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Clinical significance of *c-KIT* mutations in Children with Acute Myeloid Leukemia: An Indian Study

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Abstract

Background: *c-KIT* is a receptor tyrosine kinase (RTK) which has a pivotal role in regulation of hematopoiesis. Even though *c-KIT* mutations have prognostic significance in adult Acute Myeloid Leukemia (AML) patients, role of *c-KIT* mutations in the prognostic prediction of pediatric AML patients still remains abstruse. **Aims**: The present study, aims to understand clinical significance of *c-KIT* mutations in pediatric AML patients. **Methods:** In the study we performed mutational analysis of *c-KIT* gene (exon 8, exon 17) in 61 pediatric de novo AML patients with median age of 9 years. Mutation Analysis of *c-KIT* was performed in all cases by Polymerase Chain Reaction and Single Strand conformation Polymorphism followed by Sequencing. **Results and Conclusions:** Mutations were observed in 9.8% of the cases and among the study group exon 8 mutations prevailed. Statistical analysis showed that *c-KIT* mutations were associated with haemoglobin level at diagnosis (exon 8 mutations) (P=0.03) and age at diagnosis (exon 17 mutations) (P=0.02). Overall survival and event free survival of the patients were not associated with *c-KIT* mutation status (P>0.05). Even though *c-KIT* mutations lacks prognostic significance, high frequency of this mutation among children with AML makes it a candidate for targeted therapies by RTK inhibitors.

Keywords
Acute myeloid leukemia, c-KIT mutations, Prognosis, targeted therapy.

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INTRODUCTION

Acute myeloid leukaemia (AML) is an aggressive cancer that originates from bone marrow and this disease occurs as a result of transformation of haematopoietic precursors by acquisition of genetic alterations. Long-term survival in childhood AML is

found to be below 70% [1]. Identification of biomarkers for effective risk stratification is necessary for clinical management of this heterogeneous disease in children. Gene mutations are one of the root causes leading to leukemogenesis. Different studies revealed prognostic relevance of gene mutations in AML [2]. *c-KIT* is a proto-oncogene belong to class III receptor tyrosine kinase (RTK) family. *c-KIT* plays a major role in proliferation, differentiation and survival of haematopoietic progenitor cells. The expression of *c-KIT* was reported in 10% of blast cells in around 64% De novo AML patients. Mutation of this gene results in the constitutive phosphorylation and activation of the *c-KIT* receptor in a ligand independent manner [3,4].

Frequency of *c-KIT* mutations varies among children and adults with AML. As per previous studies, among adult AML patient's exon 8 mutations were most common whereas in paediatric AML exon 17 mutations were found to be more common [5]. In addition, *c-KIT* ITD mutations were rare in juxta membrane domain (JMD) in adults and reported in exon 11 and 12 of paediatric AML patients [5].

Chromosomal abnormalities are recurrent events in paediatric AML patients. Common chromosomal abnormalities in AML like inv (16) (p13q22) and t (8;21) (q22; q22), distort genes encoding subunits of core-binding factor (CBF) which is a transcription factor that regulates the process of haematopoiesis. For this reason, AML cases showing abnormalities like inv (16) (p13q22) and t (8;21) (q22; q22) were termed as core-binding factor AML (CBF AML) [6].

c-KIT mutations were found in a higher frequency (25-52%) in CBF AML patients and have adverse prognostic effect in adult CBF AML patients [7]. However, there were controversial reports on the prognostic significance of *c-KIT* mutations in Paediatric CBF AML patients [8, 9].

Prognostic significance of c-KIT mutations in children remains a debatable issue until now. Prevalence and clinical significance of *c-KIT* mutations were less studied among paediatric AML patients in India. Present study aims to find the frequency of *c-KIT* mutations (exon 8 and exon 17), their association with different clinical parameters, FAB subgroups and their prognostic significance among children with de novo AML in south India.

