

Original Research Article

The role of Pim-2 in apoptotic signal transduction pathway of hepatocellular carcinoma

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Received: 05 February 2017

Accepted: 02 March 2017

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ABSTRACT

Background: Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors. The objective was to investigate the role of serine/threonine kinase Pim-2 in apoptosis signal transduction pathway, because there is little study about its contribution to apoptosis in hepatocellular carcinoma.

Methods: The Pim-2 gene and protein expression were examined by qRT-PCR, Western blot and immunohistochemical stain in HCC tissues and normal liver tissues. The plasmid pCI-neo-Pim2 was transfected into human hepatoma cell line SMMC7721 by lipofectamine. Total RNAs were extracted from SMMC7721 cell in logarithm growth phase. The mRNA expression of Pim-2, Akt-1 (protein kinase B), 4E-BP1 (translation repressor of mammalian target of rapamycin), SOCS-1 (repressor of cytokine), Bad (Bcl-xL/Bcl-2 associated death promoter, Bim (Bcl-2 interacting mediator of cell death) and Puma (p53 upregulated modulator of apoptosis) were identified by qRT-PCR. The cell cycle of post-transfected SMMC7721 cells was assessed by flow cytometry.

Results: Pim-2 expression was enhanced in HCC. In post-transfected SMMC7721 cells, Pim-2 mRNA expression was up-regulated, level of Bad mRNA was attenuated, furthermore, the transcription level of Akt-1, SOCS-1, 4E-BP1, Bim and Puma gene wasn't variety. Up-regulated Pim-2 can't cause distinct change of cell cycle or apoptosis in hepatoma cell.

Conclusions: The serine/threonine kinase Pim-2 plays an important role in the development of HCC, Pim-2 dependent maintenance of cell size and survival correlated with its ability to maintain down-regulated expression of the BH3 protein Bad. Pim-2 is not a trigger in cell-autonomous survival or inhibiting apoptosis in hepatocellular carcinoma. Pim-2 is a redundancy pathway of survival signaling.

Keywords: Apoptosis, Hepatocellular carcinoma, Pim-2

INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most frequent malignant tumors.^{1,2} Its occurrence and development is closely related to cell apoptosis.³ The apoptosis of hepatocyte is regulated by several genes, transcription factors, cytokines and hormones. Recent research have found that the genes and kinase related to the regulation of HCC cell apoptosis include Bcl-2

family, survivin, p53, P21, Fas/FasL, TRAIL, Caspase-3, TGF- β , C-myc RASSF1A, STAT1, Clusterin, MAPK and PI3K/Akt, etc.⁴⁻⁸

Pim-2 is one of the members in Ser/Thr kinase Pim family. It belongs to calcium/calmodulin-regulated kinase (CAMP) and is highly conservative in cell evolutionary process.⁹ It can inhibit cell apoptosis and promote cell survival by phosphorylating substrates, thus it is proved

to play an important part in the tumorigenesis of B-cell lymphoma, sarcoma and prostate cancer.^{10,12} Pim-2 has also been proved to be relevant to the tumorigenesis of hepatocellular carcinoma (HCC), but up to now there is little study of the relationship between Pim-2 and apoptosis signal transduction pathway of hepatoma cell.^{13,14} Liver has special structure and function in the body and hepatocyte is rich in blood supply and cytokines. It offers a proper circumstance for Pim-2 to exert its biological effect. Therefore, it is of great importance to study Pim-2 to illuminate its role in inhibiting apoptosis, because it may offer a new target for the gene therapy of HCC.

METHODS

Human HCC tissue and normal liver tissue

Eleven HCC tissues were obtained from surgery sample in the second affiliated hospital of Chongqing Medical University and the affiliated hospital of Luzhou medical college from July 2005 to May 2006. Nine samples of

normal liver tissues were obtained from the abdominal surgery sample of non-liver disease volunteer patients in the affiliated hospital of Luzhou medical college in the same period. All diagnoses were confirmed with pathology. Each sample was cut into two small pieces sized 2.0cm×2.0cm×0.5cm. One dipped in 10% formalin solution for pathological section and the other one wrapped with tinfoil paper and then preserved in nitrogen canister for later use.

Pim-2 and apoptotic protein mRNA detection by qRT-PCR

For real-time PCR, total RNA was extracted from the cells using Trizol reagent. 1ug RNA was reverse transcribed into cDNA with PrimeScript RT Master Mix. All the qRT-PCR samples were performed using SYBR Green PCR Master Mix on CFX96 Real-Time PCR Detection System (Bio-Rad). The target gene expression levels for each experiment were detected and calculated using the $\Delta\Delta C_t$ comparative method. The RT-PCR primer sequences are showed in Table 1. They were both synthesized by Jikang Biotechnology Ltd. in Shanghai.

