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CALR Mutations Type 1 and Type 2 in Unmutant JAK2 Myeloproliferative Neoplasms in Sudanese Patients

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Abstract ***Background:*** During the previous 12 years, key improvement has been gifted with the encounter of activating mutations that are associated with the majority of BCR-ABL negative human myeloproliferative neoplasms (MPNs). In 2005 the discovery of JAK2 V617F generated great attention in the JAK2-STATpathway. Followed by detection of mutation in the thrombopoietin receptor (MPL) is a hallmark of MPNs. Later in 2013, mutations in the gene coding for the chaperone calreticulin were described in 20–30% of essential thrombocythemia and primary myelofibrosis patients. Consequently, genotyping for CALR mutation represents a novel, useful tool for an accurate diagnosis, prognosis and therapeutic relevance. In addition to that, CALR mutations are expected to be included in the diagnostic criteria for MPN (especially ET and PMF) in the upcoming WHO classification. Now, we will address the question: what do we know about calreticulin that could help us understand its role in MPNs? What is the frequency of this mutation among UN mutant JAK2 MPNs Sudanese patients? ***Material and methods:*** 53 male, 47 female was included in this study. A well-structured questionnaire was used to collect the data. DNA was extracted from blood samples using standard techniques, according to the manufacturer's instructions. CALR mutations were analyzed using allele specific PCR. Data were analyzed by SPSS software (ver. 23). ***The results:*** Among the participants, the total JAK V617F negative patients were 37 (36.6%), The overall CALR mutations were detected in 15 patients (40.5%) in which CALR mutations were detected in 4/37 (10.8%) and 11/37 (29.7%) with CALR mutation type 2. Of the 37 patients tested. 8 of the patients (53.3%) of CLAR positive individuals, were diagnosed with ET, 5/15 (33.4%) diagnosed with MF and 2/15 (13.3%) diagnosed with PRV. ***Conclusions:*** CALR could be a worthwhile diagnostic tool for JAK2 -or MPL -negative ET or PMF patients. CALR mutation may be a diverse disease group, with different hematological characteristics than that of JAK2 -positive patients.

Keywords Myeloproliferative neoplasms, CALR, JAK2, Sudan

1. Introduction

The human myeloproliferative neoplasms (MPNs) are a group of clonal stem-cell malignancies characterized by overproduction of mature blood cells. [1, 2]. According to the World Health Organization (WHO) classification of tumors of the hematopoietic and lymphoid tissues, [3] the MPN subtypes: polycythemia Vera (PV) essential thrombocythemia (ET) and primary myelofibrosis (PMF) [4].

The calreticulin (CALR) gene, found on chromosome 19p13.3, Functionally, CALR is believed to contribute in Ca2+ homeostasis as a calcium-binding protein, handling misfolded proteins, cell adhesion, immune response to cancer, and phagocytosis [5].

In 2013, somatic *CALR* mutations were identified in most *JAK2* unmutated patients with essential thrombocythemia or primary myelofibrosis patients. Multiple *CALR* mutations that generated a +1 bp frame shift and resulted in mutant proteins with a novel C-terminus were verified in exon 9. One insertion (K385fs*47) and one deletion (L367fs*46) mutation were particularly common (together constituting 85% of *CALR* mutations) [6, 7]

The role of *CALR* in the pathogenesis of MPN is largely unknown. Klampfl and colleagues and Mongolia and colleagues smartly established that *CALR* mutations are acquired, early in the major clone. [8]. Mutant CALR activates JAK/STAT signaling henceforth generating a proliferative signaling [8]. In addition, the forced expression

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of mutant *CALR* in hematopoietic cell lines resulted in MPL-dependent activation of MAPK signaling. Accordingly, patients with *CALR* mutations showed increased MAPK activity in blood cells and in CD34+ cells, leading to enhanced megakaryopoiesis and pro-platelet production. Recently it was also shown the IL3 independent growth of *CALR* mutant cells [9-11].

CALR mutations have been described as mutually exclusive with *JAK2* and *MPL* mutations and are present in a percentage of patients ranging from 56 to 88% of *JAK2/MPL*-negative cases [12]. The evaluation of CARL mutations increases the diagnostic accuracy in patients without other molecular markers and could represent a new therapeutic target for molecular drugs [13]. The aim of this study was to determine the prevalence, biological characteristics, and clinical correlations of these novel *CALR* mutations in patients with ET and PMF.

DNA extraction:

DNA purification kits (analytikjena, Germany) were used to isolate the DNA according to manufacturer's instructions.

