



## **Calreticulin and JAK2 Exon 12 Mutation Screening in Patients with Myeloproliferative Neoplasms' in Jeddah Region, Saudi Arabia**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

### **Article Information**

DOI: 10.9734/JPRI/2021/v33i47A33029

Editor(s):

(1) Dr. Dharmesh Chandra Sharma, G. R. Medical College & J. A. Hospital, India.

Reviewers:

(1) Theoni Kanellopoulou, Onassis Cardiac Surgery Center, Greece.

(2) Vladareanu Ana Maria, Carol Davila University of Medicine and Pharmacy Bucharest, Romania.

Complete Peer review History: <https://www.sdiarticle4.com/review-history/76035>

**Original Research Article**

**Received 12 August 2021**

**Accepted 25 October 2021**

**Published 27 October 2021**

### **ABSTRACT**

**Background:** The JAK2 V617F mutation's discovery has largely facilitated the comprehension of the myeloproliferative neoplasms'(MPNs) pathogenesis. In recent times, calreticulin (CALR) mutations have been detected in patients with JAK2V617F negative primary myelofibrosis (PMF),

and essential thrombocythemia (ET).

**Methods:** This study analyzed the impact of JAK 2 Exon 12 and CALR common mutations in 65 patients with JAK2V617F negative MPN from the Jeddah region. An allele-specific polymerase chain reaction (PCR) method was used to screen four common mutations on Exon 12 and direct sequencing and PCR analysis were utilized to screen all patients for CALR.

**Results:** None of the patients were positive for the Exon 12 mutation and eight patients were positive for CALR mutations.

**Conclusions:** This is the first Saudi Arabian research that focused on screening CALR hotspot mutations and this mutation exists. This fact highlights the importance of implementing diagnostic screening of CALR on MPN patients, in general, and patients with high platelet count, in particular. Further screening of other predisposing genetic markers might facilitate the identification of an important genetic variant, which could aid in the understanding of disease pathogenesis.

*Keywords: Myeloproliferative neoplasms; JAK2; CALR; primary myelofibrosis and essential thrombocythemia.*

## 1. INTRODUCTION

Myeloproliferative neoplasms (MPNs) are a heterogeneous group of hematopoietic stem cell (HSC) and progenitor cell associated disorders, which involve chronic myeloid neoplasms and have the potential of disease progression to acute leukemia. An initial hit in HSC in the context of MPNs results in independence from normal cytokine regulation or hypersensitivity. In turn, this causes the excessive mature cell production with one or more blood cell lineages [1]. The new WHO classification of lymphoid and hematopoietic tissue-specific tumors specifies that BCR-ABL1-negative MPNs comprise a family of three hematological malignancies, which include essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF) [1,2]. The discovery of a frequent point mutation in exon 14 (V617F) of Janus kinase 2 (JAK2) [3,4], which occurs in 95% of cases of PV and 60% of cases of PMF and ET significantly advanced the characterization of classic MPNs' genetic background in 2005 [3-8]. The unique JAK2V617F mutation plays an important role in MPNs' pathogenesis, there by resulting in the constitutive activation of JAK/STAT signaling and the subsequent stimulation of differentiation and proliferation of the myeloid lineages [9]. Furthermore, evidences prove that JAK2 exon 12 mutations are present in approximately 5% of PV, who were negative for JAK2 (V617F) [10,11]. Moreover, 5-10% of JAK2 negative PMF and ET patients have point mutation on exon 10 of the thrombopoietin receptor gene (MPL) [12-16]. However, approximately 30 to 45% of BCR-ABL negative MPNs patients do not have MPL and JAK2 V617F mutations [17].

The aim of this study is to investigate the prevalence of CALR and JAK 2 exon 12 mutations in JAK2 non-mutated patients with suspected MPN from Jeddah City.

