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Article

Deregulated expression of *A1, Bcl-2, Bcl-x_L,* and *Mcl-1* antiapoptotic proteins and *Bid, Bad,* and *Bax* proapoptotic genes in polycythemia vera patients

Elainy Patricia Lino Gasparotto[1], Raquel Tognon[1], Aline Fernanda Ferreira[1], Gislane Lelis Vilela Oliveira[1], Patrícia Vianna Bonini Palma[4], Maria Aparecida Zanichelli[2], Elizabeth Xisto Souto[2], Carlos Eduardo Engel Velano[3], Belinda Pinto Simões[3], Rita de Cassia Viu Carrara[4], Simone Kashima[4], Dimas Tadeu Covas[3,4], Fabíola Attie de Castro[1], Ana Maria de Souza[1,*]

[1]Department of Clinical Analyses, Toxicology and Food Sciences, Faculty of Pharmaceutical Sciences of Ribeirão Preto, University of São Paulo; [2]Brigadeiro Hospital, São Paulo[3], Department of Clinical Medicine, Faculty of Medicine of Ribeirão Preto, University of São Paulo; [4]Hemotherapy Center of Ribeirão Preto, School of Medicine of Ribeirão Preto, University of São Paulo

Apoptosis deregulation might have a role in the pathophysiology of polycythemia vera (PV). This study evaluated *Bcl-2* molecule expression in $CD34^+$ cells and leukocytes in 12 PV patients. Gene expression was investigated by real time PCR using SybrGreen Quantitect kit and protein expression was evaluated by western-blotting. JAK2 V617F mutation was detected according to Baxter et al (2005). $CD34^+$ cells from PV patients presented higher levels of *A1* and *Mcl-1* expression (median: 22.6 and 5.2, respectively) in comparison with controls (0.9 and 0.5, p=0.004 and p=0.020); while *Bcl-2* and *Bcl-x_L* expression decreased in PV patients (0.18 and 1.19) compared with controls (1.39 and 2.01, p=0.006 and p=0.020). $CD34^+$ cells in PV patients showed an elevated *Bid* expression (14.4) in comparison with healthy subjects (1.0; p=0.002). Patients' leukocytes showed an *A1* augmentation (7.41, p=0.001) and a reduced expression of *Bax* (0.19; p=0.040) and *Bad* (0.2; p=0.030). There was no correlation between JAK2 V617F allele burden and molecular expression. PV patients showed alterations in *Bcl-2* members' expression, which may interfere with control of apoptotic machinery and contribute to disease pathogenesis.

Uniterms: Polycythemia vera. Gene mutation. Gene expression. Apoptosis. Bcl-2 family members.

A desregulação da apoptose parece participar da fisiopatologia da policitemia vera (PV). Este estudo avaliou a expressão das moléculas da família Bcl-2 em células hematopoéticas CD34 + e leucócitos de 12 pacientes com PV. Foram realizados: a quantificação da expressão gênica por PCR em tempo real utilizando kit Sybrgreen Quantitect, avaliação da expressão de proteínas por *western-blot* e detecção da mutação JAK2 V617F segundo Baxter *et al.* (2005). Células CD34 + dos pacientes com PV apresentaram maior expressão de *A1 e Mcl-1* (mediana: 22,6 e 5,2, respectivamente) em comparação com controles (0,9 e 0,5, p = 0,004 e p = 0,020) e expressão de *Bcl-2 e Bcl-x_L* diminuída nestes pacientes (0,18 e 1,19) em relação aos controles (1,39 e 2,01, p = 0,006 e p = 0,020). Células CD34 + dos pacientes com PV mostraram expressão elevada de *bid* (14,4) em comparação aos controles (1,0; p = 0,002). Leucócitos dos pacientes mostraram aumento de *A1* (7,41, p = 0,001) e expressão reduzida do *Bax* (0,19; p = 0,04) e *Bad* (0,2; p = 0,030). Não houve correlação entre percentagem de alelos JAK2 V617F mutados e expressão molecular. Pacientes com PV apresentaram alterações na expressão de moléculas Bcl-2 que podem interferir no controle da apoptose e contribuir para a patogênese da doença.

Unitermos: Policitemia vera. Mutação gênica. Expressão gênica. Apoptose. Membros da Família Bcl-2.

Correspondence: A. M. Souza. Departamento de Análises Clínicas, Toxicológicas e Bromatológicas, Faculdade de Ciências Farmacêuticas de Ribeirão Preto – USP. Avenida do Café s/n - Monte Alegre - 14040-903 - Ribeirão Preto - SP, Brasil. E-mail: amsouza@fcfrp.usp.br.

INTRODUCTION

The 2001 WHO classification considered that the major myeloproliferative disorders (MPDs) are: chronic myelogenous leukemia (CML), polycythemia vera (PV), essential thrombocythemia (ET), and idiopathic myelofibrosis (IMF). The discovery of a mutation in the Janus kinase led to an understanding of the pathogenesis of the classic MPDs. In 2008 the WHO classification revisions reorganized the MPDs into myeloproliferative neoplasms (MPNs) (Tefferi, Vardinam, 2008; Wadleigh, Tefferi, 2010).

Polycythemia vera is a clonal disorder arising in a multipotent hematopoietic progenitor cell, exhibiting accumulation of normal red and white cells, platelets, and their progenitors in the absence of a definable stimulus. The hallmark of PV is the trilineage hematopoietic cell hyperplasia, but erythrocytosis is its most remarkable clinical manifestation (Spivak, 2002). According the 2008 WHO guidelines, the major PV diagnostic criteria are: hemoglobin levels > 18.5 g/dL in men, 16.5 g/dL in women or other evidence of increased red cell volume and presence of the Janus kinase 2 mutation (JAK2 V617F), or other functionally similar mutation such as JAK2 exon 12 mutation. The minor PV diagnostic criteria are: bone marrow biopsy showing hypercellularity with trilineage myeloproliferation; serum erythropoietin (EPO) level below the normal reference range; *in vitro* endogenous erythroid colony (EEC) formation. Diagnosis de PV requires meeting either both major criteria and one minor criterion or the first major criterion and two minor criteria (Tefferi, Vardinam, 2008; Wadleigh, Tefferi, 2010).

The JAK2 V617F somatic mutation has been detected in 95% of PV patients and in 40 to 60% of ET or IMF patients. It is responsible for the activation of downstream target genes of JAK/STAT pathways, which seem to be linked to cell apoptosis impairment. Furthermore, there are indications that there may be an association between the mutation status, PV pathogenesis, and prognosis. The JAK2 V617F homozygous mutation could promote the development of a polycythemic phenotype, since homozygosis for the JAK2 mutation occurs in roughly 30% of patients with PV (Baxter *et al*., 2005; James *et al*., 2005; Kralovics *et al.*, 2005; Levine *et al.*, 2005; Levine *et al.*, 2007).

Notwithstanding the new knowledge about PV and the description of new prognostic and diagnostic markers, the etiology and pathogenesis of this disease remains unclear. The mechanisms involved in PV molecular basis are still elusive. It seems that PV is a myeloaccumulative disorder rather than a myeloproliferative disorder (Spivak, 2002). In this context, we postulate that apoptosis deregulation may have a role in its pathophysiology.

