Bioengineering and Technology, Gauhati University, Guwahati, Assam 781014, India

³ Department of Haematology, All India Institute of Medical Sciences, New Delhi, India

⁴ Department of Statistics, Dibrugarh University, Dibrugarh, Assam, India

Introduction

Acute Myeloid Leukaemia (AML) is a type of blood borne malignant disease, originates from faulty haematopoietic progenitor cells as a result of acquired genetic alterations [1]. It is a complex multistep process where cooperation of these genetic alterations of different classes is required to obtain full-blown leukaemia [2]. Alterations responsible for causing AML includes Class I mutations such as FMS-like tyrosine kinase 3 (FLT3), KIT and RAS which

activates signal transduction pathways resulting proliferation and survival potential of haematopoietic progenitors. On the other hand t(8; 21), inv(16) and t(15; 17) are designated as Class II mutations (translocations) that affect transcription factors and impair hematopoietic differentiation [3]. Recently Nucleophosmin 1 (NPM1) mutation has been considered to be a Class II mutation affecting hematopoietic transcription and differentiation [4].

FLT3 mutation is one of the most commonly detected mutations in AML. Almost 30% AML patients with normal karyotype harbour FLT3 mutation but the risk stratification depends on some other factors also such as the nature of the mutation, mutational burden, its interaction with other mutation(s), their expression level(s) etc. FLT3 mutation is classified into two categories including internal tandem duplication (FLT3-ITD) in the juxtamembrane (JM) domain and point mutation in the tyrosine kinase domain (FLT3-TKD) [5]. FLT3-ITD mutation is already known to be a poor prognostic indicator whereas the prognostic value of FLT3-TKD or FLT3-D835 mutation is still not clear [6].

NPM1 mutation in exon 12 is the most frequently detected mutation in cytogenetically normal AML patients with favourable prognosis in terms of response to chemotherapy or disease free survival (DFS). About 35% patients harbour this mutation irrespective to any subtypes and highly associated with FLT3 [7]. There are several NPM1 mutation types detected so far such as type A through type F which causes due to either insertion or deletion of new four nucleotides in C-terminal region making NPM1 mutant-mediated cytoplasmic delocalization of nuclear proteins [8].

Acute Promyelocytic Leukaemia (APL) is one of the unique subtypes in AML characterized by a reciprocal translocation involving the promyelocytic leukaemia (PML) gene from chromosome 15 and the retinoic acid receptor alpha (RAR α) gene from chromosome 17 or t(15; 17)(q22; q12) that results in the PML–RAR α fusion transcript. This oncoprotein impairs myeloid differentiation, essential to leukaemogenesis and forms different subtypes referred to as long (L or bcr1), variant (V or bcr2) and short (S or bcr3). APL represents less than 10% of all AML and is associated with good prognosis [9].

Acute Myeloid Leukaemia 1-Eight Twenty One (AML1-ETO) fusion transcript is the result of reciprocal translocation involving the AML1 gene from chromosome 21 and the ETO gene from chromosome 8 or t(8; 21) (q22; q22), suggested to function as a transcriptional repressor and is usually associated with good prognosis. The prevalence of AML1-ETO in all AML cases is 5–10% and in French–American–British-M2 (FAB-M2) it is 10–22% [10].

INV(16) or Core Binding Factor Beta-Myosin Heavy Chain 11 (CBF β -MYH11) fusion transcript is the result of core-binding factor, β subunit gene at 16q22 and myosin,

heavy chain 11, smooth muscle gene at 16p13 [t(16; 16)(p13; q22) or inv(16)(p13.1q22)] and is associated with favourable prognosis. The prevalence of CBF β -MYH11 in AML is approximately 5–7% [11]. Over expression of epidermal growth factor receptor (EGFR) is already known in solid tumours but very few data are available in AML. Sun et al. have shown that the expression of EGFR in AML is approximately 33% from M1 to M7 subtypes and reported that EGFR confers poor prognosis in AML [12]. Immunophenotyping plays a great role in detecting AML and classifying AML subtypes [13].

There is paucity of data from North East India on the prevalence and clinical significance of FLT3 and NPM1 mutations in AML. Co-prevalence and clinical significance of other genetic alterations in patients with FLT3 and NPM1 mutation are also very rare. In this study we have reported the prevalence and clinical significance of FLT3 and NPM1 mutations. The possible influence of other common chromosomal translocations such as t(15; 17), t(8; 21), t(16; 16) and expression of EGFR and flow markers were also documented in these patients.

Materials and Methods

Selection Criteria of Patients

Ethical approval was obtained from institutional ethics committee to conduct the study. Bone marrow aspirate or peripheral blood was collected from 165 de novo AML patients in the Department of Clinical Haematology, Gauhati Medical College and Hospital, Guwahati, Assam from August 2013 to February 2016. Signed written informed consent was obtained from each patient before collecting the samples. These include 90 males and 75 females from the age of 1–84 years. Confirmed diagnosis of AML was done based upon complete blood count and blood smear, cytogenetics and flow cytometry. Peripheral blood or bone marrow aspirates were collected in 2 ml K2EDTA tubes (Becton–Dickinson, Franklin Lakes, NJ, USA), transported to the laboratory on ice as soon as possible and processed within 24 h of collection.

Cytomorphology, Cytogenetic Analysis, Flow Cytometry

Following FAB and World Health Organization (WHO) criteria, smears from each bone marrow and peripheral blood samples were stained, including May–Grunwald–Giemsa stains and myeloperoxidase (MPO) [14]. Karyotyping was done for all cases according to the International System for Human Cytogenetic Nomenclature to detect chromosomal abnormalities [15], and for flow

cytometry diagnosis from peripheral blood or bone marrow aspirate was done according to the presence and availability of blast cells as described [16]. Monoclonal antibodies used for flow cytometry (BD FACSCANTO II) were MPO-FITC, CD13-PE, CD33-PE-Cy7, HLA-DR-V500, CD34-PerCP, CD45-APC-H7, CD117-APC, CD56-APC, CD123-PE, CD7-FITC, CD15-BV421 and CD19-APC which were obtained from Becton–Dickinson Biosciences, USA.

