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Haematological & molecular profile of acute myelogenous leukaemia in India

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Background & objectives: **Recurrent balanced translocations are generally recognized to be a major parameter for prognostication in acute myeloid leukaemia (AML). The chromosomal translocation t(15;17) results in *PML/RARα* fusion gene, t(8;21) results in *AML1/ETO* fusion gene and Inv 16 generates *CBFβ/MYH11* fusion gene. Patients with these mutations have a good prognosis unlike abnormalities in chromosome 5 or 7 or *FLT3* genes. Therefore, we screened the AmL patients for known specific genetic abnormalities that could lead to more definitive prognoses.**

***Methods*: A total of 113 AML patients were evaluated at diagnosis based on routine morphology and cytochemistry and classified according to the WHO criteria. The distribution of AML subtypes was M1(1), M2(32), M3(57), M4(14), M5(1), M6(1) and seven cases where morphological subtype could not be classified. RT-PCR was performed to identify *PML/RARα*, *AML1/ETO*, *CBFβ/MYH11* and *FLT3* internal tandem duplication (ITD).**

***Results*: Of the 57 patients with M3 subtype, 55 had the *PML-RARα* fusion transcript. The prevalence of *bcr3* (short isoform) was higher (62%) than that of *bcr1* (long isoform) (38%) and no correlation was found with age, sex or white blood cell count. *FLT3* internal tandem duplication (ITD) mutations were more frequent in patients with APL than in other AML subtypes (17.5 vs. 8.9%), the frequency greater in patients with *bcr3* isoform (70%) than in those with in *bcr1* isoform (30%). Patients with *FLT3/ ITD* mutations had a significantly higher median white cell count than those without these mutations (55×10^9/l vs. 6.3×10^9/l; $P<0.001$). More patients with *FLT3/ITD* mutations died early (53%) than those without these mutations (16%) ($P<0.01$). *AML1-ETO* fusion transcript was detected in 16 of 56 patients with no correlation with clinical or haematological parameters.**

Interpretation & conclusion: **The results of the present study showed presence of *bcr3* (short isoform) higher than *bcr1* (long isoform). *FLT3* internal tandem duplication (ITD) mutation was predominant in acute promyelocytic leukaemia patients with *bcr3* isoform. Thus, patients with APL who have *FLT3* mutation appear to have a poorer prognosis. Therefore, rapid identification of specific translocations at diagnosis is important for prognostic purposes and their detection should be incorporated into routine assessment.**

Key words Acute myeloid leukaemia - reverse transcription - polymerase chain reaction - *PML-RARα* - *AML1-ETO* - *CBFβ/MYH11*- *FLT3/ITD*

Chromosomal abnormalities are the most important predictors of prognosis in acute myelogenous leukaemia (AML)[1-3]. The chromosomal translocation t(15;17) generates the *PML/RARα* fusion gene which plays an essential role in leukaemogenesis. The most common abnormality in acute promyelocytic leukaemia (APL) is *PML/RARα*, however 2 per cent of APL can have other translocations, *e.g. RARA/PLZF*. Based on the genomic breakpoint in PML gene, three different PML/RARα isoforms are recognized: intron 3, intron 6 and exon 6[1]. Breakpoints within intron 3 (known as BCR3) produce a short (S) *PML/RARα* isoform (PML exon 3: RARα exon 3) whereas breakpoints within intron 6 (BCR1) of *PML* results in a longer (L) form (PML exon 6: RARα exon 3)[1]. The chromosomal translocation t (8; 21) results in the *AML1/ETO* fusion gene and is associated mainly with the M2 French American British (FAB) subtype of AML; nevertheless, it is also seen in patients with the M1 and M4 subtypes. Inversion 16 (inv16) generates the *CBFβ/MYH11* fusion gene and is strongly correlated with, but not uniformly restricted to, *AML-M4Eo*[4].

