RESEARCH ARTICLE

Characterization of CEBPA Mutations and Polymorphisms and their Prognostic Relevance in De Novo Acute Myeloid Leukemia Patients

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Abstract

The CCAAT/enhancer-binding protein-alpha (CEBPA) is a transcriptional factor that plays a crucial role in the control of proliferation and differentiation of myeloid precursors. This gene was recognized as the target of genetic alterations and were associated with clinical complexity among AML. We here analyze the frequency and types of CEBPA mutations and polymorphisms in a de novo AML patients from South India and tried to find out associations of these variations with different clinical parameters and the prognostic significance in AML. Study was carried out in 248 de novo AML patients, cytogenetic analysis was performed from the bone marrow samples and was karyotyped. PCR-SSCP analysis and sequencing was performed for the detection of CEBPA gene variations. All the statistical analysis was performed using SPSS 17 (statistical package for social sciences) software. Pearson Chi-square test, Mann-Whitney U test, Kaplan-Meier survival analysis and log rank tests were performed. CEBPA mutations were detected in 18% and CEBPA polymorphisms were detected in 18.9% of AML cases studied. Most of the mutations occured at the C terminal region. Polymorphisms were detected in both N and C terminal region. with most common being, c.584_589dup ACCCGC and c.690G>T. A significant association was not observed for the mutation and polymorphism with respect to clinical and laboratory parameters. Survival advantage was observed for the mutated cases compared to non mutated cases, especially for the normal karyotype groups. Polymorphisms has no effect on the survival pattern of AML patients. CEBPA mutation and polymorphisms were observed with similar frequency and was identified in all the FAB subtypes as well as in cytogenetic risk groups in our study population, but CEBPA mutations alone confer a prognostic value for NK AML patients.

Keywords: CEBPA-AML-mutation-polymorphism-normal karyotype-cytogenetic risk group

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Introduction

Gene mutations and aberrant chromosomal translocations provide a selective growth advantage to the leukemic stem cells (Frohling et al., 2005). Among the oncogenes that affects proliferation and cell death, the most important is those that are involved in the haematopoietic differentiation pathways (Tenen, 2003). CEBPA gene, a single exon gene located on chromosome 19q13.1 coding for 358 aminoacids, forming a 42-KDa protein belongs to the CCAAT/enhancer binding protein family, which is involved in the balance between cell proliferation and terminal differentiation (Leroy et al., 2005). CEBPA protein, localises to the nucleus and acts as transcription factor that posses four principal domains: basic leucine zipper domain (bZIP), composed of a basic region able to interact with specific DNA sequences (DBD-DNA binding Domain) and a leucine zipper domain mediating a homo or hetro dimerization in the COOH terminus and two trans activation domains TAD 1 and TAD 2 in the NH2 terminus (Friedman et al., 1990). CEBPA gene can be translated to two isoforms, a p42 and a p30 protein of which the later one lack the normal CEBPA functions due to the lack of TAD1 functional domain and had a dominant negative effect on p42 wild-type protein (Pabst et al., 2001).

CEBPA play a crucial role in granulocytic differentiation and could block the progression from the G1 to S phase thus by inducing the terminal differentiation of haematopoietic cells (Friedman et al., 2002; Cammenga et al., 2003). Deregulation of CEBPA activity is widely known to contribute to the myeloid transformation via reducing the differentiation potential (Wang et al.,

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2003). CEBPA mutations were detected in 7% to 15% of patients with AML (Pabst et al., 2001; Preudhomme et al., 2002; Frohling et al., 2004). CEBPA polymorphisms were also discovered at higher incidence than mutations and occurred across all cytogenetic subgroups. There are two major types of CEBPA mutations, one comprises of the mutations that affect the CEBPA DNA binding via alteration of the COOH terminal bZIP, and the other comprises of frame shift mutation in the NH2 terminal affecting the normal translation producing a 30KDa protein which has the capacity to further reduce the activity of wild type protein.

