Diversities of Calreticulin Gene Mutations in Macedonian Patients With Essential Thrombocythemia

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Abstract

A wide range of different calreticulin mutations were detected in 150 Macedonian patients with essential thrombocythemia; these mutations were associated with a distinct clinical phenotype with a milder clinical course of the disease compared with that in patients with a JAK2 V617F mutation. Polymerase chain reaction/capillary electrophoresis is the method of choice for the analysis of calreticulin mutations.

Background: Acquired calreticulin (CALR) gene mutations are one of the molecular hallmarks of essential thrombocythemia (ET). It has been suggested that patients with ET with CALR mutations are associated with a distinct clinical phenotype. **Patients and Methods:** We evaluated the clinical and molecular features of 150 patients with ET followed over a period of 15 years. The screening for the presence of insertion/deletion mutations in CALR exon 9 was done with a fluorescent polymerase chain reaction/capillary electrophoresis procedure. Sanger sequencing of CALR exon 9 was used for the characterization of mutations and for the analysis of triple-negative patients. **Results:** CALR mutations were detected in 42 (28%) patients. The most common CALR mutations were type 1 and type 2, which were present in 11 (26.2%) and 20 (47.6%) patients, respectively. Additionally, 10 different small insertion/deletions (3 known and 7 new) were detected in 11 patients, resulting in an altered calreticulin C-terminal end. The clinical characteristics of all CALR+ patients with ET were in line with previously published data for this subset of patients. **Conclusion:** Our results showed that a wide range of different CALR mutations are associated with a distinct ET clinical phenotype that is associated with the male gender, younger age at diagnosis, higher platelet and lower leukocyte and erythrocyte counts and lower hemoglobin level, and a milder clinical course. The relatively high frequency of new insertion/deletion mutations indicate that the use of fluorescent polymerase chain reaction followed by capillary electrophoresis is the method of choice for the analysis of these defects.

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Introduction

Essential thrombocythemia (ET) is a clonal "classical" myeloproliferative neoplasm (MPN) characterized with megakaryocytic hyperplasia, splenomegaly, and sustained peripheral blood thrombocytosis (> 450,000/mm^3).[1] The clinical course of ET is complicated by hemorrhagic and thrombotic episodes, as well as the potential for transformation to myelofibrosis.[2] For many years, there were no specific histologic, cytogenetic, or molecular markers for ET, and the diagnosis was one of exclusion.[3] The discovery of the unique gain-of-function somatic JAK2V617F mutation in the group of myeloproliferative disorders changed the diagnostic landscape of MPNs.[3-5] The mutation was found with a frequency of 55% to 65% in patients with ET and was adopted as a molecular criterion for the diagnosis of those patients.[2] In addition, in 2006,

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activating mutations in the thrombopoietin gene (MPL) were found in 5% to 10% of the patients with JAK2-negative ET.[6]

The clonal marker was missing for one-third of the patients with ET until 2013, when somatic mutations in the calreticulin (CALR) gene, an endoplasmic reticulum (ER) chaperon, were detected in nearly 70% of JAK2- and MPL-negative patients with ET.[7,8] CALR is a multifunctional chaperon protein that plays a role in Ca^{2+} homeostasis and prevents the secretion of glycoproteins from the ER. Outside of the ER, CALR plays a role in transcription regulation, proliferation, apoptosis, phagocytosis, cell adhesion, and immune response.[9,10] The mechanism with which CALR mutations induce MPNs was unknown until recently, when Chachoua et al showed that CALR mutations specifically activate the thrombopoietin receptor and JAK2, confirming that the basic characteristic of the MPNs is the constitutive activation of the JAK-STAT pathway.[7]

All described mutations were small insertions or deletions in exon 9 of the CALR gene, resulting in translational frameshift and, hence, alteration in the C-terminal part of the protein with a loss of the terminal KDEL (lysine, aspartic acid, glutamic acid, leucin) motif, which is a major determinant for the retention of the protein in the ER, and generation of a common 44 amino acids positively charged C-terminal stretch, which is a highly potent glycan binding site. Analyses of the potential effects of the CALR mutation on the disease phenotype showed that CALR mutations are associated with a distinct clinical phenotype of ET, characterized by a preference for the male gender and younger age, higher thrombocytes and lower leucocytes and erythrocytes at diagnosis, and generally, a more benign course of the disease compared with other mutations.[8,11]

In order to extend further these observations, we conducted a prospective-retrospective study aimed at determining the frequency and pattern of CALR mutations and their clinical correlates in patients with ET from the Republic of Macedonia.

