Roczniki Naukowe Polskiego Towarzystwa Zootechnicznego, t. 17 (2021), nr 1, 13-21
DOI: 10.5604/01.3001.0014.7548

Molecular basis of essential thrombocythaemia in humans and dogs – a review

Nicola Padzik[#], **Małgorzata Szewczuk**

West Pomeranian University of Technology in Szczecin, Department of Ruminant Science, Laboratory of Biostatistics, ul. Janickiego 29, 71-270 Szczecin, Poland

SUMMARY

A potential cause of essential thrombocythaemia can be seen as the V617F point mutation within Janus kinase 2. This mutation occurs in 60-70% of patients with this disease and is located in the domain acting as an inhibitor. It increases the enzymatic activity of *JAK2* kinase and induces intensified sensitivity of cells to cytokines. Identification of mutations in the *JAK2* gene has made it possible to describe the molecular pathogenesis of myeloproliferative syndromes, which has enabled more accurate diagnosis and assisted in effective treatment. The significant similarity of the clinical, laboratory and morphological features of myeloproliferative syndromes (including essential thrombocythaemia) in animals and humans suggests that common signalling pathways within the *JAK2* gene may be involved in the development of these diseases.

KEY WORDS: myeloproliferative syndromes, *JAK2*-V617F, essential thrombocythaemia

INTRODUCTION

Chronic malignant hyperplasia of the megakaryocytic (platelet) line in the bone marrow is called idiopathic thrombocytosis. It was first described in 1934 by Epstein and Goedel (Harrison et al., 1999). In humans, it most often occurs between 50 and 60 years of age. It is estimated that there are between 1,5 and 2,4 cases per 100,000 people. Among patients with thrombocythaemia, conversion to acute myeloid leukaemia has been reported in approximately 5% of cases. Essential thrombocythaemia can be asymptomatic (30% of cases) or occur as thrombotic and haemorrhagic complications. Symptoms and their severity depend mainly on the number of platelets, which is on average above 600,000/μL. A platelet count exceeding 1,000,000/μL is often the cause of bleeding, especially in the mucous membranes and gastrointestinal tract (Hellmann and Bieniaszewska, 2000; Tomita et al., 2000). Moderate spleen enlargement is observed in some patients (10-15%).

The pathomechanism of the processes taking place in the bones and blood is the subject of

[#] Corresponding author e-mail: nicola.padzik@zut.edu.pl
Received: 11.01.2021
Received in revised form: 04.02.2021
Accepted: 08.02.2021
Published online: 10.03.2021

research focusing on finding markers enabling effective diagnosis of the disease. Patients have been shown to have an increased percentage of deletions of the short arms of chromosome 17 (17p) (Sterkers et al., 1998). A significant increase in CD36 (membrane platelet glycoprotein - GPIV) expression has been demonstrated in most patients with essential thrombocythaemia. CD36 on the cell surface is considered a receptor for thrombospondin, an adhesive glycoprotein released from platelets after their activation and promoting aggregation (Thibert et al., 1995). The V617F point mutation was found within the *JAK2* gene in 60-70% of patients, which provides grounds for seeking the cause of the disease within this gene (Dzietczenia and Kuliczkowski, 2007).

Essential thrombocythaemia is rarely diagnosed in young dogs and cats, usually affecting older individuals, irrespective of gender or breed (Valli et al., 2016; Weiss, 2006a; Weiss, 2006b).

The primary diagnostic measure is persistent thrombocytosis (above 1000 G/l), with the presence of abnormalities in the size and shape of thrombocytes (usually degranulation) and an increase in cytoplasmic basophilia. In addition, atypical forms of leukocytes, primarily promyelocytes and megakaryocytes (mainly micromegakaryocytes with irregular edges and with abnormal division into lobes) may be present in the blood. There is an increase in the percentage of megakaryocytes in the bone marrow, with normal or abnormal morphology – usually abnormally large megakaryocytes (Facklam and Kociba, 1985). In the differential diagnosis of essential thrombocytosis, all possible causes of a reactive increase in thrombocyte count, such as inflammatory reactions, infections, or iron deficiency, should be ruled out (Juopperi et al., 2011). According to the handbook of veterinary oncology (Valli et al., 2016), the diagnosis of chronic myelogenous leukaemia is based on the finding of a persistently significant increase in the number of cells of a given series of haematopoiesis, without the need to perform any additional specific tests of the cells of the established hyperplasia. Myeloproliferative neoplasms are characterized by a relatively long period without clinical symptoms, but in more advanced cases non-specific clinical symptoms such as lack of appetite or weight loss appear, and clinical examination and imaging results often indicate splenomegaly and hepatomegaly. Overall, the prognosis for chronic myelogenous leukaemia in animals is favourable, and the survival times of untreated or adjuvant patients may be several years (Mochizuki et al., 2014).

