The Egyptian Journal of Medical Human Genetics 19 (2018) 315–319

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Contents lists available at ScienceDirect

The Egyptian Journal of Medical Human Genetics

journal homepage: www.sciencedirect.com

Original article

Study of DNA methyl transferase 3A mutation in acute myeloid leukemic patients

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ARTICLE INFO

Article history:
Received 25 April 2018
Accepted 17 May 2018
Available online 14 June 2018

Keywords:
AML
DNMT3A
ASB-PCR
PCR-RFLP
Endonuclease restriction

ABSTRACT

Background: Recent studies have shown that somatic mutations in DNA methyltransferase (*DNMT3A*) might affect the prognosis of AML.
Objective: The aim of this work was to investigate the frequency and prognostic impact of the most frequent mutation of *DNMT3A*, R882H mutation in AML using simple and rapid molecular techniques.
Patients and methods: We have used allele-specific blocker (ASB-PCR) and endonuclease restriction for the detection of *DNMT3A* R882H mutation in 56 adult patients with AML.
Results: *DNMT3A* R882H mutation was detected in 7/56 (12.5%) of patients. R882H mutation positive patients were older compared to the wild-type AML ($p = 0.08$). No association was found with initial laboratory parameters including white blood cells (WBC), hemoglobin (HGB) and Bone marrow (BM) blasts ($p > 0.05$). Thirty-two patients (57.1%) achieved complete remission (CR), 11/56 (19.6%) died before day 28 induction death (ID) and 13/56 (23.2%) had resistant disease (RD). *DNMT3A* R882H positive patients were not different regarding the response to induction chemotherapy (CR) compared to the negative group (wild-type) ($p > 0.05$). Median follow-up period for all patients was 1.6 months, Overall survival (OS) was 65%, and the median was 9.89 months. OS of DNMT3A positive patients was not statistically significant compared to wild-type patients ($p = 0.09$). Disease free survival (DFS) was 54.6% for all patients, with no difference between wild and mutants (0.59) patients.
Conclusion: *DNMT3A* R882H is a frequent mutation in adult de novo AML. The frequency of the mutation tends to increase with age. The two methods used in the study are easy to interpret and are recommended for rapid detection of the mutation required for risk stratification.

1. Introduction

DNA methyltransferase controls methylation of DNA; *DNMT3A*, *DNMT3B*, *DNMT3L*, *DNMT1* and *DNMT2* [1] DNMTs catalyze the addition of a methyl group to C5 of cytosine in DNA forming 5-methyl cytosine [2]. DNA methylation occurs at cytosine in any context of the genome, however locus-specific modification at the CpG dinucleotide 5¢ end is common. Aberrant methylation in cancer cells occurs in the form of global hypomethylation or hypermethylation at promotor associated CpGs clusters [3]. The 130 kDa protein *DNMT3A* is encoded by 34 exons on 2p23 chromosome with two transcripts, Dnmt3a1 and Dnmt3a3 [2]. Methylation

Abbreviations: ASB-PCR, allele- specific blocker PCR; *DNMT3A*, DNA methyltransferase; BM, bone marrow; WBC, white blood cells; HGB, hemoglobin; CR, complete remission; ID, Induction death; OS, Overall survival; DFS, disease free survival; MPN, myeloproliferative neoplasms; MDS, myelodysplastic syndromes; CMML, chronic myelomonocytic leukemia; PCR-RFLP, polymerase chain reaction restriction fragment length polymorphism; *FLT3*-ITD, FLT3 internal tandem duplication; *NPM1*, nuclophosmine; FAB, French American British.

Peer review under responsibility of Ain Shams University.

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https://doi.org/10.1016/j.ejmhg.2018.05.005
1110-8630/

activities of *DNMT3A* require interaction with histone methyltransferases and histone deacetylases which are needed for regulation of gene expression. [1].

DNMT3A mutations are distributed among both myeloid and lymphoid malignancies. In the myeloid group, mutations are detected in AML, Myeloproliferative neoplasms (MPN), myelodysplastic syndromes (MDSs) and chronic myelomonocytic leukemia (CMML) [4–6]. Across the lymphoid group, mutations are detected in T cell leukemias and lymphomas. Most of mutations are found in adult AML (14–35%) followed by T-ALL (17%) [2].

