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β-catenin and PPAR-γ levels in bone marrow of myeloproliferative neoplasm: an immunohistochemical and ultrastructural study

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ABSTRACT

In accordance with increased proliferation in myeloproliferative neoplasm (MPN), the goal is to evaluate the immunoexpression of: β-catenin, PPAR-γ and Ki67 protein, to compare them with bone marrow ultrastructural characteristics in patients with MPN. Immunoexpression and electron microscopy of bone marrow was analyzed in 30 Ph-negative MPN patients, including per 10 patients with polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). The quantity of β-catenin immunoreactive cells was significantly higher in PV then in ET ($p < 0.01$) or PMF group of patients ($p < 0.01$) and also in ET versus PMF group of patients ($p < 0.01$). Erythroid lineage showed absent β-catenin staining without immunoreactivity in nucleus. In contrast, immunoreactivity for PPAR-γ was localized mostly in megakaryocytes and the highest number of PPAR-γ immunopositive cells was detected in PMF group of patients. In addition, the proliferative Ki67 index was significantly increased in the PMF and PV patients compared to patients with ET. Also, the megakaryocytes showed abnormal maturation in PMF group of patients as determined by ultrastructural analysis. These results indicated that PV dominantly expressed β-catenin and proliferation marker Ki67 in bone marrow, while PMF is linked preferentially to PPAR-γ immunopositive megakaryocytes characterized by abnormal maturation.

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Introduction

Myeloproliferative neoplasms (MPNs), including polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), are clonal bone marrow stem cell disorders characterized by a proliferation of one or more of the myeloid, erythroid or megakaryocytic cell lines. This proliferation results in increased numbers of granulocytes, erythrocytes or platelets in the peripheral blood. The bone marrow of PV patients displays panmyelosis and therefore an increase in cellularity with slightly increased reticulin fibrosis.[1] The presence of reticulin is extremely rare in ET patients and very few patients (<10%) develop myelofibrosis, known as post-ET myelofibrosis.[2] PMF is described by bone marrow fibrosis and extramedullary hematopoiesis. The clonal proliferation of hematopoietic stem cells produces growth factors leading to fibrosis of the bone marrow. Initially, the bone marrow is a hypercellular, but normal hematopoiesis is diminished as the bone marrow becomes fibrotic and patients become pancytopenic.[3]

It has been shown that β-catenin perform two functions: it has a crucial role in cell-cell adhesion in addition to a central effector molecule in the Wnt signaling pathway.[4] The Wnt pathway has important roles in lymphoid neoplasms, such as proliferation, migration and invasion.[5] The dysregulation of the Wnt/β-catenin signaling pathway is suggested to induce tumorigenesis in normal cells, modulate the cancer microenvironment and supports cancer cells proliferation and survival.[6] The signaling activity of β-catenin is mediated through its interaction with the T cell factor (TCF) family of transcription factors and subsequent activation of target genes. β-catenin provides the transcriptional activation domains,

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while TCFs are sequence-specific DNA binding proteins. The activity of the complex is regulated, at least in part, through the ubiquitin/proteasome-mediated degradation of β-catenin.[7] Aberrant β-catenin signaling plays an important role in tumorigenesis, involved in various cancer types, including melanoma, breast, myeloma and acute myeloid leukemia.[8,9]

Peroxisome proliferator-activated receptor gamma (PPARγ) is a multifunctional transcription factor with important regulatory roles in differentiation, inflammation, cell growth and apoptosis.[10] PPARγ expression has been found in monocytes/macrophages, dendritic cells, granulocytes, mast cells, T and B cells and also in platelets.[11] Several studies have shown the opposite interaction between β-catenin and PPARγ. This opposite interaction involves the TCF/LEF factor-binding domain of β-catenin and a catenin-binding domain of PPARγ.[12] The antagonism between the β-catenin pathway and PPARγ has been reported in various cell types, such as adipocytes[13] and hepatocytes.[14]

Although the role of β-catenin in various hematological disorders has been shown, as well as expression of PPARγ in numerous leukemias and lymphomas, their role in MPNs has not been investigated. The aim of our study was to evaluate immunoexpression of β-catenin, PPARγ and Ki67 protein and to compare them with bone marrow ultrastructural characteristics in patients with MPN.

