Molecular Screening of Exon 12 of Janus Kinase 2, Exon 9 of Calreticulin Genes in Polycythemia Vera Patients with Unmutated Janus Kinase 2 V617F

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Sofi *et al.*: Molecular Screening of Exon 12 of Janus Kinase 2 and Exon 9 of Calreticulin Genes

The calreticulin gene has nine exons and most of the frequent mutations screened and observed in the 9th exon in myeloproliferative neoplasm patients, especially essential thrombocythemia and primary myelofibrosis. In the current study, an uncommon calreticulin mutation was observed in a 51 y old man with a polycythemia vera phenotype. Somatic novel homozygous mutations affect exon 9 of the calreticulin gene. These recent genetic variations were not reported in previous studies. Molecular screening of calreticulin may be important for polycythemia vera patients that are negative for the Janus kinase 2 V617F and exon 12 Janus kinase 2. A total of 11 negative Janus kinase 2 V617F polycythemia vera patients were previously diagnosed by the amplification refractory mutation system-polymerase chain reaction technique. Sanger sequencing was performed to screen exon 12 of the Janus kinase 2 gene and the last exon of the calreticulin gene. In addition, all 11 negative V617F patients were wild for exon 12 of the Janus kinase 2 gene. The calreticulin lesion in myeloproliferative neoplasms as a second biomarker, particularly in essential thrombocythemia and primary myelofibrosis, is more informative for diagnosis. Molecularly, in negative Janus kinase 2 V617F and exon 12 patients with polycythemia vera phenotypes, genotyping calreticulin may be useful.

Key words: Myeloproliferative neoplasms, calreticulin, exon 12, Janus kinase 2

The Philadelphia-negative Myeloproliferative Neoplasms (MPNs) are defined by the clonal proliferation of an aberrant hematopoietic stem/progenitor cell involving Polycythemia Vera (PV), Essential Thrombocythemia (ET) and Myelofibrosis (MF)[1]. In general, more than 95 % of PV cases carried with Janus Kinase 2 (JAK2) V617F mutation at nucleotide position c.1849G>T in 617th amino acid results substitution of a valine for a phenylalanine. While mutations in exon 12 of the JAK2 gene were heterogeneous and variable, including substitutions, deletions and duplications, they were approximately found in 3 % of PV patients without JAK2 V617F[2,3]. In addition, incidence of JAK2 V617F ranges from 51 %-55 % in ET and 50 %-65 % in Primary Myelofibrosis (PMF)[2,4]. Clinically, PV patients had a higher level of hemoglobin and hematocrit. In contrast, thrombocytosis was significantly increased in ET than in PV and PMF patients, which acted as a risk factor for the occurrence of thrombosis[5]. Annual incidence of PV and ET were

relatively similar 1.0-2.0 per 100 000 people while, frequency of PMF was rare 0.3 per 100 000 people[6]. Moreover, life expectancy was low in PV as found in ET, 15 to 18 y, respectively[7]. The diagnosis of V617F mutation in JAK2 greatly advanced our understanding of the biology of these disorders[8]. Molecularly, mutations in the JAK2 gene are mostly associated with PV in all cases. In contrast, ET can be driven either by JAK2, Calreticulin (CALR) or Myeloproliferative Leukemia (MPL)-mutated, as well as triple negative[9]. In the 32 various types of CALR mutations, a 52 base pair (bp) deletion called "type 1" and a 5 bp insertion called type 2 mutation, were the most frequent variants identified in negative JAK2 V617F MPNs. All of them conferred frameshift and produced unique c-terminus[10]. In multigene screening among 27 relevant genes, CALR was considered the second-most prevalent target gene in ET, while in PV patients it was not identified[11]. Normally, wild-type CALR participates in hematopoiesis, including megakaryocytic/erythrocytic differentiation

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and Haematopoietic Stem Cells (HSCs) self-renewal[12].

