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
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.1 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 Oncohematology 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 95°C for 3 minutes, then 35 cycles of 95°C for 45 seconds, 61°C for 50 seconds, and 72°C for 50 seconds, then by 72°C 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, 0.5 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.

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 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.
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): ACAGGACGAGGACGAGGAG <br> F (mutant allele-specific primer): CGAGGACGAGGACGAGGAG <br> R: GGCTGAAAGGAATCAAAGAT | 403 bp |
| CALR | rs765476509 | F (wild type allele-specific primer): GAGGCAGAGCAAGGAGAG <br> R: ACAGAGGCAAGAAAAGATGA <br> R (mutant allele-specific primer): CCTCATCATCCTCCGACA <br> F: GGTGGTCCTTGTCTTCTCT | 392 bp |
| JAK2 | V617F | F (wild type allele-specific primer): ATTTGGTTTTAATTATGGAGTACGTG <br> F (mutant allele-specific primer): ATTTGGTTTTAATTATGGAGTACGTA <br> R: CTGTTAAATTATTAGTTTACCTGACAC | 156 bp |
| ACTB (internal control) | | F: CTCCCTCAGATCATTGGCTCCT <br> R: GTACAATCACCCTGTTCCA | 317 bp |

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 \textit{JAK2} and \textit{CALR} mutation analysis (Figure-3). Interestingly, 2 of 3 \textit{CALR} mutations in CML cases were negative for \textit{JAK2} mutation (\textit{CALR+}/\textit{JAK2}-). A review of the CML clinical profile of patients with \textit{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 \textit{CALR} mutation analysis, BCR/ABL had been non-detectable in them.

**Discussion**

The presentation of \textit{JAK2} mutation mainly clarifies the molecular basis and diagnosis of MPNs. Most recently, \textit{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]. \textit{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 \textit{CALR} gene mutations and \textit{JAK2 V617F} mutation prevalence in Iranian MPNs patients. In all patients, especially in Philadelphia negative cases, the \textit{JAK2} gene mutation frequency was high according to other studies [16, 17], but the rate of \textit{CALR} mutation was lower than in other studies. The lower rate of \textit{CALR} mutations in this study compared to European patients (20–25%) and in Brazilian and Argentinian patients may be related to multiple factors [18-20]. Genetic differences of the population may explain the very low \textit{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 \textit{CALR} gene and report a summation of all mutations in this gene. However, our study relied on previous reports that mentioned \textit{rs1450785140} and \textit{rs765476509} as the most common \textit{CALR} mutations in patients, presented in 80% of all patients with \textit{CALR} mutation [10-12, 21].

Thus, concerning the finding of this study, the \textit{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 \textit{CALR} gene can further clarify this finding [7, 19, 20]. However, the low rate of \textit{CALR} mutation did not allow us to analyze the correlation of \textit{CALR} mutation with \textit{JAK2} mutation.

| Table 3. The Frequency of JAK2 Gene Mutation and CALR Gene Mutations in the Patient Group. |
|---------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| **Mutations** | **CALR +** | **JAK2 +** | **CALR+ /JAK2+** | **CALR+/JAK2-** | **Both negative** |
| **MPN types** | **ET (n=49)** | **PV (n=20)** | **PMF (n=9)** | **Total (n=78)** | **CML (n=52)** |
| Philadelphia negative patients, n (%) | 4 (8.2) | 25 (51) | 3 (6.1) | 22 (44.9) | 1 (2) | 23 (46.9) |
| Philadelphia positive patients, n (%) | 1 (5) | 18 (90) | 0 (0) | 18 (90) | 1 (5) | 1 (5) |
| Total (n=78) | 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) |

*P<0.001 between PV and other groups and also between Philadelphia- vs. Philadelphia+ groups.
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-negative cases. It is worth mentioning that

Figure 3. Prevalence of CALR JAK2 mutation in MPN patients.
Haunstrup et al. CALR mutation analysis showed a reverse prevalence of type 1 CALR versus 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.

