

FLT3 and NPM-1 mutations in a cohort of acute promyelocytic leukemia patients from India

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Background: Acute promyelocytic leukemia (APL) with t (15;17) is a distinct category of acute myeloid leukemia (AML) and is reported to show better response to anthracyclin based chemotherapy. A favorable overall prognosis over other subtypes of AML has been reported for APL patients but still about 15% patients relapse.

Methods: This study evaluated the presence of Famus like tyrosine kinase-3 (FLT3) and nucleophosmin-1 (NPM1) gene mutations in a cohort of 40 APL patients. Bone marrow/peripheral blood samples from patients at the time of diagnosis and follow-up were processed for immunophenotyping, cytogenetic markers and isolation of DNA and RNA. Samples were screened for the presence of mutations in FLT3 and NPM1 genes using polymerase chain reaction followed by sequencing.

Results: Frequency of FLT3/internal tandem duplication and FLT3/tyrosine kinase domain was found to be 25% and 7% respectively. We observed a high frequency of NPM1 mutation (45%) in the present population of APL patients.

Key words: Acute promyelocytic leukemia, famus like tyrosine kinase-3, nucleophosmin-1

alpha (PML-RAR α) t (15;17),^[4] and is the most frequently curable acute leukemia in adults if promptly diagnosed and adequately treated.^[5] Many studies have correlated the presence of activating mutations in Famus like tyrosine kinase-3 receptor (FLT3 mutation) with an adverse outcome in APL patients.^[6] This receptor has been known to enhance the survival and proliferation of hematopoietic progenitors in response to its ligand.^[7] Mostly, the mutations in FLT3 are the result of internal tandem duplication (ITD) of the intracellular juxtramembrane region of this molecule^[8] which is known to contribute toward its constitutive expression;^[9] FLT3/ITD has also been associated with leukocytosis in APL patients.^[10] Many activating point mutations within the “a loop” of tyrosine kinase domain (TKD) have also been reported in AML patients^[11-13] and their prognostic significance has been described.^[14,15]

Nucleophosmin-1 (NPM1) is a molecular chaperon of proteins, which facilitates the transport of ribosomal proteins through the nuclear membrane.^[16] It is located in the nucleolus at a steady state^[17] and known to contribute toward importantly cellular functions through protein-protein interactions. Mutation in NPM1 gene results in its cytoplasmic dislocation which has been implicated to play a critical role in leukemogenesis.^[18,19] Evaluating transcript levels of NPM1 mutation has been suggested as a promising tool for minimal residual disease (MRD) monitoring in AML patients.^[20]

Presence of FLT3/ITD has been observed in 20-35% of APL cases^[10,15,21,22] where it has been correlated with shorter overall survival and poor postrelapse survival.^[15] FLT3-D835Y has been reported in 7-20% APL patients^[10,15,23] but its prognostic significance

Introduction

Acute promyelocytic leukemia (APL) is a distinct subtype of acute myeloid leukemia (AML) and is treated differently than other types of AML.^[1] It is characterized by the blast morphology,^[2] specific coagulopathy,^[3] fusion gene transcript promyelocyte leukemia retinoic acid receptor

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remains unclear in this subgroup of AML. Even though, about one-third patients with AML have been observed to be presented with mutation in NPM1 gene,^[24] no significant incidence of NPM1 mutation in APL patients is reported.^[19,25,26]

In the present study, we analyzed mutations in FLT3 and NPM1 gene in a cohort of 40 patients diagnosed with APL using combination of molecular techniques. This data were further analyzed for frequency of these mutations and then correlated with clinical characteristics at presentation, treatment and follow-up.

Materials and Methods

Patients and specimens

Overall, 54 patients were diagnosed with APL out of 276 AML patients referred to Department of Hematology, KEM Hospital, Parel, Mumbai from year 2004 to 2008 with approval of Institutional Ethical Committee. After informed consent, 3-5 ml bone marrow (BM) or/and peripheral blood specimen was collected in ethylenediaminetetraacetic acid tubes. Patients were classified according to French-American-British and WHO classification (>20% blasts in BM as established with morphological techniques; as the diagnostic criteria for acute leukemia). Cytogenetics was carried out as per standard protocols for identification of PML-RAR α . Total 40 patients were included for the molecular diagnosis of FLT3 and NPM1 mutations.