Material and methods

Patients

This prospective study is based on analysis of newly diagnosed and previously untreated 30 Ph- negative MPN patients, including per 10 patients with PV, ET and PMF. Patients were diagnosed at the Clinic for Hematology, Clinical Center of Serbia and only patients who fulfilled the WHO criteria from 2016 have been included. The study was approved and performed according to the regulations of the Local Research Ethics Committee and informed consent was obtained from each patient in accordance with the Declaration of Helsinki. For this study, the data from their medical records were extracted and double-checked. The baseline assessment included standard hematological and biochemical parameters.

Immunohistochemistry

Bone marrow biopsy specimens from the posterior superior iliac spine, were fixed in 10% neutral formalin solution for 24–36 hours, then decalcified in EDTA buffer for 3 hours and embedded in paraffin. The tissue sections were cut at 5 μm, heated at 56°C for 60 min, then deparaffinized in xylene, rehydrated through a series of alcohols followed by an epitope retrieval step. Samples were treated with 3% H2O2 solution in PBS to block endogenous peroxidase activity. The next step was incubation with the anti-β-catenin antibody (Thermo Fisher Scientific, cat.no. MA1-301, dilution 1:100), anti-PPARγ antibody (Santa Cruz Biotechnology, cat.no. sc-7273, dilution 1:25) and anti-Ki67 antibody (Novocastra Laboratories Ltd, Newcastle, UK, dilution 1:100) in a humidity chamber over night at room temperature. Immunostaining was performed using the streptavidin-biotin technique (LSAB+/HRP Kit, DAKO). Immunoreactivity was visualized with DAKO Liquid DAB+ Substrate/Chromogen System (code no. K3468) counterstained with Mayer's hematoxylin (Merck, Whitehouse Station, NJ). For the negative control samples, normal serum and TBS buffer (1:500) were pipetted without primary antibodies. Analysis and score of the immmunoreactive cells was conducted at ten fields in each sample using a computer-supported imaging system connected to the light microscope (Olympus AX70, Hamburg, Germany) with an objective magnification of x 40.

Morphometry

Quantification of labeled (Ki67+) cells in the bone marrow was determined using a computer-supported imaging system (analysis Pro 3.1) connected to a light microscope (Olympus AX70, Hamburg, Germany) with an objective magnification of 40 X. The area of the bone marrow was calculated according to the following formula:

$$P = p \times d^2/10^6,$$

Where, P is the surface area, p is the number of grid points in the bone marrow, and d is the size of the

square network at an objective magnification of 40 X. The number of Ki67+ cells per mm^2 of bone marrow was calculated according to the expression

$$N = n/P$$

Where n is the number of Ki67+ cells on the presented surface, and P is the analyzed surface area of bone marrow. The number of proliferating (Ki67+) cells was expressed per mm^2 of bone marrow. In addition to the number of Ki67 positive cells/mm^2, the ratio between dividing cells and the total number of cells per unit area (proliferation index), expressed as percentage is given. This enabled a comparison to be made between the two manners ofpresentation.

Electron microscopy of bone marrow

The bone marrow biopsy specimens were decalcified in 10% EDTA buffer for 3 hours and subsequently fixed in 2% glutaraldehyde in 0.1M sodium cacodylate buffer (pH 7.4) for 24 hours at 4°C. The fixed specimens were washed for 12 hours at 4°C in 0.5M sodium cacodylate buffer. Post fixation was done in 1% osmium tetroxide buffered with cacodylate buffer, pH 7.4 for 90 minutes at 0°C. The samples were then dehydrated in a graded series of ethanol, and embedded into Araldite (Fluka, Seelze, Germany) according to the procedure recommended by the manufacture. Semi fine sections (1 to 0.5 μm) stained by toluidine blue were inspected by light microscopy (Olympus AX70, Hamburg, Germany). Thin sections were cut on a Reichert UltratomeNove (LKB, Sweden) and double-stained with uranyl acetate and lead citrate. The sections were examined with a Morgagni 268 (D) electron microscope (FEI, Hillsboro, OR).

Statistical analysis

The results are expressed as the mean ± SE. Values were compared using the nonparametric Manne-Whitney U test in the program SPSS 10 for Windows. Differences at $p < 0.05$ were accepted as the level of significance.

Results

Patient's baseline demographic and laboratory characteristics

The main demographic and laboratory parameters of 30 MPN patients at the time of diagnosis are summarized in Table 1. There is an increased number of red blood cells ($p < 0.01$) in PV patients compared to ET and PMF patients. As expected, the hematocrit was significantly increased in PV patients compared to patients with ET ($p < 0.05$) and PMF ($p < 0.01$), while increase of hemoglobin was significant only in relation to the group of patients with PMF ($p < 0.05$, Table 1).

