RESEARCH ARTICLE

Molecular Evaluation of DNMT3A and IDH1/2 Gene Mutation: Frequency, Distribution Pattern and Associations with Additional Molecular Markers in Normal Karyotype Indian Acute Myeloid Leukemia Patients

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Abstract

Mutations in the DNMT3A and IDH genes represent the most common genetic alteration after FLT3/NPM1 in acute myeloid leukemia (AML). We here analyzed the frequency and distribution pattern of DNMT3A and IDH mutations and their associations with other molecular markers in normal karyotype AML patients. Forty-five patients were screened for mutations in DNMT3A (R882), IDH1 (R132) and IDH2 (R140 and R172) genes by direct sequencing. Of the 45 patients screened, DNMT3A and IDH mutations were observed in 6 (13.3%) and 7 (15.4%), respectively. Patients with isolated DNMT3A mutations were seen in 4 cases (9%), isolated IDH mutations in 5 (11.1%), while interestingly, two cases showed both DNMT3A and IDH mutations (4.3%). Nucleotide sequencing of DNMT3A revealed missense mutations (R882H and R882C), while that of IDH revealed R172K, R140Q, R132H and R132S. Both DNMT3A and IDH mutations were observed only in adults, with a higher frequency in males. DNMT3A and IDH mutations were significantly associated with NPM1, while trends towards higher coexistence with FLT3 mutations were observed. This is the first study to evaluate DNMT3A/IDH mutations in Indian patients. Significant associations among the various molecular markers was observed, that highlights cooperation between them and possible roles in improved risk stratification.

Keywords: Molecular markers - DNMT3A - IDH - normal karyotype AML - risk stratification

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Introduction

Acute myeloid leukemia (AML) represents one of the most biologically and clinically diverse subtypes among various hematological malignancies. The diagnostic karyotype of AML patients still remains to be the most reliable prognostic marker deciphering patients into different risk group (Grimwade et al., 1998) however the major challenge for prognosticfication occurs when the patient shows apparently normal karyotype (NK-AML). These NK-AML patient accounts for nearly 45% of all cases and have been assigned into "intermediate risk group" because of their uncertain clinical outcome (Slovak et al., 2000). The absence of cytogenetic abnormalities in a considerable proportion of patients argues for refinement of the classification of AML. Infact, identification of molecular markers in these NK-AML patients have been the focus of most of the research groups across the globe. At the present time, the most well known molecular markers in AML are mutations in FLT3, NPM1, CEBPA, WT1 and MLL which are reported to be associated with different clinical outcome (Gaidzik et al., 2008; Mrozek et al., 2008). Interestingly, few of these markers such as FLT3, NPM1 and CEBPA have been already included in the revised version of World Health Organization classification of leukemias (Swerdlow et al., 2008).

In search of additional molecular markers in AML, several breakthrough researches leveraging the power of recently identified novel technologies, such as massively parallel DNA sequencing and next-generation sequence techniques have eased the systematic efforts to identify molecular markers with valuable prognostic significance (Mardis et al., 2009; Ley et al., 2010). The outcome of such advanced studies has led to the identification of DNA methyltransferase 3a (DNMT3A) mutations (Ley et al., 2010), ten-eleven-translocation oncogene family member 2 (TET2) (Delhommeau et al., 2009), and mutations and isocitrate dehydrogenase (IDH) 1 gene mutations (Mardis et al., 2009) in AML patients. Of these mutations, two markers viz., DNMT3A and IDH mutations have gained tremendous attention in recent times, and have been extensively evaluated by different research groups across

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the globe (Paschka et al., 2010; Thol et al., 2011; Chotirat et al., 2012; Marcucci et al., 2012).

