Original Article

JAK2 V617F, MPL W515L and JAK2 Exon 12 Mutations in Chinese Patients with Primary Myelofibrosis

Jun Xia, Mi-ze Lu, Yuan-qiang Jiang, Guo-hua Yang, Yun Zhuang, Hong-li Sun, Yun-feng Shen*

Department of Hematology, Wuxi People's Hospital, Nanjing Medical University, Wuxi 214023, China

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ABSTRACT

Objective: JAK2 V617F, MPL W515L and JAK2 exon 12 mutations are novel acquired mutations that induce constitutive cytokine-independent activation of the JAK-STAT pathway in myeloproliferative disorders (MPD). The discovery of these mutations provides novel mechanism for activation of signal transduction in hematopoietic malignancies. This research was to investigate their prevalence in Chinese patients with primary myelofibrosis (PMF).

Methods: We introduced allele-specific PCR (AS-PCR) combined with sequence analysis to simultaneously screen JAK2 V617F, MPL W515L and JAK2 exon 12 mutations in 30 patients with PMF.

Results: Fifteen PMF patients (50.0%) carried JAK2 V617F mutation, and only two JAK2 V617F-negative patients (6.7%) harbored MPL W515L mutation. None had JAK2 exon 12 mutations. Furthermore, these three mutations were not detected in 50 healthy controls.

Conclusion: MPL W515L and JAK2 V617F mutations existed in PMF patients but JAK2 exon 12 mutations not. JAK2 V617F and MPL W515L and mutations might contribute to the primary molecular pathogenesis in patients with PMF.

Key words: Primary myelofibrosis; JAK2 V617F; MPL W515L; JAK2 exon 12; mutation

INTRODUCTION

Primary myelofibrosis (PMF) is a clonal stem cell disorder characterized by chronic myeloproliferation, atypical megakaryocytic hyperplasia, and bone marrow fibrosis. The disorder manifests clinically as anemia, splenomegaly due to extramedullary hematopoiesis (EMH), leukoerythroblastosis, and constitutional symptoms. Based on the seminal editorial by Dameshek in 1951[1], PMF is classified as one of the prototypic myeloproliferative disorders (MPD), along with polycythemia vera (PV) and essential thrombocytosis (ET). In 2005, using different approaches, several independent groups virtually simultaneously reported on a recurrent point mutation in the JAK2 tyrosine kinase (JAK2 V617F) in several MPD, including PV, ET, and PMF[2-6]. The allelic frequency of JAK2 V617F is upwards of 90% in PV, and roughly 50% in ET and PMF, as estimated by sensitive detection methodologies[7, 8]. The mutation has been described as a specific marker of MPD, neither detectable in healthy subjects nor in reactive proliferations[9, 10], although it could be present in rare cases of other clonal myeloid disorders[11, 12]. While JAK2 V617F is the predominant disease-associated allele in MPD, approximately 10% of patients meeting the clinical criteria for PV and 50% for ET and PMF do not present such mutations, and genetic analysis is still under progress to elucidate the responsible oncogenic events. More recently, two other related mutations have been described in patients with JAK2 V617F-negative PMF/ET (MPL W515L/K mutation) or PV (JAK2 exon 12 mutations).

The mutation involved in MPL 515, either W515L or W515K (MPL W515L/K), occurs in 8.5% of JAK2 V617F-negative ET patients[13], about 10% of JAK2 V617F-negative PMF patients[14-16], and 0% of PV[15]. Scott, et al. recently described mutations of JAK2 exon 12 in JAK2 V617F-negative patients with PV or

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*Corresponding author.
E-mail: yfshen21cn@yahoo.com.cn

idiopathic erythrocytosis[17]. Their findings have been confirmed by other studies[18-20].

