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CALR Gene Mutation

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FREQUENCY OF CALR GENE MUTATION IN MYELOPROLIFERATIVE NEOPLASMS

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ABSTRACT

Objective: To detect the calreticulin gene mutation in myeloproliferative neoplasms and its clinicohaematological correlation.

Study Design: Cross sectional study.

Place and Duration of Study: Armed Forces Institute of Pathology, from Jun 2017 to Jun 2018.

Methodology: A cross sectional study was conducted at Department of Haematology, Armed Forces Institute of Pathology from June 2017 to June 2018. A total of 48 newly diagnosed JAK2V617F patients with negative myeloproliferative neoplasma were enrolled in the study. Clinico-haematologic features were noted. DNA was extracted from bone marrow samples. Molecular analysis was performed for Calreticulin gene by Sanger Sequencing. Results were analysed by using Genetic Analyser HITACHI 3130.

Results: Of 48 newly diagnosed JAK2V617F negative myeloproliferative neoplasms patients, 38 were male and 10 were females with M:F ratio of 3.8:1. Mean age was 43.5 years (standard deviation ± 15). 9 (18.8%) were diagnosed as polycythemia vera, 21 (43.8%) as Essential thrombocythemia and 18 (37.5%) as having Primary myelofibrosis. CALR mutation was detected in four (8%) of myeloproliferative neoplasms cases. Out of four CALR positive cases, three were diagnosed to have Primary myelofibrosis while only one had a diagnosis of Essential thrombocythemia.

Conclusion: We conclude that CALR mutation was an important molecular marker in JAK2V617F negative patients. Primary myelofibrosis shares a high rate of CALR mutation as compared to polycythemia vera and essential thrombocythemia.

Keywords: Calreticulin gene, Essential thrombocythemia, Myeloproliferative neoplasms, Polycythemia vera.

INTRODUCTION

Myeloproliferative neoplasms (MPNs) are characterized by clonal expansion of terminally differentiated myeloid cells driven by somatic mutations[1]. MPNs encompass wide spectrum of disorders such as polycythemia vera (PV), essential thrombocythemia (ET), primary myelofibrosis (PMF), chronic myeloid leukaemia (CML), Chronic eosinophillic leukaemia (CEL) and Myeloproliferative neoplasms-unclassified (MPN-U)[2].

Over the years it has been learned that aberrant activation of JAK-STAT signaling is a hallmark of MPNs. In normal JAK-STAT signal transduction, the binding of ligands to cell surface receptor activates intracellular kinases from the JAK family. These kinases phosphorylate STAT, after dimerization, it translocates to nucleus to regulate genes involved in survival, proliferation and differentiation[3]. This led to the identification of JAK2V617F as novel mutation associated with these disorders[4].

However, in recent years, better understanding of the biology of disease, has led to the discovery of novel molecular findings in addition to JAK2V617F that may have an important role in pathogenesis and clinical presentation of these patients. These include CALR, MPL and JAK2 exon 12 mutations[5].

Mutations in exon 9 of (Calreticulin) CALR gene were recently implicated in the pathogenesis of majority of JAK2V617F-negative ET and PMF cases[6]. The most frequent mutations are type 1 variant 52 base pair deletion and type-2 5 base pair insertion[2]. Calreticulin is a protein that resides in the lumen of the endoplasmic reticulum, it acts as a molecular chaperone for

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glycoproteins, helps in their folding and has role in calcium homeostasis. It has 3 main domains; an N terminal lectin binding domain, a proline rich P domain, and a C-terminal acidic domain that contains multiple calcium binding sites necessary for binding to the endoplasmic reticulum[7]. The C-terminus of CALR contains divalent calcium ion binding property which is involved in other mechanisms also such as immune response to tumor, cell-cell interaction, phagocytosis and cellular signaling. All CALR genetic variants cause a loss of sequence of 27 amino acids leading to loss of most of C-terminal acidic domain and KDEL sequence necessary for function of CALR protein and binding to endoplasmic reticulum[8,9].