The gene DNMT3A is located on chromosome 2p23 locus, encodes the enzyme DNA methyltransferase (DNMT) 3A, which catalyzes the addition of methyl groups to the cytosine residue of CpG dinucleotides in DNA (Brenner and Fuks, 2006; Chen and Li, 2006). Recently, somatic mutations in DNMT3A gene were first discovered by massively parallel DNA sequencing (99.6% diploid coverage) from the genome of cells of a NK-AML patient. Different types of mutations such as missense, nonsense, frameshift, splice-site mutations and deletions were detected, of which missense mutations especially R882 was the most recurrent mutations occurring within dimer or tetramer interface of DNMT3A (Ley et al., 2010). Overall, the frequency of DNMT3A mutations varies from 14% to 35% in AML globally (Ley et al., 2010; Marcucci et al., 2012; Hou et al., 2012; Renneville et al., 2012) Ribeiro et al., 2012; Patel et al., 2012). More interestingly, similar to FLT3 mutations, several recent studies have consistently reported association of DNMT3A mutation with poor clinical outcome in AML patients (Ley et al., 2010; Thol et al., 2011; Marcucci et al., 2012).

Furthermore, the gene IDH encodes a nicotinamide adenine dinucleotide phosphate+-dependent enzyme for oxidative decarboxylation of isocitrate, and has an essential role in the tricarboxylic acid cycle as well as involved in the cellular defence of oxidative damage (Lee et al., 2004). Similar to the discovery of DNMT3A mutations, molecular alterations of IDH1 were first reported in AML by massively parallel DNA sequencing of an entire NK-AML genome (Mardis et al., 2009). Further studies found recurrent mutations in IDH2 gene indicating that both IDH1 and 2 are frequently mutated in AML patients (Paschka et al., 2010; Marcucci et al., 2010). The overall combined frequency of IDH mutations varies from 15-22% of all AML patients (Patel et al., 2011; Abbas et al., 2010; Zou et al., 2010) and 25-30% of NK-AML patients have either of IDH1 or IDH2 mutations (Marcucci et al., 2010; Paschka et al., 2010). Nevertheless, the true clinical impact of IDH mutations is still debatable, and it varies considerably among the different type of mutations detected, and also by the presence of concurrent mutation in other clinically relevant genes.

Most reports of DNMT3A and IDH mutations come from Western countries, while there are few reports from Asia (Zou et al., 2010; Lin et al., 2011; Chotirat et al., 2012; Hou et al., 2012). At the present time, no precise published data exist in India with respect to the frequency and distribution patterns of DNMT3A and IDH mutations, despite the fact that AML is one of the most common hematologic malignancies associated with the greatest mortality and morbidity in this country. In this study, we performed mutation analysis of the DNMT3A and IDH genes in Indian AML patients with an objective to explore their frequency and distribution patterns, and correlate results with the clinical features and cytogenetic findings and other molecular markers. To the best of our knowledge, this study represents the first series of DNMT3A and IDH mutations ever reported from India.

Materials and Methods

The present study was conducted at the Research and Development Division of SRL Ltd., Mumbai, India. The study included 45 *de novo* normal karyotype AML (NK-AML) samples, of whome 36 were described in our recent report on CEBPA gene mutations (Ahmad et al., 2012) and an additional 9 newly diagnosed NK-AML patients were included. Genomic DNA was available from mononuclear cells from the bone marrow and/or whole blood. DNA yield and purity were determined by measuring the absorbance at 260/280 nm. The presence of other molecular markers, such as FLT3, NPM1, WT1, KIT and CEBPA gene mutations were screened in additional nine cases as per our previous reports (Ahmad et al., 2009; Ahmad et al., 2010; Ahmad et al., 2011). The study was carried out in accordance with the Declaration of Helsinki, and written consent was obtained from each subject. Treatment and outcome were not analyzed in the current study.

Screening of DNMT3A gene mutation

High molecular weight genomic DNA was amplified for missense mutation R882 of the DNMT3A gene using the following primer sets: DNMT3A_F: 5'-TCCTGCTGTGTGGTTAGACG-3' and DNMT3A_R: 5'- TCTCTCCATCCTCATGTTCTTG-3' as described earlier (Fiied et al., 2012). Briefly, the polymerase chain reaction (PCR) was performed in a 50 μl volume containing 50 ng of genomic DNA, 1.5 mmol/L $MgCl_2$, 0.2 mM dNTPs, 20 pmol of each oligonucleotide primer, and 1.5 U of Taq polymerase (Invitrogen Life Technology, Sao Paulo, Brazil). The PCR conditions consisted of an initial denaturation step at 98ºC for 3 min followed by 35 cycles at 94ºC for 30 s, 58ºC for 30 s, 72ºC for 45 s, and a final step at 72ºC for 10 min. Amplified products were visualized on 2% agarose gel and directly sequenced.