2. PCR

Allele specific PCR was carried out to detect type 1 and type 2 CALR mutations using the following primers forward primer 1: 5′-GCA GCA GAG AAA CAA ATG AAG G-3′ for type 1 mutation, forward primer 2: 5′-GCA GAG GAC AAT TGT CGG A-3′ for type 2 mutation, and reverse primer: 5′-AGA GTG GAG GAG GGG AAC AA-3′ as a general primer. The PCR was performed in one step (single tube) in a 25 µl final volume using introns Maxime PCR PreMix Kit (I-Taq) (Korea). An initial preheating at 94°C for 5 min was followed by denaturation at 94°C for 30 Sec, annealing at 63°C for 30 Sec, and extension at 72°C for 50 Sec for 40 cycles followed by a final extension at 72°C for 5 min performed on a thermocycler. After PCR amplification, gel electrophoresis was performed in a 2% agarose gel at 130 V for 30 min to detect the amplified regions of DNA, and agarose gels were exposed under UV light in gel documentation system. Interpretation was done by comparing bands to the expected product size (wild type CALR: 357 bp, CALR type 1 mutation: 302 bp, and CALR type 2 mutation: 272 bp).

Statistical analysis: The statistical analysis of the results was done using the SPSS (vs. 23) statistical software. The Chi-Squared test was used to compare the frequencies of the categorical variables. A value of $p<0.05$ was considered statistically significant.

3. Result

Among the participants, the total JAK_{2V617f} negative mutation was 37 (36.6%), in which 4/37 (10.8%) was found to be CALR type 1 mutation and 11/37 (29.7%) with CALR mutation type 2. From the 37 patients tested, CALR mutations were detected in 15 patients (40.5%), 8 patients (53.3%) of CLAR positive individuals, were diagnosed with ET, 5/15 (33.4%) diagnosed with MF and 2/15 (13.3%) diagnosed with PRV (table 1).

Those with CALR positive mutations, gender was not a factor with 8 (53%) and 7 (47%) were females and males respectively. (Fig. 1).

A total of two types of CALR mutations was detected in this study (Fig. 2).

Table 1

Variables		PV	ET	PMF
JAK2V617F	Wild type	28(43.7%)	07 (29.1%)	02 (15.3%)
	Mutated	37(56.3%)	17 (70.9%)	10(84.7%)
CALR mutation	Type 1	01(1.5%)	07(29.1%)	03(23%)
	Type 2	01(1.5%)	01(4.1%)	02 (15.3%)

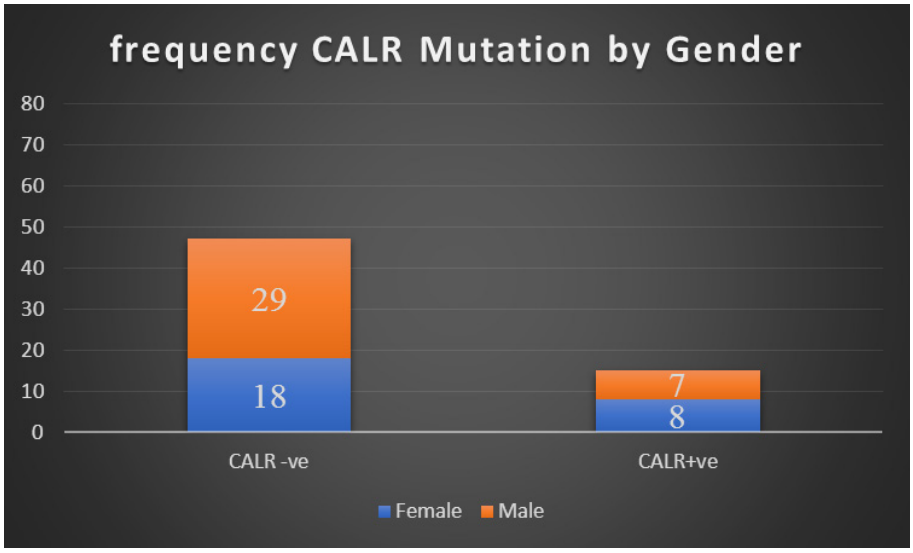
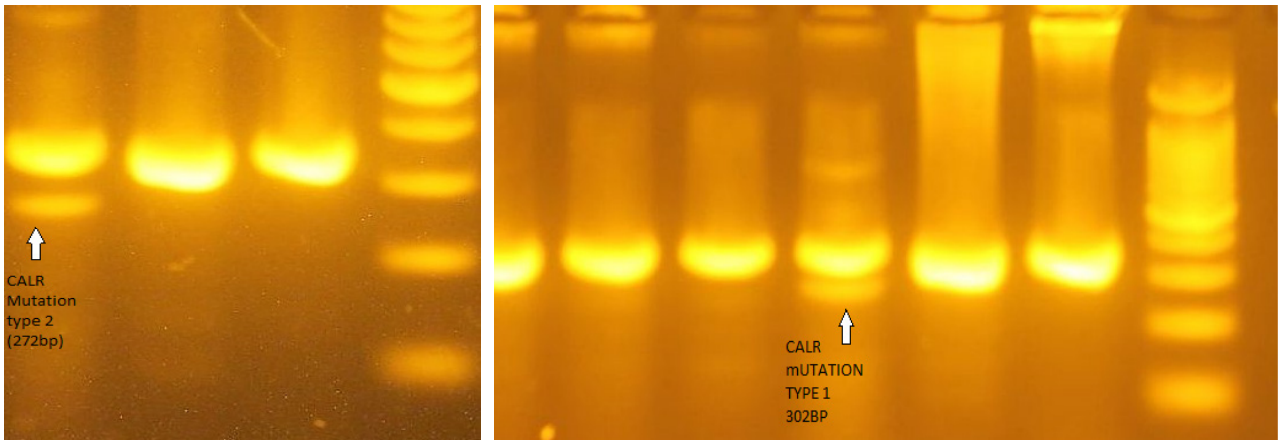


