Comprehensive FLT3 Analysis in Indian Acute Myeloid Leukaemia

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Abstract

Aim: Activating mutations of FLT3 are commonly found in AML patients and reported to be associated with poor clinical outcome. We aimed to evaluate the incidence of FLT3 mutations along with FLT3 mRNA and CD135 protein expression in AML patients of western India and their role in prognosis of disease.

Method: Analysis for the detection of FLT3 internal tandem duplication (ITD), Tyrosine kinase domain (TKD) point mutations and quantification of mRNA level was carried out in total 174 de novo patients diagnosed with acute myeloid leukemia (AML), myelodysplastic syndrome (MDS) and aplastic anemia using PCR and RT-PCR methods. FLT3 protein was quantified by flow cytometry on leukemic blasts.

Results: The incidence of FLT3 ITD, FLT3 TKD mutations and CD135 protein expression was found to be 19%, 7% and 62% respectively in AML patients. In MDS, only FLT3 ITD mutation and CD135 protein over expression could be analyzed, incidence of which was 22% and 60%. In aplastic anemia, FLT3 mutations, FLT3 mRNA and protein over expression were not detected. FLT3 mutations as well as FLT3 mRNA and protein over expression were prominently noted in AML subtypes associated with myelo-monocytic lineage. CD135 protein over expression was significantly associated with reduced Disease Free Survival (DFS) whereas WBC and blasts emerged as poor prognostic factors with respect to Disease Free Survival (DFS) and Overall Survival (OS) respectively in multivariate analysis.

Conclusion: Our data suggest that CD135 receptor protein over expression is a potential prognostic marker as well as molecular target for FLT3 inhibitors in AML patients.

Keywords: FLT3 ITD; FLT3 TKD mutation; FLT3 mRNA; CD135 protein; AML

Introduction

Fms-like tyrosine kinase 3 (FLT3), also known as stem cell tyrosine kinase 1 (STK1) [1] or fetal liver kinase 2 (flk1) [2], belongs to the group of class III receptor tyrosine kinases along with structurally similar growth receptors such as c-kit, PDGF-R and c-fms [3]. In normal bone marrow, the expression of FLT3 receptor is confined to early hematopoietic progenitor cells [4,5] and mediates stem cell proliferation and differentiation [6]. In normal hematopoiesis, FLT3 ligand binding to the FLT3 receptor causes dimerization of the receptor resulting in the subsequent autophosphorylation, activation of the receptor and induction of multiple intracellular signaling pathways [5].

FLT3 gene is localized on chromosome 13q12 and consists of 24 exons [7]. Internal tandem duplication (ITD) in the juxtamembrane domain – coding sequence, in exons 14 and 15 of FLT3 gene [5], was first identified by Nakao et al. in 1996 [4]. The length of these mutations varies but they are always in frame, thus producing a functional protein [8]. Such insertions leads to FLT3 ligand-independent kinase activation, stimulating proliferation and inhibition of apoptosis and in vitro studies particularly show proliferation of Acute Myeloid Leukemia (AML) cells [9-11]. This mutation is found in approximately 20 – 30% of adult AML patients with lower incidence of 5 – 22% in pediatric AML patients [5,10]. In addition to FLT3 ITD, mis-sense point mutation of D835 in the tyrosine kinase domain (TKD)-coding sequence in exon 20 of FLT3 gene has been identified that also leads to constitutive tyrosine phosphorylation [12,13]. These point mutations have been reported in 7% of AML patients [12,13].

Various study groups including few Indian studies have reported the association of activating mutations of FLT3 ITD, with the poor clinical outcome in AML patients [5,14-18], with only few studies reporting absence of association of FLT3 ITD with disease prognosis [19,20].

Studies on FLT3 TKD point mutations show varying results, with few suggesting the role of FLT3 TKD in poor prognosis of AML patients [16,21] while, of few one suggest good prognosis in AML [22]. Very few studies have evaluated the possible association among FLT3 mutations, RNA expression and protein expression [19,23-24]. In our initial study, clinical relevance of FLT3 receptor protein on intact leukemic blasts by flow cytometry was evaluated in Acute Leukemia and found frequent over expression of FLT3 protein in Acute Myeloid Leukemia and B-Acute Lymphoid Leukemia [25]. Further, in a small series of AML, the incidence of protein expression higher than FLT3 mutations was seen. In the present study, we evaluated FLT3 ITD and FLT3 TKD mutations, FLT3 mRNA and correlated with surface FLT3 receptor protein (CD135) in de novo AML, Myelodysplastic Syndrome and Aplastic Anemia. Further, its correlation was evaluated with clinical and haematologic parameters, disease status and treatment offered in AML.

Material and Method

Patients

In this study, 174 patients with de novo AML (N=144; AMLM1 [N=27], AMLM2 [N=36], AMLM3 [N=12], AMLM4 [N=18], AMLM5a

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[N=17], AMLM5b [N=14], AMLM6 [N=2], AMLM7 [N=1], unclassified [N=17]), Myelodysplastic Syndrome (MDS, N=9; RARS [N=1], RAEB-II [N=4] unclassified [N=4]), and Aplastic Anemia [N=21] who were referred to Gujarat Cancer and Research Institute for diagnosis and treatment from 2009 to 2011 were enrolled in the study. Bone marrow (BM) or peripheral blood (PB) samples were collected in ethylene-diamine tetraacetic acid (EDTA) vacutette (BD Vacutainer K2 EDTA, BD Franklin Lakes, NJ, USA) at the time of diagnosis from acute leukemia patients as well as from patients with pancytopenia or anemia. Total BM and PB samples obtained from AML were 74 and 70, from MDS, 6 and 3 and from Aplastic anemia patients, 2 and 19, respectively. Follow up samples from 19 patients were available post induction therapy [BM (N=67) and PB (N=12)] where 6/19 patients showed relapse and 13/19 patients showed remission.

The diagnosis and classification of the acute leukemia samples were based on morphology and cytochemistry according to the French-American-British (FAB) classification and on the presence of maturation and differentiation of antigens as determined by routine immunophenotyping criteria of the European Group for the Immunological Characterization of Leukemias [26]. The patients’ detailed clinical history (age, gender, hematological findings, treatment given, etc.) were noted from the case files maintained at the Medical Record Department of the Institute. This study was approved by the Institutional Scientific Review Board and Ethics Committee. Patients provided informed consent to use their sample for the study.