## 2. MATERIALS AND METHODS

### 2.1 Patient Samples

Sixty-five patients were included in the study whose bone marrow or blood samples were referred to Center of Excellence in Genomic Medicine Research, King Abdulaziz University, Jeddah, Saudi Arabia for the molecular screening of JAK2V617F, due to suspected MPN. The patients were all JAKV617F negative and had the following diagnosis: 25 cases non MPN-related thrombosis (38.46%), 17 cases ET (26.15%), 4 cases PMF (6.15%), 9 cases PV (13.85%), 9 cases unclassified MPNs (13.85%), and 1 case CMML (1.53%) who were diagnosed after the 2008 WHO classification of myeloid neoplasms. The patients' median age at diagnosis was 60 years. Furthermore, the patients were categorized and diagnosed using the WHO 2008 criteria [15].

This study involved diligently documenting the patients' information, which comprises their hematological and diagnostic findings, that is, white blood cells WBC, platelet, and red blood cell RBC count as well as hemoglobin levels.

### 2.2 Mutation Detection using Sanger Sequencing for CALR and JAK2 exon14 (V617F)

Genomic DNA was isolated from PB and BM using QIAmp DNA Blood mini kit (Qiagen,

Germany). Subsequently, the concentration was quantified by Nanodrops 2000 spectrophotometer (Thermo Scientific, USA). JAK2 V617 and CALR Exon 8 and 9 were independently polymerase chain reaction (PCR) amplified independently by employing the following primer: CALR forward (5'-ACAACCTTCCTCATCACCAACG-3'), reverse (5'-GGCCTCAGTCCAGCCCTG-3'), Exon 14 forward JAK2 Exon14 forward (5'-TTCTTTGAAGCAGCAAGTATGATGA-3'), and AK2 Exon14 reverse (5'-CTGACACCTAGCTGTGATCC-3').

The PCR was performed in 20 µl volume, which comprised 1 µl of dNTPs, 2 µl of 10x buffer, 1 µl forward primer and reverse primer each, 13.8 µl of nuclease water, 0.2 µl hot start DNA polymerase (5u/µl), and 1 µl (100 to 300 ng) of DNA template. The cycle protocol used in the PCR was as follows: initial denaturation at 95°C for 15 minutes followed by denaturation at 95°C for 30 seconds. Subsequently, the mixture was annealed with different temperatures for each gene for 40 cycles and this was followed by extension at 72°C for 10 minutes. After amplification, the product quality and size were visualized on 2 percent agarose gel, which was stained with ethidium bromide under ultraviolet (UV) light by utilizing the gel documentation system. The PCR product bands' molecular size were determined by extrapolating the 50 base pair (bp) scale DNA ladder loaded alongside to

the bands. The aforementioned primers, an ABI 3730 XL automatic sequencer (Applied Biosystems), and the Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA) were used to directly sequence the PCR products.

### 2.3 Allele-specific PCR (AS-PCR) of JAK2 Exon 12

The presence molecular evidence of BCR/ABL fusion transcripts and/or translocation t(9;22) (Philadelphia chromosome) as well as JAK2 V617F were analyzed in the Center of Excellence in Genomic Medicine Research for all cases, and all the results were negative. Sequence analysis of CALR and JAK2 exon 12 was conducted for all 65 cases.

Multiplex PCR reactions, was used to amplify exon 12 using the following primers: **E12F**: 5'-CTCCTCTTTGGAGCAATTCA-3', **E12R**: 5'-GAGAAGTTGGGAGTTGCGATA-3', **K539L**: 5'-CATATGAACCAAATGGTGTTCCTACTT-3', **N542-E543del**: 5'-CAAATGGTGTTCACAAAATCAGAGATT-3', **F537-K539delinsL**: 5'-CATATGAACCAAATGGTGTTAATC-3', **H538QK539L**: 5'-CATATGAACCAAATGGTGTTCCTCAATT-3'. The reaction was performed using genomic DNA template 0.5 µl, and following the amplification reaction as illustrated in Table 1.