Apoptosis could be triggered by mitochondrial (intrinsic) and death receptor (extrinsic) pathways. In the intrinsic pathway, cytochrome c is released upon stimulation by a variety of cell-death triggers such as ultra violet light, cellular stress, chemotherapy, virus infection, cytokine deprivation, and DNA damage. Cytochrome c release, in turn, leads to activation of *Apaf-1* (apoptosis protease activating factor-1), caspase-9, and caspase-3 (Borner, 2005; Jin; El Deiry, 2005; Yan, Shi, 2005).

The extrinsic apoptotic pathway involves the interaction of death receptors such as *Fas* and caspase-8, which lead to caspase-3 activation and cell death by apoptosis (Fulda; Debatin, 2006). Cell apoptotic machinery is mainly regulated by *Bcl-2* family members (Borner, 2005), probably acting through the control of mitochondrial membrane permeability. *Bcl-2* members could be divided in pro- (*Bid, Bik, Bim, Bmf, Bok, Puma, Noxa, Bak, Bad,* and *Bax*) and anti- apoptotic (*A1, Bcl-2, Bcl-x_L, Mcl-1*, and *Bcl-w*) molecules (Borner, 2005; Sharper, Arnoult, Youle, 2004).

The association between PV pathogenesis and apoptosis has been poorly investigated but its role in other myeloproliferative diseases was well established. The *A1* protein seems to have an important role in the pathogenesis in several B-cell malignancies and acute leukemias (Simpson *et al.*, 2006; Brien *et al.*, 2007). The literature reports the association of the overexpression of the *Mcl-1* antiapoptotic molecule with solid tumors, chronic lymphocytic leukemia, and chronic myeloid leukemia pathogenesis and with resistance to chemotherapy (Kitada, Reed, 2004). Economopoulos *et al*. (2008) described an increase in *A1* and *Mcl-1* mRNA levels in blastic cells of myelodysplastic syndrome patients. Del Poeta *et al.* (2008) reported an increase in the expression of *Bcl-2*, *Bcl-x_L* and *Mcl-1* in acute myeloid leukemia. Zhang *et al.* (2004) showed that $CD34^+$ cells from ET patients presented a high expression of *Bcl-x_L* in the initial differentiation phase of megakaryocytes induced by thrombopoietin.

Zeuner *et al.* (2006) reported that PV patients had abnormal erythropoiesis, EPO independence or hypersensitivity, and c-Flip overexpression, which could have contributed to apoptosis resistance and erythropoiesis alteration in some patients with the disease. Garçon *et al.* (2006) observed that only a sustained level of *Bcl-x_L* is capable of giving rise to Epo-independent erythroid colony formation suggesting that, in PV patients, JAK2 V617F may induce EEC via the STAT5/*Bcl-x_L* pathway; the JAK2/STAT5/*Bcl-x_L* pathway seems to be crucial to erythropoiesis and implicated in cell proliferation and

survival. Deregulated erythropoiesis in PV involves EPO hypersensitivity and apoptosis resistance of erythroid precursor cells associated with abnormally increased activation of RAS-ERK and phosphoinositide-3 kinase-AKT pathways (Laubach *et al.*, 2009).

These literature observations are leading investigators to focus on studies to understand how cell death deregulation is relevant to the neoplasm's pathophysiology and to develop more effective therapeutic strategies. Therefore, the objective of this study was to investigate the expression of genes and proteins of *Bcl-2* family members in CD34+ hematopoietic stem cells and leukocytes in PV patients and healthy subjects. The potential correlation between gene and protein expression with JAK2 mutation allele burden and lymphocyte resistance to apoptosis is also analyzed.

SUBJECTS AND METHODS

Patients and controls

Twelve PV patients, not treated, were evaluated in this study and included only when PV diagnosis was confirmed according to the 2008 WHO criteria. Their demographic and clinical-laboratorial parameters are described in Table I. The patients' mean age was 63.4 years old (range 50-84 years). Three males and nine females were included in the study.

Peripheral blood samples were provided by healthy subjects recruited at the Faculty of Pharmaceutical Sciences, University of São Paulo (USP) at Ribeirão Preto. Marrow donors were obtained at the Bone Marrow Transplantation Unit of the University Hospital, Faculty of Medicine of Ribeirão Preto-USP. The samples were collected from 19 healthy subjects, whose mean age was 38.4 years old (range 14-54 years), 12 males and 7 females. Leukocytes were obtained from 14 healthy individuals, 5 males and 9 females, whose mean age was 54.4 years (range 41-80 years). The local ethics committee approved the study and all samples were collected after the subjects signed the Informed Consent Form.

Peripheral blood mononuclear cell and leukocyte separation

Peripheral blood mononuclear cells (PBMC) were isolated by the Ficoll-Hypaque method (Histopaque®-1077, Sigma, St. Louis, Missouri, USA) according to instructions by the manufacturer and as described by Boyum (1976).

Peripheral blood leukocytes were obtained from patients and controls using Haes-Steril® reagent (Voluven®, Fresenius Kabi, Campinas, Brazil). Briefly, the peripheral blood is mixed to Haes reagent in a 3:1 proportion, the mixture is decanted for 120 minutes at room temperature and leukocytes are recovered from the supernatant by centrifugation at 1800xg for 15 minutes. The cells were resuspended in PBS-ACD buffer (1mL) for counting and viability determinations with 0.4% Trypan Blue (Sigma-Aldrich Chemie, Steinheim, Germany).

TABLE I - Demographic features, hematological parameters, and JAK2 V617F allele burden from PV patients

Patient	Age	Sex	Skin Color	Hb (g/dL)	Ht (%)	WBC (/uL)	Plt (x10^9/L)	JAK2 Allele Burden (%)
1	50	F	B	19.1	57.9	8.08	207	Negative
2	66	F	W	20.1	60	5.20	247	13
3	80	F	W	15.9	44.5	8.80	541	90
4	54	F	B	25.0	65	5.10	169	13
5	59	M	W	19.7	60.2	20.00	568	87
6	59	M	W	15.6	46.6	8.75	330	25
7	55	F	W	22.4	66.8	7.47	276	52
8	61	F	B	18.7	61.1	9.04	412	50
9	69	F	W	18.1	57.3	6.35	255	40
10	84	F	W	16.0	53.9	21.30	420	94
11	52	M	W	17.4	53.5	5.50	229	Negative
12	72	F	W	17.9	56	8.85	299	55

Abbreviations: PV, polycythemia vera; M, male; F, female, Hb, hemoglobin; Ht, hematocrit; WBC, white blood cell count; Plt, platelets; W, white; B, black.

CD34+ hematopoietic stem cell isolation

Bone marrow mononuclear cells were separated by Ficoll-Hypaque centrifugation (Histopaque®-1077, Sigma, St. Louis, MO, USA) and CD34+ hematopoietic stem cells (HSC) were obtained by positive selection using an immunomagnetic column according to the manufacturer's protocol (MACS, Milteny Biotec, Bergisch Gladbach, Germany).

After selection, the purity of CD34+ HSC was verified by flow cytometry. CD34+ HSC population's purity was more than 91.80% in bone marrow transplantation donors and 87.06% in PV patients.