Screening of IDH gene mutations

Genomic DNA was amplified specifically for IDH1 (R132) and IDH2 (R140 and R172) gene mutations using the following sets of primers: IDH1_F: 5'-AGCTCTATATGCCATCACTGC-3', IDH1_R: 5'-AACATGCAAAATCACATTATTGCC-3'; IDH2_F: 5'-AATTTTAGGACCCCCGTCTG-3', IDH2_R: 5'-CTGCAGAGACAAGAGGATGG-3'as described earlier (Marcuccii et al., 2010). Briefly, for both IDH1 and IDH2, the polymerase chain reaction (PCR) was performed in a 50 μl volume containing 50 ng of genomic DNA, 1.5 mmol/L $MgCl_2$, 0.2 mM dNTPs, 10 pmol of each oligonucleotide primer, and 1.5 U of Taq polymerase (Invitrogen Life Technology, Sao Paulo, Brazil) in separate PCR tubes. The PCR conditions consisted of an initial denaturation step at 98ºC for 3 min followed by 35 cycles at 94ºC for 30 s, 59ºC for 30 s, 72ºC for 1 min, and a final step at 72ºC for 10 min. Amplified products were visualized on 2% agarose gel and directly sequenced.

Statistical analysis

Data were analyzed using Student's t-test and chi-square test to calculate the significance of association

between DNMT3A/IDH mutations and other discrete variables among subgroups of patients. p-values <0.05 were considered to be significant.

Results

Frequency and type of DNTM3A and IDH1/2 mutations

In the current study, we screened four different missense mutations, viz., DNMT3A (R882), IDH1 (R132) and IDH2 (R140 and R172) genes by direct sequencing. Among the 45 cases of *de novo* NK-AML, DNMT3A and IDH mutations were observed in 6 (13.3%) and 7 (15.4%) of cases respectively. Patients with isolated DNMT3A mutations were seen in 4 cases (9%), isolated IDH mutations in 5 cases (11.1%), while interestingly, two cases showed both DNMT3A and IDH mutations (4.3%). As shown in Figure-1 (A and B), nucleotide sequencing of the DNMT3A exon 23 revealed two types of missense mutations, viz., the predominant c.G2645A; p.R882H mutation observed in 5 cases (83%), and less frequent c.C2644T; p.R882C mutation in only one case (17%) (Table 1). Similarly IDH mutations were predominantly found in IDH2 (n=5) as compared to IDH1 (n=2). Of the five mutations observed in IDH2, three of them showed c.G515A; p.R172K (60%), while the remaining two revealed c.G419A; p.R140Q (40%) (Figure-1 (C to F), Table 1). In contrast to IDH2, two cases showed c.G395A; p.R132H and c.C394A; p.R132S mutations in the IDH1gene. All patients with different mutations were heterozygous and retained a wild-type allele (Figure 1). No patients concurrently carried both IDH1 and IDH2 mutations suggesting that these mutations were mutually exclusive. However, interestingly case#38 did showed simultaneous mutations of DNMT3A and IDH mutations together (Table 1), indicating the probability of existence of cooperation between the two genes in AML pathogenesis.

DNMT3A and IDH mutations and their relation to clinical characteristics

The presence of DNMT3A and IDH mutations were correlated with clinical and laboratory parameters. Both DNMT3A and IDH mutations were predominantly present in male patients in comparison to female patients (15.2% *vs* 8.3% for DNMT3A and 18.1% *vs* 8.3% for IDH) (p>0.05). The current study included pediatric (n=8) as well as adult AML patients (n=37), of which both DNMT3A and IDH mutations were observed exclusively in adults (16.2% (6/37) for DNMT3A and 23.3% (7/37) for IDH), while none of the eight pediatric cases showed any evidence of mutations indicating that these mutations are less frequent in pediatric groups. Nevertheless, none of these differences were statistically significant (p>0.05). Interestingly, the median age of patients with either DNMT3A or IDH or both mutations (n=11) were significantly higher with respect to those without these mutations (n=34) (45 years *vs* 32.5 years, p<0.05). Furthermore, DNMT3A and IDH mutations were not associated with any FAB subtypes (p>0.05). There was no statistical correlation with other clinical parameters, including white blood cell (WBC) count, hemoglobin, and platelet count between patients with and without either of the mutations (p>0.05).