In this study, we aimed to detect the frequency and types of CEBPA mutations in de novo AML patients from South India. Interestingly CEBPA polymorphisms were also detected similar to that of mutation rate. Clinical and laboratory characteristics, cytogenetic parameters and FAB subtypes of patients were compared with CEBPA polymorphism and mutations. The prognostic significance of these variations was also studied. To our knowledge this was the first report from South India regarding the CEBPA mutation and polymorphism and its association with clinical and pathological characters and prognostic association of these variations in de novo AML patients.

Materials and Methods

The study included 248 de novo AML patients (aged 18-70yrs), who attended the medical oncology clinic of Regional Cancer Centre, Trivandrum between January 2009 and May 2011. Bone marrow/peripheral blood samples (2ml) were collected from the patients at the time of diagnosis after getting the written informed consent. The leukemia subtypes were established according to the FAB classification and the diagnosis were pathologically confirmed.

Cytogenetic analysis

The bone marrow samples (0.5ml) were cultured in the RPMI 1640 medium supplemented with fetal bovine serum (Panbiotech). Colchine was used to arrest the cells in the metaphase stage followed by hypotonic treatment, fixation, washing and slide preparation. Giemsa Trypsing Giemsa (GTG) banding and karyotyping was performed (Henagariu et al., 2001). The chromosomal abnormalities are described according to the International System for Cytogenetic Nomenclature (Shaffer et al., 2009), cytogenetic risk stratification was established according to the refined Medical Research Council (MRC) criteria (Grimwade et al., 2010). The study was approved by the ethical Committee of Regional Cancer Centre. The clinical and biological characteristics of AML patients including age, sex, WBC count, blast percentage, Hb %, LDH levels, platelet count etc were noted from the case file.

Molecular analysis

CEBPA is an intron less gene. Genomic DNA was isolated form Bone marrow/peripheral blood samples and four overlapping primer pairs were used to amplify the entire CEBPA coding region giving 306bp, 309bp, 366bp and 266bp for the four fragments respectively (Gombart et al., 2002). PCR-SSCP analysis was performed for mutation detection. 25ul PCR reaction was performed for all the fragments. PCR amplification of fragment one, two and three were carried out using 200ng DNA, 10pmol of each primer, 10mmol/L each dNTPs, 6% dimethyl sulphoxide (DMSO) and 4% formamide as additives facilitating amplification of GC-rich template and 2.5U Taq polymerase were used. The cycling condition include 94°C, 2minute; 35 cycles of 95°C, 30 seconds; 57°C, 50 seconds; 72°C for 50 seconds and final extension of 72°C for 7 minutes. For PCR amplification of the fourth fragment in addition to the common reagents MgCl2 and 6% DMSO were used. PCR conditions were as follows: 95°C, 5minutes; 35 cycles of 94°C 30 seconds; 66°C for 30seconds; 72°C for 30 seconds and a final extention of 72°C for 5 minutes. The PCR products were then subjected to SSCP analysis at 400V for 18-20hrs. After post staining and drying the band shifts were noted and the samples with different migrations were subjected to direct sequencing (Bangalore Genei) and analyzed for mutations or polymorphisms.

Statistical analysis

Statistical analysis was done using SPSS 17 (statistical package for social sciences) software. Quantitative variables were summarized using median (range). Patient characteristics were analyzed using the Mann-Whitney test for continuous variables and Pearson chi-square test for the categorical variables. For analyses, a P value of less than 0.05 was considered statistically significant. Survival analyzes was carried out using the Kaplan Meier survival and assessed using the log rank test. For overall survival (OS) time from diagnosis up to death from any cause or last follow up was calculated. Overall survival up to 36 months was calculated.

Results

Incidence of CEBPA mutations and polymorphisms in AML and association with FAB subtypes

CEBPA mutations were detected in 45/ 248 (18%) cases analyzed and occur in all FAB subtypes except M6. The highest incidence of mutation was detected in M1 patients with 26% followed by Mo (25%), M2 (23%), M5 (16%), M3 (13%), M4 (11%) respectively. . 14% of the mutations were also detected in AML unclassified group. As like mutations polymorphism in this gene were

