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ORIGINAL ARTICLE

Effects of Ruxolitinib on Immune Checkpoint Molecule Expression in JAK2 V617F-Positive Cells

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SUMMARY

Background: **This study aimed to explore the clinical significance of ruxolitinib and its effects on the proliferation and apoptosis of human erythroleukemia (HEL) cells and the expression of immune checkpoint molecules programmed death-1 (PD-1), programmed death-ligand 1 (PD-L1), and regulatory T cells (Tregs) in HEL cells and JAK2 V617F-positive patients with myeloproliferative neoplasms (MPNs).**
Methods: **JAK2 V617F-positive patients with MPNs admitted to the Baoding No. 1 Hospital from January 2016 to September 2023 were recruited, including 30 patients for the newly diagnosed group and 10 for the treatment group. Additionally, 15 healthy volunteers were selected as the control group. JAK2 V617F mutation was detected by using fluorescence quantitative PCR, and the expression levels of phosphorylated JAK2 (p-JAK2), PD-1, and PD-L1 in fresh bone marrow were examined by immunohistochemistry. HEL cells were treated with ruxolitinib at different concentrations (0, 50, 100, 250, 500, and 1,000 nmol/L). Cell viability was detected by CCK-8 assay. The mRNA expression levels of JAK2, PD-1, and PD-L1 were determined by using fluorescence quantitative PCR. The protein expression of p-JAK2 was detected by Western blot and those of PD-1 and PD-L1 were evaluated by flow cytometry. The expression of PD-1, PD-L1, and Tregs after the 48-hour co-culture of primary bone marrow cells and HEL cells were also analyzed by flow cytometry.**
Results: **In the newly diagnosed group, the bone marrow myeloid cells highly expressed p-JAK2, PD-1, and PD-L1. The Tregs expression in their peripheral blood increased and was significantly higher than those in the treatment and control groups (all $p < 0.05$). Ruxolitinib at different concentrations could inhibit the proliferation of HEL cells and was positively correlated with treatment time and dose. Additionally, ruxolitinib could reduce p-JAK2, PD-1, and PD-L1 expression in HEL cells and Tregs expression.**
Conclusions: **Ruxolitinib reduces the expression of p-JAK2, PD-1, and PD-L1 in JAK2 V617F-positive cells by specifically inhibiting the JAK2 signaling pathway, thereby suppressing the progression of MPNs.**
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KEYWORDS

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INTRODUCTION

Myeloproliferative neoplasms (MPNs) are a group of clonal malignant hematologic disorders arising from bone marrow-derived hematopoietic stem cells and are characterized by aberrant proliferation of one or more myeloid lineages. The main members of this group are

polycythaemia vera (PV), primary myelofibrosis (PMF), and primary essential thrombocytosis. Mutations in Janus kinase-2 (JAK2), myeloproliferative leukaemia virus oncogene homologue (MPL), and calreticulin (CARL) are identified in most patients with MPNs [1,2].

Programmed death-1 (PD-1), also known as CD279, is an important immunosuppressive molecule and a member of the CD28 superfamily. Immunomodulators targeting PD-1 are of great significance in fighting against tumors, infections, and autoimmune diseases and for organ transplant survival [1]. Programmed death-ligand 1 (PD-L1), also referred to as CD274, is a ligand for PD-1 that is mainly expressed on antigen-presenting cells (APCs) and tumor cells [2]. Under physiological conditions, PD-1 recognizes antigens through the T cell (antigen) receptor and regulates the function of peripheral T cells and consequently the immune response of the body. After the T cell surface receptor PD-1 binds to its ligand PD-L1, src homology region 2 domain-containing phosphatase-1 and -2 are recruited to produce inhibitory signals that suppress the phosphorylation of the PI3K/Akt pathway and the activation of mammalian target of rapamycin (mTOR) and extracellular signal-regulated kinase 2, thereby promoting the differentiation of CD4+ Foxp3- T cells into CD4+ Foxp3+ T cells [3], also known as regulatory T cells (Tregs). Under pathological conditions, PD-1 is mainly expressed on activated T cells to inhibit the activation of anti-tumor cells. The tumor microenvironment can induce infiltrating T cells and tumor cells to highly express PD-1 and PD-L1, respectively. The binding of these ligands constantly activates the PD-1 pathway in the tumor microenvironment, inhibits T cell function, and enhances Tregs expression, ultimately resulting in failure to kill tumor cells.

JAK2 is a non-receptor tyrosine kinase that plays a major role in cytokine signal transduction and promotes the growth, survival, and differentiation of many types of cells. A somatic point mutation in JAK2 (JAK2 V617F mutant) frequently occurs in BCR-ABL-negative patients with MPNs [4]. Ruxolitinib is a selective JAK1/2 inhibitor currently used to treat medium- and high-risk patients with PMF and secondary MF and patients with PV intolerance/resistance to hydroxyurea or interferon, especially those with accompanying disease-related splenomegaly and disease-related symptoms. Human erythroleukemia (HEL) cells exhibit a natural JAK2 V617F point mutation. Ruxolitinib can inhibit the angiogenesis and migration of HEL cells by inhibiting the JAK2 pathway [5,6]. At present, no relevant reports are available on the effects of ruxolitinib on the PD-1/PD-L1 signaling pathway. Therefore, this study preliminarily explored the expression of PD-1/PD-L1 in HEL cells, the effects of ruxolitinib on PD-1/PD-L1 expression, and the *in vivo* and in vitro effects of ruxolitinib on PD-1/PD-L1 and Tregs expression in JAK2 V617F-positive MPN cells before and after treatment. The findings provide a new theoretical basis for MPN diagnosis and treatment.