AML is classified into three major groups; AML with recurrent cytogenetic abnormalities, AML with myelodysplasia related changes and therapy related AML. Risk stratification of AML is based on cytogenetics and molecular abnormalities. Patients with normal cytogenetics (CN-AML) are classified with the intermediate-risk group. However, incorporation of molecular abnormalities to the risk classification has improved the risk of CN-AML from the intermediate to the favorable risk group for patients with mutated nuclophosmine (*NPM1*) or biallelic enhancer binding protein alpha (*CEBPA)* in the absence of FLT3 internal tandem duplication (*FLT-3*-ITD*)* mutation [7].

Somatic mutations of *DNMT3A* are classified into R882 versus non R882. The R882 mutations constitute 60% of all DNMT3A mutations and affect the methyltransferase domain of the *DNMT3* protein. Mutations at R882 are missense mutations and affect the amino acid at R882 with different variants: R882H, R882C, R882P, and R882S. The non R882 group includes, nonsense and frame shift mutations across the *DNMT3A* gene [8,9]. *DNMT3A* mutations are usually heterozygous. Homozygous bi-allelic mutations are confined to the T-lymphoid R882 and to the non-R882 mutations [2,5,9].

There is still some controversy regarding the prognostic impact of *DNMT3A* R882H mutations on adult AML [8–10]. Some reports have studied the prognostic effect of the association between *DNMT3A* mutation and other mutations such as *NPM1* and *FLT3*-ITD [2].

Direct sequencing has been used frequently in the past few years, for the detection of somatic mutations, although it is very informative but expensive when compared to other molecular techniques.

The aim of this study was to use simple and rapid molecular techniques; endonuclease restriction analysis and ASB-PCR, to investigate the frequency and prognostic impact of *DNMT3A* R882H mutation in de novo adult AML.

2. Patients and methods

2.1. Patients

In a period of 6 months, between January and June 2016, initial bone marrow samples from 56 Adults with de novo AML patients 18–60 years, were recruited for this study. All patients were referred to the outpatient clinic of the National Cancer Institute (NCI), Cairo University. Patients with secondary or previously treated AML and patients with AML (M3) positive for t (15;17) were excluded from the study. Diagnosis of AML was established according to the standard morphology, cytochemistry, and French American British (FAB) criteria [11]. The work has been carried out in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans and was approved by the ethical committee of the NCI (IRP number 2015160282).

All patients received the standards 3 and 7 protocols AML protocol of the NCI. Induction chemotherapy consisted of adriamycin 30 mg/m2 for 3 days and cytosine arabinoside (AraC) 100 mg/m2 by continuous infusion for 7 days. Further treatment of AML patients was according to their risk group [7].

2.2. End points

CR was defined as a normocellular BM containing less than 5% blasts. Failure of CR as either, partial remission (5%–15%) blasts or resistant disease (RD) (>15% blasts in the BM). Deaths within 30 days of entry were classified as induction death (ID). OS end points were measured from the date of diagnosis to death or last follow-up. For patients achieving CR, DFS points were measured from the date of documented CR to relapse.

2.3. Methods

2.3.1. DNA extraction

DNA was extracted from BM samples using the QiaAmp DNA extraction Mini kit (Qiagen, Hilden, Germany) according to manufacturer instructions. Genomic DNA was examined for quality and quantity on Nanodrop spectrophotometer.

2.3.2. Analysis of DNMT3A R882H

DNMT3A R882H mutation is the most frequent R882H mutation in AML. Two primers were used for amplification of exon 23 (NM_022552.4). Reaction mixture and primers were described previously, [12]. PCR products were analyzed on standard 1.5% agarose gels, expected PCR fragment after amplification was 444 bp. For mutation detection, 10ul of PCR product was digested by 10 units of Fnu4HI restriction enzyme (Thermo Scientific) and incubated overnight at 37 °C. The Digested product was separated by agarose gel electrophoresis and stained with ethidium bromide.

Custom labeled primers, probes, and blocker (Applied Biosystem) were prepared for the ASB-PCR. The PCR assay was performed on step one real-time PCR machine (Applied Biosystems). The sequence of primers, probes, and blocker was described previously [13]. One positive and one negative control samples were added to each run. The analytic specificity and sensitivity of ASB-PCR were tested. Sanger-based sequencing was performed on 3500 genetic analyzer, (Applied Biosystem) to confirm all positive cases [14].

2.4. Statistical methods

SPSS package version 17.0 was used for data management. Mann-Whitney was used to compare numerical variable in two independent groups. Chi-square tested proportion independence. Kaplan-Meier method was used to estimate survival and log-rank compared curves. P values ≤ 0.05 were considered significant.