Though JAK2617F and MPLW515L/K mutations in Chinese patients has been reported in several studies, JAK2 exon 12 mutations has been rarely researched in Chinese PMF patients. To investigate the prevalence of JAK2 V617F, MPL W515L and JAK2 exon 12 mutations in Chinese patients with PMF, we introduced allele-specific PCR (AS-PCR) and gene sequencing to screen these mutations in 30 PMF patients.

MATERIALS AND METHODS

Patients

Between January 2006 and June 2008, a total of 30 Chinese PMF patients and 50 healthy controls were enrolled in the present study. Nineteen patients were male and 11 were female (male to female ratio, 1.7), and the median age at diagnosis was 54 years (range, 32–77 years). All samples were taken on the same date for analysis of JAK2 V617F MPL W515L and JAK2 exon 12 mutations. A total of 2 ml peripheral blood samples of PMF patients were obtained at the time of initial diagnosis with informed consent, and genomic DNA was isolated with the use of the QIAmp DNA Blood Mini Kit (Qiagen, Hilden, Germany). The diagnosis of PMF was established according to 2001 World Health Organization (WHO) diagnostic criteria[21]. The patients were confirmed as BCR/ABL fusion transcript negative in contrast to CML as BCR/ABL positive.

JAK2 V617F and MPL W515L Mutations Analysis

AS-PCR was used for screening JAK2 V617F point mutation and MPL W515L mutation. PCR amplification was performed according as we previously described[22, 23]. For JAK2 V617F mutation, the electrophoresis showed that mutant allele is two bands including 203 bp and 364 bp but wild-type only 364 bp. For MPL W515L mutation, the electrophoresis showed that mutant allele is two bands including 279 bp and 409 bp but wild-type only 409 bp. The positive samples were all amplified only with the outer primer pair again and confirmed by sequence analysis.

JAK2 Exon 12 Mutation Analysis

We performed AS-PCR using DNA from total peripheral blood. The primers included JAK2 exon 12 control primers, and primers specific for the alleles containing the K539L mutation (leading to the replacement of lysine at position 539 with a leucine), the N542-E543del mutation (causing the deletion of asparagine at position 542 and glutamic acid at position 543), the F537-K539delinsL mutation (leading to the replacement of phenylalanine at position 537 through lysine at position 539 by a single leucine), or the H538QK539L mutation (causing a substitution of glutamine for histidine at position 538 and leucine for lysine at position 539) (Table 1)[17]. Each 25 μl PCR reaction solution contained approximately 25 ng of DNA template, 12.5 μl 2× taq PCR Master Mix (TIANGEN, KT201), and 0.4 μl (10 mmol/L) common reverse primer as well as 0.4 μl (10 mmol/L) two forward primers. PCR cycling parameters were: 94˚C for 4 min; 35 cycles of 94˚C for 30 s, 62˚C for 30 s and 72˚C for 60 s; followed by 72˚C for 10 min. The electrophoresis, on an ethidium bromide- impregnated 1.5% agarose gel, showed that mutant allele has two bands including 348 bp/342 bp and 496 bp but wild-type only 496 bp. The positive samples were all amplified only with the outer primer pair again and confirmed by sequence analysis using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Bio-Systems, Foster City, California, USA) on an ABI Prism 3700 DNA analyzer (Perkin-Elmer BioSystems). In addition, sequencing was also performed in patients whose AS-PCR electrophoresis had only one band in 496 bp.

Statistical Analysis

All statistical analyses were performed using the SPSS program for Windows (version 15.0) (SPSS Inc., Chicago, IL, USA). Comparisons between continuous variables were performed using the *t*-test, while categorical variables using the Chi-square test. The continuous variables included age and the counts of platelets and megakaryocytes in bone marrow. The categorical variables included gender and the incidence of thrombosis. $P<0.05$ was considered statistically significant.

