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Screening for Janus kinase-2 exon 12 mutations among Janus kinase-2 V617F-negative polycythemia vera suspected Iraqi patients

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Abstract:

BACKGROUND: Diagnosis of myeloproliferative neoplasms including polycythemia vera (PV) has been greatly improved following the identification of driver mutations at exon 12 and exon 14 of the Janus Kinase 2 (JAK2) gene. There are limited academic studies that report the frequency of the JAK2 exon 12 gene with limited number of patients.

OBJECTIVES: The aim of this study was to evaluate the frequency of JAK2 exon 12 mutations among JAK2V617F-negative PV-suspected Iraqi patients.

PATIENTS, MATERIALS AND METHODS: This is a retrospective study conducted at the National Center of Hematology/Mustansiriyah University from December 2019 to January 2022; patients with suspicion of PV were enrolled in this study. Screening for JAK2 exon 14 (JAK2V617F) mutations was done for all 323 patients. Out of them, there were 102 patients with JAK2V617F-negative who retrospectively screened for JAK2 exon 12 mutations by direct sequencing (Sanger sequencing).

RESULTS: Initial evaluation for the total 323 patients suspected with PV revealed that 84 cases were mutated for the JAK2V617F gene, and 239 were unmutated. The frequency of JAK2 exon 12 was about 1% among JAK2V17F-negative patients. The median age for all patients was 35 years. Among the total cases, 21 cases (20.59%) showed splenomegaly at the time of clinical onset sequencing of these cases revealed only one case mutated with JAK2 exon 12 (E543-D544del).

CONCLUSION: The current study showed that the frequency of JAK2 exon 12 mutations in JAK2V617F-negative cases was very low. Despite PV patients with JAK2 exon 12 mutations represent a small proportion of total PV cases, JAK2 exon 12 mutations should be routinely screened in JAK2V617F-negative cases.

Keywords:

Janus kinase 2 exon 12, Janus kinase 2V617F negative, polycythemia vera

Introduction

BCR-ABL1-negative myeloproliferative neoplasm (MPN) is a heterogeneous group of clonal disorders that originate from stem cells and affect myeloid lineage. According to the WHO classification, MPN is classified into three main disorders including polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF).[1,2] Besides laboratory and clinical observation, diagnosis of MPN disorders is mainly dependent on the genetic study of a group of driver mutations. It has been shown that about 90% of MPN disorders are characterized by the presence of mutations in at least one of three genes including Janus Kinase 2 (JAK2), calreticulin (CALR), and myeloproliferative leukemia proto-oncogene.[3]

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In particular, PV is characterized by uncontrolled overproduction of myeloid linage mainly red blood cells (erythrocytosis) as a hallmark feature.[1] The major advance in the diagnosis of MPN, especially PV was achieved in 2005 by the discovery of the JAK2V617F mutation in exon 14 of the JAK2 gene.[4] It has been shown that about 95% of PV patients harbor the JAK2V617F mutation.[2,5,6] Therefore, JAK2V617F has been included as one of the major criteria for PV diagnosis according to the WHO classification of MPN disorders.[1,2]

JAK2V617F is a gain-of-function mutation that involves guanine-to-thymine substitution at position 1849, leading to the replacement of valine for phenylalanine.[4,6] This leads to tyrosine kinase-mediated overactivation of the JAK/STAT signaling pathway, causing abnormal production of blood cells.[5]

Since 2007, many mutations in exon 12 of JAK2 gene have been reported in JAK2V617F-negative PV patients.[7,8] It has been reported that 3% of PV patients have mutations at exon 12 of the JAK2 gene.[9,10] While the JAK2V617F mutation is not restricted to PV patients and is found in other MPN entities, the JAK2 exon 12 mutations have been reported in PV patients only. Another difference between JAK2V617F and JAK2 exon 12 mutant PV patients is that the former might show simultaneous overproduction of leukocytes and platelets (PLTs), while the latter patients show only erythrocytosis.[11,12] The identification of JAK2 exon 12 mutations is more difficult than JAK2V617F mutation because the former is heterogeneous and occurs in different forms (more than 40 different mutations characterized in literature so far). In addition, JAK2 exon 12 mutations exist in low allele burden in PV patients compared with JAK2V617F mutation.[9,13,14] For these reasons, screening for JAK2 exon 12 in the routine diagnostic algorithm for PV is limited and hence, there is so limited data about the incidence and related clinical outcome of this mutation. For these reasons, the current study was performed to address the frequency and characteristics of patients with JAK2 exon 12 mutations.