β-catenin and PPAR-γ expression in bone marrow of MPN patients

β-catenin immunostaining was detected on the cell membrane and/or in the cytoplasmic region of megakaryocytes, immature myeloid lineage cells, and vascular endothelial cells. Immunoreactivity in nucleus was not detected. Erythroid lineage cells were characterized with absent or minimal β-catenin staining. According to our results, the highest number of β-catenin immunoreactive cells was detected in PV group of patients (Figures 1 and 2), while the lowest in PMF patients (Figure 1). Statistical analysis showed that differences in β-catenin positive cells were significant between PV and ET (Figure 1, a – $p < 0.01$), PV and PMF

Table 1. Demographic and laboratory characteristics of the patients with myeloproliferative neoplasms at the time of diagnosis.

	MPN		
Parameters	PV	ET	PMF
White blood cells	0.98 ± 2.7	10.68 ± 4.5	9.24 ± 3.2
Red blood cells	6.1 ± 0.7**	4.84 ± 0.75	4.56 ± 0.83
Hemoglobin	161.54 ± 11.2*	145.09 ± 22.1	128.00 ± 19.2
Hematocrit	0.50 ± 0.03	0.43 ± 0.06[a]	0.40 ± 0.05[b]
Mean corpuscular volume	81.45 ± 7.2	90.00 ± 5.1	82.01 ± 19.7
Platelets	784.09 ± 211.9	798.73 ± 290.5	779.36 ± 210.8
Lymphocytes	25.25 ± 6.9	24.75 ± 6.6	28.16 ± 8.8
Monocytes	7.35 ± 1.6	6.8 ± 2.6	6.75 ± 2.1
Granulocytes	61.81 ± 7.1	64.07 ± 8.4	61.18 ± 8.7
Gender/Male	6	3	4
Gender/Female	5	8	7
Age	59.63 ± 10.53	51.18 ± 10.68	61.81 ± 11.12

The results are expressed as the mean ± SD; *$p < 0.05$ PV vs PMF; **$p < 0,01$ PV vs ET and PMF; [a]$p < 0.05$ PV vs ET; [b]$p < 0.01$ PV vs PMF.

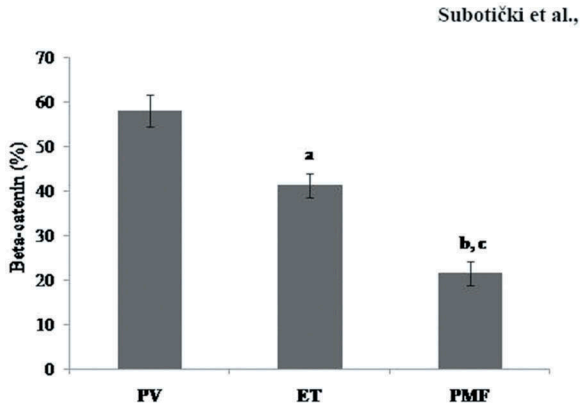

Figure 1. Number of β-Catenin immunopositive cells in bone marrow tissue section of patients with ET, PV and PMF. (n = 10) Values are mean ± SEM. PV vs. ET $^{a}p < 0.01$; PV vs. PMF $^{b}p < 0.01$; PMF vs. ET $^{c}p < 0.01$.

(Figure 1. b – $p < 0.01$) and also between PMF and ET (Figure 1, c – $p < 0.01$) group of patients.

In contrast to β–catetin, PPAR-γ showed opposite pattern of expression. Namely, the highest number of PPAR-γ immunopositive cells was detected in PMF patients, while the lowest number was confirmed in ET patients (Figure 2). Immunoreactivity for PPAR-γ was localized mostly in megakaryocytes (Figure 2).

Proliferation marker Ki67 expression in bone marrow of MPN patients

Number of Ki67 positive cells in the bone marrow of patients with PV and PMF was significantly increased compared to the values obtained in patients with ET (Table 2; Figure 3). The proliferative index, calculated from the ratio of the number of Ki67 positive cells and the total number of cells per mm^2, was significantly increased in the

