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ORIGINAL PAPER

Hippo pathway-related genes expression is deregulated in myeloproliferative neoplasms

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Abstract

Myeloproliferative neoplasms (MPN) are hematological disorders characterized by increased proliferation of precursor and mature myeloid cells. MPN patients may present driver mutations in *JAK2*, *MPL*, and *CALR* genes, which are essential to describe the molecular mechanisms of MPN pathogenesis. Despite all the new knowledge on MPN pathogenesis, many questions remain to be answered to develop effective therapies to cure MPN or impair its progression to acute myeloid leukemia. The present study examined the expression levels of the Hippo signaling pathway members in patients with polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF), as well as the role that they play in disease pathogenesis. The Hippo pathway is a tumor suppressor pathway that participates in the regulation of cell proliferation, differentiation, and death. Our main finding was that the expression of tumor suppressor genes from Hippo pathway were downregulated and seemed to be associated with cell resistance to apoptosis and increased proliferation rate. Therefore, the decreased expression of Hippo pathway-related genes may contribute to the malignant phenotype, apoptosis resistance, and cell proliferation in MPN pathogenesis.

Keywords Myeloproliferative neoplasms · Hippo signaling pathway · Apoptosis · Cell proliferation · LATS2

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Introduction

Polycythemia vera (PV), primary myelofibrosis (PMF), and essential thrombocythemia (ET) are Philadelphia-negative myeloproliferative neoplasms (MPN) characterized by increased proliferation of mature myeloid cells from one or more hematopoietic series: granulocytic, erythrocytic, and megakaryocytic. The MPN neoplastic cells are resistant to death and present exacerbated proliferation rate [1, 2].

Deregulated expression of pro- and antiapoptotic genes is an important mechanism of myeloid cell death and accumulation in MPN. Apoptosis, one of the best-characterized mechanisms of programmed cell death, clearly plays a role in hematopoiesis control. However, the exact pathways that are affected in BCR-ABL1-negative MPN have not yet been fully clarified [3]. Expression of the anti-apoptotic genes *A1*, *BCL2*, *BCL-X*$_L$, and *BCL-W* is upregulated while expression of the pro-apoptotic genes *BID* and *BIM*$_{EL}$ is downregulated in leukocytes from ET patients [4]. The JAK2V617F allele burden correlates with *BAX*, *BIK*, *BAD A1*, *BAX*, *BIK*, and *PRV1* gene expression [4].

Abnormal cytokine production by both malignant and nonmalignant cells is associated with increased JAK2-STAT3 activation and accounts for apoptosis inhibition and enhanced myeloproliferation, which create an environment that favors MPN clone maintenance and expansion [3].

The landmark of the MPN pathophysiological findings is description of the JAK2V617F mutation, present in about 95% of PV patients and in approximately 50% of PMF and ET patients [5, 6]. The JAK2V617F mutation is caused by the replacement of the amino acid valine by phenylalanine at codon 617 (JAK2V617F), with consequent constitutive activation of the JAK-STAT signaling pathway that stimulates proliferation of myeloid hematopoietic precursors [7]. The JAK2V617F mutation leads to accumulation of irreparable DNA damage that triggers the apoptotic cascade, with high BNIP-3 positivity, eliciting megakaryocyte apoptosis [3]. This process is counteracted by phosphorylated STAT5 (pSTAT5). Phosphorylated STAT3 (pSTAT3) induces BCL-X_L expression, which confers resistance to apoptosis to myeloid cells [8].

The MPL mutation results from the replacement of tryptophan at codon 515 by arginine, leucine, lysine, or alanine [9]. This mutation occurs in approximately 10% of JAK2V617F-negative PMF and ET patients [9]. Tryptophan at position 515 prevents MPL dimerization in the absence of cytokines; however, its replacement by other amino acids enables dimerization and leads to the constitutive activation of the receptor and the JAK-STAT pathway, with consequent exacerbated proliferation of myeloid cells [10]. The antiapoptotic effect remains after cytokine withdrawal in MPLW515K/L-expressing cell lines characterized by high levels of BCL-X_L expression [11].

CALR *indel* mutations are located in the exon 9 of the gene and result in a C-terminus that loses the endoplasmic reticulum retention motif. The mutated CALR constitutively activates the MPL receptor, even in the absence of thrombopoietin, and induces cell transformation, probably by stimulating MPL-JAK2 signaling [12]. Mutation in the CALR gene induces activation of the JAK2/STAT/phosphatidylinositol-3'-kinase and mitogen-activated protein pathways via MPL, leading to exacerbated proliferation of myeloid cells [13]. CALR mutants have weaker pro-apoptotic signals downstream the unfolded protein response, which leads to accumulation of misfolded proteins in endoplasmic reticulum and resistance to apoptosis induced by the unfolded protein response [14].

Despite the latest knowledge about the molecular and cellular mechanisms of MPN pathogenesis, the only curative treatment for this disease is bone marrow transplantation, but most patients are ineligible for the procedure. In this sense, our research team have investigated how the Hippo signaling pathway participates in MPN physiopathology.

The Hippo signaling pathway is a tumor suppressor pathway that plays a role in regulation of cell proliferation, differentiation and death [15]. The Hippo signaling cascade is composed of MST1/MST2, SAV1, LATS1/LATS2, MOB1A/MOB1B, YAP, and TAZ proteins. Activation of the classical pathway begins with MST1/MST2 and its adapter protein SAV1 phosphorylation by different regulatory signals. MST1/2 proteins phosphorylate and activate LATS1 and LATS2 in a process that requires interaction with the small proteins MOB1A, MOB1B, MOB2A, and MOB2B. Activated LATS1/2 proteins may phosphorylate different serine/threonine residues from YAP and TAZ transcriptional factors. When YAP is phosphorylated at serine 127 or 381 and TAZ is phosphorylated at serine 89 or 311, they bind to the 14.3.3 protein complex in the cytoplasm, which inhibits its transcriptional activity and promotes the retention and cytosolic degradation of YAP/TAZ [16, 17].

