ORIGINAL ARTICLE

Prevalence of *JAK2 V617F*, *CALR*, and *MPL W515L* Gene Mutations in Patients with Essential Thrombocythemia in Kurdistan Region of Iraq

Bestoon Muhammad Saeed¹, Hisham Arif Getta², Najmaddin Khoshnaw¹, Goran Abdulqader², Aveen M. Raouf Abdulqader², Ali Ibrahim Mohammed²¹Department of Hematology, Hiwa Hemato-Oncology Center, Sulaymaniyah, Iraq²Department of Pathology, College of Medicine, University of Sulaymaniyah, Sulaymaniyah, Iraq

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ABSTRACT

Essential thrombocythemia (ET) is a clonal bone marrow stem cell disorder, primarily involving the megakaryocytic lineage. The WHO 2016 guidelines include the molecular detection of *JAK2*, *MPL*, and *CALR* mutations as a major diagnostic criterion for ET. This study aimed to determine the frequency of *JAK2 V617F*, *MPL W515L*, and *CALR* mutations in Iraqi Kurdish patients afflicted with ET, and to analyze their clinical and hematological features. A total of 73 Iraqi Kurdish patients with ET were enrolled as subjects, and analysis was achieved utilizing real-time PCR. The frequency of *JAK2 V617F*, *CALR*, and *MPL W515L* mutations was determined to be 50.7%, 22%, and 16.4%, respectively. No statistically significant difference was obtained when considering the age and gender among different genotypes. The *JAK2 V617F* mutated patients had significantly higher white blood cell counts and hemoglobin levels than the *CALR*-positive patients (P -value=0.000, 0.007, respectively), *MPL W515L*-positive patients (P -value=0.000, 0.000, respectively), and triple negative patients (P -value=0.000, 0.000, respectively). Also, the *JAK2 V617F* mutated patients showed higher platelet count as compared to the *MPL W515L*-positive patients (P -value=0.02) and triple negative patients (P -value=0.04). Furthermore, significantly lower white blood cell count and hemoglobin levels were associated with *CALR* positivity (P -value=0.000, 0.01, respectively), *MPL W515L*-positivity (P -value=0.001, 0.000, respectively), and triple negativity (P -value=0.000, 0.000, respectively), as compared to patients with combined mutations. In conclusion, apart from a relatively high frequency of *MPL W515L* mutation, our data is comparable to earlier reports, and highlights the importance of genotyping the *JAK2 V617F*, *MPL W515L*, and *CALR* mutations for accurate diagnosis of patients with ET.

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INTRODUCTION

Essential thrombocythemia (ET) is an acquired chronic myeloproliferative neoplasm (MPN) that basically involves the megakaryocytic lineage. It is characterized by constant elevation of peripheral blood platelet count and increased numbers of large, mature megakaryocytes in the bone marrow and clinically by the tendency to thrombosis or hemorrhage [1]. To diagnose patients with ET, other reactive causes of high platelet count must be excluded such as inflammatory or infectious diseases, hemorrhage, and other MPNs. However, a major insight into the genetic lesions for the enhanced myeloproliferation and clonal dominance that characterizes the Philadelphia-negative MPNs including polycythemia vera (PV), primary myelofibrosis (PMF), and ET has been reported [2]. Mutations that disrupt the protein tyrosine kinase signaling are found

Corresponding author: Aveen M. Raouf Abdulqader
Department of Pathology, College of Medicine, University of Sulaymaniyah,
Franso-Meteran Street, Sulaymaniyah 46001, Iraq
E-mail: aveenm80@yahoo.com
ORCID: <https://orcid.org/0000-0002-4301-6470>