MATERIALS AND METHODS

Present study was carried out in 61 children with de novo AML, aged 1-14 years. Relapsed AML cases and treatment related AML cases were excluded from the study. The study was approved by Institute review board and Ethics committee of the institute and have been performed in accordance with the ethical standards as laid down in the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. Bone marrow / peripheral blood samples (1- 2 ml) from the patients were collected in heparinized vacutainers after obtaining written informed consent of parents of the patients. Mutation analysis of exon 8 and exon17 of *c-KIT* gene was performed in all cases.

Detection of *c-KIT* mutations

Genomic DNA of patient samples was isolated as previously described [10] and amplified using specific primers for exon 8 and exon 17 of *c-KIT* gene as reported in earlier study [11]. Polymerase Chain Reaction (PCR) was performed with 25µl PCR reaction mixture containing 100 ng of template DNA, 10 pmol of each primer, 0.2 mM of each mix dNTPs, 1X reaction buffer, 1.5mM $MgCl_2$ and 0.5 U of Taq polymerase enzyme (Genei, Bangalore) in a Thermocycler (Applied Biosystems, 2720 Thermal Cycler). Cycling conditions for exon 8 of *c-KIT* gene is as follows, Initial denaturation at 95°C for 1 minute and denaturation at 94°C for 30 seconds, followed by annealing at 57°C for 30 seconds, and extension at 72°C for 1 minute, repeated for 35 cycles followed by a final extension step at 72°C for 6 minutes using the primers, forward '5- GAGTGAAGTGAATGTTGCTGAG-3' and reverse '5- CAAGTGAATTGCAGTCCTTCC- 3' obtained 241 bp long PCR products. Exon 17 of *c-KIT* was amplified using the following conditions, Initial denaturation at 94°C for 1 minute denaturation at 94°C for 30 seconds, followed by annealing at 54°C for 30 seconds, and extension at 72°C for 1 minute, repeated for 35 cycles followed by a final extension step at 72°C for 6 minutes using the primers, forward '5- AAAAGTTAGTTTTCAC TCTTTACAAGT-3'and reverse '5- GAAACTAAAAATCCTTTGCAGGAC- 3' obtained 243 bp long PCR products and the products obtained were tested out on 1.5% agarose gel electrophoresis. PCR products were screened for mutations using single strand conformation polymorphism (SSCP) in a 39:1(8.5%) polyacrylamide gel electrophoresis for 16 hours. The samples that showed a band shift in SSCP were purified and sequenced to confirm mutation.

Statistical analysis

All statistical analyses was performed using SPSS software (IBM SPSS statistics V11.0). To find the association between the *c-KIT* mutation and other variables Fisher's exact test (for categorical variables), students T-test (for normal continuous variates) and Mann-Whitney U test (for non-normal continuous variates) were used. The effect of

mutation on the overall survival (OS) and event free survival (EFS) was estimated using Kaplan-Meier method and assessed by the log rank test. For estimating Overall Survival, patients have been followed up for three years. Overall survival was calculated from the time of diagnosis to death, and EFS was calculated from time of diagnosis to death or Relapse. A P-value of <0.05 was considered statistically significant.

RESULTS

The study was carried out in 61 children with de novo AML. Among them 33 were males and 28 were females (Male: Female; 1.1:1). The median age of the group was 9 years. Of the 61 cases, four (6.6%) showed *c-KIT* exon 8 mutation and two cases (3.3%) showed exon17 mutation. All together six (9.8%) cases showed *c-KIT* mutation. Three cases with exon 8 mutation of *c-KIT* showed deletion-insertion around codon 419 and one case with exon 8 mutation showed a point mutation in codon 439 in Sanger sequencing analysis. Both the *c-KIT* exon 17 mutated cases showed point mutation in codon 816. Mutations were more frequent in males than in females but there was no significant association between gender and *c-KIT* mutation status. *c-KIT* mutations were more frequent in age group 10-14years, 66.7% of mutations occurred in this age group. Even though exon 8 mutations did not show any association with age, exon 17 mutations showed a significant association with higher age at diagnosis (P=0.02) (Table.1). Among the study group, mutations were more frequent in M1 and M4 FAB subtypes of AML (33.3% each). Another two cases with mutations belonged to AML-M2 and AML-M5 subtypes respectively. Statistical analysis did not show any significant association between FAB subtypes and incidence of *c-KIT* mutations (Table.1, Table 2). Association of *c-KIT* mutations with clinical parameters (WBC count, platelet count, haemoglobin level, LDH level, Peripheral blood blast percentage, and bone marrow blast percentage) were evaluated by statistical analysis. A significant association was found between low haemoglobin level at diagnosis and *c-KIT* exon 8 mutations (P=0.03) (Table.2). No other clinical parameters showed any significant association with occurrence of *c-KIT* mutations. (Table.1, Table 2)

