

## FLT3 Mutation as a Significant Prognostic Marker in de novo Acute Myeloid Leukemia Patients: Incidence, Distribution and Association with Cytogenetic Findings in a Study from South India

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### Abstract

**Background:** Fms-like tyrosine kinase 3 is a tyrosine kinase receptor that plays an important role in proliferation and differentiation of hematopoietic stem cells. Internal tandem duplication and tyrosine kinase domain mutation are the two most common types of fms-like tyrosine kinase 3 mutations frequently reported in acute myeloid leukemia associated with pathogenesis of this disease. The present study investigates the prevalence and distribution pattern in different acute myeloid leukemia sub- and cytogenetic groups, the association with clinical parameters and the prognostic importance of these mutations in acute myeloid leukemia patients from South India.

**Methods:** Mutation analysis was performed in 276 de novo acute myeloid leukemia patients by polymerase chain reaction restriction fragment length polymorphism using specific restriction enzymes followed by sequencing to confirm the mutations. Kaplan-Meier survival analysis was performed to detect the prognosis.

**Results:** Fms-like tyrosine kinase 3 internal tandem duplication mutations were observed in 20%, tyrosine kinase domain mutation in 4% and dual mutations in 0.3% of the analyzed cases. The internal tandem duplication mutations ranged from 15-107 nucleotides with the majority at the juxta membrane domain of the receptor. Three types of tyrosine kinase domain point mutations were identified: D835Y, D835H and D835V. We observed a significant association between fms-like tyrosine kinase 3 mutations and increased WBC and LDH counts ( $P < 0.001$ ) and blast percentage but not with age, gender and FAB subtypes. A significant association with normal karyotype was observed for the mutants ( $P = 0.002$ ). Survival analysis revealed that the fms-like tyrosine kinase 3 gene mutation was a negative prognostic marker for acute myeloid leukemia patients. The risk stratified analysis showed the mutation to be a risk factor for the intermediate karyotype group, especially for those with normal cytogenetics.

**Conclusion:** Our results indicate that the presence of an fms-like tyrosine kinase 3 mutation can serve as a valuable prognostic marker in this subgroup of patients, allowing stratification for risk-directed therapy.

**Keywords:** Acute myeloid leukemia, FLT3-ITD, FLT3-TKD, Prognosis, Cytogenetics

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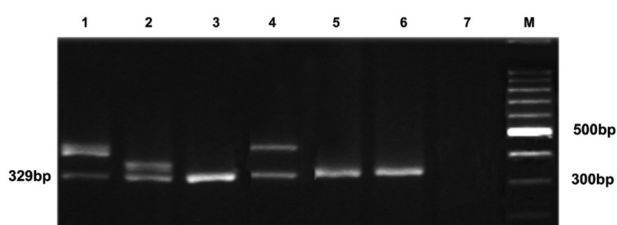
## Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of neoplastic disorders with great variability in clinical course and response to therapy, as well as in genetic and molecular basis of pathology. Cytogenetic studies of AML patients have contributed to the understanding of the remarkable histopathologic, immunophenotypic and clinical heterogeneity of AML. Cytogenetic analysis is considered as the single most important factor in determining prognosis in AML.<sup>1</sup> Large numbers of AML patients have specific chromosomal lesions known to be associated with particularly favorable or unfavorable prognoses.<sup>2</sup> The WHO classification subdivides AML predominantly according to karyotype because recurrent chromosomal abnormalities identify distinct leukemia entities and have a major impact on prognosis. In 50%-60% of patients with de novo AML clonal cytogenetic abnormalities that are either structural or numerical can be identified, though not all have been defined at the molecular level. In the remaining cases gene mutation may play a major role in the pathogenesis, which are undetectable in the cytogenetic analysis and have an impact on prognosis in the different cytogenetic subgroups.<sup>3</sup>

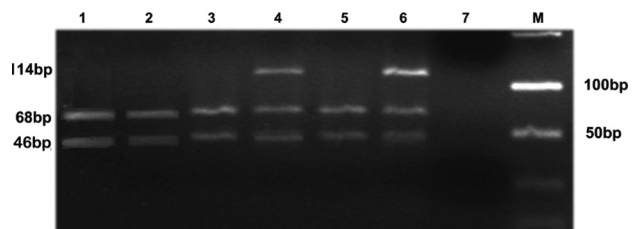
Fms-like tyrosine kinase 3 (FLT3) mutations are one of the most frequent somatic alterations in AML. Two major mutations in this gene are the most common internal tandem duplication (ITD) and a missense point mutation or small insertions or deletions within the activation loop of the second tyrosine kinase domain (TKD), called the

TKD mutation.<sup>4</sup> ITD occurs as duplication of nucleotide sequences as head to tail orientation of variable length in exons 14 and 15 (previously known as exons 11 and 12) which lead to the addition of a repeated peptide that maps to the juxta membrane domain (JMD) in the cytoplasm, seen in 20%-27% of adult AML and 10%-16% of childhood AML.<sup>5-8</sup> ITDs that affect the JMD are called length mutations (LM). The length of the duplicated JMD region varies from 3 to 400 nucleotides and is usually in-frame. Prevalence of FLT3/ITD is highly age dependent, with 5% to 10% seen from ages 5 to 10 years, 20%-30% in adults, and greater than 35% in AML patients older than 55 years.<sup>9</sup> FLT3-ITD have been reported in all FAB subtypes of AML with the highest reported frequency (30%-35%) in cytogenetically normal AML and in the M3 subtype with t(15;17), which is seen in 30%-50% of cases<sup>8,10</sup> and less frequency in the M2 subtype.<sup>6</sup> FLT3-ITD mutations have also been detected in myelodysplastic syndrome (MDS) at a frequency of 3%,<sup>11</sup> occasionally in acute lymphoid leukemia (ALL),<sup>12</sup> and chronic myeloid leukemia (CML),<sup>13</sup> but not in non-Hodgkin's lymphoma, multiple myeloma, chronic lymphoid leukemia and in normal individuals.<sup>14,15</sup> These studies reveal a strong disease specificity of this mutation for AML.

