Original Article

Analysis of DNA Methyltransferase 3A Gene Mutations in Patients with Philadelphia-negative Myeloproliferative Neoplasms

Neda Ketabchi, Mostafa Paridar, Javad Mohammadi-Asl[1], Alireza Abooali, Maria Kavianpour, Najmaldin Saki

Research Center of Thalassemia and Hemoglobinopathy, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, [1]Department of Medical Genetics, School of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

Abstract

Context: Philadelphia (Ph)-negative myeloproliferative neoplasms (MPNs), including essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF) from a group of disorders characterized by dysregulated JAK-STAT functionality, abnormal hematopoiesis, as well as increased production of proliferative cytokines. In addition to $JAK2^{V617F}$ mutation, additional gene alterations that are involved in epigenetic mechanisms, particularly *de novo* DNA methyltransferase 3A (DNMT3A), have been described in Ph-negative MPN biology. **Aims:** The aim of this study is to evaluate the H/C/S/P mutations in codon R882 of DNMT3A gene among patients with Ph-negative MPNs. **Subjects and Methods:** This study was conducted on 64 newly diagnosed patients with PV, ET, and PMF who referred to Shafa Hospital, Ahvaz, Iran. In the beginning, 5 mL whole blood was drawn from each patient, and the DNMT3A R882 codon mutations were investigated following the isolation of peripheral blood mononuclear cells by DNA amplification protocol using polymerase chain reaction and DNA sequencing techniques. **Results:** The R882H G > A mutation, which results in an amino acid substitution at position 882 of DNMT3A gene from arginine (R) to histidine (H), was observed in two patients (3.1%) with $JAK2^{V617F}$ positive PV and $JAK2^{V617F}$ negative PMFs. **Conclusions:** Based on the results, DNMT3A-R882 mutations occur at a low frequency in patients with Ph-negative MPNs. To the best of our knowledge, this is the first study to specifically estimate the prevalence of DNMT3A mutations among Ph-negative MPN patients living in the Middle East. It is recommended to investigate these mutants as a secondary defect along with common major complications in such patients.

Keywords: *DNA methyltransferase 3A, epigenetics, myeloproliferative neoplasm*

Introduction

Myeloproliferative neoplasms (MPNs) are hematopoietic stem cell (HSC) malignancies that are associated with uncontrollable proliferation and development of myeloid lineages, causing progress toward bone marrow (BM) failure and myelofibrosis, ineffective hematopoiesis, or acute leukemia.[1] The identification of common genetic events, including mutations or rearrangements of genes encoding protein tyrosine kinases that are involved in a number of signaling pathways, has significantly changed the diagnostic approach of Philadelphia (Ph)-negative MPNs, especially polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF).[2] As the most important defect in this type of disorders, $JAK2^{V617F}$ mutation is found in over 95% of PV cases, as well as 50% of ET and PMF patients.[3] Nowadays, epigenetic studies have shown that the incidence of a somatic mutation is not sufficient by itself for the pathogenesis of Ph-negative MPN and is not the only critical factor for the progress of such diseases toward acute leukemia.[4] In addition, a significant number of patients with ET and PMF may have uncommon mutations of unknown clinical significance.

Epigenetic mechanisms, including DNA methylation, histone modifications, and noncoding RNAs act synergistically to regulate chromatin structure and gene expression. Meanwhile, DNA methylation is a fundamental factor to determine the fate of cells through controlling the balance between HSC self-renewal and differentiation, since the disruption of cellular balance leads to proliferative neoplasia.[5] The mammalian DNA methyltransferase (DNMT) family are responsible for this process, and in particular, *de novo* DNA methyltransferase 3A (DNMT3A) is reported to be highly expressed in $CD34^{+}$ cells of BM, but its

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Address for correspondence:
Dr. Najmaldin Saki, Research Center of Thalassemia and Hemoglobinopathy, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran. E-mail: najmaldinsaki@gmail.com

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expression level is reduced following the differentiation of hematopoietic progenitor cells.[6]