**Therapy**

Out of 144 AML patients enrolled in the study, 76 patients underwent treatment out of which 57 were adults and 19 were pediatric patients. Total follow up period was in the range of 1 to 25 months. Mean (±Standard Error) and median Disease Free Survival (DFS) were 4.3 (±0.73) and 0.75 months while mean and median Overall Survival (OS) was 5.39 (±0.69) and 2.50 months respectively. Treatment protocol was decided by the clinicians of our Institute. Treatment protocol for AML patients were 7+3 protocol, Berlin Frankfurt Munster (BFM) 93, All Trans Retinoic Acid (ATRA) and / or Arsenic trioxide in combination with anthracycline and Palliative chemotherapy. In 7+3 protocol, induction was given with daunorubicin for 3 days and cytarabine arabinoside for 7 days, followed by consolidation with cytarabine arabinoside for 5 days every month for 3 months. In BFM 93 protocol, induction was given with Ara-C, daunorubicin and etoposide for 8 days, followed by consolidation with thioguanine, prednisolone, vincristine, doxorubicin, Ara-C, and cyclophosphamide for 6 weeks in low-risk patients and block of Ara-C and mitoxantrone in high risk patients, followed by an intensification block with Ara-C and etoposide for 5 days, followed by cranial irradiation and maintenance with thioguanine, and pulses of Ara-C, for 18 months. In AML M3 FAB subtype, induction was given with daunorubicin for 3 days along with All-trans Retinoic acid (ATRA) / Arsenic trioxide. For consolidation, two blocks each of 21 days including daunorubicin for 3 days with ATRA for two weeks or with Arsenic trioxide, followed by maintenance with 6MP continuous daily, MTX weekly along with ATRA for 15 days every 3 months or with Arsenic trioxide. Palliative chemotherapy included oral chemotherapy drugs such as - Thioguanine (6TG), 6-Mercaptopurine (6MP) and Hydroxyurea or their combinations along with prednisolone and supportive care.

**Detection of FLT3 ITD mutation by Polymerase chain reaction (PCR)**

Deoxyribonucleic acid (DNA) was extracted from fresh BM or PB samples with QIAamp DNA mini kit (Qiagen, Germany) according to manufacturer's protocol. For the study of FLT3 ITD mutation, Exons 14 and 15 of FLT3 gene were amplified from genomic DNA using mutation specific primers, Exon 14 Forward (F) 5’- tgtaaacagcgcctcgacatatgtgagaagc-3’ and Exon 15 Reverse (R) 5’- gaggagacagttgaccttcagctttggtggaggccacac-3’ (Bangalore Genei, India). β-Actin served as internal control, amplified with following primer sequences F5’- cgcctgctgcgtggagttgc-3’ and R 5’- cccttcgatcctgggca-3’ (Bangalore Genei, India) and exhibited amplified product of 350 base pair (bp) on agarose gel electrophoresis. Approximately 100ng of genomic DNA was added to PCR mix containing 5 µl (10X) PCR buffer, 10 µl (5X) Q solution, 2 µl 25 mM MgCl2, 1.5 µl dNTP (10mM each) and 0.5 µl Taq DNA polymerase of PCR core kit (Qiagen, Germany) and 5 µl (0.8 µM final concentration) each of forward as well as reverse primers, to make a final volume of 50 µl. PCR was carried out in Mastercycler gradient (Eppendorf, Germany) with conditions consisting of initial denaturation step at 95°C for 2 minutes followed by 40 cycles each of denaturation at 95°C for 30 seconds, annealing at 70°C for 1 minute and extension at 72°C for 1 minute and final extension step at 72°C for 10 minutes. Amplified products were resolved on 2% agarose gel stained with ethidium bromide and visualized on Gel Documentation System (Alpha Innotech Corp., USA). An amplified product of wild type FLT3 was obtained at 366 bp. Additional band longer than the wild type bp was considered as FLT3 ITD positive. The additional mutated bands were obtained in the range of approximately 375 to 550 bp. Quantity of wild type and mutated fragments was measured as integrated density value (IDV) on Gel Documentation System (Alpha Innotech Corp., USA) using Alphaview software.

**Detection of FLT3 point mutations by Reverse transcriptase - Polymerase chain reaction (RT-PCR)**

Ribonucleic acid (RNA) was extracted from the same samples with RNA blood kit (Qiagen, Germany) according to Manufacturer’s protocol. To study FLT3 point mutations, Exon 20 of FLT3 gene was amplified from total RNA using primers, Exon 20 F 5’- cgcctgctgacgtgc-3’ and Exon 20 R 5’- atgccgacacaggttacctc-3’ (Bangalore Genei, India). β-Actin served as internal control with following primer sequences F 5’- gctcgtcgtcgacaacggctc-3’ and R 5’- ccacagatcctggcatctctc-3’ (Bangalore Genei, India) and amplified product of 353 bp. Approximately 1 µg of total RNA was added to RT-PCR mix containing 10 µl (5X) RT PCR buffer, 10 µl (5X) Q solution, 2 µl dNTP (10mM each) and 2 µl Enzyme mix (Sensiscript and Omniscript Reverse Transcriptases) from One-Step RT PCR kit (Qiagen, Germany) and 5 µl (1.5 µM final concentration) each of forward and reverse primers, to make a final reaction volume of 50 µl. RT-PCR was carried out in Mastercycler gradient (Eppendorf, Germany) with conditions consisting of initial reverse transcription step at 60°C for 30 minutes, activation step at 95°C for 15 minutes followed by 30 cycles each of denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute and extension at 72°C for 1 minute and final extension step at 72°C for 10 minutes. The 25 µl of amplified product was then subjected to digestion with 10 Units of EcoR V restriction endonuclease enzyme (New England Biolabs, USA) and incubated at 37°C, overnight. Amplified undigested as well as digested products were resolved on 2% agarose gel in presence of ethidium bromide and visualized on Gel Documentation System (Alpha Innotech Corp., USA). The undigested product was obtained at 238 bp while the digested product bands were obtained at 174 and 64 bp. In case of D835 point mutations, undigested product at 238 bp was obtained in addition to the two digested fragments. Quantity of wild type and mutated fragments was measured as integrated density value (IDV) on Gel Documentation System (Alpha Innotech Corp., USA) using Alphaview software.
Flow cytometry analysis

Surface FLT3 receptor protein (CD135) was studied on intact leukemic blasts using bone marrow or peripheral blood samples, by flow cytometric analysis along with immunophenotypic study as described earlier [17].

Statistical analysis

Statistical analysis was carried out using SPSS statistical software version 17 (SPS Inc, Chicago, IL). Mean, standard error (SE) and median were calculated along with correlation between two parameters which was calculated by Pearson's correlation coefficient (r) method. P value less than 0.05 were considered significant. Disease free survival (DFS) and Overall survival (OS) was calculated according to Kaplan and Meier analysis. Chi square statistics was used to assess the prognostic significance of DFS, OS and significance between two subgroups of various parameters. Multivariate analysis to evaluate the prognostic significance was carried out using Cox regression analysis using method – Forward stepwise (Likelihood Ratio).

Results

In a total of 144 de novo AML, FLT3 ITD mutation was found in 19% (27/144) patients. While FLT3 TKD point mutation was observed in 7% (9/130) patients.

The incidence of FLT3 ITD was higher as compared to FLT3 TKD mutations. Both the mutations were found in only one patient with AML M5b subtype.

Correlation of FLT3 ITD with clinical and hematological parameters

In relation to clinical parameters (Table 1), similar incidence of FLT3 ITD was noted in three sub groups of age (<15 years, ≥15 to <60 years and ≥60 years). However, incidence of FLT3 ITD was found to be higher in females (22%, 13/58) as compared to males (16%, 14/86).

