FOLIA HISTOCHEMICA ET CYTOBIOLOGICA
Vol. 49, No. 2, 2011
pp. 267–271

ORIGINAL STUDY

VIA MEDICA

JAK2 mutation status, hemostatic risk factors and thrombophilic factors in essential thrombocythemia (ET) patients

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Abstract: The recently discovered JAK2 V617F point mutation, found in 50–60% of ET patients, has been reported to be associated with a higher risk of thrombotic events. In this study, we explored if JAK2 V617F mutation, or coexisting thrombophilic and hemostatic risk factors, contributed to these complications. We examined 32 patients with ET, and looked for pathogenetic JAK2 V617F mutation and prothrombotic genes mutations: factor V Leiden, prothrombin and MTHFR. We also evaluated plasma levels of fibrinogen, factors VIII and XII, AT, protein C, protein S and serum level of homocysteine. Urokinase concentration was assessed in patients' plasma as well as platelet lysates. There was no difference in the number of thrombotic complications between ET patients with and without JAK2 mutation. However, we found a number of thrombophilic and hemostatic risk factors that could contribute to thrombotic complications in ET patients. (*Folia Histochemica et Cytobiologica 2011; Vol. 49, No. 2, pp. 267–271*)

Key words: JAK2 mutation, ET, thrombophilic factors

Introduction

The recently discovered tyrosine-kinase activating JAK2 V617F point mutation is found in 50–60% of essential thrombocythemia (ET) patients. An increased risk of thrombosis in patients with this mutation, especially the homozygous type, has been reported. Other factors which can increase the risk of thrombosis arising from ET itself are: hereditary thrombophilic factors like antithrombin, protein C and protein S deficiency, the mutation of the prothrombin gene, the Leiden mutation of factor V gene, the MTHFR gene mutation, decreased FXII level, hyperhomocysteinemia, as well as increased levels of some coagulation factors such as FVIII, vWF, and fibrinogen.

In this study, we explored whether the JAK2 V617F mutation or coexisting thrombophilic and hemostatic risk factors contribute to thrombotic complications in ET patients.

Material and methods

We examined 32 patients with ET (24 females, eight males, mean age 56.0 ± 14.2 years). The 32 comprised ten untreated patients, ten treated with anagrelide, nine with hydroxyurea and three treated with both drugs. The control group (CG) consisted of 20 healthy volunteers (14 females, six males, mean age 41.4 ± 8.3). We searched for pathogenetic JAK2

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V617F mutation and prothrombotic factors such as: Factor V Leiden, prothrombin gene and MTHFR gene mutations. We also evaluated plasma levels of fibrinogen, factor VIII, factor XII and AT using coagulometric methods (reagents from Dade Behring) and protein C and free protein S using coagulometric methods (reagents from HemosIL™). Urokinase concentration was assessed in plasma and in platelet lysates using ELISA (IMUBIND uPA kit from American Diagnostica Inc.). Homocysteine level was evaluated using the immunoenzymatic method (kit from DPC). Platelet activation was studied using monoclonal mouse anti-human: CD42b/RPE,CD61/FITC,CD62P/RPE antibodies from Dako and flow cytometry to detect CD61/CD42b and CD61/ /CD 62P protein expression on the platelet surface.

JAK2 V617F point mutation was detected using allele-specific-PCR (Roche) [1]. Peripheral blood genomic DNA was isolated using QIA amp DNA Blood Mini KIT (QIAGEN). The tetra-primer PCR method was used to genotype point V617F mutation [2].

To evaluate prothrombotic mutations, blood samples for deoxyribonucleic acid (DNA) analysis were collected into tubes containing disodium ethylenediaminetetraacetic acid (EDTA). DNA was extracted from blood samples by the salting out procedure devised by Miller et al. [3]. PCR products were screened for Leiden mutation using MnL1 restriction enzyme according to Bertina et al. [4]; for prothrombin gene mutation G20210A acc. to Poort et al. [5] using HindIII restriction enzyme; and for MTHFR C677T acc. to Frosst et al. [6] using HinfI restriction endonuclease.