**Table 1. Master Mix Components for JAK2 Exon 12 Multiplex PCR**

Reagent	Set1		Set2		Set3	
	1 test	X 10	1 test	X 10	1 test	X 10
Injection water	18.8	188	19.3	193	19.3	193
10X PCR Buffer	2.5	25	2.5	25	2.5	25
10Mm dNTPs	0.5	5	0.5	5	0.5	5
Forward Primer E- 12	0.5	5	0.5	5	0.5	5
Reverse Primer E- 12	0.5	5	0.5	5	0.5	5
K539	0.5	5	-	-	-	-
H538QK539 L	0.5	5	-	-	-	-
N542-E543del	-	-	0.5	5	-	-
F537-K539 delinsL	-	-	-	-	0.5	5
Taq polymerase	0.2	2	0.2	2	0.2	2
Sub total	24µl	-	24µl	-	24µl	-
DNA	0.5	-	0.5	-	0.5	-
TOTAL volume	24.5µl		24.5µl		24.5µl	

The PCR procedure amplifies the exon 12 using primer set following the described protocol in Scott research [11]. The PCR was performed in 20µl volume, which comprised 2 µl of 10xbuffer, 13.8 µl of nuclease water, 1µl of dNTPs, 1 µl forward primer, 0.2 µl hot start DNA polymerase (5u/µl), 1 µl reverse primer, and 1 µl (100 to 300 ng) of DNA template. The PCR used the following cycle protocol initial activation at 95°C for 4 minutes followed by 35 cycles of denaturation at 94°C for 30 seconds and then annealing at 55°C for 30 seconds and extension at 72°C for 30 sec min). There is a final extension at 72°C for 10 minutes.

### 2.4 Statistic Analysis

All statistical analysis were carried out with SPSS software version 20.0 (SPSS, Chicago, IL), P values < 0.05 were considered significant.