Patients, Materials, and Methods

This is a retrospective study enrolled patients who consulted the National Center of Hematology (NCH), Mustansiriyah University in Baghdad, from December 2019 to January 2022. Inclusion criteria for patients are shown in Table 1, while those excluded from the study either met WHO criteria of PV by confirmation of JAK2V617F mutation as shown in Table 2 or had a diagnosis of ET or PMF or had incomplete patient information. This study followed the guidelines and regulations of the 1964 Helsinki Declaration and its later amendments and was approved by the committee of research ethics at NCH (reference: Nch-erc-19-11).

Table 1: Inclusion criteria for enrolled patients in the study

Inclusion criteria
Splenomegaly
Unexplained erythrocytosis
Hb ≥16.0 g/dL or HCT ≥48%
Patient admission between December 2019 and January 2022

Hb=Hemoglobin, HCT=Hematocrit

Table 2: World Health Organization criteria for diagnosis of polycythemia vera

Major criteria	Minor criteria	Diagnosis
Hb level >16.5 g/dL (male), 16 g/dL (female) and/or HCT >49% (male), >48% (female)	Low erythropoietin levels	Meet 3 of major criteria or 2 major + minor criteria
Morphological abnormality of bone marrow		
Presence of JAK2V617F or JAK2 exon 12 mutations		

Table was adopted from reference number 1. Hb=Hemoglobin, HCT=Hematocrit, JAK2=Janus kinase 2

Being a retrospective study, patients' confidentiality and privacy were strictly maintained throughout data collection and analysis. Patient's data including clinical and laboratory parameters (age, body mass index, hemoglobin (Hb), hematocrit (HCT), white blood cell (WBC), PLT, Chest X-ray, abdominal ultrasound, and other demographic data) were retrospectively analyzed. JAK2V617F mutation was screened in all patients. Cases who met inclusion criteria [Table 1] were tested for JAK2 exon 12 mutations.

DNA extraction

DNA was extracted from blood samples using either ReliaPrep blood gDNA extraction kit (Promega, USA, cat. No. A5081) or EasyPure blood genomic DNA kit (Transgenbiotech, China, cat. No. EE121) as described by the manufacturer's instruction. DNA samples were then immediately kept at −20°C until use.

Janus kinase 2V617F mutation screening

JAK2V617F mutation screening in PV-suspected cases was performed by allele-specific polymerase chain reaction (AS-PCR) method as previously described.[15] In this method, four primers were used yielding three amplicons: 463 bp (internal control), 279 bp (mutant allele), and 229 bp (wild allele). Primers sequence is 5`-TCCTCAGAACGTTGATGGCAG-3` as (forward outer), 5`-ATTGCTTTCCTTTTTCACAAGAT-3` as (reverse outer),5`-GCATTTGGTTTTAAATTATGGAGTATATG-3` as (forward wild-specific), and 5`-TCCTCAGAACGTTGATGGCAG-3` as (reverse-mutant-specific). PCR amplification was performed using C1000 thermal cycler (Bio-Rad, USA). The following protocol was used for the PCR reaction: (1) 94°C for

5 min (initial denaturation), (2) 38 cycles of 94°C for 30 s (denaturation), 56°C for 30 s (annealing), and then 72°C for 30 s (extension), and (3) 72°C for 5 min (final extension). PCR products were then analyzed by 2% agarose gel electrophoresis.

