International Journal of Medical Laboratory 2020;7(4):258-266.

Original Article

Frequency of *DNMT3A* Mutations in Patients with Acute Leukemia in Mashhad

Parisa Bagheri[1] M.Sc., Farhad Zaker[2] Ph.D., Mohammad Hadi Sadeghian[3] M.D., Ph.D., Soodeh Namjoo[2] Ph.D., Saeedeh Hajebi Khaniki[4] Ph.D., Zahra Chehreghani[1] M.Sc., Maryam Sheikhi[3] M.Sc., Hossein Ayatollahi[3*] M.D., Ph.D.

[1] Department of Hematology and Blood Banking, Faculty of Medicine, Mashhad University of Medical Sciences, Mashhad, Iran
[2] Department of Hematology and Blood Banking, School of Allied Medical Sciences, Iran University of Medical Sciences, Tehran, Iran
[3] Cancer Molecular Pathology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
[4] Student Research Committee, Department of Biostatistics, School of Health, Mashhad University of Medical Sciences, Mashhad, Iran

Article history
Received: 14 Oct 2019
Accepted: 28 Apr 2020
Available online: 30 Nov 2020

Keywords
Acute lymphoblastic leukemia
Acute myeloid leukemia
DNMT3A mutation

ABSTRACT

Background and Aims: DNA methyltransferase3A (DNMT3A) is necessary for the adjustment of gene expression, and the mutations in the *DNMT3A* gene are reported in a variety of leukemia cases. *DNMT3A* mutations are during cancer progression and cause poor prognosis in many leukemias. Thus, this gene can be a target for new treatments. This study aimed to examine the distribution of *DNMT3A* mutations in Iranian acute leukemia patients.

Materials and Methods: In this study, diagnostic samples from 45 patients with *de novo* acute leukemia, including 22 acute myeloid leukemia (AML) patients, and 23 acute leukemia lymphoblastic (ALL) patients were screened, from April 2017 to March 2018 for the incidence of *DNMT3A* mutations by polymerase chain reaction and direct sequencing.

Results: A total of 2 (9.1%) AML cases and 1 (4.34%) ALL cases were found to have the *DNMT3A R882H* mutation. It was found that a total of 22.7% and 21.7% of patients with AML and ALL had polymorphism *rs368516543*, respectively. *DNMT3A* mutations were considerably associated with higher age in AML patients.

Conclusions: The findings suggest that the *DNMT3A* mutations are probably a new biomarker in the early examination and treatment of acute leukemia, even though further studies are needed.

[*]**Corresponding Author:** Cancer Molecular Pathology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran. **Tel:**+989153033394, **Email:** ayatollahih@mums.ac.ir

Introduction

Acute leukemia includes acute lymphoblastic (ALL), and myeloid leukemia (AML) are heterogeneous malignancies in which undeveloped and dysfunctional hematopoietic progenitors grow and accumulate in the bone marrow [1]. Acute leukemia accounts for <3% of all cancers [2], and an estimated 18700 novel cases of acute leukemia are forecasted for 2008 in the United States alone [3]. An accounted for 6000 novel cases of acute lymphoblastic leukemia are recognized annually in the USA [4]. Almost three-fourths of childhood leukemia ages are ALL. AML accounts for approximately 25% of all leukemias in adults in the Western world [5]. AML is the most common form of acute leukemia in adults [6]. Globally speaking, the highest prevalence of AML is in the U.S., Australia, and western Europe [7].

The methylation of DNA is an epigenetic modification that plays a considerable role in controlling gene expression, cellular disinclination, X-inactivation, imprinting, silencing of transposable elements, and gene regulation [8]. DNA methyltransferase catalyzes the transfer of a methyl group from S-adenosyl-methionine at CpG sites in the genome. In various types of malignancies, abnormal methylation of CpG islands in the promoter region has been reported, resulting in their expression's silencing [9]. Mutations in *DNMT3A* were first reported by Ley et al. [10]. Mutations in *DNMT3A* gene has been described in different hematologic malignancies, including AML in 14-34% of cases [11], 5-15% of myelodysplastic syndromes cases [12], 10% of chronic myelomonocytic leukemia (CMML) patients [13], 5.7% of primary myelofibrosis cases [14], 12% of cases with systemic mastocytosis [15], and about 18% of T cell acute lymphoblastic leukemia patients [16]. In previous studies reported the poor prognostic impact of *DNMT3A* mutations [17], and *DNMT3A* mutations were associated with reduced overall survival, increased risk of relapse [11], and more frequent in patients with normal cytogenetics in AML patients and poor outcome [10]. *DNMT3A* mutations are highly correlated with mutations in the *NPM1* gene, *FLT3 ITD* and *TKD* gene, *IDH1/2* gene and older age, and FAB M5 type [11]. Several studies reported that *DNMT3A* mutations are stable during the disease. So, those abnormalities could be a potential marker for minimal residual disease [18]. This study aimed to examine the distribution of *DNMT3A* mutations in Iranian *de novo* acute leukemia patients for better risk categorization and targeted therapy in the Iranian population.