DNMT3A and IDH mutations and their association with other molecular markers

The presence of mutations in NPM1, FLT3, WT1, KIT and CEBPA genes were known in 36 of these cases, as per our previous report (Ahmad et al., 2012), while additional 9 cases were screened for these mutations in this study as

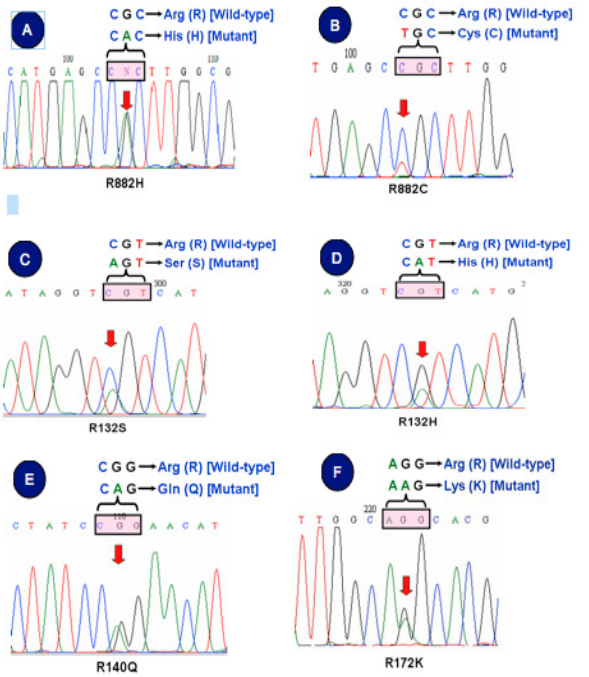

Figure 1. Representative Sequencing Figures of the DNMT3A and IDH Mutation Patterns. (A) R882H (DNMT3A); (B) R882C (DNMT3A); (C) R132S (IDH1); (D) R132H (IDH1); (E) R140Q (IDH2); and (F) R172K (IDH2)

Table 1. Distribution Patterns of DNMT3A and IDH Mutations in Patients with *de novo* NK-AML

Sr No.	Case ID	Age (Years)	Sex	FAB	DNMT3A		IDH1		IDH2		Other gene mutations detected (FLT3-ITD, FLT3-D835, NPM1, CEBPA, WT1, KIT)
					Nucleotide change	Amino acid change	Nucleotide change	Amino acid change	Nucleotide change	Amino acid change	
1	42	28	F	M4	c.C2644T	R882C	None	None	None	None	FLT3-ITD, NPM1
2	6	40	M	M2	None	None	c.C394A	R132S	None	None	FLT3-ITD, FLT3-D835, NPM1
3	8	46	M	M1	c.G2645A	R882H	None	None	None	None	FLT3-ITD, NPM1
4	7	45	M	M3	None	None	None	None	c.G419A	R140Q	FLT3-ITD, NPM1
5	38	45	F	M2	c.G2645A	R882H	None	None	c.G515A	R172K	FLT3-ITD
6	2	43	M	M4	c.G2645A	R882H	c.G395A	R132H	None	None	FLT3-ITD, NPM1
7	9	32	M	M3	c.G2645A	R882H	None	None	None	None	NPM1
8	4	54	M	M2	None	None	None	None	c.G515A	R172K	NPM1
9	10	33	M	M6	None	None	None	None	c.G515A	R172K	NPM1
10	37	63	M	M4	c.G2645A	R882H	None	None	None	None	None
11	39	50	M	M4	None	None	None	None	c.G419A	R140Q	None

Table 2. Molecular Characteristics According to DNMT3A and IDH Mutational Status in de Novo NK-AML