Results

Patients' characteristics

Mean platelet count was 785 ± 32 G/l for ET patients and 250 ± 54 G/l for the control group. The difference between both groups was statistically significant ($p < 0.001$). White blood cell count (WBC) was also higher in ET patients than in the control group: 8.3 ± ± 3.7 G/l and 5.4 ± 1.4G /l respectively, $p < 0.001$. The concentration of uPA was higher in patient plasma compared to the control group (0.635 ± 0.232 ng/ /ml vs. 0.447 ± 0.115 ng/ml respectively, $p < 0.05$). Mean uPA concentration measured in platelet lysates was similar in both groups (ET 0.317 ± 0.135 ng/10^9 platelets, CG 0.290 ± 0.065 ng/10^9 platelets). Median homocysteine serum level was similar in both groups (ET 9.77 μmol/l, P 25–75% 8.11–12.4 vs. CG 9.21 μmol/l, P 25–75% 7.94–10.60 μmol/l). In the ET group, hyperhomocysteinemia was detected in 31% of patients, and only in two persons simultaneously with MTHFR gene mutation. In two patients from this group, the level of homocysteine was extremely high. One of them, a 76 year-old woman, underwent heart infarct three times. Her other risk factors were hypertension and JAK2 mutation. Her homocysteine level was extremely high (24.6 μmol/l), without MTHFR gene mutation. Coronarography revealed normal coronary arteries. The other patient, a 79 year-old man with a very high homocysteine level (21.7 μmol/l) had obliterative atheromatosis diagnosed at the same time as ET. No JAK2 and MTHFR gene mutations were detected.

In 11 patients from the ET group, 19 MPD-related thrombotic vascular events occurred (Table 1). There were ten (50%) arterial thrombosis events including: ischemic strokes (three events), myocardial infarctions (five events), femoral artery embolism (one event) and obliterative atheromatosis (one event).

Events involving the venous system were: deep vein thrombosis (four events), toe vein thrombosis (one event), superficial phlebitis (two events), stillbirth (one event) and portal and splenic vein thrombosis (one event). In the latter case, diagnosed with portal and splenic vein thrombosis in 1999 when she was 38 years old, ET was only discovered seven years later. Her platelet count in 1999 was normal. These thrombotic events were preceded by DVT that occurred probably because the patient received birth control pills. Consequently, in 2004 the patient underwent a splenectomy. After the splenectomy, platelet count continued to increase and in 2006, after bone marrow trephine biopsy, ET was finally diagnosed. This patient carries a JAK2 V617F mutation which was diagnosed in 2007, but at the time of the occurrence of portal and splenic vein thrombosis we had no way of evaluating it. Other thrombotic risk factors which still exist in this patient are hypercholesterolemia and a high level of antinuclear antibodies. Only in 2010 was a homozygous genotype of MTHFR T/T determined with normal homocysteine level.

Thrombotic complications were noticed in two persons in the control group (Table 1).

Table 1. Specification of thrombotic complications

Group	Thrombotic complication (number)	Thrombotic complication — specification
ET	11 (6 with and 5 without JAK mutation)	Stillbirth (1), DVT (1), portal vein thrombosis (1), heart infarct (2), superficial thrombophlebitis (1), cerebro-vascular event (2), cerebrovascular event + DVT (1), great toe vein thrombosis (1), peripheral arterial disease (1)
Control	2	DVT after delivery (1), minor cerebrovascular event (1)

Table 2. Specification of bleeding complications

Group	Bleeding complication	Bleeding complications — specification
ET	7 (3 with and 4 without JAK mutation)	Alveolar hemorrhage (1), thigh hematoma after trauma (2), hypermenorrhea (2), foot hematoma after trauma (1), gastrointestinal bleeding (1)
Control	0	None

Table 3. Summary of mutation status in ET and control groups

Group	JAK2 mutation	MTHFR mutation	G 20210A mutation	Leiden mutation	Leiden + + MTHFR mutation	JAK2 + + MTHFR mutation
ET (32)	18 (60%) Heterozygous	10 (37%) Heterozygous 1 (3.6%) Homozygous	0	2 (7%)	2 (7%)	6 (20%)
Control (20)	0	12 (60%) Heterozygous 1 (5%) Homozygous	0	2 (10%)	1 (5%)	0

Bleeding episodes were noticed in seven ET patients (Table 2). In two patients these episodes (alveolar hemorrhage and gastrointestinal bleeding) occurred when the platelet count was very high (in the first patient 2,300 G/l, in the second 1,100 G/l). Because of these clinical manifestations, ET was diagnosed. In three previously diagnosed patients, hemorrhages after trauma were observed and the platelet count was below 1,000 G/l. In the last two patients, hypermenorrhoea was observed long before the diagnosis of ET was made, but at that time platelet count exceeded the normal limit.

