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Original Article

Serine threonine kinase Pim-3 regulates STAT3 pathway to inhibit proliferation of human liver cancers

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Abstract: Objective: This study aimed to investigate the effects of serine threonine kinase Pim-3 on the growth of HepG2 cells and to explore the role of STAT3 signaling pathway. Methods: Synthetic Pim-3shRNA and negative control shRNA were independently transfected into HepG2 cells in the presence of Lipofectamine™ 2000. Cells were divided into 4 groups: Pim-3 shRNA group, negative control group, liposome control group, and blank control group. Flow cytometry was performed to detect the apoptosis of these cells; RT-PCR was employed to detect the mRNA expression of Pim-3; Western blot assay was done to measure the protein expression of Pim-3, STAT3, pSTAT3^{Tyr705}, Bcl-Xl, Bad and pBad^{Ser112}. Results: When compared with blank control group, liposome group and negative control group, the apoptosis index increased and the protein expression of Pim-3, pSTAT3^{Tyr705}, Bcl-Xl and pBad^{Ser112} and the Pim-3 mRNA expression reduced in the Pim-3 shRNA group, but the protein expression of STAT3 and Bad was comparable among groups. Conclusion: Pim-3 shRNA may down-regulate pSTAT3^{Tyr705} and pBad^{Ser112} protein expression to inhibit the proliferation of liver cancer cells and Pim-3 may serve as a target for the treatment of liver cancer.

Keywords: Serine threonine kinase Pim-3, liver tumor, STAT3, apoptosis

Introduction

Primary liver cancer originates from hepatocytes or intrahepatic biliary epithelial cells and hepatocellular carcinoma (HCC) accounts for 90% of primary liver cancer [1]. Primary liver cancer is the fifth leading common malignancy and the third leading cause of cancer related death [2]. The prevalence of HCC is at a high level in Asia and Africa, but at a low level in North America, North Europe and Australia [3]. International cancer research agency reports that there are about 670000 new cases every year and the incidence of primary liver cancer is still increasing over year [4]. Currently, it is accepted that the etiology and pathogenesis of primary liver cancer are related to the hepatitis C or B virus infection, drinking, intake of aflatoxin B1, hepatitic cirrhosis, non-alcoholic fatty liver disease and genetic factors [5]. With the progression in the early diagnosis, treatment and surgical approaches of primary liver cancer, the overall therapeutic efficacy increases. However, the long-term prognosis of primary liver cancer is still poor due to high prevalence of liver cancer. Thus, to develop new methods for the prevention and treatment of liver cancer have been hot topics in studies.

Pim (proviral integration site for Moloney murine leukemia virus) family is named after being found in the Moloney murine leukemia virus during the proviral integration. Later, it was confirmed as a group of Ca/calmodulin-dependent protein kinases (CaMK) [6]. Pim family members include Pim-1, Pim-2 and Pim-3 and have serine threonine kinase activity in mammalians. Pim family members have homology to kinases and can phosphorylate some specific substrates playing important roles in regulating proliferation, differentiation and apoptosis of cells [7-10]. Pim-1 was first identified as an oncogene and closely related to mouse lymphoma [11, 12]. Pim-3 may be induced by membrane depolarization and thus is also known as depolarization-induced gene (KID) [13] and later as Pim-3. Oncogene Pim-3 gene is mapped to 22q13 [14], and its expression is significantly different from that of Pim-1 and Pim-2. Pim-1 and Pim-2 expression is high in thymus tissues