We evaluated the association of cytogenetic profiles of patients with c-Kit mutation status. *c-KIT* mutations were exclusively found in patients with normal karyotype (3/6 cases, 50%) and in cases with t (8;21) (q22; q22) (1/6 case, 16%) and inv (16) (p13q22) (2/6 cases, 33.3%). There is no significant association between cytogenetic profile and *c-KIT* mutation status (Table.3). In our study, group there were 11 CBF AML cases and *c-KIT* mutations were observed in three (27.3%) of them. The mutations were only found in exon 8 of *c-KIT* among the CBF AML cases.

Prognostic relevance of *c-KIT* mutation

The patients in the study, group have been followed up for 36 months, but adequate follow-up proportion is obtained only for 12 months. Hence survival analysis was performed only for 12 months. We separately analyzed the survival of patients with *c-KIT* exon 8 mutations and *c-KIT* exon 17 mutations. Survival analysis was performed using Kaplan-Meier analysis and compared by the log rank test. Overall survival probability among *c-KIT* exon 8 mutated cases were similar to that of non-mutated cases (75 ± 21.7 % in mutated cases vs 73.2 ± 6.4%) and we observed a higher overall survival probability (100% in mutated vs 73 ± 6.2 %) and event free survival probability (100% in mutated cases vs 59.1 ± 6.9%) among *c-KIT* exon 17 mutated cases compared to non-mutated cases. However, the difference in survival was not statistically significant and this could be due to small patient numbers in this infrequent paediatric cancer (Table.4).

We further evaluated the influence of *c-KIT* mutations in prognosis of different cytogenetic groups. Based on previous studies different cytogenetic profiles have different prognostic effect. The patients were classified into three cytogenetic risk groups based on their cytogenetic profiles and only those patients having cytogenetic data (n=56) were included in this grouping. The three groups are good risk group (including t (15;17), t (15;17) with additional abnormality, t (8;21), inv (16) with additional abnormalities) n=16), intermediate-risk (Normal Karyotype, numerical and other structural abnormalities, t (8;21) with other abnormalities, n=38); and poor risk (t (9;22) with additional abnormalities, case with structural and numerical abnormalities other than favourable, n=2) (Table.3).

Out of the six *c-KIT* mutated cases, mutations were exclusively found in good risk group (50%, 3/6) and intermediate risk group (50%, 3/6). There is no significant association between cytogenetic risk groups and *c-KIT* mutation status (Table.3). Only *c-KIT* exon 8 mutations were found in the good risk group. Overall survival probability of *c-KIT* exon 8 mutated cases was similar to that of non-mutated cases in good risk group and the event free survival probability was high in mutated cases compared to non-mutated cases (66.7±2.2% in mutated vs 58.7±14.2 %, P = 0.934). Out of the 3 *c-KIT* mutated

cases in intermediate risk group two cases were having mutation in exon 17 and one case was having mutation in exon 8. The overall survival probability was higher in mutated cases (100% vs 72.5 ± 8.4% in mutated cases, P= 0.363) but the event free survival was similar for both mutated and non-mutated cases (Table.5).