Recognizing the effect of aberrant methylation causing the development of several malignancies, including acute myelogenous leukemia (AML),[7] acute lymphoblastic leukemia (ALL),[8] myelodysplastic syndromes (MDS),[9] and MPNs, the impaired expression of DNMT3A may account for the etiology of such disorders through induction of aberrant DNA methylation patterns. Several mutations have been detected in the gene encoding DNMT3A, in which the exon 23 missense variants, especially at position 882, are the most common type of mutations converting arginine to histidine (R882H), cysteine (R882C), serine (R882S), and phenylalanine (R882P).[10] Since there has been no detailed investigation monitoring the Ph-negative MPN patients in Iran, assessment of H/C/S/P mutations in codon R882 of DNMT3A gene may provide valuable information regarding early diagnosis, prognosis, and progression of the PV, ET, and PMF patients toward acute leukemia.

Subjects and Methods

Sample collection

This study was conducted on 65 newly diagnosed patients with Ph-negative MPNs who referred to Shafa Hospital of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran during 2014–2015. Based on the WHO criteria, all the patients were assigned to three groups of PV, ET, and PMF.[11] The initial diagnosis was based on morphological examination of peripheral blood (PB) and BM aspiration in addition to the information obtained from laboratory assessments and clinical examination. Five ml ethylenediaminetetraacetic acid-anticoagulated PB sample was drawn from all the patients before the initiation of therapy. This study was approved by the Local Ethics Committee of the Ahvaz Jundishapur University of Medical Sciences, and written informed consent was obtained from all the studied patients.

DNA extraction

DNA was extracted from PB cells according to instructions of QIAamp DNA Blood Mini Kit (Qiagen, Germany). To ensure the quality of extracted DNA, the absorbance (optical density) of purified samples was confirmed at 260 and 280 nm by a spectrophotometer with purity in the range of ≤1.8. The extracted samples were stored at −80°C for polymerase chain reaction (PCR).

Polymerase chain reaction and sequencing

For DNMT3A mutation analysis, exon 23 of the target gene was amplified by PCR; then, the PCR products were sequenced. Briefly, PCR was performed in a 20 μL volume containing 1× PCR buffer, 0.6 mmol/L of deoxynucleotide triphosphates, 1 mmol/L of $MgCl_2$, 0.5 mmol/L of forward and reverse primers, 0.5 U of Taq DNA polymerase, and 1 μL of genomic DNA. The primers of DNMT3A exon 23 were as follows: 5'-GAACTAAGCAGGCGTCAGAGG-3' (forward) and 5'-CTGGGTGCTGATACTTCTCTCC-3' (reverse). PCR reactions were carried out on an ABI 2720 Thermal Cycler (Applied Biosystems, USA). After denaturing at 95°C for 5 min, the amplification was conducted for 35 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, followed by reextension for 5 min at 72°C. The PCR products were loaded onto 1.5% agarose gel containing ethidium bromide and were electrophoretically separated. After purification, the PCR products were directly sequenced in both directions using ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, USA) to screen for the presence of mutations.

Statistical analysis

Data were analyzed using SPSS (Statistical Package for Social Service Inc., Chicago, IL, USA) software version 19.0. In this study, descriptive statistics was used to show the results, including mean ± standard deviation and median.

Results

In this study, 64 newly diagnosed patients with Ph-negative MPN (30 men and 34 women) whose disease was confirmed by clinical and laboratory experiments were selected with a mean age of 52 years (age range of 21–78 years). There were 37 cases of PV (57.81%), 23 cases of ET (35.93%), and 4 cases of PMF (6.25%).

The clinical presentations of patients were evaluated, among whom only eight patients with PV, five patients with ET, and all the four patients with PMF had splenomegaly. Moreover, hepatomegaly was observed in three patients with PV, ET, and PMF. Hematologic parameters examined in this study included white blood cells, hemoglobin, and platelets [Table 1]. $JAK2^{V617F}$ mutation was positive in 35 PV patients, five ET patients, and three PMF patients.

