Effect of **DNMT3A** R882H Hot Spot Mutations on **DDX43** Promoter Methylation in Acute Myeloid Leukemia

Tahere Tabatabaei, Mohammad Reza Rezvany, Bahare Ghasemi, Farzane Vafaei, Masoumeh Kiani Zadeh, Farhad Zaker and Arash Salmaninejad

1Department of Hematology and Blood Transfusion, School of Allied Medical Sciences, Iran University of Medical Sciences, Tehran, Iran
2Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Isfahan, Iran
3Blood Transfusion Research Center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran
4Regenerative Medicine, Organ Procurement and Transplantation Multi-Disciplinary Center, Razi Hospital, School of Medicine, Guilan University of Medical Sciences, Rasht, Iran
5Department of Medical Genetics, Faculty of Medicine, Tehran University of Medical Sciences, Tehran, Iran

Correspondence should be addressed to Arash Salmaninejad; arash.salmany@yahoo.com

Received 16 February 2024; Revised 21 April 2024; Accepted 6 May 2024; Published 21 May 2024

Copyright © 2024 Tahere Tabatabaei et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Epigenetic alterations have been observed in many hematological malignancies, including acute myeloid leukemia (AML). Many of these alterations result from mutations in DNA methyl transferase (DNMT) enzymes, disabling them to methylate target genes in a proper way. In this case-control study, we investigated the association between R882H mutation in **DNMT3A** gene and **DDX43** gene methylation in patients with AML. 47 AML patients and 6 controls were included in this study. After DNA extraction, amplification refractory mutation system (ARMS)-PCR was used to evaluate R882H mutations in **DNMT3A** gene. The high-resolution melting (HRM) method was used to determine the methylation changes of the **DDX43** gene promoter. R882H mutation was only found in 10.6% (5 out of 47) of AML patients. The frequency of **DDX43** gene methylation was significantly higher in patients without R882H mutations compared to patients with R882H mutations \( (P < 0.05) \). The **DNMT3A** R882H mutation is typically present in a minority of AML patients. Nevertheless, this mutation is associated with a reduced frequency of methylation in the **DDX43** promoter region.

1. Introduction

Acute myeloid leukemia (AML) is an aggressive malignancy of monocyte or granulocyte cells contributing to about 80% of all blood cancers in adults, and it is a type of leukemia characterized by the uncontrolled growth of abnormal precursor cells and halted differentiation. Until 2017, it was the most prevalent leukemia subtype in adults in the US but was later surpassed by CLL (chronic lymphocytic leukemia). In 2019, an estimated 21,450 adults were expected to be diagnosed with AML, with males comprising 11,650 cases and females comprising 9,800 cases. AML is responsible for the largest proportion (62%) of leukemia-related deaths [1, 2]. The incidence of this malignancy is 4.3 per 100,000 annually in the United States (US). The five-year survival rate of people with AML is dependent on several factors, varying from less than 30% (for people ≥ 20) to 70% (for people older than 20). Given the aggressive entity of this malignancy and its considerable incidence, it is crucial to understand the mechanisms underlying this cancer [1].

