Acta Haematologica Polonica 2021
Number 4, Volume 52, pages 284–290
DOI: 10.5603/AHP.2021.0055
ISSN 0001–5814
e-ISSN 2300–7117

REVIEW ARTICLE

Calreticulin, a multi-faceted protein: thrombotic and bleeding risks in CALR mutation positive essential thrombocythemia

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Abstract

Essential thrombocythemia (ET) is a clonal disorder of a multipotent hematopoietic progenitor cell. In most patients, a driving mutation of Janus kinase 2 gene, calreticulin gene or myeloproliferative leukemia virus oncogene is detected. The occurrence of thrombotic and/or bleeding complications is very typical in manifestations of ET, with many cases of both occurring in the same patient. The thrombotic or bleeding phenotype can be a consequence of the coexistence of driving and non-driving molecular mutations and polymorphisms, affecting the platelet number and function. This paper discusses the nature of this disease, paying special attention to calreticulin gene function.

Key words: CALR, JAK2, MPL, acetylsalicylic acid, thrombosis, bleeding, ERp57, calnexin pathway, store-operated calcium entry, platelet function

Acta Haematologica Polonica 2021; 52, 4: 284–290

Introduction

Essential thrombocythemia (ET) is a clonal disorder of a multipotent hematopoietic progenitor cell. In 75–89% of ET cases, the driving mutations of Janus kinase 2 gene (*JAK2*), calreticulin gene (*CALR*) or myeloproliferative leukemia virus oncogene (*MPL*) are detected with frequencies of 61–65%, 13–22%, and 1–2%, respectively [1–6]. All of the mutations (*JAK2*, *CALR*, *MPL*) identified to date share the common characteristics of constitutive activation of tyrosine kinase-dependent signaling pathways and cytokine independent cellular proliferation [7, 8].

The clinical disease manifestation differs depending on the driving mutations and co-operating mutations in the myeloid genes status. ET patients are at risk of polycythemic transformation (*JAK2*V617F positive cases) and myelofibrotic transformation (*CALR* mutation positive cases, and patients with co-operating mutations in the myeloid genes). Leukemic transformation is rare, but possible due to the 'transforming' mutations acquisition (*TP53*, *RUNX1)* or overexpression of MDM2/MDM4 by hematopoietic progenitor cell(s) [9–11]. The leukemic transformation risk is also higher in ET patients with extreme thrombocytosis [12].

The main factors influencing the overall survival of ET patients are a previous thrombosis episode, leukocytosis, and the presence of co-operating mutations in the myeloid genes [13].

The risk of thrombosis is especially high in ET patients with *JAK2* mutation, a history of previous thrombosis, and advanced age (≥60 years). It has been also postulated that *JAK2* variant allele frequency (VAF) can influence venous thromboembolism [14]. A detailed analysis of thrombotic risk, depending on the type of driver mutation status, showed 5-year thrombosis-free survival rates of 93%, 91% and 88% for patients carrying the *JAK2*V617F, *MPL*

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Received: 25.04.2021 Accepted: 12.05.2021

and *CALR* mutation, respectively [15]. Recently published data documents a lower risk of venous thrombosis in ET patients carrying the *CALR*-type 2 mutation [16]. Interestingly, also the lower incidence of arterial thrombosis is consistent mostly with *CALR* mutations and/or extreme thrombocytosis [17].

In 3–18% of ET patients, bleeding occurs as an initial presenting symptom [18, 19]. The annual frequency of bleeding and major bleeding complications amounts to 4.6 and 0.79 per patient per year, respectively [20]. A report by Stuckey et al. [21] showed that the major bleeding complications frequency after an average follow-up period of 87.7 months was 6.6% (91 hemorrhagic events in 1,380 patients observed). Of 249 patients from the very-low risk group, 12 with an unknown driver mutation status experienced severe bleeding (4.8%). Interestingly, nine of them (75%) were treated with an anti-aggregatory drug.

The results of large clinical trials have revealed that major bleeding during follow-up occurs in 6% of WHO-ET defined patients, at the rate of 0.79% of patients per year. A detailed analysis of the WHO-ET patients confirmed previous hemorrhage and acetylsalicylic acid (ASA) as independent risk factors for bleeding complications. It should be mentioned that the study did not include the mutational status of the ET patients studied (*JAK2* vs. *CALR* vs. *MPL* positive) [19]. In a post hoc survey of 311 patients diagnosed with ET (mostly included in the prospective UK-PT1 trial) and receiving ASA with either hydroxyurea or anagrelide, an increased grade of bone marrow reticulin fibrosis predicted higher rates of major hemorrhage during the follow-up [22, 23].