Materials and Methods

Patients

Forty five acute leukemia patients were selected, including 23 cases of ALL and 22 cases of AML admitted to Ghaem Hospital of Mashhad University of Medical Sciences, Iran, May 2017 to February 2018. Sample size calculation was done based on a meta-analysis of *DNMT3A* mutation in AML patients [11]. According to the *DNMT3A* mutation prevalence of 34.2%, type I error of 0.05, and 85% of precision using the required sample size formula was 40. Mutation bone marrow and peripheral blood cells were

gained from these patients at diagnosis. Laboratory data including French–American–British (FAB) classifications, bone marrow blast percentage, complete blood count, hemoglobin rate, and gene mutations were collected.

Polymerase chain reaction (PCR) amplification and direct sequencing

Mononuclear cells from bone marrow or peripheral blood at diagnosis were accumulated in ethylenediaminetetraacetic acid (EDTA)-containing tubes. Genomic DNA was extracted by DNA extraction kit (FABGK001) and stored at -70˚C in a refrigerator. The PCR amplification of exon 23 in the DNMT3A gene was done using forward primer 5'-TCCCAGTCCACTATACT GACGTCTC-3'and reverse primer 5'-TCTCTCCATCCTC ATGTTCTTGG-3' [19]. PCR was carried out in a 50 µL mixture containing 2×PCR mixture (A290401) 25 µL, primers 10 pmol for each, and genomic DNA 100 ng. PCR cycles inclusive pre-denaturation at 95˚C for 5 min, denaturation at 95˚C for 30 s, annealing at 65˚C for 35s, extension at 72˚C for 30 s, final extension at 72˚C for 5 min and the product was kept at 4˚C. Direct sequencing was performed with forward and reverse primers after PCR amplification. They were sequenced on a Genetic Analyzer (3130xl, Applied Biosystems, USA). Gene Codes Sequencher v5.4.6 analyzed the sequencing results. The Ethical Committee approved this study at the Mashhad University of Medical Sciences.

Statistical analysis

In this study, the relationship between gene mutations and other variables was investigated. Fisher's exact test was used to compare the differences between categorical variables. Mann-Whitney was performed to compare the differences between continuous variables. All data analyses were performed using SPSS version 16.0, and $p<0.05$ was considered statistically significant.

Results

In this study, a total of 45 patients were studied, of which 23 (51%) had ALL and 22 (49%) patients with AML. The mean age of all patients was 17.8 ± 15.2 years. The age also ranged from 0.09 to 20 and from 0.5 to 65 years for ALL and AML, respectively. Besides, among ALL patients, the frequency of males and among AML patients, females' frequency was more. The PCR product is shown in Fig. 1.

The most and least frequent form of leukemia in patients with AML was M1 and M4, respectively. In patients with AML, *NPM1* mutation has been studied in 15 patients, which was positive in 7 (46.7%) cases. *CEBPA* mutation has been evaluated in 12 patients, which was positive in 3 (25.2%) cases. Furthermore, *FLT3ITD* and *FLT3KTD* were also studied in 14 patients and were positive in 2 (14.3%) cases. In ALL patients, the blast percentage was 86.7±17.3 percent (ranged from 30 to 100 %), and in AML patients, on average, it was 11.6±83.1% (the range of variations was from 50 to 95%). 22.7% and 21.7% of patients with AML and ALL had polymorphism *rs368516543*, respectively (Fig. 2A). Among all 45 patients, *DNMT3A* mutation in exon 23 was found in 3 cases (6.6%) (Table 1). All three mutants were *R882H* mutation (c.2645G>A) (Fig. 2B).