DNA, RNA Extraction and c-DNA Preparation

DNA was extracted from peripheral blood or bone marrow aspirates by using the HiPurA Blood Genomic DNA Miniprep Purification Kit (HiMedia Laboratories Pvt. Ltd, Mumbai, India) following the manufacturer's instructions. Total RNA was extracted from peripheral blood or bone marrow aspirates by using the QIAamp RNA blood mini kit (Qiagen GmbH, Hilden, Germany) following the manufacturer's instructions. Superscript III First Strand Synthesis System for RT-PCR kit (Thermo Fisher Scientific Waltham, MA USA) was used to prepare c-DNA from the extracted RNA. Peripheral blood was collected from patients with WBC $\geq 10,000/\mu\text{L}$ whereas bone marrow aspirates were considered for patients with WBC $< 10,000/\mu\text{L}$ and was maintained throughout the study for blood or bone marrow aspirate collection. A marrow or blood blast count of minimum 20% was considered for a diagnosis of AML as well as for the sample collection.

Detection of FLT3 and NPM1 Mutations

FLT3-ITD mutations (exon 14–15) were analysed on genomic DNA by PCR amplification as previously described [17]. In the absence of FLT3-ITD a fragment of 329 bp was detected in wild-type FLT3 gene whereas additional upper band (higher molecular weight band) was observed in cases with FLT3-ITD mutation (Fig. 1a). FLT3-D835 mutation detection was accomplished using the forward primer of 5'-GCCAGGAACGTGCTTGTCACC-3' and the reverse primer of 5'-CCACAGTGAGTGCAGTTGTTTACC-3'. The amplification reaction was carried out using 5 min of initial denaturation at 95 °C, 35 cycles of 1 min at 95 °C, 45 s at 66 °C, 45 s at 72 °C with a final extension at 72 °C for 5 min. Amplified products were then digested with EcoRV restriction endonuclease (HiMedia Laboratories Pvt. Ltd, Mumbai, India) as per manufacturer's instructions. The digested PCR products were visualized on a 2.5% agarose gel. The undigested band size for FLT3-D835 was 185 bp and after digestion 2 bands were observed for wild-type samples (125 and 60 bp). After digestion any sample with 3 bands (185, 125 and 60 bp) was considered as FLT3-D835 mutant (Fig. 1b.).

NPM1 mutations were analysed on genomic DNA by PCR amplification and DNA sequencing was performed to detect different types of NPM1 mutations as previously described [18]. PCR analysis revealed that NPM1 samples produced a band of 560 bp (Fig. 2a). Sequencing of the amplified DNA revealed 3 types of NPM1 mutations-type A, type B and type D (Fig. 2b.). Genomic DNA after isolation was measured for quality and quantity by agarose gel electrophoresis (Major Science Co., Ltd., Taiwan) and Multiskan GO Microplate Spectrophotometer (Thermo Fisher Scientific Waltham, MA USA) respectively. For further PCR amplification the DNA samples having concentration between 50 and 400 ng/ μL and purity of 1.8–2 were only considered.

Detection of Fusion Transcripts and EGFR Gene Expression

Using inventoried Taq-man assays (Taqman Universal Master Mix II with UNG from Thermo Fisher Scientific Waltham, MA USA) qRT-PCR was performed to detect PML–RAR α (ID: Hs03043651_ft for bcr1 and ID: Hs03024794_ft for bcr3), AML1–ETO (ID: Hs03024752_ft), CBF β –MYH11 (ID: Hs03460064_ft) fusion transcripts and EGFR (ID: Hs01076078_m1) expression as per StepOnePlus (StepOnePlus–Real Time PCR system, Thermo Fisher Scientific Waltham, MA USA) pre installed protocol. All samples were used for relative quantitative PCR to detect fusion transcripts and EGFR expression where Glyceraldehyde 3-phosphate dehydrogenase or GAPDH (ID: Hs99999905_m1) served as a reference gene (endogenous control).

Treatment Protocol

All patients were treated as per standard protocol. Following diagnosis for patients with less than 12 years of age BFM 87 (Berlin–Frankfurt–Munster) protocol was followed. Adult patients (less than 50 years) received remission induction therapy “7 + 3 regimen” of cytarabine (100 mg/ m^2) for 7 days plus daunorubicin (60 mg/ m^2) for 3 days. As a consolidation 3 cycles of high dose cytarabine 3 g/ m^2 every 12 h on days 1, 3 and 5 were given. Low-dose chemotherapy regimens were given for older patients as per requirement. In addition, fluconazole prophylaxis and antibiotic coverage were given to those patients as per requirement.