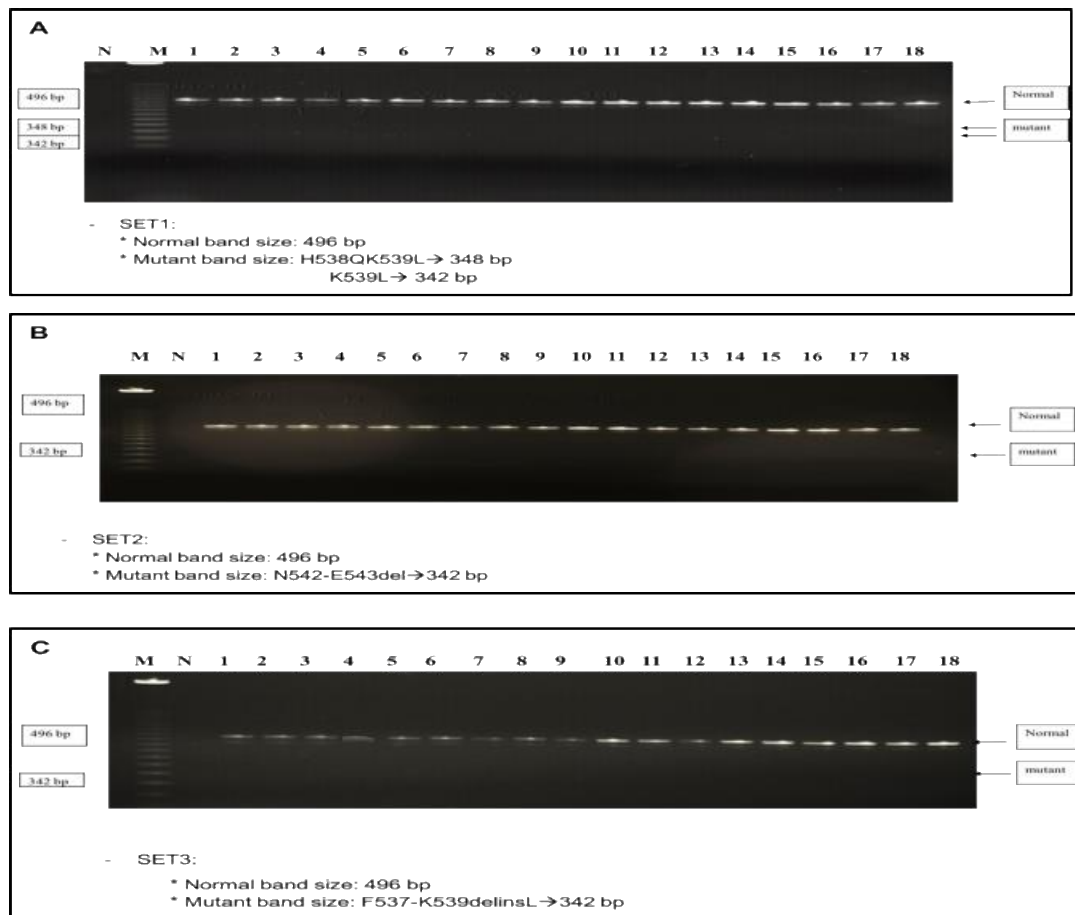
## 3. RESULTS

### 3.1 Clinical Characteristic of Patients

This study was conducted by including 65 MPN suspected patients, which comprises other non MPN with thrombosis 25 (38.46%), 17 cases ET (26.15%), 4 cases PMF (6.15%), 9 cases PV (13.85%), 9 cases unclassified MPNs (13.85%), and 1 case CMML (1.53%) (Fig. 1).

### 3.2 Mutation Screening Of JAK2EXON 12 by Allele-Specific PCR

JAK2 exon 12 mutations (N542-E543, K539L, H538QK539L, and K539delinsl) were investigated in all 65 MPN patients' cohort. The result showed that non-exon 12 mutations were detected in all MPN patients who were investigated (Fig. 1).



**Fig. 1.** PCR amplification of exon 12 DNA using multiplex PCR. (A) Represents Set 1 [the specific primers were K539L (342 bp) and H538QK539L (348 bp)]. (B) Set 2 [the specific primer was N542-E543 (342 bp)]. (C) Set 3 [the specific primer was K539delinsl (342 bp)]. N: Negative control; M: DNA marker (50 bp), Lanes (1–18): Amplified DNA from 18 samples

### 3.3 CALR Exon 9 Screening

CALR frameshift mutation types 1 and 2 were investigated in all patients' cohort. The sequencing results detected two in-frame deletion mutations within CALR exon 9 8 patients 12.3% of patients who are JAK2 V617F and exon 12 negative; 5 patients with ET 29.4% , 2 patients with thrombocytosis and non MPN in current diagnosis 8% thrombosis patients, and in 1 CMML patient (Fig. 2).Amongst the CALR frameshift mutations, four patients had typical type 1 mutations (L367fs\*46) and a 52-bp deletion, while four patients had type 2 mutations (K385fs\*47) 5 bp insertion. TYPE-1 mutation (L367fs\*46) was detected in two out of five ET (11.5%) and 1 non-MPN with thrombosis patients. On the other hand, TYPE-2 mutation (K385fs\*47) was detected in three out five ET patients, 1 CMML patient, and 1non-MPN with thrombocytosis patient.

### 3.4 CALR Mutation with Clinical Characteristics

The patients with CALR mutations were Saudi adults who had been diagnosed with thrombocytosis or MPN and their molecular screening results for JAK2 V617F and BCR/ABL were negative. Patients suffering from ET and patients with CALR frameshift mutations tend to have a higher platelet counts with normal hemoglobin levels. Moreover, the diseases of patients with ET who had CALR mutations did not progress to accelerated or blast phase disease in comparison with other MPNs. No differences in mutation rates were detected amongst men and women and there were no age-restricted differences.