In relation to hematological parameters, according to FAB classification, incidence of FLT3 ITD was found to be highest in M6 (50%, 1/2) followed by M1 (30%, 8/27), M5b (29%, 4/14), M3 (25%, 3/12), M2 (11%, 4/36) and M5a (6%, 1/17). One patient with AML M7 subtype did not express FLT3 ITD mutation. Further, higher incidence of FLT3 ITD significantly correlated with blasts ≥61% (median as cutoff value) (27%, 20/73, P = 0.007) and low platelet count (<1.5 x 10^5/µl) (21%, 27/128, P = 0.048) as compared to their respective counterparts. A trend of high FLT3 ITD incidence was noted in low Hemoglobin level (<9.0 gm/dL) (21%, 27/128, P = 0.048) and low RBC count (<3.8 x 10^6/µl) (20%, 27/134), altered M:E ratio (19%, 18/94), moderate nuclear to cytoplasmic ratio (30%, 3/10) and weak staining of Sudan Black B (cytochemical stain) (24%, 4/17) whereas similar incidence of mutation was observed in subgroups of WBC count. When correlated with disease condition present at the time of diagnosis (N=132), incidence of FLT3 ITD was found to be highest in patients with pulmonary infiltration (100%, 2/2) followed by fluid accumulation (pleural, pericardial or ascitic) (29%, 27/134), absence of associated disease condition (20%, 20/100) and hepatosplenomegaly (9%, 1/11). No mutation was found in patients presented with lymphadenopathy, gum hypertrophy, granulocytic sarcoma or multiple symptoms.

Correlation of FLT3 ITD mutation to wild type ratio with clinical and hematological parameters

In patients having FLT3 ITD, ratio of mutant (ITD) to wild type...
With respect to hematological parameters, higher mutation ratio (≥0.66) was observed in AML FAB subtypes M4 (100%, 2/2), M5a (100%, 1/1) and M6 (100%, 1/1), followed by M5b (75%, 3/4), M1 (50%, 4/8) and M2 (50%, 2/4) and FLT3 ITD mutation ratio <0.66 in subtypes M3 (100%, 3/3). Further, FLT3 ITD mutation ratio was significantly high in patients having low blast count (<61%) (86%, 6/7, P=0.03), whereas a trend of high ratio was observed in normal WBC count (4 – 11 x 10^9) (67%, 2/3) and normal Hemoglobin level (≥9.0 gm/dL) (75%, 3/4). Further, all mutated cases had low platelet (<1.5 x 10^10/µl) and low RBC count (<3.8 x 10^12/µl). In correlation with disease condition present at the time of diagnosis, incidence of mutation ratio was high in patients with hepatosplenomegaly (100%, 1/1) followed by patients with fluid accumulation (pleural, pericardial or ascitic) (50%, 1/2), pulmonary infiltration (50%, 1/2) and absence of associated disease condition (50%, 10/20).

**Correlation of FLT3 TKD point mutation with clinical and hematological parameters**

In relation to clinical parameters (Table 1), higher incidence of FLT3 TKD mutation was observed in age group ≥60 years (18%, 3/17) whereas; similar incidence was noted in case of gender subgroups.

In relation to hematological parameters, incidence of FLT3 TKD mutation in AML subtypes was found to be high in M5b (23%, 03/13) and M4 (22%, 4/18) while M5a (6%, 1/16) and M2 (3%, 1/30) showed low incidence and M1, M3 and M6 did not show TKD mutation. High incidence of FLT3 TKD mutation was noted in low blast count (<61%) (10%, 6/62), high WBC count (>11000/µl) (8%, 9/109), low Hemoglobin level (<9.0 gm/dL) (8%, 8/101), low RBC count (<3.8 x 10^12/µl) (7%, 9/123), increased M:E ratio (19%, 3/16), moderate nuclear to cytoplasmic ratio (25%, 2/8) and in subgroups of Sudan Black B (cytochemical stain) weak positive (7%, 1/15) and positive (6%, 4/69). Similar incidence of FLT3 TKD mutation was noted in the subgroups of platelet count. When correlated with disease condition present at the time of diagnosis, incidence of FLT3 TKD mutation was high in patients with fluid accumulation (pleural, pericardial or ascitic) (33%, 1/3) followed by hepatosplenomegaly (12%, 1/8) and patients with absence of associated disease condition (5%, 5/95).

**Incidence of FLT3 RNA transcript level and correlation with the clinical and hematological parameters**

FLT3 RNA was studied in 130 patients and all of them showed FLT3 mRNA expression. Upon quantification it was found in the range of 5 x 10^5 to 19 x 10^5 with mean ± SE of 27 x 10^5 ± 28 x 10^5 as well as median of 16 x 10^5. The patients were sub grouped considering median level as cutoff level (<16 x 10^5 and ≥16 x 10^5) (Table 1).

In relation to clinical parameters, incidence of RNA level was found to be similar in subgroups of age and gender.

In relation to hematological parameters, incidence of RNA levels ≥ 16 x 10^5 in AML subtypes was found to be highest in M5b (62%, 08/13) followed by M3 (60%, 6/10), M1 (52%, 13/25), M2 (52%, 16/30), M4 (50%, 9/18) and M5a (44%, 7/16). Two cases of AML M6 expressed FLT3 RNA level < 16 x 10^5. Further, high incidence of RNA levels ≥ 16 x 10^5 was noted in high blast count (≥ 61%) (54%, 37/68), normal WBC count (4 to 11 x 10^9/µl) (57%, 8/14), low platelet count (< 1.5 x 10^10) (50%, 59/117), low Hemoglobin level (< 9.0 gm/dL) (52%, 53/101), high RBC count (≥ 4.8 x 10^12/µl) (100%, 1/1), increased M:E ratio (69%, 11/16), moderate nuclear to cytoplasmic ratio (75%, 6/8) and in negative subgroup of Sudan.
|                | N    | FLT3 ITD N (%) | FLT3 ITD/WT ratio ≥ 0.66 N (%) | FLT3 TKD mutation N (%) | mRNA transcript level ≥ 16 x 10^5 N (%) |
|----------------|------|----------------|---------------------------------|-------------------------|---------------------------------------|
| **Total Patients** | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| Age            |      |                |                                 |                         |                                       |
| <15 years      | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| 15 to 59 years | 19   | 20 (19)        | 19 (10)                         | 88 (50)                 | 88 (43)                               |
| ≥ 60 years     | 20   | 20 (19)        | 10 (53)                         | 25 (49)                 | 25 (49)                               |
| Gender         |      |                |                                 |                         |                                       |
| Male           | 86   | 14 (16)        | 14 (16)                         | 130 (9)                 | 130 (9)                               |
| Female         | 58   | 13 (22)        | 13 (22)                         | 91 (53)                 | 91 (53)                               |
| **Subtypes**   |      |                |                                 |                         |                                       |
| M1             | 27   | 14 (52)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| M2, M2EO, M2B  | 36   | 14 (40)        | 36 (21)                         | 130 (9)                 | 130 (9)                               |
| M3             | 12   | 10 (83)        | 12 (10)                         | 88 (50)                 | 88 (43)                               |
| M4, M4EO      | 18   | 10 (55)        | 18 (10)                         | 88 (43)                 | 88 (43)                               |
| M5a            | 17   | 10 (58)        | 17 (10)                         | 88 (43)                 | 88 (43)                               |
| M5b            | 14   | 10 (71)        | 14 (10)                         | 88 (43)                 | 88 (43)                               |
| M6             | 2    | 10 (50)        | 2 (10)                          | 88 (43)                 | 88 (43)                               |
| M7             | 1    | 10 (50)        | 1 (10)                          | 88 (43)                 | 88 (43)                               |
| **Unknown**    | 17   | 10 (59)        | 17 (10)                         | 88 (43)                 | 88 (43)                               |
| **Blasts (Median= 61%)** |      |                |                                 |                         |                                       |
| < 61%          | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| ≥ 61%          | 73   | 42 (57)        | 42 (57)                         | 130 (9)                 | 130 (9)                               |
| **WBC count (x 10^3/µl)** |      |                |                                 |                         |                                       |
| < 4000         | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| 4000 to 11000  | 96   | 19 (20)        | 19 (20)                         | 130 (9)                 | 130 (9)                               |
| > 11000        | 20   | 14 (70)        | 20 (10)                         | 130 (9)                 | 130 (9)                               |
| **Platelet count (x 10^5/µl)** |      |                |                                 |                         |                                       |
| < 150000       | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| ≥ 150000       | 73   | 42 (57)        | 42 (57)                         | 130 (9)                 | 130 (9)                               |
| **Hemoglobin (gm/dL)** |      |                |                                 |                         |                                       |
| < 9000         | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| ≥ 9000         | 73   | 42 (57)        | 42 (57)                         | 130 (9)                 | 130 (9)                               |
| **M:E Ratio**  |      |                |                                 |                         |                                       |
| Normal (2:1 – 4:1) | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| Altered        | 73   | 42 (57)        | 42 (57)                         | 130 (9)                 | 130 (9)                               |
| Increased      | 17   | 10 (59)        | 17 (10)                         | 88 (43)                 | 88 (43)                               |
| Decreased      | 14   | 10 (71)        | 14 (10)                         | 88 (43)                 | 88 (43)                               |
| **N:C ratio**  |      |                |                                 |                         |                                       |
| Moderate       | 10   | 5 (50)         | 10 (50)                         | 88 (43)                 | 88 (43)                               |
| High           | 87   | 5 (50)         | 87 (50)                         | 88 (43)                 | 88 (43)                               |
| **Cytochemical stain** |      |                |                                 |                         |                                       |
| SBB Negative   | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| SBB weak positive | 73   | 42 (57)        | 42 (57)                         | 130 (9)                 | 130 (9)                               |
| SBB Positive   | 73   | 42 (57)        | 42 (57)                         | 130 (9)                 | 130 (9)                               |
| **Clinical Characteristics** |      |                |                                 |                         |                                       |
| Lymphadenopathy | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| Hepatosplenomegaly | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| Pneumocystis carinii pneumonia | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| Gum Hypertrophy | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| Pulmonary infiltration | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| Granulocytic sarcoma | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| Multiple symptoms | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |
| No associated abnormality | 144  | 27 (19)        | 27 (14)                         | 130 (9)                 | 130 (9)                               |

