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Prevalence of *JAK2V617F*, *CALR* in Philadelphia Positive and Negative Myeloproliferative Neoplasm

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Abstract

Background: Myeloproliferative neoplasms (MPNs) are heterogeneous disorders with a variety of genetic abnormalities. We aim to assess the prevalence of *Calreticulin (CALR)* and *JAK2* mutations in Iranian MPNs. **Materials and Methods:** In a cross-sectional study, *CALR* and *JAK2* mutations among 130 MPNs patients, including 78 Philadelphia chromosome-negative (MPN-) and 52 Philadelphia chromosome-positive (MPN+) as well as 51 healthy control subjects, were investigated by GAP-PCR. **Results**: In MPN- group *JAK2* and *CALR* gene mutations were found in 64.1% and 7.7%, respectively, that 5.1% were positive for both mutations, and 2.6% had only *CALR* mutation. In polycythemia vera (PV) patients 90% had *JAK2* mutation, which was significantly higher than other MPN- or MPN+ patients. Most of the MPN+ patients had neither mutation in *CALR* nor *JAK2* (70% *CALR-/JAK2-*). Among all patients' groups, the prevalence of *CALR+* mutation in either *rs1450785140* (4 cases) or *rs765476509* (5 cases) position was not statistically different. **Conclusion:** These results showed a low prevalence of *CALR* mutations in all types of MPNs in the Iranian population that its frequency may influence by ethnicity and genetic diversity. *CALR* mutation may be seen in *JAK2* negative cases, also. The PV had the highest *JAK2* mutation with a 90 percent positivity rate among MPNs cases. **[GMJ.2021;10:e2127]** **DOI:10.31661/gmj.v10i0.2127**

Keywords: Myeloproliferative Neoplasms; Genetic Abnormality; *CALR*; *JAK2*; Philadelphia Chromosome

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Introduction

Based on the World Health Organization (WHO) classification criteria, the classic myeloproliferative neoplasms (MPNs) include chronic myeloid leukemia (CML), essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF) [1].
MPNs are known as disorders of clonal hematopoietic stem cells arising from over-production of myeloid lineage independent from normal cytokine regulation [2]. Clinical manifestations of MPNs are heterogeneous, ranging from asymptomatic to severe constitutional symptoms and thrombotic disease, or changing to each other or secondary acute myeloid leukemia (AML) [3, 4].
Sometimes, the MPNs are divided into Philadelphia chromosome-positive (CML) or Philadelphia chromosome-negative (ET, PV, and PMF) subjects. The understanding of the pathogenesis of Philadelphia negative MPNs was expanded by describing *Janus kinase2 (JAK2 V617F*) mutation on chromosome 9 in 2005 [5]. *JAK2 V617F* mutation is detectable in nearly 95% of PV patients and around 50-60% of those with PMF or ET. Also, another somatic mutation in exon 12 of *JAK2* is detected in 3-5% of PV patients [6]. Following *JAK2* gene mutation, two other recurrent somatic gene mutations, including *myeloproliferative leukemia virus (MPL)* and recently *Calreticulin (CALR)* gene mutation, have been detected in patients diagnosed with MPNs, which seems to have an impact on pathogenesis and clinical manifestation of these patients [7]. *CALR* mutations are deletions or insertions in the terminal exon 9 DNA sequence sub-classified in two subtypes known as type one and two (52bp deletion [p.l 367fs*46; *rs1450785140*] and 2, 5-bp TTGTC insertion [p. k385fs*47; *rs765476509*] consequently). These are detected in more than 80% of patients with this gene abnormality [8]. Somatic *CALR* gene mutations are found in less than 25% of PMF or ET with *MPL* and *JAK2* wild type [9]. The presented study aimed to explore the mutations of *CALR* and *JAK2* genes in an Iranian population of MPN disorder and compare their frequency with worldwide data.

