TET2, DNMT3A, IDH1, and JAK2 Mutation in Myeloproliferative Neoplasms in southern Iran

E. Abedi[1], M. Ramzi[1], M. Karimi[1], R. Yaghobi[2], H. Mohammadi[3], E. Bayat[4], M. Moghadam[1], F. Farokhian[1], M. Dehghani[1], H. A. Golafshan[5], S. Haghpanah[1]*

[1]*Hematology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[2]*Transplant Research Center, Shiraz University of Medical Sciences, Shiraz, Iran*
[3]*Department of Pediatrics, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*
[4]*Department of Biochemistry, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran*
[5]*Department of Laboratory Sciences, Shiraz Paramedical School, Shiraz University of Medical Sciences, Shiraz, Iran*

ABSTRACT

Background: Five epigenetic regulator mutations are considered in myeloproliferative neoplasms (MPN) that have prognostic and therapeutic values.

Objective: We aimed to evaluate these mutations in MPNs among the Iranian population.

Methods: We selected 5 mutations in 4 epigenetic regulatory genes [TET2, DNMT3A, IDH1 (rs147001633& rs121913499), and JAK2)] and evaluated 130 patients with MPNs including 78 Philadelphia chromosome negative (49 ETs, 20 PVs, and 9 PMFs) and 52 Philadelphia chromosome-positive patients as well as 51 healthy controls.

Results: Eight patients (6.5%) carried the DNMT3A mutation, 35 (27%) were positive for TET2 mutation and 64 (49.3%) had the JAK2V617F mutation. In the healthy controls, 16 (31.4%) cases had the TET2 mutation (15 Heterozygote + 1 Homozygote) and one had heterozygote JAK2 mutation. There was no statistically significant difference between patient groups for any of these mutations, except for JAK2. The JAK2 mutation rate was 18 (90%), 25 (51%), 7 (77.8%), 14 (26.9%) in polycythemia vera, essential thrombocythemia, primary myelofibrosis, and chronic myelocytic leukemia, respectively. Patients aged 60 and older were more likely to carry the TET2 mutation (23% vs. 39% in younger and older than 60 years old individuals, p=0.025). IDH1 was not detected at all and PV had the highest TET2 mutation 7(35%). Two PMF patients had a history of bone marrow transplantation that were negative for IDH1and DNMT3A and one was positive for TET2 mutation.

Conclusion: In the normal Iranian population, the heterozygote form of TET2 mutation is significant, especially in the elderly. No association was found between JAK2 and TET2 mutations. Both of them are more prevalent in the age group of 60 years and older. DNMT3A mutation has a low prevalence and occurs in both positive and negative MPNs.

KEYWORDS: Myeloproliferative neoplasms; TET2; DNMT3A; IDH1; Philadelphia chromosomes

INTRODUCTION

Myeloproliferative neoplasms (MPN) are the clonal expansion of mature myeloid cells, characterized by Jak2, MPL, and CALR mutations [1]. MPNs differentiate into polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF) (negative Philadelphia chromosome), and chronic myeloid leukemia (CML) in Philadelphia positive cases that can be detected due to overexpression of pluripotent hematopoietic stem cells, characterized by the oncogenic fusion of the BCR and ABL genes, t (9; 22) [2, 3]. Survival is much better in ET and PV in comparison with PMF.

*Correspondence: Sezaneh Haghpanah, MD, MPH, Hematology Research Center, Shiraz University of Medical Sciences, Nemazee Hospital, Shiraz, Iran
ORCID: 0000-0002-8666-2106
Tel: +98-713-6122-263
E-mail: haghpanah@sums.ac.ir