to be associated with these disorders [3]. The Janus Kinase 2 gene (*JAK2*) *V617F* mutation was first discovered in MPN patients in 2005 [4]. *JAK2* is a cytoplasmic tyrosine kinase with a central role in signal transduction from multiple hemopoietic growth factor receptors [4]. *JAK2 V617F* is an acquired gain-of-function mutation in exon 14 of the gene, causing valine to phenylalanine substitutions at position 617 located in the JH2 pseudokinase domain, interrupts the domains autoinhibitory effect and results in constitutive tyrosine phosphorylation activity and enhances downstream JAK2-STAT signaling pathway leading to increase cellular proliferation [4]. This point mutation is frequently detected in the three clinically distinct MPN, being identified in ~95% in PV, ~50~60% in ET, and ~50~60% in PMF [1, 3, 5]. Furthermore, 5~10% of patients with ET carry a somatic activating mutation at exon 10, codon 515 of myeloproliferative leukemia (*MPL*) virus oncogene encoding the thrombopoietin receptor that is essential for megakaryogenesis, platelet production, and hematopoietic stem cell homeostasis [6-9]. The diagnosis of patients with findings suggestive of ET but negative for *JAK2* and *MPL* mutations, usually based on the clinical exclusion of reactive thrombocytosis and morphological changes in megakaryocytes in the bone marrow. Histopathological assessment is inherently vulnerable to inter-observer variation, needs an experienced hematopathologist, and lacks standardization. Therefore, ET patients negative for *JAK2 V617F* and *MPL* mutations have been found to carry novel mutations of the calreticulin gene (*CALR*) [7]. *CALR* is a functionally complex Ca^{2+} -binding protein and plays a substantial role in a variety of biological systems including calcium dynamic equilibrium regulation, cell proliferation, differentiation, and apoptosis [10]. The *CALR* mutations located in exon 9 are somatic insertions or deletions. There are two major variants: type 1 (L367fs*46), resulting from a 52-bp deletion, and type 2 (K385fs*47), from a 5-bp (TTGTC) insertion [11]. This mutation has been reported in 25~30% of cases of ET [12]. The aim of this study was to determine the

prevalence, and clinical correlations of these three driver mutations *JAK2 V617F*, *MPL W515L*, and *CALR* in Iraqi Kurdish patients with ET.

MATERIALS AND METHODS

1. Patients and samples

This cross-sectional study was conducted following approval by the local institutional ethical committee (approval No. 59/6.2019). Informed consent was obtained from all enrolled subjects. A total of 73 patients diagnosed with ET at Hiwa Hemato-Oncology Hospital, Sulaymaniyah Province, Kurdistan Region of Iraq were included in this study between June to November 2019. The patients were identified as ET based on the 2016 WHO diagnostic criteria. Briefly, the patients were diagnosed as ET when they had unexplained thrombocytosis, megakaryocytic proliferation, and did not meet WHO criteria for other MPNs. Bone marrow aspiration and biopsy was performed for all patients through posterior iliac spine and the aspirate smears were stained with Leishman stain (Merck/ Darmstadt, Germany). A Trephine biopsy specimen of 1.5 cm in length placed in 10% buffered neutral formalin and sent to histopathology laboratory. Following overnight fixation, biopsy specimens were slowly decalcified with ethylene diamine tetra acetate (EDTA). Three micrometer thick sections were cut from the paraffin wax blocks with the rotary microtomes and stained with H&E. Figure 1 through 4 show the characteristic megakaryocyte in patients with ET. Clinical and hematological information were collected by reviewing the medical records. Genomic DNA for the analysis of the three driver mutations was isolated from peripheral blood samples using a fully automated magnetic bead nucleic acid extraction system (Zinexts, Taiwan). The Zinexts DNA extraction kit was used for the separation of genomic DNA by using 400 μ L of whole blood with a final 100 μ L DNA elute according to the manufacturer's instruction. The isolated DNA was stored at -20°C until the time of analysis by real-time PCR with

Figure 1. Characteristic morphology of bone marrow aspirate of a patient with ET (×40).

Figure 3. Bone marrow biopsy section in a patient with ET (×10).

Figure 2. Characteristic Megakaryocyte morphology in bone marrow aspirate of a patient with ET (×100).

Figure 4. Higher power bone marrow biopsy section in a patient with ET (×40).

LineGene9600PLUS (Bioer, China). Five different fluorescence dyes attached to the DNA probe running in four different channels were used to detect wild type alleles, mutant type alleles for the three examined genes, and the internal control in each patient. For the *JAK2 V617F* mutation Hex-BHQ dye was used, for the *MPL W515L* mutation Cy5-BHQ was used, for the *CALR* mutations Texas Red-BHQ and FAM-BHQ were used, while for the internal control Cy5.5-BHQ was used. The

extracted DNA samples were quality and quantity checked by the spectrophotometer (Eppendorf Bio Photometer, Germany).

