RESEARCH COMMUNICATION

Fms Like Tyrosine Kinase (FLT3) and Nucleophosmin 1 (NPM1) Mutations in De Novo Normal Karyotype Acute Myeloid Leukemia (AML)

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Abstract

Mutations in FLT3 and NPM1 are important prognostic factors in AML, influencing outcome in normal karyotype cases. We here analysed incidences of *FLT3/ITD, D 835 and NPM1* mutations in patients with de novo normal karyotype AML using PCR and gene sequencing, along with laboratory parameters and treatment outcomes. There were 128 patients with a median age of 45 years (range, 19-65). *FLT3/ITD* mutations were detected in 26 (20.3%), *FLT3/D835* in 8 (6.2%) and NPM1 in 22 (17.1%). The incidence of *FLT3/ITD* was higher in those with elevated lactate dehydrogenase (LDH) and peripheral blasts (*p=< 0.002, < 0.001*) while *NPM1* mutations or both *NPM1* and *FLT3/ITD* was more common in elevated total leukocyte counts (TLC), LDH and peripheral blasts (*p=<0.0001*). Complete response and disease free survival were lower in those with *FLT3/ITD* mutations (*p=0.04, 0.03*). The incidence *of FLT3* and *NPM1* mutations was found to be low in Indian patients with normal karyotype AML.

Keywords: *NPM1 - FLT3-ITD - D 835* mutations - normal karyotype acute myeloid leukemia

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Introduction

The most important prognostic factor in acute myeloid leukemia (AML) is the cytogenetics at diagnosis. Patients with normal karyotype who form 45-50% of all AMLs are classified into the intermediate risk category and are candidates for allogenic stem cell transplantation. Although patients with normal karyotype are a heterogeneous group with varying event free and overall survivals, all classified into the same risk category (Estey et al., 2006).

In the recent years mutations in AML which can redefine the risk of patients with normal karyotype AML have been reported (Bullinger et al., 2004; Mrózek et al., 2007). These fall into 2 broad categories: Type 1 includes mutations in RAS, fms like tyrosine kinase internal tandem duplication *(FLT3/ITD) and D835* point mutation of the *FLT3* tyrosine kinase domain *(FLT3/D835)* that activate signal transduction pathways and Type 2 like nucleophosmin *(NPM1)* and CCAAT enhancer binding protein alfa *(CEBPA)* that affect transcription factors or transcriptional co-activation (Schlenk et al., 2008).

FLT3 is a member of the class III receptor tyrosine kinase which plays crucial role in the growth control of pluripotent haemopoitic cells, early progenitor cells and immature lymphocytes. An internal tandem duplication of *FLT3* results in ligand independent dimerization of the mutant *FLT3* receptor leading to constituent tyrosine kinase activity. Nucleophosmin is a ubiquitously expressed nucleolar phosphoprotein that acts as a molecular chaperone playing diverse roles in cellular processes and tumour suppressor pathways by binding with *TP53* and p19.Mutations in this gene result in genomic instability, an initiating event in the leukemiogenesis (Schlenk et al., 2008).

These mutations have an impact on the complete response rates, event free and overall survival of patients with AML. Patients who harbour *FLT3/ITD* have been shown to have a worse prognosis irrespective of the karyotype while those with NPM1 or CEBPA have been shown to have a favourable outcome (Dohner et al., 2008).

There is no data on incidence of mutations in normal karyotype AML from India. The primary objective of the study was to estimate the incidence of *FLT3/ITD, FLT3/D835* and *NPM1* mutations in adult patients with de novo AML and normal karyotype. Secondary objectives were to analyse the association of mutations with clinical and lab parameters, complete response (CR) and disease (DFS) free survival.

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Materials and Methods

Leukaemia samples

This prospective analysis was done on patients with AML diagnosed and treated between Jan 2005-Dec 2007. Patients over the age of 18 were included if they had a newly diagnosed de novo AML with normal karyotype. Patients with secondary leukaemias were excluded. The WHO criteria were used for making a diagnosis of acute leukaemia (Lee Harris et al., 1999). At diagnosis, standard G banding techniques were employed to karyotype the leukemia. Informed consent was taken from all patients prior to testing. The institutional ethics committee and the research committee of the hospital approved the protocol.