[15, 16]. Pim-3 expression is at a high level in the brain, heart, lung, kidney and muscles, but absent in the normal endoderm derived organs (such as liver, pancreas, stomach and small intestine) [17]. Pim-1 and Pim-2 have been found to be cancerogenic and play important roles in the initiation of lymphoma and prostate cancer. However, Pim-3 has a high expression in the endoderm-derived cancers (such as liver cancer [17], pancreatic cancer [18] and colon cancer [19]). Popivanova et al [19] found that Pim-3 expression increased in colon cancer tissues and cancer cell lines, and the Pim-3 expression in well-differentiated adenocarcinoma and colon cancer cells was higher, positive rate of Pim-3 expression in adenoma was higher than that in adenocarcinoma, and normal colonic tissues had no Pim-3 expression. These findings suggest that Pim-3 functions at the early stage of cancers. Nowadays, it is widely accepted that Pim-3 exerts its biological effects in following ways: 1) it inactivates Bad via phosphorylation to transmit survival signals; 2) it regulates the cell cycle progression; 3) it regulates protein synthesis; 4) it regulates the transcriptional activity of Myc [6]. In this study, we aimed to investigate the effects of serine threonine kinase Pim-3 on the growth of HepG2 cells and analyze its potential mechanism.

Materials and methods

Cell line and shRNA

Human liver cancer cell line (HepG2 cells [17]) was stored in the Key Lab of Molecular Medicine in Jiangxi. Pim-3 shRNA (sequence [18]: 5'-G C A C G U G G U G A A G G A G C G G-3') and negative control shRNA (sequence: 5'-T T C T C C G A A C G T G T C A C G T-3') were synthesized in the Shanghai Genechem Biotech Co., Ltd.

Main instrument

Super clean bench, flow cytometer (BD FACSCalibur), thermal cycler (Bio-Rad), Western Blotting Instruments (Bio-Rad), and low temperature centrifuge machine (SORVALL Biofuge PrimoR) were used in the present study.

Antibodies

Rabbit anti-human Pim-3 polyclonal antibody (Abgent, USA), rabbit anti-human STAT3 polyclonal antibody (Santa Cruz, USA), rabbit anti-human Phospho-STAT3 (Tyr705) monoclonal antibody, rabbit anti-human Phospho-Bad (Ser112) monoclonal antibody (CST, USA), rabbit anti-human Bcl-Xl polyclonal antibody, rabbit anti-human Bad polyclonal antibody (Proteintech, USA), mouse anti-β-actin antibody, horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody, HRP conjugated goat anti-rabbit secondary antibody (Beijing Transgen Biotech Co., Ltd) were used in the present study.

Cell culture

Human liver cancer cells (Hep G2) were maintained in DMEM (Solarbo, Beijing) containing 10% fetal bovine serum (FBS; Hyclone, USA) at 37°C in an environment with 5% CO_2. Passaging was done with 0.25% trypsin (Solarbo, Beijing) once every 2-3 days.

Grouping and transfection

Cells were divided into 4 groups: 1) blank control group; 2) Liposome group; 3) negative control group; 4) Pim-3 shRNA group. Hep G2 cells were seeded into 6-well plates at a density of 2×10^5/well, followed by culture for 24 h. When the cell confluence reached 80-90%, DMEM was refreshed with FBS free DMEM. One hour later, shRNA and Pim-3 shRNA (4 μg/10 μL) were added to negative control group and Pim-3 shRNA, respectively, in the presence of Lipofectamine™ 2000 (Invitrogen, USA). In blank control group and Liposome group, medium and Lipofectamine™ 2000 were added, respectively. Four to six hours later, the medium was refreshed with DMEM containing 10% FBS, followed by incubation for another 48 h.

Detection of apoptosis by flow cytometry

After treatment for 48 h, cells were digested with EDTA free trypsin. According manufacturer's instructions (Annexin V/FITC; Beijing Zoman Biotech Co., Ltd), binding Buffer (500 μL), Annexin V/FITC (5 μL) and PI (10 μL) were independently added, followed by incubation in dark at room temperature for 10 min. Apoptosis was detected by flow cytometry.

