Deregulation of apoptosis-related genes is associated with PRV1 overexpression and JAK2 V617F allele burden in Essential Thrombocythemia and Myelofibrosis

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Abstract

Background: Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) are Chronic Myeloproliferative Neoplasms (MPN) characterized by clonal myeloproliferation/myeloaccumulation without cell maturation impairment. The JAK2 V617F mutation and PRV1 gene overexpression may contribute to MPN physiopathology. We hypothesized that deregulation of the apoptotic machinery may also play a role in the pathogenesis of ET and PMF. In this study we evaluated the apoptosis-related gene and protein expression of BCL2 family members in bone marrow CD34+ hematopoietic stem cells (HSC) and peripheral blood leukocytes from ET and PMF patients. We also tested whether the gene expression results were correlated with JAK2 V617F allele burden percentage, PRV1 overexpression, and clinical and laboratory parameters.

Results: By real time PCR assay, we observed that A1, MCL1, BIK and BID, as well as A1, BCLW and BAK gene expression were increased in ET and PMF CD34+ cells respectively, while pro-apoptotic BAX and anti-apoptotic BCL2 mRNA levels were found to be lower in ET and PMF CD34+ cells respectively, in relation to controls. In patients’ leukocytes, we detected an upregulation of anti-apoptotic genes A1, BCL2, BCL-XL and BCLW. In contrast, pro-apoptotic BID and BIMEL expression were downregulated in ET leukocytes. Increased BCL-XL protein expression in PMF leukocytes and decreased BID protein expression in ET leukocytes were observed by Western Blot. In ET leukocytes, we found a correlation between JAK2 V617F allele burden and BAX, BIK and BAD gene expression and between A1, BAX and BIK and PRV1 gene expression. A negative correlation between PRV1 gene expression and platelet count was observed, as well as a positive correlation between PRV1 gene expression and splenomegaly.

Conclusions: Our results suggest the participation of intrinsic apoptosis pathway in the MPN physiopathology. In addition, PRV1 and JAK2 V617F allele burden were linked to deregulation of the apoptotic machinery.

Keywords: Chronic Myeloproliferative Neoplasms, Apoptosis, JAK2 V617F allele burden, PRV1, BCL2 family members

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Background

Essential Thrombocythemia (ET) and Primary Myelofibrosis (PMF) are disorders classified as Philadelphia chromosome-negative Myeloproliferative Neoplasms (MPN) [1]. ET is a clonal disease characterized by an increase in the platelet count associated with bone marrow megakaryocyte hyperplasia. Thrombosis and hemorrhagic events are the main comorbidities in ET patients. PMF is characterized by bone marrow fibrosis, as well as peripheral blood findings such as anemia, leukoerythroblastosisis and the presence of dacryocytes in peripheral blood [2].

The JAK2 V617F mutation, which leads to constitutive JAK2 activation, was shown to play an important role in MPN pathogenesis, and is found in 95% of Polycythemia Vera (PV) patients and in at least 50% of ET and PMF patients [3]. Constitutive JAK2 activation triggers several signaling pathways linked to cell survival and proliferation promoting myeloproliferation and resistance to cell death [4-8]. Other mutations have recently been described in ET and PMF patients, such as mutations in JAK2 exon 12 and in the TET2, CBL, MPL and AXSL genes [9-13]. Several studies suggest an association between MPN clinical features and the JAK2 V617F allele burden [14]. Although this knowledge and the identification of these additional mutations greatly enhanced our understanding of MPN physiopathology, a complete understanding of the cellular and molecular mechanisms involved is still lacking.