In dogs, it has been reported that non-cancerous causes of extreme thrombocytopenia may be due to iron deficiency, purulent bronchitis, and enteritis. In some cases, the disease may be caused by the administration of vincristine and immunosuppressive drugs in related immune diseases (Mandell et al., 2000; Jain, 1993).

Janus kinase *JAK2*

JAK2 kinase belongs to the signal pathways of STAT (signal transducer and activator of transcription) proteins, such as STAT3 or STAT5. With their help, its most important function is in the transmission of signals of extracellular growth factors, cytokines and hormones. It has a classic four-domain structure: JH1, JH2, SH2 and FERM (from the -C- end). The JH1 domain (amino acids 835 to 1132, PubMed NM 004972) of *JAK2* kinase is a typical eukaryotic cell catalytic domain with tyrosine kinase activity. It is responsible for binding and positioning of the phosphate donor (ATP or GTP) in a complex with divalent cations (Mg^{2+} and Mn^{2+}), binding and positioning of the peptide substrate, and transfer of γ-phosphate to the acceptor amino acid (Baxter et al., 2005; He et al., 2005). *JAK2* activity is closely controlled by several mechanisms, including protein tyrosine phosphatases, such as SHP-1, PTP1B and CD45, and signal protein cytokine suppressors that bind *JAK2*, inhibit its catalytic activity, and promote *JAK2*-mediating proteasomes (Ungurenau et al., 2002). The

constitutive activity of *JAK2* may occur in various tumorigenesis processes (Lacronique et al., 1997). By binding to the intracellular domains of receptors for type I and II cytokines, *JAK2* is involved in cytokine signal transduction (e.g. of erythropoietin) (Sędzimirska, 2007). This process is responsible for the interaction of cells that regulate differentiation and growth. These cytokinins are produced inside the cells that make up the immune system and include interferons and haematopoietic growth factors (Rane and Reddy, 2000). Their action is conditioned by a connection to a receptor that triggers the intracellular signalling cascade. Cytokinins act by activating the JAK/STAT pathway. Mammals have strong *JAK2* expression in many organs and tissues. At the cellular level, active *JAK* kinases are mainly located in the cytosol, where a relationship between *JAK2* and cytokine signalling pathways has been demonstrated (Yamaoko et al., 2004).

Molecular basis of essential thrombocythaemia

Diagnosis of essential thrombocythaemia is based on criteria established by the World Health Organization (WHO). Alternative diagnostic methods include the BCSH (British Society for Haematology) 2010 criteria (Harrison et al., 2010). If the patient has the *JAK2*V617F mutation according to BCSH criteria, additional bone marrow examination is no longer necessary (Vannucchi et al., 2008). In patients diagnosed with essential thrombocythaemia, genetic polymorphism (V617 mutation) is found in the *JAK2* gene in 60% of patients (Vannucchi et al., 2007). The mutation of the *JAK2* gene pseudokinase domain involves the substitution of valine with phenylalanine in codon 617 (*JAK2*V617F). These mutations lead to constitutive activation of signalling pathways that stimulate megakaryocyte proliferation and platelet production. A study carried out by Wieczorkiewicz-Kabut et al. (2011) showed that the *JAK2*V617F point mutation was present in 42 (55%) of 77 patients with diagnosed idiopathic myelofibrosis, fibrosis preceded by true hyperplasia, or fibrosis preceded by essential thrombocythaemia. There were no significant differences in the parameters studied between patients with or without the *JAK2*V617F mutation, except for higher bone marrow cellularity. There was no clear correlation between the presence of the *JAK2*V617F mutation and sex, age, liver size, spleen, haemoglobin concentration, leukocyte count, red blood cell count, platelet count, peripheral blood myeloblast count, myeloblast percentage, myelosuppression, LDH activity, or the degree or stage of myelofibrosis and thrombosis. *JAK2* homozygosity was also shown to be significantly more common in women, with a higher number of leukocytes at the time of diagnosis and a tendency to higher haemoglobin concentration. Extensive research has also focused on detecting the presence of the V617F mutation in myeloproliferative syndromes (MPD). Of 480 patients with this disorder, the number of positive cases per disease subtype was 30 (20%) of 152 for atypical or unclassified MPD, 2 of 134 (2%) for idiopathic hypereosinophilic syndrome, 58 of 72 (81%) in for polycythaemia vulgaris, 24 of 59 (41%) for essential thrombocythaemia (ET), and 15 of 35 (43%) for idiopathic myelofibrosis. V617F was not identified in patients with systemic mastocytosis (n = 28), chronic or acute myeloid leukaemia (n = 35), secondary erythrocytosis (n = 4) or healthy controls (n = 160). Compared with other myeloproliferative syndrome subtypes, homozygosity in essential thrombocythaemia was less common (Jones et al., 2005). Detection of the V617F mutation in most patients with myeloproliferative syndrome, as well as the predominance of homozygosity, suggests that this is probably the primary abnormality leading to myeloproliferation. Analysis of results reported by many authors indicates that therapy based on signal transduction with small molecule inhibitors could be effective in the treatment of myeloproliferative syndromes. *JAK2* plays an important role in signalling in many organs and tissues, and therefore the development of inhibitors