At diagnosis, 5 ml of peripheral blood was collected in EDTA vacutainer from 128 patients diagnosed with AML at Nizam's Institute of Medical Sciences, Genomic DNA was extracted by rapid non enzymatic method from all the blood samples. The concentration of DNA was determined by the ratio of A260/A280 and then was diluted to 1μg/1μl with water.

Screening of FLT3-ITD by DNA PCR

All DNA samples were analyzed for mutations in exon 11 of the *FLT3* gene using PCR method. Primers were designed (Sigma-Aldrich) covering the whole juxta membrane domain (JM) and the first part of the TK1-1 domain of FLT3 where most of the reported mutations are located were used. The sequence of the primers used were 11F (sense) 5´CAA TTT AGG TAT GAA AGC C´3,11R (antisense) 5´CAA ACT CTA AAT TTT CTC T´3. 50 to 100 ng of genomic DNA were amplified in a 50 μl reaction containing 200 uM of each deoxyribonucleotide triphosphate (dNTP) ,2.5 units of Taq DNA polymerase, 40 Pico moles of each primer, and 6% dimethyl sulfoxide. The PCR protocol included 35 cycles of 30 sec at 94°C for denaturation, 45 sec at 50°Cfor annealing, 1min 72°C for extension and 72°C for 7 min for final extinction. The amplified product was 133 bp in length. 10μl of PCR product was examined by 3% agarose gel electrophoresis and samples showing longer PCR products were considered positive for FLT3/ITD mutation (F.xu et al 1999).

Sequencing and Blast Analysis

PCR products were reclaimed and purified by QUIAQUICK gel extraction kit, directly sequenced with forward primer using ABI 3730 DNA sequence analyzer. They were compared with wild type sequences obtained from ensemble data base. All data were analyzed by online blast. Gene bank accession No: NG-007066.

Screening of D835Y Mutations

PCR for Detection of *D835* mutation was performed by using primers 20F 5´CCG CCA GGA ACG TGC TTG-3´ and 20R 5´- GCA GCC TCA CAT TGC CCC -3´. D835 and I836 amino acids are encoded by GATATC, which is the recognition sequence for ECORV. Mutants were detected by the loss of this enzyme restriction site. PCR was performed for 35 cycles of initial denaturation at 95°C for 9 min, denaturation at 94°C for 30 sec, annealing at 60°C for 1 min, extension at 72°C for 2min, final extension at 72°C for 10 min. Purified PCR products were digested with 5 U of *ECORV* (New England bio labs) at 37°C for 3 hrs and subjected to agarose gel electrophoresis (3%). The presence of undigested PCR product indicated the presence of a mutant. One of the mutants was confirmed by Sequencing.

Screening of NPM1 Exon 12 Mutations

PCR amplification of *NPM1* exon 12 was carried out using primers *NPM1*- F (5´ TTA ACT CTC TGG TGG TAG AAT GAA -3´) and *NPM1*-R (5´ TGT TAC AGA AAT GAA ATA AGA CGG- 3´). Samples were amplified using the following PCR reaction conditions 94°C for 3 min 35 cycles of 95°C for 1 min, 58°C 45 sec, 72°C 2min 72°C for 7 min. PCR products were purified and directly sequenced with reverse primer *NPM1*-R using ABI 3730 DNA analyzer. (Gene bank accession NO NC-000005).

Therapy and Follow Up

Patients who underwent therapy received Inj. Daunorubicin 60mg/ m^2 on Days 1, 2 and 3 along with Inj. Ara C 100mg/ m^2 as a continuous intravenous infusion over 24 hours from days 1 to 7.