SDS-PAGE and western-blot analysis

Peripheral leukocytes ($2x10^9$cel/L) from 4 healthy individuals and 4 PV patients were chosen to represent and investigate the results obtained by gene expression. The samples were chosen because we have more leukocyte aliquots from them than the others patients and because according to gene expression results they present a gene expression close to the median value of the evaluated molecule, which were washed once in ice-cold PBS, lysed directly in SDS-sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2.5% β-mercaptoethanol) and boiled for 5 min. Samples were resolved under reducing conditions for two hours at 80 volts in SDS-polyacrylamide gels. Separated proteins then were blotted onto polyvinylidene difluoride (PVDF) membranes (Pierce, Rockford, IL, USA) at 500 mA for two hours. Blots were blocked overnight in TBST buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween) containing 0.1% sodium azide and 5% non fat dried milk and then probed during two hours or overnight with an appropriate dilution of *A1* polyclonal antibody (Santa Cruz, Santa Cruz, California, USA), *Bad* monoclonal antibody (PharmingenTM BD Biosciences, San Diego, California, USA), *Bcl-*x_L polyclonal antibody (Biotechnology.Inc Santa Cruz, California, USA), and *Bim* polyclonal antibody (PharmingenTM BD Biosciences). Proteins reactions to primary antibody were secondary antibody conjugated to horseradish peroxidase (GE Amersham, Arlington, IL, USA) using enhanced chemiluminescence (GE Amersham). A semi-quantitative assessment of protein expression was performed by band relative densities detection using the Alphaease software (Alpha Inotech, San Leandro, California, USA). Protein expressions were normalized by β-actin (Sigma-Aldrich, St. Louis, Missouri, USA) or β-tubulin (Sigma-Aldrich) endogenous expression. The densitometry methodology was applied to quantify the proteins, which were expressed by integrated density value (IDV). The protein expression results are presented as supplementary data.

Total RNA extraction, cDNA synthesis and real time PCR

Leukocyte and CD34+ HSC total RNAs were obtained according to the Trizol® protocol. RNA concentration and purity was determined by measuring fluorescence at 260 nm and 280 nm. One microgram of RNA was used for cDNA synthesis by High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA), according to manufacturer instructions. Real time PCR assay was carried out in cDNA to determine proapoptotic (*Bad, Bak, Bax, Bid, Bik, Bim-*$_{EL,}$ and *Bok*) and antiapoptotic gene expression (*Bcl-2, Bcl-*x_L*, Bcl-w, Mcl-1,* and *A1*) using SybrGreen PCR Master Mix Kit (Applied Biosystems), specific primers (Table II), and ABIPrism 7700 (Applied Byosytems). Beta-actin gene was used as housekeeping genes and the results were given as $2^{-\Delta\Delta Ct}$. Beta-actin was chosen as a housekeeping gene because its expression is high in hematopoietic cells and it has a minimal expression variability between the samples evaluated in this study.

JAK2/V617F mutation detection and allele burden calculation

The JAK2 V617F mutation was detected by real time PCR according to Baxter *et al*. (2005) and the results were presented as negative or allele burden of JAK2 positive mutation.

Apoptosis resistance assay

Mononuclear cells from patients and controls were cultured with 2µg/mL of phytohemagglutinin (Sigma, St. Louis, MO, USA) in complete RPMI 1640 medium (Sigma) supplemented with 10% bovine fetal serum, 10mM HEPES, 2mM L-glutamine, 100units/mL of penicillin and 100ug/mL streptomycin, at 37 ºC in a 5% CO_2 during three days.

Then, the mononuclear cells were treated with apoptosis inducers such as: actinomycin (ACT, 1 and 5 µM, Sigma-Aldrich), cycloheximide (CHX, 25 and 50 µM, Sigma-Aldrich), cytarabine (ARA-C, 25µM, Pfizer®, Milan, Italy), etoposide (VP-16, 25µM, Bristol-Meyers-Squibb®, Mayaquez, Porto Rico), teniposide (VM-26, 25 µM, Bristol-Meyers-Squibb®), staurosporine (STS, 1 and 5 µM, Sigma-Aldrich) for twelve hours.

After incubation, mononuclear cells were recovered from the culture medium by centrifugation (240x g,

TABLE II - Primers sequences, amplicon size and their specific annealing temperature for quantitative real-time PCR

Genes	5'→ 3' sequence	Amplicon (bp)	AT (ºC)
Bax	F: CCC TTT TGC TTC AGG GTT TC R: TCT TCT TCC AGA TGG TGA GTG	500	56
Bad	F: CCG AGT GAG CAG GAA GAC TC R: GGT AGG AGC TGT GGC GAC T	205	60
Bak	F: TTT TCC GCA GCT ACG TTT TT R: TGG TGG CAA TCT TGG TGA AGT	249	60
Bid	F: GCT TCC AGT GTA GAC GGA GC R: GTG CAG ATT CAT GTG TGG ATG	203	62
Bik	F: TCT GCA ATT GTC ACC GGT TA R: TTG AGC ACA CCT GCT CCT C	193	60
Bok	F: TTT TCC GCA GCT ACG TTT TT R: TGG TGG CAA TCT TGG TGA AGT	249	59
Bim_{eL}	F: TCT GCA ATT GTC ACC GGT TA R: AAG ATG AAA AGC GGG GCT CT	196	59
Bcl-2	F: ACG AGT GGG ATG GGG GAG ATG TG R: GCG GTA GCG GCG GGA GAA GTC	204	67
Bcl-w	F: AGT TCG AGA CCC GCT TCC R: CCC GTC CCC GTA TAG AGC	308	60
Bcl-x_L	F: CTG AAT CGG AGA TGG AGA CC R: TGG GAT GTC AGG TCA CTG AA	211	60
A1	F: GGC TGG CTC AGG ACT ATC R: CCA GTT AAT GAT GCC GTC	227	50
Mcl-1	F: AGA AAG CTG CAT CGA ACC AT R: CC AGC TCC TAC TCC AGC AAC	183	56
Noxa	F: AGC TGG AAG TCG AGT GTG CT R: ACG TGC ACC TCC TGA GAA AA	167	54
Puma	F: GGA GCA GCA CCT GGA GTC R: TA CTG TGC GTT GAG GTC GTC	156	60
β-actin	F: GCC CTG AGG CAC TCT TCC A R: CCA GGG CAG TGA TCT CCT TCT	192	58

Abbreviations: AT: annealing temperature; bp: base pair; F: forward; R: reverse.

10 minutes, 4 ºC), washed once with annexin buffer (10 mM HEPES pH 7.4; 150 mM NaCl; 5 mM KCl; 1 mM $MgCl_2$; 1,8 mM $CaCl_2$) and incubated for 20 minutes with 100 µL of Annexin-V FITC. Subsequently, ten thousand cells were acquired with 40 µL of a propidium iodide solution (50 µg/mL) and the data were analyzed by the DIVA software in a FACSCanto Flow Cytometer (Becton & Dickinson, San Jose, CA, USA). The results were expressed as percentage of apoptotic cells labeled with Annexin-V-FITC.

Statistical analysis

The Mann Whitney U-test was used to determine statistical differences in gene expression and protein expression between controls and PV patients. The Spearman's correlation test was carried out to detect the associations of JAK2 V617F allele burden and gene expression, JAK2 V617F allele burden and percentage of apoptotic mononuclear cells, and gene expression and percentage of apoptotic mononuclear cells. The results were considered statistically significant when p values were < 0.05.