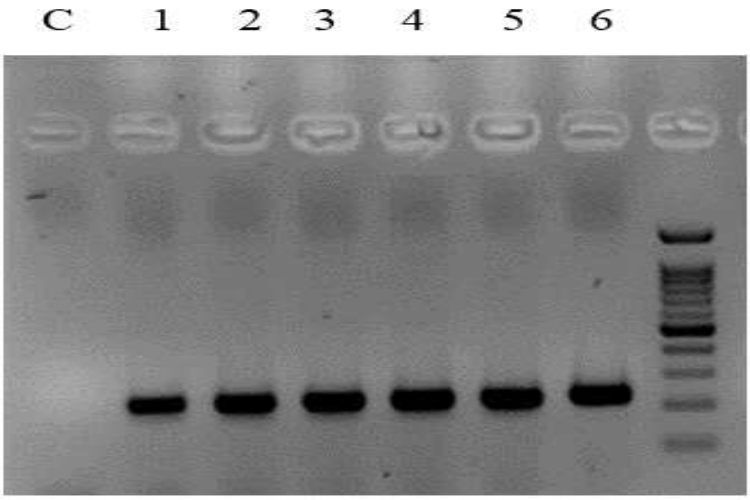

Fig. 1. Molecular analysis of *DNMT3A* mutation in Iranian acute leukemia patients. 1,2,3,4,5 and 6: different patient samples; C: Negative control; an example of PCR in exon 23 of the *DNMT3A* gene

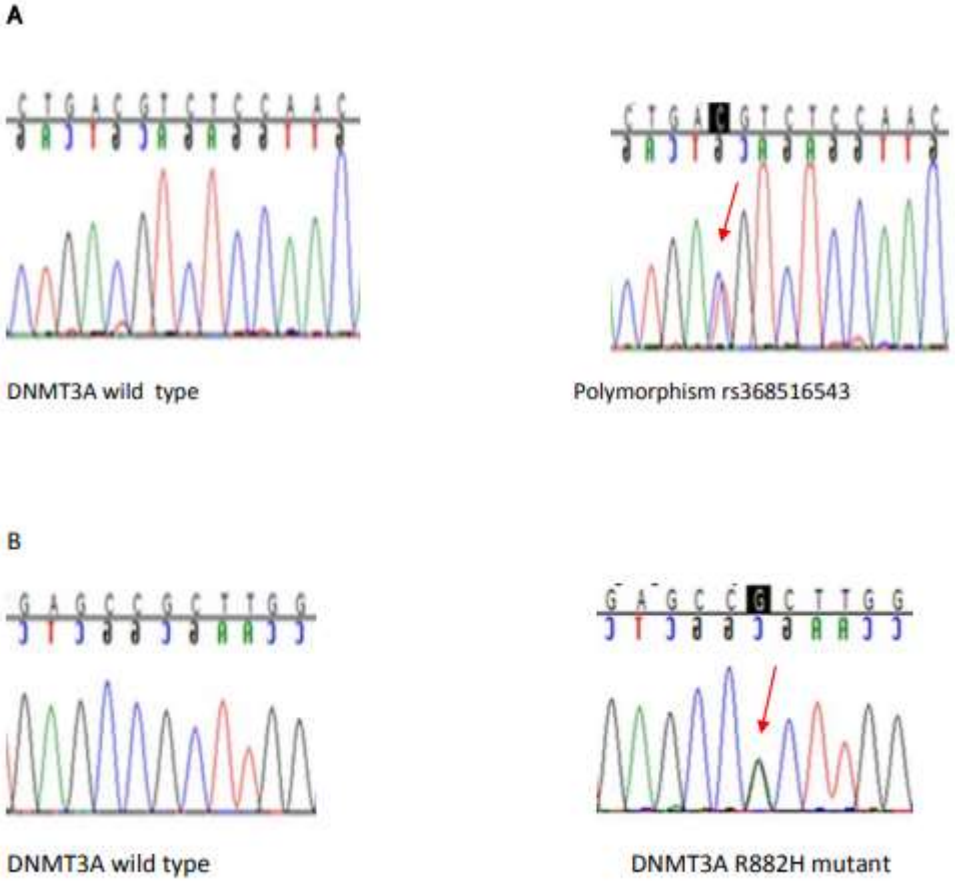

Fig. 2. A: Polymorphism *rs368516543* (ACG>ATG); B: *DNMT3A* mutant (CGC>CAC) (the red arrow)

Overall, the frequency of mutation was not statistically different between ALL and AML (p=0.608). *P.R882H* mutation was identified in 2 (9.1%) of 22 AML patients (Table 2). The basic clinical data of these patients were presented in Table 1. No relationship was observed between

p.R882H mutation and gender. The age of AML patients at diagnosis with *DNMT3A p.R882H* mutation was significantly more compared to those without mutations (Table 2). Even though the mean of white blood cell, red blood cell, hemoglobin, or platelet counts at diagnosis was higher in AML patients with *p.R882H* mutation than those without mutations, no statistical difference was seen. 1 (4.3%) heterozygous *DNMT3A p.R882H* mutation was identified in a girl aged 6 years old with ALL-L2. The difference in age and hematologic parameters was not seen confirmed between patients with and without mutations. However, this patient was recognized with normal karyotype and negative *rs368516543* polymorphism (Table 3).