a, r = 0.22, P= 0.007; b, r = -0.16, P= 0.04; c, r = -0.40, P= 0.03; d, r = 0.27, P= 0.002
P value ≤ 0.05 is significant
WBC, White Blood Cells; RBC, Red Blood Cells
M:E ratio, Myeloid:Erythroid ratio; N:C ratio, Nuclear:Cytoplasmic ratio; SBB, Sudan Black B

Table 1: Correlation of FLT3 ITD, FLT3 mutation to wild type ratio, FLT3 TKD mutation and FLT3 mRNA transcript level with clinical and hematological parameters, clinical characteristics as well as inter-correlation of FLT3 parameters.
| N | FLT3 WT/WT N (%) | CD135 Positive | N | FLT3 ITD/WT N (%) | CD135 Positive |
|---|-----------------|----------------|---|-----------------|----------------|
| Number of Patients | 117 | 46 (39) | 71 (61) | 27 | 9 (30) | 19 (70) |
| Age | | | | | | |
| <15 years | 23 | 08 (35) | 15 (65) | 05 | 02 (40) | 03 (60) |
| 15 to 59 years | 77 | 33 (43) | 44 (27) | 19 | 05 (26) | 14 (74) |
| ≥ 60 years | 17 | 05 (29) | 12 (71) | 03 | 01 (33) | 02 (67) |
| Gender | | | | | | |
| Male | 72 | 31 (43) | 41 (57) | 14 | 05 (36) | 09 (64) |
| Female | 45 | 14 (31) | 31 (69) | 13 | 05 (38) | 08 (62) |
| Subtypes | | | | | | |
| M1 | 19 | 07 (37) | 12 (63) | 08 | 01 (12) | 07 (88) |
| M2, M2E0, M2B | 32 | 14 (44) | 18 (56) | 04 | 02 (50) | 02 (50) |
| M3 | 09 | 06 (67) | 03 (33) | 03 | 07 (27) | 01 (33) |
| M4, M4E0 | 16 | 04 (25) | 12 (75) | 02 | 00 (00) | 02 (100) |
| M5 | 16 | 07 (44) | 09 (56) | 01 | 01 (100) | 00 (00) |
| M6 | 01 | 01 (100) | 00 (00) | 01 | 01 (100) | 00 (00) |
| M7 | 01 | 01 (100) | 00 (00) | 01 | 01 (100) | 00 (00) |
| Unknown | 13 | 05 (38) | 08 (62) | 04 | 00 (00) | 04 (100) |
| Blasts (Median= 61%) | | | | | | |
| < 61 | 64 | 29 (45) | 35 (55) | 07 | 01 (14) | 06 (86) |
| ≥ 61 | 53 | 17 (32) | 36 (68) | 20 | 07 (35) | 13 (65) |
| WBC count (x 10^3/µl) | | | | | | |
| < 4 | 06 | 04 (67) | 02 (33) | 01 | 01 (100) | 00 (00) |
| 4 to 11 | 14 | 08 (57) | 06 (43) | 03 | 01 (33) | 02 (67) |
| > 11 | 97 | 34 (35) | 63 (65) | 23 | 06 (26) | 17 (74) |
| Platelet count (x 10^5/µl) | | | | | | |
| < 1.5 | 101 | 40 (40) | 61 (60) | 27 | 08 (30) | 19 (70) |
| 1.5 to 4.5 | 15 | 05 (33) | 10 (67) | - | - | - |
| > 4.5 | 01 | 01 (100) | 00 (00) | - | - | - |
| Hemoglobin (gm/dL) | | | | | | |
| < 9.0 | 87 | 32 (37) | 55 (63) | 23 | 07 (30) | 16 (70) |
| ≥ 9.0 | 30 | 14 (47) | 16 (53) | 04 | 01 (25) | 03 (75) |
| RBC count (x 10^6/µl) | | | | | | |
| < 3.8 | 107 | 41 (38) | 66 (62) | 27 | 08 (30) | 19 (70) |
| 3.8 to 4.8 | 09 | 05 (56) | 04 (44) | - | - | - |
| > 4.8 | 01 | 00 (00) | 01 (100) | - | - | - |
| M:E Ratio | | | | | | |
| Normal (2:1 – 4:1) | 02 | 00 (00) | 02 (100) | - | - | - |
| Altered | 76 | 30 (40) | 46 (60) | 18 | 05 (28) | 13 (72) |
| Increased | 15 | 06 (40) | 09 (60) | 01 | 00 (00) | 01 (100) |
| Decreased | 03 | 01 (33) | 02 (67) | - | - | - |
| N:C ratio | | | | | | |
| Moderate | 07 | 01 (14) | 06 (86) | 03 | 02 (67) | 01 (33) |
| High | 72 | 31 (43) | 41 (57) | 15 | 02 (13) | 13 (67) |
| Cytochemical stain | | | | | | |
| SBB Negative | 06 | 02 (35) | 04 (65) | 01 | 00 (00) | 01 (100) |
| SBB weak positive | 13 | 07 (54) | 06 (46) | 04 | 01 (25) | 03 (75) |
| SBB Positive | 62 | 28 (45) | 34 (55) | 14 | 04 (29) | 10 (71) |
| mRNA transcript level (median as cutoff) | | | | | | |
| < 16 x 10^5 | 54 | 27 (50) | 27 (50) | 11 | 02 (18) | 09 (82) |
| ≥ 16 x 10^5 | 51 | 14 (28) | 37 (72) | 14 | 06 (43) | 08 (57) |