Thrombosis and bleeding scoring systems for risk stratification in ET patients

Thrombosis risk in ET patients can be calculated with the help of the IPSET, IPSET-thrombosis and revised IPSET thrombosis scores based on age, previous history of thrombosis, *JAK2* mutation positivity, and the presence of cardiovascular risk factors [24, 25]. Recently, a mutation-enhanced international prognostic system has been proposed (Table I).

There is as yet no final agreement regarding the bleeding risk factors in ET patients. The bleeding risk is higher in ET patients with a history of previous major bleeding and high platelet count (≥1,500 $\times 10^9$/L) [13]. The bleeding risk in individual cases however may be also influenced by acquired coagulation abnormalities. This is observed in ET patients with extreme thrombocytosis and the symptoms of acquired von Willebrand syndrome (AvWS) due to consumption coagulopathy. For this reason, the administration of ASA is not recommended, if the ristocetin cofactor activity is <30% [26, 27]. The bleeding risk assessment in ET cases should be made with caution, because AvWS symptoms can also present in patients with near-normal platelet counts [28, 29] (Table II).

In a prospective study of the myeloproliferative neoplasms (MPN) registry of the Study Alliance Leukemia, bleeding events were rarely diagnosed before the MPN diagnosis, and their frequency was constant over a period of 160 months. However, the study was limited by the fact that the analysis was performed in a group of both ET and polycythemia vera (PV) patients, independently from the driver mutation status [30]. Another unresolved problem is the issue of hemorrhagic complications severity assessment in patients with ET due to the use of different bleeding intensity scales, e.g. International Society on Thrombosis and Haemostasis (ISTH), ISTH-like, World Health Organization and Common Terminology Criteria for Adverse Events. This may be the reason for the underestimation of the low and moderate bleeding frequency in ET patients. The data concerning the severe complication frequency is more accurate, and confirms that 13.7% of deaths in ET and PV patients was caused by bleeding, especially by fatal cerebral hemorrhage [20]. The pathogenesis of bleeding complications in ET patients is likely multifactorial, including alterations of primary hemostasis (mainly related to vascular endothelial cells dysfunction), AvWS, as well as quantitative and qualitative platelets abnormalities. It has been also postulated that anti-platelets drug and/or anticoagulants administration may influence the bleeding risk in individual patients.

It has been shown that bleeding episodes are more frequently observed in MPN patients who have been treated with anti-platelet or anticoagulant drugs (61.3% at time of diagnosis vs. 72.4% at time of bleeding) [20]. The risk of bleeding with prominent thrombocytosis is even more evident than an increased risk for thrombosis [31], and major bleeding risk is higher in patients with platelet count >1,000.0 $\times 10^9$/L receiving anti-platelet therapy [32]. Recent data shows that prophylactic administration of ASA exacerbates the risk of bleeding, particularly in *CALR*-mutated ET patients, independently from the platelet count [20]. Interestingly, in *JAK2*V617F-mutated ET patients, low-dose ASA administration is associated with no effect on the risk of bleeding [32].

Clinical significance of extreme thrombocytosis in ET patients

At the time of diagnosis, extreme thrombocytosis (ExT, defined as a platelet count ≥1,000.0 $\times 10^9$/L) is present in 22% of ET patients [33, 34]. In the Mayo Clinic MPN database, 18% of adult patients (192/1,070) with ET were aged below 40 and 50% of them presented ExT at the time of diagnosis. Driver mutational status analysis revealed that young patients with ExT harbored the *CALR* gene mutation

Table I. Thrombotic risk factors and thrombosis risk categories in essential thrombocytosis patients