When the pathway is inactive, YAP/TAZ translocates to the cell nucleus and associates with transcription factors from the TEAD1-4 (TEAD/TEF) family to trigger expression of target genes such as connective tissue growth factor (*CTGF*), cysteine-rich angiogenic inducer 61 (*CYR61*), and fibroblast growth factor 1 (*FGF1*), which mediate cell proliferation and apoptosis impairment [18].

Subjects and methods

Ethical approval

The institutional review board from the School of Pharmaceutical Sciences of Ribeirão Preto (FCFRP-USP) and Ribeirão Preto Medical School Hospital (HC-FMRP-USP) at University of São Paulo approved the study protocol (CAAE 30901714.3.3001.5440). All patients and healthy subjects signed an informed written consent form that complied with the Brazilian National Health Council guidelines (law 466/2012).

Subjects

This study enrolled ET, PMF, and PV patients followed at HC-FMRP-USP and at Euryclides de Jesus Zerbini Transplant Hospital, São Paulo-SP, Brazil. For peripheral blood, ET patients' group was composed of 35 individuals (9 males, 26 females) with mean age of 59 years: 15 patients harbored JAK2V617F mutation and 9 patients harbored CALR mutation. The PMF patients' group was composed of 22 individuals (15 males, 7 females) with mean age of 62 years: 12 patients harbored the JAK2V617F mutation and 5 harbored the CALR mutation. The PV patients' group was composed of 31 individuals (14 males, 17 females) with mean age of 65 years: 30 harbored the JAK2V617F mutation and one was negative for all the three mutations. The control group comprised 60 healthy individuals (21 males, 39 females) with mean age of 56 years, who lived in Ribeirão Preto, State of São Paulo, Brazil (Table S1).

$CD34^+$ gene expression in bone marrow cells was analyzed in some samples from patients who also donated peripheral blood. The ET group was composed of 20 patients (7 males, 13 females) with mean age of 58 years; the PMF group was composed of 7 patients (6 males, 1 female) with mean age of 65 years; the PV group was composed of 15 patients (8 males, 7 females) with mean age of 64 years; and the control group was composed of 15 healthy bone marrow donors (8 males, 7 females) with mean age of 54 years (Table S2).

Blood samples

The patients' and healthy subjects' peripheral blood was collected using EDTA tubes (Vacutainer®; Becton, Dickinson, and Company). The patients' blood samples were collected at the time of diagnosis, based on the WHO 2016 [19] criteria; therefore, none of them were under therapy at the time of blood collection. Exclusion criteria for controls were the presence of neoplasms, autoimmune diseases, viral infections, HIV, or diabetes.

Clinic-hematological data

The red blood cell count (RBC), white blood cell count (WBC), platelet number, hemoglobin concentration (Hb), and hematocrit percentage (Ht) were determined using the automatic equipment ABX Micros 60 (HORIBA ABX SAS). Data from hematological parameters are reported in Table S1. Clinical data as LDH, thrombotic events, blasts percentage, and spleen size were obtained from the medical record.

Isolation of $CD34^+$ hematopoietic precursor cells

Five mL of bone marrow samples were collected from patients and healthy donors. The bone marrow mononuclear cells were isolated using the Ficoll-Hypaque (Sigma-Aldrich) method. After washing with 0.05% albumin (Sigma-Aldrich) in PBS-ACD buffer, the cells were suspended in the same buffer and centrifuged. Then, the cell pellets were sequentially treated with Fc-blocking antibody (MidiMACS®—Miltenyi Biotech) and the primary anti-CD34-hapten antibody (MidiMACS®—Miltenyi Biotech). The cells were incubated at 4 °C for 20 min in the dark, diluted with 30 mL of PBS-ACD-albumin buffer, and centrifuged at 240 × g, for 10 min, at 4 °C.

Next, the secondary anti-hapten antibody (MidiMACS®—Miltenyi Biotech) conjugated with microbeads was added to the cell pellet, followed by incubation at 4 °C for 20 min in the dark. The suspension was diluted with 20 mL of PBS-ACD-albumin buffer and centrifuged at 240 × g, for 10 min, at 4 °C. The cell pellet was suspended in 10 mL of PBS-ACD-albumin buffer, and applied on the magnetic column. The column was washed twice with the same buffer, far from the magnetic support, to release the $CD34^+$ cells. The eluate was centrifuged at 240 × g, for 10 min, at 4 °C, the cell pellet was suspended in RPMI 1640 medium (Gibco), and the cells were counted in a Neubauer chamber. The purity of the $CD34^+$ population was analyzed by flow cytometry; samples with purity above 85% were considered suitable for use. The concentration of bone marrow hematopoietic cells was adjusted to 2×10^5 in 500 µL of Trizol® (Invitrogen Life Technologies®) and the suspension was frozen for further RNA extraction.

Isolation of peripheral blood leukocytes from patients and control subjects

Isolation of peripheral blood leukocytes from patients and control subjects relied on the principle of density difference among leukocytes, red blood cells, and platelets using

Voluven (Fresenius) [20]. The protocol consists of adding four parts of whole blood to one part of Voluven, followed by 90 min of incubation. The supernatant was collected and centrifuged at 400 × *g* for 15 min. Cells were suspended in phosphate-buffer saline (PBS; pH = 7.4), diluted in 0.4% trypan blue (Sigma-Aldrich), and counted in Neubauer chamber. Leukocyte suspension at 1×10^7 cells/mL was frozen in Trizol® (Invitrogen Life Technologies®) for further RNA extraction.

Cell culture

The human cell line HEL.92.1.7 (erythroblastic cell line—HEL.92.1.7—ATCC® TIB-180™) harbors the JAK2V617F mutation. HEL.92.1.7 cells were cultured in RPMI 1640 medium (Invitrogen Life Technologies®) supplemented with 10% fetal bovine serum, 1% glutamine, 1% penicillin, and 1% streptomycin (Gibco®) under an atmosphere of 5% CO_2, at 37 °C.

