Anti-Apoptotic Pathways in Bone Marrow and Megakaryocytes in Myeloproliferative Neoplasia

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Key Words

Anti-apoptotic pathways · Bnip3 · Erk signaling · Microvessel density · Myeloproliferative neoplasia · PI3K-Akt signaling

Abstract

Objective: Proliferative activity contributes to bone marrow cellularity in myeloproliferative neoplasia (MPN). Megakaryocytes are the most important cells in MPN bone marrow pathology. *JAK2*^{V617F} mutation constitutively activates JAK2, pErk (phosphorylating extracellular signal-regulated kinase) and PI3K (phosphatidylinositol 3-kinase)-Akt signaling. Erk is involved in megakaryocyte differentiation, PI3K-Akt inhibits megakaryocyte apoptosis via Bcl-x_L and two downstream effectors (p70S6k and Bnip3). Immunohistochemic expression of phosphorylated Erk, Akt, p70S6k and Bnip3 was studied along with microvessel density (MVD) in MPN bone marrow and megakaryocytes. **Methods:** 36 essential thrombocythemia (ET), 25 polycythemia vera and 45 primary myelofibrosis patients were analyzed for pErk, pAkt, Bnip3, p70S6k and MVD expression by immunostaining bone marrow biopsy sections followed by automated image analysis. *JAK2*^{V617F} was analyzed through real-time PCR in blood samples. **Results:** pErk and pAkt were significantly higher expressed in MPN megakaryocytes, mainly in ET patients, compared to controls. Bnip3 was higher expressed in bone marrow of control patients and in MPN megakaryocytes. Mainly in

ET patients, MPN megakaryocytes showed higher p70S6k expression compared to controls. **Conclusion:** Increased bone marrow cellularity in MPN patients might be influenced by increased pErk, pAkt and decreased Bnip3 expression. A dominant role for megakaryocytes in ET patients was shown. Increased amounts of megakaryocytes in MPN patients can be due to increased pAkt and p70S6k.

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Introduction

Myeloproliferative neoplasias (MPN) are clonal bone marrow stem cell disorders originating from a multipotent hematopoietic stem cell characterized by proliferation of myeloid, erythroid and/or megakaryocytic cell lineages resulting in increased numbers of granulocytes, erythrocytes or platelets in the peripheral blood.

MPNs can be divided into chronic myelogenous leukemia carrying the Philadelphia (Ph+) chromosome as a result of t(9;22) and diseases which do not carry the Philadelphia chromosome (Ph-) [1]. The three most commonly occurring classical Ph- MPN are essential thrombocythemia (ET), polycythemia vera (PV) and primary myelofibrosis (PMF) [2, 3].

In 2005, a mutation in the *JAK2* gene was detected. The *JAK2*^{V617F} mutation, which substitutes valine for phenylalanine and disrupts the inhibitory function of the

Table 1. Clinical and laboratory findings of the patients with ET, PV and PMF and the control group

Characteristics	ET (n = 36)	PV (n = 25)	PMF (n = 45)	Control group (n = 36)
Males/females	11/25	8/17	26/19	23/13
Age, years	59±17.70	65±13.56	67±10.73	56±14.33
JAK2: wild type/mutation/unknown	15/19/2	2/17/6	7/20/18	36/0/0
White blood cell count, ×10 ⁹ /l	9.37±2.89	16.08±12.22	11.89±11.38	8.16±4.44
Range	4.4–18.30	5.70–62.00	0.90–70.60	2.80–23.80
Hemoglobin, mmol/l	8.53±1.26	9.89±1.81	7.09±1.58	8.16±1.10
Range	6.10–12.00	6.70–13.50	3.30–10.60	6.10–11.10
Hematocrit, l/l	0.43±0.06	0.52±0.09	0.35±0.08	0.39±0.05
Range	0.29–0.61	0.37–0.68	0.15–0.50	0.30–0.52
Thrombocytes, ×10 ⁹ /l	929±346	662±316	564±532	263±137
Range	327–1,862	112–1,371	15–2,644	49–585

Means ± SD, numbers and ranges are shown.

pseudokinase domain in JAK2, constitutively activates the JAK2 gene [4–7]. The activating *JAK2*^{V617F} mutation is involved in several different pathways: in the mitogen-activated protein kinase (MAPK) signaling pathway, PI3K (phosphatidylinositol 3-kinase)-Akt signaling pathway and the activation of the STAT (signal transducer and activator of transcription) family [5, 8]. Besides pathways influenced by the mutated JAK2 gene, in general both pro- and anti-apoptotic proteins could be involved in the pathogenesis of Ph– MPN.