Scale/risk	IPSET-thrombosis	Revised IPSET-thrombosis	Mutation-Enhanced International Prognostic System (MIPSS-ET)
Factors	Age >60 years =1 point Cardiovascular risk factors (tobacco use, diabetes, hypercholesterolemia, hypertension) =1 point Previous thrombosis =2 points *JAK2*V617F =2 points	Thrombosis Age *JAK/MPL* mutation	Adverse mutations *SRSF2, SF3B1, U2AF1, TP53* =2 points Age >60 years =4 points Male sex =1 point Leukocyte count ≥11.0 ×10^9/L =1 point
Category	**Low:** 0–1 point **Intermediate:** 2 points **High:** ≥3 points	**Very low** No thrombosis history Age ≤60 years No *JAK2* or *MPL* gene mutation **Low** No thrombosis history Age ≤60 years *JAK2* or *MPL* mutation **Intermediate** No thrombosis history Age >60 years No *JAK2* or *MPL* mutation **High** Thrombosis history Age >60 years *JAK2* or *MPL* mutation	**Low:** 0–1 point **Intermediate:** 2–3 points **High:** ≥4 points

Table II. Postulated bleeding risk factors in essential thrombocythemia patients

Author	Bleeding risk factor
Rumi et al. [13]	1. History of previous major bleeding 2. Platelet count ≥1,500.0 ×10^9/L
Tefferi et al. [26, 27]	3. Ristocetin cofactor activity <30%
	4. *CALR* mutation*#
	5. Clonal hematopoiesis indeterminate potential (CHIP) associated mutations — i.e. *IDH2**
	6. Germline polymorphisms predisposing for bleeding

*Postulated, #documented in the case of antiplatelet drug administration

more frequently than the *JAK2* mutation (46% vs. 35%). The frequency of arterial thrombosis and major hemorrhage rates at, or prior to, diagnosis also differs between young ET patients with and without ExT (2% vs. 8% and 15% vs. 7%, respectively). Previous data implied that ExT was an independent risk factor for leukemic transformation of ET [12]. This was confirmed by recently published results showing that ExT is an independent predictor of leukemia-free survival and overall survival in ET patients aged below 40 [35].

Role of driver mutations in pathogenesis of bleeding in patients with essential thrombocythemia

It cannot be excluded that driver mutation-specific abnormalities of platelet function play an important role in the pathogenesis of bleeding complications in ET patients. The data published so far in this field is limited. It has been documented that abnormal function of Janus kinase 2, the signal transducer and activator of transcription pathway

(*JAK2*-STAT pathway), may be responsible for abnormal platelets function in platelet aggregation studies. The phosphorylation of JAK2 in thrombin stimulated human platelets was previously reported by Rodriguez-Linares et al. [36]. Also, the regulatory role of STAT3 in collagen-induced platelet aggregation was confirmed by Zhou et al. in 2013 [37]. The involvement of *JAK2*-STAT3 pathway in the process of collagen-induced platelet activation through the activation of *JAK2*-JNK/PKC-STAT3 signaling was documented by Lu et al. [38]. The critical role of JAK2 in this process was supported by the observation that *JAK2* inhibitor AG490 (tyrphostin) attenuated collagen-induced platelet aggregation and calcium mobilization in a concentration-dependent manner [38].

Potential role of calreticulin in bleeding predisposition

CALR is made up of three protein domains: 1) an amino N-terminal lectin binding domain containing an endoplasmatic reticulum (ER) targeting signal sequence; 2) a proline-rich P-domain containing high-affinity binding sites for Ca^{2+}; and 3) a C-domain containing multiple low-affinity Ca^{2+}-binding sites and an ER retention signal (KDEL). Within the endoplasmatic reticulum, CALR participates in the control process of newly synthesized proteins (conformational dependent molecular sorting). Misfolded or unfolded proteins are retained in the ER, thereafter transported to the cytosol, and finally ubiquinated and degradated by the proteasome [39]. Due to the physiological role of CALR and the key role of calcium ions homeostasis and calcium ions flow in platelets, it is possible that abnormal cellular localization of CALR and abnormal CALR-associated cellular storage of calcium ions (including megakaryocytes and platelets) may be responsible for abnormal platelet function and an increased risk of bleeding. In 2009, Reilly et al. [40] demonstrated that calreticulin in platelets was localized to the granulomere. Co-immunoprecipitation techniques, however, did not show an interaction between calreticulin and platelet glykoprotein $\alpha_{IIb}\beta_3$ under various platelet activation states.