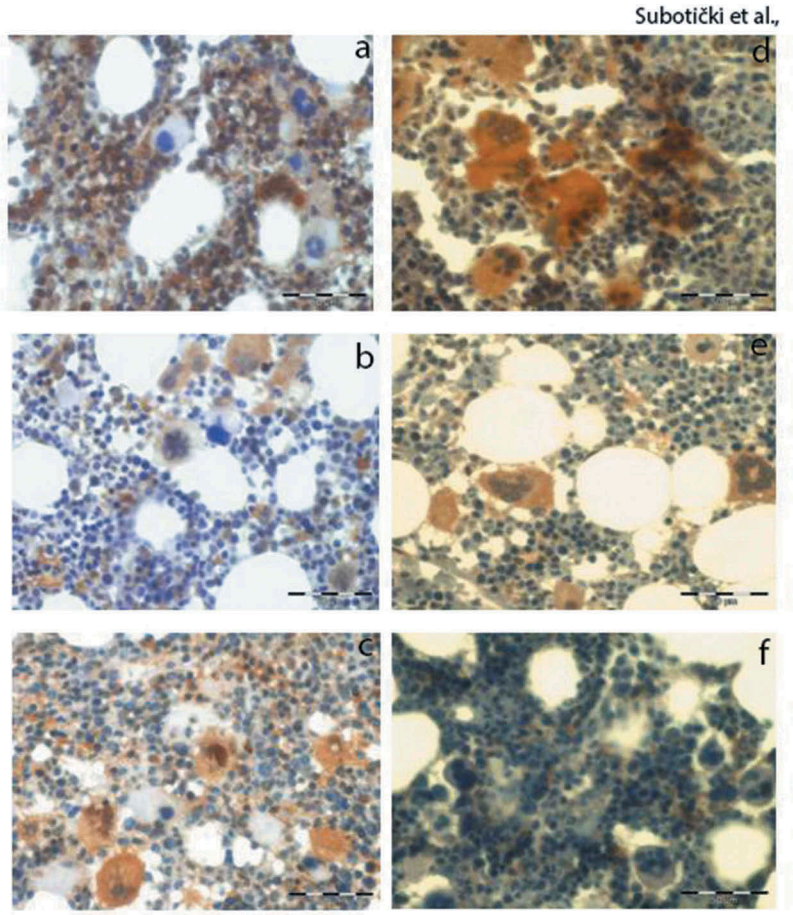

Figure 2. β-Catenin (a,b,c) and PPARγ (d,e,f) immunoexpression in bone marrow tissue section of patients with MPN. β-catenin immunoexpression in bone marrow of patients with PV (a), ET (b) and PMF (c). PPARγ immunoexpression in bone marrow of patients with PMF (d), PV (e) and ET (f). Scale bar – located in the lower right corner.

Table 2. The total number of cells and the number of Ki67 positive cells/mm^2, as well as the index of proliferation (%) in the bone marrow of patients with myeloproliferative diseases.

Groups	The total number of cells per unit area/ mm^2	The number of Ki67 positive cells/ mm^2	Proliferative index (%)
PV	69.55 ± 12.7	12.84 ± 9.1*	17.62 ± 9.9*
ET	73.54 ± 10.6	8.11 ± 6.1	11.04 ± 8.2
PMF	75.34 ± 16.1	17.89 ± 11.2***	22.81 ± 12.1***

The results are presented as mean ± SD. *p < 0.05 PV vs ET; ***p < 0.001 PMF vs ET.

PV and PMF patients compared to patients with ET (Table 2; Figure 3).

Electron microscopy analysis of the bone marrow

The ultrastructural analysis of the bone marrow of patients with a diagnosis of PV revealed the presence of numerous mature erythrocytes, nucleated erythroid precursors-normoblasts, the cell of myeloid lineage and platelets. In the nucleated erythroid cells the perinuclear space often appeared dilated. The cells of myeloid lineage were characterized by presence of segmented nuclei and the cytoplasm that contained dilated profiles of the endoplasmic reticulum and the primary granules. Platelets, which were of irregular shape, contained also dilated profiles of endoplasmic reticulum (Figure 4a,b). Megakaryocytes were seen as large cells that contained extensive and well organized cytoplasm (Figure 4c). Dilated endoplasmic reticulum was found in the cytoplasm of megakaryocytes together with numerous granules with electron dense material, vesicles and mitochondria (Figure 4d).

