Original Article

Sequencing Myeloproliferative Leukemia Exon 10 Mutations in Iranian Patients with Breakpoint Cluster Region-Abelson Murine Leukemia Viral Oncogene Homolog 1-negative Myeloproliferative Neoplasm

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Abstract

Context: Breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1 (*BCR-ABL1)*-negative myeloproliferative neoplasms (MPNs), including essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF), are distinguished by the dysregulated Janus kinase (JAK)-signal transducer and activator of transcription functionality, abnormal hematopoiesis, and spontaneous proliferation. Moreover, a mutation in *JAK2*^{V617F} as well as myeloproliferative leukemia *(MPL)* mutations have been reported in these patients, which could be important in the pathogenesis of diseases. *MPL* plays a role in the development of megakaryocytes and platelets as well as self-renewal of hematopoietic stem cells. **Aims:** The aim of the present study was to investigate the frequency of *MPL* mutations in patients with *BCR-ABL1*-negative MPNs. **Settings and Design:** This study was a cross-sectional study conducted as an analytical, descriptive review. **Subjects and Methods:** This study was performed on 54 newly diagnosed patients with *BCR-ABL1*-negative MPN (PV, ET, and PMF) who referred to Shafa Hospital, Ahvaz, Iran. Five milliliter whole blood was drawn from these patients; *JAK2*^{V617F} mutation and mutations in exon 10 of *MPL* gene were investigated using polymerase chain reaction and DNA sequencing techniques following the isolation of mononuclear cells from the blood. **Statistical Analysis:** All the data were presented as mean ± standard deviation and were analyzed by SPSS. **Results:** *JAK2*^{V617F} mutation was present in 33 patients, among whom there were 6 ET (35.3%), 7 PMF (41.2%), and 20 PV cases (100%). *MPL*$^{W515\ L/K}$ mutation was found in only one case of PMF, which was negative for *JAK2*^{V617F} mutation. The prevalence of these mutations was 1.8%, and the patient had splenomegaly with lower white blood cell counts and hemoglobin concentration than normal. **Conclusions:** Based on the results, *MPL* mutations rarely occur in patients with MPN. These mutations could be co-expressed with *JAK2* mutations and might be helpful for detecting MPN patients with no *BCR-ABL1* translocation or *JAK2*^{V617F} mutation.

Keywords: *Essential thrombocythemia, Janus kinase 2*^{V617F}*, myeloproliferative leukemia mutation, myeloproliferative neoplasms, polycythemia vera, primary myelofibrosis*

Introduction

Myeloproliferative neoplasms (MPNs) include a group of clonal hematologic disorders caused by excessive proliferation of mutated multipotent hematopoietic stem cells (HSCs).[1] Among the subgroups of these disorders, polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) show increased cellularity of bone marrow (BM), thrombosis, and bleeding, which are known as breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1 (*BCR-ABL1)*-negative MPNs due to the absence of Philadelphia chromosome.[2,3] These disorders are caused by genetic changes that usually occur in Janus kinase 2 (JAK2), calreticulin, and myeloproliferative leukemia virus oncogene (*MPL*).[4,5]

Cytoplasmic *JAK2* tyrosine kinase is one of the most important factors in intracellular signal transduction of HSCs in response to erythropoietin, thrombopoietin (TPO), and other hematopoietic cytokines stimulating hematopoiesis.[6] A point mutation in this gene was observed to result in the increased activity and response to growth factors in *BCR-ABL1*-negative MPN patients due to the substitution of valine for alanine at position 617, which was reported in 95% of PV patients, 50% of ET patients, and

How to cite this article: Kavianpour M, Far MA, Asl JM, Ahmadzadeh A, Vosughi T, Ketabchi N, *et al.* Sequencing myeloproliferative leukemia exon 10 mutations in iranian patients with breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1-negative myeloproliferative neoplasm. Clin Cancer Investig J 2017;6:86-91.

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Access this article online

Website: www.ccij-online.org

DOI: 10.4103/ccij.ccij_11_17

Quick Response Code:

45% of PMF patients.[7,8] *MPL* gene encodes TPO receptor and is the main regulator of megakaryopoiesis and platelet growth, which activates the JAK/signal transducer and activator of transcription (STAT) signaling pathway.[7,9] Similar to *JAK2* gene, acquired mutation in *MPL* causes cytokine-independent cell growth and increased sensitivity to TPO, which ultimately leads to sustained phosphorylation of JAK2, STAT3, STAT5, AKT, and ERK signaling proteins[10] [Figure 1].

