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Determining Clinical Correleation of Calr Type 1 and Calr Type 2 Gene Mutations in Chronic Myeloproliferative Disease Cases

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Abstract

Research Article

Chronic myeloproliferative diseases (CMPD) are clonal hematopoietic disorders, with cellular proliferation, where one or more of the hematopoietic cells often undergo differentiation and maturation. In the Philedelphia (Ph) (-) CMPD group, the most common ones are polistemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). In CMPD patients, CALR (Calreticulin) mutations have been identified. In this study, identification of CALR mutations in Ph (-) CMPD patients and the effects of the presence of CALR mutations on the clinical findings of patients were investigated. A total of 76 Ph (-) CMPD cases including 35 ET, 1 PMF, 4 POST-PV, 4 POST-ET MF and 32 PV were included in the study. CALR mutations were studied using DNA sequence analysis method using DNAs isolated from venous blood of patients diagnosed with CMPD. In patients with PMF, reticulin fiber increase was found higher. Splenomegaly-correlated with disease stage was higher in PMF and PV cases, and 19.7% of cases had thromboembolic events, primarily MI, SVO, Budd Chiari and DVT. There was no significant difference between groups in terms of survival. The JAK2 mutation was evaluated in 45 cases with a 20% JAK2V617F mutation in ET, 3% in PV and 33.3% in PMF ($p > 0.05$). No JAK2 mutation was detected in 76 cases evaluated for CALR type 1 and CALR type 2 mutations. In JAK 2 (-) ET and PMF patients, CALR mutations were not detected. Between two mutations, the rate of type1 CALR mutation is 45-50%, while type 2 is 32-41% in the population.

INTRODUCTION

Chronic myeloproliferative diseases (CMPD) are clonal hematopoietic disorders in which one or more of the hematopoietic cells are usually associated with cellular proliferation and the maturation continues. It is generally characterized by an increase in the cell count due to an increase in mature cells. CMPD originates from genetically transformed hematopoietic cells and it has the potential of differentiation to myelopoiesis via differentiation[1]. The term CMPD was first proposed by William Dameshek in 1951 and was used to describe four classical myeloproliferative diseases with clinical and biological similarities to each other including polycythemia vera (PV), essential thrombocytosis (ET) primary myelofibrosis (PMF) and chronic myeloid leukemia (CML)[2]. More rare diseases such as chronic neutrophilic leukemia, chronic eosinophilic leukemia, systemic mastocytosis, atypical CML were included in the scope of CMPD later[3].

CML is a neoplasm which has been known for more than 30 years, and it evolves from the clone of the Philadelphia chromosome and its oncogene BCR-ABL1. It is characterized by leukocytosis in the foreground, and the risk of leukemia transformation is about 15%. The most common ones in the BCR-ABL1 negative CMPD group are PV, ET and PMF.

JAK2 (Janus Kinase 2) gene supplies directions to cells for producing the JAK2 protein. This protein assists cell growth and division and is important for controlling blood cell production within the bone marrow.

JAK2 exon 14 V617F mutation described in 2005 and it was showed that clonal development in 95-98% of PV patients and 50% in ET and PMF patients[4-8]. Mutations in JAK2 exon 12 in PV, MPL mutations in PMF and ET. However, JAK2 and MPL mutations were negative in ET and PMF in 35-40% of patients [9].

In 2013, calreticulin (CALR) somatic gene mutation was identified in JAK2 negative ET and PMF cases. The CALR involves 9 exons and localized at 19p13.2[10] CALR chaperone activity and its role in calcium homeostasis is identified: It is a highly conserved protein in endoplasmic reticulum (ER) that takes roles in cell proliferation, differentiation and apoptosis and immunogenic cell death.

CALR has many cellular functions in the cell membrane, cytoplasm, nucleus and extracellular matrix such as

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lipid and protein synthesis, Ca^{2+} storage, post-translational modification, in and out of the ER[11-12].