Another relevant molecular alteration described in MPN patients is the overexpression of PRV1, a surface receptor from hematopoietic cells associated with cell proliferation [15] related to JAK/STAT and SRC kinase pathways [16]. PRV1 overexpression was initially described in PV patients and in some cases of ET, but was not found elevated in other malignant hematological diseases such as Chronic Myeloid Leukemia (CML) [15,17,18]. Considering that PRV1 gene expression is deregulated in MPN, it has been suggested that it may be used as a molecular marker in the diagnosis of these diseases [19]. Literature also supports the hypothesis that PRV1 overexpression contributes to MPN physiopathology, considering that there are studies showing a correlation between PRV1 expression and patients' clinical and laboratory features [15].

The apoptosis process may be triggered by two major pathways: the extrinsic or death-receptors pathway, and the intrinsic or mitochondrial pathway. The intrinsic apoptosis pathway or mitochondrial pathway is triggered by several stimuli such as ultra violet radiation, growth factor/cytokine deprivation, chemotherapeutic agents, viral infection and other stress factors related to physical and chemical injuries [20]. This pathway is mainly regulated by BCL2 family members, classified as either anti-apoptotic (BCL2, BCL-XL, BCLW, MCL1 and A1) or pro-apoptotic (BAX, BAK, BAD, BID, BIM, Bok, BIK, BMF, BOO, BCL-Xl, PUMA and NOXA) proteins [21]. The apoptotic process was found to be deregulated in several hematopoietic neoplasms, leading to resistance to therapy and progression of disease. Alterations in apoptosis have been described in CML, Myelodysplastic Syndrome (MDS), Acute Myeloid Leukemia (AML), PV, ET and PMF [20,22-24].

In this study we investigated the potential association between apoptosis deregulation, JAK2 mutation and PRV1 overexpression in ET and PMF patients. We focused on evaluating the expression of apoptosis-related genes of the BCL2 family, JAK2 V617F allele burden and PRV1 expression in ET and PMF in bone marrow CD34+ hematopoietic stem cells (HSC) and peripheral blood leukocytes from ET and PMF patients. The correlation between gene expression, JAK2 allele burden, clinical and laboratory parameters and PRV1 expression were also assessed.

We observed a deregulation in the expression of most of the studied genes in bone marrow CD34+ cells and peripheral leukocytes from ET and PMF patients in comparison to controls. Furthermore, a correlation between BAD, BAX and BIK expression and JAK2 V617F allele burden as well as between A1, BAX and BIK and PRV1 expression was detected. A negative correlation between PRV1 expression and platelet count was also observed, as well as a positive correlation between PRV1 expression and splenomegaly.

Subjects and Methods

Patients and Controls

We studied bone marrow CD34+ HSC and peripheral blood leukocytes from 26 ET patients (5 male, 21 female, mean age: 60.2 years; range: 35-80y) and 12 PMF patients (9 male, 3 female, mean age: 61.7 years, range: 41-80y), without treatment. The bone marrow control group was composed of 23 bone marrow donors from the Bone Marrow Transplantation Unit of the Clinical Hospital - University of São Paulo at the Ribeirão Preto School of Medicine, São Paulo, Brazil. This group included 12 male and 11 female, with a mean age of 35.9 years (range 14-54y). We also collected peripheral blood samples from 37 individuals with similar age, skin color and gender (14 male, 23 female with mean age of 58.5 years, range 31-80y) for leukocyte isolation. This research was approved by the local ethics committees and the consent form was signed by the patients and volunteer controls. It was not possible to match the age and sex of bone marrow donors because the local ethics committees only permit...
bone marrow samples to be obtained from bone marrow donors during the bone marrow transplantation cell collection procedure.

**JAK2 V617F allele burden detection, platelet count and spleen size determination**

Detection of the JAK2 V617F mutation and the allele burden were determined as described in Tognon et al., 2011 [24], by real time allelic discrimination PCR assay. Platelets count from ET and PMF peripheral blood was determined by the Abbott Cell Dyn 3500SL Hematology Analyzer. Spleen size was determined by ultrasonography. In order to correlate gene expression and splenomegaly, we used the increase in centimeters (cm) along the longer dimension of the patients’ spleen, considering as reference, a value of 12 cm.