Ultrastructural analysis of bone marrow samples from patients with ET showed presence of the myeloid/erythroid cells and large megakaryocytes with complex system of membranes in the cytoplasm, irregularly shaped nucleus with heterochromatin located close to the nuclear membrane (Figure 5a,b). Dilated endoplasmic reticulum, rare granules of smaller diameter, vesicles of different sizes and mitochondria with both normal and damaged structures were found in the cytoplasm of megakaryocytes. Demarcation membrane system and rare granules were located at the periphery of megakaryocytes. In the cell periphery were

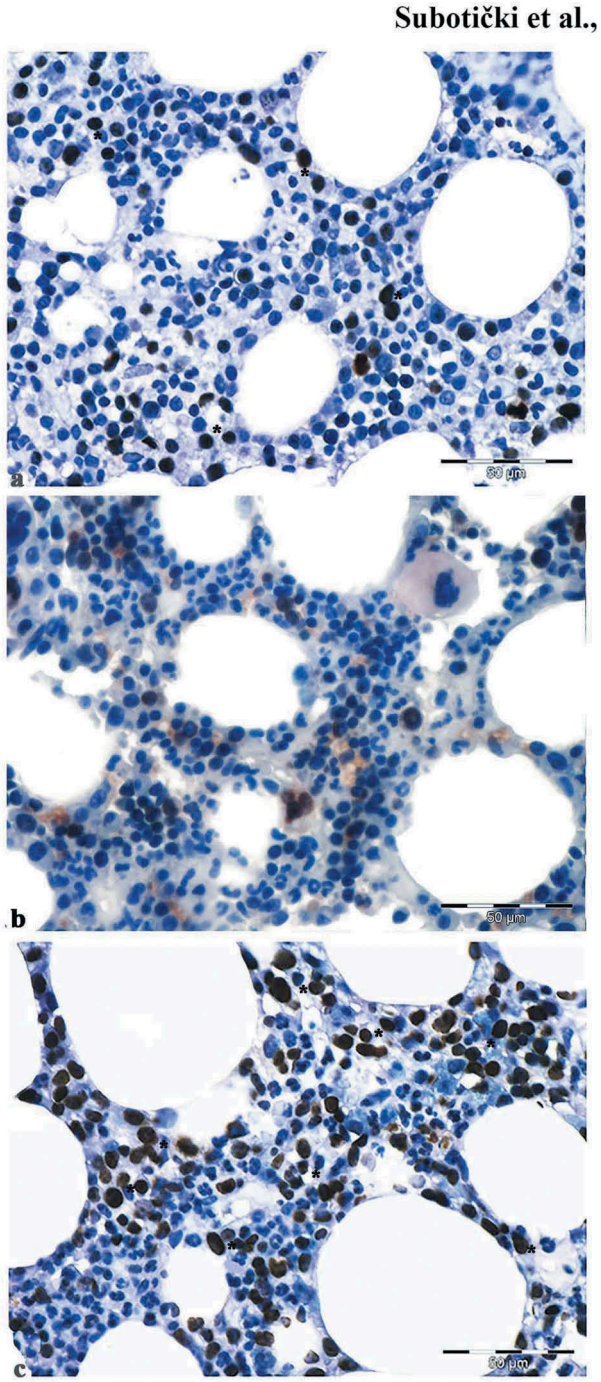

Figure 3. Ki67 immunoexpression in bone marrow tissue section of patients with MPN. Pattern of Ki67 immunoexpression in bone marrow of patients with PV (a), ET (b) and PMF (c). Legend: * – Ki67 + cells. Scale bar – located in the lower right corner.

located the demarcation membrane channels and rare granules (Figure 5c,d).

Dilated sinuses, cells of erythroid and myeloid lineage, and collagen fibers between blood elements were seen in the bone marrow of patients with PMF