Identification of these mutations compliments the diagnostic approach to MPNs as well as giving prognostic information[10]. In ET, the presence of CALR mutation identifies a subgroup of patients that present at an earlier age and have no history of thrombosis. Patients of PMF harbouring this mutation also have a good prognosis and type 1 CALR mutations confer a better prognosis and have been associated with a better overall survival in PMF as compared to type 2 CALR mutations.

In Pakistan, MPNs are not uncommon. Pakistan is a resource constraint country with limited facilities for molecular analysis. There was limited data available regarding MPNs in our population. As AFIP, is a state of the tertiary care referral center of the country, we conducted this study to determine the frequency of CALR mutations in newly diagnosed MPN patients who were JAK2v617F negative and study the clinico-haematologic features of these patients. This will help in prognostic stratification of our patients, thus aiding in making treatment decisions.

METHODOLOGY

This cross sectional study was conducted at Haematology Department, Armed Forces Institute of Pathology during June 2017 to June 2018. A total of 48 MPN patients diagnosed according to WHO 2016 diagnostic criteria were enrolled in our study. Sample size was calculated by WHO calculator[11]. Bone marrow samples were obtained by using non probability convenience sampling technique from newly diagnosed MPN patients of all ages and both genders were included. MDS-MPNs and patients on treatment were excluded in the study. After approval of Ethical committee of AFIP (Reference number: FC-HEM16-26/READ-IRB/17/379) and CPSP, infor-med consent was taken and questioner Performa was filled.

We performed Sanger Sequencing on all peripheral blood/bone marrow samples of DNA using standard protocols and conducted bioinformatic analyses to identify somatically acquired mutations. EDTA blood (3ml) was collected. DNA was isolated in molecular lab by using Solgent Genomic DNA preparation kit (column based). PCR was performed on Proflex according to manufacturers instruction. Each reaction tube contain 2µl DNA, primers 1µl (forward primer 5′- TGGTCCTGGTCCTGATGTCG -3′ and reverse primer 5′-AGAGACATTATTTGGCGCGG-3′), taq mixer 12.5µl and distilled water 9.5µl using following PCR program (table-I). Second Purification was done by Beckman coulter purification kit yields final product of 27µl. Results were analysed by using HITACHI Genetic analyser 3130.

Table-I: Polymerase chain reaction program for CALR gene mutation analysis.

CALR EXON 9	First PCR	Sequencing PCR
Initial Denaturation	94 C/01 min	96 C/05 mins
Denaturation	94 C/01 min	96 C/10 secs
Annealing	62 C /1 min 30 secs	50 C/5 secs
Extension	72 C /1 min 30 secs	60 C/4 mins
Final Extension	72 C/3 mins	72 C/3 mins

All statistical analysis were performed using SPSS program version 24. The variables like age, gender, cell count were given. The percentages and mean, medians were calculated for variables.

RESULTS

A total of 53 patients were diagnosed as MPNs. On mutation analysis of JAK2V617F, 5 (9.4%) patients were found positive for JAK2V617F and 48 (90.5%) were negative for JAK2V617F.

We then studied these 48 JAK2V617F negative MPN patients. Clinically 14 (29.16%) patients showed hepatomegaly, 32 (66.7%) had splenomegaly while only 2 (4.1%) patients presented with thrombosis.

Out of these 48 JAK2V617F negative patients, 38 (79.1%) were males and 10 (20.8%) were females with M:F ratio of 3.8:1. Mean age was 44 years (SD ± 15) years with range of 38-72 years (fig-3). On the basis of clinical features, physical examination, Blood CP, bone marrow aspiration and trephine biopsy findings; 9 (18.8%) were diagnosed as PV, 21 (43.8%) as ET, 18 (37.4) as PMF. CALR mutation was detected in 4 (8.3%) of MPN on molecular analysis. Out of 18 PMF patients 3 (16.66%) cases carried CALR mutation and only 1 (4.76%) out of 21 ET patient harboured CALR mutation. Thus overall 4 (8%) patients had mutation whereas 44 (92%) patients were triple negative. Main clinical and haema-tological features of 4 CALR positive patients in our study are shown in table-II.

