

## High fms-like tyrosine kinase-3 (FLT3) receptor surface expression predicts poor outcome in *FLT3* internal tandem duplication (ITD) negative patients in adult acute myeloid leukaemia: a prospective pilot study from India

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**Background & objectives:** Mutations in fms-like tyrosine kinase 3 (*FLT3*) receptor have significant role in assessing outcome in patients with acute myeloid leukaemia (AML). Data for *FLT3* surface expression in relation to *FLT3* internal tandem duplication (ITD) status and outcome are not available from India. The objective of the current study was to investigate adult patients with AML for *FLT3* expression and *FLT3* ITD mutation, and their association with long-term outcome.

**Methods:** Total 51 consecutive *de novo* AML patients aged 18-60 yr were enrolled in the study. *FLT3* ITD was detected by polymerase chain reaction (PCR); flowcytometry and qPCR (Taqman probe chemistry) were used for assessment of *FLT3* protein and transcript, respectively. Kaplan Meier curves were obtained for survival analysis followed by log rank test.

**Results:** *FLT3* ITD was present in eight (16%) patients. Complete remission was achieved in 33 (64.6%) patients. At 57.3 months, event free survival (EFS) was 26.9±6.3 per cent, disease free survival (DFS) 52.0±9.2 per cent, and overall survival event (OS) 34.5±7.4 per cent. *FLT3* surface expression was positive (>20%) by flow-cytometry in 38 (88%) of the 51 patients. *FLT3* surface expression and transcripts were not associated with *FLT3* ITD status. *FLT3* expression was significantly associated with inferior EFS ( $P=0.026$ ) and OS ( $P=0.018$ ) in those who were negative for *FLT3* ITD.

**Interpretation & conclusions:** This study evaluated *FLT3* ITD mutation along with *FLT3* expression in AML patients, and associated with survival. Negative impact of *FLT3* surface expression on survival was observed in AML patients who were *FLT3* ITD negative.

**Key words** Acute myeloid leukaemia - *FLT3* ITD - flowcytometry - PCR - prognosis

The fms-like tyrosine kinase 3 (FLT3) is a type III receptor tyrosine kinase (RTK) expressed on the surface of haematopoietic stem cells and plays vital role in normal haematopoiesis. *FLT3* internal tandem duplication (ITD) is associated with increased

transcript level of *FLT3* in adult acute myeloid leukaemia (AML)<sup>1,2</sup>.

*FLT3* expression elevates proliferation in myeloblasts and mitigates their apoptosis, whereas the relationship between the signal cascades through *FLT3*

is unclear in leukemogenesis. *FLT3* ITD mutations have proven association with inferior survival outcome and are, therefore, applied in risk stratification<sup>3</sup>. A few studies from Indian subcontinent have shown that *FLT3*-ITD is observed in 7-25 per cent of AML patients<sup>4-8</sup>. However, data with regard to *FLT3* surface expression in AML patients in relation to *FLT3* ITD status and survival outcome from India are lacking.

In the current study, we prospectively evaluated *FLT3* ITD mutation and *FLT3* expression by flowcytometry and real time PCR in adult patients with AML, and correlated the same with the outcome.

### Material & Methods

Total 51 consecutive *de novo* AML patients > 18 and < 60 yr of age (inclusion criteria) were prospectively enrolled from the department of Medical Oncology at Dr. B. R. A. Institute Rotary Cancer Hospital at All India Institute of Medical Sciences, New Delhi, India, from March 2008 to June 2010. Acute promyelocytic leukaemia patient were excluded. The study was approved by the Institute Ethics committee, and an informed written consent was taken prior to sampling. Five ml peripheral blood (N=32 patients, if peripheral blast count was more than 30%) or otherwise 5 ml bone marrow (N=19 patients) was collected in EDTA coated sterile vacutainer. AML was classified into good, intermediate and poor risk cytogenetic groups as per standard criteria<sup>9</sup>.