Cytogenetic analysis

Cytogenetic analysis was performed on BM/PB cells after short-term culture. Karyotypes, analyzed after G-banding, were described according to the International System for Human Cytogenetic Nomenclature.^[27] Fluorescence *in situ* hybridization investigations for specific translocations were also carried out as previously described.^[28]

Genetic analysis

DNA was isolated from BM/PB by the phenol chloroform method recommended by Sambrook *et al.*^[29] from the nucleated cells frozen at -80°C . Polymerase chain reaction (PCR) amplifications using a specific

set of primers for respective exons of each of the two genes (FLT3 and NPM1) were carried out with the appropriate set of primers (from Sigma Aldrich, USA) on PTC-225 thermal cycler (MJ Research, USA) and GeneAmp PCR System 9700 (Applied Biosystems, USA).

Screening

Famous like tyrosine kinase-3 gene screening was done by horizontal agarose gel electrophoresis as well as polyacrylamide gel electrophoresis.^[25] D835Y point mutations in the TKD were detected by PCR-restriction fragment length polymorphism with enzyme EcoRV (Fermentas). Screening for another point mutation in this exon was carried out by PCR-single strand conformation polymorphism (SSCP) technique. Screening for NPM1 gene was done using genomic PCR amplification of exon 12 followed by SSCP was used to screen for mutation in this gene.^[24]

Sequencing

The PCR product of all patients showing abnormal shifts in screening or presence of mutant bands from respective PCR of the two genes was sequenced for confirmation of the mutation.

Statistical analysis

Microsoft Excel 2007, Microsoft Inc. Washington DC and GraphPad InStat version 3.10 by GraphPad software Inc., CA, USA were used for all statistical analysis. Data have been reported as median (range). Student's *t*-test/Chi-square/Fisher's exact test has been run to demonstrate statistical significance wherever necessary. $P < 0.05$ was considered to be statistically significant.

Results

Frequency of mutations

A total of 40 patients were diagnosed with APL, out of which 21 were males, and 19 were females. There was no gender bias in any mutation frequency except FLT3/TKD where we found only three adult patients all females harboring D835Y mutation. Out of total 40 APL cases, 13 patients (30%) were found to be FLT3 mutation while 28 APL patients (70%) were FLT3wt. Frequency of FLT3/

ITD was higher (77%) than that of FLT3/TKD (33%) in the present cohort, one adult female was found to harbor both FLT3/ITD and FLT3/TKD. Total 18 APL patients were found to harbor mutations in NPM1, 8 out of 18 (44%) patients were FLT3 mutation/NPM1 mutation, while 10 patients (56%) carried NPM1 mutation without any mutation in FLT3 [Table 1].

All the patients in NPM1 mutation/FLT3 mutation group showed presence of Type A NPM1 mutation, which is the insertion of clustering-based adaptive genetic algorithm (CAGA), while this was present in about 70% patients of NPM1 mutation/FLT3wt. Two out of total 10 patients with NPM1 mutation without mutation in FLT3 had Type D mutation, that is, insertion of CAGG, complex mutation was observed in one patient [Table 2].

Clinical data

Famus like tyrosine kinase-3 mutation patients was presented with a significantly higher total white blood cell counts at the presentation when compared with FLT3wt group ($P < 0.05$, unpaired t -test, confidence interval (CI 95%). Platelet count in the former group was slightly lower than the later, but was not significantly different. Patients with a mutation in NPM1 with FLT3 mutation were observed with significantly higher total counts when compared to those without the mutation in FLT3 ($P < 0.05$, unpaired t -test, CI 95%). Blast percentage, hemoglobin and platelet count were not significantly different in any group.