The TKD mutation which is the second most common type of FLT3 mutation occurs in exon 20. This mutation is less frequent and found in 5%-10% of all AML patients.<sup>16</sup> The TKD mutation typically involves codons D835 and/or I836, and



**Figure 1.** Ethidium bromide stained 3% agarose gel picture showing FLT3-ITD mutation. Lanes 1, 2 and 4 show patients' sample positive for the ITD mutation. Lanes 3, 5 and 6 show wild type (WT) FLT3. Lane 7 is the negative control and lane M is a 100 bp marker.



**Figure 2.** Ethidium bromide stained 3% agarose gel picture showing FLT3-TKD mutation. Lanes 1-3 and 5 are patients' sample negative for the TKD mutation. Lanes 4 and 6 show patients' sample positive for FLT3-TKD mutation. Lane 7 is the negative control. Lane M is a low molecular weight DNA marker.

less frequently N841 or Y842.<sup>17</sup> The most common TKD mutation is an aspartate (Asp) to tyrosine (Tyr) substitution at codon 835, but other substitutions such as Asp835Val, Asp835His, Asp835Glu, and Asp835Asn have also been reported. Most AML patients have only one type of FLT3 mutation, but rarely some patients have both an ITD and TKD of FLT3.

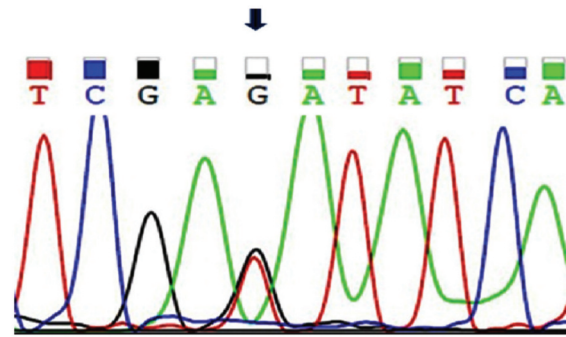
Mutations in the FLT3 JM domain and activation loop result in loss of auto-inhibitory function with subsequent constitutive activation of FLT3 kinase and its downstream proliferative signaling pathways, including RAS/mitogen activated protein kinase (MAPK), extracellular signal related kinase (ERK) pathway, PI3K/Akt pathway and signal transducer and activator of transcription 5 (STAT5) pathways.<sup>6</sup>

Several reports discussed FLT3 mutations in AML and its prognostic significance. To our knowledge no published reports were available from South India with respect to the incidence and distribution patterns of FLT3. In the current study, we investigated the ITD and TKD mutations of FLT3 in de novo AML patients in South India and correlate the mutation status with various clinical and cytogenetic parameters. The prognostic significance of these mutations was also investigated.

## Materials and Methods

### Patient samples

The present study was conducted at the Regional Cancer Centre, Trivandrum, Kerala, India. The study was carried out in 276 de novo AML patients, aged 18-70 years who attended the Medical Oncology Clinic of the Regional Cancer Centre, between January 2009 and May 2011. The diagnoses were pathologically confirmed. Bone marrow samples (2 ml) from AML patients were collected in heparinized vacutainers for cytogenetic analysis and blood samples (5 ml) was collected into EDTA vacutainers for molecular studies following written informed consent. The study was approved by the Ethical Committee of the Regional Cancer Centre. Patient's clinical data that included WBC



**Figure 3.** Representative electropherogram that shows substitution of G with T (arrow) leading to the change in codon from GAT to TAT [i.e., Asp 835 to Tyr (D835Y) amino acid change].

counts, blast percentage, platelet counts, hemoglobin, and LDH levels were noted from the case file.

### Cytogenetic and molecular cytogenetic analysis

French-American-British (FAB) classification status of the AML patients was obtained from the pathological reports. We performed cytogenetic analysis on the patient samples before the start of therapy. Conventional cytogenetic studies were performed using standard techniques.<sup>18</sup> GTG banding at approximately the 550 band level was performed and metaphases were observed under a microscope that had a 100X objective (Olympus BX 5). At least 20 metaphases were karyotyped using cytogenetic software (Cytovision Version 4.5, USA) and chromosomal abnormalities described as per the International System for Human Cytogenetic Nomenclature Guidelines (ISCN 2005).<sup>19</sup> FISH analysis was performed to determine recurrent abnormalities [t (8;21), t (15;17) and inv (16)] associated with specific FAB subtypes M2, M3 and M4, respectively using Vysis probes (Abbot Molecular Inc.).

### FLT3-ITD and FLT3-TKD mutation screening

Genomic DNA was amplified using specific primers as described previously for exons 14, 15 and 20.<sup>16, 20</sup> PCR was performed as a 25- $\mu$ L reaction containing 10 $\times$  reaction buffer with MgCl<sub>2</sub>, 0.2 mM dNTP, 10 pmol of each of the primers, 100 ng template DNA, and 0.5 units Taq polymerase. Samples were amplified using

**Table 1.** Association of demographic, clinical parameters, karyotype pattern and FAB subtype (French American British) with FLT3 mutation.

