ASXL1 MUTATIONAL ANALYSIS IN MYELOFIBROSIS PATIENTS IN KHYBER PAKHTUNKHWA

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ABSTRACT

Objectives: To determine the patients with MPNs for JAK2 mutations and to Identify ASXL1 mutation in JAK2 positive and JAK2 negative patients.

Methodology: This descriptive cross-sectional study consists of two consecutive phases. In the first phase, patients with myelofibrosis (MF) were identified. Both previously diagnosed and newly diagnosed MF cases were included. Data collection involved a comprehensive questionnaire including demographic details, clinical history, and physical examination findings. Blood samples were collected from participants for routine investigations and DNA extraction. The second phase consisted of genomic DNA extraction, and conventional PCR was performed to determine JAK-2 mutational status. Additionally, direct Sanger sequencing of ASXL1 was carried out for all patients.

Results: The study included 50 myelofibrosis (MF) patients diagnosed based on bone marrow biopsy and reticulin staining. The cohort comprised primary and secondary MF cases, including male and female participants. JAK2 mutation analysis revealed that 48 (96%) patients were JAK2-positive, while only 2 (4%) patients were JAK2-negative. Direct Sanger Sequencing analysis of ASXL1 Exon 12 identified missense variations in 2 (4%) out of 50 patients.

Conclusion: The high prevalence of JAK2 mutations (96%) among MF patients underscores its significance in disease pathogenesis and diagnosis.

Keywords: Janus Kinase 2, MPNs, CALR, ASXL1, Peshawar.

INTRODUCTION

Myelopoiesis begins when multipotent hematopoietic stem cells (HSCs) differentiate into primitive myeloid precursor cells. These cells differentiate into distinct myeloid precursors, following an intricate developmental pathway. Through carefully regulated genetic programs, these progenitor cells give rise to mature granulocytes (including neutrophils, eosinophils, and basophils) and monocytes [1]. This complex developmental process is orchestrated by specific transcription factors that regulate the expression of crucial cellular components, including adhesion molecules and hematopoietic growth factor receptors (HGFRs) [2].

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The term “myeloproliferative disorder (MFs)” was first used by William Damshek in 1951 to classify four related conditions: polycythemia vera, essential thrombocythemia, primary myelofibrosis, and chronic myeloid leukemia, which share clinical and biological feature [3]. In 2008, the World Health Organization (WHO) officially revised the terminology, adopting the term 'myeloproliferative neoplasms' (MPNs) as the new classification [4]. Philadelphia chromosome-negative chronic MPNs include three main disorders: primary myelofibrosis (PMF), Polycythemia Vera (PV) and essential thrombocythemia (ET) [5]. The global annual incidence rate of MPNs ranges from 0.44 to 5.87 per 100,000, with the lowest rates reported in Japan and Israel [6].

Classical MPNs are the most common myeloproliferative disorders and are associated with the absence of the BCR-ABL mutation. These disorders appears due to a single somatic cell mutation in hematopoietic stem cells, leading to clonal expansion and subsequent single or multilineage hyperplasia [7]. The key mutations in MPNs can be classified into four main types: JAK2, CALR, MPL, and ASXL1. Of these, CALR, JAK2 and MPL are thought to be the 'driver' mutations while ASXL1 mutations are associated with increased mortality rates [8]. The JAK2 mutation holds notable significance, being present in roughly 70% of all MPNs. Its

prevalence varies based on the specific disorder i.e., it is found in 95% of polycythemia vera cases and in 50-60% of cases of essential thrombocythemia and primary myelofibrosis. This mutation, detectable in hematopoietic stem cells, affects multiple cell lineages. It is present throughout the myeloid lineage and in specific lymphoid cells, primarily NK and B cells, with T cell involvement occurring later in disease progression. While the JAK2 mutation is generally not found in non-hematopoietic tissues, it can be found in the endothelial cells of the spleen and splanchnic veins in myelofibrosis patients. It is noteworthy that the JAK2V617F mutation occurs not only in classic MPNs, but also in some cases of refractory anemia [9].

ASXL1 (Additional Sex Combs Like 1) gene mutations were first reported in 2009 in myelodysplastic syndrome (MDS). Located on chromosome 20q11. ASXL1 mutations in myeloproliferative disorders primarily affect exon 12, with rare occurrences in other exons. These mutations, typically nonsense or frameshift, result in C-terminal protein truncation. Genetic alterations in ASXL1 leads to transformation of ET and chronic ET to PMF. Recent data suggest that ASXL1 mutations are frequently acquired in the later stages of the disease in MPN patients undergoing leukemic transformation. The study also identified acquired ASXL1 and TET2 mutations in only a single patient [10].