Induction therapy consisted of daunorubicin 60 mg/m<sup>2</sup>/days for three days with cytarabine 100 mg/m<sup>2</sup>/day for seven days; patients who were not in remission post first induction received cytarabine 100 mg/m<sup>2</sup> slow intravenous push twice daily for 10 days, daunorubicin 50 mg/m<sup>2</sup>/day for three days and etoposide 100 mg/m<sup>2</sup>/day for five days. Patients who were not in complete remission (CR) after second induction were declared refractory. Post CR, patients received three cycles of cytarabine at 18 g/m<sup>2</sup>.

*FLT3* ITD mutation detection: *FLT3* ITD was screened by amplification of the mutated gene by PCR using specific primers. DNA was isolated from 5×10<sup>6</sup> cells using DNeasy kit (QIAGEN, Crawley, UK). The quality and quantity of DNA were analyzed by NanoDrop 1000 (Thermo scientific, USA). Total 150 ng DNA was used for *FLT3* internal tandem duplication was detected by PCR using the following primers<sup>5</sup>: Forward -5'-GTAAAACGA

CGGCCAGTGCAGAACTGCCTATTCCTA-3' and Reverse-5'-CAGGAAACAGCTATGACCTGTCCTTCCACTATACTGT- 3' with 35 cycles and annealing temperature 53°C. Finally, the PCR product was resolved on 3.5 per cent agarose gel.

*Flow cytometry*: Mononuclear cells (MNCs) separated (using density gradient media) from peripheral blood/ bone marrow were evaluated for *FLT3* surface expression (CD135) on dim CD45 positive cells. Total 20,000 events were acquired and analyzed using FACS Calibur flow-cytometer and cell quest pro software (Becton Dickinson, San Jose, USA).

*Real-time polymerase chain reaction (qPCR)*: Total RNA was isolated from 10 million MNCs using TRIzol method<sup>5</sup>. RNA was evaluated for quality and quantity by spectrophotometry. cDNA was synthesized from 1 µg aliquots of total RNA in a 20 µl standard reaction mixture using reverse transcription (RT) kit (Roche Applied Sciences, Mannheim, Germany). Absolute quantification of *FLT3* transcripts was done by qPCR using TaqMan probe chemistry (Light Cycler 2.0). Standard was prepared using cloned quantified plasmids of *FLT3* amplicon (Roche Applied Sciences, Mannheim, Germany). Different concentrations ranging from 10<sup>2</sup>-10<sup>8</sup> copies of plasmid were prepared using serial dilution and four different concentrations were used in duplicate for standard curve preparation. Standards with known copies of plasmid were used in all experiments for normalization and quantification. Beta actin was used as housekeeping gene.

*Statistical analysis*: Data were expressed as median (range) and mean ± SD. The end point for the survival data was December 31, 2012. The differences between the values were determined by using Kruskal Wallis test and independent Student's t test, respectively. Median values of the quantitative variables were used as a cut-off point for categorization into high or low expression. Outcome analysis was assessed based on event free survival (EFS), disease free survival (DFS) and overall survival (OS). EFS was defined as time between the diagnosis and the first event such as failure to achieve complete remission, relapse or death. DFS was defined as the time from CR until relapse or the date of last follow up. OS was defined as time between the diagnosis and the death or the last follow up. Kaplan Meier curves were obtained for survival analysis followed by log rank test. All statistical analysis was done using STATA 11.0 (Stata Corp LP, Texas, USA).