MATERIALS AND METHODS

Ethical approval

The study was approved by the institutional Ethics Committee of Baoding No. 1 Hospital, and written informed consent was obtained from all participants.

Case data

JAK2 V617F-positive patients with MPNs admitted to the Baoding No. 1 Hospital from January 2016 to September 2023 were recruited, including 30 patients for the newly diagnosed group (14 males and 16 females) and 10 for the treatment group (5 males and 5 females). Additionally, 15 healthy volunteers (8 males and 7 females) were selected as the control group. All the patients met the 2016 WHO diagnostic criteria for hematological diseases. The ruxolitinib treatment group was given ruxolitinib phosphate at initial doses (5 - 20 mg, two times/day), corresponding to their platelet status for more than 3 months.

Main reagents and instruments

Anti-p-JAK2 and anti-PD-1/PD-L1 antibodies were purchased from ProteinTech (USA). The human peripheral blood lymphocyte separation medium was acquired from TBD sciences (Tianjin, China, item no.: 2010C 1119), and the CCK-8 reagent kit was obtained from Dojindo (Japan). Rabbit anti-PD-1/CD279 polyclonal antibody and mouse anti-PD-L1/CD274 monoclonal antibody were provided by ProteinTech (USA). Anti-PD-1 (FITC mouse anti-human CD279) and anti-PD-L1 (PE mouse anti-human CD274) antibodies were bought from BD Pharminutesgen™, and FITC-labelled anti-CD3, CD4, and CD8 monoclonal antibodies, PE-labelled anti-CD25 monoclonal antibody, and APC-labelled anti-Foxp3 monoclonal antibody were supplied by BD (USA). RNA extraction kit was purchased from Biomed (Beijing, China). Primers were designed based on the corresponding gene sequences published in NCBI and synthesized by SBS Genetech Co., Ltd. (Beijing, China). Ruxolitinib was obtained from Novartis AG (Switzerland, batch no.: SV824). ABI 7500 fluorescence quantitative PCR instrument was acquired from Applied Biosystems (USA).

Experimental methods

Cell lines and culture conditions: HEL cells were provided by the Cell Bank of Shanghai Academy of Science & Technology. HEL cells and/or primary human peripheral blood lymphocytes were cultured in an incubator at 37°C and 5% CO_2 and maintained in RPMI 1640 culture medium containing 10% fetal bovine serum (FBS). The medium was replaced every 2 - 3 days according to cell growth, and the cells at the

logarithmic growth phase were used for subsequent experiments.

HEL cells were co-cultured with peripheral blood lymphocytes. Mononuclear cells were isolated from the peripheral blood of five healthy human donors through lymphocyte separation in a sterile condition. After being washed twice with PBS, these cells were resuspended in RPMI 1640 culture medium containing 10% FBS and then added into the culture medium containing HEL cells. The cells were co-cultured in an incubator at 37℃ and 5% CO_2 and sampled at 0, 24, 48, and 72 hours for experiments.

Inhibition of cell proliferation by CCK-8 assay

HEL cells at the logarithmic growth phase were divided into experimental and control groups. The experimental group was added with ruxolitinib at different concentrations to final concentrations of 50, 100, 250, 500, and 1,000 nmol/L. The control group was not treated with ruxolitinib.

A cell suspension (100 μL) prepared by using the logarithmic growth phase cells was transferred to a 96-well plate (outer wells were filled with sterile water or PBS). A blank group, a control group, and an experimental group were constructed. The control group was added with 100 μL of cell suspension, and the experimental group was added with 100 μL of cell suspension and ruxolitinib at different concentrations, with five replicates in each group. CCK-8 reagent (10 μL) was added after 0, 24, 48, and 72 hours. After 2 - 4 hours, absorbance (OD) was measured by using a microplate reader, and growth curves were plotted. The inhibition rate of cell proliferation was calculated by using the following formula: inhibition rate of cell proliferation (%) = $(OD_{\text{control group}} - OD_{\text{experimental group}})/(OD_{\text{control group}} - OD_{\text{blank group}}) \times 100\%$.

Detection of apoptosis by flow cytometry

A cell suspension (3 μL) prepared from HEL cells at the logarithmic growth phase was seeded into a six-well plate at approximately 1×10^5 cells/well. The cells were grouped according to the experimental protocol, added with ruxolitinib at different concentrations, and incubated for 0, 24, 48, and 72 hours. The cells were collected, washed twice with PBS, and centrifugated at 1,000 r/minutes. After the supernatant was discarded, the cells were fixed in ice-cold 75% ethanol overnight and were centrifugated. With the supernatant discarded again, the cells were washed once with PBS and added with 1 mL of RNase for a temperature-constant water bath at 37℃ for 30 minutes. Afterwards, the cells were centrifugated at 1,000 r/minutes, the supernatant was discarded, and 1 mL of DNA extraction solution was added. The cells were allowed to stand at room temperature for 30 minutes, were centrifugated at 4,000 r/minutes for 5 minutes, and the supernatant was discarded. Finally, the cells were stained using 500 μL of 0.1 g/L PI staining solution in the dark at 4℃ for 30 minutes, and their apoptosis was detected by flow cytometry.

Detection of mitochondrial membrane potential changes in HEL cells by flow cytometryHEL cells treated with ruxolitinib at different concentrations for 12 and 24 hours were collected. After being washed twice with PBS, the cells were added with 5 μg/mL rhodamine 123 at 37℃ and 5% CO_2 for 30 minutes. Flow cytometry was used to quantify the fluorescence intensity of the cells in each treatment group.