Materials and Methods

Study Subjects
In this case-control study, 130 MPN patients, according to the WHO criteria, from the Oncohematolgy department at Nemazee Hospital, Shiraz, Iran, from May 2018 to May 2019 were selected. Also, 51 sex- and age-matched control group were selected. The diagnosis of patients was made based on clinical and laboratory evaluation as well as molecular analysis. For all subjects, written informed consent was obtained. The ethical committee of Shiraz University of Medical Sciences, Shiraz, Iran, approved the study (ethical code: IR.SUMS.REC 1397.535).

Molecular Analysis
DNA extraction was done on the peripheral blood cells of all subjects by a commercially available extraction kit (Gene Matrix Quick blood DNA purification kit, EURx, Gdansk, Poland), according to the manufacturing procedure. The DNA sample was stored at -80°C until analysis.

CALR Gene Mutational Analysis
Gap-polymerase chain reaction (PCR) assay was done to evaluate two major mutations that cover more than 80% of the mutations of *CALR* including *rs1450785140* (del; protein consequence: p.L367fs*46) and *rs765476509* (an ins mutation at nucleotide nomenclature: c.1154_1155insTTGTC; protein consequence: K385fs*47) in patients and control groups [10-12]. PCR mixed for *CALR rs1450785140* contained 5 µl amplicon 2x master mix, 0.2 µl forward primer and same amount reverse primers, and 0.15 µl common primer, 1 µl DNA template, 1 µl bovine serum albumin (BSA;merk-Germany), and 2.65. µl RNase, DNase-free distilled water to complete a total volume of 10 µl. PCR condition was carried out using the Thermocycler (Bio-Rad T-100, California, USA) as the following consequence: initial denaturation at 95ºC for 3 minutes, then 35 cycles of 95ºC for 45 seconds, 61ºC for 50 seconds, and 72ºC for 50 seconds, then by 72ºC for 5 minutes. PCR mixed for *CALR rs765476509* contained 5 µl

amplicon 2x master mix, forward primer (0.8 µl), 0.5 µl reverse primer and 0.5 µl common primer, 1 µl DNA template, 05 µl dimethyl sulfoxide (merk- Germany), 1 µl BSA, and 1.2 µl DNase, RNase-free distilled water to complete the final mixture volume to 10 µl. PCR condition was carried out using the Bio-Rad T-100 Thermocycler in the following order: 5 minutes initial denaturation at 95ºC, then 30 cycles include 30 seconds in 95ºC, 40 seconds in 59.5ºC and 35 seconds in 72ºC and finally by 72ºC for 5 minutes.

JAK2 Gene Mutational Analysis

JAK2 V617F was evaluated based on amplification-refractory mutation system (ARMS)-PCR by designed specific primers. Each PCR mixed contained 5 µl amplicon 2x master mix, 0.8 µl forward and reverse primers, and 0.5 µl common primer, 1 µl DNA template, 1 µl BSA, and 1.7 µl DNase, RNase-free distilled water to complete total volume 10 µl. PCR was carried out using the Thermocycler (Bio-Rad T-100, California, USA) as the following consequence: 5 minutes initial denaturation at 95ºC, then 30 cycles include 30 seconds in 95ºC, 40 seconds in 60ºC and 30 seconds in 72ºC and finally by 72ºC for 5 minutes. Table-1 shows the designed specific primers for each mutation.

PCR Products Evaluation

PCR products for gene mutations were evaluated using 2% agarose gel electrophoresis to identify the *CALR* gene *rs1450785140, rs765476509*, and *JAK2 V617F* gene mutations. The agarose gel electrophoresis result was demonstrated in Figure-1.

Statistical Analysis

Data analysis was done by SPSS software version 16 (SPSS Inc., Windows version, Chicago, USA). Descriptive data were presented as mean, standard deviation, and frequency. The chi-square test was used to compare the frequency of different genotypes between the subgroups. A P-value of less than 0.05 was considered statistically significant.