Patients who achieved complete response were administered 3 cycles of high dose Cytarabine at 3 gm/m^2 intravenously twice daily on Days 1,3, and 5.Following this, patients were followed up once every 3 months with clinical examination and complete blood counts. A bone marrow aspiration was done if there was any suggestion of a relapse on clinical examination or peripheral smear. The association between mutations and CR and DFS was analysed retrospectively for those who received treatment.

Criteria for response and evaluation of outcome were defined as per Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia 2003 (Cheson et al., 2003). The bone marrow aspiration was done on Day 28 or earlier if the peripheral blood showed recovery as evidenced by a neutrophil count of > 1.0 x $10^3/\mu$l, platelet count of > 100 x $10^3/\mu$l. No aspiration was done during the neutropenic nadir or Day 14.

Morphologic relapse was defined as reappearance of blasts after documentation of complete response (CR) in peripheral blood or >5% in the bone marrow. Disease free survival (DFS) was calculated from the date of first CR till the date of first relapse or date of death due to any cause while on follow up.

Statistical Analysis

The paired t test was used to test the significance of the difference in the clinical and lab parameters between the groups without any mutation and those with any detectable mutation. Univariate analysis was done to study the association of age (< vs > 50 yrs), sex (male vs female), lactate dehydrogenase - LDH (< vs > normal), total leukocyte count-TLC (< vs >11,000/µl) and peripheral blast percentage (< vs > 50%) with the incidence of *FLT3/ITD* and NPM1 mutations using 2x2 contingency Chi square test. A 2-tailed Fisher's p value of < 0.05 was considered significant. The effect of mutations on CR was done using Chi square test and the effect on DFS was

tested by plotting Kaplan Meier curves for patients with and without mutations and tested for significance using the log rank test. Graph pad Quickcals software was used for all the analyses except Kaplan Meier plot which were done using Graph pad Prism for Microsoft windows 2003.

Results

There were 128 patients with a median age of 45 years (range, 19-65) with 85 males and 43 females. The demographic, clinical and laboratory characters of the whole group has been summarised in Table 1. Of the whole group, 20 (15.5%) were older than 50 years, 69 (53.9%) had elevated TLC, 40 (31.2%) had peripheral blasts >50%, 81 (63.2%) had platelets <1x10^3 µ/L and 60 (46.8%) had elevated LDH at diagnosis. All patients had non M3 AML and a normal karyotype.

PCR and chomatograms are shown in Figures 1-5.

Incidence of Mutations

FLT3/ITD mutations were detected in 26 patients (20.3%), while FLT3/D835 and NPM1 were observed in 8 (6.2%), and 22 (17.1%) patients respectively. Ten (45%) of the 22 patients with NPM1 mutation also had FLT3/ITD mutation while none of the patients with FLT3/D835 had any other mutation.

Comparison of Baseline Characteristics between Groups

The baseline characteristics for individual groups are also summarised in Table 1. Patients with FLT3/ITD and those who had both FLT3/ITD and NPM1 mutations had significantly higher mean LDH, TLC and peripheral blasts compared to the group without mutations. The group with NPM1 mutations had significantly higher LDH, peripheral blasts and lower Hb% while the baseline characteristics of the group with FLT3/D835 were not significantly different from those without mutations.

Relation between Incidence of Mutations with Clinical and Lab Parameters

Patients with elevated LDH and >50% peripheral blasts were more likely to harbour an *FLT3/ITD* mutation (*p= < 0.002, < 0.001*).Patients with elevated TLC (>11,000/µl),