First, JAK2 can activate the receptor tyrosine kinase-Grb2-SOS signaling axis, activating Ras GTPase and Raf and then MEK and Erk (extracellular signal-regulated kinase) signaling. The Erk signaling pathway is not only activated by JAK2 but can also be activated by numerous extracellular signals [8–10]. Several studies have shown the involvement of the Erk pathway in megakaryocyte differentiation [11–13]. Therefore, we examined the phosphorylation status of Erk (pErk) in bone marrow trephines of Ph– MPN patients and in megakaryocytes.

Second, the PI3K-Akt signaling pathway is activated by the JAK2 mutation. Akt is an important mediator in the PI3K pathway and is involved in many cellular processes, including inhibition of apoptosis, protein synthesis and cell differentiation and metabolism. It is already known that Akt is constitutively activated in acute myeloid leukemia [14–18]. The PI3K-Akt pathway is activated by growth factors and cytokines resulting in phosphorylation of Akt, which up-regulates Bcl-x_L leading to inhibition of megakaryocyte apoptosis [19]. The mammalian target of rapamycin (mTor) is a serine/threonine kinase which is an effector protein of Akt [20]. Akt and mTor are both necessary for the activation of ribosomal

p70S6 kinase (p70S6k) [21]. Therefore, we also examined the phosphorylation status of Akt (pAkt) and p70S6k in bone marrow trephines of Ph– MPN patients.

Third, besides the Akt signaling pathway, anti-apoptotic proteins could also be important factors in the development of Ph– MPN. The Bcl-2 protein family consists of both pro- and anti-apoptotic proteins, depending on the different combinations of the Bcl-2 homology domains. Bnip3 is a pro-apoptotic protein belonging to the Bcl-2 family. Bnip3 is activated under hypoxic conditions in normal and cancer tissue upon hypoxia-inducible factor (Hif-1α) [22–24].

In the present study, we investigated the Erk and PI3K-Akt pathway and the expression of Bnip3 and p70S6k along with microvessel density (MVD) by immunohistochemistry on trephine biopsies to characterize abnormal activation of these pathways and abnormal apoptotic responses in bone marrow and megakaryocytes of Ph– MPN patients.

Design and Methods

Study Population

The study was carried out on bone marrow trephines obtained from patients recorded at the Maastricht University Medical Center, Maastricht, between January 1992 and December 2009, at the Haga Hospital, The Hague, between January 2006 and December 2009 and at the VieCuri Medical Centre, Venlo, between January 2005 and July 2010. The study was approved by the local institutional ethics committees. The study population consisted of 106 patients with a myeloproliferative neoplasm, with a mean age of 63.6 years (SD ± 14.7, ranging from 17 to 86 years) at the time of diagnosis. None of the patients received therapy when the biopsy was taken. The patient population included in the study consisted of 36 ET (33.9%), 25 PV (23.6%) and 45 PMF (42.5%) patients. All patients were clinically and histologically diagnosed according to the World Health Organization classification of 2008 [25]. As shown in tables 1 and 2,

Table 2. Clinical and laboratory findings of patients with the JAK2 mutation or with wild-type JAK2

	JAK2 positive (n = 56)	Wild-type JAK2 (n = 24)
Males/females	26/30	9/15
Age, years	64±14.96	63±14.28
White blood cell count, ×10 ⁹ /l	13.6±9.64	8.5±3.76
Hemoglobin, mmol/l	8.91±1.46	7.81±1.67
Hematocrit, l/l	0.44±0.08	0.39±0.09
Thrombocytes, ×10 ⁹ /l	728±341	907±374

Means ± SD except for the number of males/females.

61 patients (57.5%) were women and 45 (42.5%) were men. Fifty-six patients were carriers of the *JAK2*^{V617F} mutation (19 ET, 17 PV and 20 PMF patients), 24 patients were carriers of the *JAK2* wild-type gene (15 ET, 2 PV and 7 PMF patients) and in 26 patients the *JAK2*^{V617F} mutation status was unknown due to insufficient DNA to detect the *JAK2* status by PCR and because patients died prior to the availability of the *JAK2*^{V617F} mutation test.

The patients were subdivided for the grading of myelofibrosis (mf) into mf 0/1 and mf 2/3; 43 patients belonged to the mf 0/1 group (19 ET, 12 PV and 12 PMF), of which 24 were *JAK2*^{V617F} positive and 11 *JAK2* wild type; 61 belonged to the mf 2/3 group (17 ET, 12 PV and 32 PMF), of which 31 were *JAK2*^{V617F} positive and 13 *JAK2* wild type.