Patients with ET or PV might be transformed into one other or secondary acute myeloid leukemia (AML) [4, 5]. Primary myelofibrosis (PMF) or post-ET/PV myelofibrosis is one of the Philadelphia-negative MPNs with a survival rate of approximately 6 years. Although allogeneic hematopoietic stem cell transplantation (alloHSCT) can cure a significant number of patients, it is still not universally applicable due to toxicity which can lead to morbidity and mortality [6]. The best way to detect and differentiate MPNs is by detecting genetic disorders in these types of diseases. MPNs have similar clinical and laboratory features, and the discovery of the Philadelphia chromosome in CML has brought about a change in the diagnosis of these malignancies [7, 8]. The JAK2 V617 mutation was discovered in 2005 as the first molecular abnormality in ABL–BCR negative patients [9-11]. Several studies have shown the central role of JAK-STAT pathways in the pathogenesis of MPNs phenotype [12]. After the discovery of JAK2 mutation as the most important genetic aberration in MPN patients, attempts were made to detect other genetic mutations and epigenetic alterations in these patients. Mutations in several epigenetic modifiers, such as TET2, ASXL1, IDH1, and IDH2 were reported to be involved in the MPN pathogenesis [12]. In the mitochondria and cytoplasm, IDH1 and 2 (isocitrate dehydrogenase) catalyze the conversion of isocitrate into α-ketoglutarate [13]. TET2 proteins (TET1, TET2, and TET3) can change 5-methylcytosine into 5-hydroxymethylcytosine. DNMT3A is a DNA methyltransferase that adds a methyl group to the cytosine within CpG dinucleotides and is required for gene silencing [13]. Hence, this study aimed to determine the prevalence of the aforementioned mutations in some epigenetic modifiers in MPN Philadelphia negative and positive patients.

MATERIALS AND METHODS

Study Participants

The cross-sectional case-control study was performed amongst 130 myeloproliferative neoplasm patients (positive and negative MPN) including 52 CML, 49 ET, 20 PV, 9 PMF, and 51 healthy controls in hospital affiliated to Shiraz University of Medical Sciences (SUMS), between 2018-19. The diagnosis was confirmed by the hematological and molecular examination according to the WHO criteria. The study design was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (ethics code: IR.SUMS.REC1397.535).

Laboratory Assay

Five ml of whole blood was collected from the participants. Genomic DNA was extracted from the samples, using Gene Matrix Quick blood DNA purification kit (EURx, Gdansk, Poland), according to the manufacturer's instruction. DNA was stored at -80°C until analysis.

ARMS- PCR assay was performed to identify genotype-specific nucleotides in the whole blood DNA. Aiming to detect a single nucleotide polymorphism in DNMT3A rs147001633, DNA of all samples were analyzed, using tetra-primer amplification refractory mutation system (ARMS – PCR). The procedure utilizes the principle of tetra-primer PCR method and ARMS to detect two different alleles in a single PCR reaction that amplifies products followed by gel electrophoresis. Each polymerase chain reaction (PCR) was mixed for DNMT3A (rs147001633 (R882H), containing 5 µl Amplicon 2x master mix, 0.4µl Forward primer, 0.4 µl Reverse primer and 0.3 µl Common primer, 1 µl DNA template, and 3.3 µl DNase, RNase free distilled water to complete the total volume 10 µl. PCR was carried out, using the Bio-Rad T-100 Thermocycler in the following conditions: initial denaturation at 95°C for 3minutes, followed by 35 cycles of 95°C for 45 seconds, 61°C for 50 seconds, 72°C for 50 second and followed by 72°C for 5 minutes. For IDH1 rs121913499, each PCR mixed contained 5 µl Amplicon 2x master mix, 0.3 µl Forward primer, 0.3 µl Reverse primer, and 0.2 µl Common primer, 1 µl DNA template, 1 µl BSA(Bovine serum albumin), and 2.5 µl Dnase, Rnase free distilled water to complete the final mixture volume to10 µl. PCR was carried out, using the Bio-Rad T-100 Thermocy-

Table 1: Primer sequences and PCR conditions.