Table 1. Incidence of CEBPA Mutation and Polymorphisms in AML Patients

FAB Subtypes	Total (%) n=248	CEBPA Mutation n=45	CEBPA polymorphism n=47
M0	4	1 (25%)	1 (25%)
M1	46	12 (26%)	5 (11%)
M2	52	12 (23%)	10 (19.2%)
M3	31	4 (13%)	5 (16.1%)
M4	36	4 (11%)	5 (14%)
M5	69	11 (16%)	20 (29%)
M6	3	0	1 (33%)
AML Unclassified	7	1 (14%)	0

identified in 47 (18.9%) cases with a higher polymorphism rate in M5 subtype (29%) than the CEBPA mutations. Thus in M1 subtype the incidence of mutation was high compared to polymorphism and in M5 subtype incidence of polymorphism was high compared to mutation. FAB M6 subtype was observed in three cases, among them one case showed polymorphism (Table 1).

Clinical and laboratory parameters of AML patients with respect to mutations and polymorphisms

The majority of patients with CEBPA mutation and polymorphism were middle aged adults with a median age of 40 and 38 respectively. Both mutation and polymorphisms occurred in all age group with higher frequency at 40years of age. A steep decline was noted after the age of 40 in the incidence of mutation as well as polymorphism. More over CEBPA variations were somewhat equally distributed in both sex, 25 male/20 female in case of mutation and 21 male/26 female in case of polymorphism. Age or gender related statistically significant association was not detected with respect to mutated or polymorphic cases when compared to wild type. Clinical parameters such as WBC count, blast percentage, haemoglobin, platelet, LDH levels etc also did not show any correlation with CEBPA mutation compared to wild type. Significant association with FAB subtypes or karyotype pattern not observed with respect to CEBPA mutation status (Table 2).

Incidence of CEBPA mutation and polymorphism in different cytogenetic subgroups

The cytogenetic data was available for 233 patients. Fifty one patients were classified as having favourable karyotype including 19 cases of t (8; 21), 27 cases of t(15;17) and 5 cases of inv(16). Normal karyotype was detected in 138 cases. Numerical and other structural abnormalities were detected in 35 cases. Complex karyotype was detected in 9 cases.

Table 2. Association of Clinical and Laboratory Parameters with Respect to CEBPA Mutation Status

Parameters	CEBPA Mutation Negative n=203	CEBPA Mutation Positive n=45	P value
Median Age (range)	40 (13-68)	40 (17-70)	0.96
≤50	154 (76%)	34 (76%)	
>50	49 (24%)	11 (24%)	
Gender Male	99(48.8%)	25 (56%)	0.41
Female	104(51.2%)	20 (44%)	
Median WBC count x 10^9/L (range)	17.7 (0.6-297)	23.25 (770-346.4)	0.33
Median PB blast% (range)	62 (10-98)	69 (20-93)	0.44
Median BM blast % (range)	70 (4-96)	73 (20-92)	0.76
Median Platelet Count x 10^9/L (range)	42 (6-358)	31 (7-356)	0.13
Median Hb g% (range)	8.1(3-16.4)	8.2 (4.1-14)	0.25
Median LDH levels (IU/L) (range)	1250 (349-16144)	1262 (478-4565)	0.5
Karyotype pattern			0.53
Poor risk group	8 (4%)	1 (2.2%)	
Normal Karyotype	109 (57%)	30 (66.7%)	
Intermediate risk group excluding NK	28 (14%)	6 (13.3%)	
Good risk group	45 (22%)	6 (13.3%)	
Karyotype failure	13 (6%)	2 (4.4%)	
FAB Subtype			
M0	3 (1.5%)	1 (2.2%)	
M1	34 (16.7%)	12 (26.7%)	
M2	41 (20.2%)	11 (24.4%)	
M3	27 (13.3%)	4 (8.9%)	
M4	30 (14.8%)	6 (13.3%)	0.57
M5	60 (29.6%)	9 (20.0%)	
M6	2 (1.0%)	1 (2.2%)	
AML UC	6 (3.0%)	1 (2.2%)	

Table 3. Incidence of CEBPA Mutation and Polymorphism in Different Cytogenetic Subgroups