Table 1: Sequences of forward and reverse primers used for RT-qPCR study.

Gene	Gene Bank number	Forward	Reverse
Pim-2	BC018111	5'-ATGTTGACCAAGCCTCTACA-3'	5'-ACGATGGACA ACTCCACGGG-3'
Akt-1	NM_005163	5'-GGTCAAAGAA GTCAAAGGG-3'	5'-CGCCACAGAGAAGTTGTT-3'
SOCS-1	NM_003745	5'-TTCTGTAGGATGGTAGCAC-3'	5'-AAATAACACGGCATCCCAG-3'
4E-BP1	L36055	5'-TTCCTGATGGAGTGTCCGA-3'	5'-TGTCCATCTCAAACGTGTGAC-3'
Bad	NM_004322	5'-CAGAGTTTGAGCCGAGTGAG-3'	5'-GTCCACAAACTCGTCACTCA-3'
Bim	NM_000633	5'-CGGGAGATAGTGATGAAG-3'	5'-CAGTTCACAAAGGCATC-3'
Puma	AF354654	5'-CACCACCATCTCAGGAAA-3'	5'-GAATGCCAGTGGTCACAC-3'
GAPDH	BC004109	5'-AGAAGGCTGGGGCTCATTTG-3'	5'-AGGGGCCATCCACAGTCTTC-3'

The prepared HCC and normal liver tissue pieces were grinded with PBS, and the clear supernatant liquid was collected for protein extraction after the centrifugalization. The protein sample was diluted with 2×SDS loading buffer by 1:1 (v/v) and then loaded. Electrophoresis was carried out using Laemmli method. After the electrophoresis, the gel was transferred into the trans-blotting tanker and was electronically trans-blotted into a membrane in 100v for 30-60min.

The blotted membrane was reacted with the confining liquid diluted Pim-2 antibody (abcam: ab107102, 1: 500) in the room temperature for 30-60 min. Diluted horseradish peroxidase (HRP) IgG antibody (1: 2000) was added after washing the membrane and the reaction maintained 30-60 min. After washing the membrane again the radioautography was carried out for 30s-15min. Hybridization signal analysis came at last. Each group of cells were experimented twice and the average

experimental datum was taken for semiquantitative analysis.

Pim-2 protein detection by Immunohistochemistry stain(IHC)

The prepared HCC and normal liver tissue paraffin section (4μm thick) were both blocked by cony serum confining liquid and then Pim-2 antibody (abcam: ab107102, 1: 100) was added. The reaction was carried out at 4°C overnight. After washing the section with PBS for 2 min×3 times, HRP IgG antibody was added. The reaction was maintained at 37°C for 20 min and followed by a washing as above. Then the SABC agent was added and the reaction took 20 min at 37°C, also followed by a PBS washing for 5 min×4 times. DAB color reagent was used for coloration. The section was counterstained with hematoxylin followed by dehydration, clearing and mounting. Microscope observation and semi-quantitative analysis came at last.

Plasmid and strain

The plasmid pCMV-SPORT6 which contains total length of human Pim-2 sequence was purchased from Sanying Biotechnology Ltd. in Wuhan. The eukaryotic cell expression vector pCI-neo and the *E. coli* Top 10 were preserved in the genetically engineered drug laboratory of Di'ao Groups in Chengdu.

Amplification of the plasmid pCMV-SPORT6

The competent *E. coli* Top10 was prepared following the method mentioned in the reference.¹⁵ The vector pCMV-SPORT6 was amplified and small amounts of its DNA were prepared, one sample of which was sent to Jikang Biotechnology Ltd. in Shanghai for gene sequencing.

Construction of the eukaryotic cell expression vector pCI-neo-Pim-2

According to the Pim-2 gene sequence in Geng Bank (BC018111, CDs: 174.0-1109), the destination fragment is a 936bp Pim-2 cDNA total length sequence containing start codon ATG in the 5'-end. The Restriction enzyme *EcoR* I and *Sal* I sites were added into the upstream and downstream 5'-end. The primers were synthesized by Jikang Biotechnology Ltd. in Shanghai. Upstream primer: 5'-AAA GAA TTC ATG TTG ACC AAG CCT CTA CA -3'(*EcoR* I); downstream primer: 5'-AAA GTC GAC TTA GGG TAG CAA GGA CCA GG-3'(*Sal* I). The destination fragment was amplified by PCR, the vector pCMV-SPORT6 as the template. The PCR product and the eukaryotic cell expression vector pCI-neo were respectively reacted with the restriction enzyme *EcoR* I and *Sal* I and then mixed by 5:1 in volume after purified. Ligase ligation I was added as well. The reaction was carried out in 12μl reaction system at 16°C for 30min followed by transformation and culture. The vector was reacted with restriction enzyme *EcoR* I and *Sal* I and the positive vectors containing total length of Pim-2 cDNA sequence, namely pCI-neo-Pim2, were picked out. One of positive clones was sent to Ding'an Biotechnology Ltd. in Shanghai for gene sequencing.