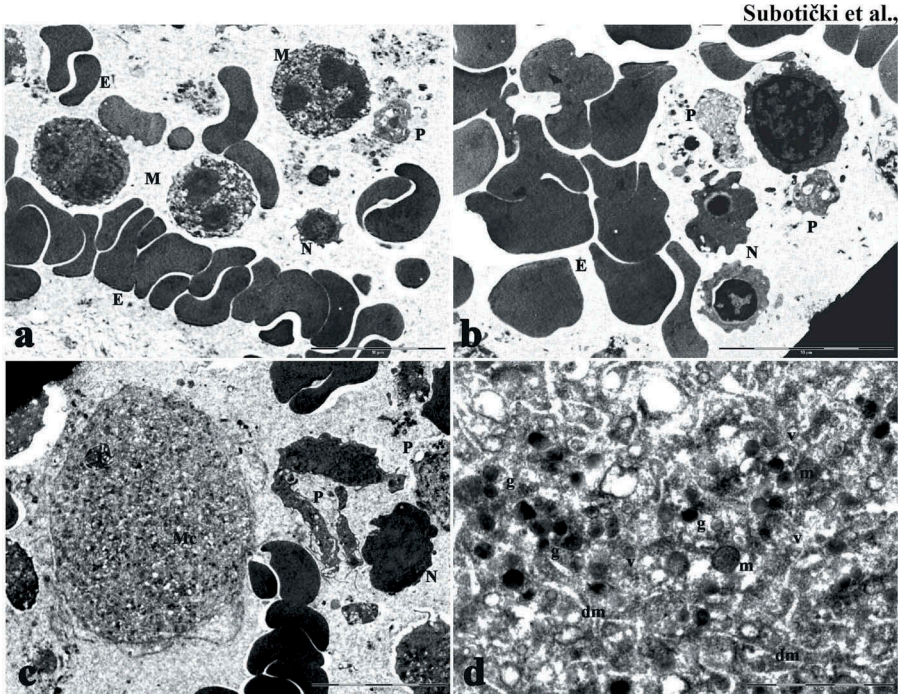

Figure 4. Electron micrographs of bone marrow from PV patients. Cells of erythroid and myeloid lineage together with the platelets were seen in bone marrow samples (a, b). Megakaryocytes contained extensive and well organized cytoplasm (c). Dilated endoplasmic reticulum was found in the cytoplasm of megakaryocytes(d). Legend: E- erythrocyte, M – myeloid cells, ME – megakaryocytes, N- normoblasts, P – platelets, g- granules, dm- dilated membranes of endoplasmic reticulum, v- vesicles, m- mitochondria. Scale bar – located in the lower right corner.

(Figure 6a,b). Cells of myeloid lineage were located near megakaryocytes withvisible collagen fibers. Megakaryocytes were seen as large cells, with large nuclei of increased lobularity and heterochromatin places along the nuclear membrane. Although granules were scarce in the peripheral part of the cytoplasm of megakaryocytes, demarcation membrane system was prominent (Figure 6c,d).

Discussion

In this study we examined the role of β-catenin, PPARγ and Ki67 protein expression in MPNs, as well as the bone marrow ultrastructural characteristics. Our results showed that β-catenin expression was increased in the cytoplasmic region of megakaryocytes of PV and ET patients, suggesting that PV and ET may share a common pathogenesis involving the Wnt/β-catenin signaling pathway in megakaryopoiesis. In PMF, the strongest staining pattern for β-catenin, besides megakaryocytes, was detected in vascular endothelial cells, which may suggest that Wnt pathway has an angiogenic role in the pathogenesis of PMF. In previous studies, β-catenin has been found in both cytoplasmic and nuclear localization in non-hematologic neoplasm's.[15] In our study, although cytoplasmic β-catenin staining was strong in most MPNs, we did not observe a nuclear staining pattern. These results are very similar to study of cutaneous lymphomas, where β-catenin was found to be cytoplasmic in all cases.[16] β-catenin-mediated Wnt signaling has been suggested to be critically involved in hematopoietic stem cell maintenance, primarily in the ability of hematopoietic cells to proliferate and to avoid differentiation.[17] *In vivo*, transplanted hematopoietic stem cells with transduced β-catenin reconstituted the myeloid and lymphoid lineages.[18] Also, it has been found that β-catenin gene expression is increased in acute myeloid leukemia.[19]

Although the role of PPARγ was investigated in different hematological malignancies,[20] so far its role in MPNs has received no attention. Our data show that in contrast to β-catenin expression, PPARγ has opposite pattern. The highest number