Parameters	Wild type (WT) (n=209)	ITD only (n=55)	TKD only (n=11)	All mutants (n=66)	P-value
Median age (years)	40	40	37	38	
Age (years)					
≤50	158 (76)	43 (77)	10 (83)	52	0.909
>50	51 (24)	13 (23)	2 (17)	15	
Male	109 (52.2)	27 (48.2)	7 (58.3)	33 (49.3)	0.703
Female	100 (47.8)	29 (51.8)	5 (41.7)	34 (50.7)	
Median White Blood cell Count (x 10 <sup>9</sup> /L)	12.5	70.1	48	62.8	<0.001
Median Peripheral Blood Blast (%)	61	74	64	71	0.012
Median Bone Marrow Blast (%)	69	78	70	72	0.016
Median platelet (x 10 <sup>9</sup> /L)	40.3	41	40	40	0.345
Median Hb (g/dL)	8.2	7.9	8.5	8	0.445
Median Lactate Dehydrogenase levels (IU/L)	1139	1900	2000	1900	<0.001
Karyotype pattern					
Poor risk	9 (4.3)	1 (1.8)	0	1 (1.5)	0.264
Normal karyotype	99 (47.4)	42 (75)	8 (66.7)	49 (73.1)	
Intermediate risk	31 (14.8)	6 (10.7)	2 (16.7)	8 (11.9)	
Good risk	49 (23.4)	6 (10.7)	2 (16.7)	8 (11.9)	
Karyotype unknown	21 (10)	1 (1.8)	0	1 (1.5)	
Normal karyotype	99 (47)	42 (75)	8 (67)	49 (73.1)	0.017
Aberrant karyotype	89 (43)	13 (23)	4 (33)	17 (25.4)	
Karyotype unknown	21 (10)	1 (2)	0	1 (1.5)	
FAB subtype					
M0	7 (3.3)	0 (0)	0	0	0.710
M1	35 (16.7)	13 (23.2)	2 (16.7)	14 (20.9)	
M2	50 (23.9)	8 (14.3)	2 (16.7)	10 (14.9)	
M3	26 (12.4)	8 (14.3)	0	8 (11.9)	
M4	28 (13.4)	11 (19.6)	3 (25)	14 (20.9)	
M5	53 (25.4)	14 (25.0)	5 (41.7)	19 (28.4)	
M6	4 (1.9)	0 (0)	0	0	
AML	6 (2.9)	2 (3.6)	0	2 (3)	

ITD: Internal Tandem Duplication; TKD: Tyrosine Kinase Domain Mutation; AML: Acute Myeloid Leukemia; Hb: Hemoglobin.

standard PCR conditions (95°C for 5 min; 35 cycles at 94°C for 30 s, 60°C for 1 min and 72°C for 2 min; 72°C for 10 min) which produced a 329

bp product. PCR products were subjected to 3% agarose gel electrophoresis for better resolution and we considered samples that showed longer

**Table 2.** Comparison of cytogenetic aberrations and FLT3 mutations in patients with acute myeloid leukemia (AML).

Cytogenetics	Total (%)	FLT3 mutation (%)		P-value
		ITD or TKD	FLT3 WT (%)	
<b>Total</b>	276	67	209	
<b>Karyotype</b>				
Unavailable	22 (8)	01 (5)	20 (95)	0.002
Normal karyotype (XX, XY)	148 (54)	49 (33)	99 (67)	
Aberrant karyotype	106 (38)	17 (16)	89 (84)	
<b>Individual aberrations</b>				
t (8;21)	21 (7.6)	03 (14)	18 (86)	
t (15;17)	30 (10.8)	05 (17)	25 (83)	
Inv (16)	06 (2)	0	06 (100)	
Numerical abnormality	31 (11)	06 (19.4)	25 (80.6)	
Other structural abnormalities	08 (3)	02 (25)	06 (75)	
Complex karyotype	10 (3.5)	01 (10)	09 (90)	

WT: Wild Genotype; ITD: Internal Tandem Duplication; TKD: Tyrosine Kinase Domain Mutation.

PCR products to be positive for the FLT3-ITD mutation.

PCR-RFLP assay was performed for the D835 mutation because D835 and I836 codons were encoded by the nucleotide GATATC, which forms the Eco RV restriction site. We added 3% of DMSO as a PCR additive. The PCR conditions included an initial denaturation at 95°C for 9 min; 35 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min with a final extension at 72°C for 10 min. Amplified products of 114 bp were digested with 5 U of Eco RV (Bangalore Genei) at 37°C for 3 h and subjected to agarose gel electrophoresis (3%). In case of wild type (WT) allele, two restriction fragments of 46 bp and 68 bp were obtained. The presence of an undigested product was indicative of a mutant which was subsequently confirmed by direct sequencing (Bangalore Genei).

### Statistical analysis

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS) software. Quantitative variables were summarized using median (range). For continuous variables we used the Mann-Whitney U test. Qualitative data were summarized with frequencies and percentages. The Pearson chi-square test was used to calculate the significance of an association between the FLT3 mutation and other discrete variables among subgroups of patients. The effect of mutation on the overall survival (OS) was

estimated using Kaplan-Meier survival and assessed by the log rank test. For OS, the time from diagnosis until death from any cause or last follow up to a maximum of 30 months, was calculated. A *P*-value of <0.05 was considered statistically significant.