Variable	**Total (n=61)**	***c-KIT* exon 17 mutated (n=2)**	***c-KIT* exon 17 wild type (n=59)**	**P-value**
Male/Female Ratio	33/28 (1.17:1)	1/1 (1:1)	32/27 (1.18:1)	1.00
Median age, Years (range)	9 (0 -14)	14 (14)	9 (0-14)	**0.02**[e]
WBC count[a] ($X10^9$/L), Median,Range	2.8 (.16 - 43.69)	8.3 (2.05-14.6)	2.8 (.16-43.69)	0.595
Platelet count ($X10^9$/L), Median, Range	3.4(.8 - 37.7)	3.5(.8-6.3)	3.4(.8-37.7)	0.481
Hemoglobin(g/L), Mean±SD[b]	7.8 ± 2.04	7.6 ± 3.81	7.8 ± 2.03	0.878
Bone marrow Blast%, Mean±SD	61.9 ± 28.03	67.50 ± 17.67	61.61 ± 28.60	0.786
Peripheral Blood Blast %, Mean±SD	54.25 ± 29.46	44.50 ± 14.84	54.57 ± 30.10	0.641
LDH (U/L) [c], Median, Range	1229 (432 –19127)	2101(1841-2361)	1184(432-19127)	0.325
FAB[d] subtypes				
AML-M0	1.6%(1/61)	0% (0/2)	1.7% (1/59)	0.816
AML-M1	11.5%(7/61)	0% (0/2)	11.9% (7/59)	
AML-M2	18%(11/61)	0% (0/2)	18.6% (11/59)	
AML-M3	16.4%(10/61)	0% (0/2)	16.9% (10/59)	
AML-M4	14.8%(9/61)	50% (1/2)	13.6% (8/59)	
AML-M5	26.2%(16/61)	50% (1/2)	25.4% (15/59)	
AML-M6	1.6%(1/61)	0% (0/2)	1.7% (1/59)	
AML-M7	3.3%(2/61)	0% (0/2)	3.4% (2/59)	
AML-other	6.6%(4/61)	0% (0/2)	6.8% (4/59)	

Table.1: Association of *c-KIT* exon 17 mutations with different clinical parameters and FAB subtypes
a) WBC-white blood cell, b) SD-Standard Deviation, c) LDH-Lactate dehydrogenase, d) FAB- French-American-British classification, e) significant.

Table.2: Association of *c-KIT* exon 8 mutations with different clinical parameters and FAB subtypes

Variable	Total (n=61)	*c-KIT* exon 8 mutated (n=4)	*c-KIT* exon 8 wild type (n=57)	P-value
Male/Female Ratio	33/28 (1.17:1)	1/3 (.3:1)	32/25 (1.28:1)	0.325
Median age, Years (range)	9 (0 -14)	9.5 (3-14)	9 (1-14)	0.725
WBC count[a] ($X10^9$/L), Median,Range	2.8 (.16 - 43.69)	2.8 (.16-43.69)	2.2 (.8-13.6)	0.836
Platelet count ($X10^9$/L), Median, Range	3.4(.8 - 37.7)	4.6 (1.4 - 7.1)	3.4 (.8-37.7)	0.916
Hemoglobin(g/L), Mean±SD[b]	7.8 ± 2.04	5.6 ± 1.7	7.9 ± 1.9	**0.03**[e]
Bone marrow Blast%, Mean±SD	61.9 ± 28.03	53.50 ± 31.76	62.49 ± 27.97	0.540
Peripheral Blood Blast %, Mean±SD	54.25 ± 29.46	45.25 ± 27.28	54.88 ± 29.73	0.532
LDH (U/L) [c], Median, Range	1229 (432 –19127)	1205(612-3967)	1229(432-19127)	0.702
FAB[d] subtypes				
AML-M0	1.6%(1/61)	0%(0/4)	1.7%(1/57)	0.306
AML-M1	11.5%(7/61)	50%(2/4)	8.7%(5/57)	
AML-M2	18%(11/61)	25%(1/4)	17.5%(10/57)	
AML-M3	16.4%(10/61)	0% (0/4)	17.5%(10/57)	
AML-M4	14.8%(9/61)	25%(1/4)	14.2%(8/57)	
AML-M5	26.2%(16/61)	0% (0/4)	28.2%(16/57)	
AML-M6	1.6%(1/61)	0% (0/4)	1.7%(1/57)	
AML-M7	3.3%(2/61)	0% (0/4)	3.5%(2/57)	
AML-other	6.6%(4/61)	0% (0/4)	7%(4/57)	