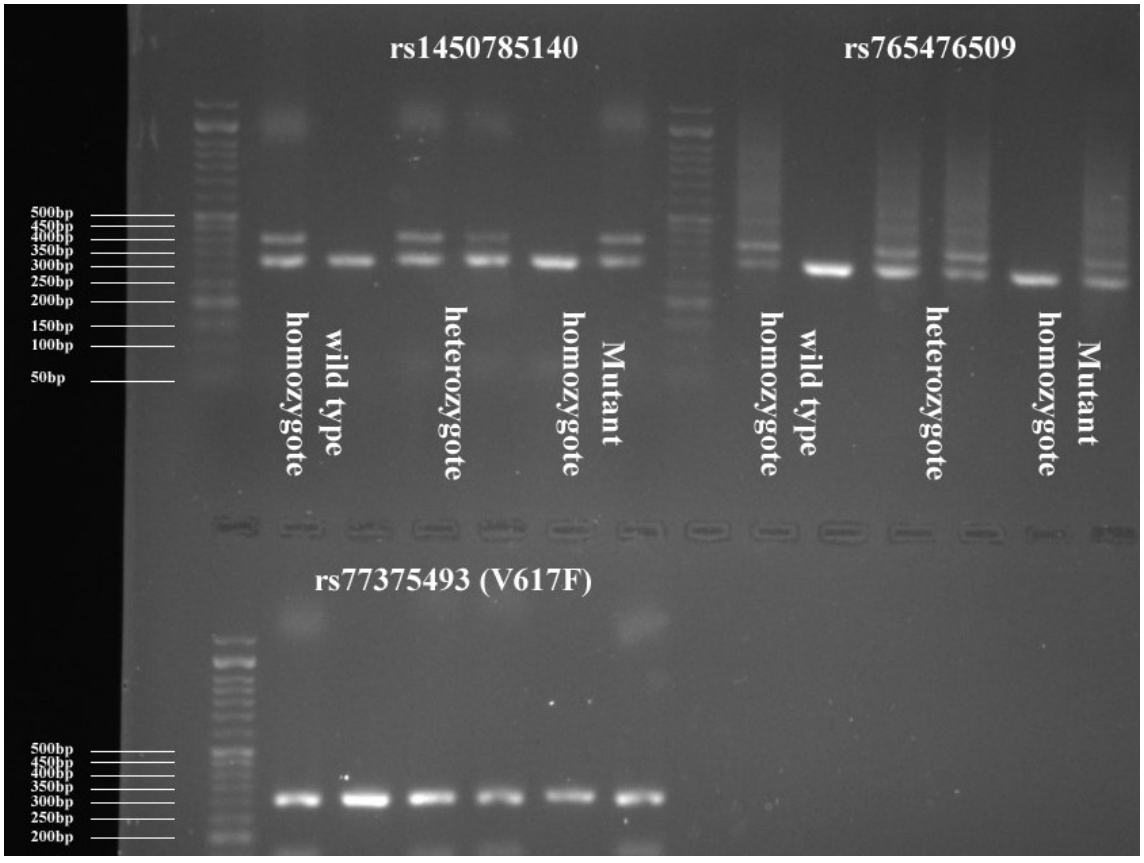

Figure 1. Agarose gel electrophoresis.

Table 1. Primers Designed for Genotype Analysis for *CALR* Gene Mutations and *JAK2 V617F*.

Gene mutations	SNP	Primer sequences	Product size
CALR	*rs1450785140*	F (wild type allele-specific primer): ACAGGACGAGGAGCAGAG	403 bp
		F (mutant allele-specific primer): CGAGGAGCAGAGGCAGAG	
		R: GGCTGAAGGAGAATCAAAGAT	
	rs765476509	F (wild type allele-specific primer): GAGGCAGAGGACAAGGAG	392 bp
		R: ACAGAGGCAAGAAAAGATG	
		R (mutant allele-specific primer): CCTCATCATCCTCCGACA	420 bp
		F: GGTGTTCCTTGTCTTCTCT	
JAK2	V617F	F (wild type allele-specific primer): ATTTGGTTTTAAATTATGGAGTACGTG	
		F (mutant allele-specific primer): ATTTGGTTTTAAATTATGGAGTACGTA	156 bp
		R: CTGTTAAATTATAGTTTACACTGACAC	
ACTB (internal control)		F: CTCCTCAGATCATTGCTCCT	317 bp
		R: GTCACCTTCACCGTTCCA	