Detection of caspase-3/7 activity in cells

Approximately 5,000 HEL cells treated with ruxolitinib at different concentrations for 12 and 24 hours were collected into a 96-well plate, with three replicates in each group. The cells were incubated with 100 μL of a mixture containing caspase-Glo 3/7 substrate and buffer for 4 hours. OD was measured at a wavelength of 405 nm, and cell viability was calculated.

JAK2/PD-1/PD-L1 mRNA expression by RT-PCR

1) Quantitative detection of the JAK2 gene in patients with MPNs: Fresh bone marrow (4 mL) was collected and anticoagulated with heparin. Genomic DNA was extracted by using a DNA extraction kit. Real-time fluorescence quantitative PCR was conducted with a reaction volume of 25 μL under the following reaction conditions: 1 cycle of 50℃ for 2 minutes and 95℃ for 10 minutes and 40 cycles of 95℃ for 15 seconds and 60℃ for 1 minute. A standard group and a blank control group were also established. The fluorescence signal in the first 3 - 15 cycles of the PCR was used as the background signal, and the baseline was adjusted to an appropriate level as the threshold. The number of cycles required by each fluorescence signal to reach the set threshold was recorded as the Ct value. The absolute copy numbers of JAK2 and JAK2 V617F were calculated based on the standard, and the JAK2 V617F/JAK2 ratio was also calculated. The PCR primers and probes were as follows: JAK2, 5′-CAG CAA GTA TGA TGA GCA AGC TTT-3′ (forward) and 5′-TGA ACC AGA ATA TTC TCG TCT CCA C-3′ (reverse); MGB-probe, 5′-FAM-TCA CAA GCA TTT GGT TTT-MGB-3′; and JAK2 V617F, 5′-CCA GAA TAT TCT CGT CTC CAC TGA A-3′ (reverse).

2) Quantitative detection of genes in HEL cells: Total RNA was extracted from the cells in each group by using TRIzol reagent, and RNA purity and quantification were determined by electrophoresis. cDNA was synthesised by reverse transcription using the Vazyme Hiscript synthesis kit. The PCR system (25.0 μL) contained 2 μL of cDNA template, 0.5 μL of forward primer, 0.5 μL of reverse primer, 1.25 μL of 20 × SYBR stain, and 10 μL of 2.5 x Real Master Mix. The PCR amplification conditions were as follows: 30 cycles of 94℃ for 5 minutes, 94℃ for 45 seconds, and 60℃ for 1 minute. The relative expression levels of the detected genes were calculated as:

$\triangle Ct = Ct_{\text{target gene}} - Ct_{\beta\text{-actin}}$ and $\triangle\triangle Ct = 2^{-\triangle Ct}$. Calculation was repeated three times in each group, and averages were taken. The PCR primers were as follows: JAK2, 5′-CAG CAA GTA TGA TGA GCA AGC TTT-3′ (forward) and 5′-TGA ACC AGA ATA TTC TCG TCT CCA C-3′ (reverse), internal reference β-actin, 5′-CTG GCA CCA CAC CTT CTA CAA T-3′ (forward) and 5′-AAT GTC ACG CAC GAT TTC CCG C-3′ (reverse), PD-1, 5′-TGT CAC ACA ACT GCC CAA-3′ (forward) and 5′-TTC TCT CTG TCA CCC TGA GC-3′ (reverse), PD-L1, 5′-GCT GCA CTA ATT GTC TAT TGG GA-3′ (forward) and 5′-AAT TCG CTT GTA GTC GGC ACC-3′ (reverse). The amplified fragment length was 101 bp for JAK2, 164 bp for PD-1, 228 bp for PD-L1, and 382 bp for β-actin.

Detection of p-JAK2/PD-1/PD-L1 protein expression by Western blot

Total protein was extracted from the cells in each group. According to the protein quantification results, the proteins were packaged and boiled in boiling water for denaturation for 5 minutes. After concentrated gels were prepared, the proteins were loaded and separated by electrophoresis, transferred to a PVDF membrane, and then blocked in 5% skimmed milk powder in an oven at 37℃ for 2 hours. The primary antibodies were diluted in antibody diluent for incubation overnight at 4℃. After the membrane was washed with TTBS six times (5 minutes/time), labelled secondary antibodies were diluted in antibody diluent for incubation in an oven at 37℃ for 2 hours. After being washed with TTBS three times and TBS once, the PVDF membrane was mixed with a chemiluminescent reagent for scanning and image analysis using the Alpha Innotech system.

Immunohistochemical staining

Waxed tissue blocks were cut into sections of 4 μm thickness, baked at 45℃ for 3 hours, dewaxed, and rinsed twice with distilled water. Following antigen retrieval, 3% hydrogen peroxide was added to block endogenous catalase activity. The sections were then washed twice with PBS and added with primary antibodies (anti-p-JAK2, anti-VEGF, and anti-CD105:1: 200, 1:200 and 1:50) overnight at 4℃. After washing with PBS three times for 5 minutes each, reagent A (amplifier) and reagent B (enzyme-labelled sheep anti-mouse/rabbit IgG polymer) of the Elivision plus kit were dropwise added, followed by PBS washing and colour development with DAB. The presence of brownish-yellow particles on the membrane, cytoplasm or nucleus of the cell was regarded as positive staining. Restaining was conducted with hematoxylin. After being acidified with 0.1% hydrochloric acid and rinsed with tap water, the sections were dehydrated in gradient alcohol, dried, and then sealed with neutral gum. The presence of brownish particles in the cell cytoplasm was considered immunohistochemically-positive. Three high-power fields of view (× 400) were selected to calculate the proportion of positive cells to total cells.