CYTOGENETICS	Total (%)	CEBPA Mut (%)		Polymorphism (%)	
Total	248	45 (18%)		47 (18.9%)	
Un Known	15 (6%)	02 (13%)		2 (13%)	
Normal Karyotype	138 (56%)	30 (22 %)		32 (23%)	
Aberrant Karyotype	95 (38%)	13 (14%)	*P*=0.119	15 (15.7%)	*P*=0.071
t (8;21)	19 (7.6%)	01 (5%)		1 (5%)	
t (15;17)	27 (10.8%)	04 (15%)		5 (18.5%)	
Inv (16)	05 (2%)	01 (20%)		0	
Numerical abnormality	28 (11.2%)	04 (14%)		4 (14.2%)	
Other structural abnormality	07 (2.8%)	02 (29%)		2 (29%)	
Complex karyotype/≥3 clonal abnormalities	9 (3.6%)	01 (11%)		1 (11%)	

CEBPA mutations were identified across all the cytogenetic subgroups with slight increase in the normal compared (22%) to abnormal karyotype (14%), statistical significance not reached. CEBPA polymorphism was also distributed in all cytogenetic groups with increased incidence in normal karyotype (23%) compared to aberrant karyotype (15.7%), but statistical significance not obtained.

Among the favourable cytogenetic group, polymorphism was not detected in the inv(16) cases. Both mutation and polymorphisms were detected in 5% of t (8;21) and 15% and 18.5% of t(15;17) cases, respectively . In 1/5 cases with inv (16) carried CEBPA mutation. The distribution of mutation and polymorphism was somewhat equivalent in both normal and abnormal karyotypes (Table 3).

Even though the incidence of mutation does not show any statistical significance in the normal karyotype group, the majority of the mutated cases (67%) comprises of the normal karyotype. In CEBPA polymorphic cases also normal karyotype (68%) form the major category compared to other groups.

Table 4. CEBPA Mutations and Polymorphisms and Predicted Amino Acid Changes Observed in the Study

Domain	Nucleotide change	Amino acid change	Effect	Previous Reports	References
SINGLE CEBPA ALTERATIONS					
Upstream of TAD1	c.68-69ins C	p.H24fs*84	Truncated Protein	Yes	Dufour et al., 2012
TAD 1	c.287-311del25	p.G96fs*56	Truncated Protein	Yes	Szankasi et al., 2011
TAD 1	c.238delG	p. D80fs*80	Truncated Protein	No	
TAD 2	c.389G>T	p.G130V	Missense Mutation	No	
bZIP	c.963-964insATT	p.N321-D322insN (homozygous)	Aminoacid insertion	No	
bZIP	c.949-950insGTC	p.E316-L317insR (homozygous)	Aminoacid insertion	Yes	Ahmad et al., 2012
Upstream of bZIP region	c.811G>A	p.A271T	Missense Mutation	No	
DOUBLE CEBPA ALTERATIONS					
TAD 1	c.232delC	p.L78fs*82	Truncated Protein	Yes	Wouters et al.,2009
bZIP	c.963-964insATT	p.N321-D322insN	Aminoacid insertion	No	
B/w TAD1 & TAD 2	c.323-324insACTA	p.108Yfs*63	Truncated Protein	No	
TAD 1	c.247 del C	p.Q83fs*77	Truncated Protein	Yes	Szankasi et al., 2011
bZIP	c.955-956ins24	p.T318-S319 ins KLQVLELT	8Aminoacid insertion	No	
Upstream of TAD1	c.64-74 del 11	p.P22fs*82	Truncated Protein	No	
bZIP	c.939-940insAAG	p.K313-V314insK	Aminoacid insertion	Yes	Greif et al., 2012
TAD 1	c.226del G	p.E76fs*84	Truncated Protein	No	
Upstream of TAD1	c.197-198delCC	p.A66fs*94	Truncated Protein	No	
bZIP	c.1036 C>T	p.P346S	Missense Mutation	No	
Upstream of bZIP region	c.661C>T	p.Q221X	Truncated protein	No	
Upstream of TAD1	c.117-118insCC	p.P40fs*121	Truncated Protein	No	
bZIP	c.912-913insTTG	p.K304-Q305insL	Aminoacid insertion	Yes	Kim et al., 2012
Upstream of TAD1	c.113-114insG	p.G38fs*70	Truncated Protein	No	
B/w TAD1 & TAD 2	c.318-319insT	p.D107X	Truncated Protein	No	
bZIP	c.914A>C	p.Q305P	Missense Mutation	No	
TAD 2	c.555G>A	p.P85P	Silent Mutation/ Polymorphism?	No	
Upstream of bZIP region	c.811G>A	p.A271T	Missense Mutation	No	
POLYMORPHISM ALONG WITH MUTATIONS OF CEBPA OBSERVED IN AML PATIENTS					
bZIP	c.936-937insCAG	p.Q312-K313insQ	Aminoacid insertion	Yes	Dufour et al., 2012
Upstream ofbZIP region	c.811G>A	p.A271T	Missense Mutation	No	
	c.690G>T	p.T230T	Polymorphism	Yes	
Upstream of bZIP region	c.811G>A	p.A271T	Missense Mutation	No	
	c.690 G>T	p.T230T	Polymorphism	Yes	
Upstream of bZIP region	c.811G>A	p.A271T	Missense Mutation	No	Pabst et al., 2001; Fuchs et al., 2009
TAD 2	c.573C>T	p.H191H	Polymorphism	Yes	
MAIN POLYMORPHISMS OF CEBPA GENE OBSERVED IN THE STUDY					
Upstream of bZIP region	c.690G>T	p.T230T	Polymorphism	Yes	Frohling et al., 2004; Leecharendkeat et al., 2008
TAD 2	c.584-589dup ACCCGC	p.P194-H195dup	Polymorphism	Yes	
TAD 2	c.573C>T	p.H191H	polymorphism	Yes	Pabst t et al., 2001; Fuchs et al., 2009