Detection of Pim-3 mRNA expression in Hep G2 cells by RT-PCR

On the basis of published mRNA sequences of Pim-3 and β-actin, Primer Premier 5 was used to design primers which were synthesized in

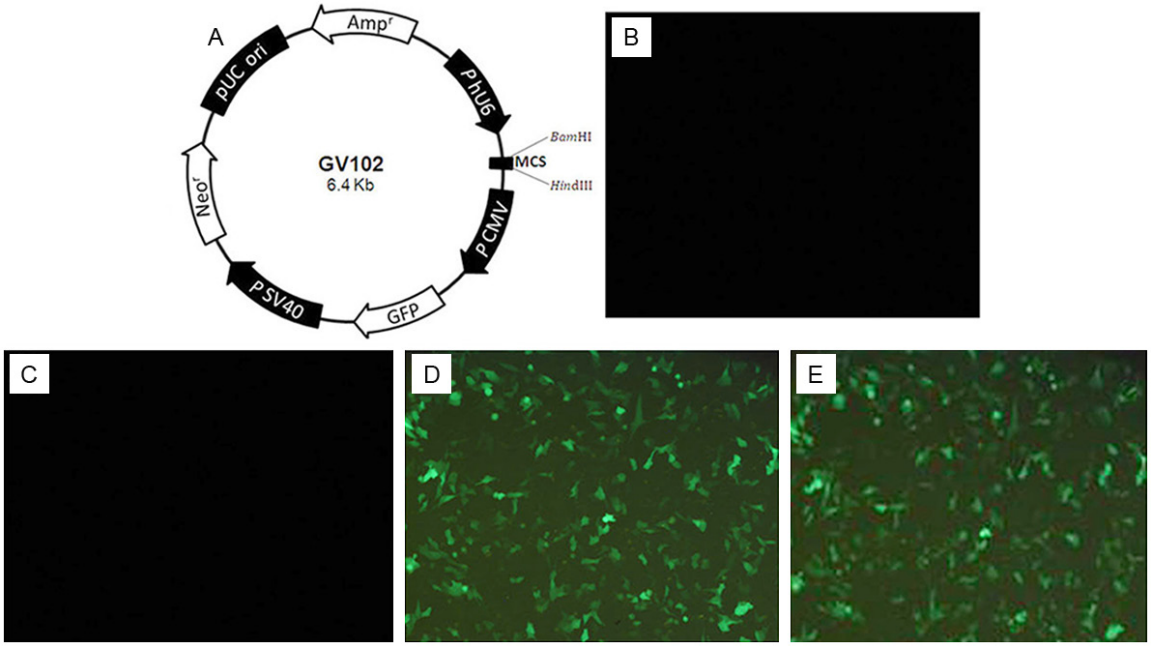

Figure 1. Expression of Green Fluorescence Protein: A: Vector; B: Blank control group; C: Liposome group. D: Negative control group; E: Pim-3 shRNA group. Medium and Lipofectamine™ 2000 were independently added to blank control group and Liposome group. Cells were transfected with shRNA and Pim-3 shRNA (4 μg; 10 μL/well) independently in the presence of LipofectamineTM 2000. 24 h, 48 h and 72 h after transfection in the negative control group and Pim-3shRNA group, and results showed the intensity of fluorescence was the highest in group D and E at 48 h.

Nanchang Purcell Scientific Instrument Co., Ltd. Pim-3: 5'-T C T C C A A G T T C G G C T C C C T-3' (forward), 5'-T C A C C C G C T C C T T C A C C A C-3' (reverse), length of product: 222 bp; β-actin: 5'-G T G G A C A T C C G C A A A G A C-3' (forward), 5'-A A A G G G T G T A A C G C A A C T A A-3' (reverse), length of product: 302 bp. Total RNA was extracted with TRIzol (Invitrogen, USA) and reverse transcribed into cDNA (1 μL). Then, 1 μL of cDNA, 1 μL of forward primer, 1 μL of reverse primer, 12.5 μL of 2x TaqPCR MasterMix, and 9.5 μL of ribozyme free water were mixed to prepare a mixture (25 μL). Pim-3 gene amplification was done with PCR and β-actin served as an internal reference. The reaction conditions for Pim-3 were as follows: pre-denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 57°C for 45 s and extension at 72°C for 45 s and a final extension at 72°C for 7 min. The reaction conditions for β-actin were as follows: pre-denaturation at 94°C for 5 min, 33 cycles of denaturation at 94°C for 40 s, annealing at 56°C for 40 s and extension at 72°C for 55 s and a final extension at 72°C for 7 min. Thereafter, 5 μL of products was subjected to 1.5% agarose gel electrophoresis, and bands were photographed under the ultraviolet.