Table-II: Main clinical and haematological features of 4 CALR positive patients on mutation analysis.

	Patient 1	Patient 2	Patient 3	Patient 4
Age (Year)	48	62	70	26
Gender (M/F)	Male	Male	Male	Male
Wbc count (x10^9/l)	4.2	20.4	26	10.5
Haemoglobin level (g/dl)	7.6	9.4	10.8	10.1
Platelet count (x10^9/l)	175	410	252	1856
Spleen size (cm) below left costal margin.	21cm	14cm	3cm	-
Diagnosis	Primary myelofibrosis	Primary myelofibrosis	Primary myelofibrosis	Essential thrombocythemia

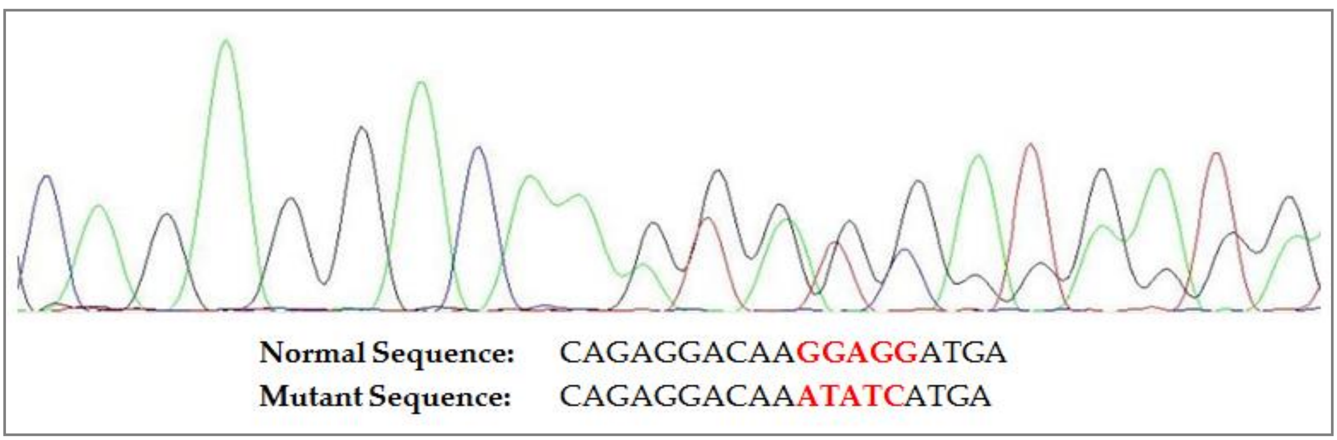

Figure-1: Electropherogram of one of our patient showing positive CALR mutation type,it shows 5 bp deletional/insertion type of mutation.

DISCUSSION

Our understanding of the genetic basis of myeloproliferative neoplasms has led to the identification of driver mutations such as CALR[14]. Hence, the identification of these driver mutations are essential for diagnosis of JAK2V617F negative MPNs[15]. WHO 2016 has incorporated CALR in the diagnostic criteria of MPN's[17].

Thus, molecular diagnosis is essential in newly diagnosed MPN patients. In developing countries like Pakistan, modern molecular diagnostic facilities are limited[16]. AFIP is a tertiary care referral center that caters patients from all over the country, thus helping in appropriate diagnosis and early management. We have conducted this study to determine the frequency of CALR mutation and their clinicohaematological parameters in our population.