Bidirectional sequencing analysis of Janus kinase 2 exon 12

To detect JAK2 exon 12 mutations in patient samples, exon 12 of the gene was first amplified by PCR using primers previously designed and used.[11] The primers 5'-CTCCTCTTTGGAGCAATTCA-3' (forward sequence) and 5'-GAGAACTTGGGAGTTGCGATA-3' (reverse sequence) amplify a region of 496 base pairs containing the 128 bp Jak2 exon 12 sequence. Primers were constructed and ordered from Alpha ADN, Montreal, Canada. PCR amplification was performed by thermal cycler C1000 thermal cycler (Bio-Rad, USA) using the following program: (1) 94°C for 5 min (initial denaturation), (2) 36 cycles of 94°C for 30 s (denaturation), 60°C for 30 s (annealing), and then 72°C for 30 s (extension), and (3) 72°C for 10 min (final extension). PCR products were confirmed by visualization on 2.5% agarose gel electrophoresis stained with RedSafe stain (iNtRON Biotechnology, Korea).

Samples were then sent to (Macrogen, Korea) for Sanger sequencing. Sequencing was performed using the Big-Dye terminator cycle sequencing kit (Applied Biosystems, USA) on the ABI 3730XL DNA analyzer (Applied Biosystems, USA) using the above-described primers.

The resulting sequences were compared with GeneBank sequence databases (website: http://www.ncbi.nlm.nih.gov/genebank) using BioEdit Sequence Viewer software (website: http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Statistical analysis

GraphPad Prism version 10.0.0 for Windows, (GraphPad Software, Boston, Massachusetts USA) was used for statistical analysis.

Results

The total number of patients who were referred to the NCH for the suspicion of PV disorder from December 2019 to January 2022 was 323 patients. Those patients were screened for the presence of JAK2V617F mutation. An example of samples screened by AS-PCR as described in the method section is shown in Figure 1. Among the samples screened, 239 (74%) were negative and 84 (26%) were positive.

AS-PCR was used for JAK2V617F screening then analyzed by (2%) agarose gel electrophoresis and visualized by DNA staining with Red safe stain. Lane numbers 1 and 4 are positive samples and lane numbers 2, 3, 5, 6, and 7 are negative.

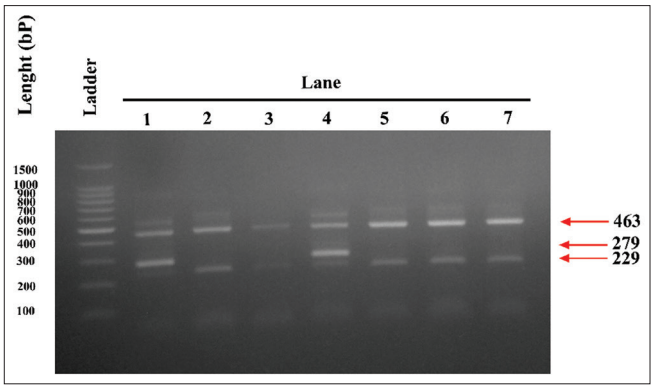

Figure 1: JAK2V617F screening by allele-specific polymerase chain reaction

Of total JAK2V617F-negative cases, there were 137 (57.4%) patients who identified with secondary conditions (e.g., inflammation, anemia, surgery, or smoking) which were confirmed by further detailed history and proper investigations. The total number of patients who met the inclusion criteria as described in Table 1 was 102 cases (42.6%). Demographic and laboratory parameters of those patients are shown in Table 3. The median age was 35 years. Those patients were manifested with high Hb and HCT levels and normal leukocyte and PLT counts. Among the total cases, 21 cases (20.59%) showed splenomegaly at the time of presentation. The frequency of JAK2 exon 12 mutations among patients with negative JAK2V617F mutation in this study was approximately 1%.