bp: Base pair

Results

A total of 130 MPNs patients, including 78 Philadelphia chromosomes negative (49 ETs, 20 PVs, and 9 PMFs), 52 Philadelphia chromosome-positive patients, and 51 healthy subjects, were included. Their mean age was 53.2±15 years in the patients' groups versus 48.8±16.4 years in the control group (P=0.054) with an equal male to female ratio. Among the patient's group, PV patients had the highest mean age (63.4±13.9 years, P=0.021, Table-2). Table-2 shows *CALR* gene mutations frequency in all groups. None of the control groups had any *CALR* mutation. *CALR rs1450785140* was found in four patients with MPNs (one heterozygous and three homozygous states), and 2 out of 4 mutations in this position were detected in Philadelphia chromosome-positive patients (CML cases). *CALR rs765476509* was found in five MPNs patients (three heterozygous and two homozygous states); one homozygous state was related to a CML patient. Statistical analysis revealed no any difference among MPN patients based on a diagnosis. It may be related to a small number of *CALR* mutations in this study. Table-3 shows the frequency of *JAK2* gene mutation and its association with *CALR* mutation in different MPN diagnoses. Figure-2 graphically demonstrates these findings and clearly shows the highest prevalence of *JAK2* mutation in PV cases (90% in PV vs. 77.8% in PMF and 51% in ET, P=0.006). In Philadelphia negative cases, most *CALR*+ mutation was related to ET, but we had no significant statistical difference between ET, PV, and PMF cases due to the small number of *CALR* mutations, and also no relation to *JAK2* mutation could be analyzed. The lowest rate of *JAK2* mutation was related to CML cases vs.

Table 2. Lab Data and Mutational Status of the Patients and Control Groups According to *CALR rs1450785140* and *rs765476509* Mutational Status.

Groups	MPNs (n=130)	PV (n=20)	ET (n=49)	PMF (n=9)	CML (n=52)	Healthy control (n=51)
Age*, y (mean±SD)	53.2±15	63.4±13.9	52±15	56.7±20.2	49.7±12.8	48.8±16.4
Sex, (male/ female)	65/65	12/8	24/25	5/4	24/28	26/25
CALR rs1450785140* (4 cases), n (%) *						
Wild-type	126 (96.9)	20 (100)	47 (95.9)	9 (100)	50 (96.2)	51 (100)
Heterozygous	1 (0.8)	0(0)	1 (2)	0 (0)	0 (0)	0 (0)
Homozygous	3 (2.3)	0 (0)	1 (2)	0 (0)	2 (3.8)	0 (0)
CALR rs765476509* (5 cases), n (%) **						
Wild-type	125 (96.2)	19 (95)	47 (95.9)	8 (88.9)	51 (34.6)	51 (100)
Heterozygous	3 (2.3)	0 (0)	2 (4.1)	0 (0)	1 (1.9)	0 (0)
Homozygous	2 (1.5)	1 (5)	0 (0)	1 (11.1)	0 (0)	0 (0)

MPNs: Myeloproliferative neoplasms; **CML:** Chronic myeloid leukemia; **ET:** Essential thrombocythemia; **PMF:** Primary myelofibrosis; **PV:** Polycythemia vera.
* P=0.09 between patient and control group (no significant statistical difference); but mean age PV vs. other patients' groups have significant statistical difference (P=0.004).
** P-value=0.688 between the patients groups.
*** P-value=0.05 between the patients group (Total positive case were limited.)

