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EXPLORING JAK2 GENE MUTATION *(V617F)* THROUGH REAL TIME PCR IN POLYCYTHEMIA VERA PATIENTS IN KPK.

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ABSTRACT

BACKGROUND: Polycythemia Vera (PV) is a chronic myeloproliferative neoplasm, the condition arise when the excessive number of red blood cell produced within the bone marrow due to the mutation occurs at the myeloid stem cells in protein named JAK2 kinase particularly within JH2 domain. A somatic point mutation occurs in the JAK2 gene on chromosome 9 due to the transversion of G-C to T-A which develops the replacement of valine by phenylalanine at codon 617. According to the WHO JAK2 V617F is responsible for about 95% of PV cases. The annual incidents for Polycythemia Vera are 1-3 individual per 100,000 populations. The aim of the study was to investigate the prevalence of the JAK2 gene mutation by using Real-Time PCR among the suspected PV patients in Khyber Pakhtunkhwa, providing visions into the frequency of this mutation within the province. **METHODS:** A total of 300 blood samples were collected from suspected PV patients and various screening test were conducted to confirm the diagnosis, including: Hematologic parameters such as Red blood cell count (RBCs), Hemoglobin level (HB), Hematocrit (HCT) and total leukocyte count (TLC) were measured. Peripheral blood smear were examined to detect the abnormalities in the morphology of red blood cell, differential eosinophil and basophil counts and presence of blast as well. Bone marrow aspirate and biopsy samples were obtained and examined to assess cellularity, panmyelosis, megakaryocyte morphology, erythroid and myeloid series cells, bone marrow eosinophilia, bone marrow iron stores, and reticulin fibrosis grade. Genomic DNA was extracted from the collected blood samples using the MN kit. Real-Time PCR analysis was then performed to detect the JAK2 V617F mutation, a hallmark of PV. This molecular analysis provided confirmation of PV diagnosis and enabled precise identification of individuals carrying the JAK2 mutation **RESULTS:** Among the suspected patients, 211 were confirmed positive through CBC, 144 through special smear, and 102 through bone marrow examination. Real-Time PCR analysis of DNA samples revealed the existence of the JAK2 V617F mutation in approximately 97 (95%) of the confirmed PV patients. **CONCLUSION:** In our study, we consider Real-Time PCR as a sensitive and accurate method for detecting the JAK2 V617F mutation in Polycythemic (PV) patients based on its demonstrated performance in previous studies and its widespread adoption in clinical laboratories. The high prevalence of this mutation, detected in nearly 95% of PV cases, highlights its significance in the pathogenesis of the disease within the Khyber Pakhtunkhwa population.

KEYWORDS: Polycythemia Vera, JAK2 V617F Mutation, Real-Time PCR, Diagnosis

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INTRODUCTION

Polycythemia, also referred to as Polyglobulia, is a rare type of cancer distinguished by heightened levels of hemoglobin, hematocrit, and Total red blood cell count. Polycythemia Vera (PV) is categorized under clonal myeloproliferative neoplasms (MPNs), chronic ailments that stem from hematopoietic stem cells, culminating in continuous overproduction of blood cells.[1] Alongside elevated red blood cell counts, symptoms of MPNs include splenomegaly and reduced erythropoietin levels. Polycythemia Vera arise when an error causes in JAK2 gene. The primary function of the JAK2 gene is to regulate the production of red blood cell within the bone marrow. When JAK2 loses its function due to a mutation arising on chromosome 9, it results in excessive production of blood cells. [2] This point mutation, characterized by the translocation of G-C to T-A, results in a gain of function within the JAK2 gene. Specifically, it leads to the substitution of valine with phenylalanine at codon 617 within the JH2 domain, which serves as an auto-inhibitory domain of the JAK2 gene. [3] The mutation, known as JAK2 V617F, activates JAK-STAT and signaling molecules, such as extracellular signal-regulating kinase, even when it lack cytokines, leading to erroneous cell proliferation. [4] In most of the cases JAK2 genes is a reason of Polycythemia Vera.[5]