Statistical Analysis

The focus of the statistical data analysis in our study was to estimate and determine the prevalence and clinical significance of FLT3, NPM1 mutations. Quantitative data such as age, WBC count, platelet count, haemoglobin level and

Fig. 1 Agarose gel electrophoresis of PCR assays to identify FLT3-ITD (**a**) and FLT3-D835 (**b**) mutation. **a** Lane 1 50 bp ladder, Lane 2 positive control, Lane 3 normal control, Lane 4 negative control, Lanes 5–13 different AML samples. Note Lane 10 is FLT3-ITD

positive. **b** Lane 1 FLT3 wild-type, Lanes 2, 5, 9 FLT3-D835 before digestion, Lanes 3, 6, 10 FLT3-D835 after digestion, Lanes 4, 8 = FLT3-ITD positive, Lane 7 100 bp ladder. Note Lane 10 is FLT3-D835 positive

blast count percentage were analysed using non-parametric test, Mann–Whitney U tests. Kaplan–Meier Product Limit Estimator was used to study the survival of patients having FLT3, NPM1 mutations. Log-rank was used to compare the survival pattern of patients having FLT3 and NPM1 mutations with FLT3 and NPM1 wild-type patients. Overall survival (OS) was calculated using the Kaplan–Meier method to estimate overall and group wise median survival. To be statistically significant, a two-sided P value <0.05 was considered. All statistical analyses were done using statistical packages SPSS 17.0 (SPSS Inc. Chicago, IL) and Epi Info 2000 (Centers for Disease Control and Prevention, Atlanta, GA).

Results

Clinical characteristics of patients with FLT3 and NPM1 mutation in the studied group are summarized in Table 1 and clinical characteristics of patients with NPM1 mutation (Live vs. Dead) in the studied group are summarized in Table 2. FLT3 mutations were detected in 27/165 (16.40%, 95% CI 10.45–22.35) patients (FLT3-ITD in 24/27 patients and FLT3-D835 in 3/27 patients). NPM1 mutations were detected in 46/165 (27.90%, 95% CI 20.75–35.05) patients. Seven patients with both NPM1 and FLT3 mutations were detected. Type A mutation was the most common (41/46 = 89.20%) types of NPM1 mutation followed by type B (3/46 = 6.50%) and type D (2/46 = 4.30%). PML–RAR α fusion transcripts were detected in 5/27 (19%) patients with FLT3 mutation (all were detected in FLT3-ITD). Among 5 PML–RAR α + / FLT3-ITD+ patients, bcr3 were detected in 3 patients and bcr1 were detected in 2 patients respectively and the survival time was comparatively less in bcr3 patients. In

patients with FLT3 mutation the presence of PML–RAR α ($P = 0.89$) and EGFR ($P = 0.52$) expression were insignificant compared to FLT3 wild-type. However AML1-ETO fusion transcripts were detected only in FLT3 wild-type patients and not in patients with FLT3 mutation ($P = 0.02$). PML–RAR α and AML1-ETO fusion transcripts were detected in 11/46 (24%) and 7/46 (15.50%) patients with NPM1 mutation respectively. The prevalence of PML–RAR α ($P = 0.18$) and AML1-ETO ($P = 0.88$) were insignificant in patients with NPM1 mutation compared to patients with NPM1 wild-type. However the expression of EGFR was significantly higher in patients with NPM1 wild-type compared to patients with NPM1 mutation ($P < 0.01$).

Out of 27 patients with FLT3 mutation 22 patients were cytogenetically normal (other 5 patients were detected with t(15; 17) translocation) whereas out of 46 patients with NPM1 mutation 28 patients were cytogenetically normal [other 11 and 7 patients were detected with t(15; 17) and t(8; 21) translocations respectively]. No CBF β -MYH11 fusion transcript was detected in our study. No Chromosomal deletion was detected in patients with FLT3 and NPM1 mutation. However 11.50% (19/165) patients (patients without FLT3 and NPM1 mutation) had complex karyotype such as monosomies (–3, –12, –13, –15, –18, –20, –21), trisomies (+6, +8, +9, +11, +13, +14, +15, +16, +17, +18, +20, +21) and showed poor survival compared to patients with normal karyotype ($P < 0.01$).

The expression of EGFR was detected in 35% patients in our study, where 3/27 (11.50%) patients were with FLT3 (only in FLT3-ITD) mutation and 13/46 (28.50%) patients were with NPM1 mutation. All patients were MPO positive. Strong and common expressions of CD13, CD33 were detected in all patients with FLT3 and NPM1 mutation. The expression and the effect of flow markers (except

Fig. 2 **a** Agarose gel showing PCR amplified product for NPM1 Lane 1 100 bp ladder, Lane 2 normal control, Lane 3 negative control (water only), Lane 4 sample of a AML patient. **b** Sequence analysis of the NPM1 mutations (insertion of 4 bases)

CD13 and CD33, as they uniformly expressed) were analysed in patients with NPM1 mutation (Table 2). As FLT3-ITD positive patients survived for a very less period of time (maximum 3 months) we have not performed the same analysis for FLT3-ITD. The expression of HLA-DR (20/27 = 74.5%), CD7 (14/27 = 52%), CD34 (15/27 = 56%) and CD123 (18/27 = 67%) were more compared to CD117 (10/27 = 37%), CD56 (11/27 = 41%), CD19 (0/27) and CD15 (0/27) in patients with FLT3 mutation.

Treatment and Follow Up

Of a total 27 patients with FLT3 mutation, 20 patients received intensive, cytarabine-based induction chemotherapy. For 7 patients induction therapy could not be completed and died before the treatment due to severe bleeding. Out of 20 patients 4 patients died during the induction therapy. Total 16 patients underwent induction and consolidation therapy. None of the FLT3-ITD positive patients (including NPM1+/FLT3-ITD+, PML-RAR α +/FLT3-