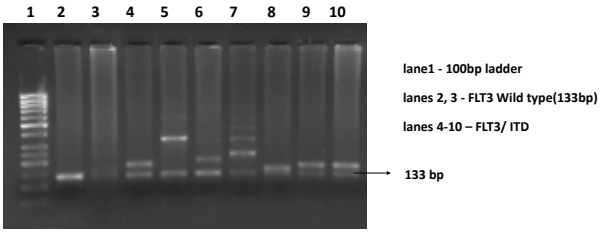

Figure 1. Detection of *FLT3/ITDs by PCR*

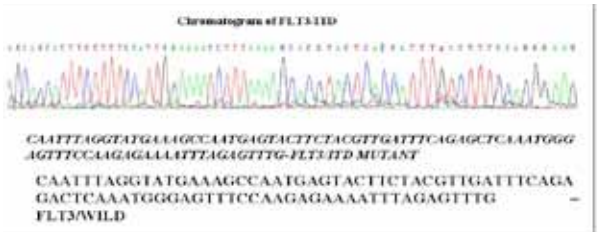

Figure 2. Chromatogram of *FLT3/ITD*

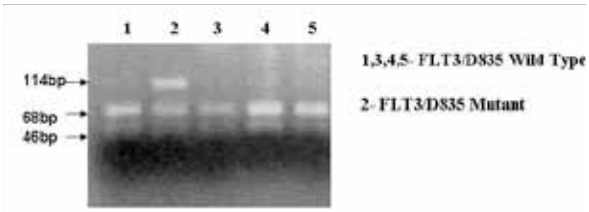

Figure 3. Detection of *FLT3/D835Y by PCR*

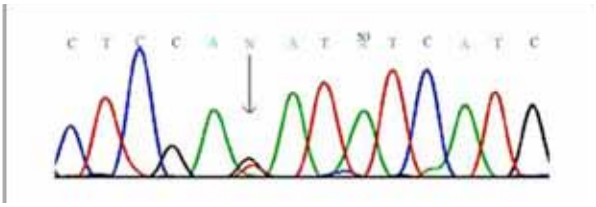

Figure 4. Chromatogram of *FLT3/D835*

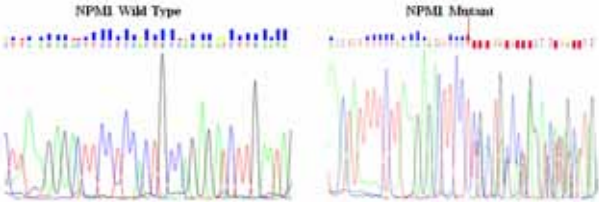

Figure 5. Chromatogram of *NPM1* mutations

Table 1. Comparison of Baseline Characteristics Between Groups

Parameter (range)	No mutations (n=82)	FLT3/ITD (n=26)	FLT3/D835 (n=8)	NPM1 (n=22)	NPM1 & FLT3/ ITD (n=10)	All (n=128)
Age (years)	33.5 (19-65)	36.12(18-62) (p=0.8954)	27.4(19-65) (p=0.8439)	33.4 (20-62) (p=0.359)	38.6 (22-62) (p=0.4338)	45 (19-65)
Male:Female	1.6:1	1.8:1	2:1	0.83:1	0.6:1	1.95:1
Hb (gm/dl)	9.29 (4-15.8)	8.7 (4.1-12.6) (p=0.0591)	7.9(7.3-10.8) (p=0.2555)	8.6 (7-12.6) (p=0.0186)	8.14 (4.1-12.6) (p=0.1861)	9.2 (3-15.8)
WBC (10^9/L)	23.8 (3.2-320000)	64.56 (2.12-164) (p=0.0369)	54.7(5.8-160) (p=0.1457)	61.2 (22.5-164) (p=0.0613)	65.8 (32-164) (p=0.0391)	12.5 (3.2-320)
Blasts (%)	26.3(0-94)	81.56 (53-93) (p=<0.0001)	52.3 (35-92) (p=0.0613)	80.68 (62-92) (p=<0.0001)	81.2 (67-92) (p=0.0003)	26 (0-94)
Platelets (10^9/L)	124 (10-520)	76 (10-160) (p=0.1088)	71.2(20-130) (p=0.4792)	97.31 (10-400) (p=0.6581)	52.7 (34-150) (p=0.4)	52 (10-590)
LDH (IU/L)	286.7 (145-1715)	644 (295-1269) (p=0.0005)	395(234-1046) (p=0.8107)	776.3 (460-1269) (p=<0.0001)	861.9 (625-1269) (p=0.0169)	245(145-1715)