Molecular characteristics		Total (%)	DNMT3A-wt/IDH-wt	DNMT3A-mt/IDH-wt	DNMT3A-wt/IDH-wt	DNMT3A-mt/IDH-wt	p values
	Total	45 (100)	34 (75.5)	4 (9)	5 (11.1)	2 (4.4)	0.006
NPM1	Wild-type	31 (68.9)	28 (90.4)	1 (3.2)	1 (3.2)	1 (3.2)	
	Mutated	14 (31.1)	6 (42.9)	3 (21.4)	4 (28.5)	1 (7.2)	
FLT3	Wild-type	29 (64.5)	24 (82.8)	2 (6.9)	3 (10.3)	0 (0)	>0.05
	Mutated	16 (35.5)	10 (62.5)	2 (12.5)	2 (12.5)	2 (12.5)	
WT1	Wild-type	41 (91.1)	30 (73.1)	4 (9.8)	5 (12.1)	2 (4.9)	>0.05
	Mutated	4 (8.9)	4 (100)	0 (0)	0 (0)	0 (0)	
CEBPA	Wild-type	42 (93.3)	31 (73.8)	4 (9.5)	5 (12)	2 (4.7)	>0.05
	Mutated	3 (6.7)	3 (100)	0 (0)	0 (0)	0 (0)	
Kit	Wild-type	45 (100)	34 (75.5)	4 (8.8)	5 (11.1)	2 (4.4)	>0.05
	Mutated	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	

Table 3. Comparison of Some of the Recent worldwide Incidence of DNMT3A and IDH Mutations in de Novo AML

Country	Total AML patients	Overall DNMT3A mt incidence, (%)	DNMT3A mt incidence in NK-AML, (%)	Overall IDH1 mt incidence, (%)	IDH1 mt incidence in NK-AML, (%)	Overall IDH2 mt incidence, (%)	IDH2 mt incidence in NK-AML, (%)	Reference
USA	281	22	36.6	ND	ND	ND	ND	Ley et al (2010)
Taiwan	439	ND	ND	5.5	8.8	ND	ND	Chou et al. (2010)
Germany	275	ND	ND	NA	10.9	ND	ND	Wagner et al. (2010)
Germany	272	ND	ND	ND	ND	NA	12.1	Thol et al. (2010)
Netherlands	893	ND	ND	6	10	11	15	Abbas et al. (2010)
Germany	805	ND	ND	7.6	12.1	8.7	8.3	Paschka et al. (2010)
France	520	ND	ND	9.6	15.8	3	5.6	Boissel et al. (2010)]
Germany	1414	ND	ND	6.6	10	ND	ND	Schnittger et al. (2010)
USA	358	ND	ND	NA	13.7	NA	19.2	Marcuccii et al. (2010)
USA	274	ND	ND	4.4	7.6	ND	ND	Ho et al. (2010)
Germany	489	17.8	27.2	ND	ND	ND	ND	Thol et al. (2011)
France	288	13.5	28	ND	ND	ND	ND	LaRochelle et al. (2011)
China	182	6.6	13.2	ND	ND	ND	ND	Lin et al. (2011)
China	77	11.9	18.5	ND	ND	ND	ND	Qiao et al. (2011)
China	416	ND	ND	6.7	ND	ND	ND	Zhang et al. (2011)
China	110	ND	ND	3.6	ND	ND	ND	Lin et al. (2011)
UK	1473	ND	ND	ND	ND	10	13	Green et al. (2011)
USA	199	ND	ND	6	8.2	2	3.2	Patel et al. (2011)
UK	51	NA	25.5	NA	14	NA	14	Fernandez-Mercado et al. (2012)
Czech Republic	226	NA	29.6	ND	ND	ND	ND	Marková et al. (2012)
Taiwan	500	14	22.9	ND	ND	ND	ND	Hou et al. (2012)
France	123	NA	29	ND	ND	ND	ND	Renneville et al. (2012)
USA	415	NA	34.2	ND	ND	ND	ND	Marcucci et al. (2012)
Thailand	230	ND	ND	8.7	8.7	10.4	9.5	Chotirat et al. (2012)
India	45	NA	13.3	NA	4.4	NA	11.1	Current study