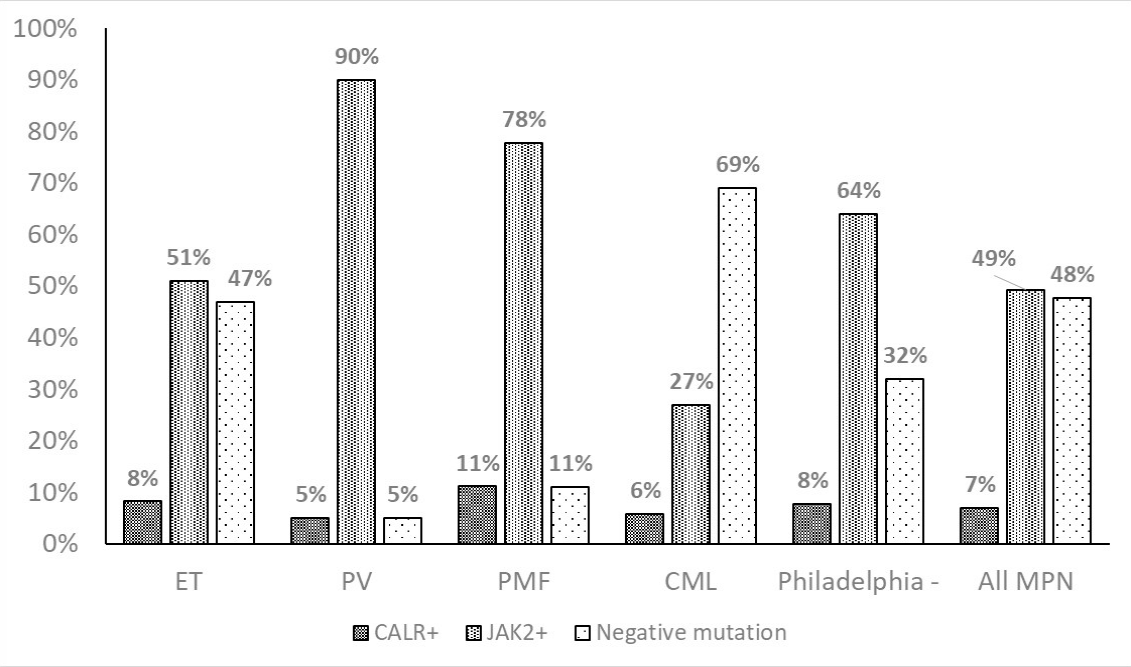

Figure 2. Prevalence of different mutations in the patient group with different diagnoses. **CML:** Chronic myeloid leukemia; **ET:** Essential thrombocythemia; **PMF:** Primary myelofibrosis; **PV:** Polycythemia vera

other MPN- cases (26.9% vs. 64%, P=0.001). Most CML cases (69%) had negative results for both *JAK2* and *CALR* mutation analysis (Figure-3). Interestingly, 2 of 3 *CALR* mutations in CML cases were negative for *JAK2* mutation (*CALR+/JAK2-*). A review of the CML clinical profile of patients with *CALR* mutation did not show any data indicative of other diagnoses, and all three patients were diagnosed based on bone marrow findings and positive BCR/ABL mutation at the initial presentation. None of these three patients developed thrombocytosis after treatment with imatinib. All of them had a good response to imatinib, and in the time of *CALR* mutation analysis, BCR/ABL had been non-detectable in them.

Discussion

The presentation of *JAK2* mutation mainly clarifies the molecular basis and diagnosis of MPNs. Most recently, *CALR* gene mutations are also presented as another important genetic abnormality, and recurrent mutation is an important factor in the pathogenesis and clinical manifestations of MPNs [13, 14]. *CALR* gene encodes Calreticulin protein, which is located in the lumen of the endoplasmic reticulum that plays a critical role in calcium homeostasis related to apoptosis and cell survival regulation, especially in cancer cells [15]. We evaluated the two most common *CALR* gene mutations and *JAK2 V617F* mutation prevalence in Iranian MPNs patients. In all patients, especially in Philadelphia negative cases, the *JAK2* gene mutation frequency was high according to other studies [16, 17], but the rate of *CALR* mutation was lower than in other studies. The lower rate of *CALR* mutations in this study compared to European patients (20–25%) and in Brazilian and Argentinean patients may be related to multiple factors [18-20]. Genetic differences of the population may explain the very low *CALR* mutation rate in the Iranian population. Another explanation may be related to the technique of analysis.