Patients with thrombotic complications were older than those without any complications (65.9 ± 10.8 vs. 47.7 ± 13.0 years, $p < 0.05$) and had higher fibrinogen and factor VIII levels (420 mg/dl vs. 302 mg/dl $p < 0.05$, and 115% vs. 88%, $p < 0.05$ respectively). Patients with thrombotic complications as compared statistically to those with bleeding episodes were also significantly older. Additionally, among ET patients with thrombotic vascular events, six were older than 60 years at the time of the first event. Analyzing the patients with regard to the relation of vascular event to the time of diagnosis, we came up with the following: four patients underwent vascular event long before ET diagnosis; in three patients the ET diagnosis was made as a result of a vascular event; and in four patients a vascular event occurred after an ET diagnosis was made. A statistically significantly lower CD61/ /42b protein expression was detected (1.74% vs. 10.00%, $p < 0.05$) in patients with thrombotic complications compared to patients without these complications.

Assessment of point mutations

The JAK2 V617F point mutation was detected in 18 (60%) tested ET patients but not in the control group. In all cases, the heterozygous genotype was determined. In 39.3% of ET patients, the MTHFR (MTHFR C677T) gene mutation was detected. In one ET patient (3.6%), the homozygous genotype was detected. This was the person who developed portal and splenic vein thrombosis. All other patients had the heterozygous genotype. In six patients, JAK2 V617F point mutation and MTHFR C677T mutation were found simultaneously.

In the control group, 13 (65%) persons presented with MTHFR gene mutation. In one person, the homozygous genotype (MTHFR T/T) was determined. This was the person with thrombotic complications (DVT) after child delivery. All the other 12 (60%) subjects had the heterozygous genotype (MTHFR C/T) and among them one had had a minor cerebrovascular event. No prothrombin gene G20210A mutation was found in either ET patients or the control group. In two patients, and in two control subjects, heterozygous genotype for Leiden mutation was detected. In two patients, and in one control subject, heterozygous genotypes for Leiden and for MTHFR gene mutations were discovered simultaneously (Table 3).

There was no difference in the number of thrombotic complications between ET patients with and without JAK2 mutation. The ET patients with JAK2 point mutation had a higher level of red blood cells (4.38 ± 0.57 T/l and 3.86 ± 0.55 T/l, $p < 0.05$ respectively), confirming hypersensitivity of mutated progenitor cells to cytokines like erythropoietin (EPO).

Assessment of coagulation factors

The activity of factor XII in JAK2 positive patients was lower than in negative ones (83.2 ± 25.5% and 109.0 ± 20.4% respectively, $p < 0.05$). In ET patients carrying the MTHFR gene mutation (compared to

wild type for MTHFR) a higher level of plasma urokinase was observed (0.747 ± 0.3 ng/l and 0.547 ± 0.2 ng/ml, $p < 0.05$), which might indicate that the fibrinolytic system is partly activated in ET patients, especially those carrying the MTHFR gene mutation, as a mechanism compensating for a hypercoagulable state present in these patients.

Discussion

In 1951, William Dameshek classified polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) as pathogenetically related myeloproliferative disorders (MPD) [7], since renamed myeloproliferative neoplasms (MPN). In 2005, somatic activating mutation in the *JAK2* nonreceptor tyrosine kinase (*JAK2 V617F* point mutation) was identified in most patients with PV (95%), and in a significant proportion of patients with ET (50–60%) and PMF (50–60%) [8]. This gain of function mutation causes hypersensitivity of JAK2 $V617F^+$ cells to cytokine stimulation [9]. In our study group, the JAK2 V617F point mutation was detected in 60% of ET patients. Campbell et al. in 2005 suggested that ET patients expressing JAK2 mutation appear to have a "PV-like" phenotype [10]. Similarly, in our ET patients with JAK2 point mutation, a statistically significantly higher level of red blood cells was found, confirming the hypersensitivity of mutated progenitor cells to cytokines like EPO and "PV-like" phenotype. Campbell also suggested that ET patients carrying JAK2 V617F point mutation seem to have an increased incidence of venous thrombosis compared to JAK2-negative subjects. Among patients with thrombotic complications, JAK2 mutation was present in six persons. We did not notice any differences in the number of thrombotic complications between ET patients with and without JAK2 mutation. The prevalence of intra-abdominal (hepatic, portal and mesenteric) vein thrombosis is unusually high among patients with PV and ET. Splanchnic (portal and hepatic) vein thromboses have been repeatedly reported in relatively young female patients and are often early symptoms which can manifest themselves long before the correct diagnosis [8]. The same situation was observed in our young female patient with portal and splenic vein thrombosis which had occurred seven years before ET was diagnosed. In this patient, JAK2 V617F mutation and homozygous type of MTHFR T/T mutation were discovered simultaneously, and although the homocysteine level was normal, the existence of two potentially prothrombotic mutations seemed to potentiate the risk of thrombotic complications. In 2009, Jamrozek-Jedlińska et al. presented seven patients tested due to idiopathic portal or splenic vein thrombosis. In six of them, JAK2 V617F mutation was discovered, suggesting a myeloproliferative disease as the primary cause of thrombosis [11]. According to Primignani et al. [12] and Kiladijan et al. [13], all subjects manifesting a splanchnic vein thrombosis should be investigated for an occult MPD and searched for the JAK2 mutation as a matter of routine.