3. Results

3.1. Characterization of the patients

We examined 56 patients with de novo AML. Median age was 38 (19–60) years, male to female ratio was 1:1. Of the examined patients, 13 (23%) presented with hepatomegaly, 6 (10.7%) with splenomegaly, and 8 (14%) with lymphadenopathy. *FLT3*-ITD mutation was detected in 12 (21.4%) patients and *FLT3*-TKD was detected in 4 (7.5%) patients. The median follow-up for survival was 1.69 (0–11.7) months. The cumulative OS at 6 months was (65%). Initial patient's characteristics are shown in Table 1.

3.2. DNMT3A R882H mutation in AML

Restriction endonuclease analysis, using Fnu4HI enzyme is represented in (Fig. 1). ASB-PCR and PCR-RFLP were 100% concordant.

Table 1
Initial patient's characteristics.

Variable	Frequency
Age (years)	
Mean SD	39.63 ± 11.87
Median (range)	38 (19–60)
Sex, n (%)	
Male	28 (50)
Female	28 (50)
TLC ($X10^9$/L)	
Mean ± SD	55.3 ± 67.16
Median (range)	24 (0.3–301)
Hb (g/dl)	
Mean ± SD	7.1 ± 1.7
Median (range)	7 (3.1–11.8)
Platelets ($X10^9$/L)	
Mean ± SD	65.7 ± 59.96
Median (range)	40 (10–246)
BM Blasts	
Mean ± SD	52.95 ± 26.80
Median (range)	51 (62–98)
FAB subtypes, n (%) (n = 53)	
M0	1 (1.8)
M1	15 (28.3)
M2	13 (24.5)
M4	18 (33.9)
M5	4 (7.5)
M7	2 (3.7)
FLT3-ITD, n (%)	12 (21.4)
FLT3-TKD, n (%)	4 (7.5)
Response to induction	
CR	29 (51.8)
RD	16 (28.6)
ID	11 (19.6)
OS	
Median, months	1.69
Cumulative at 6 months, %	56
DFS	
Median, months	5.7
Cumulative at 5 months,%	84.6

TLC: Total leukocytic count, Hb: hemoglobin, BM blasts: bone marrow blasts, FLT3-ITD: FLT3 internal tandem duplication, FLT-3-TKD, Tyrosine kinase domain, CR: complete remission, RD, resistant disease, ID: induction death, OS: overall survival, DFS: disease free survival.

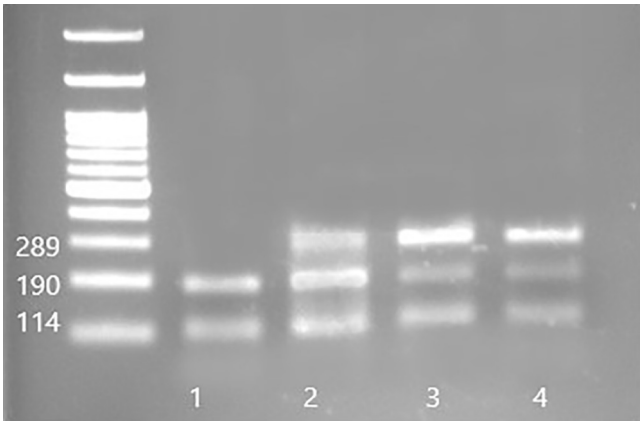

Fig. 1. PCR-RFLP for DMT3A R882H mutation. Lane 1 showing wild sample with two bands at 114 bp and 190 bp. Lanes 2,3 and 4 showing positive samples with three bands at 114 bp, 190 bp, and 289 bp.

ASB-PCR was faster and needed lower hands-on time compared to PCR-RFLP. The mean and median Ct-value for mutant and wild cases was (25.7/26 versus 33.6/34 respectively, p value = 0.001),

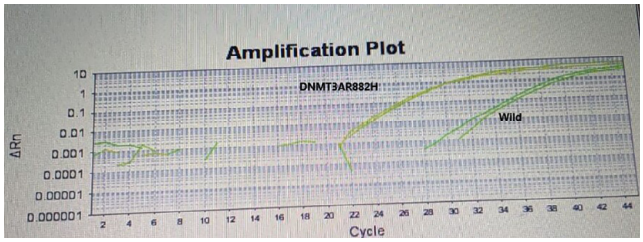

Fig. 2. Qualitative ASB-PCR showing Ct difference between wild and mutant cases.