RESULTS

Gene expression of *Bcl-2* family members in hematopoietic stem cells and peripheral leukocytes

$CD34^+$ HSC from PV patients presented higher levels of *A1* and *Mcl-1* expression (median: 22.6 and 5.2, respectively) in comparison with controls (median: 0.9 and 0.5, p=0.002 and p=0.008) (Figure 1; Supplementary Table S1). *Bcl-2, Bcl-x_L*, *Bax* and *Bad* gene expressions

were decreased in PV patients CD34+ HSC (0.18, 1.19, 6.76, 0.24) compared with controls (1.39, 2.01, 10.5 and 4.42; p=0.006, p=0.020, p=0.010 and p=0.010) (Figures 1 and 2). CD34+ HSC of PV patients showed increased *Bid* expression (14.4) in relation to normal individuals (1.0; p=0.002) (Figure 2; Supplementary Table S1).

In contrast to controls (median *A1*=1.02, *Bax*=1.25 *and Bad*=1.18), patient leukocytes showed an augmented *A1* gene expression (7.41, p=0.001) (Figure 1) and a reduced expression of *Bax* (0.19; p=0.020) and *Bad* (0.2; p=0.030) (Figure 2). No significant differences between controls and PV patients were verified regarding expression of genes *Bcl-w* (Figure 1), *Bik, Bim-$_{EL}$, Bok, Noxa, and Puma* (data not shown).

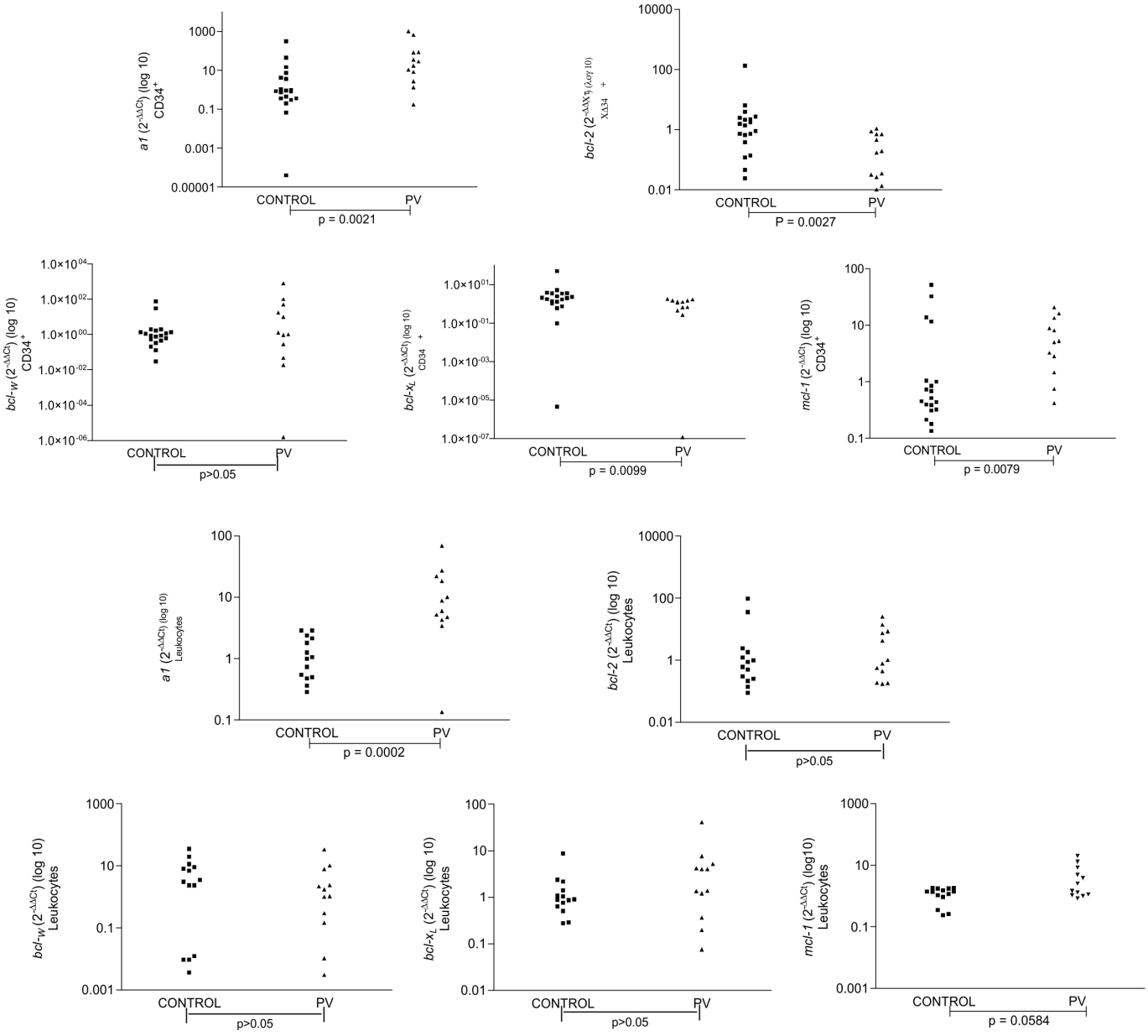

FIGURE 1 - Antiapoptotic genes expressed in CD34+ HSC and leukocytes from PV patients and controls. Beta-actin gene was used as endogenous control. The genes relative expression were detected by real time PCR and results were given as $2^{-\Delta\Delta Ct}$. The horizontal lines represent the median of gene expression in each group analyzed. *A1* and *Mcl-1* are overexpressed (median: 22.6 and 5.2, respectively) in CD34+ HSC from PV patients in comparison with controls (median: 0.9 and 0.5, p=0.002 and p=0.008). *Bcl-2* and *Bcl-x$_L$* mRNA levels were decreased in PV patients' CD34+ HSC (0.18 and 1.19) in relation to controls (1.39 and 2.01; p=0.006 and p=0.020). Patients' leukocytes also showed an *A1* gene expression augmentation (7.41, p=0.001). There were no statistically significant differences in mRNA levels of *Bcl-2*, *Bcl-x$_L$* and *Mcl-1* in patient leukocytes. No significant differences in *Bcl-w* expression between controls and PV patient CD34+ HSC and leukocytes were detected (p > 0.05).