From 2013 up to now, 50 different CALR mutations have been defined in exon 9, and all of them are frame shift mutations[13]. The most common (approximately 80%) mutation was CALR 52-bp deletion p.L367fs * 46 (Type 1 CALR mutation) and 5-bp insertion p.K385fs * 47 (Type 2 CALR mutation). These mutations were shown in 67% of JAK2 and MPL negative ET patients and 88% in PMF patients[10].

In patients with CALR (+) ET, hemoglobin (Hb) and leukocyte counts decreased compared to JAK2 (+), increased platelet count and decreased incidence of cumulative thrombosis. Although there was a decrease in the incidence of disease progression compared to the JAK2 (+) ET patients, there was a prolongation of the survey, but no other differences were detected in JAK2 (+) patients according to some other studies[10].

It was first proposed in 2014 that CALR exon 9 mutations should be in the CMPD diagnostic algorithm due to its high frequency, disease course and prognosis[28]. As of August 2015, World Health Organization (WHO) updated the diagnostic criteria for PV, ET and PMF and included CALR mutations.

The aim of this study was to determine the CALR mutations in non-CML CMPD patients who were followed-up at the Hematology Outpatient Clinic of Ege University Medical Faculty Hospital between 2015-2016. CALR mutation was determined by Sanger sequence analysis method. The demographic characteristics, clinical and laboratory data of the patients were recorded. DNA isolation from peripheral blood was performed for CALR mutations and the effects of the mutation on clinical outcomes were investigated.

MATERIALS and METHODS

Analysis of Calreticulin Gene Mutations

Polymerase Chain Reaction (PCR) for CALR 52 bp deletion (L367fs * 46) and 5 bp insertion (K385fs * 47) in 9th exon was performed after DNA isolation (High Pure PCR Template Preparation Kit, Roche Applied Science, Germany) of blood samples taken from patients. After determining PCR products by gel electrophoresis and confirming, it was evaluated by Sanger Sequence method.

Gene specific primer sequences for the amplification of the CALR gene by PCR method were selected and synthesized. Accordingly, a DNA fragment of 483 base pair (bp) was amplified with the primer pair selected to detect mutations of 52 bp deletion (L367fs * 46) and 5 bp insertion (K385fs * 47) in the 9th exon of the CALR gene.

After PCR treatment, ExoSAP-IT ™ PCR Product Cleanup Reagent was used to purify the products. DNA sequencing of the purified samples was performed according to Sanger Sequence method. The regions to be assayed were amplified by PCR using specific primers. The results were evaluated with SeqScanner 2 software.

Statistical analysis

Spearman correlation coefficient was used for correlation between Pearson Chi-square, two continuous or discrete variables, and SPSS 22.0 for Windows for all analyzes. $P < 0.05$ was considered statistically significant.

RESULTS

Clinical Background

Among 76 patients included in the study, 46.1% had ET (n = 35), 32% had PV (n = 32) and 11.8% had PMF (n = 9). When we looked at the subgroups of cases followed with the diagnosis of myelofibrosis, 4 of them were PMF, 4 of them were POST-PV MF and 4 of them were POST-ET MF. 46.1% of the patients were female and 53.9% were male and no statistically significant difference was found between the groups in terms of gender distribution (p = 0.385).

The ages of the patients ranged between 28 and 89 years, the mean age was 60.8 ± 13.7 years. When considered as age groups, 64.5% of patients are between 0-65 years of age. There was no statistically significant difference in age distribution between the groups (p> 0.05).

When the laboratory findings of disease diagnosis and follow-up were examined, the mean blood leucocyte value was 11.3x10 ^ 3 / µL in ET patients (n = 35), and 10.4x10 ^ 3 / µL in PV cases (n = 32) and in PMF (n = 9).) 12x10 ^ 3 / µL. Mean hemoglobin values were 13.4 g / dl for ET (n = 35), 17.8 g / dl for PV (n = 32) and 13.7 g / dl for PMF (n = 9). Mean platelet content was 972x10 ^ 3 / µL for ET (n = 35), 81x10 ^ 3 / µL for PV (n = 32) and 915x10 ^ 3 / µL for PMF (n = 9).