The mentioned studies use the whole exon gene analysis for the *CALR* gene and report a summation of all mutations in this gene. However, our study relied on previous reports that mentioned *rs1450785140* and *rs765476509* as the most common *CALR* mutations in patients, presented in 80% of all patients with *CALR* mutation [10-12, 21].

Thus, concerning the finding of this study, the *CALR* mutation in the Iranian population was not as frequent as other ethnicities, or these two types of mutations are not the most common mutations in the Iranian population. Another study with a whole exon analysis of the *CALR* gene can further clarify this finding [7, 19, 20]. However, the low rate of *CALR* mutation did not allow us to analyze the correlation of *CALR* mutation with *JAK2*

Table 3. The Frequency of *JAK2* Gene Mutation and *CALR* Gene Mutations in the Patient Group.

Mutations / MPN types		*CALR* +	*JAK2* +*	*CALR*+ /*JAK2*+	*CALR* / *JAK2*+*	*CALR*+ /*JAK2*-	Both negative*
Philadelphia negative patients, n (%)	ET (n=49)	4 (8.2)	25 (51)	3 (6.1)	22 (44.9)	1 (2)	23 (46.9)
	PV (n=20)	1 (5)	18 (90)	0 (0)	18 (90)	1 (5)	1 (5)
	PMF (n=9)	1 (11.1)	7 (77.8)	1 (11.1)	6 (66.7)	0 (0)	2 (22.2)
	Total (n=78)	6 (8)	50 (64)	4 (5.1)	46 (58)	2 (2.5)	25 (32)
Philadelphia positive patients, n (%)	CML (n=52)	3 (5.8)	14 (26.9)	1 (1.9)	13 (25)	2 (3.8)	36 (69.2)

*P<0.001 between PV and other groups and also between Philadelphia- vs. Philadelphia+ groups.