Cell culture

Human HCC cells SMMC7721 were offered by the tumor research institution of West China Hospital of Sichuan University. The cells were cultured in RPMI-1640 complete medium (9ml RPMI-1640+1ml calf serum+100ul 1% penicillin/streptomycin) and was placed in the 5%CO₂ 37°C hatching box. Serial subcultivation was carried out after digesting the cells with 0.25% trypsin.

Liposome transfection

The plasmid pCI-neo-Pim2 and the Lipofectamine mixture were added into the SMMC7721 cells and then the cells were cultured in 5% CO₂ 37°C condition for 4h.

The medium containing plasmid pCI-neo-Pim2 and the Lipofectamine mixture was removed and the fresh RPMI-1640 complete medium was added for continuing culturing, also in 5% CO₂ 37°C condition.

Flow cytometry

Cell cycle and apoptosis analyses were performed using Annexin V-FITC and PI staining (BD Biosciences, San Jose, CA). For cell cycle analysis, the cells were seeded in 6-well plates at 2×10⁵ cells per well. Forty-eight hours after transfection, the cells were fixed in 70% ethanol at 4°C for 24 h and stained with 50 μg/mL propidium iodide (PI) (BD Biosciences, San Diego, CA). The cell cycle distribution was analysed by flow cytometry (Epics Altra, Beckman Coulter, USA).

RESULTS

Altered expression of Pim-2 in HCC tissues

We first evaluated the expression of Pim-2 in normal human liver and HCC samples. The result of the qRT-PCR showed that the rate of Pim-2 gene expression level in HCC tissue and normal liver tissue was 1.43:1 (Figure 1A). The protein level of Pim-2 was measured by Western Blot and IHC. The Western Blot analysis showed that the expression level of Pim-2 protein in HCC tissue was 7.98 times than that in normal liver tissues (Figure 1B, Figure 1C). The IHC results indicated The Pim-2 protein was expressed mainly in cytoplasm. The expression of Pim-2 was weak in normal liver tissues, and very strong in HCC tissues (Figure 1D). All the results showed that the Pim-2 expression level in HCC tissues was obviously higher than that in normal liver tissue.

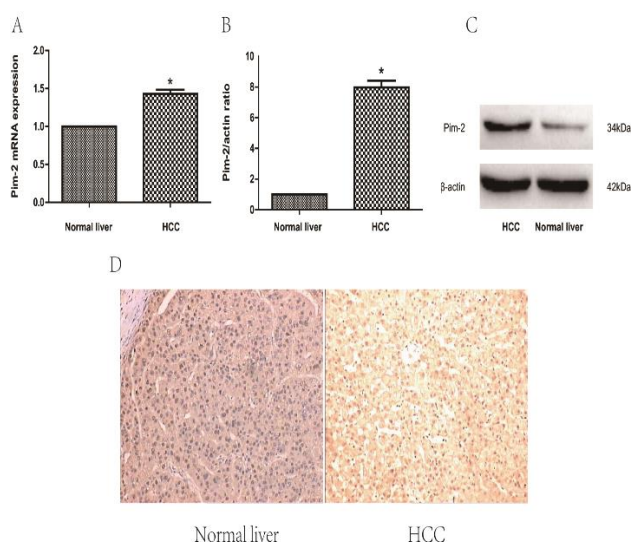


Figure 1: Pim-2 expression level in HCC and normal liver tissue. (A) qRT-PCR confirmed different Pim-2 mRNA expression. (B.C) Semi-quantitative analysis of Pim-2 protein expression using WB. (D). IHC analysis for Pim-2 expression. *P<0.05.

The variation of the apoptotic protein expression level before and after the transfection

Pim-2 was expressed in the SMMC7721 cell and its expression level was obviously enhanced after the plasmid pCI-neo-Pim2 transfection ($P < 0.01$) (Figure 2-A). There was no obviously change of mRNA expression of Akt-1, SOCS-1, 4E-BP1, Bim and Puma after the plasmid pCI-neo-Pim2 transfection (Figure 2-B). The mRNA expression of Bad was reduced after the transfection. The difference is significant ($P < 0.01$) (Figure 2-E).