Cell treatment with JAK inhibitor I

HEL.92.1.7 cells (5×10^6/mL) were treated with 10 µM of JAK Inhibitor I – an ATP-competitive inhibitor of JAK1, JAK2, JAK3, and TyK2 (Calbiochem, EMD Millipore Corp, Billerica, MA, USA) – for (i) 6, 12, and 24 h for Hippo pathway and apoptosis gene expression assays; (ii) 48 h for annexin-V assay; and (iii) 24, 48, and 72 h for cell proliferation assay. Next, part of the cell suspension was used in cell death and proliferation assays, and part was stored in Trizol® for RNA extraction and qPCR assays.

Cell death quantification

After JAK Inhibitor I treatment, 1×10^5 HEL.92.1.7 cells per tube were diluted with 500 µL of PBS and centrifuged at 240 × *g* for 10 min, at 4 ºC. The supernatant was discarded, the cell pellets were suspended in 100 µL of annexin-binding buffer (250 µg/mL), and incubated for 20 min, in the dark, at 4 °C. The cells were washed three times with PBS and suspended in 200 µL of binding buffer containing 1 µL of propidium iodide. The percentage of apoptosis (annexin-V-FITC$^+$ cells) was determined in a flow cytometer (FACSCanto, BD), based on acquisition of 10,000 events. The cell death rate was expressed as percentage of annexin-V-FITC-labeled cells.

Analysis of cell proliferation

To quantify cell proliferation rate, HEL.92.1.7 cells were labeled using the CellTrace Violet Cell Proliferation Kit (CTV—ThermoFisher Scientific). Cells (1×10^7 in 2 mL of PBS) were labeled with 1 µL of 5 µM CTV staining solution and incubated for 30 min, at 37 °C, under 5% CO_2. The cell suspension was diluted with 5 mL of RPMI and incubated for 1 min, at 37 °C, to inactivate unbound CTV. Then, cells were washed three times with RPMI, centrifuged (1500×*g*, 4 °C, 5 min), suspended in 1 mL of RPMI, distributed into 6-well plates, and treated with JAK inhibitor I for 24, 48, and 72 h. Finally, the fluorescence intensity of CTV-labeled cells was measured in a flow cytometer (FACSCanto, BD), based on acquisition of 10,000 events to quantify cell proliferation. The results were expressed as fluorescence intensity and plotted as histograms. Cell proliferation was inversely proportional to CTV labeling.

Transient expression of LATS2 gene

The plasmid pClneoMyc-LATS2 (Addgene catalog number: #66,852) was used for *LATS2* expression (Bao et al., 2011). Hel 92.1.7 cells were cultured in 75 cm^2 flasks (as described above), harvested, centrifuged (400×*g*, 5 min, 4 °C), and washed with PBS. The cells were counted in Neubauer chamber and normalized to 1×10^7/mL. The cells were pelleted (1×10^7/plasmid) and suspended in 5 µg of pClneo-Myc-LATS2 in RPMI. The cell suspensions were transferred to 4-mm sterile gap cuvettes and pulsed (250 V; 950 µF) in a BioRad GenePulser Xcell [21, 22]. As control, cells were transfected with pcDNA3.1 (ThermoFisher, catalog number: #V790-20) under the same conditions. After transfection, the cells were transferred to 75 cm^2 flasks and maintained in RPMI supplemented with 10% bovine fetal serum for 72 h, at 37 °C, and under 5% CO_2. The overexpression of *LATS2* was confirmed by RT-PCR (Fig. S1). After 72 h of culture, the transfected cells were harvested and treated with 10 µM of JAK inhibitor I for (i) 48 h to analyze cell death rate; and (i) 24, 48, and 72 h to examine whether *LATS2* overexpression affected cell proliferation.

RNA extraction and cDNA synthesis

The lysed cells in Trizol were treated with 10 µL glycogen (20 µg/µL) for 5 min, at room temperature. Then, 300 µL of cold chloroform was added to the mixture and the sample was shaken for 15 s. After centrifugation (12,000 × *g*, 15 min, 4 °C), the aqueous phase was transferred to a new tube and mixed with 500 µL of cold isopropanol. The mixture was stored at—20 °C overnight and centrifuged (12,000 × *g*, 15 min, 4 °C). The supernatant was discarded and the precipitate was washed with 70% cold ethanol and centrifuged (12,000×*g*, 10 min, 4 °C). The supernatant was discarded and the pellet (RNA) was suspended in DNAse- and RNAse- free water. RNA was quantified using the NanoVue Spectrophotometer (GE Healthcare) at 260 nM.

Reverse transcription reactions were performed using 1 µg of RNA and the High Capacity cDNA reverse

transcription (Applied Biosystems) assay kit. Polymerization of the novel complementary DNA strands (cDNA) was performed in a thermocycler apparatus (Mastercycler, Eppendorf) at 25 ºC, for 10 min, to promote primer annealing, followed by a 2-h step at 37 ºC for the extension of the novel DNA strands.

Quantification of target genes by real-time PCR

Real-time PCR assays were performed on the StepOnePlus (Thermo Fisher) using the TaqMan gene expression assay kit (Applied Biosystems®). The Hippo pathway probes used are described in Table S3. As the TaqMan probe (Hs00371735_m1) did not detect the *YAP1* gene, we used the Sybr green master mix kit (Thermo Fisher) and the primer pair sequence employed for RNA quantification described in Table S4. The Sybr green master mix kit (Thermo Fisher) was also used to analyze pro- and anti-apoptotic gene expression. The oligonucleotide sequences employed in amplification of the target genes are described in Table S4. The gene expression results were reported as relative expression unit (REU = 10,000/2Δ*Ct*) for patients or Fold Change for cell line. The ΔCT = CT of the target gene—geometric mean of the reference genes CTs. REU = 10,000 / 2deltaCt.

Statistical analyses

The groups were compared using analysis of variance (ANOVA) followed by Tukey's post-test for multiple comparisons. These models assume that their residuals have a normal distribution with a mean of 0 and a constant σ^2 variance. Transformation in the response variable were used when this assumption was not observed. All the graphs presented were plotted using the R software, version 4.0.4, while data analysis was performed using the SAS 9.4 software. For all comparisons, a significance level of 5% was adopted and correlation analyses were performed using the Pearson's correlation test. Wild type (WT), MOCK, and LATS2 differences were compared using ANOVA, with the aid of the GraphPad Prism 8.0 software (Graph-Pad Software, San Diego, CA, USA). Statistical differences were considered significant when $p < 0.05$.