According to data published by the Dynamic Internation Prognostic System for PMF, patients with ASLX1 mutations have higher progression of disease with increase mortality rate. Evidence suggests that ASXL1 mutations are associated with reduced survival in patients with PMF. ASXL1 mutations increase the risk of acute myeloid leukemia (AML) transformation in patients with chronic myelomonocytic leukemia (CMML). ASXL1 mutations accelerate the progression to acute myeloid leukemia (AML) in myelodysplastic syndrome (MDS) patients and serve as a marker of poor prognosis [11]. The current study aimed to find the prevalence of JAK2 and ASLX1 mutations in PMF patients in Khyber Pakhtunkhwa.

MATERIAL AND METHOD

A cross-sectional study was conducted from January 2021 to January 2022 to achieve the research objectives. Myelofibrosis patients were recruited from three major healthcare centers in Peshawar, i.e., Lady Reading Hospital (LRH), Hayatabad Medical Complex (HMC), and the Institute of Radiotherapy and Nuclear Medicine (IRNUM). This multi-center approach allowed for a comprehensive sampling of myelofibrosis cases in the region. Institutional Ethical approval from Khyber Medical University (KMU) approved the study before the start of the study. The participants were selected through consecutive non-probability sampling, and the sample size was determined using the World Health Organization's calculation formula [12]. All previously diagnosed myelofibrosis patients, regardless of JAK2 mutation status or gender, were included, while non-consenting patients and those with other myeloproliferative neoplasms were excluded from the study. We collected comprehensive patient data, including demographics, family and clinical history, physical examination findings, and results of previous investigations, such as bone marrow aspirate and biopsy reports. A trained phlebotomist collected 5mL venous blood samples in EDTA tubes under aseptic conditions and promptly transported them to the KMU.

For hematological analysis, 3mL of blood underwent a complete blood count (CBC) using the Sysmex KX-21 hematology analyzer (Tokyo, Japan). The remaining 2 ml of blood was stored at 4°C for extraction of DNA. Bone marrow aspiration was performed on all patients following established protocols. The posterior superior iliac spine was selected as the aspiration site due to its high concentration of mesenchymal stem cells. Using a 20mL syringe, approximately 0.5mL of bone marrow was carefully extracted. To maintain sample integrity and prevent clotting, the aspirate was immediately transferred to an EDTA tube to prevent clotting. Immediately after aspiration, the fragments were carefully transferred to clean glass slides and air-dried, preserving the cellular morphology essential for accurate analysis. These prepared slides were then subjected to reticulin staining to highlight reticulin fibers in the bone marrow, allowing for precise evaluation of fibrosis extent and pattern. An expert hematopathologist then examined these stained slides to determine the grade and stage of myelofibrosis as per the World Health Organization (WHO) classification system.

To detect JAK2 gene polymorphisms, we employed allele-specific PCR amplification consisting of a 35-cycle PCR. Each cycle included DNA denaturation at 95°C for 30 seconds, primers annealing at 60°C for 30 seconds, and a 1-minute extension at 72°C. Amplified DNA samples were sent to Macrogen, South Korea, for ASXL1 sequencing. Data were

recorded in Microsoft Excel and analyzed using SPSS version 25. Descriptive statistics were calculated for all parameters, including means, standard deviations, and percentages.

Table 1: Primers Sequence for JAK2 and ASXL1 Amplification

Gene	Primer Sequence
JAK2-6171 **JAK2-617R-G**	5'-AAA-GGG-ACC-AAA-GCA-CAT-TGT-3' 5'-GTT-TTA-CTT-ACT-CTC-GTC-TCC-ACA-CAC-3'
JAK2-617R-T	5'-GTT-TTA-CTT-ACT-CTC-GTC-TCC-ACA-CAA-3'
ASXL1-F	5'-CCA-CCC-TGG-GTG-GTT-AAA-3'
ASXL1-R	5'-TCG-CTG-TAG-ATC-TGA-CGT-3' [13]

RESULTS

A total of 50 patients with myelofibrosis were enrolled in our study, of which 41 cases were of primary myelofibrosis and 9 cases of secondary myelofibrosis. The patient cohort exhibited a diverse age range from 20 to 80 years, with a mean age of 50.58 years (median 50.0, standard deviation 13.0). To facilitate age-related analysis, we stratified the patients into two groups: an adult group (20-50 years) of 28 patients and an elderly group (51-80 years) comprising 22 patients. The gender distribution showed a predominance of males, with 34 male patients compared to 16 female patients.

Using the European Consensus grading system, we stratified patients with myelofibrosis into three grades, as shown in **Table 2**. The analysis revealed that Grade II myelofibrosis was predominant, affecting 24 patients (48%) with primary myelofibrosis, 03 (6%) with post-ET myelofibrosis, and 03 (6%) with post-PV myelofibrosis. Among patients with Grade III disease, 16 (32%) had primary myelofibrosis, and 01 (2%) had post-PV myelofibrosis. Grade I myelofibrosis was the least common in only 03 (6%) patients.