Acute myeloid leukaemias that express fusion transcripts containing *PML/RARα*, *AML1/ETO*, *CBFβ/MYH11* are associated with a more favourable prognosis[4]; however, abnormalities in chromosome 5 or 7 or duplications of the *MLL* and *FLT3* genes are associated with an adverse outcome[5]. Mutations of the *FLT3* gene are particularly prevalent in patients with *PML/RARα* fusion transcript[6-8].

As accurate diagnosis can lead to, highly specific treatment, screening for known specific genetic abnormalities by reverse transcription-polymerase chain reaction (RT-PCR) could lead to more definitive prognoses. As no studies have been reported from India on the prevalence of these translocations in patients with AML, we undertook this study to address the issue.

Material & Methods

Patients: Between January 2003 and July 2006, 113 AML patients presenting randomly to the Department of Hematology at the All India Institute of Medical Sciences (AIIMS), New Delhi were included in this study. The selection of patients was based on the availability of the sample. Ethical approval for the study was obtained from the Ethics Committee of the AIIMS, New Delhi and the patients gave their written consent for inclusion in the study. Patients who had not received prior chemotherapy and showed the presence of more than 20 per cent blasts in the marrow, were included in the study. The diagnosis was based on routine morphology and cytochemistry and classified according to the WHO criteria[9]. The distribution of AML subtypes was as follows: one case with M1, 31 with M2, 57 with M3, 14 with M4, 1 with M5, 1 with M6 and seven in which the morphological subtype could not be classified. This was because they showed increased number of convoluted cells without cytoplasmic granules or showed blasts with increased granules. Moreover, immunophenotypying was not available for them.

Cytochemistry: Peripheral blood and bone marrow smears were stained with Jenner Giemsa, and cytochemistry was performed with standard methods[10] (myeloperoxidase, Sudan black, non-specific esterase with fluoride and dual esterase). Fifty seven patients showed strong positivity for Sudan black and myeloperoxidase, and all had abnormal hypergranular promyelocytes with bilobed nuclei and Auer rods. These patients were classified as having APL. Bone marrow smears showing positivity for any stain were evaluated by the weighted score system on the basis of morphological features, as described by Andrieu *et al*[11].

Reverse transcription polymerase chain reaction(RT-PCR): Mononuclear cells were isolated from bone marrow and peripheral blood samples by Ficoll-Hypaque centrifugation (Histopaque 1077; Sigma diagnostics, USA). RNA was extracted (Qiagen, GmbH, Germany) and reverse-transcribed to cDNA with random hexamers (Roche diagnostics, GmbH Germany). The quality of cDNA was assessed by amplification of the β2 microglobulin gene. RT-PCR was performed to identify *PML/RARα* and its isoforms, *AML1-ETO*, *CBFβ/MYH11* and *FLT3* internal tandem duplication (ITD). The presence of fragment sizes of 355 base pairs (bp) for the 5′ promyelocytic leukaemia (PML) intron (*bcr3*), 685 bp or 829 bp for the 3′PML intron (*bcr1*) and 585 bp for the 3'PML exon (*bcr2*) were considered positive for *PML/RARα*[12]. Gene-specific PCR was performed for the AML1-ETO transcript with nested primers, and the presence of a fragment size of 110 bp was considered positive for this transcript[11]. The presence of a fragment size of 414 bp was considered positive for *CBFβ/MYH11*[13] and the presence of sizes greater than 366 bp (wild type) was considered positive for *FLT3/ITD*[14]. PCR reactions were set up in GeneAmp PCR system 9700 (Applied Biosystems, USA).