Table 1. The clinical and hematopoietic parameters of 3 patients with DNM3A mutations

No	Sex/age (years)	Diagnosis	WBC ($\times10^9$/L)	RBC ($\times10^{12}$/L)	Platelet ($\times10^9$/L)	Hemoglobin (g/dl)	Mutation
1	F/51	AML-M5	239.6	2.3	14.0	8.5	CEBPA, FLT3- TD, FLT3-TKD, NPM1, p.R882H
2	F/55	AML-M4	152.4	3.2	96.0	10.6	NPM1, p.R882H
3	F/6	ALL-L2	4.0	1.96	92.0	6.0	p.R882H

WBC= White blood cell; RBC= Red blood cell

Table 2. Distribution of *DNM3A R882H* mutation in AML

Variable	***R882H* mutation**	**Wild-type**	**P-value**
Acute myeloid leukemia	2	20	
Sex, male/female	0/2	10/10	0.481‡
Age at diagnosis, years	53.0±2.8	23.3±16.4	0.035†
WBC ($\times10^9$/L) at diagnosis	196.0±61.6	76.7±83.9	0.104†
RBC ($\times10^{12}$/L) at diagnosis	2.7±0.6	2.3±0.9	0.485†
Haemoglobin (g/dl) at diagnosis	9.5±1.5	7.1±2.5	0.104†
Platelet ($\times10^9$/L) at diagnosis	120.5±34.6	83.3±95.9	0.173†
FAB no.			
M0	0	1	0.459‡
M1	0	10	
M2	0	1	
M3	0	1	
M4	1	4	
M5	1	2	
M6	0	1	
NPM1, positive/negative	2/0	5/8	0.200‡
CEBPA, positive/negative	1/0	2/9	0.250‡
FLT3TKD	1/0	1/12	0.143‡
FLT3ITD	1/0	1/12	0.143‡
Blast, Percent	92.5±3.5	82.1±11.7	0.139†
***rs368516543*, Positive/Negative**	1/1	4/16	0.411‡
Karyotype classification	2/0	4/10	0.125‡
Normal / abnormal			
Cytogenetic			
t(8;21)(q22;q22);AML1-ETO	0	1	0.999‡
t(15;17)(q22;q21);PML-RARa	0	2	
Inv(16)(p13;q22);CBFB-MYH1	0	2	
Normal	2	7	

† Based on Mann -Whitney test; ‡ Based on Fisher's exact test.
WBC= White blood cell; RBC= Red blood cell

Table 3. Distribution of *DNM3A p.R882H* mutation in ALL

Variables	*p.R882H* mutation	Wild-type	P-value
Acute leukemia lymphoblastic	1	22	
Sex, male/female	0/1	15/7	0.348‡
Age at diagnosis, years	6.0	5.0± 10.2	0.522†
WBC ($\times10^9$/L) at diagnosis	4.0	37.5 ± 36.4	0.348†
RBC ($\times10^{12}$/L) at diagnosis	1.96	2.6 ± 0.8	0.485†
Haemoglobin (g/dl) at diagnosis	92.0	81.7 ± 76.9	0.609†
Platelet ($\times10^9$/L) at diagnosis	6.0	6.9 ± 2.3	0.696†
FAB no.			
L1	0	10	
L2	1	12	0.999‡
L3	0	0	
Blast, Percent	82.0	86.9 ± 17.6	0.435†
***rs368516543*, Positive/negative**	0/1	5/17	0.999‡
Karyotype classification	1/0	2/7	0.300‡
Normal/anormal			
Cytogenetic			
t(12;21)(p13;q22);TEL-AML1	0	3	
t (9;22)(q34;q11);BCR-ABL	0	1	0.999‡
Normal	1	12	

† Based on the Mann -Whitney test; ‡ Based on Fisher's exact test.
WBC= White blood cell; RBC= Red blood cell