The mechanisms contributing to the development of AML include a wide spectrum of genetic mutations and
epigenetic aberrations. Mutations in epigenetic regulators such as DNA methyl transferase (DNMT) enzymes and those involving in histone modifications can occur very early before malignancy manifestation and facilitate clonal expansion of hematopoietic stem cells [3]. Generally, DNMT enzymes direct DNA methylation transferring methyl group from S-adenosyl methionine (SAM) to cytosine on 5-carbon positions. DNA methylation causes chromatin stability and represses gene expression whereas DNA demethylation causes chromatin instability and activation of gene transcription. Dysregulation of these epigenetic mechanisms can activate oncogenes and/or inactivate tumor suppressor genes, thereby promoting cancerous phenotype [4, 5]. The members of the DNMT family include DNMT1, DNMT2, DNMT3A, DNMT3B, DNMT3L, and DNMT3C, which all cause nucleic acid methylation at different times of the cell cycle. DNMT1 concerns with maintenance methylation, ensuring the transmission of lineage-specific DNA methylation patterns during DNA replication [6, 7]. DNMT2 contributes to methylation of transfer RNA (tRNA) instead of DNA [8]. DNMT3A and DNMT3B play an important role in de novo methylation during the embryonic development. In fact, DNMT3B set genomic methylation pattern in early embryonic stages, whereas DNMT3A is expressed in the later developmental stages as well as postnatal [5, 9]. DNMT3L has an inactive catalytic domain and can only cooperate with DNMT3A in DNMT3L-DNMT3A heterodimers. Also, it is required for imprinting and spermatogenesis [10, 11]. The exact mechanism of DNMT3C in human is not yet elucidated; however, its de novo methylation activity has been shown to be necessary in protecting murine germ cells from deleterious activity of transposons [12].

Given the crucial role of DNMTs in the regulation of gene expression, mutations in them can result in inappropriate activation/inactivation of cancer-related genes, causing malignant phenotype. Early studies suggest that DNMT3A mutations are involved in hematological malignancies such as myelodysplastic syndrome (MDS), adult early T-cell precursor acute lymphoblastic leukemia (ETP-ALL), and AML [13–17]. In AML patients, more than half of the DNMT3A mutations are heterozygous missense mutations within the catalytic domain at arginine 882 (R882) [18]. Mutations in this hot spot have a dominant-negative effect and are associated with higher levels of peripheral blood hemoglobin and poor prognosis [19]. In fact, these mutations keep DNMT3A from methylating of several target genes, resulting in hypomethylation and activation of them. One such gene is DEAD (Asp-Glu-Ala-Asp) box polypeptide 43 (DDX43), also known as HAGE, a member of the cancer/testis antigen (CTAs) family [20]. DDX43 was first identified together with sarcoma antigen (SAGE) as a tumor-specific CTA gene in a human sarcoma cell line [21]. DDX43 has been identified as being overexpressed in a range of solid tumors including those affecting the salivary gland, colon, brain, lung, and prostate, as well as in hematologic malignancies like chronic myeloid leukemia and multiple myeloma [22–29]. Recently, abnormal hypomethylation of DDX43 promoter has frequently been reported in hematologic malignancies, including CML, MDS, and AML [30]. Hypomethylation of DDX43 gene promoter results in activation of its transcription, which leads to induction of RAS protein expression and signaling. In addition, studies suggest that the RNA helicase activity of this gene has a key role in the resistance of ABCB5+ malignant melanoma stem cells to IFNα treatment by promoting SOCS1 expression [31]. Given the high frequency of DDX43 hypomethylation in hematologic malignancies due to DNMT3A mutations [22], nevertheless, the pattern of DDX43 methylation has not been well studied in AML; in this study, we aim to evaluate the relation between DNMT3A R882H mutations and DDX43 methylation status in Iranian patients with AML.

2. Method and Material

2.1. Patients and Controls. In this study, 47 newly diagnosed AML patients with ≥30% blast were enrolled, who referred to Shariati Hospital from June 2020 to December 2021. The patients’ AML type was classified according to French-American-British (FAB) criteria. The clinical and laboratory characteristics of the participants are summarized in Table 1.

Peripheral blood specimens were taken from all the participants and collected in EDTA-containing tubes. For peripheral blood mononuclear cell (PBMC) separation, the blood samples were diluted 1:2 times in phosphate buffered saline (PBS) and then subjected to density gradient centrifugation (1.08 g/ml) by Ficoll-Hypaque (Pharmacia, Freiburg, Germany) in falcon 15 mL conical centrifuge tubes at 700 g for 20 min. PBMC samples were then transferred to fresh 1.5 mL microcentrifuge tubes and washed with PBS at 500 g for 10 minutes.