Results

Hippo pathway-related genes are less expressed in MPN leukocytes

The expression levels of Hippo pathway-related genes were compared among MPN categories and the control group. Compared with the control group: (i) *MST1* ($p = 0.04$), *MST2* ($p < 0.01$), *LATS2* ($p = 0.04$), and *YAP* ($p = 0.02$) genes were less expressed in PV patients (Fig. 1); (ii) *SAV1* ($p < 0.01$) and *YAP* ($p = 0.04$) genes were less expressed in ET patients (Fig. 1); and (iii) *SAV1* ($p < 0.01$) and *TAZ* ($p < 0.01$) genes were less expressed in PMF patients (Fig. 1).

MST2 gene was less expressed in PV patients than in ET ($p = 0.01$) and PMF ($p = 0.01$) patients. In contrast, *SAV1* gene was more expressed in PV patients than in ET ($p < 0.01$) and PMF ($p < 0.01$) patients. *TAZ* gene also was less expressed in PV patients when compared with ET ($p < 0.01$) and PMF ($p < 0.01$) patients. *YAP1* gene was more expressed in PMF patients when compared with PV ($p = 0.01$) and ET ($p = 0.04$) patients (Fig. 1). The expression of other Hippo pathway-related genes did not differ among the analyzed groups ($p > 0.05$).

YAP/TAZ genes are deregulated in MPN CD34$^+$ cells

TAZ expression levels were lower in PV patients than in control group ($p = 0.04$). *YAP1* RNA levels were higher in ET patients than in control ($p < 0.01$), PV ($p < 0.01$), and PMF ($p < 0.01$) groups (Fig. S2). The expression levels of other Hippo pathway-related genes did not differ among the analyzed groups ($p > 0.05$).

JAK2V617F and CALR mutation status influence Hippo pathway-related genes expression in peripheral leukocytes from ET and PMF patients

Compared with PMF JAK2V617F$^+$ patients, *MST1* ($p = 0.02$) gene was less expressed in JAK2V617F-negative patients and more expressed in CALR$^+$ PMF patients ($p = 0.04$) (Fig. S3A). *MOB1A* was more expressed in CALR$^+$ than in JAK2V617F$^+$ PMF patients ($p = 0.05$; Fig. S3A). In contrast, *MOB1B* gene was more expressed in CALR$^+$ PMF patients than in JAK2V617F$^+$ ($p < 0.01$; Fig. S3A) and CALR$^-$ ($p = 0.01$; Fig. S3A) PMF patients. Compared with CALR$^+$ ET patients, *MOB1B* ($p = 0.02$) and *SAV1* ($p < 0.01$) were less expressed in CALR$^-$ ET patients ($p = 0.02$ and $p < 0.01$, respectively; Figure S3B) and double negative (JAK2V617F$^-$/CALR$^-$) ET patients ($p = 0.01$ and $p \leq 0.01$, respectively; Fig. S3B). There was no correlation between drive mutation *status* and the expression level of the other Hippo pathway genes analyzed (Fig. 2).

Hippo pathway and apoptosis-related genes expression are correlated with hematological and clinical data in MPN patients

We used heatmap of correlation to depict the correlations among Hippo pathway and apoptosis-related genes, and patients' clinic-hematological data. The correlation profile differed among the MPN types studied. ET