Table 1 Clinical characteristics of patients with FLT3 and NPM1 mutation

Comparison between patients with FLT3 mutation (n = 27) and NPM1 mutation (n = 46)			
Demographic, laboratory and clinical characteristics	FLT3 27/165 (16.40%) n (%)	NPM1 46/165 (27.90%) n (%)	P value
Gender			
Male	17 (63)	22 (47.80)	0.21
Female	10 (37)	24 (52.20)	0.21
Sex ratio (M:F)	1.7	0.92	
	FLT3 Mean $\pm$ SD [median (range)]	NPM1 Mean $\pm$ SD [median (range)]	P value
Age (years)	33.94 $\pm$ 17.62 [38.00 (2.50–62)]	35.32 $\pm$ 19.21 [36.00 (2.50–84)]	0.86
WBC count ($\times 10^9$ /L)	110.17 $\pm$ 100.14 [91.00 (5.00–422.93)]	49.32 $\pm$ 73.90 [22.20 (5.00–422.93)]	<0.01
Platelet count ($\times 10^9$ /L)	26.09 $\pm$ 18.09 [28.00 (3.00–72.00)]	42.7 $\pm$ 38.3 [35.0 (8.00–215.00)]	0.03
Haemoglobin level ($\times$ g/dL)	6.43 $\pm$ 1.82 [6.80 (2.50–10.10)]	7.05 $\pm$ 2.12 [7.00 (2.20–12.70)]	0.25
Blast count (%)	59.41 $\pm$ 17.76 [60.00 (24.00–87.00)]	52.28 $\pm$ 17.41 [55.50 (21.00–80.00)]	0.11
	FLT3 n (%)	NPM1 n (%)	P value
PML–RAR α	5 (19)	11 (24)	0.59
AML1-ETO	0 (0)	7 (15.50)	0.09 ^a
EGFR gene expression	3 (11.50)	13 (28.50)	0.09
	FLT3 n (%)	NPM1 n (%)	P value
Early death	25 (93)	26 (56.50)	<0.01
Complete remission	2 (7.50)	20 (43.50)	<0.01

Bold values indicate $p < 0.01$

P values computed using Pearson Chi square, unpaired and Mann–Whitney U tests

^a Applying Yate's correction

ITD+) survived more than 3 months. Poor overall survival was noticed in 1/3 FLT3-D835 patients and survived less than 40 days. However 2/3 FLT3-D835 patients achieved complete bone marrow remission after both the therapy. The follow up duration was 24 months.

For patients with NPM1 mutation cytarabine-based induction and consolidation chemotherapy were used. Out of 46 patients 5 patients died due to severe bleeding and 7 patients died during the induction therapy. Bone marrow remission rate was better in patients with NPM1 mutation. During the follow up time of 35 months 26/46 (56.50%) patients died and complete remission was achieved for 20/46 (43.50%) patients. The mean survival time of patients with NPM1 mutation was 16.59 months (95% CI 11.94–21.24) whereas for patients with NPM1 wild-type

the mean survival time was 24.96 months (95% CI 22.11–27.81).

Survival Analysis

The death rate in our study was 25/27 for patients with FLT3 mutation and 26/46 for patients with NPM1 mutation and the CR rate was 2/27 for patients with FLT3 mutation and 20/46 for patients with NPM1 mutation (Table 1). Survival analysis revealed that (Fig. 3) the probability of survival of patients with FLT3-ITD ($P < 0.01$) and NPM1 mutation ($P \leq 0.01$) were significantly lower compared to patients with FLT3 wild-type and NPM1 wild-type respectively. However it was not observed in FLT3-D835 positive patients ($P = 0.80$).

Table 2 Clinical characteristics of patients with NPM1 mutation (live vs. dead group)

Comparison between NPM1 live (n = 20) and NPM1 dead patients (n = 26)			
Demographic, laboratory And clinical characteristics	<i>NPM1 Live</i> 20/46 (43.50%) n (%)	<i>NPM1 Dead</i> 26/46 (56.50%) n (%)	<i>P</i> value
Gender			
Male	10 (50%)	12 (46%)	0.80
Female	10 (50%)	14 (54%)	0.80
Sex ratio (M:F)	1	0.86	
	<i>NPM1 Live</i> Mean $\pm$ SD [median (range)]	<i>NPM1 Dead</i> Mean $\pm$ SD [median (range)]	<i>P</i> value
Age (years)	32.05 $\pm$ 16.71 [26.50 (6–65)]	37.83 $\pm$ 20.91 [39.50 (2.50–84)]	0.39
WBC count ($\times 10^9/L$)	38.15 $\pm$ 52.91 [18.15 (6.81–214.69)]	57.91 $\pm$ 86.76 [24.95 (5.00–422.93)]	0.13
Platelet count ($\times 10^9/L$)	39.35 $\pm$ 23.95 [37.50 (12.00–96.00)]	45.27 $\pm$ 46.76 [31.00 (8.00–215.00)]	0.52
Haemoglobin level ($\times g/dL$)	7.36 $\pm$ 1.77 [7.10 (4.40–10.80)]	6.82 $\pm$ 2.36 [6.80 (2.20–12.70)]	0.51
Blast count (%)	51.60 $\pm$ 16.93 [49.00 (24.00–80.00)]	52.81 $\pm$ 18.09 [57.50 (21.00–80.00)]	0.80
	<i>NPM1 Live</i> n (%)	<i>NPM1 Dead</i> n (%)	<i>P</i> value
HLA-DR	1 (5%)	8 (31%)	0.57
CD7	1 (5%)	5 (20%)	0.21
CD15	14 (70%)	9 (35%)	0.04
CD19	3 (15%)	2 (8%)	0.64
CD34	0 (0)	8 (31%)	≤ 0.01
CD56	0 (0)	0 (0)	–
CD117	4 (20%)	1 (4%)	0.15
CD123	0 (0)	0 (0)	–
PML–RAR α	5 (25%)	6 (23%)	1.00
AML1–ETO	1 (5%)	6 (23%)	0.12
FLT3–ITD	0 (0)	5 (20%)	0.06
FLT3–D835	1 (5%)	1 (4%)	1.00
EGFR gene expression	6 (30%)	7 (27%)	1.00

Bold value indicates $p \leq 0.01$

Discussion

Although cytogenetics is considered one of the most valuable prognostic determinants in AML but in patients with normal karyotype, FLT3-ITD and NPM1 mutation plays an important role in prognostic risk stratification. Therefore, correct mutation identification may help to optimize therapeutic approaches in AML. We assessed the prevalence and prognostic impact of FLT3 and NPM1 mutations in our study group of 165 AML patients. In patients with FLT3 and NPM1 mutations, the associations of other genetic alterations have also been evaluated to

understand any possible role in disease outcome. The prevalence of FLT3 and NPM1 mutations found in this population was in coherence with other studies (Asian and Western) despite geographic and ethnic differences (Tables 3, 4) [19–30].