2.2. DNA Extraction. DNA was extracted from each PBMC sample using QIAamp Blood DNA Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer’s instructions. The quality of extracted DNA was evaluated by electrophoresis on 1% agarose using loading dyes.

2.3. Bisulfite Conversion. DNA samples were subjected to modification by sodium bisulfite for methyl-sensitive high-resolution melting curve (MS-HRM). After obtaining DNA concentration by NanoDrop ND-1000 (Nanodrop Technologies, Wilmington, Delaware, USA), we used 1 mg/mL of each DNA sample for bisulfite treatment using EZ DNA Methylation-Golden kit (Zymo Research Crop. Irvine, CA, USA). The final elution volume was 20 μL.

2.4. ARMS-PCR. For detection of R882H mutation in exon 23 of DNMT3A gene, we performed tetra-primer amplification refractory mutation system (ARMS)-PCR. For this purpose, DNMT3A DNA sequence was taken from the NCBI (http://www.ncbi.nlm.nih.gov) website. Primers were designed with Primer1 online software (http://primer1.soton.ac.uk/primer1.html). Primers’ details are given in
The promoter sequence was as follows: forward 5′-GGGGTTTTTAAGTAG-3′ and reverse 5′-CGTCCAACCTCTACACACCTA-3′. The promoter sequence was obtained from https://genome.ucsc.edu and then evaluated in Metprimer online software (https://www.urogene.org/metprimer/).

Each reaction consisted of 3 μL 5X Hot FIREPOL Eva Green qPCR Mix, 0.5 μL forward primer, 0.5 μL reverse primer, and 1 μL bisulfite-modified DNA. The final volume got to 15 μL with distilled water. The protocol was set on 95°C preincubation for 15 minutes, 45 cycles of 95°C for 15 seconds, 60°C annealing temperature for 20 seconds, and 72°C extension temperature for 20 seconds, and then, 10 cycles were added to MS-HRM consisting of 95°C heating for 1 minute, 40°C for 1 minute, 65°C for 1 minute, and continuous heating to 95°C at a ramp rate of 0.05°C per second. Fully methylated and unmethylated standards were purchased from the Pasteur Institute of Iran (Tehran, Iran). For creating a standard curve, we prepared different dilutions (0, 25, 50, 75, and 100%) and used them in our run work. Sample curves were then compared with standard curves.

2.7. Statistical Analysis. Data are presented as median and mean ± SD. Data normality was assessed using the Shapiro–Wilks test. Student’s t-test and Mann–Whitney U tests were used to compare quantitative data. Fisher’s exact test and Chi-squared test were carried out to compare frequencies. P < 0.05 was considered as statistically significant. Statistical analysis was performed using STATA (v.14) software.

3. Results

3.1. Patients’ Characteristics. In this study, 38% (18/47) of the patients were women and 61% (29/47) were men. The average age of patients at the time of diagnosis was 47.91 ± 18.45, whereas the average age of the controls was 41.2. The median age of the patients and the controls was 39 and 40, respectively. AML subtypes were classified based on FAB criteria, and M4 (16/47) and M2 (10/47) were the commonest types, respectively. WBC was higher while RBC and platelets were significantly lower than healthy donors (P < 0.05) as shown in Table 1.

3.2. ARMS-PCR. According to the results from our tetra ARMS-PCR, five patients were heterozygote for R882H mutation, where A was substituted by G in nucleotide 2853 located in exon 23. This status was indicated by observing three bands on gel electrophoresis. Arginine to histidine change results in a negative domain on wild-type protein. The control band (317 bp) was observed in all samples. In samples with mutation, 2 other bands (214 bp and 160 bp) were observed in addition to the control band. Other 42 patients had only 2 bonds in 317 and 160 bp (Figure 1).