SNP	Primer Sequences
DNMT3A (rs147001633 (R882H)	DNM-FN: TATACTGACGTCTCCAACATGATCCG
	DNM-FM: TATACTGACGTCTCCAACATGATCCA
	DNM-RC: CCTCTCTCCCACCTTTCCTCTG
IDH1 rs121913499	IDH-RN: ACTTACTTGATCCCCATAAGCATTACG
	IDH-RM1: ACTTACTTGATCCCCATAAGCATTACA
	IDH-RM2: ACTTACTTGATCCCCATAAGCATTACC
	IDH-RN1: GACTTACTTGATCCCCATAAGCAAGAC
	IDH-RM3: GACTTACTTGATCCCCATAAGCAAGAG
	IDH-FC: AGCCTCTCTTAGTTCTCTTTGTAGTTG
IDH1 rs121913500	RN: ACTTACTTGATCCCCATAAGCATTACG
	RM1: ACTTACTTGATCCCCATAAGCATTACA
	RM2: ACTTACTTGATCCCCATAAGCATTACC
	RN1: GACTTACTTGATCCCCATAAGCAAGAC
	RM3: GACTTACTTGATCCCCATAAGCAAGAG
	FC: AGCCTCTCTTAGTTCTCTTTGTAGTTG
TET2 rs763480	TET2-FN: AATAACCATGTAGACTGTTTTAAaGTT
	TET2-FM: AATAACCATGTAGACTGTTTTAAaGTA
	TET2-RC: CTATTGCTGCTGGCTTTGG
Jak2 rs77375493	FN: ATTTGGTTTTAAATTATGGAGTACGTG
	FM: ATTTGGTTTTAAATTATGGAGTACGTA
	RC: CTGTTAAATTATAGTTTACACTGACAC

cler as follows: initial denaturation at 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 64°C for 40 seconds, 72°C for 35 seconds and followed by 72°C for 5 minutes. For IDH1rs121913500 each PCR was mixed, containing 5 µl Amplicon 2x master mix, 0.3 µl Forward primer, 0.3 µl Reverse primer, 0.2 µl Common primer, 0.7 µl DNA template, 1 µl BSA (Bovin serum albumin), and 2.8 µl Dnase, Rnase free distilled water to complete the total volume 10 µl. PCR was carried out using the Bio-Rad T-100 Thermocycler as follows: initial denaturation at 95°C for 3 minutes, followed by 35cycle of 95 °C for 30 seconds, 63°C for 50 seconds, 72 °C for 40 seconds and followed by 72 °C for 5 minutes. For TET2 rs763480, each PCR was mixed, containing 5 µl Amplicon 2x master mix, 1 µl Forward primer, 1 µl Reverse primer, and 1 µl Common primer, 0.7 µl DNA template,1 µl BSA (Bovin serum albumin), 0.2 µl DMSO, 0.5 µl Mgcl2 and 1.3 µl Dnase, Rnase free distilled water to complete the total volume 10 µl. PCR was done using the Bio-Rad T-100 Thermocycler as follows: initial denaturation at 95°C for 3 minutes, followed by 30 cycles of 95°C for 30 seconds, 61°C for 40 seconds, 72°C for 30 seconds and followed by 72°C for 5 minutes. For JAK2 rs77375493, each PCR was mixed, containing 5 µl Amplicon 2x master mix, 0.8 µl Forward primer, 0.8 µl Reverse primer, 0.5 µl Common primer, 1 µl DNA template, 1 µl BSA (Bovin serum albumin) and 1.7 µl Dnase, Rnase free distilled water to complete the total volume of 10 µl. PCR was carried out, using the Bio-Rad T-100 Thermocycler in the following conditions: initial denaturation at 95°C for 5 minutes, followed by 30 cycles of 95°C for 30 seconds, 60°C for 40 seconds, 72°C for 30 seconds and followed by 72°C for 5 minutes.

The designed specific primers for each mutation are shown in Table 1.

Table 2: Demographic features and gene mutation frequency in different study groups. Only the JAK2 mutation had a statistical difference amongst the patient groups.