that inhibit V617F without undesirable side effects can be challenging (Macchi et al., 1995; Russell et al., 1995).

Genetic engineering - animal models of essential thrombocythaemia (ET)

In-depth knowledge of the aetiology of essential thrombocythaemia will make it possible to devise an effective method of treatment and prevention of the disease in animals. Genetic engineering methods have been used to investigate how expression of the *JAK2* gene with the V617F mutation affects the development of myeloproliferative diseases, including essential thrombocythaemia (ET), and how this mutation contributes to pathogenesis. Mice transplanted with bone marrow cells, transduced by a retrovirus encoding *JAK2*V617F, did not initially show phenotypic features manifested in myeloproliferative diseases. After 4 months, one line showed granulocytosis. Of 43 mice, 8 (19%) showed polycythaemia and 15 (35%) showed thrombocythaemia. The second line exhibited extreme leukocytosis and thrombocytosis. Megakaryocytes dominated in the bone marrow of the animals, and splenomegaly was also observed. The expression of *JAK2*V617F mRNA in bone marrow cells was 0,45 and 1,35 for wild-type endogenous *JAK2* expression in two lines. In vitro analysis of bone marrow cells from both lines showed constitutive activation of ERK1/2, STAT5 and AKT and an increase in their phosphorylation by cytokine stimulation. The researchers concluded that expression of *JAK2*V617F in vivo causes myeloproliferative diseases, including essential thrombocythaemia (Shide et al., 2008). Genetic instability, which results in the V617F mutation in the *JAK2* gene, not only affects the occurrence of myeloproliferative diseases, but also their significant progression. Marty et al. (2013) showed that the *JAK2*V617F mutation induced accumulation of reactive oxygen species (ROS) in the haematopoietic stem cell compartment in a knock-in (KI) mouse model and in patients suffering from myeloproliferative cancer *JAK2*V617F. An increased amount of reactive oxygen species resulted in a decrease in catalase expression, with consequent increased numbers of 8-oxoguanine and double-stranded DNA breaks. There is evidence of mitotic recombination in mice resulting in the loss of *JAK2*V617F heterozygosity. Polycythaemia-like disorders were observed in mice vaccinated with 30% of bone marrow cells with the *JAK2*V617F mutation. Antioxidant treatment with N-acetylcysteine (NAC) restored blood parameters and reduced DNA damage. Researchers have shown overproduction of reactive oxygen species to be a mediator of *JAK2*V617F-induced DNA damage. Preventing the accumulation of reactive oxygen species could prevent the development of *JAK2*V617F myeloproliferative syndromes. Activation of STAT1 by IFNγ in the presence of *JAK2*V617F has been shown to reduce erythrocyte differentiation and promote megakaryocyte development, resulting in a phenotype characteristic of essential thrombocythaemia (ET). Another research team focused on the consequences of STAT1 deficiency on the action of Janus 2 (*JAK2*)-V617F kinase in vivo by crossing *JAK2*V617F-expressing mice with STAT1 knockout mice. Removal of STAT1 reduced megakaryocyte colony formation driven by *JAK2*V617F, but the modification made was not sufficient to completely normalize platelet counts. These studies confirm the role of STAT1 in increasing the number of platelets and reducing the number of erythrocytes in *JAK2*V617F-induced myeloproliferative syndromes (Duek et al., 2014). Subsequent researchers pointed out differences in *JAK2*V617F gene expression between polycythaemia (PV) and essential thrombocythaemia (ET). Higher levels of *JAK2* activation associated with homozygosity of *JAK2*V617F may be manifested in PV, while lower levels signalling activation may result in the appearance/disclosure of ET (Levine, 2008). Pardanani et al. (2007) developed therapies based on inhibition of *JAK2* kinase activity by oral administration of

TG101209 (N-tert-butyl-3-(5-methyl-2- [4-(4-methyl-piperazin-1-yl)- phenylamino]-pyrimidin-4-ylamino)-benzenesulfonamide). In the *JAK2*V617F human acute myeloid leukaemia cell line, cell cycle arrest and apoptosis as well as inhibition of *JAK2*V617F, STAT5 and STAT3 phosphorylation have been observed.