Treatment protocol: At diagnosis, 57 patients with APL were given all-trans retinoic acid (ATRA) at a dose of 45 mg/m^2 per day for 3 days. As part of the protocol, bone marrow was examined on day 28; if complete remission had not been achieved, ATRA was continued and bone marrow was examined again on day 45. After achieving remission, two cycles of consolidation, each of 28 days, consisting of daunorubicin in the same dose on days 1, 2 and 3 were given. Maintenance was given for a total of two years consisting of 6-mercaptopurine (60mg/m^2/day), methotrexate (20mg/m^2/wk) and ATRA (45mg/m^2/day) for 15 days every 3 months. All other patients received standard '3+7' induction chemotherapy, consisting of daunorubicin at a dose of 45 mg/m^2 per day for 3 days and cytarabine at a dose of 100 mg/m^2 per day as a continuous 24 h intravenous infusion for 7 days. Consolidation comprised high-dose cytosine arabinoside (3 g/m^2 every 12 h on days 1, 3 and 5; total, 18 g/m^2). All patients were treated with above protocol and followed up regularly in OPD. Patients dying within seven weeks of start of therapy were considered "early deaths" [15]. In case the day 45 bone marrow was not in remission, patients underwent a prolonged induction schedule lasting between 45-60 days following which their bone marrows were documented to be in morphological and molecular remission.

Statistical analysis: SPSS software (Stata Statistical Software, Release 9.0; Illinois, USA) was used to analyze clinical characteristics and molecular features. Qualitative clinical and laboratory features of patients with and without a *FLT3/ITD* mutation were compared by the chi-squared and Mann-Whitney tests. All tests were 2-sided, and a $P<0.05$ was considered statistically significant.

Results

Fifty five (96.5%) of the 57 patients classified as having APL were positive for PML-RARα. Two patients were negative, although they showed typical morphology of APL and a complete response to ATRA. Complete response was considered when patients achieved a morphologic leukaemia-free state[15]. *Bcr3* isoform was found in 34 (61%) patients and *bcr1* isoform in 21 (38%) (Table). There was no association between *bcr* subtype and age, sex or white blood cell count (WBC) at diagnosis.

AML1-ETO was detected in 16 of 56 patients with AML. As expected, the majority of the patients had the M2 subtype (10 of 16). In addition, this fusion transcript was found in one patient each with the M4, M5 and M6 subtypes and in three patients with unclassifiable subtypes. CBFβ/MYH11 was detected in 3 of 14 patients classified as AML M4. *FLT3/ITD* was identified in 15 of 113 patients of AML. This mutation was more frequent in patients with APL 10 of 55 (18.1%) than in those with other AML subtypes 5 of 57 (8.7%). Of the 10 APL patients, 7 had the *bcr3* isoform; 5 (71.4%) of these died early due to sepsis, one relapsed while on ATRA maintenance and one

Table. Clinical and molecular characteristics of 74 patients identified as having cytogenetic anomalies by RT-PCR

Clinical characteristic	Molecular characteristic			
	PML-RARα		AML1-ETO	CBFα/MYH11
	bcr1	*bcr3*		
No. of patients	21	34	16	3
Median age, yr (range)	29 (11–50)	26 (14–62)	36 (13–55)	31 (24–46)
Male/female	8/11	21/13	9/3	3/0
Median white [blood cell count x 10^9/l (range)	6.4 (1.2–160)	8.5 (1.0–170)	7.2 (2.1–43)	8.4 (2.1–15.7)
Median platelet count x 10^9/l (range)	26 (9–194)	40 (4–178)	26.5 (4–185)	40 (20–121)
Median haemoglobin (g/dl) (range)	6.8 (3.2–9)	7 (2.9–11.2)	5.2 (3.2–12)	7.8 (5.6–8.1)
FLT 3 mutations		*FLT3*-positive (n = 15)	*FLT3*-negative (n = 98)	
Median white blood cell count (x 10^9 /l)		55**	6.3*	
Induction complete remission No. (%)		4 (26)	60 (61)*	
Early death No. (%)		8 (53)	15 (16)*	

*$P<0.01$ compared to *FLT3* – positive patients
**$P<0.001$ compared to *FLT3* – negative patients

was continued on maintenance therapy. Of the three patients with the *bcr1* isoform, one was lost to follow up, one relapsed during consolidation therapy, and one developed sepsis and died early. The median WBC count was significantly (P<0.001) higher in patients with *FLT3/ITD* mutations than in those without (55.0 x 109/l vs. 6.3 x 109/l; P<0.001). Complete remission was achieved by 61 per cent of patients without a *FLT3/ITD* mutation and by 40 per cent of patients with the mutation. 'Early deaths' were observed in more patients with *FLT3/ITD* mutations than in those without these mutations (53 vs. 16%, P<0.01). In all, 10 patients were lost to follow up (Table).