Following DNA sequencing of exon 23 PCR products, DNMT3A-Arg882His (R882H) mutation was found in only two patients with PV and PMF, resulting in 882 R (C**G**C)→H (C**A**C) substitution [Figure 1]. These two mutated patients with PMF and PV were negative and positive for $JAK2^{V617F}$ mutation, respectively, and both had splenomegaly but showed no other evidence of hepatomegaly at the time of diagnosis [Table 2].

Discussion

Ph-negative MPNs, including PV, ET, and PMF, are caused by a clonal proliferation of malignant HSCs. Despite numerous attempts to identify the etiology and molecular mechanisms of these disorders, there has been no comprehensive understanding with respect to the pathogenesis of this type of hematologic malignancy

Table 1: Laboratory parameters of the study subjects

Hematological parameters	PV			ET			PMF		
	Median	Range	Mean±SD	Median	Range	Mean±SD	Median	Range	Mean±SD
WBC (10^3/mm^3)	8.6	2-17	9±3.9	10.6	2.9-18.8	11.2±4.2	11.5	3.1-18.4	11.1±7.1
Hb (g/dL)	17.8	14.9-22.9	18±1.6	11.4	7.4-15.6	11.3±2.3	9	8.3-14.4	10.2±2.8
Plt (10^3/mm^3)	399.1	116-1052	311±24.2	933.3	574-1927	892±28.9	284.5	131-547	311.7±173.1

WBC: White blood cell, Hb: Hemoglobin, Plt: Platelet, PV: Polycythemia vera, ET: Essential thrombocythemia, PMF: Primary myelofibrosis, SD: Standard deviation

Table 2: Basic characteristics of two DNA methyltransferase 3A mutated patients

Sex	Age	Disease	BCR-ABL1 mutation	JAK2^{v617f} mutation	WBC ($\times10^3$/mm^3)	Hb (g/dl)	Plt ($\times10^3$/mm^3)	Clinical findings
Female	46	PV	Negative	Positive	13.5	16.8	436	Splenomegaly
Male	62	PMF	Negative	Negative	3.1	8.3	131	Splenomegaly

WBC: White blood cell, Hb: Hemoglobin, Plt: Platelet, PV: Polycythemia vera, PMF: Primary myelofibrosis

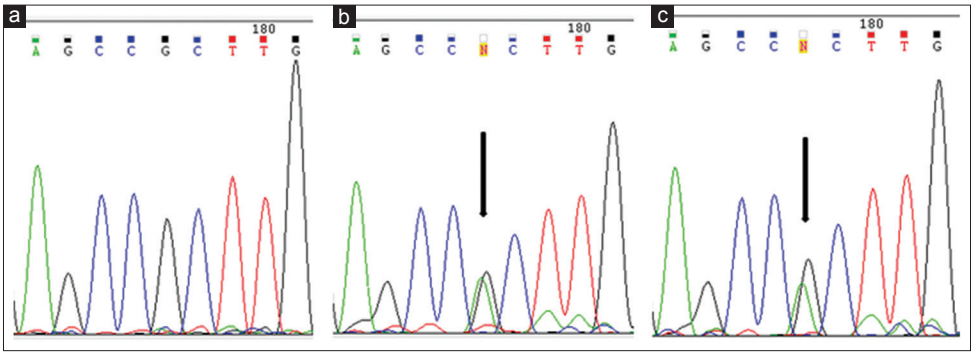

Figure 1: The DNA methyltransferase 3A mutation and wild type.(a) Wild-type DNA methyltransferase 3A, (b) polycythemia vera patient with DNA methyltransferase 3A mutation (G>A, p.R882H), and (c) primary myelofibrosis patient with DNA methyltransferase 3A mutation (G>A, p.R882H)