The majority of mutations in this gene occur in juxtamembrane region of the receptor, including $MPL^{W515\ L}$ (leucine to tryptophan) and MPL^{W515K} (lysine to tryptophan) in position 515,[7,11] which have been observed in 5%–10% of MPN patients,[12] involving 0%–10% of PMF, and nearly 0%–5% of ET cases.[13] In this study, we chose exon 10 mutations of *MPL* gene because mutations of other exons are uncommon in patients with *BCR-ABL1*-negative MPNs (PV, ET, and PMF). This mutation is analyzed along with molecular studies such as $JAK2^{V617F}$ mutation and clinical features in these patients. This was the first study to investigate *MPL* gene mutations in Khuzestan Province in southwest of Iran.

Subjects and Methods

Sample collection

A total of 54 patients (17 ET, 17 PMF, and 20 PV patients) were referred to Shafa Hospital of Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran, between 2014 and 2015 and were diagnosed with MPN according to the World Health Organization criteria.[14] The initial diagnosis was based on peripheral blood (PB) cell morphology and BM aspiration as well as the information obtained by laboratory tests and clinical examinations. Five milliliter ethylenediaminetetraacetic acid-anticoagulated PB sample was drawn from all the patients before starting the treatment. This study was approved by Local Ethics Committee of Ahvaz Jundishapur University of Medical Sciences, and written informed consent forms were signed by all the patients (IR.AJUMS.REC.1394.344).

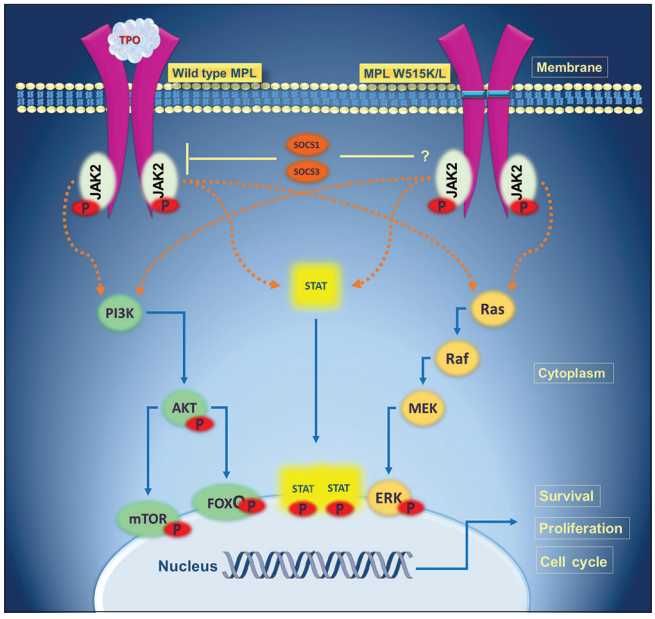

Figure 1: Thrombopoietin receptor signaling pathway. After thrombopoietin binding to myeloproliferative leukemia, phosphorylation of Janus kinase 2 in the cytoplasmic domain of the receptor activates PI3K, signal transducer and activator of transcription, and RAS/MAPK pathways, and the transcription factors are recruited to the nucleus to enhance the expression of genes involved in survival, proliferation, and cell cycle. If myeloproliferative leukemia$^{W515\ L/K}$ mutations are present, spontaneous activation of this receptor leads to uncontrollable overactivity, which may result in myeloproliferative disorders

DNA extraction

Genomic DNA was isolated from mononuclear cells using a spin-column DNA isolation kit (Roche Diagnostics, Mannheim, Germany). To ensure the quality of extracted DNA, optical density of purified samples was confirmed at 260 and 280 nm wavelengths by a spectrophotometer (260/280 ratio ~ 1.8). The extracted samples were stored at −80°C until polymerase chain reaction (PCR) test was performed.

Polymerase chain reaction and sequencing

For $JAK2^{V617F}$ mutation, PCR was performed in 20 μL volume, containing 5.5 μL distilled water, 10 μL PCR buffer (×1), 1 μL MgCl2, 0.5 μL dNTPs, 0.5 μL of each forward and reverse primers, 1 μL Taq polymerase, and 1 μL DNA sample. The amplification process for $JAK2^{V617F}$ mutation included initial denaturation at 95°C for 5 min, 30 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 1 min and a final extension at 72°C for 5 min. $JAK2^{V617F}$ primers included *JAK2* common reverse (R), 5′-CTGAATAGTCCACAGTGTTTTCAGTTTCA-3′; *JAK2* mutant 617 A-202 forward (F), 5’-AGCATTTGGTTTTAATATGGAGTATATT-3’; and *JAK2* wild-type 363 bp (F), 5’-ATCTATAGTCATGC TGTTAGTAGGAGAAAG-3’.