Patients harbouring ≥ 3 acquired chromosome aberrations in the absence of three prognostically favourable recurrent chromosomal translocations namely t(15; 17), t(8; 21), t(16; 16), are categorised into a separate group known as AML with complex karyotype [31]. Approximately 10–14% of all AML patients has karyotype complexity and is associated with poor prognosis [32]. The

Fig. 3 **a** Kaplan–Meier survival analysis between FLT3-ITD positive and FLT3-ITD negative patients (Log-rank test $P < 0.01$). **b** Kaplan–Meier survival analysis between FLT3-D835 positive and FLT3-

D835 negative patients (Log-rank test $P = 0.80$). **c** Kaplan–Meier survival analysis between patients with NPM1 mutation and NPM1 wild-type (Log-rank test $P \leq 0.01$)

Table 3 Prevalence of FLT3 mutation in AML in various studies

Study	Country	Number of AML cases	Prevalence of FLT3 mutation		Total prevalence (%)
			FLT3-ITD	FLT3-D835	
Present study	India (North East)	165	24	3	27 (16.40%)
Wang et al. [19]	China	371	59	15	74 (20%)
Cuervo-Sierra et al. [20]	Latin America (Mexico and Colombia)	138	24	4	28 (20.30%)
Dunna et al. [21]	India	128	26	8	34 (26.50%)
Park et al. [22]	South Korea	201	45	5	50 (24.90%)
Koczkodaj et al. [23]	Poland	50	4	—	4 (8%)
Al-Mawali et al. [24]	Australia	39	12	—	12 (31%)
Gari et al. [25]	Saudi Arabia	129	15	11	26 (20.20%)

prevalence rate of karyotype complexity was found similar in our study. Our analysis also demonstrated that patients with complex karyotype showed significantly poor survival compared to patients with normal karyotype and the reason could be the absence or presence of specific chromosome and/or gene alterations which may differently respond to therapy.

In AML, if samples are diluted with no malignant hematopoietic elements it will lower the sensitivity of the assay, therefore blast percentage plays a central role in diagnosis and sample collection [5]. Earlier studies have shown the independent poor and favourable prognostic impact of FLT3-ITD and NPM1 mutations respectively [33, 34]. Presence of FLT3-ITD was associated with poorer

Table 4 Prevalence of NPM1 mutation in AML in various studies

Study	Country	Number of AML cases	Total prevalence (%)
Present study	India	165	46 (27.90%)
Thiede et al. [26]	Germany	1485	408 (27.50%)
Wei et al. [27]	China	389	55 (14.10%)
Boonthimat et al. [28]	Thailand	400	105 (26.30%)
Chauhan et al. [3]	India	161	34 (21%)
Marshall et al. [29]	South Africa	160	12 (7.50%)
Zidan et al. [30]	Egypt	55	12 (21.80%)

prognosis compared to patients with FLT3 wild-type and patients with NPM1 mutation and was statistically significant. However we did not find that APL or NPM1 (which are considered to be good prognostic markers) mutation has any favourable prognostic impact on FLT3-ITD patients as none of the patients survived. This could be due to higher ITD ratios comparison to NPM1 mutant level or PML–RAR α transcript level. One drawback of our study was because of financial constraints no FLT3-ITD patient underwent transplantation which could affect their survival. The clinical significance of FLT3-D835 is still under debate and more studies are required for a concrete conclusion [35]. In our present study the numbers of FLT3-D835 patients were too small to draw a firm conclusion.

The association of FLT3-ITD with APL patients is well known but the prognostic impact of FLT3-ITD in APL is still controversial [36–38]. In our study PML–RAR α +/FLT3-ITD+ patients were associated with higher WBC count, hypogranular variant morphology (M3v) and breakpoint cluster region 3 (bcr3) isoform. The prognosis of bcr3 patients were poorer compared to breakpoint cluster region 1 (bcr1) in APL patients [37]. That could be one more reason for lower survival rate of APL patients with FLT3-ITD in our study. The highest survival period observed was 3 months for APL patients with FLT3-ITD mutation. However no FLT3-D835 association with APL patients was observed.

Earlier studies reported that the low prevalence of FLT3-ITD in AML1-ETO patients [3], or presence of FLT3-ITD in AML1-ETO patients with significantly higher relapse rate and shorter leukaemia free survival (LFS) [39]. FLT3-D835 was reported to be lower in AML1-ETO patients [40]. No association was observed between FLT3 mutants (FLT3-ITD and FLT3-D835) and AML1-ETO in our study.

Common fusion transcripts such as PML–RAR α , AML1-ETO, CBF β -MYH11 were reported to be absent in NPM1 mutants [41]. One study from India showed that the prevalence rate of NPM1 mutation in APL was higher (45%) [42]. In our study we have also noticed the similar association but with lower prevalence. This striking observation indicates that in Indian population PML–

RAR α might be frequent in patients with NPM1 mutation. Previous study showed that in India the prevalence of AML1-ETO in patients with NPM1 mutation was very low [3]. In our study the prevalence of AML1-ETO in patients with NPM1 mutation was higher which is a rare observation. However no significant prognostic impact of PML–RAR α and AML1-ETO were observed in patients with NPM1 mutation.