Hemoglobin values of patients with PV were higher than PMF and ET. The amount of platelets was found to be higher in the PMF group as well as in the ET diagnosis and in the MF secondary to ET. No statistically significant difference

was found in the statistical analysis of the distribution of these values in the diagnostic groups (p> 0.05). Although sedimentation and LDH values were higher than expected in PMF, no statistically significant difference was found between the groups.

It was observed that the levels of reticuline increase were low in patients with ET and PV, whereas in patients with PMF, the degree of reticulin increase was high. 42.9% (n = 15) of the ET 35 cases were grade 0, 22.9% (n = 8) grade 1, 22.9% (n = 8) grade 2 and 11%, 4 (n = 4) were identified as grade 3. Among 32 PV cases, 43.8% (n = 14) were grade 0, 34.4% (n = 11) were grade 1, 21.9% (n = 7) as grade 2 It was. 11.1% (n = 1) of the 9 PMF cases were grade 0, 22.2% (n = 2) were grade 4. Reticuline fiber increase was found to be higher in patients with PMF due to the increase in fibrosis, and it was statistically significant between the groups. (p = 0.001).

When the distribution of the presence of splenomegaly in the diagnosis groups in patients with CMPD was analyzed, splenomegaly was observed in 22.9% (n = 8) of the ET cases (n = 35) and 77.1% (n = 27) were not observed. While splenomegaly was observed in 0.6% (n = 13) of the PV cases (n = 32), it was not detected in 59.4% (n = 19). While splenomegaly was observed in 88.9% (n = 8) of PMF cases (n = 9), no was found in 11.1% (n = 1).

The distribution of splenomegaly rates among the diagnostic groups was statistically significant (p = 0.001). The distribution of hepatomegaly according to Table 1 is given below; according to the diagnosis, no statistically significant difference was found within the groups (p> 0.05).

Table 1: The distribution of hepatomegaly and splenomegaly according to the diagnosis (p>0.05)

Diagnosis	Hepatomegaly		Splenomegaly	
	Yes	No	Yes	No
ET (n=35)	3 (8,6%)	32 (91,4%)	8 (22,9%)	27 (77,1%)
PV (n=32)	6 (18,8%)	26 (81,2%)	13 (40,6%)	19 (59,4%)
PMF(n=9)	3 (33,3%)	6 (66,7%)	8 (88,9%)	1 (1,11%)
P*	0,16		0,001	

19.7% of the patients had a history of thromboembolic event that is common in CMPD patients. 17.1% of ET patients, 21.9% of PV patients and 22.2% of PMF patients had a history of thromboembolic event, especially MI and SVO, as shown in Table 2. However, no significant difference was found between the groups in terms of statistical significance (p = 0.871).

Table 2: Distribution of thromboembolic events according to diagnosis

Diagnosis	Thromboembolic Event	
	Yes	No
ET (n=35)	6 (17,1%)	29 (82,9%)
PV (n=32)	7 (21,9%)	25 (78,1%)
PMF(n=9)	2 (22,2%)	7 (77,8%)
P*	0,871	

The clinical follow-up period of the patients was 12 months for ET, 107 months for PV and 148 months for PMF. In the follow -up of patients, 1 patient with PV was diagnosed to AML and the patient was lost. High allogeneic stem cell transplantation was performed in 2 patients with 1 PMF and 1 Post-ET MF.

In the follow-up, 21.1% (n = 16) of the cases were ex due to transformation to 1 AML and other causes. However, there was no statistically significant difference between the groups in terms of survival rates (p = 0.139).