## 4. DISCUSSION

In the context of the classification of MPNs and MPN/myelodysplastic syndrome with thrombocytosis and ring sideroblasts, JAK2V617F, CALR, and JAK2 Exon 12 mutations are critical biomarkers. At present, general guidelines for JAK2 and CALR molecular testing in MPNs are unavailable. Several suggested indicators for these mutations' molecular testing in screening and diagnosis involve the following work-up:(1) clinically suspected PV, PMF, ET, and MDS/MPN with thrombocytosis and ring sideroblasts, (2) unexplained leukocytosis, and (3) unexplained splanchnic vein thrombosis [18].

Mutational testing does not only play an important role in the diagnosis and prognosis of the diseases but it also provides significant prognostic and other critical information. For instance, type 1 CALR mutations in PMF have been associated with superior survival rates than type 2 CALR and JAK2 mutations [19]. Across all MPNs, JAK2 mutations are related with higher hemoglobin, older age, leukocytosis, increased thrombotic events, and lower platelets [19,20]. The JAK2 V617F, JAK2 exon 12 and CALR mutations are assessed by various molecular methods. This includes allele-specific (assessing for hotspot mutations) and sequencing-based methods.

We employed Sanger sequencing to detect JAK2 V617F and CALR in this study, and allele-specific PCR was utilized to detect known mutations at exon 12.

No mutations were detected amongst the 65 patients who were screened for exon 12 mutations. Although exon 12 mutations' prevalence is uncommon, the method used for detection of mutations might not have the sensitivity margin to detect exon 12 mutations. Allele-specific tests, which assess hotspot mutations, might fail to detect other relevant variants that are less common, for example, JAK2 exon 12 mutations, and those that are found in approximately 3% of PVs, thereby giving false negatives. In addition, some allele-specific methods might be affected by near variant interferences, where in the hotspot mutation's detection might be hindered by the interference of second nearby single nucleotide variant.

The sensitivity of some techniques is less sensitive than that of others. Moreover, some MPNs (for example, ET) can present with low mutant allele fractions. Newer methods are more sensitive than Sanger sequencing, as it has the typical detection limit of 20% mutant allele fraction [21]. No universally accepted cutoffs are available for a positive result, even though some recommend the analytical sensitivity range between 1-3% mutant allele fraction for molecular assays [22,23].

In 2013, Klampfl *et al.* and Nangalia *et al.* described how somatic mutations recurrently and exclusively affect the exon 9 of the calreticulin (CALR) gene [24,25]. CALR mutations were mutually exclusive in the MPL and JAK2 non-mutated patients' subset [14]. This, in turn,

confirms an MPN diagnosis. The impact of CALR in prognosis and clinical presentation continues to be partially comprehensible. Although several additional studies have described the presence

of low frequencies of CALR mutations in different MPN related diseases, they have not specified this presence with respect to other hematologic diseases.