**Cell Isolation, RNA extraction and cDNA synthesis**

Cell isolation, RNA extraction and cDNA synthesis were performed as described in Tognon et al., 2011 [24]. Briefly, bone marrow CD34+ HSC were separated using the Ficoll-Hypaque protocol followed by Miltenyi CD34 isolation kit MidiMacs CD34+ Isolation Kit (MACS; Miltenyi Biotec, Bergisch Gladbach, Germany) and peripheral leukocytes were obtained by the HaesSteril method. Total RNA from CD34+ HSC and leukocytes was obtained according to the Trizol™ (Invitrogen Life Technologies, CarlsBAD, California, USA) method. One microgram of RNA was used to synthesize cDNA using the High Capacity™ Kit from Applied Biosystems Life Technologies (Foster City, California, USA).

**Quantification of apoptosis-related gene and PRV1 expression**

The expression of anti-apoptotic genes A1, MCL1, BCL2, BCL-XL and BCLW and pro-apoptotic genes BAD, BAX, BAK, BID, BIK and BIMEL was evaluated by real time PCR in duplicate. For gene expression quantification, we used the SYBR Green PCR Master Mix Kit (Applied Biosystems) and specific oligonucleotides (Invitrogen Life Technologies) (Table 1) on the 7500 Real Time PCR System (Applied Biosystems) and specific oligonucleotides (Invi- technology) (Foster City, California, USA).

| Gene | Primer Sequence |
|------|-----------------|
| A1   | F: GCC CTC ACC ACT ACT ATC | R: CCA GTT AAT GAC GTC |
| MCL1 | F: AGA AAG CTT CAG TAG ACC AT | R: CCA AGC TCC TAC TAC GAC AAG |
| BCL2 | F: ACG AGT GGG GTG CAG ATG TG | R: GCG GTA GCG GCG GCG GAA GAA GTC |
| BCLW | F: AGT TCG ACC CCA GCT TCC | R: CCC GTC CCC GTA TAG AGC |
| BCL-XL | F: CTG AAT CCG AGA TGG AGA CC | R: TGG GAT GTC AGA TGA TGG AGT |
| BID  | F: GCT TCC AGT GTA GAC GGA GC | R: GTG CAG ATT CAT GTT GTC AGT |
| BIK  | F: TCT GCA ATT GTC ACC GGT TA | R: TTG AGC ACA CCT CTC C |
| BIMEL | F: GCC CCT ACC TCC CTA CAG AC | R: AAG ATG AAA AGC GGG GAT CT |
| BAD  | F: CCG AGT GCG GAA GAC GTT | R: GGT AGG AGC TGG GTC TAA |
| BAK  | F: TCT GCC CCT ACA GGT CTA CC | R: ACA AAC TGG CCC AAC AGA AC |
| BAX  | F: CCC TTG TTC ATG AGT TTG | R: TCT TCT TGA AGT TGG TGA |
| β-actin | F: GCC CTG AGG CAC TCT TCC A | R: CCA CAG CAG TGA TCT CCT TCT |
| GAPDH | F: GCC TCA GATCAC TATC AGA TGC | R: CATGAGCTGTGCTGCTGCTT |