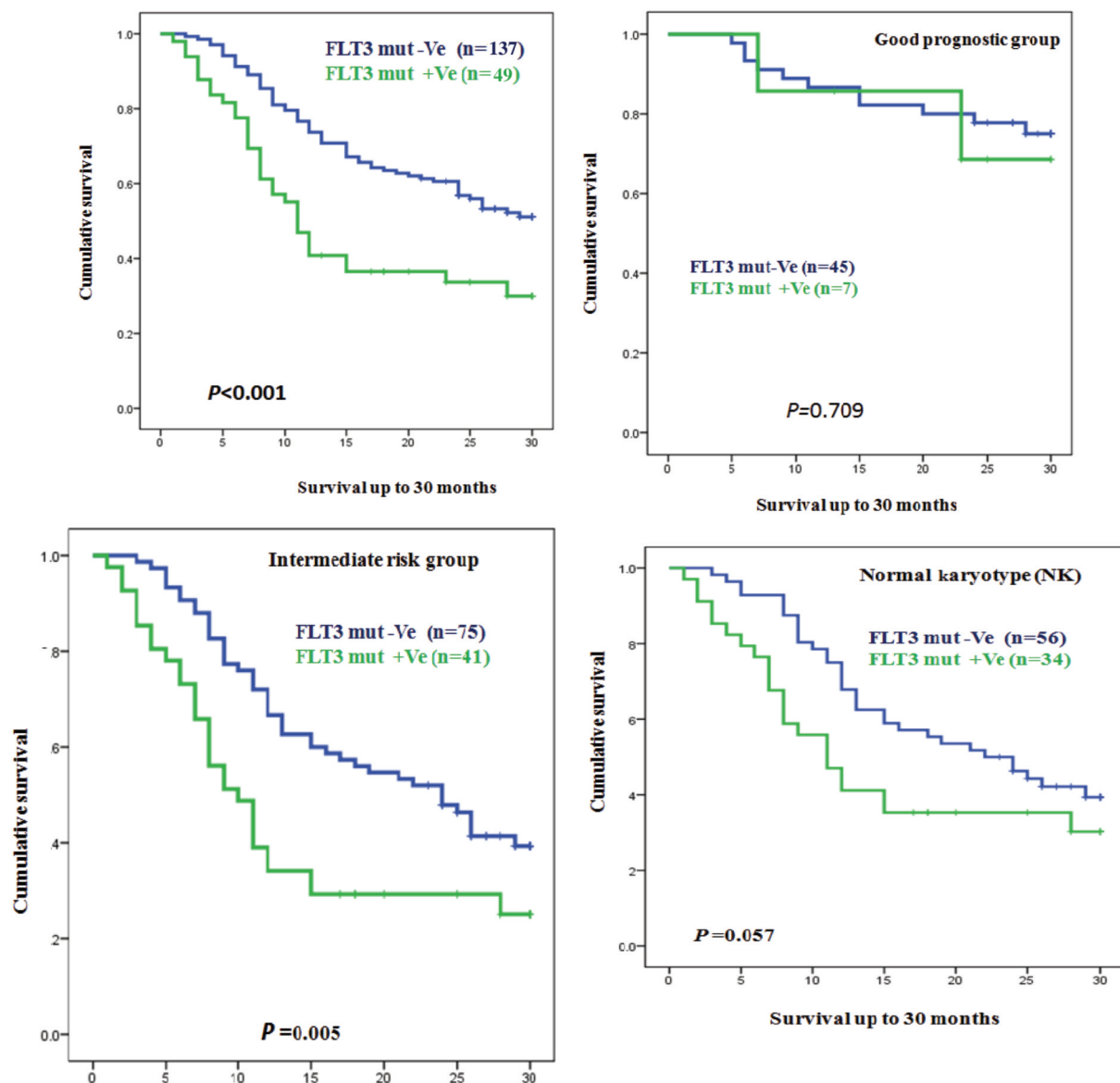
### Results

The incidence of FLT3-ITD and -TKD were determined in 276 de novo AML cases. FLT3 gene mutations were detected in 67 (24.3%) cases, while the remaining 209 (75.7%) showed normal WT alleles. Patients with isolated FLT3-ITD mutations were found in 55 (20%) cases, the TKD (Asp 835) mutation in 11 (4%) cases, and in one (0.3%) case we observed a combined mutation. All mutants (ITD and TKD) were heterozygous for the mutation (Figures 1 and 2). FLT3 gene mutations were somewhat equally distributed in both males (49%) and females (51%). The same was observed for isolated ITD, with 48% of males and 52% of females. The TKD mutation showed a slight predominance in males (58%) compared to females (42%), which was not significant (*P*=0.626). Interestingly, the incidence of FLT3 mutations (ITD or TKD) showed a gradient decrease with increased age. In patients with either ITD or TKD mutation more than 75% have been observed in cases less than 50 years of age, with 52% of the mutation observed between the ages of 31 and 50 years. The median ages of FLT3 mutated (40 years) and non-mutated (38

years) cases were approximately the same. There was no significant association between patient age and gender wise distribution of mutated and non-mutated cases.

We compared clinical parameters such as WBC count, hemoglobin, peripheral and bone marrow blast percentages, platelet count and LDH values between FLT3 mutated and non-mutated cases. There was a significant association between the presence of FLT3 mutations and markedly increased WBC counts and LDH levels compared to their WT FLT3 ( $P < 0.001$ ). Median WBC counts

increased from  $12.5 \times 10^9/L$  in patients without the FLT3 mutation to  $48 \times 10^9/L$  in those with the FLT3 TKD mutation and were highest in patients with FLT3-ITD ( $70.1 \times 10^9/L$ ), which was significant ( $P < 0.001$ ) across the three groups. Pair-wise comparison showed that the difference between patients with no FLT3-ITD mutation and those with FLT3-ITD was statistically significant ( $P < 0.001$ ). In contrast, there was only borderline significance for the patients with and without the FLT3 TKD mutation ( $P = 0.04$ ). The combined ITD and TKD mutations were observed



**Figure 4.** Kaplan-Meier survival curves showing an association of the FLT3 mutation with overall survival (OS) of (A) de novo acute myeloid leukemia (AML) patients, (B) good risk cytogenetic group, (C) patients in the intermediate risk group, and (D) normal karyotype (NK).

**Table 3.** Type and frequency of exon 20 kinase mutations in South Indian adult acute myeloid leukemia (AML) patients.

Nucleotide variation	Amino acid change	Mutated cases (n=12)
835 GAT-TAT	Aspartate (Asp) – Tyrosine (Tyr)/(D835Y)	07 (58.3%)
835 GAT-CAT	Asp – Histidine/(D835H)	04 (33.3%)
835 GAT-GTT	Asp – Valine/(D835V)	01 (8.3%)

in one patient who had a very high WBC count of  $116.2 \times 10^9/L$ . This case also had a very high increase in peripheral blood (88%) and bone marrow blast (96%) compared to the ITD or TKD cases. The pair-wise comparison for peripheral blood and bone marrow blast count between ITD positive and negative cases showed an increased blast percentage for the mutated ( $P=0.003$  and  $P=0.002$ , respectively), but no significant relation was observed for the TKD positive cases. Serum LDH levels were significantly higher in ITD positive and TKD positive patients compared to non-mutated cases ( $P<0.001$ ) across the three groups. Pair-wise comparison was also significant. The clinical characteristics of 275 AML patients with and without the FLT3 mutation are summarized in Table 1. The one case that had both ITD and TKD mutations was not included for analysis and is not shown in Table 1. There was no significant difference with respect to other clinical characteristics such as hemoglobin, platelet count and mutation status.