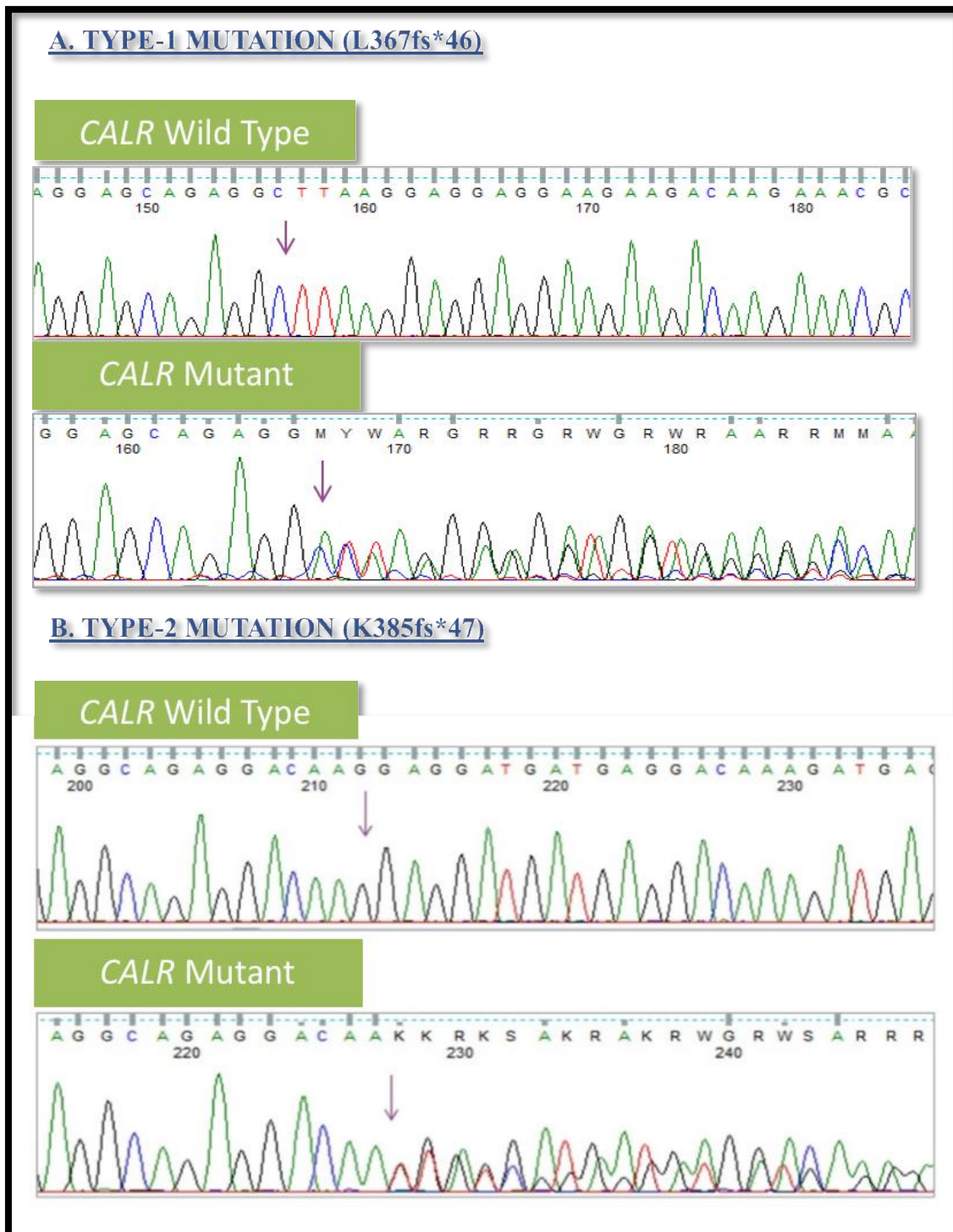


Fig. 2. Sanger sequencing for CALR exon 9 mutation; (A) Normal control vs Positive samples with in frame deletion mutation (L367fs\*46), (B) Normal control vs Positive sample with in frame deletion mutation (K385fs\*47)

Mutation advent in the CALR gene changed the MPN landscape. Klampfl *et al.* in 2013 first recognized it as a somatic mutation in those patients who had MPNs but with no mutations in either MPL or JAK2 [25]. CALR is a protein found in cytoplasm, endoplasmic reticulum, or cell surface. It maintains calcium hemostasis and regulates cell proliferation, apoptosis, and phagocytosis while facilitate accurate glycoprotein folding [26].

CALR mutation was identified in eight out of the total 65 patients who presented with thrombocytosis and had been suspected to have MPN. Amongst patients who had the CALR mutation, six patients were diagnosed as suffering from ET based on the 2016 WHO classification and diagnostic criteria for MPNs. Consequently, the CALR mutation types 1 and 2 were equally identified amongst those patients who were diagnosed as suffering from ET. The other two patients in which CALR mutations were detected were diagnosed as suffering from systemic lupus erythematosus (SLE) and chronic myelomonocytic leukemia (CMML). No discernible difference could be detected in the disease's clinical phenotype amongst those with CALR and ET mutations.