Statistical analysis

All data were expressed as mean ± standard deviation. *t*-test and paired *t*-test were used for the comparison of means between two samples, analysis of variance was used for the comparison of means among multiple samples, and Q-test was used for pairwise comparison. Pearson's linear correlation was employed to analyze the correlation between two variables. Data analysis and processing were performed with SPSS 19.00.

RESULTS

Expression of JAK2 V617F mutation in patients

All the newly diagnosed patients were positive for JAK2 V617F mutation, as determined through quantitative PCR. The mutation frequency was (51.98 ± 18.37)% in the newly diagnosed group (n = 30), which was significantly higher than the (29.86 ± 23.26)% in the ruxolitinib treatment group (n = 10) ($p < 0.05$). No JAK2 V617F mutation was detected in the control group.

Detection of p-JAK2/PD-1/PD-L1 expression in patients with MPNs by immunohistochemical staining

p-JAK2/PD-1/PD-L1 protein expression in bone marrow: p-JAK2, PD-1, and PD-L1 proteins were all expressed on the cell membrane and in the cytoplasm. Their positive rates in bone marrow-derived cells were the highest in the newly diagnosed group (80.13 ± 23.35, 50.29 ± 24.37, and 54.52 ± 21.15, respectively), followed by those in the treatment group (56.34 ± 22.46, 21.19 ± 14.28, and 27.65 ± 19.35, respectively) and the lowest in the control group (40.53 ± 13.72, 6.09 ± 3.12, and 5.16 ± 3.64, respectively), with statistically significant differences (all $p < 0.01$; Figure 1, Table 1).

Inhibitory effect of ruxolitinib on HEL cell proliferation as detected by CCK-8 assay

CCK-8 assay showed that with the increase in ruxolitinib concentration and treatment time, the inhibition rate of HEL cell proliferation also increased in a time- and dose-dependent manner. For the groups treated with ruxolitinib at different concentrations (0, 50, 100, 250, 500, and 1,000 nmol/L), the inhibition rates of HEL cell proliferation were (23.9 ± 3.09)%, (33.96 ± 3.14)%, (39.94 ± 2.71)%, (40.73 ± 2.86)%, and (42.14 ± 2.32)% at 24 hours, respectively; (29.86 ± 3.39)%, (40.29 ± 3.34)%, (47.2 ± 2.61)%, (56.7 ± 2.96)%, and (61.44 ± 2.15)% at 48 hours, respectively; and (44.17 ± 3.43)%, (52.37 ± 3.12)%, (63.57 ± 2.98)%, (68.95 ± 2.78)%, and (85.52 ± 2.86)% at 72 hours, respectively, as show in Figure 2.

Detection of apoptosis by flow cytometry

Flow cytometry revealed that after the HEL cells were treated with ruxolitinib at different concentrations (50, 100, 250, 500, and 1,000 nmol/L) for 48 hours, the apoptotic rates of HEL cells were (8.83 ± 0.421)%,

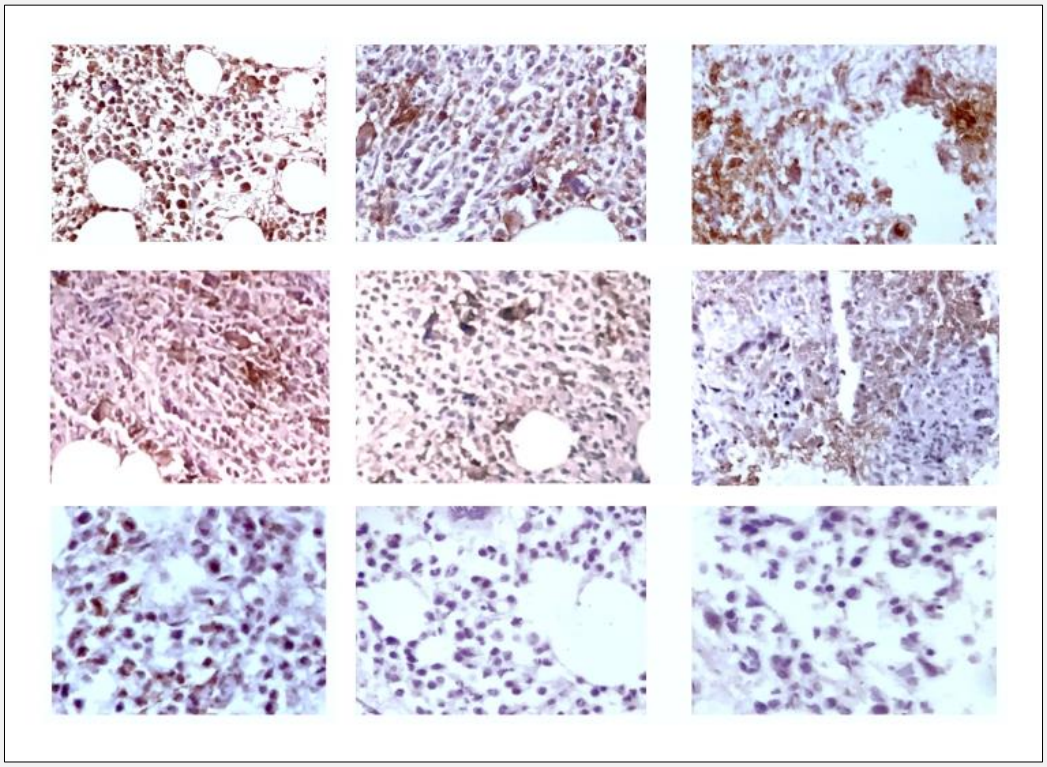

Figure 1. Immunohistochemical staining analysis of the expression levels of p-JAK2, PD-1, and PD-L1 protein (× 400).