*p value ≤ 0.05 is significant; NC=Non Computable

WBC, White Blood Cells; RBC, Red Blood Cells
M:E ratio, Myeloid:Erythroid ratio; N:C ratio, Nuclear:Cytoplasmic ratio; SBB, Sudan Black B

Table 2: Correlation of CD135 protein expression within FLT3 WT/WT and FLT3 ITD/WT groups with clinical and hematological parameters, clinical characteristics and FLT3 mRNA transcript level.

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Black B (cytochemical stain) (62%, 5/8). When correlated with disease condition present at the time of diagnosis, incidence of RNA level ≥16 x 10^5 was high in patients with fluid accumulation (pleural, pericardial or ascitic) (100%, 3/3) and granulocytic sarcoma (100%, 1/1) followed by multiple symptoms (67%, 2/3), absence of associated disease condition (49%, 25/52), lymphadenopathy (43%, 3/7) and hepatosplenomegaly (36%, 13/36). The incidence of CD135 over expression was higher in FLT3 ITD group was noted in the age group of 15 to 59 years (74%, 14/19) and in males (79%, 11/14) over negative group. Further, incidence of CD135 over expression (≥20%) in FLT3 ITD group was higher in age group of 15 to 59 years (74%, 14/19) and in males (79%, 11/14) over negative group.

Incidence of FLT3 receptor protein and its correlation with clinical and hematological parameters

FLT3 receptor protein (CD135) levels were estimated by flow cytometer method in 144 AML patients. CD135 over expression (≥20%) was found in 62% (90/144) of AML patients. In CD135 over expressing group, FLT3 ITD was found in 21% (19/90) of patients whereas; in CD135 negative group the mutation was found in 15% (8/54) patients (Table 1). To determine whether the role of protein over expression in disease progression was dependent on FLT3 ITD, the FLT3 ITD positive and negative groups were further divided into CD135 negative (<20%) and positive (≥20%) groups for comparison in their respective correlation with the clinical and hematological parameters (Table 2).

With respect to clinical parameters, no significant correlation was obtained when CD135 over expression of FLT3 ITD group was compared with that of FLT3 ITD negative group. But trend of higher incidence of CD135 over expression in FLT3 ITD group was noted in the age group of 15 to 59 years (74%, 14/19) and in males (79%, 11/14) over negative group.

With respect to hematological parameters, the number in each AML subtype was too small to evaluate statistical significance between CD135 overexpressing FLT3 ITD group and FLT3 ITD negative group. Further, incidence of CD135 over expression was higher in FLT3 ITD group having low blast count (<61%) (86%, 6/7), normal to high WBC count (4 to 11 x 10^3/µl) (70%, 19/27), low and normal Hemoglobin level (<9 gm/dL and ≥9 gm/dL) (70%, 16/23 and 75%, 3/4). Log Rank=3.90, df=1, P=0.04* Log Rank=2.61, df=1, P=0.10 Log Rank=0.04, df=1, P=0.84 Log Rank=1.75, df=1, P=0.18 Log Rank=0.002, df=1, P=0.96 Log Rank=2.55, df=1, P=0.11 Log Rank=4.57, df=1, P=0.03* Log Rank=5.47, df=1, P=0.008 Log Rank=6.87, df=1, P=0.000 Log Rank=1.07, df=1, P=0.75 Log Rank=0.05, df=1, P=0.62 Table 3: Correlation of FLT3 ITD, FLT3 TKD mutation, mRNA transcript level and CD135 protein expression with clinical outcome.

With respect to clinical parameters, no significant correlation was obtained when CD135 over expression of FLT3 ITD group was compared with that of FLT3 ITD negative group. But trend of higher incidence of CD135 over expression in FLT3 ITD group was noted in the age group of 15 to 59 years (74%, 14/19) and in males (79%, 11/14) over negative group.
13/20 vs. 59%, 47/80) whereas incidence of CD135 (≥20%) was lower in case of hepatosplenomegaly (0%, 0/1 vs. 50%, 5/10) as compared to the CD135 over expression in FLT3 ITD negative group. Both patients with pulmonary infiltration had mutation with CD135 over expression (≥20%) (100%, 2/2) and other subgroups could not be compared due to absence of mutation.

**Correlation of FLT3 ITD, TKD mutation with RNA transcript level and protein levels**

The incidence of FLT3 ITD was correlated with RNA transcript level and CD135 protein where, a trend of high incidence of FLT3 ITD was observed in transcript level ≥ 16 x 10^5 (22%, 14/65) and CD135 ≥ 20% (21%, 19/90) as compared to their respective counterparts (Table 1).

FLT3 ITD to wild type ratio was correlated with RNA transcript level and CD135 protein where, a trend of higher incidence of mutation to wild type ratio ≥0.66 was observed in transcript level <16 (64%, 7/11) and CD135 ≥20% (58%, 11/19) as compared to their respective counterparts (Table 1).

Further, the incidence of FLT3 TKD mutation was correlated with RNA transcript level and CD135 protein where, FLT3 TKD mutation was significantly high in transcript level ≥16 x 10^5 (14%, 9/65, P=0.002) while incidence was similar in case of both subgroups of CD135 expression levels (Table 1).

Next, the incidence of RNA level was correlated with CD135 protein where, a trend of high incidence of CD135 protein over expression (≥20%) was found (56%, 45/81) in patients with RNA level ≥16 x 10^5 (14%, 9/65, P=0.002) while incidence was similar in case of both subgroups of CD135 expression levels (Table 1).

**Correlation of FLT3 mutations, FLT3 RNA transcript level and CD135 protein with CD34 expression and leukemia associated immunophenotype (LAP)**

FLT3 expression is mainly found on immature and precursor cells and therefore a correlation of FLT3 ITD as well as TKD mutations, RNA level and CD135 with CD34 (a marker of immature cells) was evaluated. Incidence of FLT3 ITD was significantly high in CD34+ AML (28%, 16/57, P=0.02) than CD34+ (13%, 11/87) AML. While incidence of FLT3 TKD mutation, RNA level and CD135 protein expression was found similar in the subgroups of CD34- and CD34+ (data not shown).