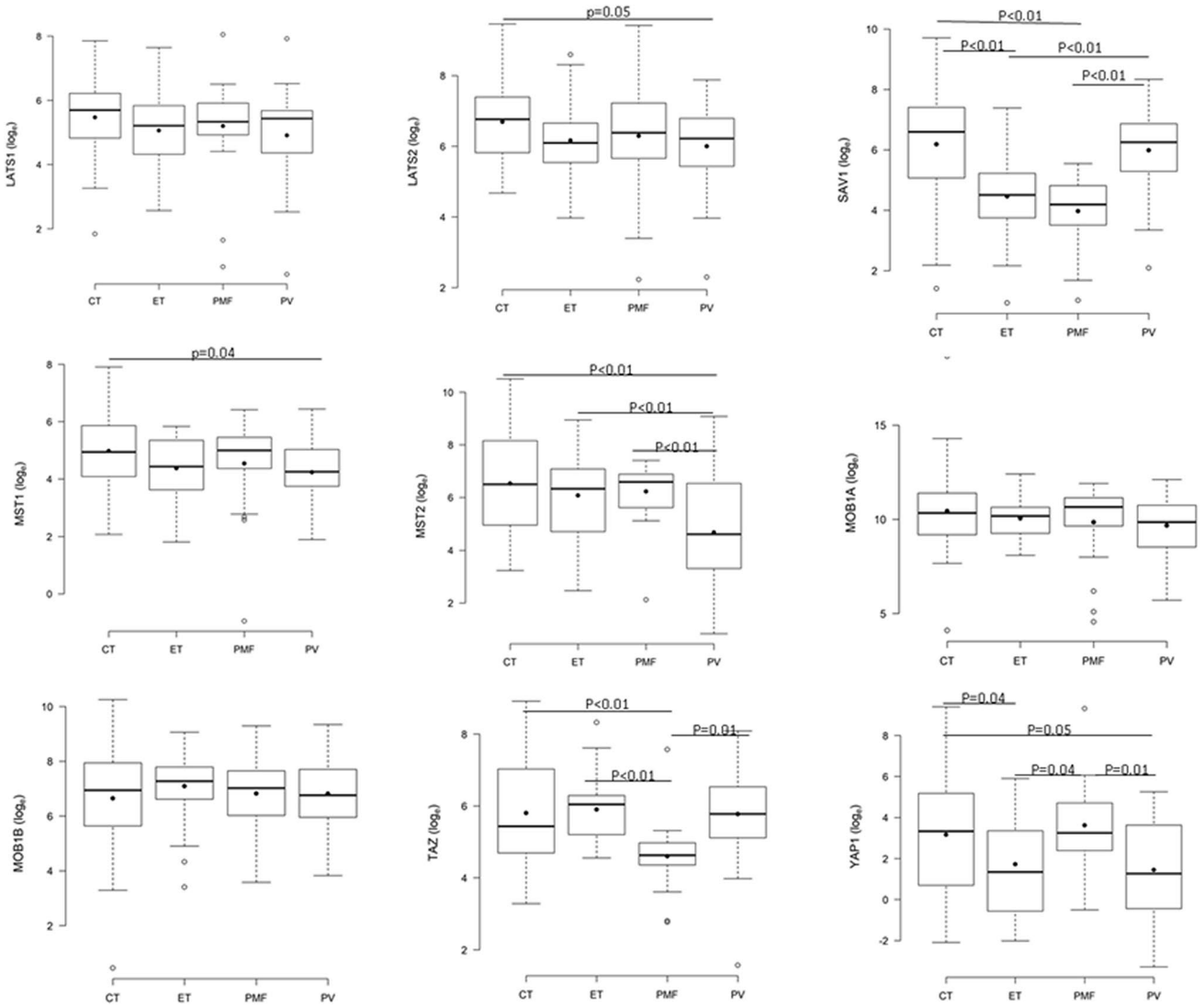

Fig. 1 Expression of Hippo pathway-related genes in leukocytes from patients with polycythemia vera (PV) and control subjects (CTRL). The *MST1*, *MST2*, *LATS2*, *SAV1*, *YAP1*, and *TAZ* gene expression levels in MPN patients were lower than in the CTRL group. Results are expressed as relative expression

patients presented negative correlations between *BCL-X*$_L$ expression and WBC count; thrombotic events and *MST1*, *MOB1A*, and *YAP1* expression; LDH level and *BAD* expression (Fig. S4). PV patients presented negative correlations between platelet counts and *LATS1*, *LATS2*, *MST1*, and *MST2* gene expression and positive correlations between *BCL-X*$_L$ and RBC count and between blasts percentage and *MST1* and *MST2* expression (Fig. S5). PMF presented negative correlations between platelets and *MST1*, *MOB1A*, *BAD*, *BLC-2*, and *BCL-X*$_L$ expression; and between WBC and *MST1*, *MST2*, *MOB1A*, *MOB1B*, *SAV1*, BAK, *BLC-2*, and *BCL-X*$_L$ expression (Fig. S6).

The apoptosis-related genes expression is deregulated in MPN patients' leukocytes

Compared with the control group, the pro-apoptotic *BAD* gene was less expressed in PV, ET, and PMF patients ($p < 0.01$; Fig. 3A), while the pro-apoptotic gene *BAK* was less expressed in PV ($p < 0.01$) and ET ($p < 0.01$) patients (Fig. 3B). The anti-apoptotic gene *BCL-2* was more expressed in ET patients than in control group ($p < 0.01$), PV ($p = 0.01$), and PMF ($p = 0.02$) patients (Fig. 3C), while the anti-apoptotic gene *BCL-X*$_L$ was more expressed in PV ($p < 0.01$) and ET ($p < 0.01$) patients than in control group and PMF patients ($p < 0.01$) (Fig. 3D).