For analysis of $MPL^{W515K/L}$ mutations, exon 10 of *MPL* gene was amplified by PCR, and PCR products were subsequently sequenced. Briefly, PCR was performed in 20 μL volume, containing 10 μL distilled water, 6 μL PCR buffer (×1), 0.5μL dNTPs, 1 μL MgCl2, 0.5 μL of each forward and reverse primers, 0.5 μL SmarTaq DNA polymerase, and 1 μL genomic DNA.

The exon 10 primers of *MPL* were as follows: F: 5’-ACCCAACTAGGGTGGAGACC-3’ and R: 5’-AGAGGTGACGTGCAGGAAGT-3’. PCR reactions were carried out on an ABI 2720 Thermal Cycler (Applied Biosystems, USA). After denaturing at 95°C for 3 min, the amplification was conducted for 32 cycles at 95°C for 1 min, 60°C for 1 min, and 72°C for 1 min, followed by re-extension for 5 min at 72°C. PCR products were loaded onto a 1.5% agarose gel and were electrophoretically separated. After purification, 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

All data were presented as mean ± standard deviation; Statistical Package for the Social Sciences (IBM) was used in statistical analyses to evaluate the findings of this study.

Results

In this study, 54 newly diagnosed patients (27 men and 27 women) with a mean age of 54 years (age range of 29–78 years) with *BCR-ABL1*-negative MPN whose disease was confirmed by clinical and laboratory studies were selected. There were 17 ET patients (31.4%), 17 PMF patients (31.4%), and 20 PV patients (37.2%).

The hematologic parameters examined in this study included white blood cells (WBC), red blood cells, and hemoglobin (Hb) concentration [Table 1]. The patients were evaluated for clinical symptoms, among whom 6 ET patients, 6 PMF patients, and 4 PV patients had splenomegaly, but hepatomegaly was observed only in one PMF patient.

The $JAK2^{V617F}$ mutation was positive in 6 ET patients, 7 PMF patients, and all the PV patients [Table 2]. After sequencing of PCR products in exon 10, only one patient with PMF showed the considered mutation of MPL^{W515L} type, which is a function of single nucleotide substitution of TGG >TTG (Trp/Lue) in codon 515 [Figure 2]. This patient was negative for $JAK2^{V617F}$mutation and showed splenomegaly as well as a normal liver function at the time of diagnosis [Table 3].

Discussion

PV, PMF, and ET are clonal disorders of HSCs. This group of heterogeneous disorders is defined by the increased proliferation and maturation of erythroid, myeloid, and megakaryocyte lineages.[15] In recent years, MPN has been classified according to molecular characteristics, which can be effective in the management of these disorders. Molecular analysis revealed the $JAK2^{V617F}$ mutation in *BCR-ABL1*-negative MPN patients and attracted the attention of researchers.[16] *JAK2* and *MPL* genes play an important role in cell signaling and proliferation of myeloid cells.[17] Mutation in these genes results in the sustained activation of JAK/STAT pathway, as well as other signaling pathways, eventually leading to the proliferation and differentiation of several lineages.[15] Exon 10 of *MPL* gene at position 515 encodes tryptophan (W), which can be converted to five other amino acids such as leucine (L) or lysine (K) due to the mutation.[13,18] There is a higher frequency of MPL^{W515K} and $MPL^{W515\ L}$ mutations, but the less prevalent MPL^{W515R} and MPL^{W515A} mutations have been reported in one and two patients, respectively.[19] Since the position 515 plays a key role in the formation and spontaneous inactivity of the receptor, mutations in this exon affect the severity of anemia, leading to a higher platelet count and proliferation of BM megakaryocytes.[20] In different studies, MPL^{W515L} and MPL^{W515K} mutations have

Table 1: Laboratory parameters of the study participants

Hematological parameters	ET (*n*=17)			PMF (*n*=17)			PV (*n*=20)		
	Mean±SD	Range	Median	Mean±SD	Range	Median	Mean±SD	Range	Median
WBC (10^3/mm^3)	9.1±3.4	3-14.7	10.2	280.4±4.6	2.9-18.4	19.6	14.4±9.9	7-55	13.1
Hb (g/dL)	13.6±2.0	10-18	13.2	10.6±2.0	8-14	9.8	17.9±1.5	16-23	17.5
Plt (10^3/mm^3)	876.1±340.5	262-1927	800.0	809.9±235.2	30-887	760.0	439.9±192.2	116-975	428.5