P1-polymorphism 690 G>T according to GenBank Accesion No. NM_004364.2 and 1281 G>T according to GenBank Accesion No. U_34070. P2-polymorphism 584_589 dup according to GenBank Accesion No. NM_004364.2 and 1175_1180dup according to GenBank Accesion No. U_34070. P3-polymorphism 573 C>T according to GenBank Accesion No. NM_004364.2 and 1164 C>T according to GenBank Accesion No. U_34070.

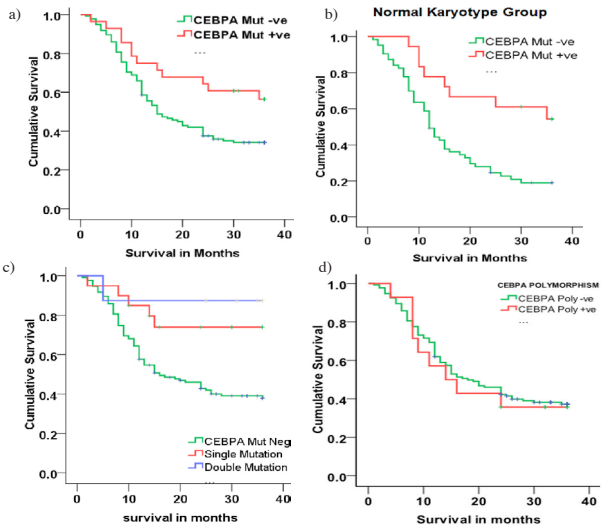

Figure 1. Kaplan Meier Survival Curves Showing Association of (a) CEBPA Mutation with Overall Survival of De Novo AML Patients (b) CEBPA Mutation and Overall Survival of De Novo AML Patients with Normal Karyotype (c) Single and Double Mutations of CEBPA and Overall Survival of AML Patients, (d) CEBPA Polymorphism with Overall Survival of De Novo AML Patients

Characterization of CEBPA variations

By sequencing the CEBPA coding regions, that showed variation in the migration pattern of SSCP, twenty-five distinct type of nucleotide changes and three polymorphisms were identified among the 45 (18%) mutated patients including 17 novel and 8 known mutations (Table 4). Among the novel mutations observed one is a substitution mutation without causing any amino acid change (silent mutation). Of the 45 mutated patients, 10 patients had the combination of N- and the C-terminal type of mutation, referred here as double alterations/ mutations, 4 cases had a single N-terminal mutation, 4 had a single C terminal mutation and 27 cases had both C-terminal type of mutation and polymorphism, out of which one case had double alteration/mutation at the C-terminal region along with a polymorphism. In two cases C terminal mutation was observed along with TAD2 polymorphism, while in the remaining cases both the mutation and polymorphisms were observed at the same regions ie upstream of bZIP.