The prognostic impact of NPM1 in the absence of FLT3-ITD is well known but according to a very recent large study it was reported that type A mutation confers poor overall survival in patients with NPM1 mutation [43]. This could be one of the reasons which affected the overall survival in patients with NPM1 type A mutation in our study group. Previous studies demonstrated that high WBC count, cytogenetic abnormalities, older age, association with FLT3-ITD [44, 45], expressions of HLA-DR, CD34 and CD7 [46] also have a significant impact on survival in patients with NPM1 mutation. Due to our limited number of cases of patients with NPM1 type B and type D mutation we could not statistically compare the above parameters with NPM1 type A mutation. However we could analyse those parameters in patients with NPM1 mutation (live vs. dead group). Our analyses implied that WBC count, cytogenetic abnormalities, age, association with ITD, HLA-DR and CD7 were insignificant in these 2 groups. However CD34 expression significantly showed poor prognosis in patients with NPM1 mutation (dead group) and CD15 expression was significantly higher in patients with NPM1 mutation (live group). Kaplan–Meier survival analysis showed that the probability of survival in patients with NPM1 mutation was significantly lower compared to patients with NPM1 wild-type. Larger prospective studies with more data are required for clearer understanding.

The expression and clinical outcome of EGFR in AML is still under controversy [12, 47]. In our study EGFR expression was almost similar with the earlier report [12] and the expression was more in patients with NPM1 mutation compared to patients with FLT3 mutation although it was statistically insignificant. Our study also confirmed that EGFR expresses in AML. Although abnormal EGFR expression shows unfavourable prognosis

in solid cancers, the effect of EGFR expression in patients with FLT3 and NPM1 mutation was insignificant.

Conclusion

In summary, our study indicates that FLT3-ITD and NPM1 are common genetic alterations in Indian adult patients with AML. FLT3-ITD mutation uniformly confers poor overall survival with an aggressive presentation but the prognostic impact of FLT3-D835 mutation remains unclear. A larger study with more FLT3-D835 patients is needed to confirm. Although NPM1 and APL indicates good prognosis, but association with FLT3-ITD results in poor outcome in those patients. The maximum survival time in patients with NPM1 mutation is recorded more than 30 months till the possible follow up time but the probability of survival is significantly poorer compare to patients with NPM1 wild-type. The association of PML–RAR α and AML1-ETO fusion transcripts is observed with NPM1 mutations. EGFR expression is more common in patients with NPM1 mutation compared to patients with FLT3 mutation without showing any adverse effect.

Acknowledgements The authors are thankful to Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India for their financial assistance for this project.

Funding This study was financially supported by a grant from Department of Biotechnology (DBT), Ministry of Science and Technology, Government of India (Grant Number BT/348/NE/TBP/2012).

Author Contributions All authors listed have contributed to the work and agreed to submit the manuscript for publication.

Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interests.

Research Involving Human Participants and/or Animals This study involves blood and bone marrow aspirates from AML patients. Ethical approval was obtained from institutional ethics committee (GUEC-12/2015) to conduct the study.

Informed Consent Informed consent was obtained from all individual participants included in the study.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional committee.

References

- Zhong L, Jia YQ, Meng WT, Ni X (2012) FMS-like tyrosine kinase 3 internal tandem duplication and the patterns of its gene sequence in 207 Chinese patients with De novo acute myeloid leukemia. *Arch Pathol Lab Med* 136:84–89
- Takahash S (2011) Current findings for recurring mutations in acute myeloid leukemia. *J Hematol Oncol* 4:36
- Chauhan PS, Ihsan R, Singh LC, Gupta DK, Mittal V, Kapur S (2013) Mutation of NPM1 and FLT3 genes in acute myeloid leukemia and their association with clinical and immunophenotypic features. *Dis Mark* 35:581–588
- Kao HW, Liang DC, Wu JH, Kuo MC, Wang PN, Yang CP, Shih YS, Lin TH, Huang YH, Shih LY (2014) Gene mutation patterns in patients with minimally differentiated acute myeloid leukemia. *Neoplasia* 16:481–488
- Levis M (2013) FLT3 mutations in acute myeloid leukemia: what is the best approach in 2013? *Hematology. Am Soc Hematol Educ Progr* 2013:220–226
- Elyamany G, Awad M, Fadalla K, Albalawi M, Shahrani MA, Abdulaaly AA (2014) Frequency and prognostic relevance of FLT3 mutations in saudi acute myeloid leukemia patients. *Adv Hematol*. doi:10.1155/2014/141360
- Shamaa S, Laimon N, Aladle DA, Azmy E, Elghannam DM, Salem DA, Taalab MM (2014) Prognostic implications of NPM1 mutations and FLT3 internal tandem duplications in Egyptian patients with cytogenetically normal acute myeloid leukemia. *Hematology* 19:22–30
- Licínio MA, Silva MCS (2010) Importance of detecting FLT3 and NPM1 gene mutations in acute myeloid leukemia—World Health Organization Classification 2008. *Rev Bras Hematol Hemoter* 32:476–481
- Wakim JJ, Tirado CA (2012) Acute promyelocytic leukemia lacking the classic translocation t(15; 17). In: Koschmieder S, Krug U (eds) *Myeloid leukemia—clinical diagnosis and treatment*. InTech, Shanghai, pp 219–234
- Reikvam H, Hatfield KJ, Kittang AO, Hovland R, Bruserud Ø (2011) Acute myeloid leukemia with the t(8; 21) translocation: clinical consequences and biological implications. *J Biomed Biotechnol*. doi:10.1155/2011/104631
- Schwind S, Edwards CG, Nicolet D, Mrózek K, Maharry K, Wu YZ, Paschka P, Eisfeld AK, Hoellerbauer P, Becker H, Metzeler KH, Curfman J, Kohlschmidt J, Prior TW, Kolitz JE, Blum W, Pettenati MJ, Dal Cin P, Carroll AJ, Caligiuri MA, Larson RA, Volinia S, Marcucci G, Bloomfield CD (2013) inv(16)/t(16;16) acute myeloid leukemia with non-type A CBFB-MYH11 fusions associate with distinct clinical and genetic features and lack KIT mutations. *Blood* 121:385–391
- Sun JZ, Lu Y, Xu Y, Liu F, Li FQ, Wang QL, Wu CT, Hu XW, Duan HF (2012) Epidermal growth factor receptor expression in acute myelogenous leukaemia is associated with clinical prognosis. *Hematol Oncol* 30:89–97
- Angelescu S, Berbec NM, Colita A, Barbu D, Lupu AR (2012) Value of multifaced approach diagnosis and classification of acute leukemias. *Maedica (Buchar)* 7:254–260
- Selicean EC, Patiu M, Cucuiaru A, Dima D, Dobreanu M (2013) Correlation of cytomorphology with flowcytometric immunophenotyping in acute myeloid leukemia. *Rom J Lab Med* 21:333–341
- Mir Mazloumi SH, Appaji L, Madhumathi DS, Kumari P (2013) G-banding and fluorescence in situ hybridization in childhood acute myeloid leukemia from South India. *Arch Iran Med* 16:459–462
- Dong HY, Kung JX, Bhardwaj V, McGill J (2011) Flow cytometry rapidly identifies all acute promyelocytic leukemias with high specificity independent of underlying cytogenetic abnormalities. *Am J Clin Pathol* 135:76–84
- Pazhakh V, Zaker F, Alimoghaddam K, Atashrazm F (2011) Detection of nucleophosmin and FMS-like tyrosine kinase-3 gene mutations in acute myeloid leukemia. *Ann Saudi Med* 31:45–50
- Kassem N, Hamid AA, Attia T, Baathallah S, Mahmoud S, Moemen E, Safwat E, Khalaf M, Shaker O (2011) Novel