Polycythemia Vera (PV) belongs to the category of myeloproliferative neoplasms (MPNs) that do not involve the Philadelphia chromosome. [6, 7] The incidence of PV is higher in males compared to females across various races and ethnicities, with rates approximately 1.3 per 100,000 women and 2.8 per 100,000 men. This condition typically manifests between the ages of 40 and 60, although it can occur at any age. [8] Annual incidents of PV in Pakistan are approximately 1.5 per 100,000 inhabitants.

PV remains asymptomatic for many years; however, symptoms typically develop gradually, often first indicated by elevated hematocrit (Hct). When Polycythemia Vera (PV) progresses, it often presents a spectrum of symptoms, including shortness of breath, fatigue, headaches, weakness, dizziness, itchiness, and joint pain. This condition instigates a rise in blood viscosity, which occurs when red blood cells multiply excessively. This heightened viscosity, akin to a thickened fluid, sets the stage for thrombosis, increasing the risk of blood clots. Consequently, the quality of life for individuals with PV is compromised, leading to a reduced life expectancy. [9]

The JAK2 gene mutation is detected in 95% of Polycythemic patients. [10] It has been observed that the JAK2 gene mutation is also responsible for other MPNs; approximately 50% of cases of myelofibrosis and essential thrombocythemia may have the JAK2 V617F mutation. [11] According to the World Health Organization (WHO), the JAK2 gene mutation is responsable for almost 80-90% of patients with Polycythemia Vera. [12] The World Health Organization provides proper guideline criteria for the diagnosis of Polycythemia Vera. [13] It mainly concerns elevated erythrocytes and establishes principles accordingly, whereas a British committee (PCSG) focuses on the hematocrit value and the mass of RBCs. The criteria have major and minor both, which are based on laboratory tests for erythrocyte measurement, cell morphology, and detection of the mutation.

The primary objective of this research endeavor was to provide invaluable support to the relevant authorities and establish comprehensive guidelines for individuals affected by Polycythemia Vera (PV). In addressing the unique healthcare needs of the Khyber Pakhtunkhwa (KPK) region, our study employed Real-Time PCR technology for the first time to detect the JAK2 V617F mutation in polycythemic patients. The aim of this research was to facilitate the concern authorities and put the guidelines for the affected patients. We explored this diagnostic tool for detection of JAK2 V617F mutation in Polycythemic patients in KPK, such diagnostic facilities were never existence in the province before. We aimed to ease the problem of PV patients, who used to travel so far for the diagnostic centers for tests and treatments.

METERIALS AND METHODS

This study was approved by the ethical committee of the department of Biotechnology, Abdul Wali Khan University Mardan, Approximately 300 patients in KPK region were selected for the current study. Among them most of the patients were already enrolled in Hayatabad Medical Complex (HMC), that's why it was the most preferable area of concern, Shaukat Khanum Memorial Cancer Hospital and research center and some private clinics

were also selected for the study, The sampling of whole Blood was accomplished in the department of hematology in the HMC Peshawar and Real Time PCR Lab Dabgari Garden. Each patient provided approximately 5 ml of peripheral blood with full informed consent, with the help of disposable syringe and were stored in EDTA tube at -4^{o}c for further analysis.

These collected blood was then endured to a series of laboratory test to evaluate the various parameters. These tests included Complete Blood Count (CBC), examination of blood smears, and bone marrow analysis. The laboratory analyses were conducted with meticulous attention to detail and adherence to standardized protocols to ensure the accuracy and reliability of the results.

CBC tests were performed through Sysmex Hematology analyzer, as per the WHO criterion 2016, the suspected PV male would have hemoglobin (HB) higher than 16.5 g/dl and all the suspected PV female would have hemoglobin Hb higher than 16.0 g/dl, as well as all the suspects would have elevated Hematocrit > 49 in male and > 48 in female.