Treatment and follow-up

Of a total 40 APL patients, 25 underwent chemotherapy while 15 did not receive treatment at our center. Treatment regimen for these patients included arsenic trioxide as described earlier for an Indian study.^[30] Patients who were untreated either could not opt for treatment due to insufficient finances or they chose to take treatment at other centers and, therefore, follow-up of these patients could not be done. Mostly follow-up was done till the first remission. Frequency with which FLT3 mutation patients achieved complete remission (22%) was significantly lower than that of FLT3wt (69%) patients ($P < 0.05$, Fisher's exact test, CI 95%, the relative risk 1.6). Presence of NPM1 mutation with FLT mutation showed a better complete remission frequency of 40% that was quite comparable to the NPM1 mutation group without mutation in FLT3 (50%) [Table 3].

Discussion

Despite of a favorable cytogenetic risk group, patients with APL still relapse with an approximate rate of 15-20%.^[1] Evaluation of MRD by quantifying transcript levels of PML-RAR α or NPM1 been sought by research groups.^[20,31] Characterization of recurring molecular defects such as mutations in FLT3, NPM1, Ras etc., has enhanced our understanding toward basic mechanism of leukemogenesis in past few years. The present study evaluates a cohort of 40 APL patients from

Table 1: Frequency and clinical data of 40 APL patients across respective mutation groups

Mutation	FLT3			NPM1		
	FLT3mut	FLT3wt		NPM1mut/FLT3mut	NPM1mut/FLT3wt	NPMwt/FLT3wt
Total number						
Overall	10	3*	28	8	10	18
Pediatric	4	0	8	3	1	7
Adults	6	3	20	5	9	11
Gender distribution						
Male/female	5/5	0/3	16/12	4/4	4/6	12/6
Median age (in years)						
Median (range)	22.5 (5-60)	45 (27-60)	32.5 (4-80)	22.5 (5-50)	42 (4-40)	31.5 (5-60)
Hb at diagnosis (g/dl)						
Median (range)	6.8 (5-11.3)	5.4 (5.4-6.5)	7.5 (3.8-13)	6.8 (5.2-11.3)	8.5 (5.8-11.9)	6.6 (3.8-13)
WBC at diagnosis ($\times 10^3/\mu\text{l}$)						
Median (range)	93.8 (7.3-384.1)	49.2 (7.1-79.8)	10.8 (1.1-229) [†]	90.4 (7.3-260)	11.2 (2.5-110) [‡]	10.4 (1.1-229.9)
Platelets ($\times 10^3/\mu\text{l}$)						
Median (range)	34 (20-120)	53 (20-90)	57.5 (10-210)	42.5 (20-120)	65 (40-150)	46.5 (10-210)
Blast %						
Median (range)	92 (19-35)	94 (60-95)	71.5 (5-96)	93.5 (70-95)	62 (5-96)	74 (22-95)

Frequency and clinical data N=40 APL patients, mut: mutated, wt: wild type, *: FLT3/TKD was found to be female biased where all 3 patients were adult females while one carried both ITD and TKD, †: FLT3mut has significantly higher WBC count ($P < 0.05$), ‡: Total counts were higher in NPM1mut patients with mutation in FLT3, APL: Acute promyelocytic leukemia, FLT3: Famus like tyrosine kinase-3, NPM: Nucleophosmin

Table 2: Types of NPM1 mutation in APL patients with and without mutation in FLT3

Type	Insertion	Percentage of NPM1mut/FLT3mut	Percentage of NPM1mut/FLT3wt
Type A	Insertion CAGA	100.0	70.0
Type B	Insertion CATG	0.0	0.0
Type D	Insertion CAGG	0.0	20.0
Complex not determined	Insertion not determined	0.0	10.0

Types of NPM1 mutation: N=8 APL patients with NPM1/FLT3mut, where all were observed with 'Type-A' mutation; N=10 NPM1mut/FLT3wt APL patients, APL: Acute promyelocytic leukemia, FLT3: Famus like tyrosine kinase-3, NPM: Nucleophosmin