Detection of protein expression in hep G2 cells by western blot assay

After treatment for 48 h, total proteins were extracted from cells in different groups with a protein extraction kit (Beijing Applygen Tech Co., Ltd) and then subjected to SDS-PAGE. These proteins were transferred onto PVDF membrane, which was blocked in 5% non-fat milk (Beijing Applygen Tech Co., Ltd) for 2 h and incubated with primary antibody at 4°C overnight. The membrane was washed with TBST thrice (10 min for each), and then incubated with HRP-conjugated secondary antibody at room temperature for 2 h. Visualization was done with ECL, and representative images were captured.

Statistical analysis

Data were expressed as mean ± standard deviation, and statistical analysis was performed with SPSS version 20.0 for Windows. Comparisons were done with one-way analysis of variance among groups, followed by LSD-t test. A value of $P<0.05$ was considered statistically significant.

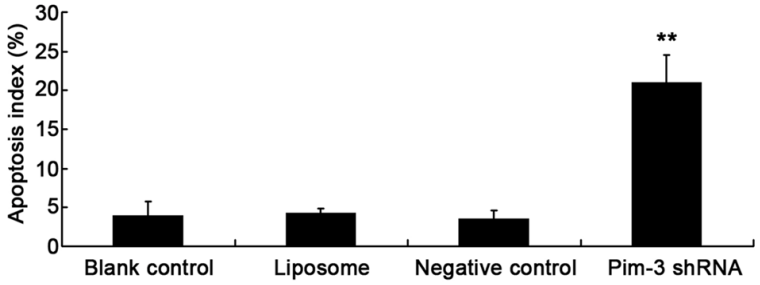

Figure 2. Apoptosis of HepG2 cells in different groups: 1: Blank control group; 2: Liposome group; 3: Negative control group; 4: Pim-3 shRNA group. The number of apoptotic cells in early phase was comparable among blank control group, liposomes group and negative control group, but significantly lower than that in Pim-3 shRNA group; **, P<0.001.

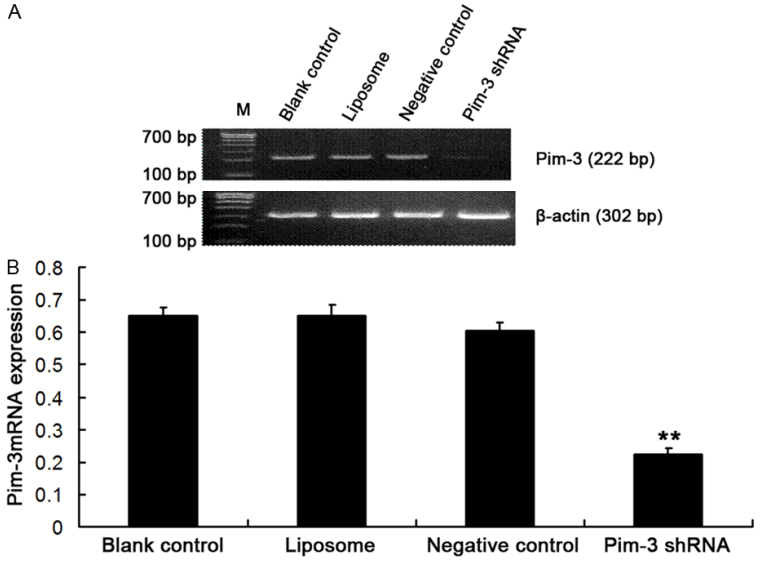

Figure 3. Pim-3 mRNA expressions in different groups. A: Pim-3 mRNA and β-actin mRNA in Hep G2 cells after gel electrophoresis (0: Marker, 1: Blank control group; 2: Liposome group, 3: Negative control group; 4: Pim-3 shRNA group). B: 1: Blank control group; 2: Liposome group, 3: Negative control group; 4: Pim-3 shRNA group. Pim-3 mRNA expression was comparable among blank control group, liposomes group and negative control group, but significantly higher than that in Pim-3 shRNA group; **P<0.001.