MATERIALS AND METHODS

3 ml of blood from 11 suspected PV patients were collected in an Ethylenediamine Tetraacetic Acid (EDTA) tube from Hiwa Hemato Oncology Hospital in Sulaimani city. The total genomic Deoxyribonucleic Acid (DNA) was extracted from peripheral blood samples using the (AddbioPrep) Genomic DNA extraction kit according to the manufacturer's protocols and the quality of DNA samples was assessed using NanoDrop™ One (Thermo Scientific) and stored at -20° for later downstream applications. The negative samples for the V617F mutation were screened for mutations in exon 9 of the CALR gene. Primer for CALR exon 9 forward 5'-GCTATCGGGTATCACCTCTGAC-3' and CALR reverse 5'-AGTTCTCGAGTCTCACAGAGAC-3'. Exon 12 JAK2 gene primer include forward 5'-CAAAGTTCAATGAGTTGACCCC-3' and reverse 5'-CTAACATCTAACACAAGGTTGGC-3'. 40-80 ng of patient DNA were used for Polymerase Chain Reaction (PCR) amplification (513 bps) and (463 bps) of the CALR and JAK2 genes respectively. Using AddStart Taq DNA Polymerase kit (Addbio) according to the manufacturer's protocol, 3 µl buffer, 2 µl Magnesium Chloride (MgCl$_2$), 2 µl deoxynucleotide Triphosphate (dNTP), 1 µl of each primer, 0.4 µl Taq DNA polymerase, 13.6 µl PCR grade water and 2 µl template DNA were mixed in PCR tube and amplified in the following PCR conditions for CALR and JAK2 genes. Initial denaturation for 5 min at 94° followed by 35 cycles at 94° for 30 s, 61° for 30 s and 72° for 30 s, followed by final extension 72° for 7 min and PCR amplification products were separated using 1.5 % agarose gel electrophoresis at 90 V, 100 mA, 9 W for 1 h. The PCR products were purified and sequenced by the Sanger sequencing method using the 3130 Genetic Analyzer (Applied Biosystems, Hitachi High-Technologies, Tokyo, Japan) in Immunogene Center, Erbil, Iraq. The study was authorized by Salahaddin University's medical ethics committee.

RESULTS AND DISCUSSION

In the present study, we performed Sanger sequencing for the exon 12 of JAK2 gene and PCR product (513 bps) as outlined in fig. 1 for exon 9 of CALR gene. The results revealed only in a 51 y old male PV patient harboring compound mutations in the CALR gene. In contrast all 11 patients for exon 12 with wild type (fig. 2) presented normal sequence. 11 distinct unique homozygous variations observed and distributed at several positions that affected exon 9 is demonstrated in fig. 3. Among these mutations, the first two rare homozygous frameshift CALR lesions (c.1129Adel and c.1153Adel) were critical as shown in Table 1, which led to generate stop codons, producing truncated protein and eliminating almost last negative amino acids that modified the normal C-terminus and loss of the K-Lysine, D-Aspartic acid, E-Glutamic acid, L-Leucine (KDEL) signal. Hematologic parameters of a PV patient had a higher values for hemoglobin level, 16.5 g/dl and hematocrit, 52.5 % while red blood cells, white blood cells and platelet counts were normal at $5.82 \times 10^6/\mu l$, $10.5 \times 10^3/\mu l$, $177 \times 10^3/\mu l$ respectively.
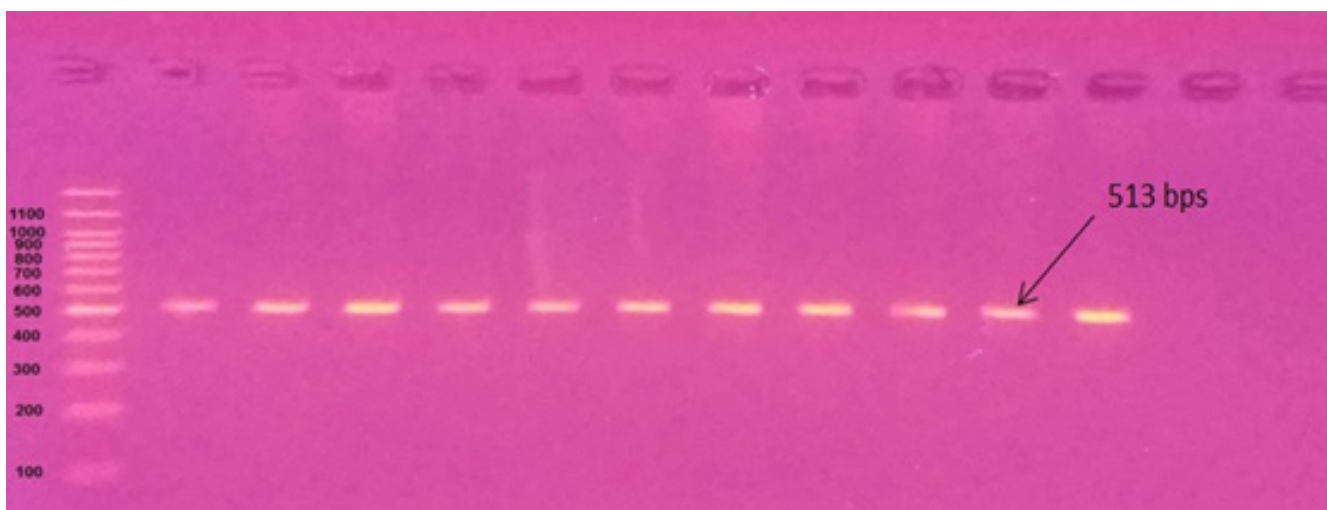

