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Research paper

Increased Lipocalin 2 level may have important role in thrombotic events in patients with polycythemia vera and essential thrombocythemia

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ABSTRACT

The aim of our study was to evaluate the possible effect of lipocalin 2 on thrombotic events in polycythemia vera and essential thrombocythemia patients.

The samples of 89 patients were collected and RNA based method was used to evaluate the relative gene expression level of lipocalin 2.

74 samples were available for evaluation. Drawing a cut off point at level 30 relative expression rate, 13 patients with elevated lipocalin 2 expression had thrombotic events during the course of their disease.

Based on these data high lipocalin 2 expression level seems to have strong positive predictive value on thrombotic events in patients with polycythemia vera and essential thrombocythemia. Lipocalin 2 may be useful in thrombotic risk stratification in these patients.

1. Introduction

Polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF) are phyladelphia- negative chronic myeloproliferative neoplasms (MPN) characterized by thrombophilic behavior with excess cardiovascular mortality [1–4].

In the European collaboration study on low dose Aspirin in polycythemia (ECLAP), which was the largest epidemiologic study in PV, cardiovascular mortality accounted for 41% of all deaths (1.5 deaths per 100 persons per year), mainly due to coronary heart disease (15% of all deaths), congestive heart failure (8%), nonhemorrhagic stroke (8%), and pulmonary embolism (8%). The cumulative rate of nonfatal thrombosis was 3.8 events per 100 persons per year, without a difference between arterial and venous thrombosis [5].

According to the ET studies [6–8], the rate of fatal and nonfatal thrombotic events ranged from 2% to 4% patientyears and the incidence of arterial events was 2–3 times higher than that of venous events in ET patients.

The relatively low thrombotic rate in PMF could depend on other competing events, such as acute leukemia development or other noncardiovascular major complications, including early death [9].

The thrombophilic state manifests in microcirculatory disturbances and arterial and venous thromboses. The relationship between these clinically distinct entities of MPNs and clinical manifestations of thrombophilia has been extensively investigated over the last decades. The pathogenesis of thrombophilic state in patients with MPN is currently believed to be multifactorial in origin. Clinical factors such as age, obesity, hypertension, previous history of thrombotic events, and hyperlipemia are known as poor prognostic factors in thrombosis. The incidence of thrombotic events in PV or ET increases with age and history of thrombosis, but not with platelet count [10–12]. Advanced age has also been identified as one of several risk factors in PMF-associated thrombosis [9].

Additional contributing factors may also play role in thrombotic events of MPNs such as circulating procoagulant microparticles that express platelet and endothelial markers, impaired endothelial function and repair system [13,14], blood hyperviscosity [15], increased activation of neutrophil, monocyte or platelet [16,17], and acquired activated protein C (APC) resistance [18].

Some studies has drawn attention to leukocytosis, which contribute to increased risk of thrombosis in these patients [19,20].

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Activated neutrophils and monocytes could promote a hypercoagulable state. The possible mechanisms are the formation of leukocyte-platelet aggregates [21], upregulation of neutrophil or monocyte tissue factor expression, and abnormal release of procoagulant cellular proteins [22].

Despite of the abundant theories of the mechanisms underlying the thrombotic state of MPNs, there is no clear and definite answer to this question. The data from earlier studies are often conflicting or difficult to interpret in regard of their clinical or pathogenetic significance [9].

Recently the scientific interest is focused on the neutrophil gelatinase-associated lipocalin (lipocalin-2; LCN2), which has been implicated in the pathobiology of MPNs [23]. Increased LCN2 gene expression was shown in CD34 cells isolated from primary MF (PMF) and polycythemia vera (PV) patients, and LCN2 levels were elevated in the plasma of MPN patients [24]. Kagoya and collegues demonstrated in a mouse model that JAK2V617F-positive cells elaborate LCN2, which results in DNA damage in neighboring normal cells and cells belonging to the malignant clone by generating reactive oxygen species (ROS) [25]. It is well known that ROS are affecting endothel cells impaired in their function, therefore disrupt the balance between antithrombotic and thrombotic factors. We therefore have examined whether the LCN2 level has a predictive value in the thrombotic events in patients with MPN.