Discussion

In the present study, detection of *PML-RAR*α fusion transcript was found to have a definitive role not only in diagnosis but also in prognostication of APL. Detection of *AML1-ETO* fusion transcript or *CBF*β/*MYH11* also helped in diagnosis of AML subtypes. The prognostic role of *FLT3/ITD* mutation was observed in patients with APL as compared to other subtypes of AML. The prevalence of the *bcr3* isoform in APL patients was considerably higher than that reported in other studies[16-18]. We found a similarly higher prevalence of the *bcr3* isoform in our previous study of 36 patients with APL[19]. Several investigators[16,20] have sought correlations between *bcr* isoform and clinical characteristics at presentation and found that the WBC count at presentation was associated with the hypogranular variant (M3v) morphology and the short (*bcr3*) isoform. We found no association between isoform status and WBC count, age, sex or platelet count at presentation.

Patient, population in this study may not be representative of Indian AML population and may represent referral bias as patients with better prognosis (*e.g*. APL) may be referred in preference compared to other AML patients. Hence, the samples were based on availability, perhaps resulting in a higher prevalence of APL than of other subtypes.

It is noteworthy that within the good-risk cytogenetic group, there are distinct biologic entities which showed marked variation in the incidence of *FLT3* mutations. The frequency was high in patients with PML-RARα, and low in AML1-ETO fusion transcript and in patients without translocations. It is possible that the clinical impact of a *FLT3/ITD* also varies between these different cytogenetic entities. However, there were very few patients with *AML1-ETO* fusion transcript or *CBF*β/*MYH11* and a *FLT3/ITD* to define their prognosis. Consistent with previous reports[7, 8] we found that *FLT3/ITD* was prevalent in APL patients with *PML-RAR*α, fusion transcript. We also found that it was significantly associated with increased WBC count at presentation and the short (*bcr3*) isoform of *PML-RAR*α, which is a well established prognostic factor for APL[14, 21-23].

The relative incidence of *FLT3/ITD* in patients with APL differs in Europe and North America from that in Asia[6,7,23]. The overall incidence in our study was similar to some studies[22,24] and also to our previous study[25] but lower than that reported by others[13,26]. Different studies have reported controversy regarding the prognostic significance of *FLT3/ITD* mutations. Our results showed that *FLT3/ITD* was associated with a significantly higher rate of early death. The effect of *FLT3/ITD* mutation on the incidence of induction deaths has not been reported in other studies on patients with APL[14,22,23], except in one study of 42 patients[27]. The reasons for a higher incidence of 'early death' among patients with this mutation are, however, unclear. In our series, patients with *FLT3/ITD* underwent prolonged induction chemotherapy. The high frequency of the *FLT3/ITD* mutation in patients with APL, particularly those whose WBC counts exceeding 10 x 10^9/l, has led to considerable interest in the potential clinical value of targeted therapy for this disease with FLT3 inhibitors.

Patients with the AML1-ETO fusion transcript achieved complete remission and successfully completed therapy, except for three who died during induction therapy. Thus, patients with an AML1-ETO transcript at presentation are deemed to have good risk disease, with high rates of complete remission, disease-free survival and overall survival. The sample size of patients was small for multivariate analysis to understand the effect of compounding factors.

Rapid identification of specific translocations at diagnosis is important in the decision-making process and identifies breakpoints that may have potential prognostic significance. Overall, our study showed that patients with APL who have a *FLT3/ITD* mutation, appeared to have a poorer prognosis. Therefore, the ability to identify those patients with a high risk of relapse would be a valuable step which may lead to the introduction of alternative forms of therapy in this group of patients. It can also be used in monitoring remission in these patients. Hence, molecular analysis

can provide rapid results to aid their detection and should now be considered as part of the routine work-up of patients with AML at diagnosis, with a view to introducing this parameter into risk stratification and patient management.

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