In the retrospective review of patients' data, 59.2% of the patients had JAK2 mutations. JAK2 V617F mutation was found in 20% (n = 7) of the ET (n = 20) cases, while 14.3% (n = 5) were found to be mutant (wild) type. While the JAK2 V617F mutation was detected in 34.4% of the cases (n = 11), it was found that 28.1% (n = 9) were wild type. JAK2 V617F mutation was detected in 33.3% of PMF cases (n = 7), while 11.1% (n = 1) were found to be wild type. The presence of JAK2 V617F mutation was not found to be statistically significant among the diagnostic groups. (p = 0.294)

In our study, the most common (approximately 80%) mutation in JAK2 (-) ET and PMF patients was CALR 52-bp deletion p.L367fs * 46 (Type 1 CALR mutation) and 5-bp insertion p.K385fs CALR mutation was not detected in our cases. Because of these results, no evaluation was made for clinical correlation.

DISCUSSION

Chronic myeloproliferative diseases (CMPD) are clonal hematopoietic disorders in which one or more of the hematopoietic cells, together with cellular proliferation, usually undergo diffe-

rentiation and maturation. The result is usually characterized by an increase in cell count due to an increase in mature cells [1]. The most common ones in the BCR-ABL1 negative CMPD group are PV, ET and PMF. In 2005 JAK2 mutation was defined and studies on the genetic factors involved in pathogenesis increased.

With the JAK2 V617F mutation described in 2005, it was possible to show clonal development in 95-98% of patients with PV and 50% of ET and PMF patients[4-8]. Then, JAK2 exon -12 mutations in PV, MPL mutations in PMF and ET have been described [9]. After all exom sequencing studies in patient group with JAK2 and MPL mutations (-), CALR exon 9 somatic mutations have been identified by Klampfl et al in 2013, and later on by Nangalia et al [10,13].

Klampfl et al showed that 67% of ET and 88% of CALR mutations in PMF were found in JAK2 and MPL (-) patients[10]. In the same year, in all exome sequencing studies conducted by Nangalia et al. The mutations in all these studies were in the form of insertion and deletion in exon 9 [10,13].

From 2013 up to now, 50 different CALR mutations have been defined in exon 9 and all were frameshift mutations [13,29]. The most common (approximately 80%) mutations are CALR 52-bp deletion p.L367fs * 46 (Type 1 CALR mutation) and 5 -bp insertion p. 385fs * 7 (Type 2 CALR mutation). Among these two mutations, Type 1 CALR mutation is seen 45 -50%, Type 2 CALR mutation is 32-41% of the population[13]. There are many studies about hemogram values, risk of thrombosis and survival in patients with mutations in the literature for these mutations.

In accordance with the literature, in our study male gender for PV and PMF cases and female gender for ET cases were higher; however, there was no statistically significant difference between the groups.

Similar to a study conducted with 1545 patients by WHO-linked Myeloproliferative Neoplasms International Study Group on Research and Treatment (IWG-MRT)[23], in our study, the mean Hb was 15.3 g / dl, the leukocyte count was 11900 / μL, and the platelet count was 758.000 / Ml. Hemoglobin values of patients with PV were higher than PMF. The amount of platelets was found to be higher in the PMF group due to MF cases secondary for ET as expected. Hemoglobin and erythrocyte increase in the PV, thrombocyte increase among the primary diagnostic features for ET is the expected cause of this result. No statistically significant difference was found in the distribution of these values in the diagnostic groups ($p > 0.05$).

According to Italian Polycythemia Vera Group data, 19% of patients have a history of thrombosis[15]. Ischemic stroke, myocardial infarction and transient ischemic attack are the most common thrombotic events. Deep vein thrombosis, pulmonary embolism and peripheral vascular occlusion are common in patients. A serious thrombotic event associated with PV is Budd-Chiari syndrome caused by hepatic venous or inferior vena cava thrombosis[16-21]. Among our cases, 19.7% of cases had a history of frequent thromboembolic event in MP patients. 17.1% of ET patients, 21.9% of PV patients and 22.2% of PMF patients had a history of thromboembolic event, especially MI, SVO, DVT and Budd-Chiari. As expected, no statistical significance was found between these groups.