N terminal mutations were detected in 14/45 (31%) cases and form the sole CEBPA abnormality in 4 cases, which include substitution mutation c.389G>T (p.G130V), formed due to substitution of G to T at codon 130 causing an aminoacid change from glycine to valine. In one patient a one nucleotide deletion is observed at position c. 238delG (p. D80fs*80) leading to the formation of truncated protein. Both these mutations were not reported and considered as novel variations. In other two patients an already reported mutation was observed, which include an insertion C [c.68-69ins C, p.H24fs*84] and a 25 nucleotide deletion [c.287-311del25, p.G96fs*56] both leading to the formation of truncated protein. C terminal mutation was observed in 41/45 (91%) cases. Among them in 4 cases C terminal mutation form the sole CEBPA variation, which include two novel mutations like c.811G>A and c.963-964insATT and already reported mutation of insertion GTC, c.949-950insGTC. In the remaining cases one case showed two mutations, c.811G>A at upstream of bZIP leading to substitution affecting aminoacid and c.936-937insCAG with in the bZIP region leading to aminoacid insertion along with a polymorphism c.690G>T in patient F47. In 24 cases a C terminal mutation c.811G>A at upstream of bZIP region was observed together with polymorphism c.690G>T. In two cases c.811G>A was observed with TAD 2 polymorphism c.573C>T). In the remaining cases C terminal mutation was observed along with N terminal mutation (double alterations).

The most common variation observed in the study groups was the c.811G>A mutation (66.6%, 30/45). It was observed as a sole abnormality in two cases. This mutation was observed in one double alteration cases, where this was seen along with silent mutations at the TAD2 region c.555G>A. In the remaining 27 cases this mutation was observed along with polymorphisms like c.690 G>T (25 cases) and c.573C>T (2 cases). Thus this CEBPA gene variation c.811G>A was repeatedly observed either single or in combination with other alterations (30/45) and form the major C-terminal missense mutation that leads to an aminoacid change from alanine to threonine. This was not reported in the previous studies so far and is considered novel. Further studies are needed to validate the importance of this particular mutation in AML. The other novel mutations observed in our study was detailed in the table. Sequence numbering given is in accordance with Gene Bank accession number NP_004364.2. All the alterations obtained in the CEBPA gene were heterozygous except for two, c.963-964insATT (novel) and c.949-950insGTC. Most of the N terminal mutation lead to formation of truncated protein, which include the TAD1 mutation, consisted of insertions and deletions, C terminal mutation at bZIP region include the aminoacid insertions and the bZIP upstream mutations include the missense mutations.

In the present study three different polymorphism has been identified, the most common being the c.690G>T in the upstream of bZip region with frequency of 10% (25/248) and c.584-589dup ACCCGC observed in the TAD2 region in 8% (20/248) of the study population. Another polymorphism c.573C>T, which also occurred at the TAD2 region was observed in only 2 cases (0.8%). All these polymorphisms are non pathogenic and are reported. Altogether 47 (18.9%) cases showed polymorphism. Details regarding the mutations and polymorphisms were shown in Table 4.

Survival analysis

The prognostic impact of CEBPA coding region mutation in a well defined group of 163 AML patients who have undergone standard treatment were subjected to survival analysis. As both mutation and polymorphisms were observed in similar frequency in the study group, survival effect of both mutation and polymorphism were analysed in the AML patients and also in different cytogenetic groups. Out of 163 cases 28 cases showed mutation. Survival up to 3years was analyzed. The