Factors \ Groups		MPN (n=130)	PV (n=20)	ET (n=49)	PMF (n=9)	CML (n=52)	Healthy Individuals (n=51)
Age (years)*		53.2 ± 15	63.4 ± 13.9	52 ± 15	56.7 ± 20.2	49.7 ± 12.8	48.8 ± 16.4
Gender (M/F)		65/65	12/8	24/25	5/4	24/28	17/17
DNMT3A (rs147001633) (R882H)	Wild-type	122 (94%)	18 (90%)	47 (96%)	9 (100%)	48 (92.3%)	51 (100%)
	Heterozygous	5 (4%)	1 (5.0%)	1 (2.0%)	0 (0.0%)	3 (5.8%)	0 (0.0%)
	Homozygous	3 (2.5%)	1 (5.0%)	1 (2.0%)	0 (0.0%)	1 (1.9%)	0 (0.0%)
	Total mutant variant	8 (6.5%)	2 (10%)	2 (4%)	0	4 (7.7%)	0
IDH1 Wild-type homozygous	rs121913499	130 (100%)	20 (100%)	49 (100%)	9 (100%)	52 (100%)	51 (100%)
	rs121913500	130 (100%)	20 (100%)	49 (100%)	9 (100%)	52 (100%)	51 (100%)
TET2 (rs763480)	Wild-type homozygous	95 (73%)	13 (65%)	34 (69.4%)	7 (77.8%)	41 (78.8%)	35 (88.6%)
	Heterozygous Variant	24 (18.5%)	5 (25.0%)	10 (20%)	2 (22.2%)	7 (13.5%)	15 (29.4%)
	Homozygous	11 (8.5%)	2 (10%)	5 (10.2%)	0 (0.0%)	4 (7.7%)	1 (2.0%)
	Total mutant variant	35 (27%)	7 (35%)	15 (30.2%)	2 (22.2%)	11 (21.2%)	16 (31.4%)
Jak2 rs77375493**	Wild-type	66 (50.7%)	2 (10%)	24 (49%)	2 (22.2%)	38 (73.1%)	50 (98.0%)
	Heterozygous	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (2.0%)
	Homozygous	64 (49.3%)	18 (90%)	25 (51%)	7 (77.8%)	14 (26.9%)	0 (0.0%)
	Total mutant variant	64 (49.3%)	18 (90%)	25 (51%)	7 (77.8%)	14 (26.9%)	1 (2.0%)

*P= 0.09 between the patients and control groups (no significant statistical difference); however, the mean age of PV vs. other patient groups had a statistically significant difference (P= 0.004).

**P <0.001

Statistical Analysis

Data were analyzed, using SPSS (Statistical Package for Social science) software version 23 (SPSS Inc., Chicago, IL, US). Descriptive data are presented as mean, standard deviation, and frequency. Chi-square and Fisher's exact test were used to compare the frequency of different genotypes between the subgroups. To compare the age between the two groups, student t-test was used. P-value less than 0.05 was considered to be statistically significant.

RESULTS

One hundred and thirty patients with MPNs including 78 Philadelphia chromosomes negative (49 ETs, 20 PVs, and 9 PMFs), 52 Philadelphia chromosome-positive patients along with 51 healthy controls were included in this study. The mean age of patients was 53.2±15 years versus 48.8±16.4 years in the control group (p=0.054) with an equal male to female ratio. In the patient groups, PV patients had the highest mean age (63.4±13.9 years; p=0.021) (Table 2).

Amongst the overall study population, 5 mutations in the 4 selected genes were analyzed, and are shown in Table 2 and Fig 1.

As reported in Table 2, eight patients (6.5%) carried the DNMT3A mutation, 35 (27%) carried the TET2 mutation and 64 (49.3%) carried the JAK2V617F mutation. Interestingly, among the healthy controls, we detected 16 cases with TET2 mutations (15 heterozygot

Table 3: Comparison of age, based on the TET2 mutation

Age (years) / TET2	Patient group (n=130)	P value	Control group (n=51)	P value	Total (n=181)	P value
Wild-type homozygous	52.7 ± 14.4	0.27	48.2 ± 50.2	0.1	51.5 ± 14.8	0.072
Mutant genes	54.2 ± 16.5		50.2 ± 18.6		53 ± 17.1	

+ 1 homozygote) and one with heterozygote JAK2 mutation (Table 2). Except for JAK2, none of the mutations were statistically different. The highest prevalence of JAK2 mutations was found in PV followed by PMF, and the lowest prevalence was found in CML patients.

IDH1 rs121913499 and IDH1 rs121913500 SNPs were not detected in any of the patients or healthy individuals, showing wild-type homozygous in all cases.

In the eight patients with DNMT3A mutations, none of them belonged to the PMF group, and two of them also had a background of bone marrow transplants. The highest prevalence of TET2 mutation was detected among PV patients (35%).

Tables 3 and 4 compare of age in JAK2 and TET2 mutation. The mean age of TET2 mutant cases was slightly more than TET2 negative cases, but the difference was not statistically significant (51.5±14.8 vs. 53±17.1; p=0.07). Then, we categorized cases into two age groups, accordingly patients aged 60 years or older had higher rates of JAK2 mutations, however was not statistically significant, which was likely due to a small number of positive cases. When the control group was included (overall 181 samples) in this analysis, both JAK2 and TET2 had a significantly higher prevalence at the age of 60 years or older (Table 4).