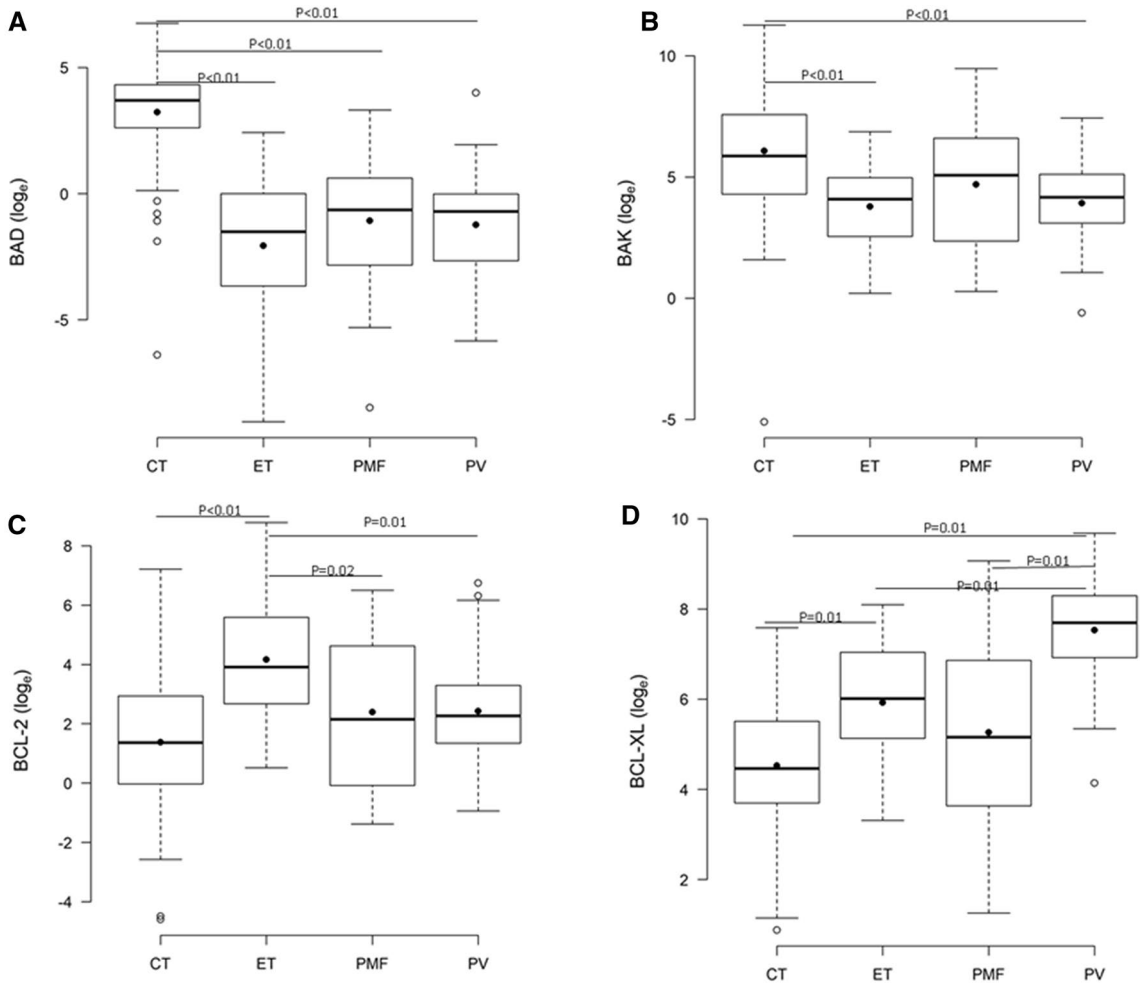

Fig. 2 Expression levels of the proapototic genes *BAD* (**A**) and *BAK* (**B**) and the anti-apoptotic genes *BCL-2* (**C**) and *BCL-XL* (**D**) in leukocytes from patients with myeloproliferative neoplasms. Data from control subjects (CT) and patients with essential thrombocythemia (ET), primary myelofibrosis (PMF), and polycythemia vera (PV) are expressed as relative expression. Mann–Whitney statistical test

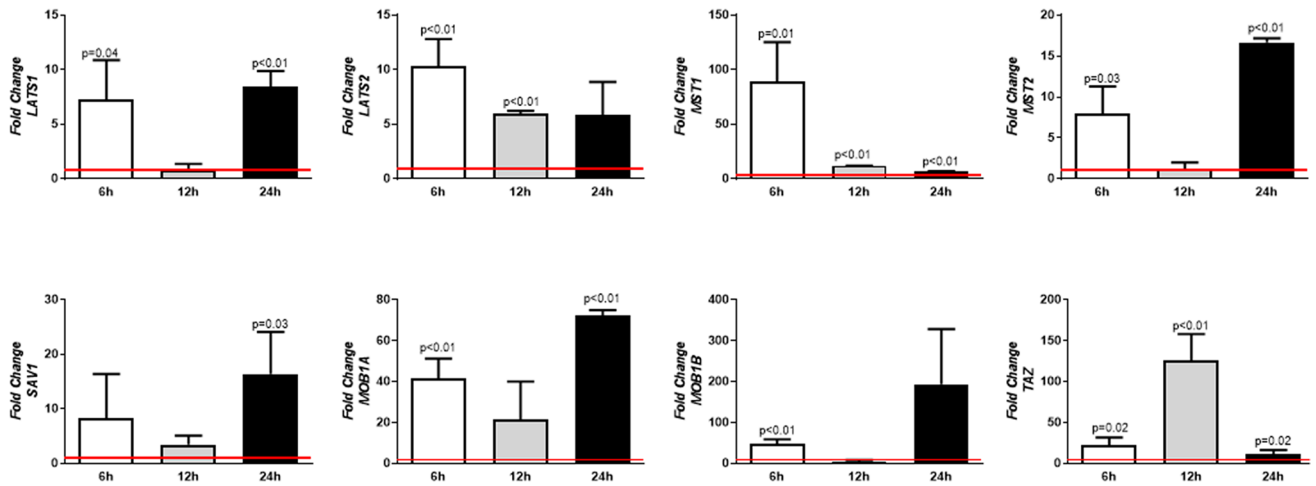

Fig. 3 Expression of Hippo pathway-related genes in HEL.92.1.7 cells treated with JAK inhibitor I (JII). *LATS1, LATS2, MST1, MST2, MOB1A, MOB1B*, and *TAZ* gene expression increased after 6 h of treatment. *LATS1, MST1, MST2, SAV1, MOB1A*, and *TAZ* gene expression increased after 12 h of treatment. The red line represents untreated cells. Data are expressed as Fold Change. Unpaired test

JAK inhibitor I affects the expression of Hippo pathway- and apoptosis-related genes

HEL.92.1.7 cells treated with JAK inhibitor I exhibited increased expression of: (i) *LATS1* ($p=0.04$ and $p<0.01$, respectively), *MST2* ($p=0.03$ and $p<0.01$, respectively), and *MOB1A* ($p<0.01$ for both) after 6 and 24 h of treatment; (ii) *LATS2* after 6 and 12 h of treatment ($p<0.01$ for both); (iii) *MST1* ($p=0.01$, $p<0.01$, and $p<0.01$, respectively) and *TAZ* ($p=0.02$, $p<0.01$, and $p=0.02$, respectively) after 6, 12, and 24 h of treatment; (iv) *SAV1* after 24 h of treatment ($p=0.03$); (v) *MOB1B* genes after 6 h of treatment ($p<0.01$) (Fig. 4).

After treatment with JAK inhibitor I, expression of the following apoptosis-related genes was upregulated: (i) *BAD* after 12 h of treatment ($p=0.02$); (ii) *BCL-2* after 6 and 24 h of treatment ($p<0.01$ for both); and (iii) *BCL-X_L* after 6 h of treatment ($p=0.01$). In contrast, JAK inhibitor I downmodulated *BAK* and *BCL-X_L* gene expression after 12 h ($p=0.03$ and $p<0.01$, respectively) and 24 h ($p=0.04$ and $p<0.01$, respectively) of treatment (Fig. 5).

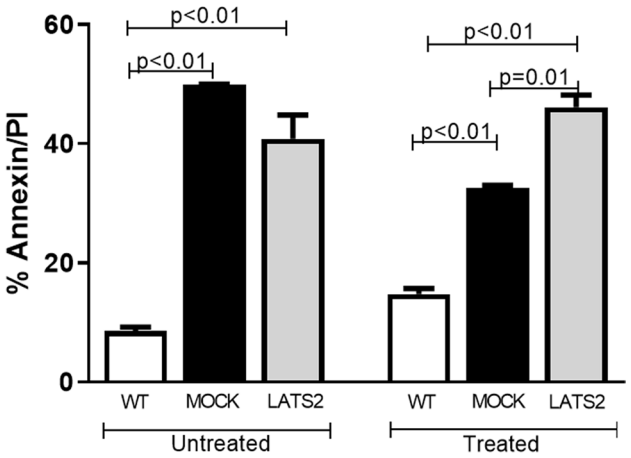

Fig. 5 Apoptosis rate of HEL.92.1.7-Wild type (WT), HEL.92.1.7-LATS2, and HEL.92.1.7-MOCK cells treated with JAK Inhibitor I (JII) for 48 h. The treatment killed more HEL.92.1.7-LATS2 cells than HEL.92.1.7-MOCK cells. Data are expressed as mean ± SD of the percentage of annexin/FITC-labeled cells. Mann–Whitney statistical test