Usually, the patients in whom heterozygous CALR mutated PMF is detected are men whose age is comparatively younger than that of patients with JAK2 mutated cases. Furthermore, myeloproliferation in such cases is more specific to the megakaryocytic lineage and, therefore, presents with a more pronounced thrombocytosis. They usually have low white cell count and hemoglobin. Longer survival rates and low incidence of thrombotic complications are reported in this patient group. The prognostic impact of CALR on PMF is limited to type 1 mutation while the prognosis in case of type 2 is similar to that of JAK2 mutated PMF [27].

The widespread impact of CALR mutation on MPNs, baseline characteristics, disease outcome, a patient's clinical behaviors, and benefits and risks in the long term warrants further exploration, as the impact of CALR mutation on MPNs is a recent scientific discovery. Prospective studies have to be conducted in order to outline the manner in which CALR mutation influences MPNs by following a detail-oriented approach toward the mutation's homozygous pattern. Several publications have analyzed and elucidated the CALR mutation screening methods, and it has even been

suggested by some authors that fragment analysis determination might sufficiently fulfill the needs of routine diagnosis and aid in the development of real-time PCR detection methods [28]. Such screening methods do not facilitate precise characterization. Therefore, determining the accurate size of insertion or deletion might sometimes prove to be difficult through fragment analysis. This is an important issue, as this study has show cased that in-frame indel polymorphisms could be misinterpreted as mutations in case they are improperly characterized. Sanger sequencing was employed in this study, as mutation characterization is a critical factor in not only determining whether the alteration belongs to clinically relevant types, namely, 1 or 2, or to the type-1/2-like but also differentiating polymorphisms from point/nonsense mutations, which might play an important role in diagnosis.

Rare nonsense mutations, which indicate the loss of a variable number of negatively charged amino acids of the C-terminus, have been reported. This includes p.E380X, p.E374X, and p.K391X [29]. We consider both the simultaneous assessment of multiple mutations by next-generation sequencing and sequential testing algorithms as valid testing approaches. NGS facilitates the holistic assessment of targeted genes that are relevant to myeloid neoplasms. The genes that were included in this regard differ from one another based on the specific panel. Moreover, larger insertions and deletions (notably, type 1 mutations in CALR exons 9) might be missed based on the data analysis types used for the panel [30]. NGS' comprehensive nature will enable the reporting of less well-characterized variants of uncertain significance at other loci as well as pathogenic variants.

Our understanding of the molecular landscape of PV, PMF and ET is rapidly evolving. Recent studies have implicated other genes and pathways in disease progression and MPN pathogenesis [20]. Although many amongst such mutations are not specific to MPNs, they represent additional biomarkers that can be utilized to provide additional prognostic-specific information or prove clonality [20]. Molecular testing is simultaneously becoming increasingly complex—in terms of the associated technologies as well as the produced information. The facilitation of strong and effective communication between pathologists, clinicians, and molecular diagnosticians is

important to properly integrate molecular data with clinical and pathologic findings.

## 5. CONCLUSIONS

This is the first Saudi Arabian research that focused on screening CALR hotspot mutations and this mutation exists. This fact highlights the importance of implementing diagnostic screening of CALR on MPN patients, in general, and patients with high platelet count, in particular. Further screening of other predisposing genetic markers might facilitate the identification of an important genetic variant, which could aid in the understanding of disease pathogenesis.

## CONSENT

As per international standard or university standard, patient's written consent has been collected and preserved by the author(s).

## ETHICAL APPROVAL

Approval for this work was sought from CEGMR Bioethical committee with ethical no. HA-02-J-003

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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