DNA samples of these cases were sequenced using primers spanning the entire exon 12 of the JAK2 gene. Among the total samples analyzed, only one case showed JAK2 exon 12 mutations (E543-D544del). This mutation involved heterozygous deletion of six nucleotides (code for glutamic acid and aspartic acid) [Figure 2].

Genetic information about this mutation is summarized in Table 4. The mutated case was a 35-year-old female. Hematological parameters of this patient manifested overcrowded red cells, mild leukocytosis, and thrombocytosis (Hb = 15.9, HCT = 52, WBC = 11.2, and PLT = 764). Bone marrow examination showed hypercellular fragments and traits. The absence of CALR mutation in this patient was also confirmed. Consequently, the diagnosis of this patient with PV was confirmed.

Samples were tested for JAK2 exon 12 mutations as described in the method section. The upper panel shows the chromatogram of normal cases and patients with JAK2 exon 12 mutations. The lower panel shows the

Table 3: Demographic and laboratory parameters of JAK2V617F-negative patients enrolled in the study

Characteristics	Analyzed patients (*n*=102)
Age at onset, median (range)	35 (10–70)
Sex, *n* (%)	
Male	92 (90.2)
Female	10 (9.8)
BMI, mean±SD	28.45±5.7
Smoking, *n* (%)	
Yes	11 (10.78)
No	91 (89.22)
Splenomegaly	
Yes	21 (20.59)
No	81 (79.41)
HB level (g/dL), median (range)	17.2 (14.3–20.8)
HCT level (%), median (range)	50.9 (8.14–61.8)
WBCs count (×10³/μL), median (range)	7.9 (4.3–64.4)
PLTs count (×10³/μL), median (range)	236 (51.6–1890)

Hb=Hemoglobin, HCT=Hematocrit, BMI=Body mass index, WBCs=White blood cells, SD=Standard deviation, PLTs=Platelets

Table 4: Genetic characteristics of Janus Kinase 2 exon 12 mutations

Genetic character	Details
Transcript	NM_004972.4
gDNA	Chr9: g. 5070038_5070043del GAAGAT
cDNA	c. 1627_1632del GAAGAT
Protein	p.E543_D544delED
Source database	GeneBank (accession number NG_009904.1)
Reference genome	GRCh/hg38

gDNA=Genomic DNA, cDNA=Complementary DNA

sequence of both wild and JAK2 exon 12 mutants with the deletion of 6 nucleotides (code for glutamic acid and aspartic acid).

Discussion

In the current study, patients suspected with PV were screened for JAK2V617F mutation using AS-PCR. Although the majority of PV patients harbor a high incidence of JAK2V617F mutation which reaches up to 95% of cases, the small proportion <5% of patients with negative JAK2V617F mutation need further testing for JAK2 exon 12 to rule out PV, especially with those patients presented with erythrocytosis as some mechanism suggest that it upregulate erythropoietin signaling.[16] It might be explained also by the differential coupling of mutant JAK2 with cytokine receptors in which exon 12 mutations can result in powerful ligand-independent signaling through JAK2 and generate higher levels of JAK2, ERK1, and ERK2 phosphorylation than does the V617F mutation.[11]

The entire exon 12 of the JAK2 gene of 102 patients was sequenced using bidirectional Sanger sequencing. Only one case was JAK2 exon 12 mutated was detected which represents about 1% of JAK2V617F-negative