Fig. 1: Agarose gel electrophoresis PCR-product (513 bp) CALR gene exon 9
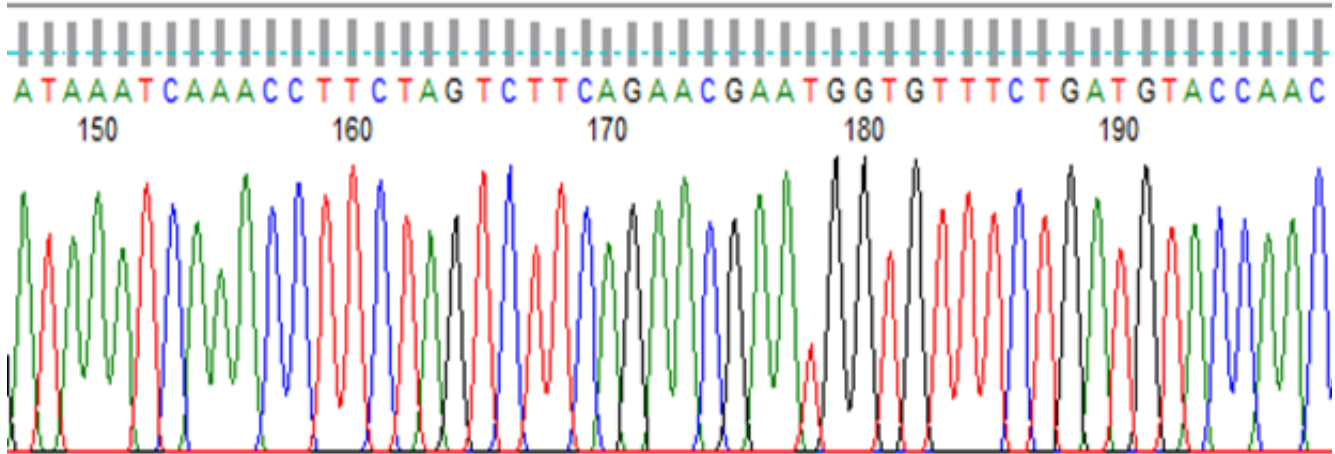
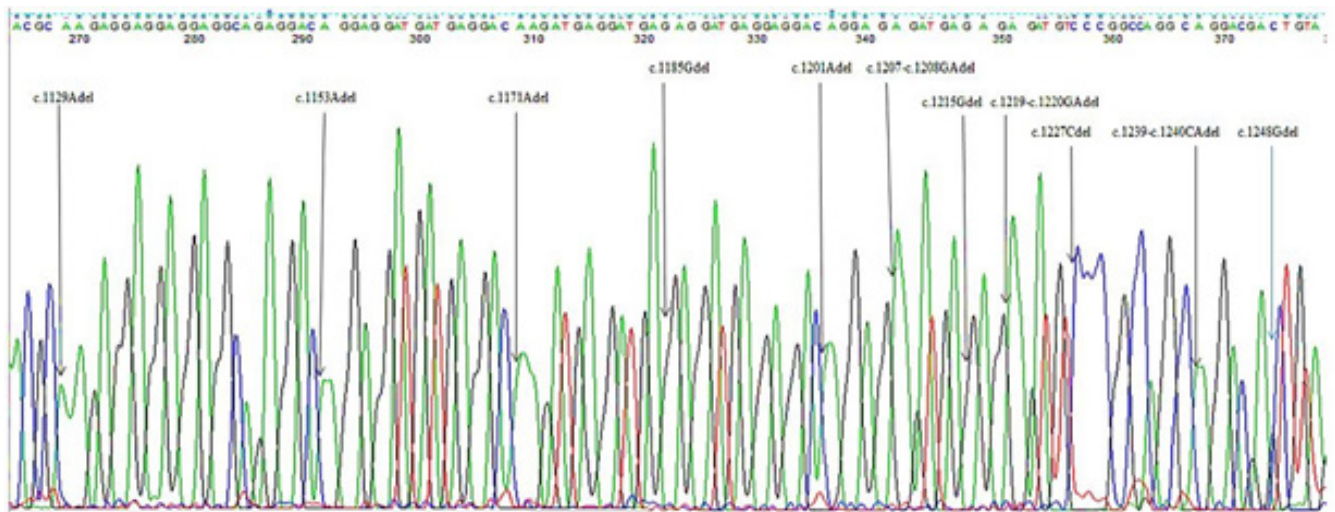**Fig. 2: Partial sequence of exon 12 JAK2 gene wild type**