**Western Blot**

One million leukocytes were lysated in 100 μL of sample buffer (5% mercaptoethanol, 4% sodium dodecyl sulfate - SDS, 20% glycerol and 100 mM Tris-HCl-pH 6.8), boiled at 100°C for 5 minutes and kept at -20°C until the Western-Blot analysis was performed. Twenty-five ml of patient and controls’ lysate were loaded in polyacrylamide gel, the proteins were separated on 15% SDS-PAGE and transferred onto polyvinylidene difluoride (PVDF) membrane (Amersham, GE Healthcare Life Science). The membranes were incubated with the primary antibody anti-BCL-XL (1:200 dilution, H-62, SC7195, Santa Cruz Biotechnology®) or anti-BID (1:500 dilution, # 615258, BD Pharmingen) diluted in TBS-Tween (20 mM Tris, 137 mM NaCl, 0.01% Tween-20) with 5% non-fat milk for 16 hours. As a control for sample loading, the blot was probed with anti-γ-tubulin (1:2000 dilution, T3320, Sigma-Aldrich). Horseradish peroxidase (HRP) conjugated secondary antibodies and ECL Plus® (Amersham, GE Healthcare Life Science) were used for protein expression detection. Protein expression was measured by densitometry analysis performed by ImageQuant TL (Image Analysis Software, 2005, Amersham). To express the proteins densitometry results, firstly we calculated the ratio between protein test IDV (Integrated density value) and tubulin IDV, and
then we calculated the protein expression ratio between patient and controls (IDV of patients protein expression divided by controls protein expression).

**Statistical analysis**
The Mann-Whitney test (t-test, one-tailed) was used to compare the values of gene expression among the studied groups. The correlations between apoptosis-related gene expression, JAK2 allele burden and PRV1 gene expression were carried out by Spearman tests using Prisma 5.0 Software. A p value < 0.05 was taken as significant.

**Results**
**Gene expression of apoptosis-related BCL2 family members is deregulated in ET and PMF patients**
Gene expression analysis in CD34+ HSC from ET patients showed an increased expression of anti-apoptotic genes *A1* and *MCL1* and of pro-apoptotic genes *BID* and *BIK* (median = 39.25, 2.07, 5.69 and 2.99, respectively) in comparison to controls (0.986, 0.431, 1.15 and 1.36, respectively) (p < 0.0001, p = 0.0349, p = 0.0128 and p = 0.0279, respectively) (Figure 1A-D). The mRNA level of the pro-apoptotic gene *BAX* was found to be lower (0.19) in patients than controls (5.08) (p = 0.0319) (Figure 1E).

In PMF CD34+ HSC the anti-apoptotic genes *A1*, *BCLW* and pro-apoptotic *BAK* expression were significantly increased (21.11, 1.55 and 11.67, respectively) compared to controls (0.99, 1.01 and 1.67, respectively) (p = 0.0002, p = 0.0125 and p = 0.0460, respectively) (Figure 1A, F and 1G). *BCL2* mRNA levels were downregulated in these cells (0.18) in comparison to controls (1.99) (p = 0.0086) (Figure 1H).

In ET patients’ leukocytes we found an overexpression of the anti-apoptotic genes *A1*, *BCL2*, *BCL-XL* and *BCLW* (16.10, 2.21, 2.71 and 2.21, respectively) when compared with controls (0.49, 0.88, 0.80 and 1.06, respectively).

![Figure 1 Gene expression in Control, ET and PMF CD34+ HSC](image-url)

(A) anti-apoptotic gene *A1* was increased in ET and PMF compared to control; (B) anti-apoptotic gene *MCL1* was increased in ET compared to control; (C) pro-apoptotic gene *BID* was increased in ET compared to control; (D) pro-apoptotic gene *BIK* was increased in ET compared to control; (E) pro-apoptotic gene *BAX* was decreased in ET compared to control; (F) anti-apoptotic gene *BCLW* was increased in PMF compared to control; (G) pro-apoptotic gene *BAX* was increased in ET compared to control; (I) anti-apoptotic gene *BCL2* was decreased in PMF compared to control. The significant "p values" are shown in the figure. Otherwise, p > 0.05. The horizontal bars show the median 2^ΔΔCt for each group.
respectively) \((p < 0.0001, p = 0.0218, p = 0.0043\) and \(p = 0.0342\), respectively) (Figure 2A-D). The expression of pro-apoptotic genes BID and BIMEL was lower in ET leukocytes (0.62, 0.53) than in controls (1.43, 0.86) \((p = 0.0004, p = 0.0116)\) (Figure 2E, F).