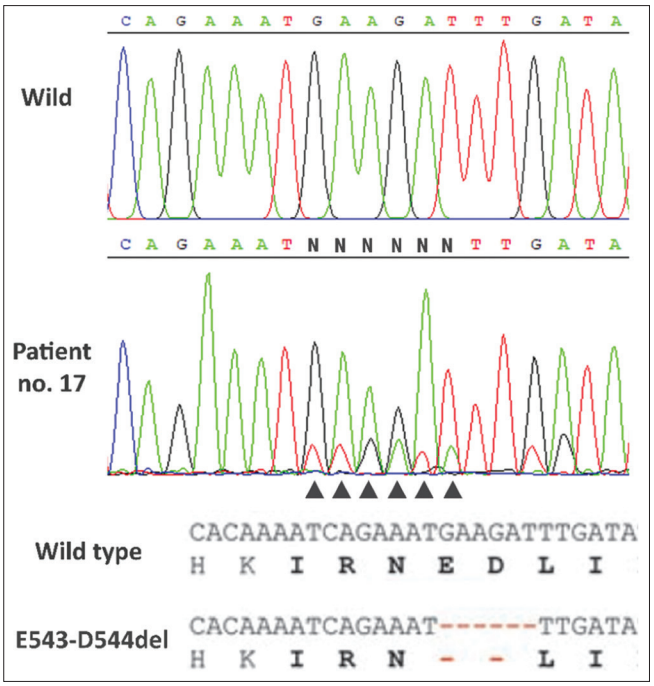

Figure 2: JAK2 exon12 screening by Sanger sequencing

cases. This finding was also confirmed using quantitative PCR analysis. This case was initially referred due to suspicion of PV, her bone marrow examination showed hypercellular fragments and traits. Despite the low frequency, the type of exon 12 mutations detected in our study (E543-D544del) was consistent with findings from other studies.[13] There is a wide range of variability in regard to the frequency of JAK2 exon 12, for example, some studies including the current study reported a low frequency of JAK2 exon 12 among JAK2V617F-negative patients in addition to similar findings as reported zero percent by Zhang *et al*.,[17] (3.7%) by Schnittger *et al*.,[7] and (12%) by Park *et al*.,[18] or it may be high frequency as reported to reach (40%) by Akram *et al*.,[11,19] and even (50%).[9] The variability of the above results is due to a number of reasons one of these is the number of patients enrolled in each study another reason might be the demographic allele distribution in the affected gene, but this cannot be confirmed because of the very limited data about the incidence of JAK2 exon 12 in Iraqi patients. To the best of our knowledge, no previous study reported JAK2 exon 12 incidences in JAK2V617F-negative patients in the Iraqi population. Various frequencies reported might also be due to variability in diagnosis procedures followed. An important reason for variability in literature is mutation screening technique. There is a wide range of methods used in this regard. These include quantitative PCR followed by high-resolution melting-curve analysis (HRM),[7] Sanger sequencing,[18,19] allele-specific PCR followed by sequencing or genotyping by digestion with *Ase*I,[11] or direct sequencing and cloning method.[9] Detection sensitivity of these methods ranges

from >2% (allele-specific PCR) to 5% (PCR with HRM), and 10% (Sanger sequencing).[20] Due to low allele burden of JAK2 exon 12 mutations as previously mentioned,[14] sensitivity of the detection method is important in this regard. Other reasons such as the presence of mutations other than JAK2V617F and exon 12 as well as a low proportion of exon 12 mutated myeloid cells in peripheral blood are also possible and have been mentioned in the literature.[9]

In our study, the mutation detected involved heterozygous deletion of amino acid (glutamic acid and aspartic acid) residues at position 543-544 (E543-D544del) [Figure 2]. This agrees with previous studies reported that JAK2 exon 12 mutations are clustered within the 537–544 codon region.[10,11,21] This mutation was reported in previous studies. For example, in one study, six cases were E543-D544del-mutated and all of them were confirmed PV.[10] Another study reported 2 E543-D544del-mutated cases; one of them was familial.[7] This mutation was also detected in one patient with idiopathic erythrocytosis.[22] Unfortunately, due to only one PV case was confirmed with JAK2 exon 12 mutations in the current study, we could not compare laboratory and clinical parameters of JAK2 exon 12-mutated with JAK2V617F-mutated PV cases.

Conclusion

Despite PV patients with JAK2 exon 12 mutations represent a small proportion of total PV cases, JAK2 exon 12 mutations should be routinely screened in JAK2V617F-negative suspected PV patients. Furthermore, using different methods with high detection sensitivity is highly recommended for genetic diagnosis of PV patients.

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Conflicts of interest
There are no conflicts of interest.

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