to be able to eliminate the leukemic stem cells (LSCs). In addition to molecular defects in cytosolic signaling molecules and transcription factors controlling hematopoiesis, there is evidence on the important role of mutations in epigenetic regulators involved in the development of hematological malignancies.[12] DNA methylation is an important epigenetic mechanism, controlling the expression of a wide range of genes in the human body. Aberrant expression or structural mutations of DNMT genes can lead to a number of malignancies associated with altered DNA methylation patterns in the form of hypomethylation or hypermethylation.[13] Several studies have indicated a high prevalence of DNMT3A mutation among AML-M4/M5 patients, especially in a hotspot fragment of the R882codon, which is associated with an older age and hyperleukocytosis in such patients.[14] However, this type of mutation has rarely been reported in other hematologic malignancies such as ALL, MPN, and MDS. Grossmann *et al*. indicated a strong correlation between DNMT3A mutation with decreased survival and poor prognosis of T-ALL patients.[8] Moreover, Wang *et al*. showed that this type of mutation has a poorer prognosis relative to JAK2^{V617F} among MPN and MDS/MPN patients.[15] Obviously, the occurrence of mutations with a poor prognosis should be a diagnostic priority in such patients; however, the low prevalence of these genetic complications and the absence of adequate studies complicates this matter.

DNMT3A mutation in patients with Ph-negative MPNs was first studied by Stegelmann *et al*.[16] They were only able to identify two PV and three PMF cases out of 115 MPN patients harboring mutations in DNMT3A exon 2–23 together with JAK2^{V617F} somatic mutation [Table 3].

Preliminary results highlighted that these mutations might play a role in the development of malignancy, but other effective mutations are needed for the persistence of malignant conditions. In this study, DNMT3A mutation was assessed in 64 patients with PV, ET, and PMF, among whom only two patients with PV and PMF were detected to harbor the mutation. R882H was the observed nucleotide sequence alteration in both JAK2^{V617F} positive PV and JAK2^{V617F} negative PMF patients. Considering the fact that DNMT3 mutation occurs with or without JAK2^{V617F} mutation, it cannot be regarded as an independent risk factor for the development of these diseases Nangalia J *et al*.[17] On the other hand, unlike AML patients, the role of DNMT3A mutations in the prediction of disease severity and progression toward acute phase remains to be unknown in Ph-negative MPN population. In this regard, DNMT3A mutation does not seem to be a common finding in early stages of MPNs in comparison to patients progressing

Table 3: DNA methyltransferase 3A mutations among patients with Philadelphia-negative myeloproliferative neoplasms

Reference	Investigated exons	Type of mutations		
		PMF percentage (*n*/total)	ET percentage (*n*/total)	PV percentage (*n*/total)
Stegelmann *et al*.[16]	All coding exons (2-23)	1.5 (3/20)[#] E523*/W305fs/P264fs	-	7 (2/30)[#] R882H/R488fs
Abdel-Wahab[18]	All coding exons (2-23)	2 (1/46)[##] R882H	-	-
Brecqueville *et al*.[19]	Exons 15-23	4 (1/25)[##] R882S	-	3 (1/33)[#] R749C
Rao *et al*.[20]	Exon 23	-	-	3 (2/75)[#] M880V/R882C
Brecqueville *et al*.[21]	Exons 15-23	3 (1/30)[#] R882S		3 (1/30)[#] R749C
Lin *et al*.[22]	Exon 23	-	1 (1/91)* R882H	-
Wang *et al*.[15]	Exon 23	4 (1/24)[#] R882C	-	-
Our study	Exon 23	25 (1/4)[##] R882H	-	3 (1/37)[#] R882H