overall survival (OS) for CEBPA mutated cases was found to be more compared to wild type with mean survival 26.59±2.34 for mutated *vs* 20.11±1.09 months for the wild (P=0.028), providing a survival advantage for the CEBPA mutated cases (Figure 1a). In cytogenetically good prognostic group, CEBPA mutation does not showed a significant variation in the survival pattern. In intermediate cytogenetic risk group, CEBPA mutation confer a better survival for the mutated with a mean survival of 26.41 compared to non mutated having mean survival of 16.48 (*P*=0.004). The survival analysis in normal karyotype group showed a significant result, with increased survival for mutated cases with mean survival 16 vs 27 months (*P*=0.003) for wild and mutated respectively (Figure 1 b). The patients with numerical and other structural abnormality didn't showed much difference in the survival pattern (*P*=0.681). In our study CEBPA mutation was significantly associated with the normal karyotype (NK) than the other groups. Double CEBPA mutations were analyzed for the survival in comparison with single CEBPA mutation and wild type cases. Here both double and single mutations showed similar prognosis, but the mean survival was slightly higher for the double mutation cases with 32.12±3.62 compared to single mutation cases having mean survival 29.23±2.64 . When compared with the wild type genotype of CEBPA, the mutated either single or double CEBPA mutation showed significantly higher survival (*P*=0.005) (Figure 1c). This shows a significant association for the CEBPA mutations (single as well as double mutations) in the prognosis of AML).

CEBPA polymorphism was also analyzed for the survival and found that polymorphism was not associated with overall survival of AML patients (*P*=0.788). The different cytogenetic risk group did not differ among the survival rates with respect to the polymorphism status (Figure 1d).

Discussion

In the present study, we aimed to detect the frequencies and characteristics of CEBPA mutation in de novo AML patients from India. CEBPA mutations were detected in 18% (45/248) of the cases. At the same time in 18.9% CEBPA polymorphisms were also detected. The frequency of CEBPA mutation was reported to be between 6%-17.9% in various reports (Pabst et al., 2001; Frohling et al., 2004; Lin et al., 2005; Kim et al., 2012). A higher frequency of polymorphism 24.3% and 25.6% was reported by Leecharendkeat et al. (2008) in Southeast Asian population and Su L et al in Chinese population (Leecharendkeat et al., 2008; Su et al., 2013).CEBPA mutation was reported at a frequency of 26.3% in de novo AML cases with 36% of the mutation in NK-AML in the Chinese population. In the elderly Chinese patients the mutation frequency was reported to be 23.3% (Su et al., 2013; 2014). Study by Ruan et al. (2014) showed a 6.7% of CEBPA mutation in paediatric AML patients from China. Report from an Indian study showed 8.3% of CEBPA mutation in NK AML patients (Ahmad et al., 2011).

We came across 3 types of polymorphisms, c.584_589dup ACCCGC, c.690G>T and c.573C>T. These three polymorphism has been reported in other studies (Frohling et al., 2004; Lin et al., 2005; Leecharendkeat et al., 2008; Fuchs et al., 2009; Kim et al., 2012). The c.584-589dup ACCCGC leading to p.P194_H195dup (proline-histidine duplication) was initially reported as mutation but later found as a germline polymorphism (Frohling et al., 2004; Lin et al., 2005; Resende et al., 2007; Wouters et al., 2007). This polymorphism was reported in 3.1-39% in normal healthy controls (Pabst et al., 2001; Lin et al., 2005; Resende et al., 2007) and 3.2-20% in AML (Lin LI et al., 2005; Resende C et al., 2007; Leecharendkeat et al., 2008). In a recent study by Kim et al. (2012) ACCCGC duplication was observed in 30% and c.690G>T in 6.7%. Frohling et al. (2004) reported a higher frequency of c.690G>T polymorphism (32%). In our study, the frequency of these polymorphism was 10% (25/248) for G>T and 8% (20/248) for ACCCGC which was lower when compared to other studies. Another variation (silent mutation) observed in two cases (c.573C>T) was also reported as polymorphism by Fushs O et al in 2009. The polymorphisms observed in our study were non pathogenic, which leads to duplication of two amino acids and/or substitution without affecting the amino acid (silent). Thus the frequency of polymorphism in our study group become 18.9% (47/248).