#### *Distribution of FLT3 mutation in different FAB subtypes*

FLT3 mutations were associated with a wide spectrum of FAB subtypes. The incidence of FLT3 mutations in different FAB subtypes showed a slight predominance in M4 (33%) followed by M1 (28%), M5 (26%), M3 (23.5%) and M2 (16.6%). There was no significant difference in the distribution of FLT3 mutations between Acute Promyelocytic Leukemia (APL) (17%) and non-APL (25%) cases. A statistically significant correlation was not observed for a specific FAB subtype with respect to the FLT3 mutations.

#### *Association of FLT3 mutation with cytogenetic findings*

Among the 276 cases selected for FLT3 mutation analysis, successful cytogenetic data

were available for 254 cases. In the remaining cases analyzable metaphases were not available. Normal karyotype (NK; n=148), favorable group [t(8;21), t(15;17) and inv(16)] (n=57), numerical and other structural abnormalities (n=39), cases with more than three clonal abnormalities that included 10 cases (aberrant karyotype, n=106). In 22 cases the karyotype was unknown. FLT3 gene mutations were associated with a wide spectrum of cytogenetic groups and not equally distributed among the different subtypes. Among the NK group (49/148) 33% of the cases showed an FLT3 mutation compared to 17/106 aberrant karyotype (16%), which showed an increased incidence of mutation in the normal cytogenetic group ( $P=0.002$ ; Table 2). Among aberrant karyotypes, there were 8 cases in the favorable karyotype group, 5 cases in t(15;17), 3 cases in t(8;21), 8 cases in the numerical and other structural abnormality group (including +8, -18, -16, 21q+ etc) and one case in the complex karyotype group. When pair-wise comparison was performed for ITD and TKD with respect to the WT, we observed a significant association with the NK only for ITD.

#### *Sequencing analysis for FLT3-ITD and TKD mutated cases*

We performed sequencing in the ITD mutated cases to detect the length of the duplications, which ranged from 15-107 nucleotides. The majority of the mutations occurred at the JMD (exon 14) of the receptor, but in a few cases the intron was also involved in the duplication. PCR products from all 12 TKD mutated cases were subjected to direct sequencing which showed three types of point mutations in the TKD domain (exon 20); all were confined to codon 835. As seen in Table 3 and Figure 3, these mutations included Asp835Tyr (n=7), Asp835His (n=4) and

Asp835Val (n=1). Ile836 was not affected in any of the mutated cases. The majority of mutation forms comprised the typical asparagine to Tyr change (58.3%).

### *Prognostic significance of FLT3 mutations in Acute Myeloid Leukemia (AML)*

We performed survival analysis in patients with de novo AML who underwent FLT3 mutation analyses. Only patients who had undergone conventional treatment protocols were included (n=186/276). Those who received palliative care were excluded from the survival study. In order to include all cases with the above criteria, we limited survival analysis to 2.5 years. Overall survival of up to 30 months was performed for all patients as a group. Additionally, for survival analysis we divided patients into three cytogenetic subgroups: good-risk [t(8;21), t(15;17), Inv(16)]; intermediate-risk (NK, numerical and other structural abnormalities); and poor risk (>3 clonal abnormalities). There were 186 patients included in the survival analysis. From these, 52 patients were considered to have good risk, 116 were classified as intermediate risk, 2 had with poor risk, and 16 patients were classified into the karyotype unknown group. For stratified analysis, we considered only the good and intermediate prognostic groups.

We separately analyzed patients with ITD and TKD mutations who had undergone treatment in terms of OS, irrespective of the risk group. ITD and TKD mutations were considered together in the survival analysis for the good and intermediate risk group. In the poor risk group, treatment was carried out in only two cases, one of which was positive for ITD, therefore we were unable to perform survival analysis in this group. Survival curves stratified by FLT3 mutation were estimated by Kaplan-Meier plots and compared by the log rank test.

Survival analysis in 186 cases showed significantly worse survival for those with the FLT3 mutation ( $P<0.001$ ) irrespective of the risk group with a mean survival of 15 months versus 21 months for non-mutated cases (Figure 4a). ITD positive cases were found to have a

statistically significant decrease in OS than TKD mutated cases ( $P=0.001$  for ITD vs.  $P=0.171$  for TKD) compared to its non-mutated genotype. No difference was found in median OS between FLT3-mutated and FLT3 WT patients in the good risk group ( $P=0.709$ ; Figure 4b). In the intermediate risk group, survival analysis showed worse survival for the mutated versus wild FLT3 genotype ( $P=0.005$ ) with a mean survival of 14 months for the mutated versus 20 months for the non-mutated cases (Figure 4c). ITD and TKD, when analyzed separately, showed significant results for both in the intermediate risk group with decreased survival ( $P=0.033$  and  $P=0.010$ , respectively). TKD showed a significant association when all cases with FLT3-ITD were excluded ( $P=0.002$ ). Mean survival time for ITD mutated compared to its WT was 15 months versus 20 months. For TKD, cases with the mutation had an 8-month mean survival versus 18 months for non-mutated cases. Survival analysis in the NK showed a significant association of FLT3 mutated cases with decreased OS compared to the wild genotype ( $P=0.057$ ; Figure 4d). Stratified analysis of ITD and TKD separately compared to the wild genotype with no mutation showed a significant association for ITD and OS in the intermediate risk group excluding the NK ( $P=0.006$ ) compared to the NK group alone ( $P=0.113$ ). TKD had a significant association with NK ( $P=0.014$ ) compared to the intermediate risk group excluding NK ( $P=0.062$ ).