**Table I.** Relationship of baseline characteristics of patients with *FLT3* ITD mutation

A. <i>FLT3</i> expression with <i>FLT3</i> ITD mutation			
Variables	<i>FLT3</i> ITD positive (8)	<i>FLT3</i> ITD negative (43)	<i>P</i> value
<i>FLT3</i> (>20% ; CD 135 Positive)	5 (62.5%)	38 (88%)	0.09
CD135 (mean $\pm$ SD)	47.23 $\pm$ 31.99	64.58 $\pm$ 32.07	0.162
<i>FLT3</i> transcripts (mean $\pm$ SD)	75.9 x 10 <sup>6</sup> $\pm$ 124.6 x10 <sup>6</sup>	76.9 x 10 <sup>6</sup> $\pm$ 143.8 x 10 <sup>6</sup> (N=40)	0.985
B. Relationship of baseline patients characteristics with <i>FLT3</i> ITD mutation			
Median Age in years (range)	33 (19-52)	35 (19-59)	0.547
Sex (Male: Female)	1.6: 1	1.3:1	0.877
Median Hemoglobin (g/dl) (range)	7.7 (3.3 - 10.0)	7.7 (2.0 - 12.2)	0.541
Median WBC (per $\mu$ l) (range)	67150 (1000-257000)	19700 (1000-685000)	0.165
Median platelets (per $\mu$ l) (range)	24500 (17000-124000)	37000 (10000-266000)	0.471
Cytogenetics (n=37) <sup>†</sup>			
Good risk	2 (40%)	4 (12%)	0.293
Intermediate	3 (60.0%)	23 (72%)	
Poor risk	0	5 (16%)	
FAB AML subtype			
M0	1 (12%)	1 (2%)	0.910
M1	-	5 (12%)	
M2	6 (76%)	25 (58%)	
M4	-	6 (14%)	
M5	1 (12%)	4 (9%)	
M7	-	2 (5%)	
C. Survival outcome of the patients in relation to <i>FLT3</i> ITD mutation			
CR	4 (50%)	29 (67%)	0.287
EFS* $\pm$ S.E (CI) (%)	25.0 $\pm$ 15.3 (3.7 - 55.8)	27.2 $\pm$ 6.9 (14.8 - 41.1)	0.570
DFS* $\pm$ S.E (CI) (%)	50.0 $\pm$ 25.0 (5.78 - 84.4)	52.08 $\pm$ 9.9 (31.32 - 69.31)	0.816
OS* $\pm$ S.E (CI) (%)	37.5 $\pm$ 8.24 (18.95 - 50.19)	39.70 $\pm$ 7.12 (25.88 - 53.19)	0.738
Median EFS (months)	3.9 months	7.1	
Median DFS (months)	10.9 months	-	
Median OS (months)	13.3 months	19	
*Survival at 57.3 months. WBC, white blood cells; FAB, French-American-British; <i>FLT3</i> ITD, <i>FMS</i> like tyrosine kinase 3 internal tandem duplication; CR, complete remission; EFS, event free survival; DFS, disease free survival; OS; Overall survival ; S.E, standard error; C. I., 95% confidence interval			
<sup>†</sup> Cytogenetics data were not available in 14 patients			

## Results

*FLT3* ITD was present in eight (16%) patients. None of the baseline patients characteristics was associated with *FLT3* ITD. The median WBC count in

*FLT3* ITD positive patients was three times higher as compared to patients whose blasts were negative for *FLT3* ITD (Table IB). CR was achieved in 33 (64.6%) patients. For all 51 patients at 57.3 months, EFS was

**Table II.** Detailed presentation of patients with *FLT3* ITD mutation (N=8)

S. No	Median <i>FLT3</i> expression (High/Low)*	Age (yr)	Cytogenetics risk group	Remission	EFS (Months)	DFS (Months)	OS (Months)	Status
1	52.88 (low expression)	35	Not available	No	1.1	-	13.3	Died (Refractory)
2	87.32 (High expression)	46	Intermediate risk	No	1.4	-	4.4	Died (Refractory)
3	87.09 (High expression)	35	Good risk	No	0.8	-	0.8	Died (Refractory)
4	12.15 (low expression)	52	Not available	Yes	43.6	41.9	43.6	CR
5	10.43 (low expression)	24	Intermediate risk	Yes	42.8	41.5	42.8	CR
6	13.42 (low expression)	19	Not available	No	0.7	-	0.7	Died (Refractory)
7	55.69 (low expression)	31	Good risk	Yes	12.3	10.9	15.5	Relapse LFU#
8	58.91 (low expression)	21	Intermediate risk	Yes	3.9	0.9	5.1	Died (Relapsed)

\*Median value of entire group (71.06) was used as cut-off for high and low expression of *FLT3*; #LFU lost to follow up  
EFS, event free survival; DFS, disease free survival; OS, overall survival; CR, complete remission

26.9±6.3 per cent, DFS 52.0±9.2 per cent, and OS 34.5±7.4 per cent. *FLT3* ITD status did not predict EFS, DFS and OS (Table IC).