Each blood sample were prepared for blood smear examination, slides were made and were examined under microscopy, their morphology was observed accordingly. Patients whose RBC morphology (shape, size, coloure) were normal were excluded and those patients whose morphology were abnormal were included.

The bone marrow biopsy revealed hypercellularity appropriate for the patient's age, characterized by trilineage growth (panmyelosis). This growth pattern included prominent proliferation of erythroid, granulocytic, and megakaryocytic cell lines. Additionally, mature megakaryocytes were observed with pleomorphic features, exhibiting differences in size.

The MN kit (MACHEREY-NAGEL, GERMANY) was used to extract the DNA from that of each whole blood sample. These genomic DNA were further used for amplification in the detection of JAK2 V617F mutation. DNA extractions were done according to the protocols available in the manufacturer's booklet.

For the purification of DNA, a 2ml microcentrifuge tube was first taken, to which 200 µl of blood and 25 µl of proteinase K were added. It was then vortexed for 10 to 20 seconds and incubated for about 10 to 15 minutes at 70°C. Subsequently, the DNA binding step was carried out as per the protocol, adjusting the condition for DNA binding by adding 210µl ethanol to each sample. These samples were centrifuged for 60 seconds at 11,000 x g.

This was followed by washing of the silica membrane containing bound DNA. Washing was performed in two steps: Wash 1 involved adding about 500 µl of Buffer BW to each sample and centrifuging for 1 minute, followed by Wash 2 with the addition of 600 µl of B5 to each sample and centrifugation. Further centrifugation of the sample was done for 1 minute at 11,000 x g to separate the residual ethanol and dry the silica membrane.

Afterward, 100 µl prewarmed buffer BE was added to each sample tube on the silica membrane, followed by a two-minute wait and then centrifugation for 2 minutes at 8000 rpm. Finally, DNA in elution buffer was obtained and stored at -70°C until further use.

For Real-Time PCR, amplification was carried out using the oncoplex® JAK2 Mutation kit (Gene first, UK). Real-Time PCR was performed to detect the JAK2 V617F mutation in specific samples. The Oncoplex JAK2 Mutation Kit consisted of primers (wild type and mutant type) specific for the JAK2 V617F mutation, along with cycling conditions for the wild-type normal allele and mutant allele. This facilitated the detection of rare mutations alongside high non-mutated DNA.

All these phases of master mix preparation were done on ice with least exposure to light. The working mix and master mix were fully melted and vortexed. About 19 µl reaction mix was added to each tube according to the number of samples, with 0-5µl of the test sample and 1 µl each of positive control (PC) and negative control added to separate tubes labeled as positive and negative control. These tubes were then placed in the PCR machine, the lid closed, and thermal protocols initiated by clicking the run button.

Table 1: Components required for the reaction of PCR/Mater Mix

Colour	Volume per single reaction	Name of ingredients
Amber tube cap	6 ul	Working Mix
Blue tube cap	8 ul	Master Mix
	19 ul (0-5 ul as required)	H2O
AND		
Yellow	1ul	Negative control
Or		
Red	1ul	Positive control
Or		
N/A	1-6 ul	Test sample
Or		
NTC	1 ul	Not template control

Table 2: Oncoplex® JAK2 mutation Kit thermal cycling conditions

Stage	Cycle	Temperature	Duration	Data collection
Activation stage	1	50	2 min	
		95	3 min	
Amplification stage	10	95	4 sec	
		52	30 sec	
		72	20 sec	
Amplification stage	42	95	6 sec	
		56	30 sec	Hex, FAM and ROX
		60	30 sec	

The mutational status was analyzed by measuring the amplification curves from the ROX and FAM signals and Threshold Cycle (Ct) values. We measured the ROX signals and FAM signals in each reaction to confirm the accuracy of the Ct values' reaction. The detection limit of the assay was 1% of wild DNA (Figure.1).