2. Materials and methods

2.1. Eligibility criteria

Eligibility criteria included: age 18 years or over; previous pathological confirmation of PV and ET. PV and ET were diagnosed according to WHO 2008 criteria [26]. No patients had received aspirin or any cytoreductive (hydroxiurea or anagrelide) treatment for ET or PV over the previous 7 days of blood sampling. Anticoagulant treatment was maintened in patients who had previously a thrombotic event. None of the patients had symptoms of active infections, inflammatory diseases, or kidney failure at the time of investigation. No patient was studied during the acute phase of thrombosis. We have collected laboratory (WBC, Hb, PLT, CRP) and anamnestic data (familiar thrombofilia, dyslipidemia, hypertension and diabetes mellitus) at the time of thrombotic events, which could have a role in development of thrombotic events.

2.2. Diagnosis of thrombosis

Vascular thrombosis was defined as a history of occlusive vascular events [27] . Major events included peripheral thrombosis (peripheral arterial thrombosis, deep venous thrombosis, and pulmonary thromboembolism), cardiac vascular complications (angina pectoris and myocardial infarction), central nervous system vascular complications (transient ischemic attack, stroke, and retinal vessel thrombosis), and intraabdominal vascular complications (splenic-portal thrombosis and Budd-Chiari syndrome). Minor events were superficial thrombophlebitis of the extremities. Visual complaints, headache, dizziness, tinnitus, or acroparesthesias were not considered thrombotic events.

2.3. Ethics and study management

The study was conducted according to good clinical and laboratory practice rules and the principles of the Declaration of Helsinki. Informed written consent was obtained after the purpose, nature, and potential risks were explained to the subjects.

2.4. Measurement of LCN2 mRNA levels using quantitative real-time PCR (Q-PCR)

Total RNA was extracted from peripheral blood samples using Trizol reagent (Ambion) as recommended by the manufacturer. Two micrograms of RNA were reverse transcribed with the High- Capacity cDNA Reverse Transcription Kit (Life Technologies) according to the manufacturer's instructions. The Q-PCR analysis was performed using a Taqman probe based gene expression assay (Hs01008571_m1, Life Technologies) according to standard protocols with GUSB (Hs99999908_m1) used as endogenous control. The displayed values were calculated by the ΔC_T method and represent relative LCN2 expression values normalized to GUSB expression.

2.5. Statistical analysis

Commercially available statistical software (Social Science Statistics; www.socscistatistics.com) was used to calculate statistics. Student *t*-test was used to evaluate significance of variables between thrombosis positive and negative subgroups, even as between LCN2 < 30 and LCN > 30 relative expression level subgroups. This statistical method was used to evaluate the significance of lipocalin 2 relative gene expression levels between JAK-2 positive and negative groups and ET and PV subgroups.

Crude odds ratio (OR) and 95% CI for thrombotic risk were estimated for high and low LCN 2 relative gene expression. A LCN2 relative gene expression cutoff of 30 level was set up to distiguish between high and low thrombotic risk. In a multivariate analysis logistic regression was performed to adjust for sex, age, diagnosis, Jak2V617F mutation, LCN2 30 cutoff level, blood parameters, BMI, CRP, and cardiovascular risk factors (such as presence of dyslipidemia, hypertension or diabetes mellitus). Adjusted OR values (and 95% CIs) were also calculated.

3. Results

3.1. Patient characteristics

89 patients with PV and ET were enrolled to this prospective study. LCN 2 relative gene expression analysis was conducted in case of these 89 patients. Measurement of 15 samples were missed because of technical problems. These patients were excluded from the data analysis. Data of 37 patients with ET and 37 patients with PV were assessed. 35 patients had a thrombotic event in the past. The JAK-2 mutation analysis showed positivity in 19 cases (51%) of ET patients. All patients with PV were positive for JAK-2 mutation (37 patients; 100%). The main characteristics of the patients are shown in Table 1.

3.2. LCN 2 relative gene expression results

The LCN 2 relative gene expression level did not differ among JAK-2 positive and JAK-2 negative patients. The mean lipocalin 2 relative gene expression value was 15.33 (ranges: 0.47–109.14) in patients with ET and it was not significantly higher in patients with PV (24.87; ranges: 7.38–419) (Table 1.).

Samples of patients with thrombotic events showed higher LCN 2 relative expression values. Therefore we have choosen a cut-off point at 30 LCN 2 relative gene expression level, where the crude OR was 4.487 for thrombotic risk and the p-value was 0.034. This result is significant at $p < 0.05$.

We compared characteristic variables and laboratory values of thrombosis positive and thrombosis negative subgroups, including LCN2 relative genexpression level >30 and LCN2 relative gene expression level <30 subgroups (Tables 2 and 3)

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