A: p-JAK2, B: PD-1, C: PD-L1; 1: newly diagnosed group, 2: treatment group, 3: control group.

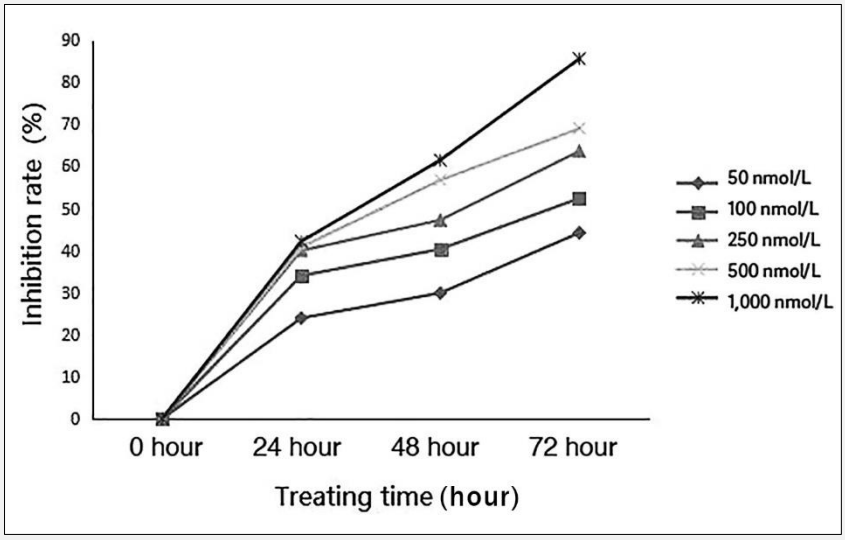

Figure 2. Inhibition rate of HEL cell proliferation by ruxolitinib at different concentrations and different time points as detected by CCK-8 assay.

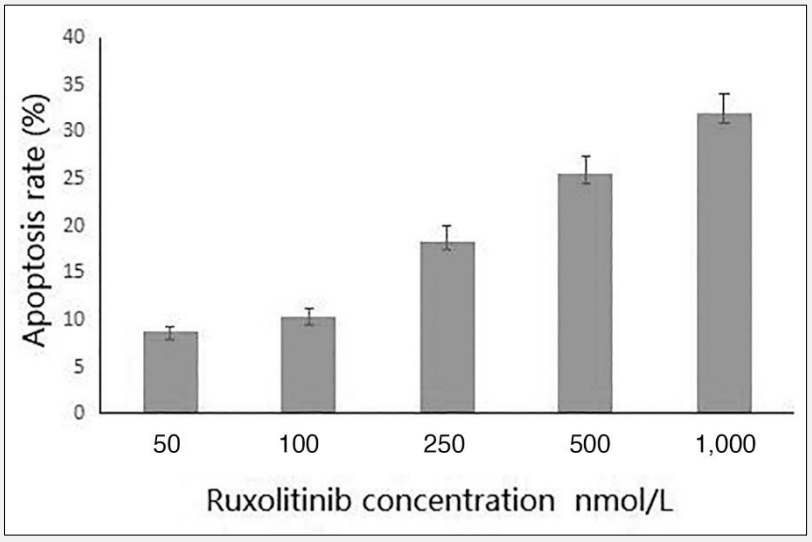

Figure 3. Apoptotic rate of HEL cells treated with ruxolitinib at different concentrations as detected by flow cytometry.

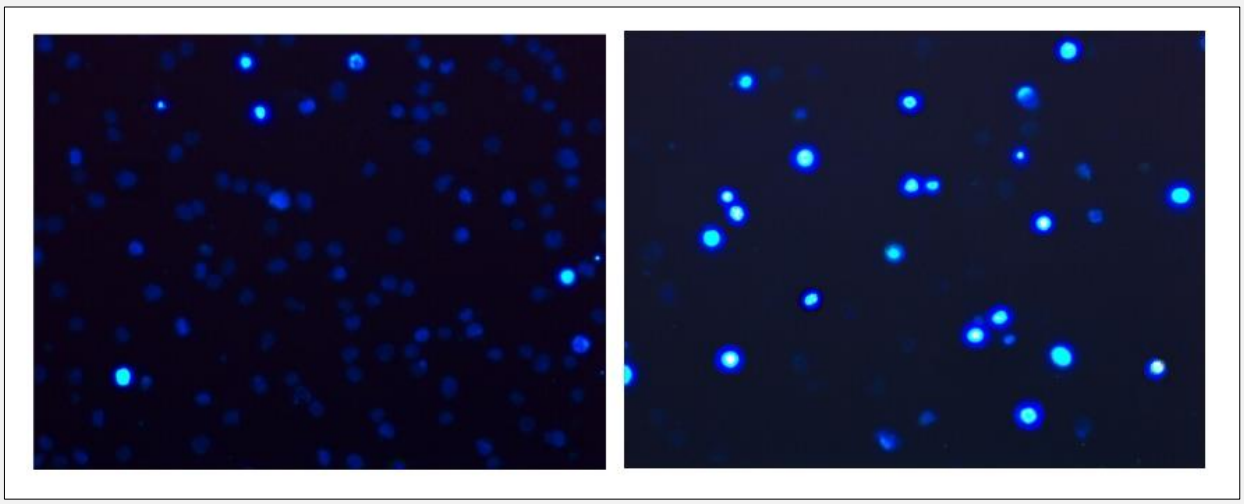

Figure 4. Apoptotic morphology of HEL cells after treatment with ruxolitinib for 48 hours as detected by fluorescence staining with Hoechst 33342.