LATS2 transient expression and JAK2 inhibitor alter HEL.92.1.7 cell death and proliferation rates

HEL.92.1.7-LATS2 cells were less resistant to JAK inhibitor I-induced cell death than HEL.92.1.7.-pcDNA3.1 (MOCK) cells ($p=0.01$) and wild-type cells ($p<0.01$) (Fig. 6). HEL.92.1.7-LATS2 cells were less resistant to cell death than wild-type cells and MOCK cells, since the transfection process itself caused death of part of the cells (Fig. 6).

Treatment with JAK2 inhibitor for 24 h decreased the cell proliferation rate of HEL.92.1.7-LATS2 cells when compared with MOCK-transfected cells (Fig. 6). LATS2-transfected cells treated or not with JAK2 inhibitor for 12 or 24 h had lower proliferation rate than wild-type cells.

Discussion

The Hippo signaling pathway has emerged as a complex signaling network related to molecular mechanisms involved in cancer development and progression [23, 24]. In mammals, Hippo pathway deregulation contributes to the

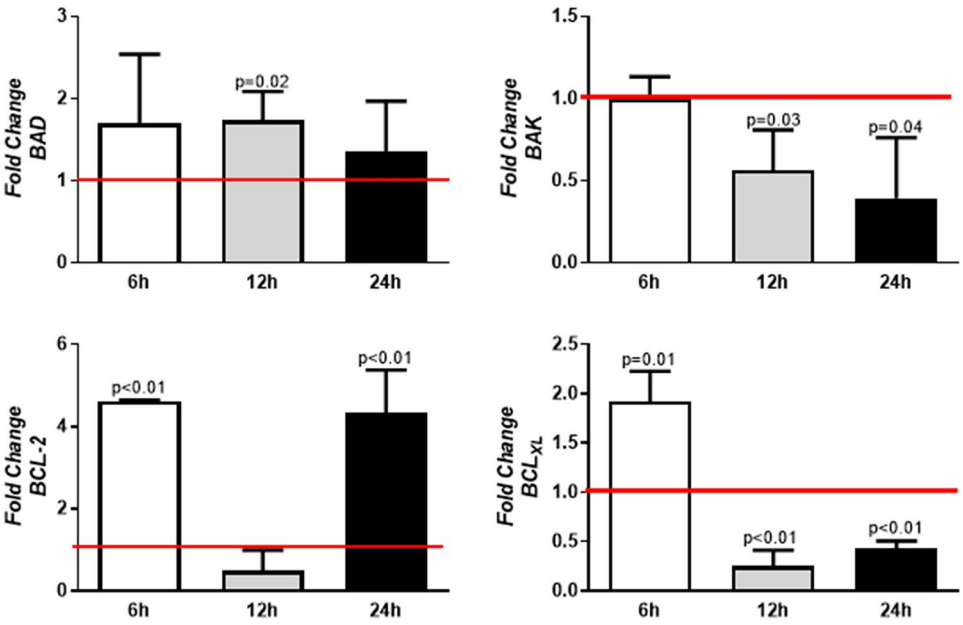

Fig. 4 Expression of apoptosis-related genes in HEL.92.1.7 cells treated with JAK Inhibitor I (JII). The treatment upregulated *BCL-2* and *BCL-X_L* expression after 6 h; upregulated *BAD* expression and downregulated *BAK* and *BCL-X_L* expression after 12 h; decreased *BAK* and *BCL-X_L* expression and increased *BCL-2* expression after 24 h. The red line represents untreated cells. Data are expressed as Fold Change. Unpaired test

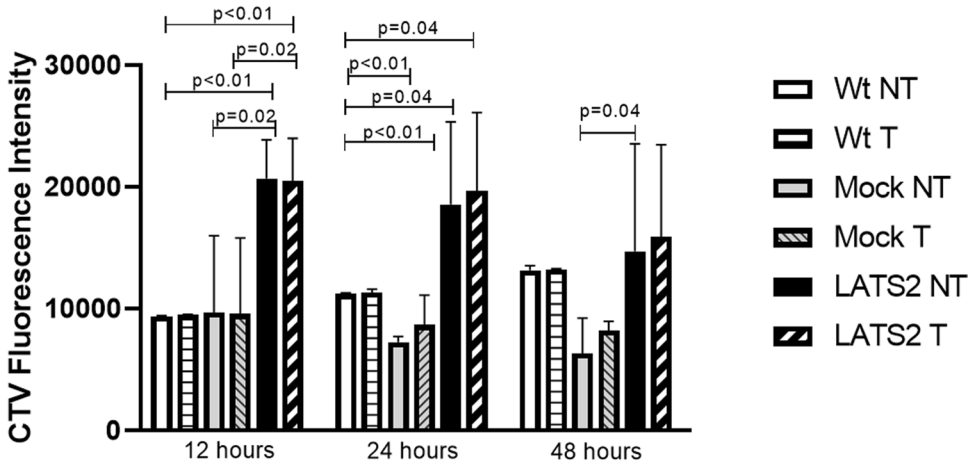

Fig. 6 Proliferation rate of the LATS2-transfected cell line HEL.92.1.7-LATS2 and HEL.92.1.7-MOCK cells treated with JAK inhibitor I. CTV fluorescence intensity was inversely proportional to the cell proliferation rate. HEL.92.1.7-LATS2 cells proliferated slower than HEL.92.17-MOCK cells in the three time periods analyzed

pathophysiology of various clinical disorders. The altered expression of Hippo pathway-related genes is associated with tumorigenesis of colorectal cancer [25], lung cancer [26], gliomas [27], thyroid gland neoplasms [28], and hematological neoplasms such as acute myeloid leukemia, acute lymphocytic leukemia, mantle cell lymphoma, multiple myeloma, and myelodysplastic syndromes [29–32].