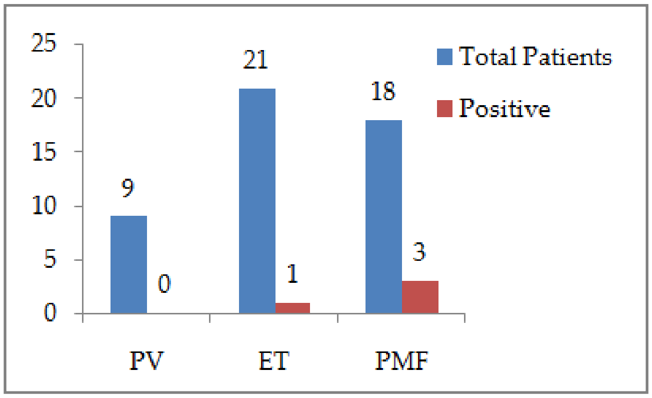

Figure-2: Graphical representation of positive samples.

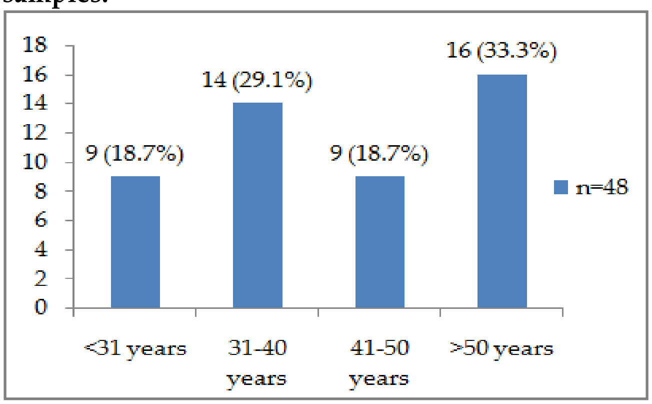

Figure-3: Age distribution of Myeloproliferative neoplasms patients included in our study.

In this study we found that CALR mutation is present in 4 (8.3%) cases of MPNs. Among these patients CALR was found in 3 cases of PMF and in 1 ET patient. Ojeda[18] in a study conducted in the Argentinian population, has reported CALR mutation frequency of 12.3%. These findings were similar to our results. However, a study conducted at Fondazione IRCCS Ojeda *et al*[19], Italy between 1982 to 2014 on a total of 1282 patients has reported a much higher frequency of 24%. Singdong *et al*[20] in his study conducted in Thailand reported CALR mutation in 35.7% of patients with JAK2 negative ET and 33.3% with JAK2 negative PMF. However, as disease biology in different populations maybe different, the much lower frequency in our population may be due to ethnic and geographic differences.

Studying patients of ET harboring the CALR mutation, Pietra *et al*[21] in his study conducted in Italy has reported main clinical and haematological parameters of patients with ET with CALR mutation as follows; median age at diagnosis was 40 years, haemoglobin level 13.8g/dl, WBC count 8.1x10^9/l, Platelet count 982 x10^9/l. While our ET patients with CALR mutation presented with much higher median WBC count of 15.4 x 10^9/l, much lower haemoglobin and platelet count of 9.7g/dl and 331x199/l respectively. The reason for higher platelet count and lower haemoglobin level may be due to the fact that in our country patients have less awareness and less accessibility to tertiary care centers and present at a later and more advanced stage of disease.

According to international study conducted in 2013 CALR was present in 20 to 25% ET patients where as 25 to 30% MF patients. Our data was quite different from international data due to small sample size and our own population and patient characteristics. However further studies on large size sample should be conducted to determine exact frequency of CALR mutation in our population.

CONCLUSION

We conclude that CALR mutation was an important molecular marker in JAK2V617F negative patients. Primary myelofibrosis shares a high rate of CALR mutation as compared to polycythemia vera and essential thrombocythemia.

CONFLICT OF INTEREST

This study has no conflict of interest to be declared by any author.

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