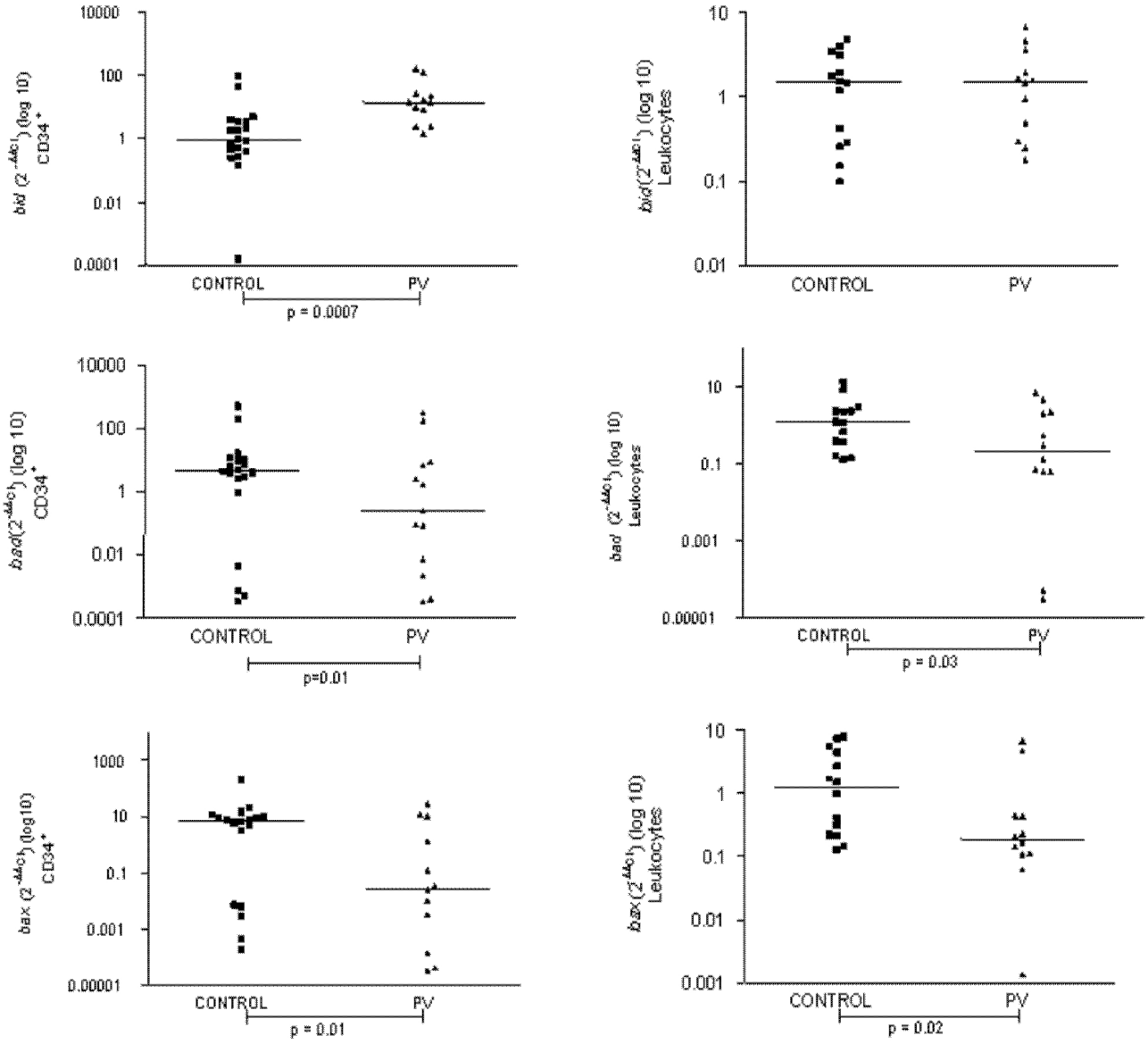

FIGURE 2 - mRNA levels of *Bid, Bax* and *Bad* proapoptotic molecules in $CD34^+$ HSC and leukocytes from PV patients and controls. Beta-actin gene was used as endogenous control. Gene relative expressions were detected by real time PCR and results were given as $2^{-\Delta\Delta Ct}$. The horizontal lines represent the median of gene expression in each group analyzed. PV patients' $CD34^+$ HSC showed *Bid* elevated expression (median=14.4, p=0.002) in relation to normal individuals. The *Bax* (median= 6.76, p=0.010) and *Bad* (0.24, p=0.010) expression were reduced in patients' $CD34^+$ HSC. The *Bad* (0.2; p=0.030) and *Bax* (0.19; p=0.020) levels were also decreased in PV patient leukocytes compared with controls.

Bad, Bim-$_{EL}$, Bcl-x_L and *A1* protein expression in peripheral leukocytes

Bad, Bim_{EL}, Bcl-x_L and *A1* protein expressions (Online Supplementary Figure S1) detected in peripheral leukocyte lysates from four healthy individuals and four PV patients were taken to represent the expression of these molecules in the whole group of patients and controls. Patient's samples were randomly chosen to support and check gene expression results.

We chose samples with gene expression values similar to median of *Bad*, Bim_{EL}, *Bcl-x_L* and *A1*. Evaluation of protein expressions was performed to confirm gene expression data. The results showed high *Bcl-x_L* and *A1* expression and low levels of *Bad* protein in PV patients in comparison to controls. *Bim-$_{EL}$* expression was performed just because it is known that this molecule is susceptible to post-transcriptional regulation mechanisms. *Bim-$_{EL}$* proteic

levels were also reduced in patients, PV leukocytes having only 35% of *Bim-$_{EL}$* normal values. *Bad* protein expression was significantly lower in PV patients in relation to controls, which were 60% higher. PV patients had triple *Bcl-x_L* expression compared with control leukocytes. Hence, *Bcl-x_L* and *Bim-$_{EL}$* protein expression are different; they were opposite from that observed in PV gene expression profiles, whereas *A1* and *Bad* data supported the mRNA level results.

JAK2 V617F mutation allele burden did not correlate with gene expression of *Bcl-2* family members

No correlation was detected between JAK2 V617F allele burden and antiapoptotic protein (*A1*, *Bcl-2*, *Bcl-w*, *Bcl-x_L* and *Mcl-1)* or proapoptotic gene expression (*Bad*, *Bak*, *Bax*, *Bid*, *Bik*, *Bim-$_{EL}$* and *Bok*) in CD34+ HSC or leukocytes (Supplementary Table S2).

Mononuclear cells from PV patients were resistant to apoptosis

Concerning assessment of mononuclear cell apoptosis, PV patients cells were more resistant to ACT-D 1μM (median = 29.95; p=0.015), CHX 25μM and 50 μM (median = 5.53 and 19.80; p=0.002 and 0.001), STS 1 μM (median = 6.59; p<0.001), ARA-C 25μM (median = 2.76; p<0.001), VM26 25 μM (median = 22.80; p<0.003) and VP16 25 μM (median = 12.25; p<0.001) than controls (median = 39.70) (Figure 3).

JAK2 mutation allele burden is not associated with the percentage of mononuclear cells apoptosis in PV patients

No significant correlation between the percentage of JAK2 V617F allele burden and mononuclear cell apoptosis