In PMF leukocytes, BCL2, BCL-XL and BCLW expression were elevated (9.17, 16.10, 27.86, respectively) compared to controls (0.88, 0.80 and 1.07, respectively) \((p = 0.0291, p < 0.0001\) and \(p = 0.0007\), respectively) (Figure 2B, C and 2D).

Pro-apoptotic BAD expression was found to be increased in ET and PMF leukocytes (3.92, 5.18) compared to controls (0.63) \((p = 0.0130, p = 0.0276)\) (data not shown).

There were no differences when we compared BCL-XL, BAD and BIMEL expression between CD34+ cells from ET and PMF patients and controls, and this was also the case for MCL1, BID, BIK and BAX expression in leukocytes \((p > 0.05)\). Furthermore, between ET and PMF leukocytes we only found a significant difference in BCLW and BCL-XL expression \((p = 0.0145, p = 0.0033)\) (Figure 2C and 2D).

**BCL-XL and BID protein levels were different between controls and PMF and ET patients**

We detected an elevated level of anti-apoptotic BCL-XL in PMF leukocytes and a decreased expression of pro-apoptotic BID protein in ET leukocytes in comparison to control subjects (Figure 3A and 3B). Densitometry quantification by Integrated Density Value (IDV) showed that BCL-XL protein level is 2.4 times higher in PMF leukocytes than in controls, and BID protein in ET patients was 0.64 times lower in ET compared to control.

**Figure 2** Gene expression in Control, ET and PMF leukocytes. (A) anti-apoptotic gene A1 was increased in ET compared to control; (B) anti-apoptotic gene BCL2 was increased in ET and PMF compared to control; (C) anti-apoptotic gene BCL-XL was increased in ET and PMF compared to control and also significantly different between ET and PMF; (D) anti-apoptotic gene BCLW was increased in ET and PMF compared to control and also significantly different between ET and PMF; (E) pro-apoptotic gene BID was decreased in ET compared to control; (F) pro-apoptotic gene BIMEL was decreased in ET compared to control. The significant “p values” are shown in the figure. Otherwise, \(p > 0.05\). The horizontal bars show the median 2-\(ΔΔCt\) for each group.

**Figure 3** Protein expression in Control, ET and PMF leukocytes. (A) anti-apoptotic BCL-XL showed higher expression in PMF leukocyte than in controls (fold change: 2.4); (B) pro-apoptotic BID showed lower expression in ET leukocyte than in controls (fold change: 0.64). Tubulin was probed as loading control and densitometry was used to confirm the fold change in the protein expression.
leukocytes is 0.64 fold decreased in relation to its expression in controls.

**JAK2 V617F allele burden is correlated with BAD, BAX and BIK expression in ET patients’ leukocytes**

In JAK2 V617F-positive ET patients we detected lower levels of pro-apoptotic BAX, BIK and BAD expression (median: 0.26, 0.45 and 1.86, respectively) in leukocytes compared to JAK2 V617F-negative ET patients (1.59, 2.96 and 11.10, respectively) (p = 0.0189, p = 0.0309 and p = 0.0055, respectively) (Figure 4A-C). In addition, BAX, BIK and BAD expression were negatively correlated with JAK2 V617F allele burden (BAX: r = -0.4522; p = 0.0102; BIK: r = -0.4067, p = 0.0196; BAD: r = -0.5966, p = 0.0006) in ET (Figure 4D-F).

**PRV1 overexpression is correlated with A1, BIK and BAX gene expression, JAK2 V617F mutation, platelet count and splenomegaly**

We observed that PRV1 was overexpressed in ET (3.04) and PMF (5.12) leukocytes in comparison to controls (0.93) (p = 0.0011 and p = 0.0009) (Figure 5A). In ET leukocytes we also found that PRV1 expression was positively correlated with A1 expression (r = 0.3409, p = 0.0442), and negatively correlated with expression of BAX (r = -0.3791, p = 0.0281) and BIK (r = -0.5009, p = 0.0046) (Figure 5B-D). Moreover, PRV1 expression was positively correlated with BCLW (r = 0.6503, p = 0.0110) expression in PMF patients’ leukocytes (Figure 5E).