(Fig. 2). All positive cases were confirmed by sanger sequencing (Fig. 3).

The frequency of *DNMT3A* was detected in 7/56 (12.5%) of patients. The mean age of patients with positive mutations tends to higher when compared to the mutation negative group (wild-type) (47 ± 7.3 vs 38.5 ± 12, p = 0.08). No association was found with initial laboratory parameters, including FLT3-ITD (p = 1), (Table 2).

3.3. Response to induction chemotherapy

Forty-five (45/56, 80.3%) patients were followed for 28 days, 29/56 (51.8%) patients achieved CR, and 16/56 (28.6%) had RD. Eleven patients 11/56 (19.6%) died during induction chemotherapy (ID). DNMT3A R882H mutation positive patients did not show statistical different response to induction chemotherapy when compared to the wild-type group (20% vs 79.3, p = 0.2), (Table 2).

3.4. Survival analysis

The median follow-up period for the whole group was 1.6 (0–11.7) months. The cumulative OS at 6 months was 65%, and DFS was 84%. *DNMT3A* mutation had no effect on the OS and DFS (100% vs 60%, p = 0.09 and 80% vs 85%; p = 0.59 respectively), (Table 2).

4. Discussion

In the present study, we identified *DNMT3A R882H* mutations in 12.5% of patients with M3 negative de novo AML. This frequency is very close to that (13%) reported by other studies, [12]. Higher frequency (27%) of *DNMT3A* mutation could be attributed to the selection of specific cytogenetic subgroups such as CN-AML versus a cohort of a non-AML M3 group in our study [15,16]. *DNMT3A* mutations are frequently associated with intermediate risk cytoge-

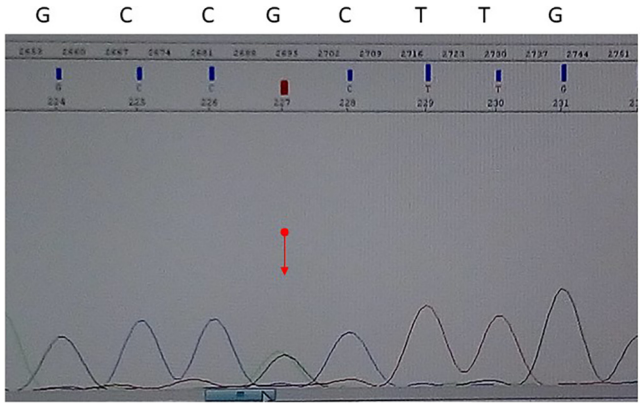

Fig. 3. Analysis of ASB-PCR specificity using sanger sequencing. The arrow is pointing to the mixed peak G > A.

Table 2
DNMT3A mutation association with initial patient's characteristics.

Variable	DNMT3A wild (n = 49)	DNMT3A mutant (n = 7)	P value
Mean age (years)	38.5 ± 12	47 ± 7.3	0.08
Sex, n (%)			
Male	25 (51)	3 (42.9)	
Female	24 (49)	4 (57.1)	1
Mean TLCx10^9/L	58.42 ± 69.77	36.25 ± 47.06	0.42
Mean HGB (g/dl)	7.09 ± 1.72	7.31 ± 2.20	0.76
Mean PLTx10^9/L	64.65 ± 58.43	72.5 ± 73.6	0.36
Mean BM blasts n (%)	52.67 ± 26.89	54.57 ± 28.31	0.87
Hepatomegaly, n (%)	10 (20.4)	3 (42.9)	0.34
Splenomegaly, n (%)	4 (8.2)	2 (28.6)	0.16
Lymphadenopathy, n(%)	7 (14.3)	1 (14.3)	1
FLT3-ITD, n (%)			
Wild	39 (79.6)	5 (71.4)	
Mutant	10 (20.4)	2 (28.6)	1
FLT3-TKD, n (%) (n = 53)			
Wild	43 (91.5)	4 (8.5)	1
Mutant	6 (100)	0	
FAB subtypes, n (%)			
M1 + M2	24 (85.7)	4 (14.3)	
M4 + M5	20 (90.9)	2 (9.1)	
Response to induction			
CR	23 (79.3)	6 (20.7)	
RD	15 (93.8)	1 (6.3)	0.2
ID	11 (100)	0	
OS			
Cumulative	60	100	0.09
DFS			
Cumulative	85	80	0.59