Material and Methods

Patients

The study group consisted of 150 unrelated adult (> 15 years) patients diagnosed with ET in the last 15 years at the University Clinic of Hematology. The diagnosis of ET was made according to the 2008 World Health Organization criteria.[1] The median follow-up was 49 months (range, 13-227 months). JAK2V617F and MPL (W515L and W515A) mutations were previously detected in 78 (52.0%) and 5 (3.3%) patients, respectively. All patients, regardless of their JAK2V617F and MPL mutation status, were tested for CALR mutation from the stored genomic DNA samples. Clinical and laboratory data at diagnosis and during follow-up were obtained from patient charts according to the protocol approved by the Institutional Review Board of the University Clinic of Hematology.

Molecular Methods

Fluorescent polymerase chain reaction was used for the amplification of exon 9 of the CALR gene according to the protocol described by Nangalia et al[9] using a forward primer labeled with 6-fluoroscein. The products obtained were analyzed with a capillary electrophoresis on an ABI 3500 Genetic Analyzer (ThermoFisher). All samples that showed additional (smaller or larger) picks other than the normal 321 bp fragment were further analyzed with Sanger sequencing using BigDye Terminator, version 3.1, Cycle Sequencing Kit (ThermoFisher). Exon 9 of the CALR gene was also analyzed with the Sanger sequencing in the 25 patients negative for either the JAK2 V617F, CALR, or MPL mutations.

Statistical Analyses

Statistical comparison between categorical variables was performed with the Student *t* test, χ^2 statistics, or the Fisher exact tests using SPSS 18 software.

Results

The molecular analyses showed that CALR mutations, which were mutually exclusive with JAK2 and MPL mutations, were present in 42 (28%) patients. All mutations were small insertion/deletions in exon 9 of the CALR gene. Further analysis with the Sanger sequencing of this region did not reveal the presence of a point mutation in any of the patients that were considered as triple-negative. The most common CALR type 1 [52-bp deletion (c.1092_1143del)] and CALR type 2 [5-bp insertion (c.1154_1155insTTGTC)] mutations were present in 11 (26.2%) and 20 (47.6%) patients, respectively. In addition, 10 different CALR mutations, including 6 insertions and 4 deletions, were detected, 7 of which were not described previously. All mutations led to a frameshift that resulted in a protein with an altered C-terminal, which includes the deletion of the terminal KDEL sequence.

The clinical and laboratory features at diagnosis and the long-term prognosis of CALR+, JAK V617F+, MPL+, and triple-negative patients with ET are presented in Table 1. Correlation of the data showed that patients with the CALR mutation exhibited a statistically significant preference toward the male gender ($P = .002$) and had a higher platelet count at the time of diagnosis compared with both patients with mutated JAK2 ($P = .047$) and with triple-negative ($P = .024$) patients. Patients with CALR type 2 had a statistically significant higher platelet count compared with patients with CALR type 1 mutation ($P = .04$). We also found that patients who are CALR+ have a lower leukocytes count ($P < .001$) and decreased risk of thrombosis, both arterial ($P = .04$) and venous ($P = .01$), compared with patients with a JAK2 V617F mutation. Survival curve analyses of patients with different mutations showed marked disparity in the overall survival, but statistically significant difference has not been reached as yet (Figure 1). However, there was a trend for better survival for patients with CALR+ compared with those with JAK2 V617F and the patients who were triple-negative, which was most prominent for patients with CALR type 2 mutations.

Discussion

Our study shows that CALR mutations have an overall frequency of 28% in Macedonian patients with ET. They were mutually exclusive with JAK2 V617F and MPL mutations, as described previously for most patients with MPN,[8-13] and their frequency in JAK2- and MPL-negative patients with ET was 63%. This is slightly lower from the initially reported frequency of 73%, but similar to the one reported in published studies.[8-13] Overall, JAK2 V617F, CALR, and MPL mutations were detected in 84% of patients with ET from our cohort, which has important implications