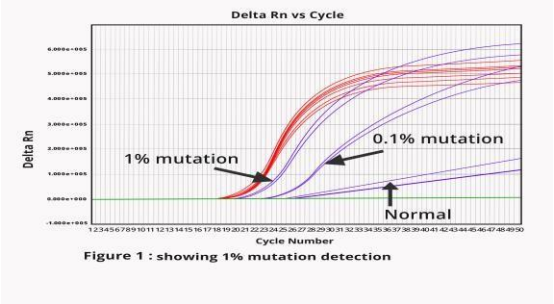

Figure 1 : showing 1% mutation detection

RESULTS

Peripheral blood was taken from 300 PV suspected patients from various hospitals in Peshawar. Among these patients, 183 (61.8%) were men and 117 (39%) were women. The CBS test were performed for each blood sample. Approximately 211 (70.3%) suspects tested positive for PV due to higher HB and Hct according to the WHO criteria 2016. Out of these 211 suspects, 82 (38.8%) were female and 129 (61%) were male and, while the remaining 29.6% tested negative due to normal Hct and HB levels according to the WHO criteria.

Blood smear tests were then performed on these 211 samples, Blood smear finding were different due various stages of diseases. There were normochromic and normocytic red blood cells, some were hypochromic other were microcytic. Few of them were Anisocytosis and Poikilocytosis. Approximately 144 (68.24%) patients' samples were of abnormal morphology, 96 (66.6%) were male suspects and 48 (33.33%) were female suspects. Subsequently, bone marrow aspiration was done on these 144 samples, with approximately 102 (70.8%) samples suspected for PV. Among these, 69 (67.6%) were male suspects and 33 (32.35%) were female suspects, while the some were found negative.

Real-Time PCR was then accomplished for advance validation to detect the JAK2 V617F mutation. After carrying out PCR on the remaining 102 samples, approximately 97 (95%) samples were positive for the JAK2 mutation, while the 5% were negative as they lacked the mutation. From these obtained 97 positive samples 31 (32%) were female and, 66 (68%) were male.

Table 3: Gender distribution among the suspected patients

Test	Patients (n=300)	Male	Female	Mean age
CBC	211 (70.3%)	129 (61%)	82 (38.8%)	55
Blood Smear	144 (68.24%)	96 (66.6%)	48 (33.33%)	55
Bone marrow aspiration	102 (70.8%)	69 (67.6%)	33 (32.35%)	55
RT PCR	97 (95%)	66 (68%)	31(32%)	55

DISCUSSION

Our study aimed to elucidate the epidemiology and molecular characteristics of PV among individuals in the Khyber Pakhtunkhwa (KPK) region, Pakistan. We collected 300 whole blood samples from suspected PV patients, with 211 (70.3%) exhibiting clinical features indicative of PV. Consistent with previous research, we witnessed a high proportion of male patients, with a male to female ratio of 2:1. This finding aligns with global trends and underscores the gender-specific predisposition to PV.

In this study World Health Organization (WHO) diagnostic criteria were used to confirm PV. According to WHO threshold an elevated level of hematocrit, and hemoglobin in addition with abnormal morphology of red blood cell indicates polycythemia Vera (PV). Our study completely line up with the WHO diagnostic criteria, underlying the significance of Complete Blood Count (CBC) and Blood smear examinations as crucial tools for PV diagnosis. All the patients were have elevated level of hemoglobin (HB) and hematocrit (HCT).