Results

Findings after transient transfection

The synthesized hU6-MCS-CMV-GFP-SV40-Neomycin (with Green Fluorescent Protein expression) was used to carry Pim-3 shRNA and negative control shRNA. Once Pim-3 shRNA and negative control shRNA were successfully transfected into Hep G2 cells, green fluorescence may be observed under a fluorescence microscope. In the presence of Lipofectamine™ 2000, Pim-3 shRNA and negative control shRNA were independently transfected into Hep G2 cells. About 48 h later, these cells were observed under a fluorescence microscope (the intensity of fluorescence was the highest at this time point), and cells with successful transfection in negative control group and Pim-3 shRNA group showed green fluorescence. In blank control group and liposomes group, no green fluorescence was observed (**Figure 1**).

Apoptosis of hep G2 cells

After treatment for 48 h, cells in different groups were harvested following digestion with EDTA free trypsin, and Annexin V/FITC was added according to manufacturer's instructions. The apoptotic cells were detected by flow cytometry. Results showed the number of apoptotic cells in early phase was comparable among blank control group, liposomes group and negative control group, but significantly lower than that in Pim-3 shRNA group (**Figure 2**).

Detection of Pim-3 mRNA expression

After treatment for 48 h, total RNA was extracted with TRIzol for RT-PCR. The products of Pim-3 and β-actin gene amplification were 222 bp and 302 bp, respectively, in length. Results showed the Pim-3 mRNA expression was comparable among blank control group, liposomes group and negative control group, but significantly higher than that in Pim-3 shRNA group. The mRNA expression of Pim-3 was normalized to that of β-actin as the relative expression (**Figure 3**).

Protein expression

After treatment for 48 h, total proteins were extracted with a kit according to manufacturer's instructions and Western blot assay was