3.3. Sanger Sequencing. As stated in materials and methods, we performed Sanger sequencing on five mutated and three unmutated samples to confirm the results from the ARMS-PCR method. Our sequencing results confirmed the genotyping results of ARMS-PCR and demonstrated that 5
patients showed A > G transition at nucleotide 2853, which results in R882H missense mutation. This mutation is located in the catalytic domain and results in a negative domain and the lack of activity (Figure 2).

We also evaluated the associations between R882H mutations and clinical and laboratory characteristics of the patients, including WBC, PLT, RBC, and AML subtypes. Three of DNMT3A R882H-mutated patients were in M4 subtype, one in M2 subtype, and one in M1 subtype. Therefore, M4 and M2 are the commonest subtypes in R882H-mutated patients, as were in unmutated patients. Three patients with the mutation were male, and 2 were female.

All of them were normal in karyotypes, and only one had unfavorable risk del (5)(q22; q34) with higher median WBC and PLT. Also, there was not any association with R882H mutation and other clinical and laboratory characteristics.

3.4. HRM Assay. HRM assay can distinguish a 0.01°C difference in the melting temperature of DNA molecules. Serial dilutions of fully methylated and unmethylated standards were prepared, and each of the 25, 50, 75, and 100% graphs was compared with the unmethylated graph. The melting curves are given in Figure 3.
Figure 3: Continued.
3.5. The Relation between DNMT3A R882H Mutation and DDX43 Promoter Methylation. Subsequent to genotyping and evaluation of the methylation status, we compared these two states to see whether there is any association between R882H mutation in DNMT3A gene and promoter methylation in DDX43 gene.

The data regarding the distribution of patients (with or without R882H mutation) and controls in various methylation status groups are given in Table 3. As shown in this table, the major number of patients without R882H mutation (30 out of 42) showed 75-100% methylation in DDX43 promoter, whereas only 1 out of 5 patients with R882H mutation showed this methylation status. Therefore, the distribution of mutated and unmutated patients in the methylation status groups was significantly different (P < 0.05). In addition, none of the healthy controls revealed 75-100% methylation, demonstrating a statistically significant difference between patients and controls (P < 0.05).

4. Discussion

In this study, using ARMS-PCR and Sanger sequencing, we evaluated the frequency of R882H mutations in exon 23 of DNMT3A gene in newly diagnosed AML patients and healthy controls. In addition, we assessed the association between R882H mutations and methylation status in DDX43 gene promoter. Our results demonstrated that 10.6% of the patients showed R882H mutation, and this mutation was significantly associated with decreased methylation in DDX43 gene (P < 0.05), suggesting the possible role of DNMT3A function in promoter methylation and transcriptional inactivation of DDX43. Our result may shed a light on the mechanism of action of DNMT3A mutation R882H, which is a prognostic factor for AML patients.

DNMTs play an important role in genomic methylation during the cell cycle. DNMT-mediated methylation of gene promoters results in transcription repression and reduced gene expression. Aberrant methylation of various genes has been demonstrated in solid tumors and various hematological malignancies such as MDS, CML, and AML [32].

DNMT3A is an important member of DNMTs and plays an important role in de novo methylation of DNA [5, 9]. Mutations in DNMT3A gene have been reported frequently in AML, and R882H mutations are the most frequent ones [33]. R882H mutations disappear after complete remission in AML patients [34]; therefore, this factor can be used for therapeutic monitoring and even the type of treatment choice.