In the PVSG (Polycythema Vera Study Group) study, the platelet count closest to a thrombotic event did not predict its occurrence. The same information was provided by the ECLAP study. Major thrombosis occurred in 8.3% and 9.3% of patients whose baseline platelet count was greater or lower than 400 G/l, respectively [14].

Given such findings, it is not surprising that thrombocytosis is not considered among the criteria for thrombosis risk assessment in MPD. In our study group, most MPD-related thrombotic events (15 out of 19) occurred when platelet count was greater than 400 G/l. And nine of them occurred before a diagnosis of ET had been made. In our opinion, this was due to the fact that before 2007 we used older diagnostic criteria which recommended testing for ET when the platelet count exceeded 600 G/l. On the other hand, it has been proved by some investigators that a high platelet count can contribute to vascular events only in the microcirculatory system because the platelet count reduction lowers the risk of microcirculatory disturbances [15]. The vast majority of our patients reported a disappearance of erythromelalgia, or a considerable reduction of its intensity, after decreasing platelet count.

The established risk factors for thrombosis in both ET and PV are old age (> 60 years) and previous thrombosis history [16]. We have observed that among our ET patients, thrombotic complications often occurred at older age and those patients presented more than one episode.

Among hereditary thrombophilic factors which can increase the risk of thrombosis are: antithrombin, protein C and protein S deficiency, mutation of the prothrombin gene and the Leiden mutation of factor V gene. They are known to have an important function especially in the pathogenesis of venous thrombosis. Among our ET patients, protein S deficiency was diagnosed only in one person who, besides erythromelalgia, presented also one episode of TIA. The Leiden mutation of factor V was diagnosed in two ET patients. No clinically significant thrombotic event occurred in these patients, although one of them presented infertility. The Italian guidelines for ET management recommend routine screening only in ET patients with either a personal or familial history of thrombosis [17].

In our opinion, routine screening for thrombophilia in all ET patients might be very useful, especially done simultaneously with the assessment of other thrombotic risk factors, because all these factors presented together can amplify the thrombotic risk arising from ET itself. To establish the proper role of thrombophilic factors, it would be necessary to test a larger group of patients.

In 39.3% of ET patients, and in 60% of persons from the control group, the MTHFR 677C/T gene mutation was detected. In one ET patient and in one person from the control group, the homozygous genotype was discovered and both subjects developed thrombotic complications. Khandanpour et al. [18] argued that being homozygous for the MTHFR C677T allele was associated with an increased risk of PAD (peripheral arterial disease). A few months later, McGimpsey et al. [19] stated more precisely that retinal vein occlusion was caused by elevated total homocysteine, rather than homozygosity for the MTHFR C677T genotype. We suppose that not only a higher homocysteine level, but also a homozygous mutation of the MTHFR gene, is associated with an increased risk of thrombotic complications.

Conclusions

1. In the group of patients with a heterozygous JAK2 point mutation, we have not observed an increased number of thrombotic complications.

2. To evaluate the risk of thrombosis it would be necessary to assess not only JAK2 mutational status, but also additional thrombotic risk factors. This is becoming increasingly important, because due to new diagnostic criteria of ET, we can diagnose this disorder at a younger age and nowadays the observation period before cytoreductive treatment is longer. During this observation period, it is possible not only to detect, but also to actively correct, as many thrombotic risk factors as possible.

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Submitted: 29 June, 2010
Accepted after reviews: 30 November, 2010