[#]Mutant JAK2^{V617F}, [##]Wild typeJAK2^{V617F}, *Nonsense mutation. fs: Frameshift mutation, PMF: Primary myelofibrosis, PV: Polycythemia Vera, ET: Essential thrombocythemia

to acute leukemia. Rao *et al*. have reported two M880V and R882C mutations following their exon 23 sequence analysis of 75 patients with PV.[20] They also assessed the frequency of an R882C allele in CD14$^+$, CD3$^+$, and CD19$^+$ cells, which was revealed to be limited to the myeloid lineage. Lin *et al*. have studied the lineage specificity of DNMT3A mutation in only one mutated ET patient out of 130 Ph-negative MPN cases and found that the R882H allele was limited to myeloid cells as well.[22] In fact, by providing favorable conditions, DNMT3A mutation is likely to be conducive to the growth and proliferation of the myeloid lineage. By genotyping the burst forming unit-erythroid colonies of a PV patient, Rao *et al*. observed the R882C mutation (but not the JAK2 mutation) in all the investigated colonies.[20] These findings address the hypothesis that whether the development of these epigenetic changes predisposes to other defects related to the pathogenesis of MPNs.

Identification of the exact role of DNMT3A in controlling the genes involved in hematopoiesis is an important issue in this background since the decreased or increased activity of this enzyme causes irreversible complications in myeloid precursors, as well as the incidence of malignancy.[23] Experimental results on DNMT3A gene represent mutation in R882 codon of both PV and PMF patients, which would be associated with poor prognosis in such cases. Studies investigating this mutation in different hematological disorders have suggested the aberrant methylation of tumor suppressor genes involved in such malignancies.[24,25] It seems that further studies on the evaluation of quantitative DNMT3A gene promoter methylation among MPNs, as well as the comparison of their methylation patterns with expression profiles of other genes involved in disease recurrence, could be helpful for the prediction of clinical course of the patients.

Conclusions

In addition to aberrant DNA methyltransferase activity, the probable functions of DNMT3A mutations, including the involvement in histone modifications or other regulatory epigenetic mechanisms, demand further investigations. Hence, an important question arises: is the DNMT3A enzyme responsible for the control of gene expression within LSCs or malignant precursor cells could affect the activity of genes involved in the cell cycle in ways other than epigenetic DNA methylation modification?

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Conflicts of interest

There are no conflicts of interest.

References

1. Tefferi A, Pardanani A. Myeloproliferative neoplasms: A contemporary review. JAMA Oncol 2015;1:97-105.
2. Ortmann CA, Kent DG, Nangalia J, Silber Y, Wedge DC, Grinfeld J, *et al*. Effect of mutation order on myeloproliferative