TET2 mutation was unrelated to JAK2 mutation, and we found a similar frequency of TET2 positive (48.6 percent +TET2) versus negative (51.4 percent +TET2) cases (Table 5).

DISCUSSION

Since the discovery of the JAK2V617 tyrosine kinase, several genes have been found mutated in MPNs. Patients with MPNs are prone to bone marrow failure or transform into acute myeloid leukemia (AML). Previous studies

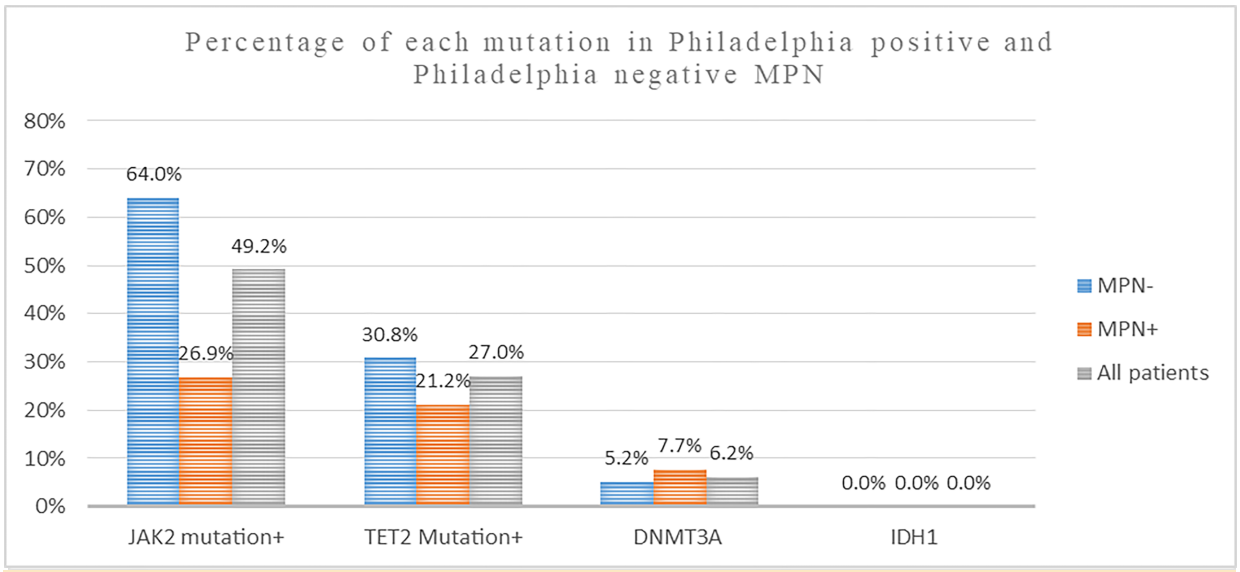

Figure 1: Percentage of each mutation in Philadelphia positive and philadelphia negative MPNs.

Table 4: Association of Jak2 and TET2 mutation with age category (less than 60 years vs. equal or more than 60 years). The prevalence of both JAK2 and TET2 mutations significantly increased after 60 years of age.

			JAK2 gene			P value	TET2 gene			P value
	Age		Wild	Mutant	Total		Wild	Mutant	Total	
MPN cases (n=130)	<60 years	Count %	51 59.3%	35 40.7%	86 100.0%	0.002	67 77.9%	19 22.1%	86 100.0%	0.065
	≥60 years	Count %	15 34.1%	29 65.9%	44 100.0%		28 63.6%	16 36.4%	44 100.0%	
Total		Count %	66 50.8%	64 49.2%	130 100.0%		95 73.1%	35 26.9%	130 100.0%	
All cases (n=181)	<60 years	Count %	87 71.3%	35 28.7%	122 100.0%	0.007	94 77.0%	28 23.0%	122 100.0%	0.025
	≥60 years	Count %	29 49.2%	30 50.8%	59 100.0%		36 61.0%	23 39.0%	59 100.0%	
Total		Count %	116 64.1%	65 35.9%	181 100.0%		130 71.8%	51 28.2%	181 100.0%	

have highlighted the importance of mutations in epigenetic modifiers in the pathogenesis of myeloid malignancies. Mutation in some epigenetic regulators such as TET2, IDH1, IDH2, ASXL1, especially in enzyme isocitrate dehydrogenase (IDH) is associated with poor outcome [14-16].