a) WBC-white blood cell, b) SD-Standard Deviation, c) LDH-Lactate dehydrogenase,
d) FAB- French-American-British classification, e) significant.

Table.3: Association of *c-KIT* exon 8 mutations with cytogenetic profiles and cytogenetic risk groups

Cytogenetic Profile	Total (n=61)	*c-KIT* exon 8 mutated	*c-KIT* exon 8 wild type	P-value	*c-KIT* exon 17 mutated	*c-KIT* exon 17 wild type	P-value
Normal	41%(25/61)	25%(1/4)	42.1%(24/57)	0.251	100%(2/2)	38.9%(23/59)	0.809
t(8;21), t(15;17),inv(16), Other structural abnormalities	23%(14/61)	25%(1/4)	22.8%(13/57)		0%(0/2)	23.7%(14/59)	
Numerical abnormalities	14.8%(9/61)	0%(0/4)	15.8%(9/57)		0%(0/2)	15.25%(9/59)	
Karyotype with Structural and numerical abnormalities	13.1%(8/61)	50%(2/4)	10.5%(6/57)		0%(0/2)	13.6%(8/59)	
Unknown	8.2% (5/61)	0%(0/4)	8.8%(5/57)		0%(0/2)	8.5%(5/59)	
Cytogenetic Risk group	**Total (n=56)**	***c-KIT* exon 8 mutated (n=4)**	***c-KIT* exon 8 wild type(n=52)**	**P-value**	***c-KIT* exon 17 mutated (n=2)**	***c-KIT* exon 17 wild type(n=54)**	**P-value**
Favourable	28.6%(16/56)	75%(3/4)	25%(13/52)	0.141	0%(0/2)	29.6%(16/54)	1.00
Intermediate	67.9%(38/56)	25%(1/4)	71.1%(37/52)		100%(2/2)	66.7%(36/54)	
Poor	3.6% (2/56)	0%(0/4)	3.8%(2/52)		0%(0/2)	3.7%(2/54)	

Table 4. Survival probability of *c-KIT* mutated cases among the pediatric AML patients.

Total AML Samples (n=51)	Overall Survival probability (%)	Standard Error (%)	P-Value	Event Free Survival probability (%)	Standard Error (%)	P-Value
c-KIT exon 8 mutated	75	21.7	0.847	75	21.7	0.304
c-KIT exon 8 wild type	73.9	6.3		67.9	6.7	
c-KIT exon 17 mutated	100	0	0.495	100	0	0.623
c-KIT exon 17 wild type	73	6.2		59.1	6.9	

Table 5. Survival probability of c-KIT mutations among different cytogenetic risk groups

Intermediate risk group Total (n=39)	Overall Survival probability (%)	Standard Error (%)	P-Value	Event Free Survival probability (%)	Standard Error (%)	P-Value
c-KIT mutated (n=3) (exon 8 mutated n=1, Exon 17 mutated n=2)	100	0	0.363	50	35.4	0.547
c-KIT wild type (n=36)	72.5	8.4		51.2	9.5	
Good Risk Group Total (n=13)						
c-KIT mutated (n=3) (exon 8 mutated n=3, Exon 17 mutated n=0)	66.7	27.2	0.884	66.7	27.2	0.934
c-KIT wild type (n=10)	67.1	13.5		58.7	14.2	