RESULTS

Through AS-PCR combined with sequence analysis, samples from 30 patients with PMF were screened for the presence of JAK2 V617F, MPL W515L and JAK2 exon 12 mutations. Fifteen PMF patients (50.0%) carried JAK2 V617F mutation (Figure 1), and only two JAK2 V617F-negative patients (6.7%) harbored MPL W515L mutation (Figure 2). None had JAK2 exon 12 mutations. Furthermore, these three mutations were not detected in 50 healthy controls. There were no significant differences in median age at presentation or gender between JAK2 V617F-positive and JAK2 V617F-negative PMF patients ($P>0.05$). Seven JAK2 V617F-positive patients had thrombosis, but only three JAK2 V617F-negative PMF patients had

it. Patients with JAK2 V617F mutation had a higher counts of platelets and megakaryocytes in bone marrow than those in patients without JAK2 V617F mutation (P<0.05). Although the statistical power of this analysis was limited by the small number of patients with PMF in our cohort, two MPL W515L-positive PMF patients exhibited leukocytosis and thrombocytosis at the time of disease presentation. One of the two MPL W515L-positive patients had a rapidly progressive clinical course, with death 28 months after initial presentation from complications related to PMF.

Table 1. Primers and length of AS-PCR products

Primer	Sequence	Length of product (bp)
Forward primer-E1	5'-CTCCTCTTTGGAGCAATTCA-3'	
Reverse primer-E2	5'-GAGAACTTGGGAGTTGCGATA-3'	496
K539L	5'-CATATGAACCAAATGGTGTTTTCACTT-3'	342
N542-E543del	5'-CAAATGGTGTTTCACAAAATCAGAGATT-3'	342
F537-K539delinsL	5'-CATATGAACCAAATGGTGTTAATC-3'	342
H538QK539L	5'-CATATGAACCAAATGGTGTTTTCAATT-3'	348

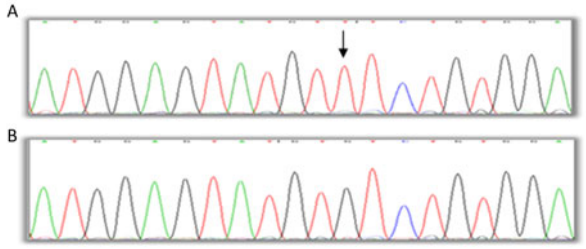

Figure 1. Sequencing chromatogram of JAK2 V617F. **A:** JAK2 V617F-positive (G→T); **B:** JAK2 V617F-negative.

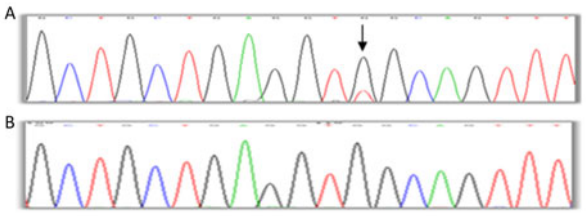

Figure 2. Sequencing chromatogram of MPL W515L. **A:** MPL W515L-positive (containing two peaks with "T" and "G"); **B:** MPL W515L-negative.

DISCUSSION

The WHO currently classifies PV, PMF, and ET together with chronic myelogenous leukemia, chronic eosinophilic leukemia, chronic neutrophilic leukemia, hypereosinophilic syndrome, and unclassifiable chronic myeloproliferative disease, under the rubric of chronic MPD[24]. However, PV, PMF, and ET have more common phenotypes with each other than with these other disorders, and this difference was confirmed genotypically by discovery of the JAK2 V617F mutation[5]. Extensive studies of JAK2 V617F have indicated that its frequency within PV, PMF and ET is variable, and is most common in PV. The JAK2 V617F mutation discovery was a big breakthrough in the history of BCR/ABL-negative MPD. Beside bringing a new pathogenetic view of these disorders and offering the potential for innovative target therapies, it provided a diagnostic and, possibly, prognostic molecular marker with high specificity[2-6]. Although the constitutively active JAK2 V617F mutant kinase is present in many patients with MPD, there are a significant proportion of PMF and ET patients who are JAK2 V617F-negative.