*FLT3* expression in relation to *FLT3* ITD status and outcome: *FLT3* surface expression was positive (>20% expression) by flow-cytometry in 38 (88%) *FLT3* ITD negative patients and median expression of the total patients was 71.0 per cent (range: 3.63-99.66%). *FLT3* surface expression and transcripts were not associated with *FLT3* ITD status (Table IA).

*FLT3* ITD positive and negative patients were grouped according to *FLT3* surface expression above and below median. In *FLT3* ITD positive subgroup high *FLT3* expression did not predict survival (Fig. 1A-C) (Table II). In *FLT3* ITD negative subgroup, *FLT3* expression was not associated with cytogenetic risk group (Table IIIA); however, high *FLT3* expression was significantly associated with inferior EFS ( $P=0.026$ ) and OS ( $P=0.018$ ) (Table IIIB); Fig. 1D-F).

It was observed that patients who were negative for *FLT3* ITD and had low *FLT3* expression had a trend towards improved OS ( $p=0.058$ ) when compared with *FLT3* ITD negative and high *FLT3* expression and those with patients who were positive for *FLT3* ITD (Fig. 2A-C).

### Discussion

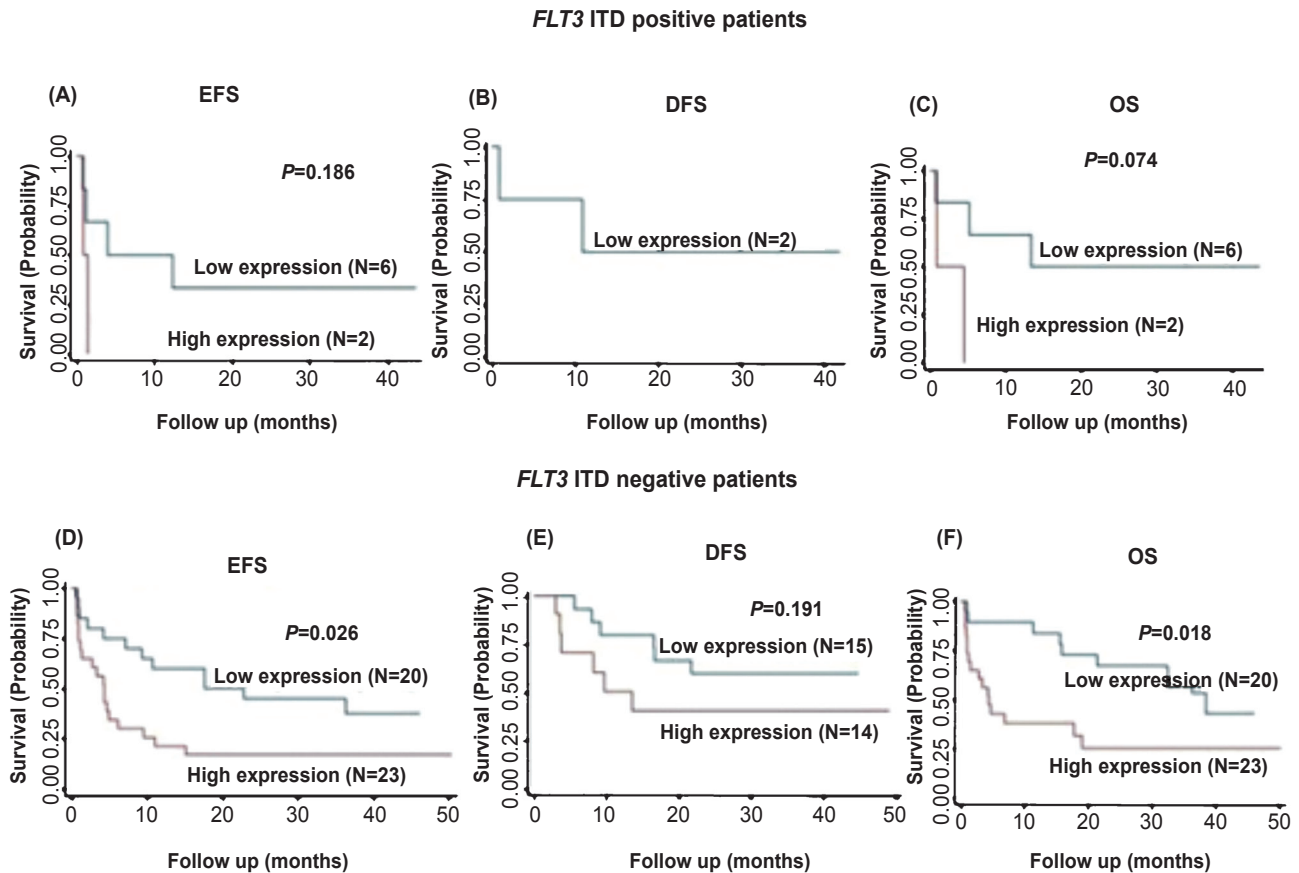
In AML, incidence of *FLT3* ITD has been reported to vary from 15-35 per cent in western countries<sup>10,11</sup>, whereas the studies from India analyzing AML patients showed 7-25 per cent incidence of *FLT3*-ITD