We detected a positive correlation between PRV1 expression and JAK2 V617F mutation allele burden in ET and PMF patients and this result corroborates the literature concerning the association between PRV1 and JAK2 V617F described in murine myeloid cells [25] and PV patients [26]. Leukocytes from ET patients harboring the JAK2 V617F mutation showed higher expression of PRV1 (median = 4.88) in comparison to those negative for the JAK2 V617F mutation (1.91) (p = 0.0074) (Figure 6A) and, consequently, a positive correlation between PRV1 expression and JAK2 V617F allele burden was observed in ET patient leukocytes (r = 0.4785; p = 0.0067) (Figure 6B).

Furthermore, PRV1 expression was negatively correlated with platelet count in ET (r = -0.3799, p = 0.0278) (Figure 7A) and PMF patients (r = -0.6713, p = 0.0084) (Figure 7B), and PRV1 expression in PMF leukocytes showed a positive correlation with the increase of the spleen size in centimeters (splenomegaly) (r = 0.6150, p = 0.0220) (Figure 7C).

**Discussion**

Our results indicate a deregulated expression of genes related to the intrinsic apoptosis pathway in CD34+ HSC and peripheral leukocytes from ET and PMF patients.

Our hypothesis was that higher expression levels of anti-apoptotic genes may contribute to the myeloaccumulation in ET and PMF. In support of this notion, in this study we found increased expression of A1, MCL1,
BCL2 and BCL-XL genes in ET and PMF patients compared to controls. Importantly, as previously described by our group, the cells from MPN patients are resistant to apoptosis induced by different drugs (actinomycin D, teniposide, etoposide, cytarabin, and staurosporin) [24]. These observations, and our findings in the present investigation, support our hypothesis that deregulated expression of apoptosis-related genes is linked to myelocaccumulation and pathogenesis in ET and PMF.

The BCL2 family proteins have a central role in the process of apoptosis control. The A1, BCL2, BCLW, BCL-XL and MCL1 BCL2-family members encode anti-apoptotic molecules, while BAD, BAX, BAK, BID, BIK, BOK, BOO, PUMA, NOXA and BIMEL encode pro-apoptotic molecules, all of them involved in the mitochondrial apoptosis pathway [21].

A1 (bfl1) expression is detected in several tissues such as hematopoietic and endothelial cells [27]. A1 gene transcription is dependent on the NF-kB pathway and its overexpression has been reported in Chronic Lymphocytic Leukemia (CLL), particularly in CLL patients who do not respond to therapy [27].

BCL2 is an oncogene, which was first identified in Non-Hodgkin lymphoma B-cells and this gene is a pivotal molecule in the mitochondrial apoptosis pathway. Castro et al. (2005) described in the American Society of Hematology. PRV1 gene expression in Control, ET and PMF leukocytes and PRV1 expression versus gene expression. (A) PRV1 expression was significantly elevated in ET and PMF leukocytes; (B) anti-apoptotic gene A1 showed positive correlation with PRV1 expression in ET leukocytes; (C and D) pro-apoptotic genes BAX and BIK showed negative correlation with PRV1 expression in ET leukocytes; (E) anti-apoptotic gene BCLW showed positive correlation with PRV1 expression in PMF leukocytes. The significant “p values” are shown in the figure. Otherwise, p > 0.05. The horizontal bars show the median 2-ΔΔCt for each group.