When we evaluated our cases in terms of reticular fibrosis with progression of disease in the bone marrow, it was observed that the majority of patients in ET and PV group had a low degree of reticulitis in accordance with the definition of disease.

The majority of PMF patients (66.6%) were found to have a high degree of reticin in Grade 2 and above. In all of these diseases, the clinical picture may be in a fibrotic appearance which may overlap with each other [25]. In the subgroup evaluation of the PMF group, it was associated with increased fiber retention of the reticular in cases of myelofibrosis secondary to PV and ET.

As stated by Swerdlow et al., splenomegaly increases in parallel with the increase in bone marrow reticle, as a sign of increased extramedullary hematopoiesis in PMF, ET and PV, especially in advanced stages [25]. This pattern was consistent with expected characteristics.

Splenomegaly was associated with a significant association between the disease groups and the presence of significant splenomegaly in the PMF group due to the advanced stage of the secondary myelofibrosis disease. In terms of the presence of hepatomegaly, significant statistical difference between the groups was not found as expected.

The transformation of myeloid metaplasia to myelofibrosis or leukemia (AML or MDS) in PV is a possible cause of mortality [15]. The results of the two clinical studies indicate that advanced age (> 70 years) and treatment with cytoreductive drugs other than hydroxyurea poses a significant risk for acute leukemia or MDS development [14,26].

Advanced age (> 60 years) and duration of the disease are risk factors for the development of secondary myelofibrosis[14,27]. The relative risk for myelofibrosis development is shown as 15.2% if the disease duration exceeds 10 years [27]. The clinical follow-up period of the patients was 12 months for ET, 107 months for PV and 1-8 months for PMF.

During the follow-up period, 1 patient with PV was diagnosed to AML and the patient was lost. 4 patients had post-polycythemic myeloid metaplasia and 4 patients had postthrombocytic myeloid metaplasia. Consistent with the literature, these patients had a history of advanced age (> 60 years) and a history of cytoreductive treatment. However, there was no statistically significant difference between the groups in terms of survival rates.

With definition of the JAK2 V617F mutation, it was possible to demonstrate clonal development in 95-98% of PV patients, 50% in ET and PMF patients (50-70% in ET, 40% in PMF) [4-8,24].

During the retrospective screening of the cases, 59.2% of the patients were evaluated by JAK2 mutation. The JAK2 V617F mutation was found in 20% of T cases, 34.4% of PV cases and 33.3% of PMF cases.

The presence of JAK2 V617F mutation was not found to be statistically significant among the diagnostic groups. When the data were compared with the literature, the rates in the literature were found to be lower. This situation was related to data loss due to long follow-up period, the fact that some cases were diagnosed before 2005 and JAK2 mutation could be examined in Hematology clinic after 2009, and the number of cases was not evaluated.

It was first proposed in 2014 that CALR exon 9 mutations should be in the CMPD diagnostic algorithm due to its frequency, disease course and prognosis.[28] As of August 2015, WHO (World Health Organization) updated the diagnostic criteria for PV, ET and PMF by adding CALR mutations.

Therefore, in our study we aimed to investigate, the most common (approximately 80%) mutations in JAK2 (-) ET and PMF patients which are CALR 52-bp deletion p. L367fs * 46 (Type 1 CALR mutation) and 5-bp insertion p.K385fs * 47 (Type 2 CALR mutation) and these mutations were not detected in our patients.

In order to reduce morbidity and mortality, studies on this subject are increasing for new treatment targets. Illumination of the pathogenesis of the disease will be associated with clinical and survival, as well as paving the way for new studies in terms of targeted therapies. We expect further studies to clarify the pathogenesis of chronic myeloproliferative disease and pave the way for new research to be planned for targeted therapies.

Conflict of Interest

The authors declare that there is no conflict of interest.

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