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

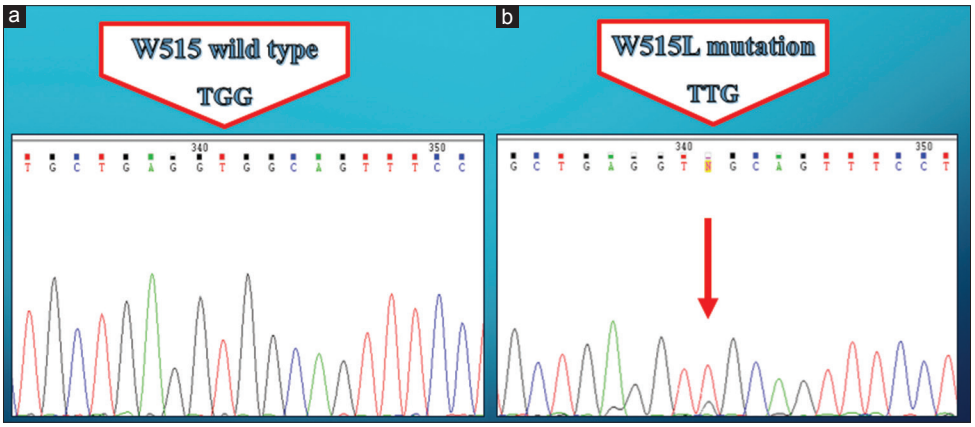

Figure 2: Sequencing of *MPL* exon 10 for screening $W^{515L/K}$. The sequencing results of myeloproliferative leukemia gene at position 515, which is observed as wild-type TGG (a), indicates the substitution of TTG (b) in myeloproliferative leukemia^{W515L} mutation, leading to translation of leucine instead of tryptophan

been observed in almost 9% and 5% as well as 5% and 1% of PMF and ET cases, respectively.[13,21] This mutation was observed in 1.8% of patients in our study, and the patient harboring MPL^{W515L}mutation was afflicted with PMF. As shown in Table 4, this type of $MPL^{W515\,L}$ mutation has been mainly observed in PMF group in other studies.[28,29]

The results of Ghotaslou *et al*.[22] investigation in Iranian people indicated that from a total of 60 patients, 34 (56.6%) and 1 (1.7%) patients had $JAK2^{V617F}$ and *MPL* mutation, respectively. Patients with $JAK2^{V617F}$mutation had higher WBC counts and Hb concentrations than those without the mutation (P = 0.005, P = 0.003). In addition, the mutation was negative in all the healthy participants of the control group. Their study revealed that unlike the $JAK2^{V617F}$ mutations, *MPL* mutations rarely occur in Iranian patients with Ph-negative MPNs, and this low mutation rate should be considered in the design of screening strategies for MPN patients.

Furthermore, Karimzadeh *et al*.[23] showed that 26 out of 30 PV patients (86%), 8 out of 13 PMF patients (61%), 8 out of 15 ET patients (53%), and none of 31 chronic myeloid leukemia (CML) patients were positive for $JAK2^{V617F}$mutation. The PV patient harboring this mutation displayed higher WBC counts (P = 0.03). Sixteen out of 26 $JAK2^{V617F}$-positive patients were female, which demonstrates the correlation between the presences of a mutant allele with gender. The differences in other groups were not significant, and the results of their study showed that a single acquired point mutation in *JAK2* is present in virtually all the patients with PV and about 50% of those with ET or PMF. However, in another study, $JAK2^{V617F}$ mutation has been detected in the vast majority of patients with PV (65%–95%), which was less frequent in patients with ET (23%–57%), PMF (23%–57%), and CML (19%, 3 out of 16 Ph-negative CML patients).

In 2011, Asghari *et al*.[24] reported that the prevalence of $JAK2^{V617F}$ mutation in patients was 58.2%, and the highest prevalence was observed among PV patients. There were significant differences in age, WBC, and PLT in PV patients regarding the prevalence of $JAK2^{V617F}$ mutation. Their study indicated a high level of association between $JAK2^{V617F}$ mutation in patients with PV, ET, and PMF in Iranian

Table 2: Frequency of Janus kinase 2^{V617F} mutation in all the participants in the study

Type of MPN	JAK2		Total (%)
	Negative (%)	Positive (%)	
ET	11 (64.7)	6 (35.3)	17 (100.0)
PMF	10 (58.8)	7 (41.2)	17 (100.0)
PV	0	20 (100.0)	20 (100.0)
Total	21 (38.9)	33 (61.1)	54 (100.0)