Twenty-five distinct type of mutations were identified among the 45 (18%) mutated patients involving both the N-terminal TAD domain which stimulate the transcriptional activity of the target genes and the C-terminal bZIP domain involved in the DNA binding and dimerization (Mueller et al., 2006). Seventeen novel mutations were reported in the present study, remaining nine mutations were already reported in previous studies (Pabst et al., 2001; Leecharendkeat et al., 2008; Wouters et al., 2009; Szankasi et al., 2011; Ahmaed et al., 2012; Dufour et al., 2012; Greif et al., 2012; Kim et al., 2012). The 29% of the mutations constitute frames shift mutations generating a truncated protein, the remaining includes the missense mutations and aminoacid insertions. Among the novel mutations identified, one variation was more frequent which is a missense variation c.811G>A at the upstream of bZIP region. This variation was observed in 30/45 (75%) mutated cases. Further studies are needed to evaluate the significance of this mutation.

Regarding the association of the mutations (single and double mutations separately and in combination) and polymorphisms of CEBPA with clinical and laboratory parameters, no significance was obtained for the age, gender, WBC count, Hb, blast percentage, platelet count and LDH levels. Other reports also show the same with absence of significant association for the age, gender and WBC count. Some reports showed a higher blast percentage and decreased platelet count for mutated when compared to wild type patients, but no significant association was observed for the CEBPA polymorphism with clinical and laboratory parameters (Leecharendkeat et al., 2008). There are reports showing an increased peripheral white blood cell counts and/or higher marrow blast percentage in CEBPA mutated cases compared to wild type (Schnittger et al., 2002; Thiede et al., 2002; Schnittger et al., 2005; Haferlach et al., 2012). Lack of

such association was reported by Su et al. (2013). An increase in the haemoglobin percentage was observed for the mutated cases by Lin et al. (2005). Increased incidence of CEBPA mutation in M2 was reported by Frohling et al. (2004). The increased incidence of CEBPA in M1, M0 and M2 observed in our (statistical significance not obtained) study was concordance with other studies. A lack of association was observed for FAB subtype and polymorphism. Studies have shown an association of CEBPA mutation with normal karyotype (Lin et al., 2005; Leecharendkeat et al., 2008). CEBPA mutation was detected in 8%-19% of NK-AML (Pabst et al., 2001; Gombart et al., 2002; Dufour et al., 2010). In the present study the incidence of CEBPA mutation was 22% in NK-AML. Among the CEBPA mutated cases, NK-AML patients form the predominant group with 67%. In another study cytogenetic analysis revealed normal karyotype in 70.5% of CEBPA mutated cases (Fasan et al., 2013). In previous studies a favourable outcome for the CEBPA mutation has been reported. CEBPA mutations improve the prognosis of AML with normal cytogenetics from intermediate to favorable (Yohe, 2015). Present study also reveals a significant favorable impact for the mutated patients with respect to wild type in overall cases (P=0.028). Stratified analysis showed CEBPA mutation as a significant favourable prognostic factor for the normal karyotype AML patients (P=0.003). No association was observed for the mutation with good risk group (P=0.149) or for the intermediate risk group with numerical and other structural abnormality excluding normal karyotype compared to its wild type (P=0.681). Several reports have linked CEBPA mutation with a favorable outcome in AML especially for the normal karyotype (Gombart et al.,2002; Preudhomme et al., 2002; Frohling et al., 2004; Bienz et al., 2005; Lin et al., 2005; Pabst et al., 2007). Fuster et al. (2011) reported a significant OS for the intermediate karyotype group with CEBPA mutation. In recent reports, it was stated that a better prognosis was observed for biallelic CEBPA mutations compared to monoallelic CEBPA mutations when subjected to a long term follow up study (Li et al., 2014; Pastore et al., 2014). In our study single and double CEBPA mutations didn't show any difference in the OS, but was significantly higher than the wild type (P=0.005) which was different from other reports, who showed a significant association for the double over single CEBPA mutation on the prognosis of AML patients (Pabst et al., 2009; Fasan et al., 2014). Polymorphisms observed in this gene on survival analysis revealed no significant association for the OS in any of the cytogenetic groups or in total cases, showing the lack of importance of these variation with respect to survival in AML patients, which was similar to that reported by (Leecharendkeat et al., 2008).

In conclusion, CEBPA mutation and polymorphism occurred with similar frequency in our study population and was identified in all the FAB subtypes as well as in cytogenetic risk groups. Even though both of them were in similar frequency, a difference in the prognosis was noted for the mutation rather than polymorphism, especially in the normal karyotype patients providing a better survival for the mutated compared to non mutated cases

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