TLC: Total leukocytic count, Hb: hemoglobin, BM blasts: bone marrow blasts, FLT3-ITD: FLT3 internal tandem duplication, FLT-3-TKD, Tyrosine kinase domain, CR: complete remission, RD, resistant disease, ID: induction death, OS: overall survival, DFS: disease free survival.

netics, especially AML with normal karyotype, and are infrequent in adverse cytogenetic, and rare in cytogenetically low risk group [17]. In addition, we have found a tendency of *DNMT3A*R882H mutation to increase with age, (p = 0.08). This finding was consistent with previous studies [18,19]. Whereas, no association was found with initial laboratory parameters including WBC, HGB and BM blasts (p > 0.05) supporting the previous findings [20–22].

With respect to AML FAB subtypes, like previous studies [22,23], we were not able to find an association of *DNMT3A* mutation with a with specific FAB subtype. However, other studies [9,22,23] reported an association between FAB M4 and M5 and *DNMT3A* mutations.

Previous studies demonstrated an association between *DNMT3A* mutation with *FLT3*-ITD mutation [9,24,25]. These molecular associations between *DNMT3A* and *FLT3*-ITD were frequently detected in patients with CN-AML [17]. The selection of non-AML M3 patient cohort in this study, could be the main factor behind the lack of association. Similar results were previously observed [15]. In addition, the characterization of *DNMT3A* mutation with high WBC count and the FAB M5 and M4 subtypes can be attributed to the frequent co-occurrence of *DNMT3A* mutation with the *FLT3*-ITD mutation which is usually presented with these two laboratory features [10]. Besides, the association between *DNMT3A* R882 mutation with *NPM1* mutation could be a part of the association between *FLT3*-ITD and *NPM1* [2,17].

The effect of *DNMT3A* R882 mutation on response to induction chemotherapy and survival showed confusing results in the literature. In accordance with most of the studies [10,15–6,22], we have found no significant influence of *DNMT3A* R882H mutation on the response to induction chemotherapy. In contrast, Gaidzic et al. [17] found a slightly favorable outcome for patients carrying the mutation (p = 0.058) in an entire AML cohort. However, it was affected by the distribution of the mutation among cytogenetics of subgroups and not linked to the mutation [15]. On the other hand, Thol et al. [19] described inferior response to induction chemotherapy in *DNMT3A* positive patients compared to wild-type.

When we revised the published literature on the effect of *DNM3A* mutations on patient's survival, we have found a very contradicting results. However, they agreed on the effect of age, coexisting molecular alterations such as *FLT3-ITD* and *NPM1* and cytogenetic subgroups. In this work, we found no significant effect for *DNMT3A* R882H mutation on OS or DFS. This finding was supported by previous studies [5,17,24]. In contrast, other studies [8,22,25,26] found a highly significant inferior outcome for *DNMT3A* R882 mutation.

In this work, we used the most common molecular techniques, ASB-PCR and PCR-RFLP methods for the detection of *DNMT3A* R882H mutation. Both techniques showed 100% concordance. ASB-PCR was more reliable when compared to endonuclease restriction for the detection of the mutation. However, both methods are easy to interpret when used for the initial screening of AML patients.

Analysis of *DNMT3A* effect on patient's outcome in this study was limited by the small number of patients positive for the mutation, short follow-up period and testing for only the R882H type of mutations. The reason behind the death of 19.6% of our group of patients before the end of induction chemotherapy (ID) should be investigated thoroughly in future studies.

In conclusion, the *DNMT3A* R882H is a frequent mutation in de novo AML and tends to increase with age. Endonuclease restriction is a useful tool for detection of *DNMT3A* R882H mutation in AML. However, ASB-PCR is more rapid and could be used for quantitation of MRD after induction chemotherapy. Larger sample size and homogenous cytogenetics and molecular group are recommended for the study of the prognostic effect *DNMT3A* R882H mutation on AML patient's outcome.

Acknowledgments

No funding was received. All authors had full access to all data and take responsibility for the complete integrity of the data and accuracy of the data analysis. All authors meet the International Committee of Medical Journal Editors (ICMJE) criteria for authorship for this manuscript, take responsibility for the integrity of the work and have given final approval to the version to be published.

Disclosure

Ghada M. Elsayed, Abd Elgawad A. Fahmi Nevine F. Shafik, Reham A.A. Elshimy Heba K. Abd Hakeem, Sara A. Attea declare that they have no conflict of interest.

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