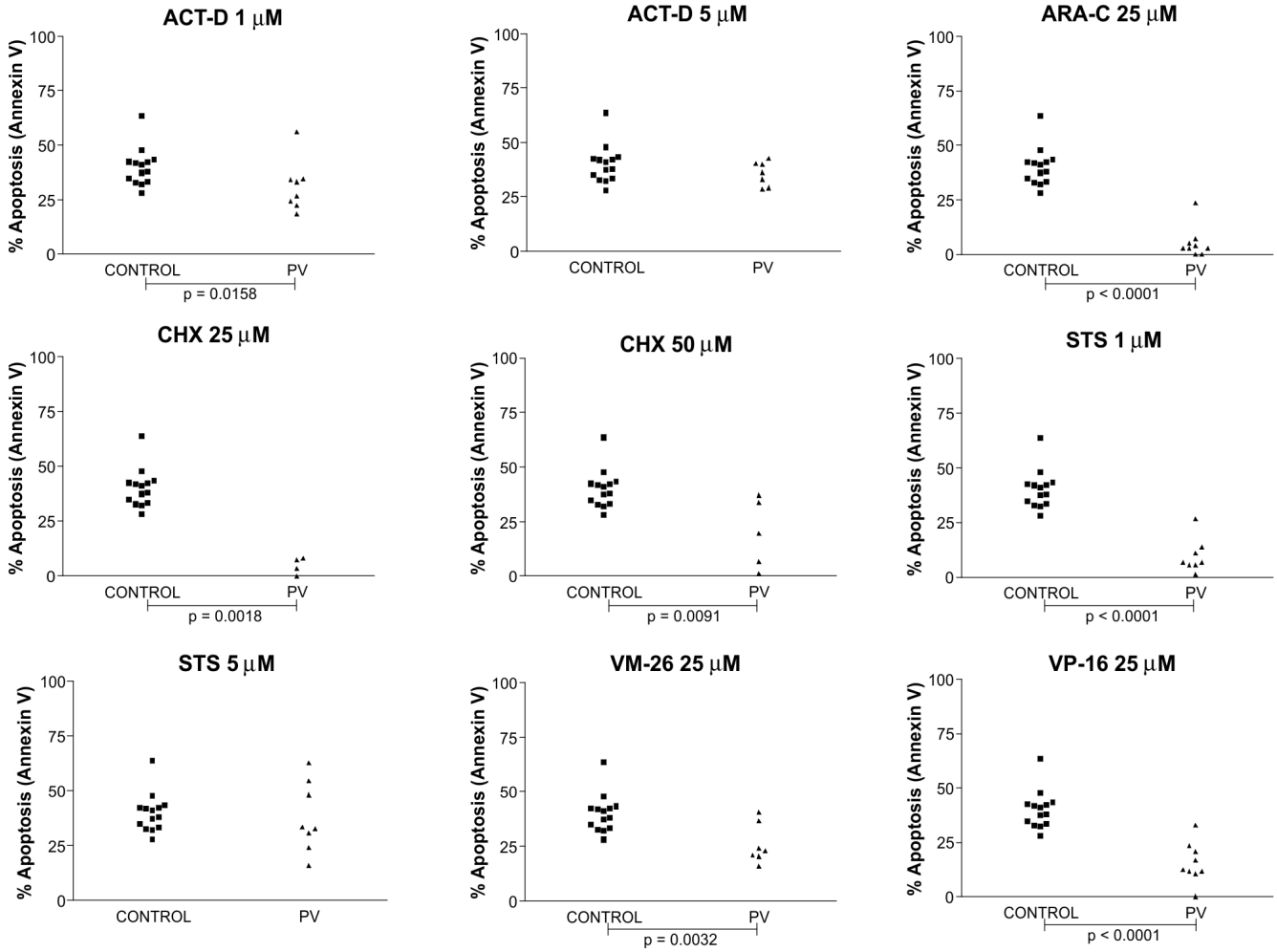

FIGURE 3 - Percentage of mononuclear cell apoptosis induced by drugs in the PV and control groups. Patient's mononuclear cells were more resistant to ACT-D 1 μM (p=0.015), CHX 25 μM and 50 μM (p=0.002 and 0.001), STS 1 μM (p<0.001), cytarabine 25 μM (p<0.001), teniposide 25 μM (p<0.003), and etoposide 25 μM (p<0.001) than controls' mononuclear cells.

induced by STS, CHX, ACT-D, ARA-C, VP16 and VM26 (data not shown) was found in PV patients.

Gene expression of *Bcl-2* family members is linked to mononuclear cells' resistance to apoptosis in PV patients

A negative correlation was detected between the percentage of PV cells apoptosis induced by ACT-D (1uM), CHX (50 μM) and ARA-C (25 μM) and *A1* mRNA levels (r=-6527, p=0.044); r=-0. 527; p=0.033 and r=-0.900; p=0.041, respectively); ACT (5 μM), STS (1 μM) and CHX (50 μM) and *Bcl-2* (r=-0.857; p=0.012; r=-0.691; p=0.038 and r=-0.9; p=0.040, respectively); ARA-C and *Mcl-1* (r=-0.728, p=0.015). Another interesting result was that *Bad* expression was positively correlated with cell percentage of apoptosis induced by VM26 (r=0.7857; p=0.024). Thus, low levels of *Bad* gene are related to a decrease in cell apoptosis percentage mediated by VM26.

These results indicate that death resistance to apoptogenic inducers in PV patients is linked to *A1, Mcl-1* and *Bcl-2* antiapoptotic protein and *Bad* proapoptotic genes levels.

DISCUSSION

Several studies have addressed the clinical and laboratorial features in chronic MPDs these diseases, but the cellular and molecular mechanisms involved in the pathogenesis of MPD cells are still unclear.

Altered apoptosis regulation seems to contribute to the physiopathology of several diseases like neoplasia, autoimmune disorders, neurodegenerative disorders, myelodysplastic syndromes, and acute and chronic leukemia (Del Poeta *et al.*, 2008; Economopoulou *et al.*, 2008; Yan, Shi, 2005). The association of apoptosis-related proteins deregulation with PV pathogenesis, examined in the present study, has been scarcely explored elsewhere.

A differential profile of *Bcl-2* family members' genes expression was verified in PV patients in relation to controls. Data suggest disequilibrium in anti- and proapoptotic gene expressions related to the intrinsic pathway, which is represented by a significant increase in *A1* and *Mcl-1* expression in marrow stem cells and *A1* in peripheral blood and by a reduction in mRNA levels of *Bad* and *Bax* molecules. In addition, the low expression of these proapoptotic molecules is related to drug-induced mononuclear cells resistance, such as topoisomerase inhibitors, cytarabine, and cycloheximide. Furthermore, our results indicate a potential augmented survival in $CD34^+$ HSC and peripheral leukocytes, leading to promoted clonal expansion and perpetuation of myeloid lineage cells characteristic of the disease.

The literature shows that inhibition of apoptosis by *A1* and *Mcl-1* genes may occur through inactivation of the *Bak* protein. Conformational change of *Bak* and mitochondrial MOMP formation are prevented by the *A1* and *Mcl-1* molecules, blocking release of cytochrome C and other apoptogenic factors, not activating caspase-3 and suppressing apoptosis (Chipuk, Green, 2008; Thomadaki, Scorilas, 2006). It is possible to speculate that the patients included in the present study showed cell resistance to apoptosis through this mechanism, as stem cells or leukocytes showed elevation of *A1* and *Mcl-1* and decrease of *Bad*, *Bim-*$_{EL}$ and *Bax* in comparison with controls.

Another finding is related only to the increase in the *Bcl-*x_L protein (supplementary data), a potent antiapoptotic molecule, which may contribute to a phenotype with a higher survival and myeloaccumulation of myeloid cells in PV patients. The findings in this investigation also include decreased levels of *Bcl-2* and increased *Bid*, a paradox, since these molecules promote apoptosis potentiating and not blocking. It is possible that these molecules do not play a relevant role in the physiopathology of PV.

In addition, since apoptosis unleashing is dependent on the interaction and equilibrium of several proteins of the *Bcl-2* family (Chipuk; Green, 2008), the increase of *A1, Mcl-1*, and *Bcl-*x_L antiapoptotic molecules and the decreased expression of *Bax* and *Bad* would be sufficient to prevent cellular death in PV.