JAK2: Janus kinase 2, MPN: Myeloproliferative neoplasm, ET: Essential thrombocytosis, PV: Polycythemia vera, PMF: Primary myelofibrosis

Table 3: Basic characteristics of myeloproliferative leukemia protein mutated patient

Sex	Age	Disease	BCR-ABL1 translocation	$JAK2^{V617F}$ mutation	Exon 10 MPL mutation	WBC ($\times10^3$/mm^3)	Hb (g/dL)	Plt ($\times10^3$/mm^3)	Clinical findings
Male	62	PMF	Negative	Negative	W515L	3.1	8.3	131	Splenomegaly without hepatomegaly

BCR-ABL1: Breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1, JAK2: Janus kinase 2, MPL: Myeloproliferative leukemia protein, WBC: White blood cell, Hb: Hemoglobin, Plt: Platelet, PMF: Primary myelofibrosis, W: Tryptophan, L: Leucine

Table 4: Myeloproliferative leukemia protein mutations among patients with breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1-negative myeloproliferative neoplasms

Sex	Age	Mutated/ total patients	JAK^{2V617F} mutation	Type of MPN	Hb (g/dL)	WBC ($\times10^3$/mm^3)	PLT ($\times10^3$/mm^3)	Clinical signs	MPL mutation	Reference
Male	60	1/60	Negative	ET	-	-	897	Splenomegaly	W515R	[22]
Female	87	2/58	Negative	1/21 PMF	6.4	8100	300	Abdominal thrombosis	W515L	[25]
Female	65		Positive	1/17 ET	13.5	14,800	748	-	W515L	
Male	61	18/217	Positive in 4 patients	PMF	10.1*	9760*	371*	-	9 W515K 9 W515L	[28]
17 males 8 females	64*	25/617	-	PMF	11.0*	8400*	307*	-	-	[26]
-	-	34/570	Just one patient with ET carried both $JAK2^{V617F}$ and MPL (W515L)	(11.1%) PV (6.6%) ET or PMF	-	-	-	-	W515L/A/R/K in ET W515L in PMF	[29]
Male	61	1/54	Negative	PMF	8.3	3100	131	Splenomegaly	W515L	Our study

*Median range. Hb: Hemoglobin, WBC: White Blood Cell, PLT: Platelet, ET: Essential thrombocytosis, PV: Polycythemia vera, PMF: Primary myelofibrosis, W: Tryptophan, K: Lysine, L: Leucine, A: Alanine, R: Arginine, BCR-ABL1: Breakpoint cluster region-Abelson murine leukemia viral oncogene homolog 1, JAK2: Janus kinase 2, MPL: Myeloproliferative leukemia protein

patients. Therefore, screening for *JAK2*^{V617F} mutation can be incorporated into the initial evaluation of patients suspected to chronic MPNs. This test can be used to determine the association between *JAK2*^{V617F} mutation with prognosis and treatment of patients with abnormal blood indices.

MPL mutation is rarely observed in PV patients or other myeloid disorders,[13] and there was no case of *MPL* mutation in PV patients in our study. The evaluation of *JAK2*^{V617F} mutation is important for the detection of simultaneous mutations in this gene and *MPL*. In the study of Dos Santos and Rumi, *JAK2*^{V617F} and *MPL*$^{W515\ L}$ mutations have been simultaneously detected in ET patients[25,26] [Table 4]. In our study, the *JAK2*^{V617F} mutation was observed in 100% of PV patients, 35.3% of ET patients, and 41.2% of PMF patients, but the patient harboring *MPL* mutation was negative for *JAK2*^{V617F} mutation, and we were not able to identify the simultaneous occurrence of these two mutations with our low sample size. Mutation in *JAK2* gene leads to erythropoiesis, but a mutation in *MPL* is associated with thrombosis, extramedullary hematopoiesis, and myelofibrosis.[27]

Conclusions

It can be concluded from the current study that *JAK2*^{V617F} and *MPL*$^{W515\ L/K}$ mutations are rarely seen in patients with MPN but might be helpful for detecting MPN patients with no *BCR-ABL1* translocation or *JAK2*^{V617F} mutation.

Acknowledgments

This work was financially supported by grant TH94/7 from the Vice Chancellor for Research Affairs of Ahvaz Jundishapur University of Medical Sciences. This paper is issued from the thesis of Maria Kavianpour.

Financial support and sponsorship

Nil

Conflicts of interest

There are no conflicts of interest.

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