A: Control group B: 100 nmol/L ruxolitinib treatment group.

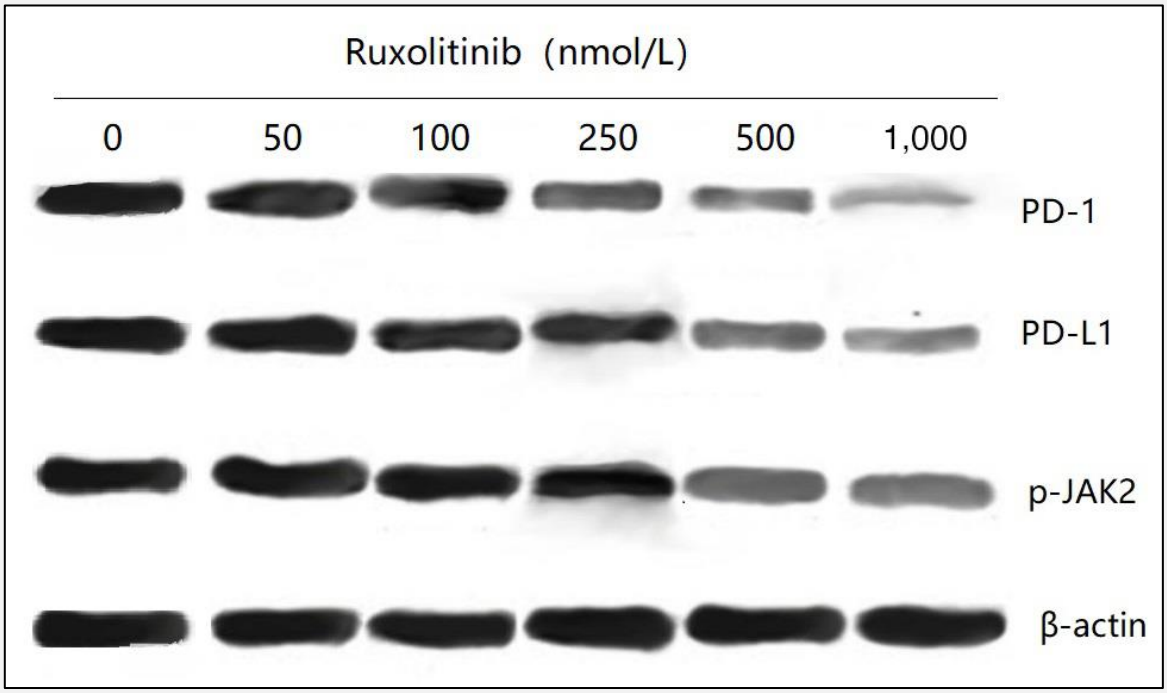

Figure 5. p-JAK2, PD-1, and PD-L1 expression in HEL cells treated with ruxolitinib at different concentrations for 48 hours as detected by Western blot.

(10.34 ± 0.768)%, (18.39 ± 1.62)%, (25.45 ± 2.01)%, and (31.98 ± 2.04)%, respectively, indicating that ruxolitinib promoted HEL cell apoptosis in a dose-dependent manner, as displayed in Figure 3.

Observation of apoptosis by fluorescence staining with Hoechst 33342

After the HEL cells were treated with 100 nmol/L ruxolitinib for 48 hours, Hoechst 33342 staining revealed that the Hoechst-stained cells in the control group were light blue with round or oval nuclei and uniform DNA distribution but no pyknosis or deformation (Figure 4A). In the treatment group, the Hoechst-stained nuclei appeared bright blue due to concentration, and pyknosis and deformation were observed, suggesting cell apoptosis (Figure. 4B). The proportion of bright blue cells in the 100 nmol/L ruxolitinib treatment group was higher than in the control group [(49.21 ± 1.80)% vs. (10.02 ± 1.40)%, $p < 0.05$].

Effect of ruxolitinib on mitochondrial membrane potential in HEL cells

After the treatment of HEL cells with ruxolitinib for different times (12 and 24 hours), the fluorescence intensity of rhodamine 123 was the highest in the control group. As the ruxolitinib concentration increased, the fluorescence intensity of rhodamine 123 weakened gradually in a dose-dependent manner (Table 2).

Detection of caspase-3/7 activity

After the treatment of HEL cells with ruxolitinib at different concentrations for 12 and 24 hours, the caspase-3/7 activity exhibited a dose-dependent increase ($p < 0.01$), as listed in Table 3.

PD-1 and PD-L1 mRNA expression detected by RT-PCR

After the treatment of HEL cells with ruxolitinib at different concentrations for 48 hours, the mRNA expression levels of JAK2, PD-1, and PD-L1 in the treatment group were lower compared with those in the control group and showed a gradually decreasing trend with the increase in drug concentration, as listed in Table 4. JAK2 mRNA expression was positively correlated with PD-1 and PD-L1 mRNA expression, with coefficients of correlation of 0.956 and 0.878, respectively. A positive correlation was found between PD-1 and PD-L1 mRNA expression, with a coefficient of correlation of 0.936.