Further, FLT3 was correlated with leukemia associated immunophenotype (data not shown). When correlated with the myeloid lineage markers, high incidence of FLT3 ITD was found in CD33- (38%, 3/8) as compared to CD33+ (18%, 24/136) AML while incidence was similar in case of CD13, MPO and CD117 sub groups. In case of FLT3 TKD mutation, high incidence was noted in CD13+ (7%, 9/124) and CD33+ (7%, 9/123) and MPO- (10%, 6/57) AML as compared to their respective counterparts while incidence of FLT3 TKD mutation was similar in both the subgroups of CD117. High incidence of RNA level was found in CD13+ (67%, 4/6), CD33- (71%, 5/7) AML as compared to their respective counterparts while incidence was similar in case of CD117 and MPO subgroups. Similar incidence of CD135 over expression was found in subgroups of CD13, CD33, CD117 and MPO.

Aberrant expression of B cell marker CD19 was noted in 19% (27/144) and incidence of FLT3 ITD, TKD, RNA level and CD135 protein was found to be similar in both the subgroups of CD19. Abrupt expression of T cell marker CD7 was found in 14% (19/140) and high incidence of FLT3 ITD (26%, 5/19) and TKD mutation (18%, 3/17) was noted in CD7+ AML while similar incidence of RNA level and CD135 protein was found between CD7+ and CD7+ AML.

**Impact of FLT3 mutations, RNA transcript level and CD135 protein expression on clinical outcome**

Out of 144 patients included in the study, 76 patients underwent treatment out of which 57 were adults and 19 were pediatric patients while 68 out of 144 patients were either lost to follow up or died within a month. Induction remission was achieved in 30% (23/76) while disease relapse was noted in 70% (53/76) of AML patients.

According to Kaplan Meier analysis (Table 3), FLT3 ITD showed no correlation with DFS but a trend of increase in incidence of death was seen in FLT3 ITD group (47%, 8/17) with a decrease in median OS (1 month) as compared to FLT3 ITD negative group (33%, 25/75 and median OS 3 months).

With regards to FLT3 TKD mutation, patients having FLT3 TKD mutation showed higher incidence of relapse (83%, 5/6) and reduced median DFS (0.50 month) as compared to patients without TKD mutation (68%, 43/63 and median DFS 1 month). No such trend was seen with OS.

RNA transcript level was correlated with disease status, where, transcript level (≥ 16 x 10^5) showed decrease in relapse rate (63%, 20/32) and increased median DFS (2 vs. 0 months) as compared to low RNA level (< 16 x 10^5) whereas no such trend was observed with OS (Table 3).

Similarly patients with CD135 over expression also showed significantly high incidence of relapse (78%, 36/46, P = 0.04) and reduced median DFS (0 vs. 3 months) as compared to CD135 < 20% expression. No such trend was seen with OS.

Further, RNA transcript was divided into FLT3 ITD positive and negative groups. In low FLT3 RNA (< 16 x 10^5) group, FLT3 ITD showed significantly high incidence of relapse (100%, 6/6, Log Rank = 4.57, df=1, P=0.03) with reduced median DFS (0 vs. 0.50 month) and high incidence of death (50%, 4/8) with reduced median OS (1 vs. 3 months) as compared to FLT3 ITD negative patients. No such trend was observed in high FLT3 RNA (≥ 16 x 10^5) group.

Next, RNA transcript level was divided into FLT3 TKD positive and negative groups. The low FLT3 RNA expression group (< 16 x 10^5) did not have FLT3 TKD mutations but in high FLT3 RNA expression group (≥ 16 x 10^5), FLT3 TKD showed higher incidence of relapse (83%, 5/6) with reduced median DFS (5.0 vs. 2 months) as compared to the FLT3 TKD negative group. No such trend was seen with OS in this group.

Further, FLT3 ITD group was divided into CD135 expression (< 20% and ≥ 20%), CD135 over expression (≥ 20%) showed trend of higher incidence of relapse (88%, 7/8 and 76%, 29/38) and reduced median DFS (0 vs. 3 months) in FLT3 ITD positive and negative groups respectively. No such trend was seen with OS.

Next, RNA transcript level was then divided into CD135 expression (< 20% and ≥ 20%). CD135 over expression (≥ 20%) showed trend of higher incidence of relapse (73%, 16/22 and 85%, 17/20) with reduced median DFS (0.50 vs. 4.50 and 0 vs. 3 months) in high (≥ 16 x 10^5) and low (< 16 x 10^5) FLT3 RNA expression groups respectively, while no such trend was observed in relation to OS.
Further, patients with wild type RNA transcript were selected with exclusion of patients with FLT3 ITD and TKD mutation. These patients were further sub grouped according to median cut off level of $14 \times 10^5$. In patients with high wild type RNA transcripts ($\geq 16 \times 10^5$), wild type CD135 protein over expression showed higher incidence of relapse (78%, 13/18, Log Rank=5.92, df=1, $P=0.01$) than their respective counterpart.

Impact of FLT3 mutations, RNA transcript and CD135 expression with treatment on clinical outcome

In case of correlation of FLT3 ITD with DFS, high remission rate was noted in patients treated with BFM93 protocol (50%, 4/8) compared to 7+3 protocol, ATRA / Arsenic trioxide protocol and palliative chemotherapy while high survival rate was seen in patients treated with BFM93 protocol (50%, 4/8) compared to 7+3 protocol, ATRA / Arsenic trioxide protocol and palliative chemotherapy (Table 4).

Multivariate Analysis of clinical and hematological parameters along with FLT3 parameters

Multivariate analysis using cox forward stepwise regression model was carried out to evaluate the prognostic significance of clinical and hematological parameters such as age, gender, AML subtype, blast count, WBC count, platelet count, RBC count and hemoglobin level along with FLT3 ITD, FLT3 TKD point mutation, RNA transcript level and CD135 protein in clinical outcome of patients (Data not shown). High WBC count (>11000/µl) was found to be significant prognostic factor ($P=0.03$) for predicting reduced DFS and had DFS shorter than 15 months whereas high blast count ($\geq 61\%$) was found as significant prognostic factor ($P=0.001$) for poor OS and had OS shorter than 21 months.

Comparison of incidence of FLT3 mutations, RNA transcript level and CD135 expression in Aplastic anemia, Myelodysplastic syndrome and Acute myeloid leukemia patients

Patients diagnosed with Aplastic Anemia and MDS were compared with the AML patients for the incidence of FLT3 mutations, RNA level and CD135 protein expression (Data not shown). With respect to CD135 over expression ($\geq 20\%$), high remission rate was noted in patients treated with BFM93 protocol (50%, 4/8) compared to 7+3 protocol, ATRA / Arsenic trioxide protocol and palliative chemotherapy while high survival rate was seen in patients treated with 7+3 protocol 96% (22/23) than BFM93, ATRA / Arsenic trioxide protocol and palliative chemotherapy (Table 4).
noted in 22% (2/9) of MDS RAEB-II and 19% (27/144) of AML patients. In case of FLT3 TKD mutations, Aplastic anemia or MDS patients did not have FLT3 TKD mutation while 7% (9/130) of AML expressed TKD mutations. Further RNA level ≤14 x 10⁴ was not found in Aplastic anemia or MDS cases while in AML it was noted in 54% (70/130). With respect to CD135 protein expression, CD135 protein over expression was not seen in Aplastic Anemia patients while CD135 positivity (≥20%) was noted in 60% (3/5) MDS patients and 62% (90/144) in AML patients.