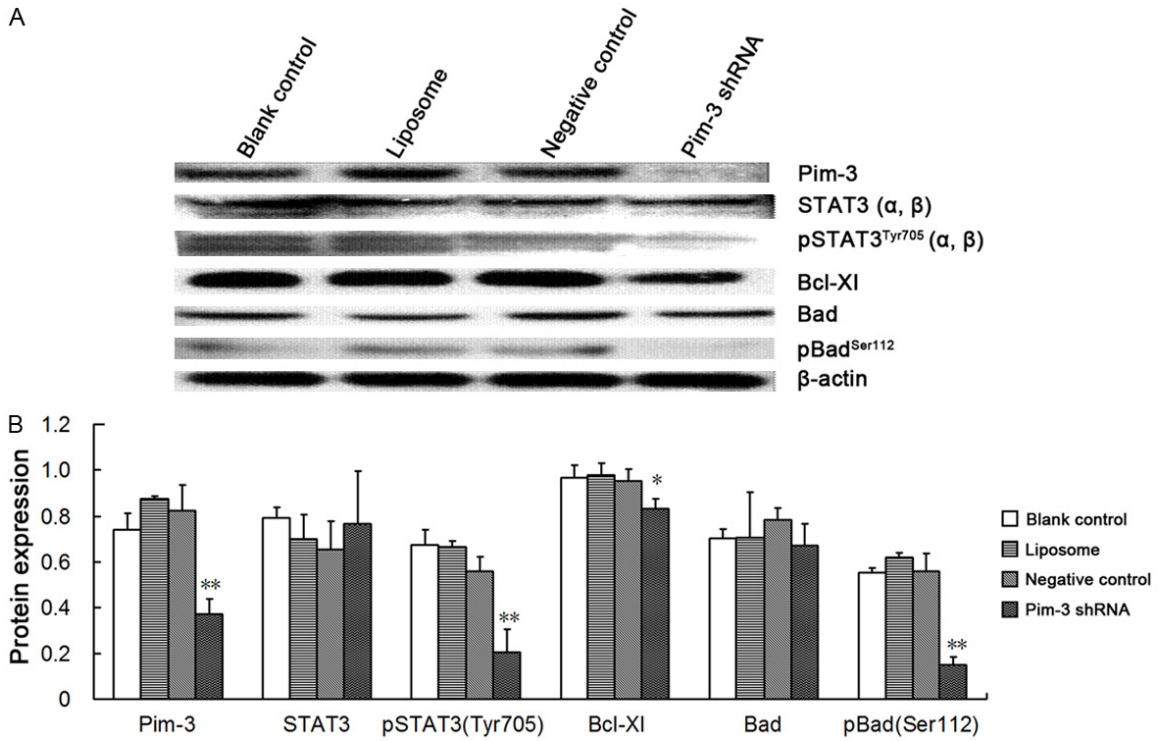

Figure 4. Related protein expression in different groups. A: Western blot belt. B: Protein expression was determined by Western blot and normalized to that of β-actin. Results showed the protein expression of Pim-3, pSTAT3^{Tyr705}, Bcl-XI and pBad^{Ser112} was comparable among blank control group, liposomes group and negative control group, but significantly higher than that in Pim-3 shRNA group. There was no significant difference in the protein expression of STAT3 or Bad among 4 groups. *P<0.05; **P<0.001.

performed to detect the protein expression. Results showed the protein expression of Pim-3, pSTAT3^{Tyr705}, Bcl-XI and pBad^{Ser112} was comparable among blank control group, liposomes group and negative control group, but significantly higher than that in Pim-3 shRNA group. In addition, there was no significant difference in the protein expression of STAT3 or Bad among 4 groups. The protein expression of Pim-3 was normalized to that of β-actin as the relative expression (**Figure 4**).

Discussion

Apoptosis is an active death process regulated by genes and characterized by the activation of apoptosis related signaling pathway and the expression apoptosis related genes. Bcl-2 family members play an important role in the apoptosis. Pro-apoptotic protein Bad (Bcl-XI/Bcl-2-Associated Death promoter) belongs to the Bcl-2 family, and contains 204 amino acids including 23 serines and 10 threonine. Bad is active in the non-phosphorylated state. Bad as a member of pro-apoptotic family neither forms homodimers nor forms heterodimer with Bax and Bak. Bad mainly replaces the Bax in the Bcl-2/Bax complex and Bcl-XI/Bax complex in a concentration dependent manner, which allows the pro-apoptotic Bax outside the mitochondrion forms homodimers. This leads to the increase in the mitochondrial permeability, release of cytochrome C and cascade activation of Caspases resulting in apoptosis [20]. After phosphorylation, Bad binds to 14-3-3 protein (as a chaperone) and stays in the cytoplasm, which leads to the imbalance between Bad and pro-survival Bcl-2 family members resulting in inhibition of apoptosis [21, 22].

To date, the serine 112, 136 and 155 of Bad protein have been confirmed as the phosphorylation sites [23-25]. In addition, the phosphorylation of Bad at serines 112 and 136 determines the pro-apoptotic property. Studies have revealed that Pim-2 and Pim-2 may inactivate Bad by phosphorylating it at serine 112 [26, 27]. In addition, Popivanova et al [19] found that Pim-3 could inactivate Bad by phosphorylating it at serine 112 (not Serine 136) and

block the interaction between Bcl-XI and Bad in pancreatic cancer and colonic cancer, which induced the apoptosis of cancer cells and promote the progression of cancers. Like in pancreas and colon, liver is also derived from entoderm, and Pim-3 gene plays important roles in the initiation and development of liver cancer, pancreatic cancer and colonic cancer. Thus, experiments showed Pim-3 in the liver cancer could phosphorylate Bad at serine 112 causing Bad inactivation in the absence of influence on total Bad protein, which promoted the occurrence and development of cancers.