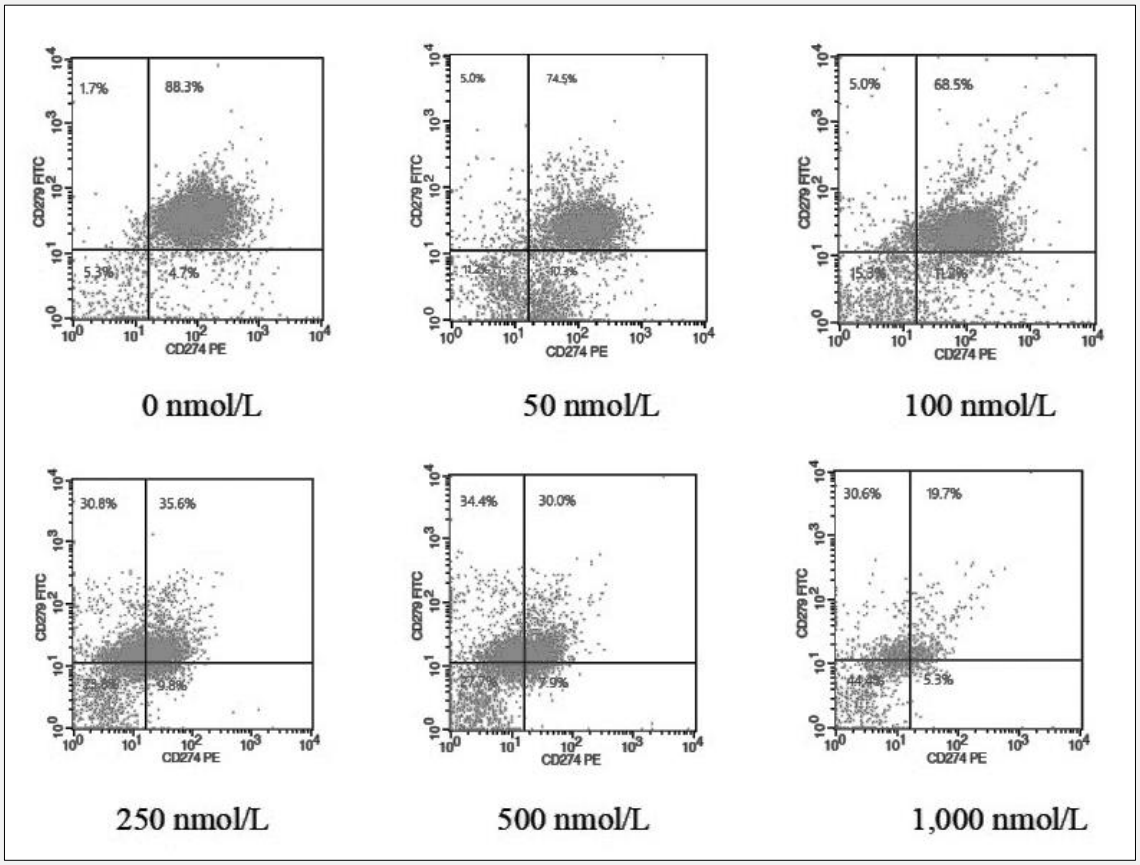

Figure 6. PD-1 and PD-L1 protein expression after treatment with ruxolitinib at different concentrations for 48 hours.

p-JAK2, PD-1, and PD-L1 protein expression by Western blot

After the treatment of HEL cells with ruxolitinib at different concentrations, the levels of p-JAK2, PD-1, and PD-L1 protein expression were reduced significantly as the treatment was prolonged. After incubation for 48 hours, the p-JAK2 protein expressions in the HEL cells treated with ruxolitinib concentration gradient were 0.969 ± 0.091, 0.517 ± 0.033, 0.348 ± 0.031, 0.256 ± 0.032, 0.133 ± 0.016, and 0.081 ± 0.025; the PD-1 protein expressions were 0.826 ± 0.087, 0.513 ± 0.027, 0.396 ± 0.029, 0.224 ± 0.033, 0.106 ± 0.011, and 0.038 ± 0.020; and the PD-L1 protein expressions were 0.886 ± 0.087, 0.625 ± 0.027, 0.401 ± 0.029, 0.376 ± 0.033, 0.128 ± 0.011, and 0.062 ± 0.020, as shown in Figure 5.

PD-1 and PD-L1 protein expression in HEL cells by flow cytometry

After the treatment of HEL cells with ruxolitinib at different concentrations (0, 50, 100, 250, 500, and 1,000 nmol/L) for 48 hours, the PD-1 and PD-L1 protein expression decreased gradually with the increase in drug concentration (93%, 84.9%, 79.7%, 45.4%, 37.9%, and 25% for PD-1 and 90%, 79.5%, 73.5%, 66.4%, 64.4%, and 50.3% for PD-L1), as shown in Figure 6.