**Discussion**

In this study, we have evaluated clinical relevance of FLT3 ITD and FLT3 TKD point mutations, FLT3 mRNA transcripts and correlated with FLT3 receptor protein determined on leukemic blasts by flow cytometry in patients with Acute Myeloid Leukemia (AML), Myelodysplastic Syndrome (MDS) and Aplastic Anemia. Overall incidence of FLT3 ITD was 19%, FLT3 TKD mutation was 7%, high mRNA transcript level was 50% and CD135 over expression was 62% in AML patients. In aplastic anemia patients, FLT3 ITD and TKD mutation, high mRNA transcript level as well as CD135 protein over expression was not seen. While, in MDS patients, incidence of FLT3 ITD was 20%, CD135 over expression was 60% while only one case was analyzed for FLT3 TKD mutation and mRNA transcript level which were negative and low, respectively.

FLT3 ITD mutations were first detected by Nakao et al. in 1996 reporting approximately 23% incidence in AML [27] followed by various study groups reporting incidence of FLT3 ITD in the range of 10 to 35% [8-10,14-16,19,23-24,28-33]. Similar to our study, Indian studies reported incidence of FLT3 ITD in the range of 18 to 21% in AML M3 [17,34,35] and 19 - 20% in AML with or without normal karyotype [18,20,36]. With regards to clinical parameters, incidence of FLT3 ITD in pediatric age group was found to be similar to that of adult age group in AML patients. Contrary to our finding other studies demonstrated low frequency of FLT3 ITD in pediatric patients within the range of 5 to 16 % [36-40] as compared to adults, while only one study demonstrated 40% incidence in pediatric AML though it was done only in 5 patients [15]. A study on pediatric AML by Iwai et al. [38] suggested that since the internal tandem duplication involved juxtamembrane domain of FLT3 gene and was in-frame in pediatric which is similar to adult group; it can be assumed that mutation in children with AML occurred in same manner as in adults. With gender, the incidence of FLT3 ITD tended to be high in females, and similar to our study, two groups show significantly high incidence in females [9,30].

In addition, among hematological parameters significant association of FLT3 ITD was confirmed with higher blast count as seen in other studies [15,19,23] as well as with low platelet count which has also been shown by the study of Schnittger et al. [41] on APL patients. In relation to AML FAB subtypes, high incidence of FLT3 ITD was noted in AML M6 (50%) followed by M1 (30%), M5b (29%), M3 (25%) while low incidence was noted in M2, M4 and M5a though difference was not significant. In AML M6 subtype, few studies have noted FLT3 ITD mutation in the range of 7% to 20% [15,22,30] while majority of the studies have not observed FLT3 ITD mutation in AML M6 [9,10,16,28,31,32,38]. High incidence in AML M1, M5b and M3 subtypes could be explained by the theory suggesting that FLT3 blocked the granulocytic differentiation of myeloblastic cells [42] while the expression remains associated with the monocytic differentiation [10,31]. As demonstrated by Munoz et al. [43], the incidence of FLT3 ITD was found more in AML subtypes associated with matured myeloid progenitor cell population which could explain significantly high incidence of FLT3 ITD in CD34 negative population in our study. Further in FLT3 ITD mutated cases, high mutation to wild type ratio was found to correlate significantly with female gender, low blast count (<61%) and a trend with normal WBC count and normal Hemoglobin level was noted. In relation to subtypes, high mutation to wild type ratio was found in AML subtypes M4 (100%), M5a (100%) and M5b (75%) and single mutation case of M6 had high mutation ratio. The high incidence of mutation to wild type ratio (≥ 0.66) in monocytic cell lineage confirms the association of constitutive activation of FLT3 in monocytic differentiation. Contrary to our finding, no significant association between FLT3 ITD mutation ratio and gender was noted by study of Gule et al. [8]. However, Kuchenbauer et al. [19] and 'Thiede et al. [31], have correlated FLT3 ITD mutation ratio with age and not the gender, and observed no significant correlation with respect to age.

Our study demonstrated incidence of FLT3 TKD point mutations in 7% of patients with AML. Various study groups have reported its incidence in the range of 4 to 11% in AML [12-13,16,21-22,31-32,44] while Indian studies have reported the incidence in the range of 3 – 6% in AML [18,20,36]. With respect to clinical and hematological parameters and CD34 expression showed no significant correlation, but a trend of high expression was noted in patients with age ≥ 60 years, moderate nuclear to cytoplasmic ratio, AML M5b and M4 subtypes. This result is in accordance with other study groups [10,21,31] which suggest the distribution of FLT3 TKD mutation in monocytic subtypes of AML. Therefore, similar to FLT3 ITD, FLT3 TKD mutations also seem to play important role in constitutive activation of FLT3 in leukemic myelo-monocytic cell differentiation and proliferation.

In this study, all AML patients showed FLT3 mRNA expression and therefore subgrouped according to median level 16 x 10⁴. No significant correlation was found with clinical and hematological parameters in these two subgroups. With AML subtypes, high incidence of FLT3 transcript level ≥16 x 10⁴ was noted in AML M5b followed by M3 subtype which is in line with the theory that FLT3 expression increases in monocytic cell lineage differentiation and proliferation. Few studies have reported high expression of FLT3 mRNA in AML compared to normal BM [19,23,24,45-46].

Further, 62% of AML over expressed CD135 protein determined by flow cytometry. Different methodologies have been used for protein detection, such as western blot [45,47-48], flow cytometry method [19,23-24,49,50] and ELIZA [51] and observed over expression of FLT3 protein in 70 to 100 % of AML cases [45,49,49]. The advantage of flow cytometry over Western blotting in protein estimation was reported by Wergeland et al. [52]. Our previous study on acute leukemia [25] was the first Indian report on CD135 protein over expression in 82% of AML, 60% of B-ALL, and 23% of T-ALL by flow cytometry. Our earlier study compared CD135 protein expression with clinical and hematological parameters, so the current study compared CD135 expression with clinical and hematological parameters in two subgroups of FLT3 ITD (mutant and wild type), as the incidence of CD135 over expression was higher in FLT3 ITD group. However, no significant correlation was obtained between these two subgroups in relation to clinical and hematological parameters. Further, in FLT3 ITD negative group, significant higher incidence of CD135 over expression was noted in WBC count > 11,000 correlates CD135 protein over expression of wild type cases with disease aggressiveness.

These markers when intercorrelated, a trend of high incidence of FLT3 ITD mutation seen in FLT3 mRNA transcript and CD135 over expressing subgroups. Similarly, few studies have shown positive correlation of FLT3 ITD mutation with FLT3 mRNA over expression [23-24] and CD135 protein over expression [23]. In case of mutation to
wild type ratio, trend of high ratio was found in CD135 over expressing group, and in low FLT3 mRNA transcript level which remained unexplained. Further, the incidence of FLT3 TKD mutation was seen higher in patients with high FLT3 mRNA transcript level while remained similar in subgroups of CD135 protein. Moreover, high FLT3 mRNA transcript level was noted in CD135 over expressing group, similarly, over expression of FLT3 mRNA along with FLT3 protein over expression was reported in AML [19,23,45]. Further in FLT3 ITD negative group, incidence of CD135 over expression tended to be significantly high in transcript level ≥ 16 x 10^5 while a trend of inverse correlation was obtained with FLT3 ITD positive group.