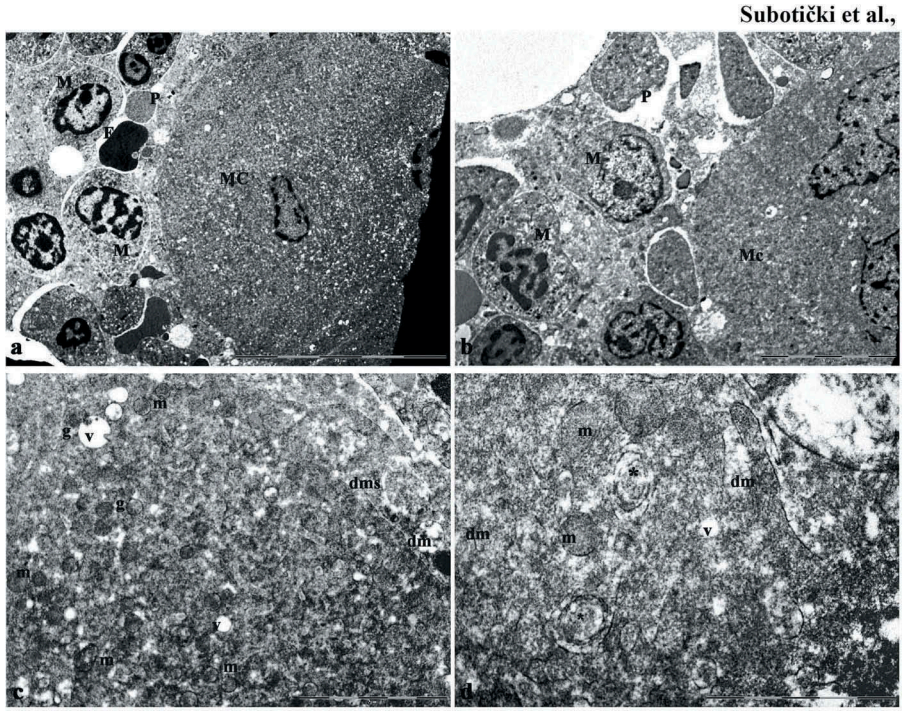

Figure 5. Electron micrographs of bone marrow from patients with ET. Cells of myeloid and erythroid lineage and large megakaryocytes were seen in bone marrow sections (a, b). Megakaryocytes had dilated endoplasmic reticulum, granules of small size, vesicles of different sizes and mitochondria with preserved and damaged structures. Intensive network of demarcation membrane system channels was located on the cell periphery (c, d). Legend: E- erythrocyte, M – myeloid cells, ME – megakaryocytes, P – platelets, n- nucleus, g- granules, dms- demarcation membrane system, dm- dilated membranes of endoplasmic reticulum, v- vesicles, m- mitochondria, asterix-damaged mitochondria. Scale bar – located in the lower right corner.

of PPARγ immunopositive cells was detected in PMF patients, while the lowest number in ET patients. Also, immunoreactivity for PPARγ was localized mostly in megakaryocytes of MPNs. Previous study showed that PPARγ is not restricted to the nucleus, but is also expressed in the cytoplasm.[21] Transcriptional activity of PPARγ is controlled primarily by ligand binding and its ligands include both synthetic and natural molecules.[22] Synthetic PPARγ ligands, including drugs of the thiazolidinedione (TZD) family, have potent insulin sensing properties, and are commonly used for the treatment of type 2 diabetes.[22,23] There are also many synthetic compounds that can function as PPARγ agonist. One of them, triterpenoid 2-cyano-3,12-dioxooleana -1,9-dien-28-oic acid (CDDO), binds to PPARγ with nanomolar affinity and displays anti-proliferative and differentiating activities, making it useful as a chemotherapeutic agent.[24] PPARγ ligands have anti-proliferative, pro-differentiated, anti-metastatic and pro-apopototic effects on several hematological malignancies, making PPARγ a promising target in therapeutic regimens designed to combat these types of cancer.[11]

Some studies suggest that β-catenin can interact with transcription factors other than those mediating the Wnt signaling pathway.[25] Additional studies suggested that PPARγ downregulates the levels of β-catenin but only in cells that contain a functional APC molecule and an intact destruction complex.[26] It has been shown that repression of β-catenin function in malignant cells by non-steroidal anti-inflammatory drugs requires high level expression of PPARγ.[27] Also, it has been demonstrated that activation of PPARγ in mesenchymal cells induces the proteosomal degradation of β-catenin, whereas expression of an oncogenic β-catenin resist such a PPARγ-associated destabilization and inhibits the ability of PPARγ to induce target genes.[28]

Distinguishing three MPN subtypes in the early phase is important, because of a different risk of thromboembolic complications for PV and the inferior survival rate of PMF compared to ET patients.[29] Our results show that Ki67 and