Figure 1



(A) (B)

Figure 2. (A) CALR mutation type 2 (272bp), the two thick bands represent normal samples of 357pb of CALR gene (B) CALR mutation type 1 (302bp)

4. Discussion

The frequencies of CALR mutation reported by previous studies

Authors	Subject	Frequency of CALR mutation			Both JAK2 (+)/ CALR(+)
		ET	PMF	PV	
Klampfl et al [1]	1,107 MPN	ET (67)	PMF (88)		
Nangalia et al [2]	151 MPN	ET (82)	PMF (80)		
Jung-Sook Ha et al [3]	168 MPN	ET(58.5)	PMF(33.3)		1 ET
Tefferi et al [4]	254 PMF		PMF (74)		1 PMF
Rotunno et al [5]	576ET	ET (48.9)			
Rumi E et al [6]	717 ET	ET (63.1)			
Li et al [7]	357 PMF		PMF (43)		
Matsumoto et al [8]	177MPN	ET(43)			
Kim et al [9]	199 MPN	ET(17.7)	PMF(14.8)		
Zhiyuan Wu et al [10]	210 MPN	ET(20)	PMF(16)		
Machado et al [11]	73 MPN	ET(41)	PMF(33)		
Lin, Y et al [12]	929 MPN	ET(22.7)	PMF(17.6)		ET(1),PMF(1)
Lavi, N et al [13]	896 MPN	ET(25)	PMF(35)		
Sun, C et al [14]	310ET	ET(59)			

Somatic insertions/deletions in the calreticulin gene have recently been found to be causative varieties in myeloproliferative neoplasms [14]. All changes prompting a frame shift to a novel perusing outline, brings about the loss of KDEL flag, which is in charge of keeping the discharge of protein from the ER, and makes an alternate C-end protein [2]. This new arrangement contains various positively charged amino acids, while the wild-sort grouping is normally contrarily charged. While the wild-type sequence is naturally negatively charged. Accordingly, proposed to disrupt the ER-signaling peptide and influence the CALR subcellular localization, stability and function inside (calcium homeostasis and protein folding inside [3] and outside ER (cell adhesion, gene nuclear transport, apoptosis, and immunogenic cell death [2]. In the current study, the allele specific method demonstrated CALR mutation in 58.5% and 33.3% of the JAK2 negV617Fative ET and PMF patients, respectively, and this at the same track with Li Bx 43% for PMF [6] and also to Rotunno et al who evaluate this type of mutation in 576 ET patient his result is 63% harboring CALR exon 9 mutation[2]. In contrast to previous studies revealed that the frequency of CALR mutation in JAK_2 V617F negative cases was 48.9-82% for ET and 43-88% for PMF [14] This variation in the frequency range owing to the difference in the biology of disease in Sudanese population, Ethnic background, sample size, and sensitivity of the recognized methods.

In the present study, the method demonstrates CALR mutations have been found not only with ET and PMF, but with PV as well. This agrees to a few reports on cases carrying both JAK2 and CALR mutations [4[.And disagreed with *Seon Young Kim et al* who reported frequent (43.9%)

CALR mutations in patients with PMF, ET, and MPN-U without JAK2 and MPL mutations but not in patients with PV without JAK2 and MPL mutations [9] CALR mutation was also detected in other hematological malignancies such as refractory anemia (RA; 9%), RA with ringed sideroblasts (RARS; 11%), and RA with excess blasts (RAEB; 12%). RARS is associated with marked thrombocytosis (RARSt; 9-12%), chronic myelomonocytic leukemia (CMMoL; 3%), and atypical chronic myelogenous leukemia (aCML; 3%) [14].

In conclusion, CALR exon 9 mutations are novel somatic mutations and the most frequently observed mutations in ET and PMF. Therefore, for patients with suspected ET or PMF without JAK2 V617F, MPL should be analyzed favorably. Combined tests of JAK2 V617F, JAK2 exon 12, MPL exon 10, and CALR exon 9 have greatly enriched the diagnosis rates of BCR-ABL1-negative MPN, specifically in ET and PMF. As CALR might be a valuable diagnostic marker and therapeutic target in ET leading to the development of innovative diagnostic criteria and therapies for MPN patients.

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