All p values are between the no mutations group (column 2) and the comparator groups (column 3-6)

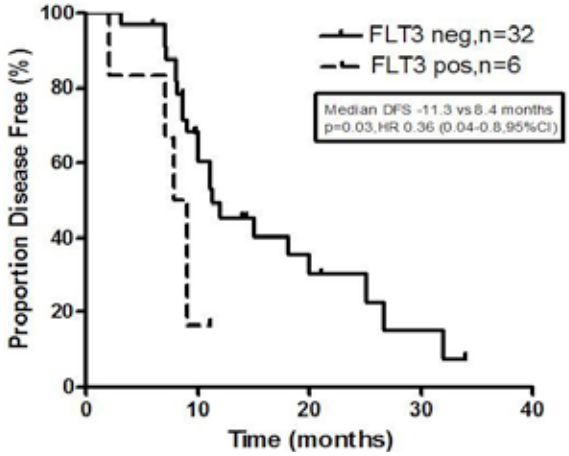

Figure 6. Kaplan Meier Curve Comparing Disease Free Survival (DFS) of Patients with and without FLT3/ITD Mutations

LDH and peripheral blasts had higher incidence of NPM1 or a combination of *NPM1* and *FLT3/ITD* mutations (*p = <0.0001*). There was no correlation between the incidence of *FLT3/ITD* mutations and age or TLC. There was no difference in incidence of mutations between sexes.

Treatment Details and Outcomes for the Whole Group

A total of 76 (59.8%) patients i.e. 16 (61.5%) of the 26 patients who had *FLT3/ITD* mutation, 6 (75%) of the 8 patients with FLT3/D835, 7 (31.8%) of the 22 with NPM1 mutation and 47 (57.3%) of the 82 patients without any detectable mutation underwent induction chemotherapy with the standard 7+3 regimen consisting of Ara C and Daunorubicin followed by 3 cycles of high dose Ara C for those who attained CR. None underwent allogenic or autologous stem cell transplantation.

For the whole group of 76 patients who received induction chemotherapy, 44 (57.8%) achieved CR. Thirty two patients (42.2%) were classified as induction failure, which included partial responses in 23 patients (30.2%) and induction deaths in 9 patients (12%). The DFS for the group was 10.7 months (range, 2-44).

In the 47 patients without any detectable mutation who underwent induction, 32 (68.5%) patients attained CR, 15 (31.5%) patients' were induction failures and the DFS was 11.3 months (range, 3-44).

Relation between CR and DFS with FLT3/ITD and FLT3/D835 Mutation

Of the 16 patients with FLT3/ITD mutation who received induction, 6 (37.5%) patients achieved CR and 10 (62.5%) were treatment failures (8 partial responses and 2 induction deaths).

The CR rate was significantly lower than the 68.5% achieved in patients who did not harbour any mutations (*p=0.04*). The DFS was significantly shorter compared to those without any mutations (11.3 vs 8.4 months, *p=0.03*) Figure 6. Of the 6 patients with FLT3/D835 who received induction, 4 (66%) patients achieved CR, which was not different from the CR rate of 68.5% for the group without mutations (*p=1.0*). Similarly the DFS between these groups were also not significant (11 vs. 11.3 months, *p=1.0*).

Relation between CR and DFS and NPM1 Mutations

Of the 22 patients who had NPM1 mutations, 7 (30.4%) received induction therapy, 4 (57%) achieved CR and 3 were induction failures. This was not significantly different from the CR rates of 68.5% for those without mutations (*p=0.67*). Similarly the differences in the DFS between the groups was not significant (11.8 vs 11.3 months, *p=0.84*).

There were only 2 among the 10 patients with both FLT3/ITD and NPM1 mutations who received induction therapy, both of whom were induction failures. They were not analysable for DFS.