The severity of anemia correlated with the progression of myelofibrosis (**Table 3**). Among Grade II myelofibrosis patients, 4 patients (8%) had mild anemia, 15 (30%) had moderate anemia, 4 (8%) had severe anemia, and 7 (14%) maintained normal hemoglobin levels. Grade III myelofibrosis patients showed a higher proportion of severe cases, i.e., 7 (14%), with 6 patients (12%) having moderate anemia and 4 (8%) showing normal hemoglobin levels. Anemia in grade I myelofibrosis was rare, with only 1 patient (2%) presenting with moderate anemia and 2 (4%) having normal hemoglobin levels.

JAK2 mutation analysis of our 50-patient cohort revealed that 48 patients (96%) were JAK2-positive, while only 2 (4%) were JAK2-negative. Among these, 3 patients (6%) with post-ET and 6 patients (12%) with post-PV were JAK2-positive, as shown in **Table 4**. Direct gene sequencing analysis in Korea identified genetic variations in two samples. Both samples were from female patients with post-PV myelofibrosis, aged 42 and 60 years, respectively. These patients presented with Grade 2 myelofibrosis (**Table 5**).

Table 2: Frequency of Patients w.r.t Grading of MF

Diagnosis	Grading of MF		
	Grade-I	Grade-II	Grade-III
PMF	1 (2%)	24 (48%)	16 (32%)
Post-ET	0 (0%)	3 (6%)	0 (0%)
Post-PV	2 (4%)	3 (6%)	1 (2%)

Table 3: Frequency of patients with Anemia w.r.t Grading of MF

Anemia	Grading of MF		
	Grade-I	Grade-II	Grade-III
Mild	0 (0%)	4 (8%)	0 (0%)
Moderate	1 (2%)	15 (30%)	6 (12%)
Severe	0 (0%)	4 (8%)	7 (14%)
Normal	2 (4%)	7 (14%)	4 (8%)

Table 4: Frequency of patients with Diagnosis and JAK-2 Status

JAK 2	PMF	Post ET	Post PV
Negative	2 (4%)	0 (0%)	0 (0%)
Positive	48 (96%)	3 (6%)	6 (12%)

Table 5: Characteristics of the Patients

S.No	Age	Gender	Hb (g/dl)	Diagnosis	Grading	Jak 2	ASXL1
1.	42 yrs	Female	8.0 g/dl	SMF	Grade-II	Positive	Missense
2.	47 yrs	Male	7.8 g/dl	PMF	Grade-III	Positive	Missense

DISCUSSION

As per the literature, the subtypes of BCR-ABL negative myeloproliferative (ET, PV, PMF) disorders share some common features exhibit unique clinical manifestations that distinguish them from one another, such as bone marrow findings and disease outcomes [14]. Despite significant advances in understanding genetic mutations over the past decade, the complete genetic pathogenesis of these disorders remains incompletely understood. Nevertheless, identified mutations are increasingly important in diagnosis and prognosis. The genetic landscape of MPNs is primarily characterized by two groups of mutations: driver mutations (CALR, JAK2, MPL) and additional mutations such as ASXL1 (additional sex combs like chromosome-1), which occur less frequently but can impact disease progression. The JAK2, CALR, and MPL mutations are considered 'driver mutations' and have been recently incorporated into the WHO diagnostic criteria for myeloproliferative neoplasms. Their prevalence varies by disease, i.e., in ET, JAK2 is found in approximately 60% of cases, CALR is observed in 22%, and MPL in 3% of cases. Interestingly, in polycythemia vera (PV), JAK2 mutations are present in approximately 98% of cases, and these mutations also play a significant role in the diagnosis of PMF [15]. These mutations also provide valuable prognostic information in myeloproliferative neoplasms. For instance, JAK2 mutations linked with increased chances of venous thromboembolism in subjects with essential thrombocythemia. Conversely, CALR mutations are predictive of better survival outcomes in patients with PMF [16]. Key molecular markers associated with poor prognosis in primary myelofibrosis (PMF) include mutations in ASXL1, SRSF2 (serine/arginine rich splicing factor-2), EZH2 (enhancer of zeste homolog-2), and IDH1/2 (isocitrate dehydrogenase 1 and 2). These genetic alterations significantly impact disease progression and patient outcomes, providing crucial insights into PMF pathogenesis [17].