mutation<sup>5,6</sup> as compared to 16 per cent positivity in the current study. Although we could not identify any baseline characteristic associated with *FLT3* ITD, it is noteworthy that those with *FLT3* ITD had a white blood cell count which was three times that of patients who were negative for *FLT3* ITD. We have previously shown that high WBC count at presentation is independently associated with poor outcome in AML<sup>4</sup>.

*FLT3* ITD mutation is involved in autophosphorylation of tyrosine domain, which results in enhanced proliferation of myeloblasts. In the present study *FLT3*-ITD mutation were evaluated along with *FLT3* transcript and surface expression in AML along with outcome. *FLT3* expression was similar whether patient was *FLT3* ITD positive or negative. Impact of *FLT3* ITD surface expression on survival was observed only in *FLT3* ITD negative patients. Clinical trials using RTK inhibitors showed promising results in AML by downregulating expression of *FLT3*<sup>12-15</sup>.

We could not demonstrate a difference in survival with respect to the presence or absence of *FLT3* ITD. Our study was limited by the fact that sample size was small, and associated mutations in *FLT3* tyrosine kinase domain and nucleophosmin which could have altered the differences in survival, were not evaluated as part of this study.

In conclusion, our study evaluated *FLT3* expression and *FLT3* ITD mutation in adult AML patients. High *FLT3* expression was associated with inferior survival outcome in *FLT3* ITD negative patients.



**Fig. 1.** High *FLT3* expression in *FLT3* ITD positive AML patients did not predict survival (A-C). In *FLT3* ITD negative AML patients high *FLT3* surface expression was significantly associated with inferior EFS [ $17.39 \pm 7.90$  (5.44 - 34.95) vs  $37.50 \pm 11.52$  (16.32 - 58.82) ( $P=0.026$ )] (D) and inferior overall survival (OS) [ $25.51 \pm 10.07$  (9.01-46.04) vs  $42.70 \pm 13.77$  (16.72-66.71) ( $P=0.018$ )] (F), but had no effect on disease free survival (DFS) (E). Median values of *FLT3* surface expression (percentage) were used as cut off point for categorization into high or low expression.