Signal transducers and activators of transcription (STATs) are a group of cytoplasmic proteins and were first identified in 1992. STAT can bind to target genes, which are downstream substrates of tyrosine protein kinase JAKs. To date, a total of 7 members of STATs have been identified and include STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b and STAT6. Generally, members of STAT family contain 750-850 amino acids and seven domains: carboxy-terminal transcriptional activation domain, DNA binding domain, tyrosine phosphorylation domain, SH2 domain, connected domain, coiled-coil domain and amino-terminal domain [28]. Studies have indicated that phosphorylated STATs are active. The activated STATs may form heterodimers or homodimers via the SH2 domain, then translocate into nucleus, and bind to the DNA regulatory region of target genes to induce signal transduction [29]. As a member of STAT family, STAT3 plays important roles in the regulation of survival, proliferation, and differentiation of cells.

Currently, STAT3 is regarded as an oncogene and may affect the survival, proliferation, differentiation and apoptosis of cells via regulating Bcl-XI, CyclinDA and c-myc [30, 31]. There is evidence showing that Bcl-XI is highly expressed in human liver cancer and liver cancer cell lines, and plays an important role in the apoptosis of liver cancer cells [32, 33]. Zhang et al proposed that the STAT3 expression in human liver cancer was significantly higher than that in adjacent normal tissues, and the increased STAT3 protein expression was related to the pathological grade and clinical stage of liver cancer: the higher the STAT3 expression, the poorer the prognosis was [34], which might be attributed to the promotion of Bcl-XI expression induced by high STAT3 expression.

In the present study, results showed silencing of Pim-3 gene could reduce Pim-3 expression, which affected the $pSTAT3^{Tyr705}$ expression but had no influence on STAT3 expression. Change et al [35] found, in prostate cancer cell line (DU-45 cells) and pancreatic cancer cell line (MiaPaCa-2 cells), Pim-2 (not Pim-1 and Pim-2) could regulate the $pSTAT3^{Tyr705}$ expression, which might be attributed to the protein tyrosine phosphatase and tyrosine kinase. These findings were consistent with ours.

Cancer growth requires a large amount of oxygen and nutrients. With growth, cancers may degenerate if there is no formation of new blood vessels. Vascular endothelial growth factor (VEGF) may stimulate the proliferation, migration of endothelial cells, and induce the angiogenesis, playing important roles in the invasion and metastasis of malignant tumors. Pim-3 plays pivotal roles in the migration of endothelial cells and angiogenesis [36]. Previous studies showed the activated STAT3 could induce VEGF expression to promote carcinogenesis [37, 38]. In addition, some investigators found that Pim-3 could promote VEGF expression in gastric cancer and pancreatic cancer, facilitating the occurrence and development of both cancers [39, 40]. It has been confirmed that STAT3 can bind to the promoter of VEGF gene to promote the VEGF expression. These findings suggest that Pim-3 may be a positive regulatory factor of STAT3 and can regulate the expression of Bcl-XI and VEGF via increasing $pSTAT3^{Tyr705}$ expression, resulting in the promotive occurrence and development of cancers.

Our results showed, in liver cancer cells, Pim-3 could exert biological effects in following ways: 1) Pim-3 could inactivate Bad (a pro-apoptotic protein of Bcl-2 family) via phosphorylating it at serine 112 and block the interaction between Bcl-XI and Bad to induce apoptosis; 2) Pim-3 could serve as a positive regulatory factor of STAT3 to induce the STAT3 signaling pathway and regulate the expression of apoptosis related genes (Bcl-XI, CyclinD1 and c-myc) and VEGF –over-expression, affecting the proliferation, differentiation and apoptosis of cancer cells and facilitating the occurrence and development of cancers. Taken together, Pim-3 plays important roles in the occurrence and development of cancers. Current, Pim-3 is regarded as a novel target for the anti-tumor therapy.

Disclosure of conflict of interest

None.

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