The molecular mechanisms of the majority of JAK2 V617F-negative MPD are unknown. It is hypothesized that the activation of JAK-STAT pathway might also occur as a consequence of activating mutations in certain hematopoietic-specific cytokine receptors, including erythropoietin receptor (EPOR), thrombopoietin receptor (MPL), or granulocyte-colony stimulating factor receptor (GCSFR). More recently, two novel somatic mutations of *MPL* gene with its coding function for MPL were detected in 8.5% of JAK2 V617F-negative ET patients[13], about 10% of JAK2 V617F-negative PMF patients[14-16], and 0% in PV[15]. So far, two different variants (the W515L and the W515K mutation) were identified, and the W515L mutation is more frequent than W515K[14, 15]. MPL W515L is an acquired mutation that induces constitutive cytokine-independent activation of the JAK-STAT pathway[14]. Although both JAK2 V617F and MPL W515L may transform hematopoietic cells to cytokine-independent growth, expression of MPL W515L in murine bone marrow causes a distinct phenotype notable for thrombocytosis, leukocytosis, and myelofibrosis, but not polycythemia. MPL W515L-positive PMF patients present with more

severe anemia than JAK2 V617F-positive patients, and in some studies, endogenous megakaryocyte colonies, but not endogenous erythroid colony formation, can be obtained from MPL W515L-positive cells[13]. These data suggest there are differences between JAK2 V617F and MPL W515 mutations in modulating clinical phenotype.

Most recently, other JAK2 mutations were described in JAK2 V617F-negative patients with PV and in "idiopathic" erythrocytosis[17]. The majority of such cases (10 of 11 in one study) were found to harbor one of four JAK2 exon 12 mutant alleles (F537-K539delinsL, H538QK539L, K539L, N542-E543del) with functional relevance that is similar to that of JAK2 V617F, and they can induce cytokine-independent/hypersensitive proliferation in EPOR-expressing cell lines and a PV-like phenotype in mice. The four newly described exon 12 mutations, which include both in-frame deletions and tandem point mutations, and appear to be specific to either PV or idiopathic erythrocytosis. Unlike the case with PV-associated JAK2 V617F, JAK2 exon 12 mutations are heterozygous but associated with stronger abnormal JAK2 activation.

AS-PCR was used to ascertain the real prevalence of these mutations in Chinese PMF patients. The first forward primer of AS-PCR is specific for the mutant allele and contains an intentional mismatch at the third nucleotide from the 3′ end. The outer primer pair including forward primer and reverse primer was designed to amplify fragments from both mutant and wild-type alleles as internal control. PCR in the number of electrophoretic bands can be straight-forward based on visual judgments. Compared with AS-PCR, the lack of internal negative reference system, complicated operation, increased pollution risk, the lack of intuitive, time-consuming and capital costs are the shortages of the traditional PCR. AS-PCR bands were confirmed with double recovery, purified and then sequenced to ensure the quality of research, but also cost savings.

In our study, applying an AS-PCR technique to a population of 30 patients with PMF, we obtained 50%, 6.7% and 0% of patients with JAK2 V617F, MPL W515L and JAK2 exon 12 mutations, respectively. MPL W515L mutation was only found in two JAK2 V617F-negative patients. These data provide genetic evidence that MPL W515L is a pathogenetic mutation in this subset of JAK2 V617F-negative PMF. We will also detect other related mutations such as MPL S505N and TET2 in Chinese PMF patients to further promote the MPN study.

The discovery of activating mutations in JAK2 and MPL has greatly improved our understanding of the pathogenesis of PMF. However, a significant proportion of patients with PMF are JAK2/MPL-negative, and the underlying cause for clonal hematopoiesis in these patients is yet to be discovered. Based on existing genetic data, we predict that additional oncogenic alleles in the JAK-STAT pathway will be identified in these patients, and the additional genomic and functional studies will provide further insight into the pathogenesis of JAK2/MPL-positive and -negative PMF.

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