**Table IIIA.** Cytogenetic risk group of patients with high and low expression of *FLT3* in *FLT3* ITD negative patients (N=43)

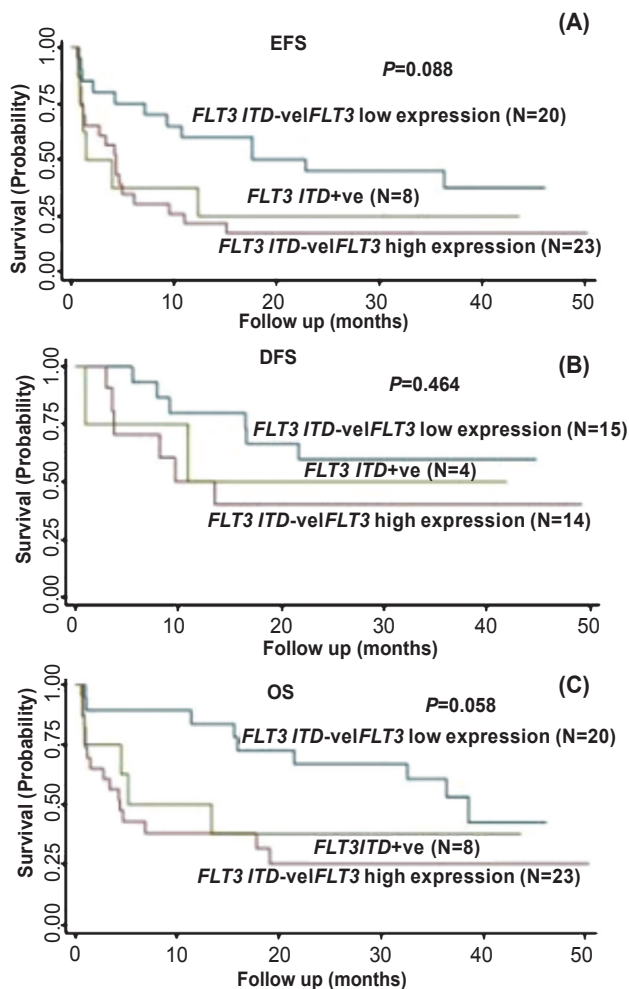
	Low <i>FLT3</i> expression (n=20) N (%)	High <i>FLT3</i> expression (n=23) N (%)	<i>P</i> value
Cytogenetics available	15 (75)	17 (73.9)	0.735
Good risk group	1 (7)	3 (18)	
Intermediate risk group	11 (73)	12 (70)	
Poor risk group	3 (20)	2 (12)	

**Table IIIB.** Survival outcome

	Low <i>FLT3</i> expression (n=20)	High <i>FLT3</i> expression (n=23)	<i>P</i> value
CR	15 (75)	14 (61)	0.324
EFS* $\pm$ S.E (CI) (%)	$37.5 \pm 11.5$ (16.3 - 58.8)	$17.3 \pm 7.9$ (5.4 - 34.9)	0.026
DFS* $\pm$ S.E (CI) <sup>#</sup> (%)	$60.0 \pm 12.6$ (31.75 - 79.65)	$40.4 \pm 15.5$ (12.46 - 67.41)	0.191
OS* $\pm$ S.E (CI) <sup>€</sup> (%)	$42.7 \pm 13.7$ (16.75 - 66.7)	$25.5 \pm 10.07$ (9.1 - 46.04)	0.018

\*Survival at 57.3 months. WBC, white blood cells; FAB, French-American-British; *FLT3* ITD, *FMS* like tyrosine kinase 3 internal tandem duplication; CR, complete remission; EFS, event free survival; DFS, disease free survival; OS, overall survival; CI, 95% confidence interval





**Fig. 2.** Survival of patients with *FLT3* ITD positive vs *FLT3* ITD negative and high *FLT3* expression vs *FLT3* ITD negative and low *FLT3* expression: [event free survival, EFS ( $P=0.088$ ) (A), disease free survival, DFS ( $P=0.464$ ) (B), and overall survival, OS ( $P=0.058$ ) (C)]. Median values of *FLT3* surface expression (percentage) were used as cut-off point for categorization into high or low expression.

**Conflicts of Interest:** None.

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