Ley et al. found R882H mutations in 37 of 281 (13.1%) AML patients with massively parallel sequencing of DNMT3A gene, of which 27 resulted in arginine to histidine at this position R882H [15]. In another study on 63 Egyptian AML patients, 17 patients (27%) showed mutations, of
which 11 patients (61.1%) were burdened with R882H mutation [35]. On the other spectrum, in a Chinese study, Yamashita et al. reported that, from 870 adult patients receiving standard induction therapy, 74 patients (8.51%) showed R882H mutations, investigated by pyrosequencing [36]. Also, Lin et al. showed that DNMT3A R882H mutations were found more frequently among monoblastic leukemia compared to non-monoblastic leukemia (P = 0.041). Their results further confirmed the specificity of DNMT3A R882H mutations in monocytic lineage; furthermore, 86.6% of R882H mutations were observed in patients with normal karyotypes [34]. The discrepancy in the prevalence of R882H mutations in AML patients may be due to differences in patients’ numbers, differences in race and ethnicity, the method used to detect the mutations, and other genetic and environmental factors. In the present study, we found that 10.6% of the patients showed R882H mutations in a heterozygous state, which is almost consistent with the previous studies, all demonstrating that <20% of the patients harbor R882H mutations.

In the present study, we also evaluated the associations between R882H mutation and clinical and laboratory characteristics of our AML patients. In line with previous studies [37], we did not find any associations between R882H and patient characteristics, including, age, gender, and WBC, PLT, and RBC counts. However, Ghannam et al. reported the association between higher age and WBC count and R882H mutations in 63 cytogenetically normal AML patients [35].

DDX43 is a new member of the RNA helicase family that contributes to pre-mRNA splicing, RNA metabolism, and RNA degeneration [38]. Other family members such as DDX51, DDX5, and DDX53 play important roles in several cancers [39, 40]. In 2014, Lin et al. reported that the DDX43 gene promoter gets hypomethylated frequently in AML and the DDX43 protein level is significantly higher in hypomethylated patients than in methylated patients. Studies have also shown that this event is a favorable prognostic factor in AML [37] as well as MDS [9, 30]. On the other hand, Roman-Gomez et al. found a poor prognosis in CML patients [26]. Given the frequent studies reporting the hypomethylation of DDX43 in hematological malignancies, and the significance of R882H mutations in DNMT3A, we hypothesized that there may be some relations between these two events. We detect a significant association between R882H and low methylation status. Given the favorable prognostics of DDX43 hypomethylation which has been reported by the previous studies [9, 30, 37] and the association of this hypomethylation with R882H mutation in DNMT3A in our study, it may be concluded that R882H mutation may be a favorable prognostic factor for AML patients. Previous studies have reported that the prognostic impact of DNMT3A R882H versus non-R882H mutations in AML is inconclusive [18, 41, 42]. A recent study showed that DNMT3A R882H mutations confer unique clinical characteristics in MDS, including a high risk of AML transformation [43]. Similar to DNMT3A R882H mutation, DDX43 has been shown to be involved in tumor cell development in many types of cancers [40]. However, DDX43-related transformation may not be so devastating as Lin et al. reported that DDX43 hypomethylation was associated with favorable/intermediate-risk groupings in AML [37]. Also, DDX43 expression increases following exposure to demethylating agent 5-aza-deoxycytidine which can help following response to therapy [37].

In conclusion, considering all of the above-mentioned studies, as well as our study, it may be concluded that DNMT3A R882H mutations, which are usually observed in <20% of AML patients, may have a role in transforming blood cells to AML, but this event is a favorable prognostic factor. The reason for this conclusion may be that this mutation may result in decreased methylation of DDX43 promoter. In summary, the DDX43 gene is activated by promoter hypomethylation and DDX43 hypomethylation may be a favorable prognostic factor in AML. Further studies, however, are needed to confirm the association between DNMT3A R882H mutation and DDX43 promoter hypomethylation and to elucidate the exact mechanism by which DDX43 functions in AML.

Data Availability

The data that support the findings of this study are available on request from the corresponding author.

Ethical Approval

This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Iran University of Medical Sciences (IR.IUMS.REC1395.9411264004).

Consent

Consent is obtained from the included patients in the study.

Conflicts of Interest

The authors declare that they have no conflict of interest.

Acknowledgments

Financial support was given by the Iran University of Medical Sciences (9411264004).

References


J. Lin, Q. Chen, J. Yang et al., “DDX43 promoter is frequently hypomethylated and may predict a favorable outcome in acute