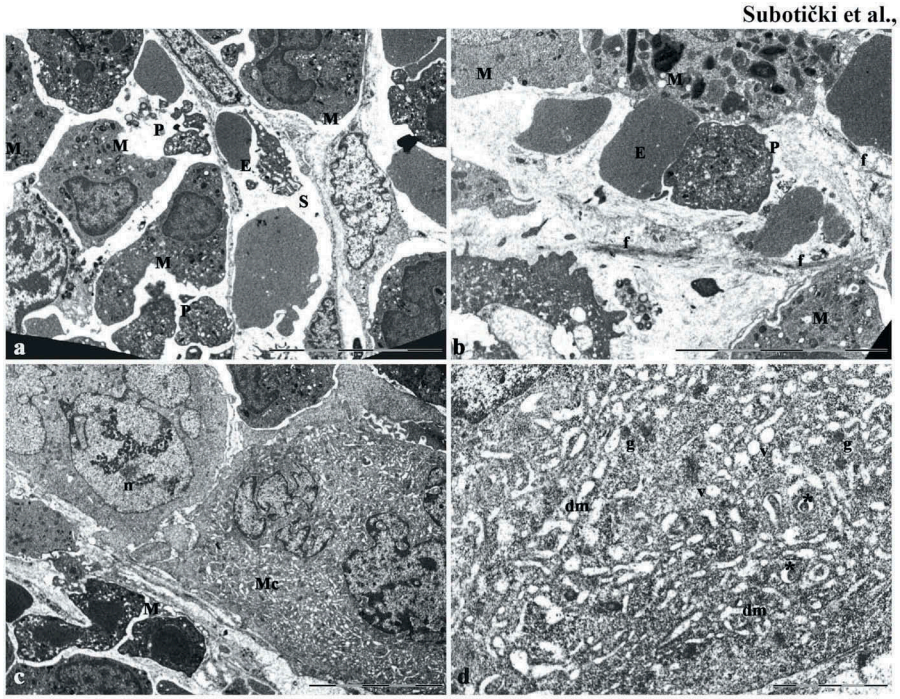

Figure 6. Electron micrographs of bone marrow patients with PMF. Cells of erythroid and myeloid lineage surrounds sinusoids (a). Fibrous fibers were seen between blood elements (b). Megakaryocyte with lobulated nucleus (c) and dilated endoplasmic reticulum profiles, damaged mitochondria, rare granules and vesicles (d). Legend: E- erythrocyte, M – myeloid cells, ME – megakaryocytes, P – platelets, s-sinusoid, f – collagen fibers, n- nucleus, g- granules, dms- demarcation membrane system, dm- dilated membranes of endoplasmic reticulum, v- vesicles, asterix-damaged mitochondria. Scale bar – located in the lower right corner.

PPARγ have the same trend of expression in MPNs, bearing in mind that proliferative index has been significantly increased in PMF and PV patients compared to ET group of patients. In contrast to those two markers, β-catenin has opposite pattern of expression in correlation with Ki67 and PPARγ. According to those statement we could conclude that patients with high level of Ki67 and PPARγ expression has more progressive form of disease (PMF) in comparison to those who has high level of β-catenin expression (PV). Electron microscopy showed that bone marrow of PV patients can be recognized by numerous mature erythrocytes, normoblast, myeloid cells and platelets. Also, megakaryocytes are pleomorphic and vary in size, but they are without maturing defects. Characteristic for ET patients are the large, hyperlobulated and mature-appearing megakaryocytes clustered loosely together. In contrast, megakaryocytes in bone marrow of PMF patients show abnormal maturation and have hyperchromatic and irregularly folded bulky nuclei which are densely clustered. Furthermore, reticulin and collagen fibrosis and often osteosclerosis are present in fibrotic PMF. Low grades of reticulin fibrosis are sometimes also found in the bone marrow of ET and PV patients.[30] PMF has the worst prognosis among the MPNs.[31] The disease can start as PMF or as the burnt out phase of PV (post-PV MF) or ET (post-ET MF).[32] In all MPNs, megakaryocytes proliferate, acquire multilobulated nuclei and exhibit clustering in the bone marrow.[33] It has been shown that mice with a megakaryocyte deficiency of *Gata 1*have elevated number of immature megakaryocytes and severe bone marrow fibrosis.[34] Megakaryocytes from PMF patients secrete increased levels of the fibrotic cytokine TGF-β.[35] However, the extent to which megakaryocytes are required for myelofibrosis and whether targeting the megakaryocyte lineage is sufficient to prevent disease has not been shown.

Concluding remarks

Our results indicate that β-catenin has different pattern of expression in different subtypes of MPNs. While in PV and ET patients Wnt/β-catenin

signaling pathway may have role in megakaryocytopoiesis, β-catenin may act as an angiogenic factor in PMF patients. Furthermore, PPARγ shows a different pattern of expression, mainly in megakaryocytes of MPNs patients, which may suggest the antagonism between the Wnt/β-catenin pathway and PPARγ in MPNs. The expression of PPARγ and Ki67 was the highest in PMF patients, which may indicate that this diagnosis is the most progressive type of MPNs. PPARγ expression was detected in megakaryocytes of MPNs patients while in those types of organelles EM analysis also showed that they were very heterogenic: in PV-pleomorphic, in ET- large and hyperlobulated and in PMF – abnormal maturation.

Further studies with a larger sample size are need to confirm our observation and to investigate the role of β-catenin and PPARγ in pathogenesis of MPNs.

Conflicts of interest

The authors declare no conflict of interest.

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