2. Mutation analysis

Newly designed primers were used in the current study. All 73 patients were examined for *JAK2 V617F*, *MPL W515L*, and *CALR* [type 1 (52-bp deletion) and type 2 (5-bp insertion)] mutations. All genomic DNA was amplified in a 40-cycle PCR reaction at an annealing

temperature of 59°C, 58°C, and 60°C for *JAK2 V617F*, *MPL W515L*, and *CALR* mutations, respectively as shown in Table 1. The specific primers and probes used to detect the corresponding mutations are shown in Tables 2~4. The internal control template, probe, and primer were designed on the Homo sapiens ribonuclease P/MRP subunit p30 (RPP30), transcript variant 2, mRNA

as shown in Table 5. Amplification was carried out in a 20 µL final volume containing 25~50 ng of genomic DNA in 1× TaqMan universal PCR master mix, 1× internal control mix, and 0.2 µM of each corresponding forward primers, reverse primers and probes. Furthermore, samples from positive (obtained from Tehran Medical Genetic Laboratory, Iran) and negative controls for each driver mutation were included in the experiment.

Table 1. PCR program for the detection of *JAK2 V617F*, *MPL W515L*, and *CALR* type 1 & 2 mutations

PCR steps	Temperature (°C)	Time	Cycle
Pre-heating	50	2 minutes	1
Pre-denaturation	95	10 minutes	1
Denaturation	95	25 seconds	40
Annealing, elongation and detection	59 for <i>JAK2 V617F</i> 58 for <i>MPL W515L</i> 60 for <i>CALR</i> type 1 & 2	25 seconds 30 seconds 20 seconds	

3. Statistical analysis

The statistical package SPSS version 25 (IBM Corp., Armonk, NY, USA) was used to analyze the data: The continuous data are summarized as the means (SD) and ranges. The Chi-Square test was applied to compare the differences in the categorical data. An independent

Table 2. Sequences of oligonucleotide primers and probe for the detection of *JAK2 V617F* mutation

Primer	Direction	Length	Sequence	Modification
JAK2-F	Forward	24 bp	5'- CTTTCTTTGAAGCAGCAAGTATGA -3'	-
JAK2-WR	Reverse wild type allele	30 bp	5' -GTAGTTTTACTTACTCTCGTCTCCACATAC -3'	-
JAK2-MR	Reverse mutant type allele	30 bp	5' - GTAGTTTTACTTACTCTCGTCTCCACATAA -3'	-
JAK2 Probe	Probe	30 bp	5'-TGAGCAAGCTTTCTCAAGCATTGTGTTT-3'	Hex-BHQ

Table 3. Sequences of oligonucleotide primers and probe for the detection of *MPL W515L* mutation

Primer	Direction	Length	Sequence	Modification
MPL-F	Forward mutant type allele	20 bp	5'- TGGTGACCGCTCTGCATCTA -3'	-
MPL-R	Reverse mutant type allele	16 bp	5' - AGTGTGCAGGAAACTG -3'	-
MPL Probe	Probe	18 bp	5'- CTGGGCTCAGCGCCGTC -3'	Cy5-BHQ

Table 4. Sequences of oligonucleotide primers and probe for the detection of *CALR* type 1 & 2 mutations

Primer	Direction	Length	Sequence	Modification
CARL-F	Forward primer	21 bp	5'- TGGTCCTGGTCTGATGTCGG -3'	-
CARL-R	Reverse primer	21 bp	5' - CCCAAATCCGAACCAGCCTGG -3'	-
CARL-M1 probe	Deletion mutant probe	22 bp	5' - CGAGGAGCAGAGACAAGGAGG -3'	FAM-BHQ
CARL-M2 probe	Insertion mutant probe	25 bp	5'- TTGTCGGAGGAAGATGAGGAGGAAG-3'	TexasRed-BHQ

Table 5. Sequences of internal control (template, probe and primer)

Primer	Direction	Length	Sequence	Modification
IC-F	Forward	21 bp	5'- CCA AGT GTG AGG GCT GAA AAG -3'	-
IC-R	Reverse	26 bp	5' -TGT TGT GGC TGA TGA ACT ATA AAA GG -3'	-
IC-Probe	Probe	26 bp	5' - CCC CAG TCT CTG TCA GCA CTC CCT TC -3'	Cy5.5-BHQ
IC-Template	DNA Template	80 bp	5'-CCA AGT GTG AGG GCT GAA AAG AAT GCC CCA GTC TCT GTC AGC ACT CCC TTC CCT TTT ATA GTT CAT CAG CCA CAA CA-3'	-

t-test was used for differences in continuous data. *P* values <0.05 were regarded as statistically significant.