The JAK2V617 was the most frequent mutation in this study among all the patients (49.2%), negative MPN or positive MPN cases. Similar to other studies we had the highest rate for PV cases up to 90% and then PMF with a 77% positivity rate. This finding has been confirmed in several studies with similar prevalence [17-19]. The lowest frequency of JAK2 mutation was related to CML cases with a 27% prevalence. Our data are on top of the published prevalence of JAK2 mutation. [17-20] and resemble to Pakistan data (44%) [18].

In this study, the mutation frequency of TET2 was higher than that observed in similar studies, with 27% among all patients, 30% among MPN negative and 21% among CMLs. Various studies have reported an incidence range of 4% to less than 20% in MPN negative cases and less than 10% in CML cases [21-26]. Soyer et al. from Turkey, reported high heterozygote mutation rate as much as 40.8% in MPN negative cases and very low frequency (1.5%) for homozygote TET2 mutation[21], however, in our study, the rate of homozygote mutation of TET2 was 8.5% among all patients. Because of population genetic diversity, the high rate of heterozygote mutation in our control group can be regarded as a possible cause of this difference. Other studies have found this somatic mutation in DNA methylation even in healthy elderly people [21]. In our study, the mean age of mutant TET2 cases was older than that of non-mutant cases, but there was no statistical difference (Table3). However, after the age of 60, the prevalence of both JAK2 and TET2 mutations increased. (Table4).

Although the rate of TET2 mutation was numerically higher in the PV (35%) and ET (30%), it was not statistically significant across diagnoses (Table2). This finding is consistent with the findings of other studies, such as Soyer et al. and Tefferi et al., who found the same frequency in PV, ET, and PMF cases.[21-23].

TET2 mutation was unrelated to JAK2 mutation, and we found a similar frequency of TET2 positive (48.6 percent +TET2) versus negative (51.4 percent +TET2) cases (table 5). Tefferi et al. found a higher TET2 mutation rate in JAK2 positive cases, but they did not account for the possible confounding role of age. As shown in Table 4, both mutations are more common in the elderly, which may interfere with their independent correlation [21]. Other studies support our findings, reporting a similar prevalence of TET2 in JAK2 positive and negative cases [23-25].

Table 5: Association between Jak2 mutation and TET2 amongst the patients. TET2 distribution was similar between patients with or without JAK2 mutation.

			TET2			
			Wild gene	Mutant	Total	P value
JAK2 In all patients	Wild-type homozygous	Count %	48 50.5%	18 51.4%	66 50.8%	0.543
	Mutant gene	Count %	47 49.5%	17 48.6%	64 49.2%	
JAK2 In ET	Wild-type homozygous	Count %	18 52.9%	6 40.0%	24 49.0%	0.333
	Mutant gene	Count %	16 47.1%	9 60.0%	25 51.0%	
JAK2 In PV	Wild-type homozygous	Count %	1 7.7%	1 14.3%	2 10.0%	0.580
	Mutant gene	Count %	12 92.3%	6 85.7%	18 90.0%	
JAK2 In PMF	Wild-type homozygous	Count %	1 14.3%	1 50.0%	2 22.2%	0.417
	Mutant gene	Count %	6 85.7%	1 50.0%	7 77.8%	

The frequency of DNMT3A mutation in our population was low (6.5 percent in all patients), which was consistent with other studies reporting a rare 0-10 percent prevalence, and this mutation worsens the prognosis of MPN [23, 26, 27]. We were unable to conduct any analysis due to the low frequency of CML cases (7%). We didn't have any cases with IDH1 mutations. This is a rare mutation in MPN cases, with a prevalence of less than 5%.[21, 23, 25, 26].

To summarize, the most common MPN mutations are JAK2V617 and TET2. The highest rate of JAK2V617 mutations was found in Polycythemia Vera. Among the Iranian population, the heterozygote form of TET2 mutation has a high prevalence, especially among the elderly.

No correlation was found between JAK2 and TET2 mutations. Both are more common among those 60 years old or older. DNMT3A mutations were rare and found in both positive and negative MPNs. IDH1 mutations were not detected in our population.

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