Table 3: Treatment and follow up data for APL patients

Group	FLT3 mut	FLT3 wt	NPM1mut/FLT3mut	NPM1mut/FLT3wt	NPMwt/FLTwt
Treated (no.)	9	16	5	8	8
Treated (median age)	31.6	33.1	39.3	39.4	23.5
Complete remission	2 (22%)	11 (68.7%)	2 (40%)	4 (50%)	7 (87.5%)
Partial remission	3 (33%)	2 (12.5)	1 (20%)	2 (25%)	0
No remission	4 (44%)	3 (18.7)	2 (40%)	2 (25%)	1 (12.5%)
Untreated (median age)	15	35.25	15	2	10
Untreated (no.)	3	12	3	65	29.3

Treatment and follow up data: Sixty percent (25/40) patients underwent chemotherapy and follow up was done till first remission, APL: Acute promyelocytic leukemia, FLT3: Famus like tyrosine kinase-3, NPM: Nucleophosmin

India belonging to heterogeneous population of different ethnicity and culture. Frequency of FLT3/ITD and FLT3/TKD (D835Y) in the present population is observed to be 25% and 7% respectively, which in accordance with the previous studies from various populations.^[10,15,21,22] However, NPM1 mutation is present in about 50% of the current population of APL patients which has never been observed as a significant incident in other reports.^[19,25,26] Median age of patients with FLT3/ITD is about 23 years, which is quite comparable to the median age value of 28 years reported by an Indian study,^[21] but not to a Japanese study where it has been reported as 56 years,^[11] median age of FLT3/TKD is 45 years as observed in the previous studies.^[10,15,21,22] However, FLT3-D835Y is completely female biased in the current cohort when compared to the reported predominance in males,^[15] reason for which could not be established.

One striking observation in the present study is the higher incident rate of NPM1 mutations, which have never been reported at this frequent rate in APL patients and suggested to be less frequent in Asians as compared to Caucasian in a Thai study.^[26] All the patients with NPM1

mutation/FLT3 mutation harbor "Type A" mutation, which is characterized by insertion of CAGA whereas patients with only NPM1 mutation without FLT3 mutation harbor "Type A" and "Type D" mutation, "Type B" mutation is not observed in the present population.

Clinical features at the presentation for the current cohort of APL patients with or without mutation in FLT3 gene are quite similar to those reported in the other studies. Moreover, presence of NPM1 mutation with FLT3 mutation shows a higher total count when compared to NPM1 mutation with FLTwt, which contributes toward the poor prognosis of former group as already been described by several studies in AML.^[17-19]

Sixty-three percent (25/40) of the total diagnosed APL patients underwent chemotherapy, follow-up data until the first remission is available and is correlated with the incidence of specific mutations. Complete remission was achieved by only 22% of FLT3 mutation patients compared with 68% of FLT3wt APL patients. This observation is quite in accordance with previous studies where complete remission was achieved by 23-40% APL patients with FLT mutation.^[21,32] A better frequency of remission is seen in patients with NPM mutation/FLT3wt which is in accordance with the previous studies deriving results from Asian APL patients^[33,34] and is as good as the remission frequency of NPMwt/FLT3wt group that is, 60% and 75%, respectively.

One of the questions from the present study is why we have lower remission rates in APL in contrast to what has been described in the literature. Though we cannot be sure but it may not be out of place to mention here that general population in large part of India are exposed to arsenic either through ground water contamination or through industrial pollution. This may have produced underlying arsenic resistance situation. However, this hypothesis needs to be conformed through larger well-designed epidemiological studies.

Conclusion

The present cohort of APL patients shows a higher frequency of FLT3/ITD and a female biased incidence of FLT3/TKD. The high frequency of mutation in NPM1 gene of APL patients here is a novel observation where

presence of NPM1 mutation with FLT3 mutation shows a better remission as observed in our previous study on AML patients, but the remission is better in APL patients with NPM1wt/FLT3wt than the group with only NPM1 mutation.^[35] We suggest that a better understanding toward mutation in FLT3 and NPM1 gene in APL patients might help in overcoming the relapse in this favorable group of AML.

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