The control group consisted of 36 morphologically normal negative staging biopsies from patients with non-Hodgkin lymphoma and Hodgkin lymphoma (mean age: 55.8 years).

Immunohistochemistry

The bone marrow biopsy specimens were decalcified using the Kristensen procedure for 1 h or EDTA decalcification for 4 h, followed by standard tissue processing and paraffin embedding. From the paraffin-embedded blocks, 3-μm sections were cut for immunohistochemical staining and mounted on StarFrost slides (Knittel Gläser, Braunschweig, Germany). All the antibodies were tested for specificity on positive and negative tumor control slides and also individually tested on decalcified control bone marrow biopsies, resulting in a variation of immunohistochemical techniques optimized for all individual antibodies.

Immunohistochemical staining of pErk, pAkt, Bnip3 and p70S6k was carried out using the antihuman rabbit monoclonal antibody phospho-p44/42MAPK (Thr²⁰²/Tyr²⁰⁴), phospho-Akt (Ser⁴⁷³), antihuman mouse monoclonal antibody anti-Bnip3 (B7931) and phospho-70S6 kinase (Thr³⁸⁹; 1A5) at a dilution of 1:100, 1:25, 1:300 and 1:100, respectively [pErk, pAkt and p70S6k (Cell Signaling Technology, Danvers, Mass., USA) and Bnip3 (Sigma-Aldrich, St. Louis, Mo., USA)]. After deparaffinization, the slides were put in 0.3% H₂O₂ (Bnip3, p70S6k) in methanol to block endogenous peroxidase activity, followed by antigen retrieval by boiling for 20 min in 10 mM sodium citrate buffer (pH 6.0) in a water bath of 100°C. For pErk and pAkt after deparaffinization and antigen retrieval by boiling for 20 min in 10 mM sodium citrate buffer (pH 6.0) in a water bath at 100°C, endogenous peroxidase activity was blocked in 3% H₂O₂ in methanol.

After blocking solution [3% BSA/PBS for Bnip3 and p70S6k and TBST (Tris-buffered saline Tween)/5% goat serum (pH 7.2–7.6) for pErk and pAkt], the primary antibody, applied in TBST/1% BSA (pH 7.2–7.6 for pErk, pAkt) or antibody diluent (for Bnip3, p70S6k; Dako, Glostrup, Denmark) was incubated overnight at 4°C (pErk, pAkt) or for 1 h at room temperature (Bnip3, p70S6k). The slides were then incubated for 30 min with a PowerVision poly-HRP-anti Ms/Rb/Ra IgG histostaining kit (ImmunoLogic, Duiven, The Netherlands: pErk, pAkt) or with EnVision [Dako Real™ EnVision™ detection system (K5007); Bnip3, p70S6k]. After developing the color with freshly made diaminobenzidine solution (Dako), slides were counterstained with hematoxylin (Merck, Whitehouse Station, N.J., USA), dehydrated and mounted in Entellan (Merck).

The 142 trephines (MPN patients plus control patients) were immunohistochemically analyzed using an automated immunostainer (Dako Autostainer Link 48) with CD34 (clone QBend 10; Dako). CD34 was incubated for 20 min at room temperature. The reaction was revealed by means of the Dako EnVision Flex Kit (Dako) according to the manufacturer's instructions.

Quantification of Staining

The numbers of cells staining positive for pErk, pAkt, Bnip3 and p70S6k (fig. 1) were quantified using an image processing and analysis system (Leica, Cambridge, UK) linked to a Leica DML3000 light microscope (Leica Quantimet). The program used in this system was QWin (Leica's Windows-based image analysis tool kit). All measurements were conducted at ×40 magnification pictures. For measuring pErk, pAkt, Bnip3 and p70S6k, 5 hot spots per slide were included to measure total tissue, total nuclei positive for pErk, pAkt, Bnip3 or p70S6k and total nuclear count. The amount of positivity was calculated as the percentage of positive nuclear pixels related to the total number of nuclear pixels.

From the 5 pictures taken, we counted the total amount of megakaryocytes, the total amount of positively stained megakaryocytes, and megakaryocytes with nuclear and cytoplasmic staining. The mean total amounts of megakaryocytes, positively stained megakaryocytes, and megakaryocytes with nuclear and cytoplasmic staining were calculated using Excel (Microsoft, Redmond, Wash., USA).