In 2013, Klampfl et al. [41] and Nangalia et al. [42] described new genetic variants of *CALR* in patients with ET and primary myelofibrosis. More than 50 different types of *CALR* exon 9 mutants have been found in ET patients. All of these mutants lead to a 1-bp frameshift and loss of the KDEL sequence (endoplasmatic reticulum retention peptide) and the original *CALR* stop codon [43]. The most frequent variants, type 1 (c.1092_1143del) and type 2 (c.1154_1155insTTGTC), account for c. 80% of all *CALR* mutations. Type 1 mutations are more frequent, accounting for c.50% of *CALR*-mutated cases of ET. Recently, it was shown that *CALR* mutations promoted the formation of abnormal protein chaperone complexes, which resulted in its mislocalization to the nucleus to enhanced *MPL* transcription due to increased recruitment of Friend leukemia integration 1 transcription factor (FLI1), ERp57, and *CALR* to the *MPL* promoter [44–47].

The abovementioned abnormalities may have resulted in an increase in platelets production. However, the role of mutant *CALR* protein on platelet function is still an open question. Recently published data has shed light on this field, stressing the role of abnormal interaction between proteins in the calnexin pathway. The calnexin pathway includes, among others, thiol isomerase ERp57 (ER protein 57, ERp57), calnexin and its soluble homolog, calreticulin, and is dedicated for N-glycosylated proteins folding in ER [48]. Under physiological conditions, ERp57 is mobilized to the surface of activated platelets, regulating their function (platelet aggregation, dense granule secretion, fibrinogen binding, calcium mobilization and thrombus formation) [49, 50]. Moreover, ERp57 modulates store-operated calcium (Ca^{2+}) entry (SOCE) activity, a key regulator of megakaryopoiesis. The abovementioned process is mediated by the C-terminal domain of *CALR* protein which is deleted in the case of *CALR* mutants [51, 52]. The regulatory role of the C-domain of *CALR* on SOCE was confirmed by experimental results documenting significantly increased SOCE in megakaryocytes positive for the *CALR* mutation [47], and interactome data confirming that *CALR*wt binds directly to ERp57, but *CALR*mut does not [44, 53].

The hypothesis that *CALR* mutants can affect not only the platelet number, but also their function, was confirmed by Hauschner et al. [54], who showed that after ADP stimulation aggregation of *CALR* mutated platelets was less pronounced that in the case of normal or *JAK2* mutated platelets. Moreover, *CALR* mutated platelets attachment to immobilized fibrinogen and the number of *CALR* mutated platelets achieving the fully spread state is lower than in the case of normal and *JAK2* mutated platelets. This is accomplished by an increased and more dispersed localization of intracellular free Ca^{2+} in the case of *CARL* mutation positive platelets. The abovementioned data may, at least in part, explain the increased bleeding frequency observed in *CALR* mutation positive MPN patients who have been treated with anti-aggregatory drugs.

Other potential molecular aberrations affecting thrombotic and bleeding risks

The occurrence of thrombotic and/or bleeding complications is very typical in manifestations of ET, with cases of both occurring in the same patient. The thrombotic or bleeding phenotype may be a consequence of the coexistence of driving and non-driving molecular mutations and polymorphisms, affecting the platelet number and function. Lindstrom et al. [55], with the help of a genome-wide association study (GWAS) and a transcriptome-wide association study (TWAS),

identified 16 novel susceptibility *loci* for venous thromboembolism. Some of them (*GP6*, *ZFPM2*) have been associated with megakaryopoiesis and platelet biology [55].

In 2020, Veninga et al. [56] documented a predisposition for thrombosis and bleeding in patients with clonal hematopoiesis of indeterminate potential (CHIP). According to this concept, in ET patients carrying the CHIP-associated gene mutations, the risk of thrombosis may be affected by elevated platelet counts (i.e. *ABCB6*, *ASXL1*, *DNMT3A*, *GATA1*, *SF3B1*, *SH2B3*) or elevated platelet counts and hyper-reactive platelet phenotype (*ABCB6* and *SH2B3*). On the contrary, the coexistence of CHIP-associated *IDH2* mutations may result in an increase in the platelet count and bleeding phenotype.

Conclusion

Thrombotic and bleeding risk assessment is an essential part of the treatment strategy in ET patients. However, laboratory and clinical data should be interpreted with caution, especially in *CALR* mutation positive individuals who can experience bleeding episodes during anti-platelet therapy. Also, molecular study results should be carefully analyzed, since data from the COSMIC database has revealed 155 different *CALR* variants, including the newly created class E (about 10% of *CALR* variants) which seems not be to associated with ET [57].

Author's contributions

KL — sole author.

Conflict of interest

None.

Financial support

None.

Ethics

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform requirements for manuscripts submitted to biomedical journals.

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