In this study, we analyzed the prevalence of JAK2 and ASXL1 mutations in 50 patients from different regions of Khyber Pakhtunkhwa (KP), Pakistan, with a mean age of 48.9 years. This finding aligns with data from India in which majority of the population suffering with PMF had a mean age of 47 years were suffering from PMF [18]. In contrast, studies from Western countries showed higher median ages: 55 years in Sweden and 57 years in Germany [19, 20]. In our analysis, primary myelofibrosis showed a marked male predominance, with 29 males (71%) as compared to 12 females (29%), yielding a male-to-female ratio of 3:1. Our finding are in accordance with another Pakistani study in which a similar gender distribution (75% males, 25% females) was observed [21]. This male predominance was also observed in Indian studies [18]. However, data from Western populations suggests an equal gender distribution, highlighting potential geographical or ethnic differences in disease presentation [22]. Primary myelofibrosis (PMF) typically exhibits a gradual onset and progression, with diagnosis predominantly occurring in individuals over 50 years of age. A previous study conducted endorses this trend, revealing that the majority of PMF patients presented within the 50-60 year age range [21].

Analysis of bone marrow fibrosis grades revealed the global distribution as MF-0 in 25% of patients, MF-1 in 32%, MF-2 in 17%, and MF-3 in 26% of patients [23]. As per our study, most of the patients (60%) were presented with grade-II myelofibrosis, followed by grade-III (34%) and grade-I (6%). Several studies have evaluated the correlation between the symptomatology of primary myelofibrosis (PMF) and the grading of bone marrow fibrosis. A recent retrospective study has provided valuable insights into the initial presentation of primary myelofibrosis (PMF). The analysis, which examined 865 newly diagnosed PMF patients, revealed that a significant majority (565 patients, approximately 65%) presented with low-grade bone marrow fibrosis (MF0/1). Notably, these patients with early-stage fibrosis

also exhibited mild anemia [24]. Similarly, in our study, there was a related correlation between bone marrow fibrosis grade and hemoglobin levels in myelofibrosis patients. Low-grade bone marrow fibrosis (MF-I) was present in 6% of our cohort, and these patients exhibited either moderate anemia or normal hemoglobin levels. While patients with higher grades of bone marrow fibrosis (MF-2/3), a clear inverse relationship between fibrosis severity and hemoglobin concentration was identified, consistently demonstrated lower hemoglobin levels.

Recent research has refined our understanding of the prevalence of JAK2 V617F mutations across myeloproliferative neoplasms (MPNs). While this mutation is nearly ubiquitous in polycythemia vera (PV), its frequency in essential thrombocythemia (ET) and primary myelofibrosis (PMF) has been reported to be lower, typically ranging from 50-60% [25]. Our study, however, revealed a notably higher prevalence of JAK2 mutations in primary myelofibrosis patients. We found that 78% of our PMF cohort harbored JAK2 mutations, with only 4% testing negative. Understanding JAK2 mutations in PMF is crucial for diagnosis, risk stratification, and treatment decisions, particularly regarding using JAK inhibitors. Furthermore, patients suffering from MPNs can be ASXL-1 positive during the disease progression. Understanding these mutations is crucial for diagnosis, risk stratification, and treatment decisions in myelofibrosis patients. While JAK2 mutations are more common and directly linked to the disease mechanism, ASXL1 mutations appear to significantly impact prognosis and disease progression. Furthermore, according to the International Prognostic Scoring System (IPSS), ASXL1 mutations are associated with patients over 65 years of age, male gender, and lower platelet counts. According to a previous study, it was found that of 64 patients with PMF, a heterozygous mutation in ASXL1 was identified in 5 cases that were all JAK2V617F negative [26]. Our study revealed intriguing findings regarding ASXL1 gene variations in myelofibrosis patients. Among our cohort of 50 patients, we identified ASXL1 variations in only two individuals. Notably, both were female patients diagnosed with secondary myelofibrosis and presented with grade II myelofibrosis. The significance of ASXL1 mutations extends beyond their frequency. These mutations are increasingly recognized as important prognostic markers and are now used as inclusion criteria in numerous clinical trials for myelofibrosis and related myeloid disorders. The impact of ASXL1 mutations is thought to be mediated through distinct methylation changes in cancer-related genes, potentially driving disease progression [27]. While ASXL1 gene polymorphisms show potential as a therapeutic marker, their utility may be maximized when combined with other genetic and clinical factors. Further research is needed to fully elucidate the role of ASXL1 in treatment decision-making for myelofibrosis patients.

CONCLUSION

The high prevalence of JAK2 mutations (96%) among MF patients underscores its significance in disease pathogenesis and diagnosis. The identification of ASXL1 missense variations in a small subset of patients (4%) suggests a potential role for this gene in MF development or progression, albeit less common than JAK2 mutations in this cohort.

Authors Contribution

Laila Bahadur (LB): Manuscript writing and Laboratory work
Humaira Taj Niazi (HN): Manuscript editing and writing
Kulsoom Bahadur (KB): Sample collection and manuscript writing
Ansa Kulsoom Rehman (AR): Manuscript writing
Saliha Syed (SS): Results compilation and statistical analysis
Sidra Humayun (GF): Data analysis and manuscript compilation.

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Conflict of Interest

The author declares no conflict of interest.

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