Discussion

In this analysis of Indian patients with AML and normal karyotype, the incidence of *FLT3/ITD* mutations was 20.4% and those who harboured the mutation had significantly worse CR and DFS rates. There was a definite positive correlation between incidence of mutation with elevated LDH and peripheral blast percentage which predict for poorer outcomes in patients with AML (Schnittger et al., 2005; Suzuki et al., 2005).

Although the mutation analysis was done for the whole group of 128 patients, due to financial constraints, almost half of these patients did not receive induction chemotherapy and none underwent a transplant. However, for those who received therapy, the induction treatment was standard and uniform. Most patients who had a relapse did not undergo further therapy and were referred back to their primary physicians for supportive care only. Since a meaningful survival analysis was not possible with most of these patients being lost to follow up, the overall survival data has not been reported.

There have been previous studies on the impact of *FLT3/ITD* mutations in Indian patients with acute promyelocytic leukaemia (Hasan et al., 2007; Mathews et al., 2007). One of these reported a negative impact of the mutation on both CR rates and overall survival while the other showed no impact on these outcomes. There has been no data reported from India on patients with AML and normal karyotype. Although the incidence *FLT3/ITD* is marginally less than the 28-34% reported in literature its negative impact on CR rates and DFS concur with previous reports. However recent evidence has shown that the poorer outcomes of these patients may not be related just to the presence of the mutation but to the expression level of the mutant allele (Kottaridis et al., 2001; Whitman et al., 2001). Levels of mutant alleles were not performed in our study.

Similarly, the 6% incidence of D835 TKD mutations in our study was lower than the 11-14% reported. Although a meta-analysis of 1160 cases with AML and FLT3/TKD mutations showed a negative prognostic impact, a retrospective review from MRC AML 10 and 12 trials showed a positive impact in patients with normal karyotype (Yanada et al., 2005; Mead et al., 2007). We could not demonstrate any association between D835 TKD mutations and laboratory parameters or outcomes.

NPM1 mutations have been reported in 45-62% of

patients with normal karyotype AML compared to the 17.1% in our study. However as reported earlier, the mean peripheral blast % and LDH values were higher in those with *NPM1* mutations (Schnittger et al., 2005; Suzuki et al., 2005). The prognostic value of *NPM1* has been controversial at least in univariate analysis where some studies have shown a positive impact on CR, DFS and OS while some have failed to show any impact.

At least 4 large studies have shown that *NPM1* mutation in normal karyotype AML is associated with favourable prognosis (Döhner et al., 2005; Thiede et al., 2007) which we were unable to demonstrate probably due to low patient numbers.

Forty five percent of patients with *NPM1* mutation also harboured a *FLT3/ITD* mutation which is similar to the 40% reported in the literature (Yanada et al., 2005; Mead et al., 2007). The significance of this is that *NPM1* mutation confers favourable prognosis only in the absence of a co-existing *FLT3/ITD* mutation (Schnittger et al 2005; Thiede et al., 2007). Conversely NPM1 did not have any impact on the adverse prognosis conferred by FLT3/ITD.

Since patients who harbour *FLT3/ITD* have dismal outcome with standard therapy and considering the fact in India most patients are unable to afford treatment, patients should be encouraged to be a part of a clinical trial wherever possible. In the future, this information could be incorporated into decision making algorithms, especially to choose patients with normal karyotype for intensive post remission therapies. Although the complete response rates in our study are comparable to published literature, even patients who did not have any mutations had a poorer DFS compared to patients reported previously who were treated on a similar protocol without stem cell transplantation (Farag et al., 2005). Hence there is a need to study the biology of these patients looking at a wider range of genes which can have an impact on the outcomes.

In conclusion, the incidence of *FLT3/ITD*, *FLT3/D835* and *NPM1* mutations is lower in Indian patients than that reported earlier. There is a significant correlation between presence of *FLT3/ITD* and *NPM1* mutations with LDH and peripheral blast.

Considering the small patient Population of our study, precludes any conclusions on the influence of these mutations on treatment and outcomes. Larger studies on a wider range of mutations to define the biology of this group of patients are warranted.

Acknowledgments

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