**References**

1. Arber DA, Orazi A, Hasserjian R, Thiele J, Borowitz MJ, Le Beau MM, et al. The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood. 2016;127(20):2391-405.
2. Wong WJ, Pozdnyakova O. Myeloproliferative neoplasms: Diagnostic workup of the cythemic patient. Int J Lab Hematol. 2019;41 suppl:142-50.
3. Zhou T, Medeiros LJ, Hu S. Chronic Myeloid Leukemia: Beyond BCR-ABL1. Curr Hematol Malig Rep. 2018;13(6):435-45.
4. O'Sullivan J, Mead AJ. Heterogeneity in myeloproliferative neoplasms: Causes and consequences. Adv Biol Regul. 2019;71:55-68.
5. James C, Ugo V, Le Couedic JP, Staerk J, Delhommeau F, Lacout C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. Nature. 2005;434(7037):1144-8.
6. Gango A, Mozes R, Boha Z, Kajtar B, Timar B, Kiraly PA, et al. Quantitative assessment of JAK2 V617F and CALR mutations in Philadelphia negative myeloproliferative neoplasms. Leuk Res. 2018;65:42-8.
7. Klampfl T, Gisslinger H, Harutyunyan AS, Nivarthi H, Rumi E, Milosevic JD, et al. Somatic mutations of calreticulin in myeloproliferative neoplasms. N Engl J Med. 2013;369(25):2379-90.
8. Cazzola M, Kralovics R. From Janus kinase 2 to calreticulin: the clinically relevant genomic landscape of myeloproliferative neoplasms. Blood. 2014;123(24):3714-9.
9. Langabeer SE, Andrikovics H, Asp J, Bellosillo B, Carillo S, Haslam K et al. Molecular diagnostics of myeloproliferative neoplasms. Eur J Haematol. 2015;95(4):270-9.
10. Nangalia J, Green TR. The evolving genomic landscape of myeloproliferative neoplasms. Hematology Am Soc Hematol Educ Program. 2014;2014(1):287-96.
11. Bilbao-Sieyro C, Florido Y, Gómez-
10. Casares MT. CALR mutation characterization in myeloproliferative neoplasms. Oncotarget. 2016;7(33):52614-7.
12. Giannopoulos A, Rougkala N, Loupis T, Mantzourani M, Viniou N-A, Variami E et al. Detection of CALR Mutations Using High Resolution Melting Curve Analysis (HRM-A); Application on a Large Cohort of Greek ET and MF Patients. Mediterr J Hematol Infect Dis. 2019;11(1):e2019009.
13. Wong WJ, Hasserjian RP, Pinkus GS, Breyfogle LJ, Mullally A, Pozdnyakova O. JAK2, CALR, MPL and ASXL1 mutational status correlates with distinct histological features in Philadelphia chromosome-negative myeloproliferative neoplasms. Haematologica. 2018;103(2):e63-e8.
14. Imai M, Araki M, Komatsu N. Somatic mutations of calreticulin in myeloproliferative neoplasms. Int J Hematol. 2017;105(6):743-7.
15. Chao MP, Jaiswal S, Weissman-Tsukamoto R, Alizadeh AA, Gentles AJ, Volkmer J et al. Calreticulin is the dominant pro-phagocytic signal on multiple human cancers and is counterbalanced by CD47. Sci Transl Med. 2010;2(63):63-94.
16. Poopak B, Hagh MF, Saki N, Elahi F, Rezvani H, Khosravipour G et al. CALR mutations screening in wild type JAK2(V617F) and MPL(W515K/L) Brazilian myeloproliferative neoplasm patients. Blood Cells Mol Dis. 2015;55(3):236-40.
20. Ojeda MJ, Bragos IM, Calvo KL, Williams GM, Carbonell MM, Pratti AF. CALR, JAK2 and MPL mutation status in Argentinean patients with BCR-ABL1-negative myeloproliferative neoplasms. Hematology. 2018;23(4):208-11.
21. Haslam K, Conneally E, Flynn CM, Cahill MR, Gilligan O, O'Shea D et al. CALR mutation profile in Irish patients with myeloproliferative neoplasms. Hematol Oncol Stem Cell Ther. 2016;9(3):112-5.
22. Dogliotti I, Fava C, Serra A, Gottardi E, Darafo F, Carnuccio F et al. CALR-positive myeloproliferative disorder in a patient with Ph-positive chronic myeloid leukemia in durable treatment-free remission: a case report. Stem Cell Investig. 2017;4:57.
23. Lewandowski K, Gniot M. Coexistence of JAK2 or CALR mutation is a rare but clinically important event in chronic myeloid leukemia patients treated with tyrosine kinase inhibitors. Int J Lab Hematol. 2018;40(3):366-71.
24. Haunstrup LM, Ebbesen LH, Hansen M, Severinsen MT, Aggerholm A. Skewed ratio between type 1 and type 2 calreticulin mutations in essential thrombocytosis patients with concomitant Janus kinase 2 V617F mutation. Exp Hematol. 2018;68:62-5.
25. Zaen-Al-Abideen Pahore TS, Shamsi MT, Tasneem Farzana SH, Nadeem M, Ahmad M, Naz A. JAK2V617F mutation in chronic myeloid leukemia predicts early disease progression. J Coll Physicians Surg Pak. 2011;21(8):472-5.
26. Tabassum N, Saboor M, Ghani R, Moinuddin M. Frequency of JAK2 V617F mutation in patients with Philadelphia positive Chronic Myeloid Leukemia in Pakistan. Pak J Med Sci. 2014;30(1):185-8.