RESULTS

As demonstrated in Table 6, out of 73 patients with ET, 37 (50.7%) patients carried *JAK2 V617F* mutation, 10 (13.7%) had *CALR* type 1 and 2 mutations, while 7 (9.6%) patients had *MPL W515L* mutation. Nineteen (26%) of the patients showed negative results for the three driver mutations (triple negative). Further, 10 patients carried more than one driver mutation, five patients had combined *JAK2 V617F*+ *CALR*, four cases had combined *JAK2 V617F*+ *MPL W515L*, and only one patient exhibit triple positivity for the three mutations. Age, gender, and main hematological features of the 73 patients with ET are shown in Table 7. The correlation

of the patients' main clinical and hematological characteristics with their mutational status are reported in Table 8. Concerning age and gender, in the current study, there was no statistically significant difference between different genotypes. Patients with mutated *JAK2* were associated with higher white blood cell count and hemoglobin level compared to patients with mutated *CALR*, mutated *MPL*, and triple-negative patients. Further, patients with mutated *CALR* and patients with mutated *MPL* were associated with significantly lower white blood cell count and hemoglobin level compared to patients with combined mutations. Also, triple-negative patients showed lower

Table 6. Frequency of the three driver mutations in 73 patients with ET

Driver Mutations	No.	%
<i>JAK2 V617F</i> ^a	37	50.7%
<i>CALR</i> type 1 & 2	10	13.7%
<i>MPL W515L</i>	7	9.6%
Triple negative	19	26.0%
Total	73	100.0%

^aIncluding the combined cases: *JAK2 V617F*+ *CALR* Type 1 & 2= 5 cases, *JAK2 V617F*+ *MPL W515L*=4 cases, *JAK2 V617F*+ *CALR* Type 1 & 2+ *MPL W515L*=1 case.

Table 7. Main clinical and hematological features of the 73 patients with ET

Parameters	Value
Gender No. (%)	
Male	37 (50.7)
Female	36 (49.3)
Age (years)	
Mean (SD)	55 (17)
Range	12~89
Leukocytes ($\times 10^9/L$)	
Mean (SD)	9.5 (3.2)
Range	4.0~15.3
Hemoglobin (g/dL)	
Mean (SD)	13.2 (1.3)
Range	11.0~15.6
Platelets ($\times 10^9/L$)	
Mean (SD)	949 (387)
Range	443~1900

Table 8. Correlation of the three mutational status with the main clinical and hematological characteristics in 73 Iraqi Kurdish patients with ET

Characteristics	<i>JAK2</i> 1	<i>CALR</i> 2	<i>MPL</i> 3	Com. Mut 4	Trip-ve 5	<i>P</i> 1 v 2	<i>P</i> 1 v 3	<i>P</i> 1 v 4	<i>P</i> 1 v 5	<i>P</i> 2 v 3	<i>P</i> 2 v 4	<i>P</i> 2 v 5	<i>P</i> 3 v 4	<i>P</i> 3 v 5	<i>P</i> 4 v 5
No. of Pt	27	10	7	10	19										
Sex M/F	14/13	7/3	2/5	7/3	7/12	0.32	0.27	0.32	0.31	0.09	1.00	0.09	0.09	0.67	0.08
Age, years (range)	56 (27~89)	61 (36~80)	53.5 (12~70)	59 (40~72)	48.1 (18~79)	0.09	0.76	0.63	0.15	0.39	0.72	0.06	0.51	0.51	0.09
WBC, $\times 10^9/L$ (range)	12 (8.0~15.3)	6.6 (4.0~10.0)	7.5 (5.0~10.0)	12 (7.0~15.0)	6.8 (5.0~11.2)	0.000	0.000	0.99	0.000	0.41	0.000	0.81	0.001	0.39	0.000
Hb, g/dL (range)	14.2 (12.0~15.6)	12.8 (11.0~15.0)	11.7 (11.0~13.0)	14.07 (13.0~15.0)	12.1 (11.0~14.0)	0.007	0.000	0.75	0.000	0.09	0.01	0.13	0.000	0.28	0.000
Platelets, $\times 10^9/L$ (range)	1084 (600~1820)	867 (550~1271)	696 (464~1357)	1049 (600~1900)	841 (443~1723)	0.11	0.02	0.81	0.04	0.23	0.22	0.86	0.06	0.40	0.19