neoplasms. N Engl J Med 2015;372:601-12.
3. Poopak B, Hagh MF, Saki N, Elahi F, Rezvani H, Khosravipour G, *et al*. JAK2 V617F mutation in Iranian patients with myeloproliferative neoplasms: Clinical and laboratory findings. Turk J Med Sci 2013;43:347-53.
4. Saeidi K. Myeloproliferative neoplasms: Current molecular biology and genetics. Crit Rev Oncol Hematol 2016;98:375-89.
5. Cullen SM, Goodell MA. Dynamic DNA methylation discovered during HSC differentiation: Comment on: Lipka DB, *et al*. Identification of DNA methylation changes at cisregulatory elements during early steps of HSC differentiation using tagmentation-based whole genome bisulfite sequencing. Cell Cycle 2014;13(22):3476-87. Cell Cycle 2015;14:693.
6. Mizuno S, Chijiwa T, Okamura T, Akashi K, Fukumaki Y, Niho Y, *et al.* Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. Blood 2001;97:1172-9.
7. Shivarov V, Gueorguieva R, Stoimenov A, Tiu R. DNMT3A mutation is a poor prognosis biomarker in AML: Results of a meta-analysis of 4500 AML patients. Leuk Res 2013;37:1445-50.
8. Grossmann V, Haferlach C, Weissmann S, Roller A, Schindela S, Poetzinger F, *et al.* The molecular profile of adult T-cell acute lymphoblastic leukemia: Mutations in RUNX1 and DNMT3A are associated with poor prognosis in T-ALL. Genes Chromosomes Cancer 2013;52:410-22.
9. El Ghannam D, Taalab MM, Ghazy HF, Eneen AF. DNMT3A R882 mutations in patients with cytogenetically normal acute myeloid leukemia and myelodysplastic syndrome. Blood Cells Mol Dis 2014;53:61-6.
10. Roller A, Grossmann V, Bacher U, Poetzinger F, Weissmann S, Nadarajah N, *et al.* Landmark analysis of DNMT3A mutations in hematological malignancies. Leukemia 2013;27:1573-8.
11. Kvasnicka HM. WHO classification of myeloproliferative neoplasms (MPN): A critical update. Curr Hematol Malig Rep 2013;8:333-41.
12. Yang L, Rau R, Goodell MA. DNMT3A in haematological malignancies. Nat Rev Cancer 2015;15:152-65.
13. Mayle A, Yang L, Rodriguez B, Zhou T, Chang E, Curry CV, *et al.* Dnmt3a loss predisposes murine hematopoietic stem cells to malignant transformation. Blood 2015;125:629-38.
14. Lin J, Yao DM, Qian J, Chen Q, Qian W, Li Y, *et al.* Recurrent DNMT3A R882 mutations in Chinese patients with acute myeloid leukemia and myelodysplastic syndrome. PLoS One 2011;6:e26906.
15. Wang M, He N, Tian T, Liu L, Yu S, Ma D. Mutation analysis of JAK2V617F, FLT3-ITD, NPM1, and DNMT3A in Chinese patients with myeloproliferative neoplasms. Biomed Res Int 2014;2014:485645.
16. Stegelmann F, Bullinger L, Schlenk RF, Paschka P, Griesshammer M, Blersch C, *et al.* DNMT3A mutations in myeloproliferative neoplasms. Leukemia 2011;25:1217-9.
17. Nangalia J, Nice FL, Wedge DC, Godfrey AL, Grinfeld J, Thakker C, *et al.* DNMT3A mutations occur early or late in patients with myeloproliferative neoplasms and mutation order influences phenotype. Haematologica 2015;100:e438-42.
18. Abdel-Wahab O, Pardanani A, Rampal R, Lasho TL, Levine RL, Tefferi A. DNMT3A mutational analysis in primary myelofibrosis, chronic myelomonocytic leukemia and advanced phases of myeloproliferative neoplasms. Leukemia 2011;25:1219-20.
19. Brecqueville M, Cervera N, Gelsi-Boyer V, Murati A, Adélaïde J, Chaffanet M, *et al.* Rare mutations in DNMT3A in myeloproliferative neoplasms and myelodysplastic syndromes. Blood Cancer J 2011;1:e18.
20. Rao N, Butcher CM, Lewis ID, Ross DM, Melo JV, Scott HS, *et al.* Clonal and lineage analysis of somatic DNMT3A and JAK2 mutations in a chronic phase polycythemia vera patient. Br J Haematol 2012;156:268-70.
21. Brecqueville M, Rey J, Bertucci F, Coppin E, Finetti P, Carbuccia N, *et al.* Mutation analysis of ASXL1, CBL, DNMT3A, IDH1, IDH2, JAK2, MPL, NF1, SF3B1, SUZ12, and TET2 in myeloproliferative neoplasms. Genes Chromosomes Cancer 2012;51:743-55.
22. Lin HC, Wang SC, Chen CG, Chang MC, Wang WT, Su NW, *et al*. Mutation and lineage analysis of DNMT3A in BCR-ABL1-negative chronic myeloproliferative neoplasms. Int J Gerontol 2013;7:186-8.
23. Hamidi T, Singh AK, Chen T. Genetic alterations of DNA methylation machinery in human diseases. Epigenomics 2015;7:247-65.
24. Ci C, Wang Y, Gu Y, Su J. The aberrant methylation sites identification and function analysis associated with DNMT3A and IDH mutations in AML. Cancer Genet Epigenetics 2015;3:1-8.
25. Lu R, Wang P, Parton T, Zhou Y, Chrysovergis K, Rockowitz S, *et al.* Epigenetic perturbations by Arg882-mutated DNMT3A potentiate aberrant stem cell gene-expression program and acute leukemia development. Cancer Cell 2016;30:92-107.