### **Discussion**

FLT3 gene mutations are one of the most prevalent somatic alterations in patients with AML.<sup>16,21</sup> In this study we have explored the incidence and type of FLT3 mutations in 276 de novo AML patients. We observed that the JM mutations (exons 14 and 15) were the most common type of alteration (20%) compared to TKD mutations at exon 20 (4%). Combined mutation was observed only in one case (0.3%). Even though the mutation was distributed in all FAB subtypes and in cytogenetic groups, increased incidence was observed for the M4/M5 subtype. An increased prevalence of FLT3 mutation in



APL cases has been observed in different studies.<sup>15,22</sup> Ahamed et al.<sup>27</sup> reported the same result in the Indian population, but in our study we did not observe this association. Incidence of the FLT3 mutation was more in the M4 subtype followed by M1 and M5 subtypes. A larger proportion of mutant FLT3 cases with M4 or M5 subtypes have been reported by Auewarakul et al.<sup>22</sup> FLT3 mutation was not detected in M0 and M6 subtypes. The incidence of FLT3-ITD was lower in the M2 subtype compared to the other subtypes. We did not observe any statistically significant association for the FLT3 mutation with age, gender or with any of the FAB subtypes even though a difference in the frequency distribution was noted. Similar results were observed in other studies.<sup>15,16,23,24</sup>

Reports from Western countries have shown that the incidence of FLT3 mutation varies from 20%-35% of all AML cases, with an increase in the NK group to 40%.<sup>15,21,25,26</sup> Ahmad et al. reported 19.1% of isolated ITD mutation in AML<sup>27</sup> which was similar to our study with 20%. A decreased incidence of ITD was observed in the Korean (13%) and Malay (16.7%) populations.<sup>28,29</sup> The frequency of isolated TKD was 4.7% and that of combined ITD and TKD mutations was 4.2% in a study by Ahmad et al.<sup>27</sup> In the present study TKD was observed in 4% and combined mutations in only 0.3% of the study subjects. Similar reports were available from several studies.<sup>5,16,26,28</sup> Altogether, the FLT3 mutation was detected in 24.3% of AML cases from south India in contrast to 28% in an Indian study, which indicated that FLT3 is an important target of mutational activation in de novo AML patients. A comparison of FLT3 mutational data with clinical parameters revealed significantly elevated WBC counts, high percentages of peripheral blood and bone marrow blasts and elevated serum LDH levels, and a lack of significance with regards to hemoglobin level and platelet count which agreed with previous reports.<sup>15,22,27,30-32</sup>

Based on the cytogenetic analysis, we identified different prognostic groups. In more than 40% of cases chromosomal abnormalities were not

identified (cytogenetically normal) and lacked markers useful for sub-classification and risk assessment. In the present study we correlated the incidence of FLT3 mutation in different cytogenetic subgroups and observed a significant association of FLT3 mutation with NK (33%) compared to those with abnormal karyotype (16%,  $P=0.002$ ). Association of this mutation with NK was also reported by other studies.<sup>22,23,27,33</sup>

Structural alterations of the FLT3 gene were detected by direct sequencing of the mutated sample and showed an internal tandem duplication that varied from 15-107 nucleotides, all of which were in frame with most confined to exon 14. Studies from different countries have reported duplications that ranged from 3 to more than 400 bp.<sup>22,23,34</sup> Ahamed et al.<sup>27</sup> found that the ITD mutation in the Indian population ranged from 3 to 165 nucleotides, all of which were in frame duplications.

Association of FLT3-ITD mutation with poor prognosis in AML patients was reported in various studies.<sup>15,16,35,36</sup> Studies on the TKD mutation showed no effect on the OS pattern of de novo AML patients compared to the wild genotype or the ITD mutant.<sup>5,16,37</sup> We investigated the prognosis of FLT3-ITD and D835 mutations in 186 de novo AML patients and found that FLT3-ITD positive cases showed decreased OS than the TKD mutated cases when compared with its wild genotype ( $P=0.001$  versus  $P=0.171$ ), which was similar to other reports. Hence, FLT3-ITD might be a strong prognostic factor in AML patients. D835 mutations were not prognostically significant in de novo AML patients. However, Schnittger et al.<sup>21</sup> reported that OS did not differ between patients with or without FLT3/ITD in their AML cohort. As the clinical outcome of patients depended largely on the cytogenetic subgroups, a stratified analysis was undertaken in the good risk and intermediate risk group patients. We observed no significant association for the good risk category with respect to the FLT3 mutation but in the intermediate risk group, FLT3 mutations decreased the OS rate ( $P=0.005$ ). Similar results were observed in a study by Santos

et al.<sup>33</sup> The results were significant when the ITD and TKD mutations were analyzed separately compared to its wild genotype. The significant association of TKD observed in our study differed from previous reports. However reports by Moreno et al. and a meta-analysis by Yanada et al. showed significantly worse outcomes in AML patients with the TKD mutation.<sup>38,39</sup> Another study showed an inferior outcome for cases with the TKD mutation in the intermediate cytogenetic subgroup, but no impact on survival in the poor cytogenetic subgroup.<sup>22,33</sup> A significant association was noted for the NK and the FLT3 mutant genotype with a *P*-value of 0.057 which agreed with previous studies.<sup>30,31</sup> ITD and TKD when analyzed independently with the WT genotype (ITD and TKD neg) in NK showed a significant result only for the TKD mutation compared to ITD, whereas ITD was significantly associated with the intermediate risk group with numerical and other structural abnormalities, excluding NK.