*ND: Not done; NA: Not applicable

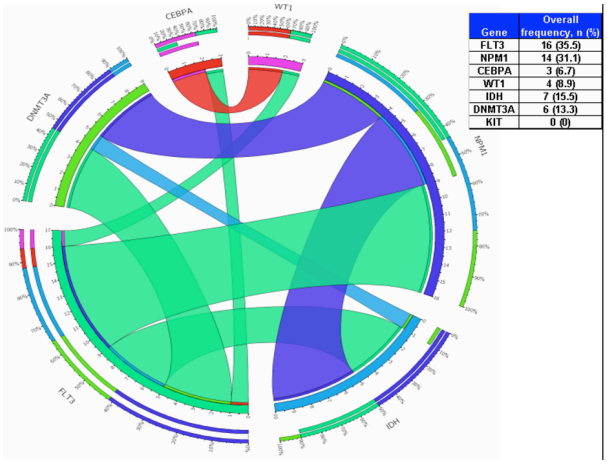

Figure 2. Circos Plot Showing the Overall Frequency of Somatic Mutations in The Entire Cohort (N=45) and the Interrelationships among the Various Mutations. The length of the arc corresponds to the frequency of mutations in the first gene, and the width of the ribbon corresponds to the percentage of patients who also had a mutation in the second gene. Pairwise co-occurrence of mutations is denoted only once, beginning with the first gene in the clockwise direction

per our previous reports (Ahmad et al., 2009; Ahmad et al., 2010; Ahmad et al., 2011). Table 2 shows the overall molecular characteristics of the entire cohort, while Figure 2 shows the interrelationships among the various mutations, as represented visually with the use of a Circos plot. The length of the arc corresponds to the frequency of mutations in the first gene, and the width of the ribbon corresponds to the percentage of patients who also had a mutation in the second gene. Pairwise co-occurrence of mutations is denoted only once, beginning with the first gene in the clockwise direction. It is worth noting that DNTM3A mutations were significantly more prevalent in NPM1 positive cases when compared to NPM1 wild type cases (28.6% *vs* 6.4%, p<0.05) (Table 2). In addition, when correlated with FLT3 mutations, though DNTM3A mutations were more dominant in FLT3 positive cases than wild type (25% *vs* 6.9%), this difference was statistically insignificant (p>0.05) (Table 2). Furthermore, the IDH mutations were present in 5 of 14 (35.7%) cases with NPM1 mutation, when compared with only 2 of 31 (6.4%) cases with NPM1 wild type genotype (p<0.05). Likewise DNMT3A mutation, IDH mutations were also

preponderant in FLT3 positive cases in comparison to those with normal FLT3 (25% *vs* 10.3%) cases, however this association failed to achieve statistical significance ($p<0.05$). Thus, it appears that NPM1 mutation appears as a major significant coexisting genetic mutation in DNMT3A and IDH-mutated cases.

In contrast, no DNMT3A and IDH mutations were observed in cases with WT1, KIT or CEBPA mutation indicating that these mutations cane be mutually exclusive in nature. Both DNMT3A and IDH mutations were observed only in WT1, KIT and CEBPA wild type cases ($p>0.05$) (Table 2).

Discussion

Research in the last decade has shown tremendous improvement in terms of identification of new molecular lesions which are of high clinical relevance in AML especially those with NK-AML. A subset of these NK-AMLs are found to harbor mutations of genes that normally function in cell proliferation, differentiation, and survival such as FLT3, NPM1, CEBPA, KIT, WT1, RAS and AML1 (Takahashi et al., 2011a; 2011b). The mutations are already known to play a significant role in the clinical outcome of AML patients. Furthermore, identification of additional molecular markers is a need, which can allow further risk stratification and effective patient management.