Regards to immunophenotypic markers, a trend of high incidence of FLT3 ITD was noted in CD33- group whereas FLT3 TKD mutations was noted in CD33+, CD13+, and MPO AML. Higher incidence of mRNA transcript level was found in CD13 and CD33 AML whereas similar incidence of CD135 over expression was found in subgroups of CD13, CD33, CD117 and MPO. In case of aberrant expression of immunophenotypic markers, incidence of FLT3 ITD and FLT3 TKD mutation were found to be high in CD7+ group while no trend could be obtained with CD19 subgroups. FLT3 ITD mutant group AML with aberrant CD7 expression showed higher relapse rate (75%) as compared to CD7 group (62%). This result is in line with studies reporting that CD7 is aberrantly expressed on blast cells in AML and is associated with a poor prognosis [53,54].

To assess the prognostic significance in univariate analysis, FLT3 mutations, FLT3 mRNA transcript level and CD135 protein expression were correlated with clinical outcome. In relation to DFS, interestingly, only CD135 mRNA transcript level was significantly correlated with increased relapse rate with shorter DFS whereas FLT3 TKD mutation and mRNA transcript showed no such trend. No such correlation was found in subgroups of FLT3 ITD. FLT3 ITD showed significant increase in relapse rate and reduced median DFS in patients with FLT3 mRNA transcript level <16 x 10^5. While FLT3 TKD mutation showed a trend of higher relapse rate and reduced median DFS in FLT3 mRNA transcript level ≥16 x 10^5 group. CD135 over expression tended to increase relapse rate and reduced median DFS in both subgroups of FLT3 ITD cases and FLT3 mRNA transcripts. Thus, our results demonstrate that in wild type FLT3 group CD135 protein expression is equally important in predicting clinical outcome. These results strengthen findings of our previous study that CD135 protein could be of potential prognostic value in AML [25]. Similarly few studies [19,24,30] have reported high wild type FLT3 expression level as a possible risk factor. In relation to OS, only FLT3 ITD mutant cases showed a trend of increase in incidence of death and decrease in OS. No such correlation was found in subgroups of FLT3 TKD mutations, mRNA transcript level and CD135 protein expression. The results concerning the clinical outcome with respect to presence of FLT3 ITD is varying among different study groups. Most of the studies, including one Indian study group showed significantly reduced DFS and OS [5,14-15,17,32] while few show no correlation with clinical outcome [19]. Some studies, similar to our study, showed no correlation of FLT3 ITD mutation with DFS but a trend of decrease in OS [9,30]. Among Indian studies, Dunna et al. [18] showed significantly reduced DFS while Chauhan et al. [20] showed no correlation of FLT3 ITD with DFS. With respect to TKD mutations, some studies, including our study suggest poor prognostic in relation to DFS [16,21], while few show trend in reduced OS [21,31] and some showed no correlation with clinical outcome [13,18,32,34]. Contrary to that only one study by Mead et al. [22] has shown better survival with FLT3 TKD mutations.

Regards to treatment, in FLT3 ITD and high mRNA transcript level groups, increased remission rate was observed when treated with ATRA / Arsenic trioxide and BFM93 protocols. In FLT3 TKD mutation group, remission rates increased with 7+3 protocol while in CD135 over expressing group, remission rate was high with BFM93 and 7+3 protocol. Overall survival improved in FLT3 ITD, FLT3 TKD mutation and mRNA over expressing groups with all treatment options except palliative chemotherapy and in CD135 over expressing group, overall survival increased with 7+3 protocol and BFM93 protocol. Our results suggest that standard 7+3 protocol remains the treatment of choice in a subset of FLT3 TKD mutation, ITD mutation, CD135 over expression and high mRNA transcript level in relation to overall survival. Some clinical trials have suggested use of FLT3 inhibitor along with standard 7+3 protocol to improve response rate though there is a need to demonstrate prolongation of CR duration and survival in patients receiving FLT3 inhibitors [55-56]. Also, in APLM patients with FLT3 ITD and RNA transcript level ≥ 16 x 10^5, ATRA / Arsenic trioxide protocol remained treatment of choice. None of the patients with TKD mutation were available for correlation while CD135 over expressing subset of patients showed higher relapse rate with ATRA / Arsenic trioxide protocol and therefore CD135 may be used as a marker for identification of an aggressive phenotype of APLM which needs to be confirmed on larger patient series. Studies related to APLM patients suggest induction with anthracycline along with ATRA as best option [57] though study by Hong et al. [58] suggest that this option does not benefit patients with FLT3 ITD and that FLT3 inhibitors along with ATRA combined with anthracycline should be the treatment option. Further, BFM93 protocol was effective in pediatric patients with FLT3 ITD mutation, high mRNA transcript level and CD135 over expression, while showed high relapse rate with FLT3 TKD mutations, although, studies on pediatric patients treated with BFM demonstrate FLT3 ITD as predictor of poor outcome and suggest need for FLT3 inhibitors to improve prognosis [59]. Therefore, a long term follow up is required to assess the duration of CR and overall survival with the given treatment options.

In multivariate survival analysis by cox forward stepwise regression model, FLT3 ITD, FLT3 TKD mutation, mRNA transcript level and CD135 protein did not turn out to be an independent prognosticator whereas among all parameters, high WBC count (≥ 11000) emerged as significant predictor of high relapse rate and shorter DFS and, high blast count (≥ 61%) was significant predictor of high incidence of death and short overall survival. These findings are consistent with studies which demonstrated WBC count as significant prognostic factor in AML [60-61] while blast count was demonstrated to be of prognostic significance for reduced OS in AML patients as shown by Amin et al. [62].

In relation to pre leukemic conditions such as MDS and aplastic anemia, FLT3 ITD incidence of 22% in MDS is contrary to the findings of few study groups including one Indian study which reported absence of FLT3 ITD in MDS [32,63,64], while two studies showed 2% incidence [65,66]. Incidence of CD135 protein (60%) was similar to that found in AML patients. We were unable to evaluate FLT3 TKD mutations or RNA transcript level as enough RNA could not be extracted from the samples due to low blood cell count. In aplastic anemia patients of our study, FLT3 ITD and FLT3 TKD mutations were absent along with low expression of mRNA transcript and CD135 protein. Further, implication of myelosuppressive cytokines in hematopoietic failure [67,68] along with activation of apoptotic pathway and down regulation of genes promoting cell proliferation and cell cycle [69] in aplastic anemia suggests that FLT3 gene expression is down regulated causing low expression of CD135 protein.

In summary, CD135 protein expression emerged as significant
predictor of DFS in only univariate analysis while WBC and blasts appeared as poor prognostic factors with respect to DFS and OS respectively in multivariate analysis. The presence of FLT3 ITD was found to be more associated with the reduced overall survival of the patients while FLT3 TKD mutation was found to be associated with increased relapse rate and shorter disease free survival. Importantly in FLT3 wild type as well as mutant group, pre-therapeutic CD135 protein over expression predicted poor DFS. Our data suggest that evaluation of CD135 protein over expression at diagnosis can help identify the high risk subset of patients which can be targeted by designing new therapies that include FLT3 inhibitor. Further, CD135 protein estimation by flow cytometry is less time consuming than other methods, it is cost-effective and can be done in association with leukemia immunophenotyping.

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