A1 protein is abundantly expressed in bone marrow cells, spleen, and lung in several lineages originating in thymus, testicle, and intestine cancers (Thomadaki; Scorilas, 2006). It has an important role in high risk leukemia and other B cell malignancies. This molecule is a direct target in the transcription of NF-κβ, which is involved in myeloid and lymphoid differentiation, in apoptosis resistance by different stimuli, like the activation of death receptors (Simpson *et al.*, 2006; Brien *et al.*, 2007).

Mcl-1 protein probably participates in the immune system development and hematopoiesis. In hematopoietic cells the protein is regulated by ERK, JAK/STAT, p38, MAPK, P13K/AKT signaling and phosphorylation of elf2α and E2F1 and may be phosphorylated by JNK, GSK-3α/β, a cyclin-dependent kinase (Inoue *et al.*, 2008). It may be inactivated and/or degraded by a caspase mediated cleavage or other proteins (ubiquitin-proteasome pathway) (Wuillème-Toumi *et al.*, 2006). In chronic myeloid leukemia (Aichberger *et al.*, 2005), expression of *Mcl-1* protein is frequently associated with resistance to chemotherapy and disease progression. In multiple myeloma (MM), chemoresistance is also caused by apoptosis deregulated pathways, with over expression of *Mcl-1* and

an insignificant expression of *Bcl-2* (Wuillème-Toumi *et al.*, 2005). Increase in *Mcl-1* levels is correlated with disease severity, turning the molecule into a potential therapeutic target in MM.

Mcl-1 protein neutralizes the proapoptotic function of *Bim* and prevents activation of death receptors (Wuillème-Toumi *et al.*, 2006). Regulating the *Mcl-1* expression is important to break the *Bim/Mcl-1* complex and induce *Bim* activation in HMCL-human myeloma and Hek-293-human embryonic kidney cell lines. It has been shown recently that *Bim*, a direct activator of *Bax/Bak*, protects the mitochondrial membrane permeability dependent on the pair of proteins by loosing its activity (Chipuk, Green, 2008). Thus, increased levels of this protein interfere with mitochondrial membrane permeability and activation of apoptosis extrinsic pathway, both contributing to the resistance profile to apoptosis shown by PV patient cells.

The results in this study suggest that *A1* and *Mcl-1* contribute to apoptosis resistance by means of direct or indirect *Bax* inhibition, by blocking the activation of *Bad, tBax* or *Bim* molecules and/or compromising the activation equilibrium of other proapoptotic proteins such as *Bok and Noxa*, which inhibit cytochrome liberation and caspase 3 activation and suppress the apoptotic cascade.

Increased amounts of *Bcl-x_L* protein in PV patients' leukocytes confirm the data in the literature. *Bcl-x_L* protein is increased in erythroid precursors and provokes cell accumulation in PV (Silva *et al.*, 1998). Other reports consider the molecule as the most potent antiapoptotic agent, its high levels being partially responsible for cell apoptosis resistance in patients with chronic myeloid leukemia (CML). It seems that *Bcl-x_L* instead of *Bcl-2* mediates the most prominent antiapoptotic effect of Bcr-abl tyrosine kinase in CML patients. Amarante-Mendes *et al.* (1998) showed that treatment with antisense oligonucleotides targeted to *Bcl-x_L* downregulates the expression of *Bcl-x* and increases Bcr-Abl positive cell susceptibility to apoptosis induced by staurosporine in CML.

The sensitive profile of PV mononuclear cells to several apoptogenic drugs (actinomycin D, staurosporine, cycloheximide, cytarabine, etoposide, and teniposide) was also evaluated in this research. Substances with diverse mechanisms of action were employed to induce cell death, aiming to analyze cell apoptotic machinery at a functional level and its relation to the expression profile of molecules of the *Bcl-2* family members.

Our results suggest that PV patients' mononuclear cells resistance to cell death is related to alterations in the gene expression profile of the molecules regulating apoptosis intrinsic pathway.

The percentage of JAK2 mutated alleles reflects the tumoral load in the disease and is linked to worse prognosis (Passamonti, Rumi, 2009; Finazzi, Barbui, 2008; Gangat *et al.*, 2008; Vannucchi *et al.*, 2007). Lack of correlation between the JAK2 (V617F) mutation and the genes in the study does not exclude the relation between the expression of these genes to the activity of JAK2-mutated tyrosine kinase or the presence of different mutations in JAK2 or other kinases. It is worth emphasizing that in the present study we only evaluated the JAK2 V617F mutation in the exon 14 in PV patients, we did not measure JAK2 activity or detect other mutations associated with PV pathogenesis, such as MPL or JAK2 mutation in the exon 12 (Vainchenker, W. *et al.*, 2011).

Nussenzveig *et al.* (2007) showed that events related to the JAK2 V617F mutation may not give rise to PV, but identification of the genetic lesion is essential to the development of therapeutic strategies, since inhibitors of active, constitutive tyrosine kinase JAK2 are not sufficient to eradicate the disease. The authors correlated the frequency of the JAK2/V617F mutant allele with clonality and presence of endogenous erythroid colonies with allele homozygosis and demonstrated that PV is not only provoked by the mutation, which seems to be a secondary genetic event. However, the primary molecular lesion responsible for the clonal hematopoiesis was not defined.

JAK2 inhibitors have been explored as the most efficient therapeutic agents in MPDs but some patients do not respond to therapy and some are JAK2 negative. Thus, the importance of describing new therapeutic targets, as inserted in this context, for Bcl-2 family members.

Apoptosis resistance to stimuli by drugs such as actinomycin D, cycloheximide, cytarabine, teniposide, and staurosporine correlates with high levels of *A1, Mcl-1* and low expression of *Bad* and *Bax.*

Actinomycin D acts inhibiting the synthesis of RNA and it seems to potentiate Fas-mediated apoptosis, prevent binding of NF-κβ to DNA (Wang *et al.*, 2007), decrease the expression of the extrinsic antiapoptotic pathway, c-Flip, and stimulate *Puma* and *Noxa* expression of p53 and APAF-1 pathway (Kalousek *et al.* , 2007). ACT-D and cycloheximide conduct cells to apoptosis via the TRAIL protein or TNF-α receptor. Both drugs are able to reduce RIP expression, a kinase having a death domain involved in TNF-R1 signaling that mediates NF-κβ activation, which, in turn, may be antiapoptotic. They also reduce XIAP expression, a member of the IAP family responsible for the regulation of the common apoptosis pathway by inactivating effector caspases (Fulda *et al.*, 2000). ACT-D and STS require *Bax* and *Bak* to induce cytochrome c liberation to take the cell to its death. These data indicate

that PV patients' lymphocyte resistance to apoptosis induced by STS and ACT-D may be a consequence of low levels of *Bax* mRNA.

Cytarabine, a cytotoxic antineoplastic agent shows cell phase specificity, initially destroying cells that synthesize DNA and, under certain conditions, blocking progression of phase G1 to phase S. The mechanism of action is not completely known, but the drug seems to inhibit DNA polymerase during replication and damage DNA, thus activating the p53 pathway. Other antineoplastic drugs tested in this study were etoposide (Vepesid) and teniposide, both inhibitors of topoisomerase II; their main effects may be related to rupture of DNA double loop by enzyme interaction or production of free radicals; the drugs increase TRAIL receptor expression and stimulate p53 pathway by damaging DNA (Karpinish *et al.*, 2002; Li *et al.*, 2006).