The role that this pathway and its components play in hematopoietic system and hematological neoplasm pathogenesis and progression remains controversial [33]. For instance, the enforced expression of a constitutively active *YAP* mutant does not affect the normal hematopoietic stem cell biology [34]. In contrast, *MST1* gene inactivation causes primary immunodeficiency characterized by susceptibility to chronic infection and frequent autoimmune manifestations [35, 36].

The low *LATS2* gene expression in adult T cell leukemia/lymphoma and natural killer leukemia cells results in decreased pro-apoptotic gene expression in leukemic cells and the consequent resistance to chemotherapy [37]. On the other hand, high *LATS2* gene expression accounts for leukemogenesis in acute myeloid leukemia [32, 38].

In our study, the expression of the Hippo pathway-related genes *SAV1* and *TAZ*, and the pro-apoptotic gene *BAD* was downregulated in PMF patients when compared with the control. PV and ET patients also presented low expression of various Hippo pathway-related genes, including *MST2, SAV1*, and *TAZ*. The low expression of these genes was associated with deregulated expression of apoptosis-related genes, upregulated expression of the anti-apoptotic genes *BCL-2* and *BCL-*X_L, and downregulated expression of *BAK* and *BAD* genes. Altogether, these data suggested that deregulation of both signaling pathways is involved in the exacerbated cell proliferation and cell death resistance in MPN.

Another relevant finding was the association between driver mutations and Hippo pathway gene expression, especially in PMF patients; those patients who harbored JAK2V617F mutation presented low expression of *MST1*, *MOB1A*, and *MOB1B*. The heterozygous *MST1/MST2* kinase inactivation leads to accelerated development of lethal myelofibrosis, associated to innate immune proinflammatory cytokine production, including IL-1β and IL-6, in a JAK2V617F model [39].

Our data also suggested the existence of association among Hippo pathway genes, apoptosis-related genes, hematological data, and clinical data. In the three disease subtypes studied herein, *MST1* correlated with platelet counts, WBC counts, or thrombotic events. *MST1* is an Mpl ligand-inducible serine/threonine kinase that induces signaling cascades and functions to potentiate polyploidization and differentiation during megakaryopoiesis [40]. Hence, the low *MST1* expression in these MPN seemed to be an attempt to counterbalance megakaryocyte proliferation, which is common in these neoplasms.

The expression of *c-myb*, *HOXA9*, *LYN*, *cystatin c*, and *LTC4s* differs between bone marrow and peripheral blood samples from patients with acute myeloid leukemia [41]. The gene expression analysis of total mRNA of all matched pairs of a group of patients with chronic lymphocytic leukemia, using Affymetrix U133A 2.0 arrays, has identified 543 genes with at least 1.5-fold higher expression in bone marrow than in peripheral blood, and 192 genes with at least 1.5-fold higher expression in peripheral blood than in bone marrow [42]. These reports are in line with the findings reported here that the expression of Hippo pathway genes differed between bone marrow and peripheral blood, and that the main changes occurred in peripheral blood.

In recent years, LATS kinases have attracted the interest of many researchers due to their broad range of biological activities in cell cycle regulation, differentiation, and motility, as well as the diverse pathological outcomes of their deregulation [43]. As the *LATS2* gene expression was decreased in PV patients, we examined its role in cells with

malignant phenotype, resistance to death, and exacerbated proliferation through the induction of ectopic expression of *LATS2* gene in HEL.92.1.7 cells. The *LATS2* gene overexpression increased the apoptosis rate and decreased cell proliferation rate in HEL 92.1.7 cells.

Ectopic expression of human *LATS2* induces apoptosis in two lung cancer cell lines, A549 and H1299 [44]. The anti-apoptotic proteins BCL-2 and BCL-X_L, but not the pro-apoptotic protein BAX, are downregulated in transduced human lung cancer cells, as demonstrated by Western blotting [44]. Overexpression of either *BCL-2* or *BCL-*X_L genes in these cells suppresses the LATS2-mediated caspase cleavage and apoptosis, confirming that *LATS2* gene induces human lung cancer cell apoptosis through downregulation of the anti-apoptotic proteins BCL-2 and BCL-X_L [44]. Overexpression of *LATS2* gene in human glioblastoma cells U-372 MG is associated with inhibition of cell proliferation, migration, and invasion, as well as with increased levels of phosphorylated TAZ and YAP proteins [45]. *LATS2* is a potential tumor suppressor gene in human glioblastoma cells, and its downregulation may contribute to the pathogenesis of this disease [45]. In this context, our data indicate that *LATS2* also acts as tumor suppressor gene in MPN.

We also analyzed the potential association between Hippo pathway-related genes and the patients' mutation *status*. The CALR mutation was associated with increased *MOB1B* and *SAV1* gene expression in ET patients, while the JAK2V617F mutation was associated with decreased *MST1, MOB1A*, and *MOB1B* expression in PMF patients, suggesting that the JAK2V617F mutation favored Hippo pathway deregulation.

In *Drosophila* sp., the integrated action of the JAK/STAT and Hippo signaling pathways controls development by independently regulating the transcription of common target genes, such as *CycE* and *E2f1* to control cell proliferation, and *Diap1* to control cell survival [46].

The heterozygous Hippo kinase *MST1* inactivation significantly accelerates myelofibrotic transformation into JAK2V617F MPN through its action on innate immune signaling and proinflammatory cytokine production [39]. No association between components of the Hippo pathway and the CALR mutation has been described so far.

Taken the results altogether, we concluded that expression of Hippo pathway-related genes is deregulated and *LATS2* is a tumor suppressor gene in MPN patients. The altered expression of Hippo pathway-related genes seems to mediate the exacerbated cell proliferation and apoptosis impairment in MPN patients.

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Author contributions All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by MdCC, JGC, LMP, MGB-C, GMB, LB, TM, RT, NdSN, and EXS. The first draft of the manuscript was written by MdCC and FAdC. The manuscript was reviewed by LLdF-P, APY, and FAdC**.** All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

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