Essential thrombocythaemia (ET) in dogs

Myeloproliferative syndromes (MPN) are very rare in animals. In one study of 67 dogs with different types of cancerous bone marrow hyperplasia, not one case of MPN was observed, while in another study, cases of chronic myeloid leukaemia accounted for 0,3% of all bone marrow abnormalities in dogs, less than 4% of all leukaemias, and 28% of chronic leukaemias (Turinelli et al., 2015). Human haematological tumours having a canine counterpart with similar mutations are known in key oncogenes such as the N-RAS and C-KIT mutations or BCR-ABL rearrangement in acute canine leukaemia (Usher et al., 2009) or chronic myelogenous leukaemia (Breen et al., 2008). Canine and human *JAK2* genes show strong homology (94% identity), in particular in the pseudo-tyrosine kinase domain. In another study, essential thrombocythaemia (ET) was found in 240 of 5,342 dogs (4,6%). Beurlet et al. (2011) investigated the presence of the V617F mutation in dogs showing signs of myeloproliferative diseases. Identical mutations of the *JAK2* gene (located in the JH2 pseudokinase domain involved in automatic kinase inhibition) have been detected in both humans and dogs, resulting in constitutively active *JAK2* kinase. As the mechanism of myeloproliferative diseases in humans and dogs is mostly likely the same, a similar diagnostic and therapeutic approach can be used in both cases.

Molecular diagnostics and treatment of myeloproliferative syndromes in dogs

Genetic markers based on the presence of the V617F mutation in the *JAK2* gene are used to diagnose myeloproliferative syndromes in humans. In order to accelerate and reduce the costs of diagnosis of myeloproliferative syndromes, it is necessary to standardize the diagnostic procedures and tests (e.g. genetic markers). The selection of methods should focus on cytomorphological, haematological and histological factors. Statistical analyses of data sets are required to identify valid prognostic markers, as well as immunophenotypic and genetic analyses to confirm clonality. Prognostic markers in dogs for myeloproliferative tumours (including essential thrombocythaemia) include clinical and haematological factors, but they are not commonly used to make a diagnosis (Juopperi et al., 2011). This is largely due to the small number of cases of dogs with MDS and the lack of a common veterinary register that collects clinical case data. The ultimate goal is to build a forecast-based classification system using diagnostic tools readily available in academic and reference laboratories. The significant similarity of the clinical, laboratory and morphological features of MPN in animals and humans suggests that common signalling pathways within the *JAK2* gene may be involved in the development of myeloproliferative syndromes.

CONCLUSIONS

The goal of treating essential thrombocythaemia is to reduce and maintain a constant platelet count and to prevent complications such as haemorrhage or thrombosis. Radiophosphorus has been found to be a safe and effective treatment for essential thrombocythaemia (ET) in humans (Facklam and Kociba, 1985). In a retrospective study, people with ET receiving P' (3,1 mCi/m^2 BSA) responded positively to treatment, with a faster response to the drug and longer remissions compared to patients receiving busulfan (Silverstein, 1968). The effectiveness of therapy based on the

administration of radiophosphorus has also been proven in dogs. Animals receiving 3,25 mCi/m^2 BSA had a sharp decrease in platelet count, indicating a positive response to treatment. Platelet normalization stabilized in most dogs over 3 to 6 months (Facklam and Kociba, 1985). An additional advantage of radiophosphorus therapy is its ease of administration and noticeable effects after the first dose. In some cases, exogenous administration of glucocorticoids may reduce spleen activity and stimulate megakaryocytopoiesis (Hopper et al., 1989). In another study, a dog with ET was treated with aspirin, radioactive phosphorus and melphalan. Eighteen months after referral, the disease developed into chronic granulocytic leukaemia and the treatment was switched to hydroxyurea. Fourteen months later, the dog was euthanized due to uncontrolled atrial fibrillation (Degen et al., 1989). Of 165 dogs, 73 (44,2%) received glucocorticosteroids (55) or vincristine (18). A slight (850-969 × 10^3 platelets/µl) or extreme (≥ 970 × 10^3 platelets/µl) increase in thrombocytes was observed in 24 (14,5%) dogs, 12 (50,0%) of which had cancer. Thromboembolism occurred in 13 (7,9%) dogs (Neel et al., 2012).

Because of the strong homology between the human and canine *JAK2* gene (94%), a similar diagnostic and therapeutic approach can be used in both cases (Athanasiou et al., 2017). More research should be done into the effect of the V617F mutation on essential thrombocythaemia in dogs to determine a reliable diagnostic and therapeutic pattern.

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