Mutations of DNMT3A and IDH were recently described in AML through a rapid whole genome sequencing approach (Mardis et al., 2009; Ley et al., 2010). The reported frequency of DNMT3A mutations varies between 14% to 35% (Ley et al., 2010; Hou et al., 2012; Marcucci et al., 2012; Patel et al., 2012; Renneville et al., 2012; Ribeiro et al., 2012;), while that of IDH mutations varies between 25-30% of NK-AML globally (Paschka et al., 2010; Marcuccii et al., 2010) (Table 3). However, most of these available reports on DNMT3A and IDH mutations are from Western countries, while those from Asian countries are few (Lin et al., 2011; Chotirat et al., 2012; Hou et al., 2012), and in India to date there is no precise published data (Table 3). To the best of our knowledge, this is the first study to evaluate the mutation status of DNMT3A and IDH genes in an Indian NK-AML patient. We have found the frequency of DNMT3A and IDH gene alterations in 13.3% and 15.4% of cases respectively, suggesting that alterations of the IDH gene are more common in comparison to mutations of the DNMT3A gene ($p>0.05$). The frequency of DNMT3A mutation (15.4%) in our NK-AML cohort was lower in comparison to some of the reported frequency from France (29%) (Renneville et al., 2012), Taiwan (22.9%) (Hou et al., 2012), Germany (27.2%) (Thol et al., 2011). The difference in the frequency of DNTM3A mutation may be explained by the fact that in the current study only R882 mutations were evaluated and mutation other than R882 were not identified. In addition to this, variable sensitivity of the detection assays, or the racial differences can also be taken into consideration.

The overall frequency of IDH1 mutations appears to vary between 2-14%, and 1-19% for IDH2 from most Western reports (Mardis et al., 2009; Boissel et al., 2010; Schnittger et al., 2010; Marcuccii et al., 2010; Gross et al., 2010; Chotirat et al., 2012). The frequency of IDH1 mutations in our NK-AML cases of 4.4% (2 of 45) was lower in comparison to that from Thailand (8.7%, 11 of 126) (Chotirat et al., 2012), Germany (7.6%, 61 of 805) (Paschka et al., 2010) and USA (14%, 49 of 358) (Marcuccii et al., 2010) when only NK-AML cases were considered. In addition to this, the frequency of IDH2 mutations (11.1%) in the present report is in agreement with most of the European (3.2-15.2%) (Patel et al., (2010; Abbas et al., (2010; Wagner et al., (2010; Thol et al., 2010; Boissel et al., 2010) and US studies (2.4-19.2%) (Ley et al., 2010; Marcuccii et al., 2010; Thol et al., 2010), notably, our frequency is marginally higher than a recent study from Thailand (9.5%, 12 of 126) (Chotirat et al., 2012).

Both DNMT3A and IDH mutations were observed only in adults (>18 years), while no evidence of mutation in either of the genes were seen in pediatric patients (≤ 18 years), though the no. of pediatric patients were lesser in the current study (n=8). This is in agreement with a recent study (Ho et al., 2011), and highlights the fact that childhood leuekemias can be very different from that of adults in pathogenesis and biological characteristics (Jeha et al., 2002), and raises the possibility that there are significant differences in the mechanisms responsible for the introduction of DNMT3A or IDH mutations in the two populations. There appears to be higher frequency of DNMT3A/IDH mutations in male patients in comparison to female patients, however the difference was statistically insignificant ($p>0.05$). No statistical association with any FAB classification was observed which is in agreement with recent literature (Qiao et al., 2011; Marková et al., 2012). With respect to correlation with laboratory parameters, both DNMT3A and IDH mutations were not associated with Hb, WBC and PC ($p>0.05$) which tallies with previous finding (Qiao et al., 2011).

Sequencing analysis of all the cases for DNMT3A R882 mutation revealed R882H (5 of 6, 83.4%) as the predominant mutation type, while R882C (1 of 6, 16.6%) mutation type was less frequently observed, which is in agreement with a recent study from Germany (Thol et al., 2011). Furthermore, sequencing of IDH gene revealed two types of amino acid exchanges in IDH1 gene (R132H and R132S), one each in two different cases. Likewise, two types of IDH2 mutations were detected, R172K (3 of 5, 60%) which was more frequent in comparison to R140Q (2 of 5, 40%). However, another study reported R140 mutations as the prevalent IDH2 mutation in comparison to R172 mutations (Marcuccii et al., 2010). Nevertheless, none of our cases showed simultaneous mutations of both IDH1 and IDH2 together which tallies with previous finding (Marcuccii et al., 2010).