In brief, to our knowledge, this is the first report of FLT3-ITD and TKD mutations in relation to de novo AML in the south Indian population. Numerous studies were available that showed the prevalence and prognosis of these mutations in AML. This was the first report from India regarding the prognostic significance of these mutations. The present study revealed the effect of these mutations in single or combination in terms of OS, as well as in different cytogenetic groups. The results showed that ITD was a more significant prognostic marker for AML. ITD itself decreased survival in all cytogenetic subgroups except for the good prognostic group. Interestingly, survival analysis in the NK group showed a decreased survival for the TKD rather than ITD positive cases which differed from other reports. We have identified TKD as an important factor that affects survival of the NK group.

In conclusion our study shows that FLT3 mutations are a common alteration in AML with ITD being the most prevalent mutation. They are associated with a poor prognosis in overall cases. In our study group, FLT3 mutations did not negatively affect prognosis in the good risk group.

We have shown an association in both ITD and TKD with a significantly worse outcome in the intermediate cytogenetic group. The FLT3 mutation, mainly TKD, forms a significant prognostic marker especially in the NK. ITD is important in the intermediate risk group, excluding NK. Thus the presence of FLT3 mutations ITD and TKD can serve as valuable prognostic markers in different subgroups of patients, allowing stratification for risk-directed therapy.

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### Conflict of Interest

No conflict of interest is declared.

### References

1. Mrozek K, Heinonen K, Bloomfield CD. Clinical importance of cytogenetics in acute myeloid leukaemia. *Best Pract Res Clin Haematol.* 2001;14(1):19-47.
2. O'Keefe CL, Tiu R, Gondek LP, Powers J, Theil KS, Kalaycio M, et al. High-resolution genomic arrays facilitate detection of novel cryptic chromosomal lesions in myelodysplastic syndromes. *Exp Hematol.* 2007;35(2):240-51.
3. Bacher U, Haferlach T, Schoch C, Kern W, Schnittger S. Implications of NRAS mutations in AML: a study of 2502 patients. *Blood.* 2006;107(10):3847-53.
4. Nakao M, Yokota S, Iwai T, Kaneko H, Horiike S, Kashima K, et al. Internal tandem duplication of the *flt3* gene found in acute myeloid leukemia. *Leukemia.* 1996;10(12):1911-8.
5. Mizuki M, Fenski R, Halfter H, Matsumura I, Schmidt R, Muller C, et al. *Flt3* mutations from patients with acute myeloid leukemia induce transformation of 32D cells mediated by the Ras and STAT5 pathways. *Blood.* 2000;96(12):3907-14.
6. Gilliland DG, Griffin JD. The roles of FLT3 in hematopoiesis and leukemia. *Blood.* 2002;100(5):1532-42.
7. Bacher U, Haferlach C, Kern W, Haferlach T, Schnittger S. Prognostic relevance of FLT3-TKD mutations in AML: the combination matters--an analysis of 3082 patients. *Blood.* 2008; 111(5):2527-37.