MVD was assessed by counting the number of CD34-positive capillary, arteriolar or sinus lumen in five 1-mm² fields at ×100 magnification and calculating the mean of these 5 fields.

Fibrosis was graded according to the European consensus on grading of bone marrow fibrosis [26].

Statistical Analysis

The data were statistically evaluated using the SPSS 15 statistical package and analyzed descriptively (descriptives, explore and crosstabs). Statistical comparisons were performed by Mann-Whitney U test for median values and by the independent t test to evaluate differences in clinical parameters between the different groups. Values of p < 0.05 were considered statistically significant. Pearson's test was performed to correlate the expression of pErk, pAkt, p70S6k and Bnip3 with MVD, and fibrosis grading with MVD.

In some cases bone marrow tissue was lost during pretreatment of the slides; for pErk we report 4 missing values, for pAkt 5, for p70S6k 20, for Bnip3 21 and for MVD 5 missing values. For the grading of myelofibrosis we report 2 missing values.

Fig. 1. Examples of pErk, pAkt, Bnip3 and p70S6k staining in MPN patients and control patients. **a** pErk (HE, 1000×) staining in MPN patient. **b** pErk (HE, 650×) staining in control patient. **c** pAkt (HE, 630×) staining in MPN patient. **d** pAkt (HE, 650×) staining in control patient. **e** Bnip3 (HE, 1000×) in MPN patient. **f** Bnip3 (HE, 650×) in control patient. **g** p70S6k (HE, 1000×) in MPN patient. **h** p70S6k (HE, 650×) in control patient.

Results

The results of staining are summarized in tables 3–7. Qualitative microscopic evaluation of pErk showed nuclear expression predominantly in the erythroblasts and

occasionally in endothelial cells and plasma cells. Granulopoiesis and myelopoiesis did not show any pErk expression. Compared to the control, pErk expression was significantly higher in the general bone marrow of ET patients ($p = 0.013$) and the total MPN group ($p = 0.028$). In

Table 3. Percentage of positively stained megakaryocytes in ET, PV and PMF patients versus the control group

Positively stained megakaryocytes	ET (n = 36)	p value	PV (n = 25)	p value	PMF (n = 45)	p value	Control group (n = 36)
pErk	80.13±26.67	0.02	84.25±25.67	0.01	75.61±30.92	0.10	63.62±32.69
pAkt	93.19±8.63	0.06	83.97±24.75	0.85	84.81±21.44	0.72	82.58±30.38
Bnip3	97.04±6.27	0.07	87.72±23.23	0.80	83.00±29.67	0.66	86.29±30.92
p70S6k	95.92±7.38	0.12	86.79±22.70	0.77	87.77±19.65	0.87	88.63±24.58
VEGF	84.59±20.97	0.57	79.32±26.00	0.80	86.80±22.67	0.35	81.13±27.67

Means ± SD. t test.

Table 4. Percent pErk, pAkt, Bnip3, p70S6k and MVD in ET, PV and PMF, all MPN and control patients in total bone marrow cells

Total bone marrow cells	ET (n = 36)	PV (n = 25)	PMF (n = 45)	All MPN (n = 106)	Control (n = 36)
pErk, %					
Mean ± SD	16.93±11.04	13.49±8.73	14.61±7.66	15.13±9.21	11.41±6.70
Range	1.88–36.68	2.10–31.44	3.24–30.23	1.88–36.68	4.09–34.32
CI	12.37–21.34	10.14–19.71	12.52–19.16	13.74–18.36	11.74–20.34
pAkt, %					
Mean ± SD	15.31±6.17	14.04±7.51	13.99±6.01	14.43±6.42	10.11±7.19
Range	4.93–29.85	0.99–29.18	0.71–27.76	2.79–29.18	1.04–27.17
CI	12.44–17.33	13.47–20.90	12.15–16.80	13.74–16.70	11.25–19.15
Bnip3, %					
Mean ± SD	15.55±5.72	18.20±6.53	15.27±5.27	16.10±5.83	20.09±5.28
Range	4.02–25.46	6.46–29.89	6.09–26.03	4.02–29.89	11.18–28.91
CI	12.79–17.42	15.70–23.01	12.30–17.07	14.35–17.36	13.48–20.57
p70S6k, %					
Mean ± SD	20.74±5.25	22.54±7.28	21.06±5.84	21.28±5.97	20.07±4.44
Range	10.87–33.63	9.33–33.27	8.54–33.23	8.54–33.27	8.71–30.16
CI	18.67–22.44	17.31–25.91	18.56–23.40	19.53–22.36	16.94–22.35
MVD, n/mm ²					
Mean ± SD	37.72±22.18	47.55±27.45	58.47±31.56	48.79±28.92	27.95±11.25
Range	3.40–89.60	5.80–111.20	12.80–122.40	3.40–122.40	5.60–57.80
CI	29.70–49.07	35.77–70.80	50.30–76.39	44.16–59.03	17.94–34.27