Figure 5 PRV1 gene expression in Control, ET and PMF leukocytes and PRV1 expression versus gene expression. (A) PRV1 expression was significantly elevated in ET and PMF leukocytes; (B) anti-apoptotic gene A1 showed positive correlation with PRV1 expression in ET leukocytes; (C and D) pro-apoptotic genes BAX and BIK showed negative correlation with PRV1 expression in ET leukocytes; (E) anti-apoptotic gene BCLW showed positive correlation with PRV1 expression in PMF leukocytes. The significant “p values” are shown in the figure. Otherwise, p > 0.05. The horizontal bars show the median 2-ΔΔCt for each group.

Figure 6 PRV1 expression versus JAK2 V617F mutation. (A) PRV1 expression was higher in ET JAK2 V617F positive patients than in JAK2 V617F negative patients; (B) a positive correlation between PRV1 expression in ET leukocytes and JAK2 V617F allele burden percentage was detected. The significant “p values” and the “r value” for correlations are shown in the figure.
Hematology Congress a decrease in BCL2 expression and an increase in BCL-XI, BCLW, A1, MCL1 and cflip expression in CML and they also demonstrated that this profile of expression was correlated with CML progression. Furthermore, many publications have shown that in neoplasms such as breast or stomach cancer, high levels of BCL2 proteins were associated with a worse prognosis.

Deregulation of MCL1 expression was described in hepatocellular carcinoma and in multiple myeloma. Furthermore, the expression of this gene was also found to be correlated with prognosis in multiple myeloma. Such deregulated expression was also verified in bone marrow blasts from patients with MDS. Del Poeta et al. reported increased levels of BCL2, BCL-XI and MCL1 expression in AML and Aichberger et al. showed that MCL1 is a BCR-ABL target gene in CML.

Among the pro-apoptotic proteins of the BCL2 family, BAX has a crucial role in apoptosis and the lack of BAX leads to apoptosis impairment and facilitates the development of B-cell lymphoma by c-Myc stimulation.

Regarding MPN pathophysiology, Zhang et al. demonstrated that BCL-XI is down-regulated early during in vitro differentiation of megakaryocytes from ET patients and this might reflect an early entry of megakaryocytes into a degenerating mature stage. There is little data in the literature regarding apoptosis deregulation in PMF. On one hand, Mesa et al. showed that the levels of the anti-apoptotic and pro-apoptotic BAX, BAK, BIM and Bmf were not different between PMF and controls. On the other hand, it was demonstrated that JAK2 inhibition in a cellular model of MPN (JAK2 V617F positive cell lineage) triggers BIM activation and leads to enhanced sequestration of MCL1, furthermore, BCL-XI and MCL1 depletion by RNAi was sufficient to compromise JAK2 V617F mutant cell viability and sensitized the cells to JAK2 inhibition, indicating an association between these apoptosis-related molecules and the aberrant JAK2 signaling in these cells.

Figure 7 Correlation of PRV1 gene expression with platelet count (PLT) and splenomegaly. PRV1 expression in ET (A) and PMF (B) leukocytes showed negative correlation with platelets (PLT); (C) PRV1 expression in PMF leukocytes showed positive correlation with the increase in centimeters of the spleen length (splenomegaly) determined by ultrasonography (reference value: 12 cm). The significant “p” values and the “r” value for correlations are shown in the figure.
It has been described that BCL2 family proteins interact with each other to control the intrinsic apoptosis pathway [37]. The balance among the activities of these proteins is very important to tightly control the apoptosis process [38]. The MPN patients enrolled in this study showed overexpression of several anti-apoptotic genes such as A1 and MCL1 but also overexpression of some pro-apoptotic genes such as BIK, BID and BAK in CD34+ cells. Furthermore, in CD34+ cells we observed a downregulation of BAX and BID, and BIM EL levels were found reduced in ET leukocytes. Therefore, we postulate that the balance among these BCL2 family members is disrupted, and may contribute to the myeloaccumulation in these patients due to the increase in cell survival.