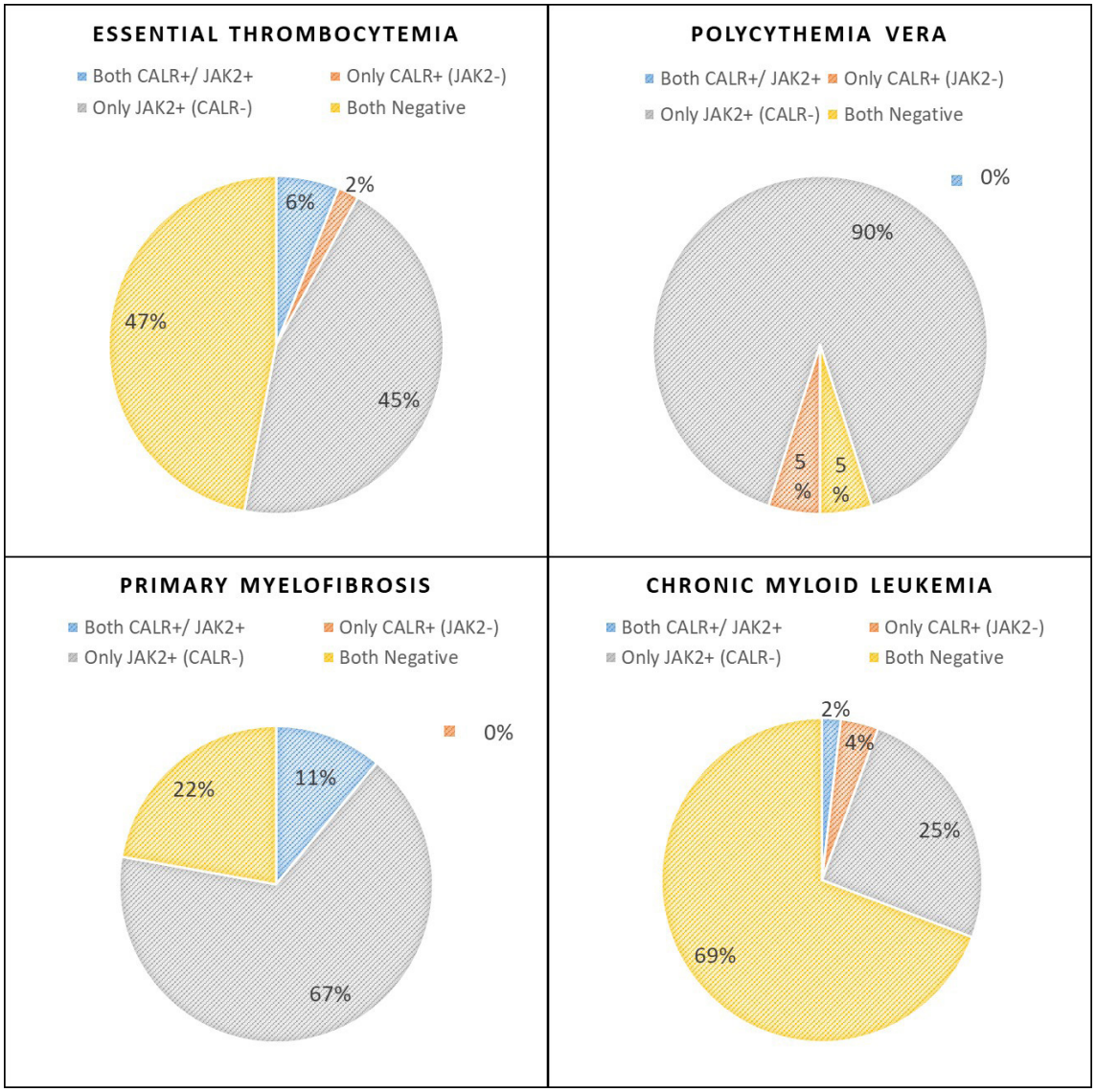

Figure 3. Prevalence of *CALR* /*JAK2* mutation in MPN patients.

mutation. Another interesting finding in this study was the three cases of CML with *CALR* mutation. This was a rare mutation in CML cases, and most reports are related to case reports with a borderline diagnosis of CML with some equivocal presentation of ET and CML [22, 23]. Their patients developed PV features after treatment of CML or had a poor response to imatinib, but we had no resistance case or thrombocytosis in three CML cases with *CALR*. The exact effect of this mutation in our patients was not clear to us. They all had a usual course of CML, and in fact, the *CALR* mutation was detected incidentally. A more detailed view of genetic screening in MPN patients was presented by Haunstrup *et al.* [24], who focused on the allele burden of *JAK2 V617F* and the frequency of *CALR* mutation in low allele burden (AB) MPN cases. They showed 11% *CALR* mutation in low AB versus no *CALR* mutation in a group of MPN patients with more than 5% AB [24]. This finding indicated the importance of other genetic screening in cases with low AB of *JAK2*. Our study with 7.7% *CALR* mutation in all MPN cases also showed the *CALR* mutation could be found even in *JAK2*- patients. Although the *CALR* mutation rate is low in MPN cases, Haunstrup *et al.*'s recommendation for extending the molecular screening could be true for both low AB *JAK2* and *JAK2* negative cases. It is worth mentioning that

Haunstrup *et al*. *CALR* mutation analysis showed a reverse prevalence of type 1 *CALR* versos type 2 mutation, which was hypothesized based on distinct MPN entity for double mutated cases [24]. The result of *JAK2* mutation analysis was in the same line with those of other studies, clearly showing the *JAK2* mutation's role in the diagnosis of PV (90% *JAK2+)*. Even this mutation was statistically more prevalent in PV rather than all other types of MPNs. The rate of *JAK2* mutation in CML cases (26.9%) was also in agreement with other studies [25, 26].

Conclusion

Our results showed that the *JAK2* gene mutation was common among MPNs, especially PV cases. On the other hand, two of the most common mutations of the *CALR* gene had a very low prevalence in Iranian MPNs but may be seen even in negative *JAK2* mutation cases. Further evaluation of the *CALR* gene by whole exon analysis of *CALR* is recommended to find the other possible mutation and its dependency on ethnicity and genetic diversity.

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Conflict of Interest

None.

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