CI = Confidence interval. Percent positive nuclei per total nuclei counted are shown except for MVD.

megakaryocytes, pErk expression was significantly higher in ET ($p = 0.000$), PV ($p = 0.000$) and PMF patients ($p = 0.000$) and in the total MPN group ($p = 0.000$) compared to the control group. As shown in table 2, there were significantly more stained megakaryocytes in the ET and PV group compared to the control group.

pAkt was expressed in the cytoplasm and nucleus of immature myeloid cells. Megakaryocytes showed cytoplasmic expression of pAkt. pAkt expression was significantly higher in megakaryocytes of ET ($p = 0.000$), PV ($p = 0.000$) and PMF patients ($p = 0.000$) and in the total MPN group ($p = 0.000$) compared to the control group.

The megakaryocytes of ET patients showed significantly higher pAkt expression compared to PV ($p = 0.035$) and PMF ($p = 0.050$) patients.

Bnip3 showed nuclear and cytoplasmic expression in myeloid cells. The megakaryocytes expressed Bnip3 in the cytoplasm and the endothelial cells in the nuclei. The expression of Bnip3 in the general bone marrow was statistically significantly higher in the control group: $p = 0.003$ versus ET patients, $p = 0.001$ versus PMF patients and $p = 0.001$ versus the total MPN group. However, megakaryocytes expressed Bnip3 significantly higher in ET ($p = 0.000$), PV ($p = 0.000$) and PMF pa-

Table 5. Percent pErk, pAkt, Bnip3, p70S6k and MVD in JAK2-positive and wild-type JAK2 patients in total bone marrow cells

Total bone marrow cells	JAK2 positive (n = 56)	Wild-type JAK2 (n = 24)
pErk, %		
Mean ± SD	17.10±9.89	13.71±7.52
Range	3.65–36.68	1.88–25.79
CI	13.58–19.41	10.82–17.53
pAkt, %		
Mean ± SD	14.82±6.40	16.89±5.45
Range	3.47–29.18	8.35–23.30
CI	13.03–16.71	13.72–17.90
Bnip3, %		
Mean ± SD	16.89±4.43	17.84±5.25
Range	4.02–28.22	7.21–26.03
CI	15.49–18.65	15.25–20.44
p70S6k, %		
Mean ± SD	22.74±5.73	21.47±4.17
Range	8.54–33.63	13.76–27.47
CI	21.21–24.46	19.46–23.48
MVD, n/mm ²		
Mean ± SD	52.77±30.58	49.01±25.98
Range	3.60–122.40	17.20–111.20
CI	44.72–63.18	40.64–65.24
Percent positive nuclei per total nuclei counted are shown except for MVD.		

Table 6. Absolute amount of positively stained megakaryocytes of pErk, pAkt, Bnip3 and p70S6k in ET, PV, PMF, all MPN and control patients

Absolute amount of positive megakaryocytes	ET (n = 36)	PV (n = 25)	PMF (n = 45)	All MPN (n = 106)	Control (n = 36)
pErk					
Mean ± SD	2.79±1.91	2.24±1.32	1.88±1.55	2.29±1.67	0.71±0.69
Range	0.00–7.00	0.60–5.60	0.00–5.20	0.00–7.00	0.00–3.40
CI	2.06–3.51	1.62–2.86	1.32–2.44	1.93–2.66	0.46–0.96
pAkt					
Mean ± SD	3.04±1.47	2.25±1.71	2.38±1.42	2.59±1.53	1.11±0.95
Range	0.80–7.60	0.00–7.80	0.40–5.80	0.00–7.80	0.00–4.00
CI	2.48–3.60	1.45–3.05	1.87–2.89	2.25–2.92	0.76–1.46
Bnip3					
Mean ± SD	2.43±1.31	1.85±1.19	1.95±1.47	2.10±1.35	0.68±0.46
Range	0.20–5.40	0.20–4.00	0.00–5.20	0.00–5.40	0.00–1.60
CI	1.94–2.93	1.29–2.40	1.42–2.48	1.80–2.40	0.51–0.85
p70S6k					
Mean ± SD	2.85±1.24	1.80±1.23	1.96±1.27	2.24±1.32	0.97±0.65
Range	0.60–5.80	0.00–5.80	0.00–6.80	0.00–6.80	0.00–2.80
CI	2.38–3.32	1.22–2.37	1.50–2.41	1.94–2.53	0.73–1.21
CI = Confidence interval.					