Our study's outcomes align closely with established literature, highlighting the significance of complete blood count (CBC) and blood smear tests as pivotal tools for diagnosing Polycythemia Vera (PV). Specifically, all patients under investigation exhibited elevated levels of hemoglobin (Hb) and hematocrit (Hct), consistent with previous research findings. [14] Our report is also in accordance to one study regarding gender ratio, from Pakistan, according to that among total of 46 subjects who were suspected to have Polycythemia Vera, about 30 (65%) were men and 16 (35%) were women with a ratio of 1.9:1. [15] These parallels underscore the reliability and reproducibility of our findings within the broader context of PV research. In most of the cases man show more prevalence than women and ratio is 2:1. [16] Similarly several studies and assessments in Pakistan indicated that women are mainly less affected as compared to man with ratio of 1.5:1. According to our report the majority were males with the ratio of 2:1, intensely supported by current report from India and Thailand just like some recent studies of India and Thailand [3, 17]. Our data is more or less related to the previously national and international literatures.

The 211 samples were screened for abnormal RBC morphology. Out of these suspected patients, 148 (68.24%) subjects were have low EPO level and so were claimed to have PV, among these, 48 (33.33%) were male and 96 (66.6%) were female (Table.3). Several international and national studies have already verified it that individual whose RBC's are not in normal size, shape or color would have Polycythemia Vera. According a report from New York, about 18 (73.8%) individual were had low EPO level (Richard T et al).

Furthermore our study shed light on the median age of PV onset, with most patients presenting in their 50s. This age distribution aligns with previous reports from both national and international studies, indicating a typical onset of PV in the sixth decade of life. [3, 18]. The observed age distribution underscores the importance of age as a risk factor for PV and informs clinical decision-making regarding diagnostic evaluations in older individuals. According to numerous reports 60 is a median age for PV is diagnoses, however the condition may causes at any age.[8, 19]. PV is considered as rare disease worldwide and there are barely few reports from Pakistan.[20] However some international reports supports our study, according to an Indian report; the mean age is 52 years [(3)] and a Thai literature mentiones that the mean age is 58. [21]

Real Time PCR was carried out for the remaining patients to confirm PV by detecting JAK2 V617F mutation in those polycythemic patients. In this regard, by using Real Time PCR, JAK2 V617F mutation were detected in approximately 97 (95%) patients (Table.3). Among them 31 (32%) were female and 66 (68%) were males. These finding is supported by most of the studies in 2005, one of the study mentioned that every polycythemic patient would have JAK2 V617F gene mutation. [22] The mutation in JAK2 shows a continuous proliferation of mature cells. [23] Several other researchers stated in their studies that, JAK2 V617F was detected in 95% of patients of PV. [24] Alongside most of the national and international reports strongly support our study.

One of the Pakistani report mentioned, that the JAK2 V617F mutation was detected in almost 92.3% PV patients. [25] Another study from Pakistan stated that the JAK2 V617F mutation were detected in about 95% to 100% PV patients. [20] Some international reports also assured the presence of JAK2 V617F mutation in PV patients. A former report from France also claimed about 95% of occurrence of JAK2V617F mutation in PV patients. [26] According to a Malaysian study JAK2 V617F mutation is accountable for 95.8% of Polycythemia Vera. One of the study from Turkey mentioned that there were 95% of JAK2 V617F gene mutation in Polycythemic patients. [27]There is boundless resemblance and association between our findings and that of national and international reports, however the little difference between the percentages of mutation existence is due to the involvement of few numbers of individuals in the study and a single centered study. Hence this may be more cleared by doing large scale study. Therefore RT PCR is a reliable and accurate technique for the detection of the mutation in PV patients which facilitate the finding and also provide guidelines in the treatment of PV.

CONCLUSIONS

In our study, Real-Time PCR emerged as a highly accurate, reliable, and sensitive technique capable of detecting even minute mutations, as low as 1%. Real-Time PCR serves as a powerful tool for monitoring PV by identifying the JAK2 V617F mutation. There are several other techniques to detect JAK2 gene mutation in PV patients but those are costly while RT PCR is affordable and easily manageable. Moreover, the insights gleaned from Real-Time PCR analyses provide invaluable guidance for tailoring treatments to meet the specific needs of affected individuals.

COMPETING INTEREST: Authors declare that they have no conflict of interest.

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