8. Renneville A, Roumier C, Biggio V, Nibourel O, Boissel N, Fenaux P, et al. Cooperating gene mutations in acute myeloid leukemia: a review of the literature. *Leukemia*. 2008;22(5):915-31.
9. Meshinchi S, Alonzo TA, Stirewalt DL, Zwaan M, Zimmerman M, Reinhardt D, et al. Clinical implications of FLT3 mutations in pediatric AML. *Blood*. 2006;108(12):3654-61.
10. Beitinjaneh A, Jang S, Roukoz H, Majhail NS. Prognostic significance of FLT3 internal tandem duplication and tyrosine kinase domain mutations in acute promyelocytic leukemia: a systematic review. *Leuk Res*. 2010;34(7):831-6.
11. Yokota S, Kiyoi H, Nakao M, Iwai T, Misawa S, Okuda T, et al. Internal tandem duplication of the FLT3 gene is preferentially seen in acute myeloid leukemia and myelodysplastic syndrome among various hematological malignancies. A study on a large series of patients and cell lines. *Leukemia*. 1997;11(10):1605-9.
12. Nakao M, Janssen JW, Erz D, Seriu T, Bartram CR. Tandem duplication of the FLT3 gene in acute lymphoblastic leukemia: a marker for the monitoring of minimal residual disease. *Leukemia*. 2000;14(3):522-4.
13. Xu B, Tian H, Zhou SY. Detection of FLT3 gene and FLT3/ITD gene mutation in chronic myeloid leukemia and its significance (Article in Chinese). *Ai Zheng*. 2004;23:1218-21.
14. Ishii E, Zaitzu M, Ihara K, Hara T, Miyazaki S. High expression but no internal tandem duplication of FLT3 in normal hematopoietic cells. *Pediatr Hematol Oncol*. 1999;16(5):437-41.
15. Kottaridis PD, Gale RE, Frew ME, Harrison G, Langabeer SE, Belton AA, et al. The presence of a FLT3 internal tandem duplication in patients with acute myeloid leukemia (AML) adds important prognostic information to cytogenetic risk group and response to the first cycle of chemotherapy: analysis of 854 patients from the United Kingdom Medical Research Council AML 10 and 12 trials. *Blood*. 2001;98(6):1752-9.
16. Yamamoto Y, Kiyoi H, Nakano Y, Suzuki R, Kodera Y, Miyawaki S, et al. Activating mutation of D835 within the activation loop of FLT3 in human hematologic malignancies. *Blood*. 2001;97(8):2434-9.
17. Kottaridis PD, Gale RE, Langabeer SE, Frew ME, Bowen DT, Linch DC. Studies of FLT3 mutations in paired presentation and relapse samples from patients with acute myeloid leukemia: implications for the role of FLT3 mutations in leukemogenesis, minimal residual disease detection, and possible therapy with FLT3 inhibitors. *Blood*. 2002;100(7):2393-8.
18. Henegariu O, Heerema N.A, Lowe Wright, Bray-Ward P, Ward D.C, Vance G.H, et al. Improvements in cytogenetic slide preparation: controlled chromosome spreading, chemical aging and gradual denaturing. *Cytometry*. 2001;43:101-9.
19. Shaffer LG, Tommerup N. Karyotype Designation. In: Shaffer LG, Tommerup N, editors. *An International System for Human Cytogenetic Nomenclature (2005)*. Korea, Karger Medical and Scientific Publishers, 2005: 39- 48.
20. Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, et al. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. *Blood*. 1999;93(9):3074-80.
21. Schnittger S, Schoch C, Dugas M, Kern W, Staib P, Wuchter C, et al. Analysis of FLT3 length mutations in 1003 patients with acute myeloid leukemia: correlation to cytogenetics, FAB subtype, and prognosis in the AMLCG study and usefulness as a marker for the detection of minimal residual disease. *Blood*. 2002;100(1):59-66.
22. Auewarakul CU, Sritana N, Limwongse C, Thongnoppakhun W, Yenchitsomanus PT. Mutations of the FLT3 gene in adult acute myeloid leukemia: determination of incidence and identification of a novel mutation in a Thai population. *Cancer Genet Cytogenet*. 2005;162(2):127-34.
23. Thiede C, Steudel C, Mohr B, Schaich M, Schakel U, Platzbecker U, et al. Analysis of FLT3-activating mutations in 979 patients with acute myelogenous leukemia: association with FAB subtypes and identification of subgroups with poor prognosis. *Blood*. 2002;99(12):4326-35.
24. Colovic N, Tosic N, Aveic S, Djuric M, Milic N, Bumbasirevic V, et al. Importance of early detection and follow-up of FLT3 mutations in patients with acute myeloid leukemia. *Ann Hematol*. 2007; 86(10):741-7.
25. Kiyoi H, Naoe T, Yokota S, Nakao M, Minami S, Kuriyama K, et al. Internal tandem duplication of FLT3 associated with leukocytosis in acute promyelocytic leukemia. Leukemia Study Group of the Ministry of Health and Welfare (Kohseisho). *Leukemia*. 1997;11(9):1447-52.
26. Liang DC, Shih LY, Hung IJ, Yang CP, Chen SH, Jaing TH, et al. FLT3-TKD mutation in childhood acute myeloid leukemia. *Leukemia*. 2003;17(5):883-6.
27. Ahmad F, Mandava S, Das BR. Analysis of FLT3-ITD and FLT3-Asp835 mutations in de novo acute myeloid leukemia: evaluation of incidence, distribution pattern, correlation with cytogenetics and characterization of internal tandem duplication from Indian population. *Cancer Invest*. 2010;28(1):63-73.
28. Bang SM, Ahn JY, Park J, Park SH, Park J, Cho EK, et al. Low frequency and variability of FLT3 mutations in Korean patients with acute myeloid leukemia. *J Korean Med Sci*. 2008;23(5):833-7.
29. Abdullah NAF, Hassan R, Abdullah AD, Yong AC, Ghazali S, Sulong S, et al. Low incidence of FLT3 gene mutation among Malay patients with Acute Myeloid

- Leukemia: A preliminary study. *As Pac J Mol Biol Biotechnol.* 2011;19:57-62.
30. Frohling S, Schlenk RF, Breitruck J, Benner A, Kreitmeier S, Tobis K, et al. Prognostic significance of activating FLT3 mutations in younger adults (16 to 60 years) with acute myeloid leukemia and normal cytogenetics: a study of the AML Study Group Ulm. *Blood.* 2002;100(13):4372-80.
  31. Wang L1, Xu WL, Meng HT, Qian WB, Mai WY, Tong HY, et al. FLT3 and NPM1 mutations in Chinese patients with acute myeloid leukemia and normal cytogenetics. *J Zhejiang Univ Sci B.* 2010;11(10):762-70.
  32. Ishfaq M, Malik A, Faiz M, Sheikh I, Asif M, Khan MN, et al. Molecular characterization of FLT3 mutations in acute leukemia patients in Pakistan. *Asian Pac J Cancer Prev.* 2012;13(9):4581-5.
  33. Santos FP, Jones D, Qiao W, Cortes JE, Ravandi F, Estey EE, et al. Prognostic value of FLT3 mutations among different cytogenetic subgroups in acute myeloid leukemia. *Cancer.* 2011;117(10):2145-55.
  34. Tan AY, Westerman DA, Carney DA, Seymour JF, Juneja S, Dobrovic A. Detection of NPM1 exon 12 mutations and F. *J Hematol Oncol.* 2008;1:10.
  35. Abu-Duhier FM, Goodeve AC, Wilson GA, Gari MA, Peake IR, Rees DC, et al. FLT3 internal tandem duplication mutations in adult acute myeloid leukaemia define a high-risk group. *Br J Haematol.* 2000; 111(1):190-5.
  36. Rombouts WJ, Blokland I, Lowenberg B, Ploemacher RE. Biological characteristics and prognosis of adult acute myeloid leukemia with internal tandem duplications in the Flt3 gene. *Leukemia.* 2000; 14(4):675-83.
  37. Sheikhha MH, Awan A, Tobal K, Liu Yin JA. Prognostic significance of FLT3-ITD and D835 mutations in AML patients. *Hematol J.* 2003;4(1):41-6.
  38. Moreno I, Martin G, Bolufer P, Barragan E, Rueda E, Roman J, et al. Incidence and prognostic value of FLT3 internal tandem duplication and D835 mutations in acute myeloid leukemia. *Haematologica.* 2003; 88(1):19-24.
  39. Yanada M, Matsuo K, Suzuki T, Kiyoi H, Naoe T. Prognostic significance of FLT3 internal tandem duplication and tyrosine kinase domain mutations for acute myeloid leukemia: a meta-analysis. *Leukemia.* 2005;19(8):1345-9.