Effects of ruxolitinib on expression of PD-1 and PD-L1 in co-cultured cells and Tregs

After the co-culture of primary lymphocytes from healthy individuals and HEL cells for 48 hours, the PD-1 and PD-L1 expression levels in HEL cells were detected. The results showed that the PD-1 expression was 7.05 ± 0.42 versus 6.85 ± 0.36 and the PD-L1 expression was 10.21 ± 0.58 versus 9.87 ± 0.49 before and after co-culture. Although their expression levels were both reduced after co-culture, no statistical differences were observed. The PD-1 expression in lymphocytes was 1.74 ± 0.88 versus 2.98 ± 0.78 and the Tregs expression was 1.10 ± 0.55 versus 3.03 ± 0.71 before and after co-culture. After the 48-hour treatment of the co-cultured primary lymphocytes from healthy individuals and HEL cells with ruxolitinib at different concentrations (0, 50, 100, 250, 500, and 1,000 nmol/L), the

Tregs proportion presented a dose-dependent downregulation with PD-1 and PD-L1 expression (3.03 ± 0.71, 3.02 ± 0.44, 2.64 ± 0.49, 1.93 ± 0.40, 1.47 ± 0.43, and 0.98 ± 0.46, respectively). Additionally, the Tregs proportion was positively correlated with PD-1 and PD-L1 expression, with coefficients of correlation of 0.978 and 0.969, respectively.

DISCUSSION

Chemotherapy and immunotherapy remain the most important means for treating hematological tumors. Negative immune regulatory molecules PD-1 and PD-L1 play important roles in the occurrence and metastasis of malignant tumors. PD-1 and its ligand PD-L1 are immune checkpoints that prevent the immune system from damaging normal cells during stress under physiological conditions. After binding to its ligand PD-L1, cancer cells overexpress PD-1 to inhibit the anti-tumor activity of immune cells and their ability to normally recognize cancer cells, resulting in an immunosuppressive microenvironment, where cancer cell growth is uncontrolled [7] and tumor immune escape occurs. The overexpression of PD-L1 and/or PD-1 is present in various solid tumors, such as lung cancer and liver cancer, leading to the enhanced vitality of peripheral blood Tregs and the inability of anti-tumor T cells, which is closely related to poor prognosis [8,9]. The overexpression of PD-1 and PD-L1 has also been confirmed in patients with hematological tumors. In 2016, the US Food and Drug Administration approved the PD-1 inhibitor nivolumab for the treatment of recurrent Hodgkin's lymphoma, and reasonable therapeutic effects have been achieved [10]. A study revealed that activating the JAK-STAT signaling pathway can upregulate PD-L1 [11]. The regulation of PD-1/PD-L1 is complex, varies among different tumors, and can occur at genetic, transcriptional, and post-transcriptional levels. The activation of common carcinogenic pathways, such as JAK/STAT, RAS/ERK, or PI3K/Akt/mTOR, and the treatment with cytotoxic drugs can both affect the expression of PD-L1 in tumors [12]. Our study showed that JAK2 V617F-positive patients with MPNs exhibited the spontaneous activation of the JAK2-STAT5 signaling pathway and the high expression of PD-1 and PD-L1. Particularly, the high expression of PD-1 and PD-L1 in HEL cells was observed at the mRNA and protein levels. In the co-culture system, HEL cells could promote PD-1 upregulation in normal lymphocytes and transformation into Tregs.

JAKs play a vital role in hematopoietic regulation and are essential signal transduction for hematopoietic/immune cytokine receptor 1 [13]. The cytokine receptor-JAK-STAT pathway is relevant to cell proliferation; that is, its activation promotes cell proliferation and inhibits cell apoptosis [14]. The activation of JAKs can also trigger mitogen-activated protein kinase, phosphatidylinositol-3′-kinase (PI3K/Akt), and mTOR [13]. In solid tumors, ruxolitinib can significantly improve the chemotherapeutic efficacy and reduce the side effects [15]. In hematological tumors, ruxolitinib can be used as a selective JAK1/2 inhibitor for the targeted treatment of MPNs and has been successfully applied in clinical practice [16]. In this study, the JAK2 V617F mutation frequency and the expression levels of PD-1 and PD-L1 were significantly reduced in the patients with MPNs treated with ruxolitinib. This drug could also dose-dependently inhibit HEL cell proliferation, induce cell apoptosis, and significantly suppress the expression of PD-1 and PD-L1, and its effects are superior to those of interferon [17,18]. In the co-culture system of primary lymphocytes from healthy individuals and HEL cells, the HEL cells could induce the lymphocytes to express PD-1 and transform into Tregs. Meanwhile, ruxolitinib could inhibit and downregulate the expression of PD-1 and Tregs. All the above findings indicated that ruxolitinib regulates the tumor immune microenvironment by controlling the PD-1/PD-L1 signaling pathway and Tregs through the suppression of the JAK signaling pathway, thus achieving tumor inhibition.

The proportion of Tregs in T lymphocytes significantly increases among patients with tumor, thereby inhibiting the anti-tumor activity of T lymphocytes [16]. The PD-1/PD-L1 signaling pathway may be one of the mechanisms by which Tregs exert inhibitory effects. Our study suggested that Tregs are significantly upregulated in patients with MPNs, and ruxolitinib treatment significantly suppresses Tregs, resulting in a significant downregulation of their expression. This finding was also supported by the results of primary cell culture. Therefore, ruxolitinib can participate in regulating the immune microenvironment of patients with MPNs by inhibiting the JAK2 signaling pathway.

In conclusion, this study preliminarily explores the expression of JAK2 V617F, PD-1, PD-L1, and Tregs in JAK2 V617F-positive patients with MPNs and their correlations with each other. The expression of PD-1 and PD-L1 in HEL cells is also detected. Results demonstrate that ruxolitinib inhibits the proliferation, induces the apoptosis of HEL cells, and suppresses the PD-1/PD-L1 signaling pathway. This work provides a theoretical basis for the role of the PD-1/PD-L1 pathway in MPNs and offers new diagnostic and therapeutic ideas for patients with poor therapeutic efficacy of interferon and intolerance to ruxolitinib.

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Declaration of Interest:
The authors declare that there is no conflict of interest.

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