In addition, we found that mRNA levels of pro-apoptotic BAX, BAD and BIK mRNAs were lower in JAK2 V617F-positive patients than in those negative, and also presented a negative correlation with the JAK2 V617F allele burden. Some reviews published in the literature have already pointed out and discussed the relationship between constitutively activated JAK2/STAT signaling and deregulation of apoptosis-related genes in CML and human tumor cell lines [39,40].

Moreover, we analyzed PRV1 mRNA expression and the correlation with JAK2 V617F mutation, and with clinical laboratorial data in leukocytes from control, ET and PMF patients. PRV1 is a hematopoietic cell surface receptor that has been shown to transduce intracellular signals leading to proliferation, involving JAK2 and Src kinases [16]. PRV1 is overexpressed in MPN patients [15,19,41,42] and seems to be associated with PV disease phenotype characterized by high erythrocyte and low platelet counts [15]. These studies also described a correlation between PRV1 expression and the JAK2 V617F allele burden, as well as between PRV1 overexpression and elevated JAK2 tyrosine kinase activity [15,19,25,41,42].

In our results we detected a correlation between PRV1 overexpression and the anti-apoptotic genes A1 and BCLW, and the pro-apoptotic genes BAX and BIK expression. We also found a differential expression of PRV1 according to JAK2 V617F status and a correlation between PRV1 expression and platelet count in ET and PMF patients, as well as splenomegaly. Thus, our results suggest a link between PRV1 and intrinsic apoptosis pathway regulation and are in good agreement with previous reports about the association of the JAK2 V617F mutation with deregulation of apoptosis and disease phenotype [25].

An illustrative overview of the gene expression results in CD34+ HSC and leukocytes from ET and PMF is shown in Figure 8. This figure highlights the complexity of the apoptosis network in MPN patients. The analysis of the interaction of genes involved in the apoptotic machinery described here implies that apoptosis is deregulated and impaired in MPN patients since the majority of anti-apoptotic genes assessed are overexpressed, while concomitantly, some pro-apoptotic genes appear to be downregulated.

These observations are in line with previous results described by our group in Tognon et al. (2011) [24], which demonstrated that mononuclear cells from MPN patients are resistant to apoptosis, considering that patients’ cells stimulated by numerous apoptotic inducers, such as actinomycin and cytarabine, showed less Annexin-V FITC staining compared to controls [24]. Therefore, deregulation of the intrinsic apoptosis pathway might contribute to ET and PMF physiopathology and myeloaccumulation.

MPN do not yet have a curative therapy so it is particularly relevant to consider the possibility of designing new drugs targeting apoptosis pathway. In this context, Zivny et al. (2010) reviewed this subject emphasizing the importance of developing new and more effective target cancer therapies with the potential of inhibiting the anti-apoptotic BCL2 family members or enhancing pro-
apoptotic proteins expression [20]. These approaches might impair evasion of tumor cell to apoptosis processes or might sensitize cells to apoptosis. Taken together our results suggest that ET and PMF treatment could not be restrict to JAK2 inhibitors, considering that there are other molecular mechanisms involved in MPN pathogenesis, in addition to JAK2 mutation. Maybe in the future, JAK2 inhibitor treatment must be associated with new target therapies, such as anti-apoptotic BCL2 family members’ inhibitors or pro-apoptotic enhancers, for better patients’ response to therapy.

Conclusion
CD34+ HSC and leukocytes from ET and PMF patients displayed a deregulation in expression levels of BCL2 family members, which are correlated with JAK2 V617F mutation and PRV1 mRNA levels. Our findings suggest that these alterations may contribute to increased resistance to apoptosis and to myeloaccumulation in ET and PMF patients.

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RT designed and performed experiments, analyzed data and wrote the paper. EPLG, RPN and PRV1 mRNA overexpression: relevance in the diagnosis of myeloproliferative neoplasms: the 2008 World Health Organization criteria and point-of-care diagnostic algorithms. Leukemia 2008, 22:14-22.

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