Abbreviations: Pt, Patients; Com Mut, combined mutations; Trip -ve, triple-negative; v, versus.

white blood cell count and hemoglobin levels in comparison to patients with combined mutations. Concerning the platelet count, apart from patients with mutated *JAK2* that were associated with higher platelet count as to patients with mutated *MPL* and triple-negative patients, there was no statistically significant difference between the other genotypes.

DISCUSSION

This is the first study to characterize the genotype profile of Iraqi Kurdish patients with ET. Using real-time PCR with specific primers and probes for the three driver mutations, we found that 54/73 (74%) of the ET patients carried *JAK2 V617F*, *CALR*, or *MPL W515L* mutations, highlighting the value of combined genetic testing for the diagnosis of ET patients. The *JAK2 V617F* was the most common mutation, being detected as a single driver mutation or in combination with the other two mutations in 37/73 patients (50.7%). Although *CALR* and *MPL W515L* mutations were detected (as single mutation) in only 10/73 (13.7%) and (7/73) 9.6%, respectively, taking into consideration the double mutational positivity and the triple mutational positivity, the *CALR* and *MPL W515L* mutations were identified in 16/73 (22%) and 12/73 (16.4%), respectively. The frequency of *JAK2 V617F* mutation in our study is in agreement with previous reports from China (57.79%) [13], (56.3) [14], Korea (57.1%) [15], and Italy (57%) [5]. While other studies reported a higher frequency such as studies from Argentine (61.2%) [16], India (92.3%) [17], and Korea (63.3%) [8]. Also, some researchers observed a lower frequency of *JAK2 V617F* mutation (31%) [18] and (35.7%) [19]. In the current series, the frequency of *CALR* (22%) is consistent with earlier reports [15, 20–24] but, higher than previous reports from Korea (17.7 %) [8], and Italy (14%) [25]. In concern to *MPL W515L* mutation, our observation (16.4%) is higher than earlier records that ranged from 1% to 14% [18, 25–30]. This variation in the frequency of the driver mutation could be related to different characteristics of the studies

cases including sample size, disparate sensitivities of the methods used, and ethnicity-based diversity in the genetic backgrounds.

In the current study, no statistically significant difference in age and gender was observed among different genotypes. This finding is in agreement with earlier reports [4, 5, 7, 8, 10, 16, 20]. Similar to previous reports, patients with mutated *JAK2 V617F* showed significantly higher hemoglobin levels, white blood cell count, and platelet count compared to patients with other driver mutations and triple negative patients [6–8, 10, 16, 20]. Further, patients with mutated *CALR*, patients with mutated *MPL W515L*, and triple negative patients had lower hemoglobin levels and white blood cell count compared to patients with combined mutations comparable to earlier records [7, 16].

In conclusion, our data reveal that the mutation profile in ET patients in our locality is in line with earlier reports. Further our findings demonstrate similarities with these records in concern to hematological features of patients with ET. However, we notice a rather higher frequency of *MPL W515L* mutation in our cohort. Lastly, our study accentuates the influence of *JAK2 V617F*, *MPL W515L*, and *CALR* genotyping for a precise diagnosis of ET patients because, in certain cases, the determination of disease-specific mutations may justify the use of mutation analysis for concluding the diagnosis, prognosis, and assessment of response to treatment.

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Conflict of interest: None

Author's information (Position): Saeed BM¹, Researcher; Getta HA², M.D.; Khoshnaw N¹, M.D.; Abdulqader G², M.D.; Abdulqader AMR², M.D.; Mohammed AI², M.D.

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