To explore if any possible correlation of DNM3A and IDH mutations coexist with other clinically relevant molecular markers, we had data for FLT3, NPM1, CEBPA, WT1 and KIT genes as per our previous findings (Ahmad et al., 2009; Ahmad et al., 2010; Ahmad et al., 2011). Both DNMT3A and IDH mutations were significantly more frequent in NPM1 mutant cases in comparison to NPM1 wild type cases (28.6% *vs* 6.4% and 35.7% *vs* 6.4%,

p<0.05) respectively (Table 2). This is in agreement with previous reports, wherein, DNMT3A and IDH mutations were significantly associated with NPM1 mutations (Marcuccii et al., 2010; Thol et al., 2011). The circos plot (Figure 2) shows the inter relationships among the various mutations, and it looks like NPM1 mutation appears as a major significant coexisting genetic mutation in DNMT3A and IDH-mutated cases. It is interesting to note that, unlike previous report (Marcuccii et al., 2010) , wherein, R172 IDH2 mutations were mutually exclusive with all other prognostic mutations (NPM1, FLT3, WT1, CEBPA and MLL-PTD), in the present study, of the three R172 IDH2 mutations detected, two of them also showed presence of NPM1 mutations and remaining one showed FLT3-ITD mutations. Additionally, cases with DNMT3A and IDH mutations were more likely to have mutations in FLT3 in comparison to FLT3 wild types cases which is in agreement with previous findings (Marcuccii et al., 2010; Thol et al., 2011), though the differences in the current study was statistically insignificant (p>0.05). In contrast to NPM1 and FLT3 mutation, both DNMT3A and IDH mutations were exclusively found in CEBPA, WT1 and KIT wild type cases (p>0.05), which corroborates previous finding (Marcuccii et al., 2010; Chotirat et al., 2012) (Table 2). Nevertheless, additional number of cases would have certainly helped to get more statistically defined information in terms of the co-occurrence and their association.

As far as the possible mechanism of DNMT3A mutation in oncogenesis is concerned, Holz-Schietinger and colleagues, recently demonstrated that the common mutations such as R882H which occurs within the dimer or tetramer interface domain disrupts the tetramerization activity of DNMT3A. The processive methylation of multiple CpG sites is disrupted when tetramerization is eliminated which may contribute to initiation of oncogenesis and its progression (Holz-Schietinger et al., 2012). Furthermore, in case of IDH mutations and its role in cancer formation, it is thought that these mutations prevent oxidative decarboxylation of isocitrate to α-ketoglutarate and confer novel enzymatic activity, facilitating the reduction of α-ketoglutarate to 2-hydroxyglutarate, a putative oncometabolite (Rakheja et al., 2012).

Finally, though the current study did not evaluated the effect of DNMT3A and IDH mutations in the clinical outcome, numerous studies have consistently reported association of DNMT3A mutation with poor clinical outcome in AML patients (Renneville et al., 2012; Marcuccii et al., 2010), usually independent of the presence of either FLT3 or NPM1 mutations (Ley et al., 2010). More recently, another interesting study reported no impact of DNMT3A mutation on outcome, but could be a predictive factor for response to idarubicin and thus, could have a direct influence in the way AML patients should be managed (LaRochelle et al., 2011). In contrast, the true clinical impact of IDH mutations is still debatable, and it varies considerably among the different type of mutations detected, and also by the presence of concurrent mutation in other clinically relevant genes (Paschka et al., 2010).

In conclusion, this is the first study to evaluate DNMT3A and IDH mutations in Indian patients, and has demonstrated that the frequency of DNMT3A and IDH1 mutation is lower to the worldwide incidence, while that of IDH2 is comparable. The present study depicts for the first time in Indian patients the involvement of DNMT3A/IDH in AML. Clearly, the list of acquired mutations with clinical value in AML is growing each day. The true clinical implementation of this multitude of markers will require integrated approaches and selection of markers to facilitate AML risk stratification in the future, especially in NK-AML.

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