The percentages of cell death detected in PV patients suggest mononuclear cells resistance to most drugs and indicate that cells may show alterations in at least one of the regulatory mechanisms of cellular apoptosis. The decreased cell susceptibility to death may be associated with cell accumulation in PV, being partially responsible for the morbidity/mortality of patients with the disease.

In conclusion, high levels of *A1* and *Mcl-1* and a reduced expression of antiapoptotic *Bcl-2* and *Bcl-*x_L and proapoptotic *Bad* and *Bax* genes in CD34 HSC were detected in PV patients. In leukocytes, *A1* expression was elevated and *Bad* and *Bax* mRNA levels were decreased. Thus, overexpression of the above antiapoptotic genes and a low mRNA level of *Bax* and *Bad* proapoptotic molecules may be associated with PV pathogenesis.

Additionally, a low expression of *Bad and Bax* proapoptotic genes also seems to be linked to PV lymphocyte resistance to apoptosis and contribute to the disease physiopathology.

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SUPPLEMENTARY MATERIAL

TABLE S1 - Percentiles 25 and 75 of gene expression $2^{-\Delta\Delta Ct}$ from *Bcl-2* family members in hematopoietic stem cells and peripheral leukocytes in PV patients and controls

Genes	CD34$^+$ HSC		Peripheral Leukocytes	
	Controls P25 - P75	PV Patients P25 - P75	Controls P25 - P75	PV Patients P25 - P75
A1	0.3596-4.212	4.068-83.69	0.4918-2.191	4.428-21.17
Bad	0.0003-9.596	0.005-7.556	0.3045-2.407	0.0582-2.053
Bax	0.007-9.659	0.0009-8.017	0.2187-4.631	0.1075-0.4351
Bcl-2	0.3801-2.539	0.0277-0.7124	0.2462-1.969	0.2535-8.265
Bcl-$_W$	0.4549-1.704	0.1067-42.67	0.0118-9.754	0.1846-6.439
Bcl-x_L	1.067-3.491	0.5042-1.457	0.6067-1.610	0.5790-4.920
Bid	0.4190-3.681	4.015-26.11	0.2778-3.286	0.3461-3.180
Mcl-1	0.3248-1.044	1.803-12.33	0.7861-1.753	1.071-7.579

HSC: Hematopoietic Stem Cells; PV: Polycythemia Vera; P: Percentile.

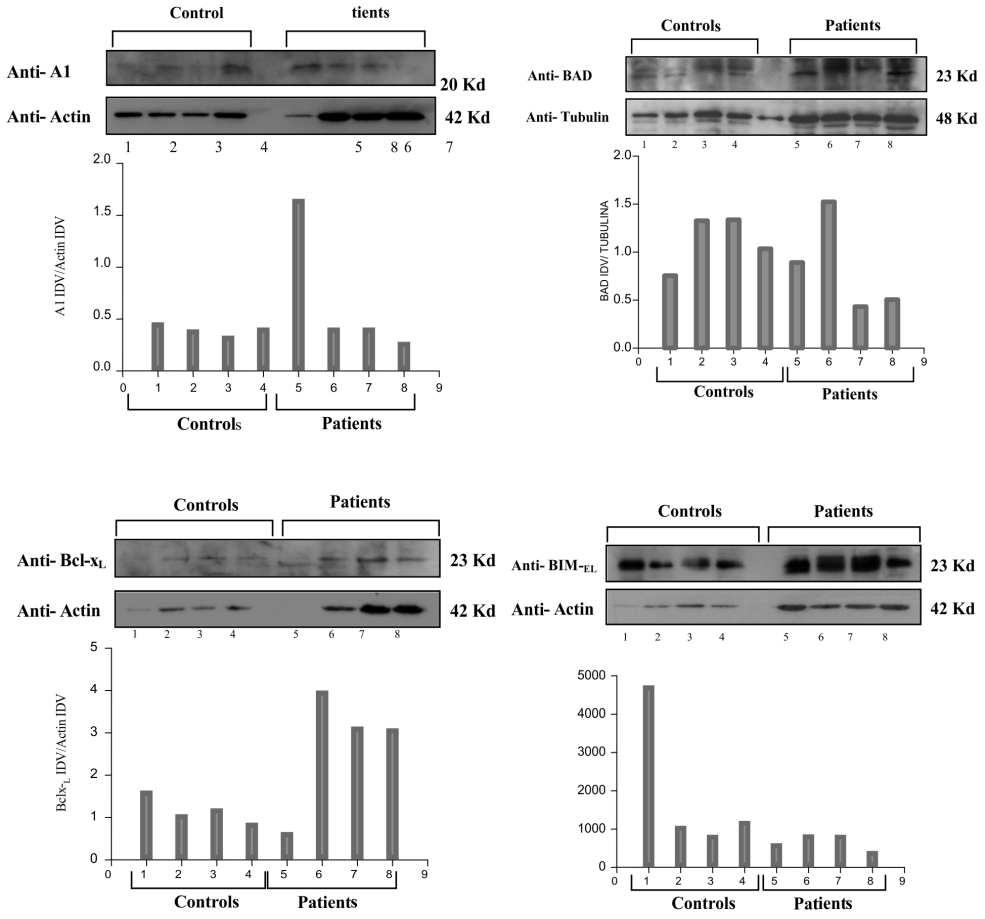

FIGURE S1 - *A1, Bcl-x_L, Bad,* and *Bim-$_{EL}$* protein expression determined in peripheral leukocyte lysates from four PV patients and four controls. *A1, Bcl-x_L, Bad,* and *Bim-$_{EL}$* expression were assessed by immunoblot with specific antibody. Protein loading is controlled by beta-actin or tubulin. Results were shown by blotting figures and protein expression quantification detected by densitometry and expressed by the ratio of target protein integrated density values (IDV) and beta-actin or tubulin IDV. *Bcl-x_L* and *A1* presented a high expression while *Bad* protein levels were decreased in PV patients in comparison with controls. *Bim-$_{EL}$* proteic levels are also reduced in PV patient leukocytes being only 35% of the values in normal individuals.

TABLE S2 - Correlation between JAK2 V617F allele burden and genes expression of *Bcl-2* family members in PV patients

Genes	$CD34^+$ HSC		Peripheral Leukocytes	
	r	p	r	p
A1	0.1763	p>0.05	-0.03647	p>0.05
Bad	-0.3465	p>0.05	-0.09726	p>0.05
Bak	-0.3404	p>0.05	-0.08113	p>0.05
Bax	-0.2979	p>0.05	-0.03647	p>0.05
Bcl-2	-0.6565	p>0.05	0.1216	p>0.05
Bcl-w	-0.1398	p>0.05	0.3536	p>0.05
Bcl-x_L	-0.2675	p>0.05	0.01824	p>0.05
Bid	-0.2614	p>0.05	-0.2349	p>0.05
Bik	-0.1094	p>0.05	-0.04863	p>0.05
Bim-$_{EL}$	-0.2675	p>0.05	-0.1702	p>0.05
Bok	-0.2371	p>0.05	-0.1885	p>0.05
Mcl-1	0.1216	p>0.05	0.3161	p>0.05

HSC: Hematopoietic stem cell.