DISCUSSION

The present study is the first Indian report on *c-KIT* mutation status in paediatric AML patients with in an age group of 1-14 years. The study analysed frequency of *c-KIT* mutations (exon 8 and exon 17) among 61 paediatric AML patients and evaluated its association with clinical parameters, cytogenetic profile and clinical outcomes of the patients. The overall incidence of *c-KIT* mutations ranges from 6-8% in adults [12] and 3-5% [13, 14, 15] in paediatric patients. *c-KIT* mutations were predominant in CBF AML cases with a frequency of 25-35% in adult [16, 17] and comparatively lower frequency of 17-25% was observed among paediatric cases [8,9]. In the present study out of 61 paediatric patients with de novo AML, 9.8% of the patients showed mutations either in exon 8 or in exon 17 of *c-KIT* gene. This is a high frequency of *c- KIT* mutations compared to previous reports [13, 14, 15]. Among the CBF AML cases in the study group also a comparatively higher number of cases (27%) showed *c-KIT* mutation. A similar trend of high incidence of *c-KIT* mutations was observed in a report on adult AML patients in north India [3, 18]. The higher incidence should be verified by large cohort studies since we have a relatively small study group. From the observations obtained from *c-KIT* mutation studies in adult and paediatric patients from India we can assume that race and ethnic variations may influence the c-KIT mutation frequencies among the Indian population.

In our study we got a higher frequency of *c-KIT* exon 8 mutations (6.6%) than exon17 mutations (3.3%) which is contradictory to previous reports on adult AML patients which claims exon 17 mutations were more frequent [16,17]. Another study describing *c-KIT* mutations among paediatric CBF AML patients

also observed a higher frequency of exon 8 mutations among paediatric patients as reported in our study [9]. Thus, Incidence of *c-KIT* mutations not only increases with increase in age but also the exons of the gene affected by mutations was also changed. Most of *c-KIT* mutations in the study group were observed in age group 10-14years, 66.7% of mutations occurred in this age group. Many studies reported that there is no association between *c-KIT* mutation status and age at diagnosis [9,19]. Among the present study group, *c-KIT* exon 8 mutations did not show any association with age but exon 17 mutations showed a significant association with age (P=0.02). Also, in the present study group, *c-KIT* mutations were more frequent in M1 and M4 FAB subtypes of AML and mutations were not prominent in AML-M2 as reported in earlier studies [12, 20].

In the present study we did not find any significant association between *c-KIT* mutation status and clinical parameters like gender, white blood cell count, platelet count, percentage of bone marrow blasts at diagnosis, percentage of peripheral blood blast at diagnosis and LDH levels**.** Similar results were observed in previous studies also [21]. As an exception, a significant association was observed in the present study between low haemoglobin levels at diagnosis and *c-KIT* exon 8 mutation status which is not reported in previous studies.

Previous reports on prognostic significance of *c-KIT* mutations were contradictory. Some studies found *c-KIT* as a poor prognostic indicator in CBF AML [9, 22] while other studies did not find any prognostic significance of *c-KIT* mutations either in overall survival or in disease free survival of AML patients [13, 23]. In the present study, *c-KIT* mutations did not show any prognostic significance either in overall survival or in disease free survival of paediatric AML patients.

CONCLUSION

In the present study, *c-KIT* mutations were observed in 9.8% of the patient group and 66.7% of the mutations were found in an age group of 10-14 years. Unlike previous studies *c-KIT* exon 8 mutations were more frequent in the study group than exon 17 mutations. *c-KIT* mutations were not associated with any clinical parameters of the patients except age at diagnosis and haemoglobin level at diagnosis. We did not find any association between survival of patients and *c-KIT* mutation status. The frequency of c-*KIT* mutations were higher in the study group than in previous studies. Comparatively high incidence of c-KIT mutations among paediatric AML patients in Indian population makes it a potential target for receptor tyrosine kinase inhibitor therapy. Such targeted therapies may augment the conventional chemotherapy which may help in improved clinical outcome in paediatric AML patients.

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