- mutations of the nucleophosmin (NPM-1) gene in Egyptian patients with acute myeloid leukemia: a pilot study. *J Egypt Natl Canc Inst* 23:73–78
19. Wang W, Wang XQ, Xu XP, Lin GW (2010) Prevalence and prognostic significance of FLT3 gene mutations in patients with acute leukaemia: analysis of patients from the shanghai leukemia co-operative group. *J Int Med Res* 38:432–442
 20. Cuervo-Sierra J, Gómez-Almaguer D, Jaime-Pérez JC, Martínez-Hernández RA, Sepúlveda RDG, Sánchez-Cárdenas M, Ortiz-López R, Ignacio-Ibarra G, Muciño-Hernández G, Arana-Trejo RM, Ruiz-Arguelles GJ, Ruiz-Delgado GJ, Lutz-Prestno J, Jiménez-Mejía A, Vásquez-Palacio G, Camargo-Guerrero M (2013) Prevalence of FLT3 mutations in acute myeloid leukemia: a multicenter Latin America study. *Blood* 122:4979
 21. Dunna NR, Rajappa S, Digumarti R, Vure S, Kagita S, Damineni S, Rao VR, Yadav SK, Ravuri RR, Satti V (2010) Fms like tyrosine kinase (FLT3) and nucleophosmin 1 (NPM1) mutations in de novo normal karyotype acute myeloid leukemia (AML). *Asian Pac J Cancer Prev* 11:1811–1816
 22. Park BG, Chi HS, Park SJ, Min SK, Jang S, Park CJ, Kim DY, Lee JH, Lee JH, Lee KH (2012) Clinical implications of non-A-type NPM1 and FLT3 mutations in patients with normal karyotype acute myeloid leukemia. *Acta Haematol* 127:63–71
 23. Koczkodaj D, Zmorzyński S, Michalak-Wojnowska M, Wąsik-Szczepanek E, Filip AA (2016) Examination of the FLT3 and NPM1 mutational status in patients with acute myeloid leukemia from southeastern Poland. *Arch Med Sci* 12:120–128
 24. Al-Mawali A, Gillis D, Lewis I (2013) Characteristics and prognosis of adult acute myeloid leukemia with internal tandem duplication in the FLT3 gene. *Oman Med J* 28:432–440
 25. Gari M, Abuzenadah A, Chaudhary A, Al-Qahtani M, Banni H, Ahmad W, Al-Sayes F, Lary S, Damanhoury G (2008) Detection of FLT3 oncogene mutations in acute myeloid leukemia using conformation sensitive gel electrophoresis. *Int J Mol Sci* 9:2194–2204
 26. Thiede C, Koch S, Creutzig E, Steudel C, Illmer T, Schaich M, Ehninger G (2006) Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). *Blood* 107:4011–4020
 27. Wei H, Zhang YQ, Lin D, Wang Y, Zhou CL, Liu BC, Li W, Rao Q, Wang M, Mi YC, Wang JX (2014) Clinical characteristics of newly diagnosed acute myeloid leukemia patients with NPM1 mutation. *Zhongguo Shi Yan Xue Ye Xue Za Zhi* 22:11–15
 28. Boonthimat C, Thongnoppakhun W, Auewarakul CU (2008) Nucleophosmin mutation in Southeast Asian acute myeloid leukemia: eight novel variants, FLT3 coexistence and prognostic impact of NPM1/FLT3 mutations. *Haematologica* 93:1565–1569
 29. Marshall RC, Tlagadi A, Bronze M, Kana V, Naidoo S, Wiggill TM, Carmona SC (2014) Lower frequency of NPM1 and FLT3-ITD mutations in a South African adult de novo AML cohort. *Int J Lab Hematol* 36:656–664
 30. Zidan M, Shaaban H, Ghannam DE (2013) Prognostic impact of nucleophosmin 1 (NPM1) gene mutations in Egyptian acute myeloid leukemia patients. *Turk J Haematol* 30:129–136
 31. Mrózek K (2008) Acute myeloid leukemia with a complex karyotype. *Semin Oncol* 35:365–377
 32. Stölzel F, Mohr B, Kramer M, Oelschlägel U, Bochtler T, Berdel WE, Kaufmann M, Baldus CD, Schäfer-Eckart K, Stuhlmann R, Einsele H, Krause SW, Serve H, Hänel M, Herbst R, Neubauer A, Sohlbach K, Mayer J, Mitdeke JM, Platzbecker U, Schaich M, Krämer A, Röllig C, Schetelig J, Bornhäuser M, Ehninger G (2016) Karyotype complexity and prognosis in acute myeloid leukemia. *Blood Cancer J*. doi:10.1038/bcj.2015.114