Fig. 3: Chromatograph sequence revealed compound variations in exon 9 CALR gene

TABLE 1: CALR GENE VARIATIONS IDENTIFIED IN A PV PATIENT

Nucleotide change	C-terminal amino acids change
Wild type CALR	....KRKEEEEAEDKEDDEDKDEDEEDEEDKEEDEEEDVPGQAKDEL
Mutant CALR	....KRKRRRRQRTGG**GQDEDERMRRTGEMREMSRPGRTTV
c.1129Adel	
c.1153Adel	
c.1171Adel	
c.1185Gdel	
c.1201Adel	
c.1207-c.1208GAdel	
c.1215Gdel	
c.1219-c.1220GAdel	
c.1227Cdel	
c.1239-c.1240CAdel	
c.1248Gdel	

Note: Wild type amino acids in black; frameshifted amino acids in red and *stop codon

The unique compound mutations as shown in Table 1 were investigated in the current study that were not resembled by previous studies, or not previously described in PV, even in ET patients. CALR has three main domains: A globular N-domain, a proline-rich P-domain and an acidic C-domain. The C-terminal domain is enriched with a large number of negatively charged residues of acidic amino acids that are responsible for the calcium regulating function of the protein. It contains an ER retention signal (KDEL) that prevents the CALR protein from leaving the ER[13-15]. In our investigation, the complex instability of CALR was gain-of-function mutations. The outcome of the present mutations was similar to type 1 and type 2 CALR variations, which cause loss of KDEL signal. According to the majority of studies, the frequency of JAK2 V617F mutation in negative-Breakpoint Cluster Region protein-Proto-Oncogene ABL (BCR-ABL) Philadelphia MPNs represented 75 %-95 % in PV, 60 %-73 % in ET and 45 %-63 % in MPF[16-20], whereas the incidence of JAK2 exon 12 lesion is 4.5 %-5.76 % with wild JAK2 V617F PV of patients[17,21]. ET and PMF patients that lack JAK2 V617F harbor CALR mutations 7.3 %-21.9 %, 5.3 %-30 %, respectively, whereas in all cases of PV had nonmutated CALR aberrations[16-20]. Type 1, 52 bp deletions were of most prevalence in ET[16,18,19,21-23], in contrast others reported type 2, 5 bp insertions[17]. Other than two major types, minor CALR variations were found in ET but not in PMF[16,19] and not observed in ET and PMF[18] but incidence were similar[17]. The previous studies for PV individuals did not agree with our investigation. Furthermore, heterozygous CALR type 1, 52 bp deletions observed in two patients who had phenotypic PV, increased hemoglobin and hematocrit with unmutated JAK2 V617F and exon 12 relatively with thrombocytosis[24]. In a proteomic study, overexpression of endoplasmic reticulum proteins such as Calnexin (CANX) and CALR was presented on the red blood cell surface of PV patients[25]. The results may be supportive and comparable with the current findings. CALR lesion occurs in a heterozygous state[26-28]. However, a recent report showed that a PMF patient carried a rare homozygous mutation[29]. In recent large cohort study frequency of CALR mutations in negative JAK2 V716F MPNs with low 4.4 % is mostly seen in type 1 in ET and PMF, 5 bp insertion was not documented in PMF, type 1 and uncommon CALR mutations identified 1:2 in MPN-Unclassifiable (MPN-U) respectively[23]. In addition, a PV case, concurrent variations in JAK2 V617F and 3 bp deletion in exon 9 of the CALR gene were demonstrated, but frameshift was not generated[30]. While type 1 (52 bp deletions) or type 2 (5 bp insertions) restricted to exon 9 induced frameshift that changed C-terminal domain of the CALR protein[31]. Furthermore, mutant CALR generate the novel C-terminus contains multiple positively charged amino acids constitutively promote activation of Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) signaling *via* phosphorylation (P) through binding, altered glycosylation which lead to the activation of thrombopoietin receptor MPL that induce megakaryocytopoiesis[32]. Mutant CALR exposed on cell surface which prevent the phagocytosis by Dendritic Cells (DC)[33]. Hematocrit and hemoglobin levels increased in PV patients due to an increase in red blood cell production[34]. PV patients harboring genetic lesions in exon 12 of JAK2 with a wild JAK2 V617F[35-37]. JAK2 V716F mutant PV was significantly older, had higher hemoglobin, white blood cell counts and lower platelet counts than those with the ET harboring CALR variants[21].

It is a possible way for the molecular screening of CALR gene as important tool for identification rather than other gene especially for patients that lack mutations in JAK2 exon 14 and exon 12.

Author's contributions:

Formal analysis was done by Jamal Sleman Sofi; project administration was taken care by Jamal Sleman Sofi and Hazha Jamal Hidayat; supervision was done by Hazha Jamal Hidayat; validation was done by Jamal Sleman Sofi and Hazha Jamal Hidayat; writing original draft was done by Jamal Sleman Sofi and review and editing was done by Hazha Jamal Hidayat.

Conflict of interests:

The authors declared no conflict of interest.

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