tients ($p = 0.000$) and in the total MPN group ($p = 0.000$) compared to the control group. Bnip3 expression was also significantly higher in megakaryocytes of ET patients compared to megakaryocytes of PV patients ($p = 0.019$).

p70S6k was mainly expressed in immature (nuclear) and mature myeloid cell lines (nuclear and cytoplasmic). Megakaryocytes expressed p70S6k in the cytoplasm and in the nucleus. Adipocytes also expressed p70S6k. p70S6k expression was significantly increased in megakaryocytes of

amount of stained megakaryocytes in ET patients compared to the control group.

Akt is phosphorylated by activated PI3K, which in turn can be phosphorylated by pSTAT5 and the *JAK2*^{V617F} mutation (fig. 2). The downstream effector of pAkt is the apoptosis inhibitor of megakaryocytes, Bcl-x_L [5, 29–33]. Megakaryocytes of ET, PV and PMF patients and the total MPN group showed higher pAkt expression compared to the megakaryocytes of control patients in our study, with the highest expression noted in ET patients. This suggests a role for pAkt in the pathological increase in megakaryocytes seen in the bone marrow of MPN patients leading to an increase in platelets in the peripheral blood. However, our results could not confirm the increased pAkt expression in *JAK2*^{V617F}-positive patients found in several previous studies [34, 35] probably due to the relatively high number of patients with an unknown JAK2 status.

Bnip3 is a pro-apoptotic protein which is activated under hypoxic conditions by Hif-1α (fig. 3) [24]. The lower Bnip3 expression in the total group of MPN patients in our study might indicate that the increased bone marrow cellularity is also a result of decreased apoptosis and not only due to proliferative activity. A discrepancy seems to exist between our results of Bnip3 expression in total bone marrow cells and the Bnip3 expression in megakaryocytes of MPN patients. However, it might also refer to the protective role against bone marrow apoptosis; Bnip3 might contribute to the increased cellularity in total bone marrow cells.

Concerning the activation of p70S6k, the formation of a complex between the regulatory subunit of PI3K (p85) and mTor is required [21] and is therefore in line with the higher pAkt expression in megakaryocytes of MPN patients in our study. Activated p70S6k phosphorylates BAD resulting in inactivation of BAD and consequently inhibition of apoptosis (fig. 3) [36]. The increased expres-

sion of p70S6k in megakaryocytes of MPN patients might indicate an inhibition of megakaryocyte apoptosis via p70S6k.

More studies were done to assess MVD in MPN patients: all show higher MVD in PMF patients compared to ET and PV patients and higher MVD in post-ET myelofibrosis and post-PV myelofibrosis compared to ET and PV, which indicates that angiogenesis is primarily involved in later stages of the disease [37–41]. We found a correlation between MVD and fibrosis, which is in line with the study by Boveri et al. [38], who found that MVD increased with the grade of fibrosis.

Higher p70S6k expression in the mf 0/1 group compared to the mf 2/3 group indicates a declining p70S6k expression with increasing myelofibrosis. This might also explain why we did not find a significant difference in p70S6k expression in bone marrow cells between MPN patients and control patients, while a higher percentage of patients in our study belonged to the mf 2/3 group.

In conclusion, the increased cellularity seen in MPN bone marrow might be influenced by the increased expression and anti-apoptotic mechanism of the pErk and pAkt pathways and a decreased expression of the pro-apoptotic protein Bnip3 in bone marrow cells in general. The increased amount of megakaryocytes seen in MPN might be due to the increased pAkt and p70S6k expression. Further, our results also suggest an important pathogenetic role for megakaryocytes in the pathogenesis of MPN patients, mainly in ET patients, as the anti-apoptotic pErk, pAkt, p70S6k and Bnip3 expression was higher in MPN megakaryocytes, mainly in ET patients, compared to the controls. Further, the increased MVD expression in patients with myelofibrosis suggests an important role of angiogenesis in the development of myelofibrosis and is therefore a potential therapeutic target in MPN patients with myelofibrosis.

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