Discussion

Gene mutations and epigenetic changes play an essential role in the pathogenesis and prognosis of leukemia. DNMT3A is a crucial enzyme in the *de novo* DNA methylation at CpG sites, and *DNMT3A* mutations have been reported in a diversity of malignancies and are one of the significant molecular aberrations associated with AML [11]. In the present study, a group of 45 de novo acute leukemia patients was investigated with a Direct Sequencing approach of 23 exons in the *DNMT3A* gene to identify the frequency of *DNMT3A* mutations as well as their associations with other molecular abnormalities. The mutation taster tools were used to evaluate DNA sequence variants for their disease-causing potential [20]. Generally, the *DNMT3A* mutation rate of 6.6% and 22.2% polymorphism *rs368516543* was observed in the total cohort. In this study, a missense mutation *p.R882H* (4.34%) was found in ALL patients. In previous studies, the frequency of *DNMT3A* gene mutations in ALL patients was reported to be between 0% to 18% [21].In this article, two missense mutation *P.R882H* (9.09%) were found in AML patients, and in previous studies, the frequency of *DNMT3A* gene mutations in AML patients was reported to be between 4% to 30%. The difference in the prevalence of *DNMT3A* mutations in various studies may be due to differences in patient populations, methods used, and ethnic history [22]. No significant correlation was found between platelet count and hemoglobin levels with *DNMT3A* mutations, which were matched with the study's results done by Ibrahem et al. in 2015. While in other studies, *DNMT3A* mutations have been reported

with higher platelet counts [23] and lower hemoglobin levels [24].

In the present study, no significant correlation was found between sex and white blood cell count with *DNMT3A* mutations, which were agreed with the results of Ibrahem et al.'s study in 2015 [23], while in Lin et al.'s study in 2011 [25] and other studies, *DNMT3A* mutations have been reported with higher white blood cell counts [26] and in the study of Liu et al., in 2015, *DNMT3A* mutations were reported with lower white blood cell counting [19]. In AML, patients with *DNMT3A* mutations were older than the patients without *DNMT3A* mutations, which were in line with the results of Lin et al.'s study in 2011 [25] and other studies. While in ALL patients, no significant difference was found between mutant patients and without mutation patients. It was in contrast with Liu's results et al.'s in 2015 [19].

In this study, a significant relationship was not found between the blast percent of bone marrow and *DNMT3A* mutations, which were matched with the results of Ibrahem et al.'s study in 2015 [23] and Paganin et al. in 2011 [27]. Thol et al. in 2011 reported *DNMT3A* mutation strongly related to *NPM1* mutations [28]. Although positive relationships were not observed between *DNMT3A* and *NPM1* mutations, both AML patients mutated had *NPM1* mutations.

There was no significant relationship between *DNMT3A* and *CEBPA* mutations. It was according to the results of Marcucci et al.'s study in 2012 [29]. In this study, a significant association between DNMT3A and FLT3mutations was not found. While in previous studies, a strong association between *DNMT3A* and *FLT3* mutations was reported [30].

Although a significant correlation between FAB classification and *DNMT3A* mutations was not discovered in this study. However, similar to previous studies, *DNMT3A* mutations in the AML population were observed in the monocyte category [10].

No significant relationship was found between cytogenetic abnormalities and *DNMT3A* mutations in AML patients, but both mutant patients had normal cytogenetics. As regards, normal cytogenetics tends to be an intermediate prognosis [31]. Therefore, patients with *DNMT3A* mutations with normal cytogenetics may also be prone to intermedia prognosis in this study, which was in line with previous results [24, 28]. The frequency of *DNMT3A* mutations in AML patients was higher than ALL patients, but further studies with larger groups are needed to compare these two groups.

22.7% and 21.7% of patients with AML and ALL had polymorphism *rs368516543*, respectively. In previous studies, the frequency of polymorphism *rs368516543* has not been reported. In mutation, tester has been reported polymorphism *rs368516543* as the cause of the disease. Consequently, further studies of the relationship between polymorphism *rs368516543* with *DNMT3A* mutations and prognosis in acute leukemia patients are recommended.

Conclusion

This study showed that *DNMT3A* mutation occurred in Iranian acute leukemia patients. *DNMT3A* mutations are probably new biomarkers in the early examination and treatment of acute leukemia, although further studies involving larger groups are needed to assess prognosis and compare AML and ALL patients.

Conflict of Interest

There is no conflict of interest to declare.

Acknowledgments

This work was supported by a grant from Mashhad University of Medical Sciences. The authors would like to acknowledge the Cancer Molecular Pathology Research Center professors and staff at Ghaem Hospital of Mashhad.

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