 33. Pratcorona M, Brunet S, Nomdedéu J, Ribera JM, Tormo M, Duarte R, Escoda L, Guàrdia R, Queipo de Llano MP, Salamero O, Bargay J, Pedro C, Martí JM, Torrealbadell M, Díaz-Beyá M, Camós M, Colomer D, Hoyos M, Sierra J, Esteve J (2013) Favorable outcome of patients with acute myeloid leukemia harboring a low-allelic burden FLT3-ITD mutation and concomitant NPM1 mutation: relevance to post-remission therapy. *Blood* 121:2734–2738
 34. Wu DP, Yan LZ, Yang L, Chen SN, Wu XJ, Liang JY (2007) A study of NPM1 and FLT3 gene mutations in acute myeloid leukemia. *J Intern Med* 262:907–910
 35. Mały E, Przyborski M, Nowak T, Nowak J, Januszkiewicz D (2010) FLT3 internal tandem duplication and FLT3-D835 mutation in 80 AML patients categorized into cytogenetic risk groups. *Postepy Hig Med Dosw* 64:466–470 (**online**)
 36. Molica M, Breccia M (2015) FLT3-ITD in acute promyelocytic leukemia: clinical distinct profile but still controversial prognosis. *Leuk Res* 39:397–399
 37. Breccia M, Loggisci G, Loggisci MG, Ricci R, Diverio D, Latagliata R, Foà R, Lo-Coco F (2013) FLT3-ITD confers poor prognosis in patients with acute promyelocytic leukemia treated with AIDA protocols: long-term follow-up analysis. *Haematologica* 98:161–163
 38. Schnittger S, Bacher U, Haferlach C, Kern W, Alpermann T, Haferlach T (2011) Clinical impact of FLT3 mutation load in acute promyelocytic leukemia with t(15; 17)/PML-RARA. *Haematologica* 96:1799–1807
 39. Kim YK, Kim HN, Lee IK, Bang SM, Jo DY, Won JH, Yim CY, Yang DH, Lee JJ, Kim HJ (2007) Prognostic significance of FLT3/ITD mutation in AML1/ETO-associated acute myeloid leukemia. *Blood* 110:3493
 40. Bacher U, Haferlach C, Kern W, Haferlach T, Schnittger S (2008) Prognostic relevance of FLT3-TKD mutations in AML: the combination matters-an analysis of 3082 patients. *Blood* 111:2527–2537
 41. Roti G, Rosati R, Bonasso R, Gorello P, Diverio D, Martelli MF, Falini B, Mecucci C (2006) Denaturing high-performance liquid chromatography: a valid approach for identifying NPM1 mutations in acute myeloid leukemia. *J Mol Diagn* 8:254–259
 42. Swaminathan S, Garg S, Madkaikar M, Gupta M, Jijina F, Ghosh K (2014) FLT3 and NPM-1 mutations in a cohort of acute promyelocytic leukemia patients from India. *Indian J Hum Genet* 20:160–165
 43. Alpermann T, Schnittger S, Eder C, Dicker F, Meggendorfer M, Kern W, Schmid C, Aul C, Staib P, Wendtner CM, Schmitz N, Haferlach C, Haferlach T (2016) Molecular subtypes of NPM1 mutations have different clinical profiles, specific patterns of accompanying molecular mutations and varying outcomes in intermediate risk acute myeloid leukemia. *Haematologica* 101:55–58
 44. Suzuki T, Kiyoi H, Ozeki K, Tomita A, Yamaji S, Suzuki R, Kodera Y, Miyawaki S, Asou N, Kuriyama K, Yagasaki F, Shimazaki C, Akiyama H, Nishimura M, Motoji T, Shinagawa K, Takeshita A, Ueda R, Kinoshita T, Emi N, Naoe T (2005) Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. *Blood* 106:2854–2861
 45. Dang H, Chen Y, Kamel-Reid S, Brandwein J, Chang H (2013) CD34 expression predicts an adverse outcome in patients with NPM1-positive acute myeloid leukemia. *Hum Pathol* 44:2038–2046
 46. Chen CY, Chou WC, Tsay W, Tang JL, Yao M, Huang SY, Tien HF (2013) Hierarchical cluster analysis of immunophenotype classify AML patients with NPM1 gene mutation into two groups with distinct prognosis. *BMC Cancer* 13:107
 47. Stegmaier K, Corsello SM, Ross KN, Wong JS, Deangelo DJ, Golub TR (2005) Gefitinib induces myeloid differentiation of acute myeloid leukemia. *Blood* 106:2841–2848