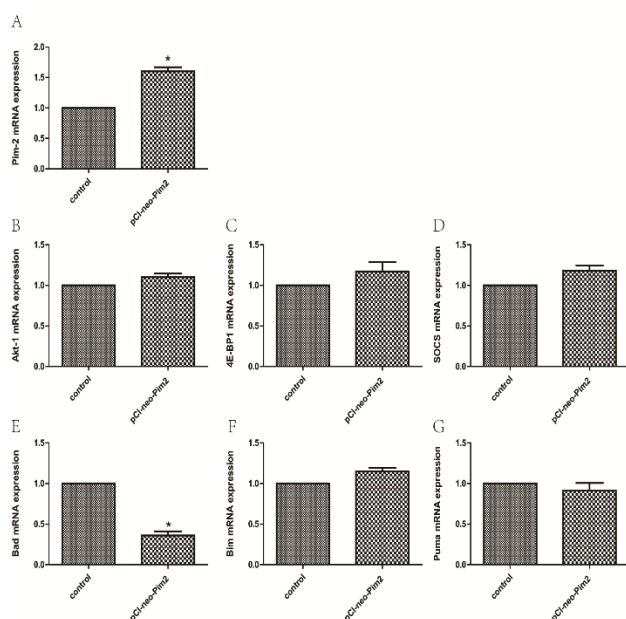


Figure 2: The mRNA expression of Bad was reduced after the pCI-neo-Pim2 transfection. (A) qRT-PCR confirmed different Pim-2 mRNA expression before and after pCI-neo-Pim2 transfection. (B.C.D.E.F.G) Semi-quantitative analysis of Akt-1, 4E-BP1, SOCS-1, Bad, Bim and Puma mRNA expression using qRT-PCR.
* $P < 0.05$

The variation of the cell cycle and the apoptotic rate before and after the transfection

There was no obvious variation in size and shape of the SMMC7721 cells after the transfection (Figure 3-A), and most of the cells stay in the G1 period both before and after the transfection. There were 58.61% of the SMMC7721 cells in the G1 period and 15.88% in the G2 period before the transfection, and the rate of G1/G2 was 2.14. While 63.04% of the cells are in G1 period and 13.65% are in G2 period after the transfection, and the rate of G1/G2 is 2.06 (Figure 3-B). The variation of the cell cycle and apoptotic rate after the transfection is of no significance ($P > 0.05$). That means the transfection of the plasmid pCI-neo-Pim2 has no obvious impact on the cell cycle and apoptosis of the SMMC7721 cells.

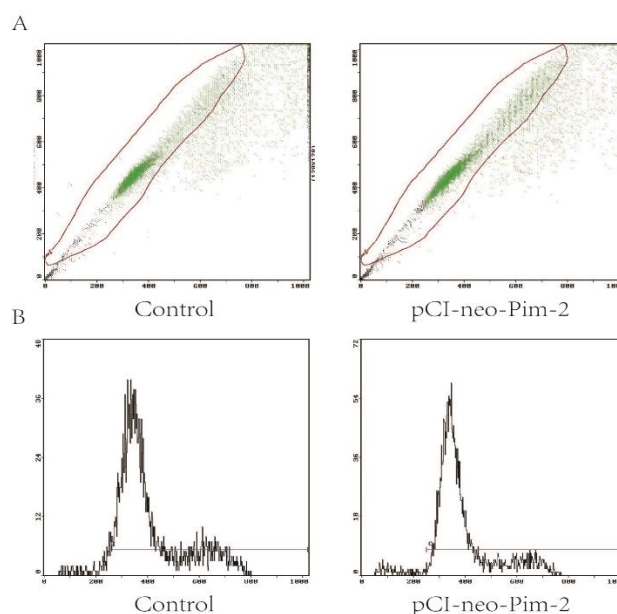


Figure 3: The variation of the cell cycle and the apoptotic rate with the pCI-neo-Pim2 transfection or not. (A) The size and shape of the SMMC7721 cells with the pCI-neo-Pim2 transfection or not. (B) The cell cycle of SMMC7721 cells with the pCI-neo-Pim2 transfection or not determined by FCM.

DISCUSSION

Pim-2 is a kind of Ser/Thr protein kinase. It can promote cell survival through phosphorylating Ser/Thr residue when stimulated by stimulus signals. At present, Pim-2 and Akt are regarded to be parallel and redundant apoptotic inhibiting pathway.^[16] It has been proved that Pim-2 has the feature of mitogen and can promote the malignant transformation of hematopoietic cells and liver cell line. And Pim-2 can cooperate with C-myc in the tumorigenesis of lymphoma.^{17,19} In order to investigate the role of Pim-2 in apoptosis signal transduction pathway of hepatocellular carcinoma (HCC). We detect the normal liver tissue and the HCC tissue in the level of gene and protein by qRT-PCR, Western-Blot and IHC. The result shows that Pim-2 expressed in both tissues but much more obvious in HCC tissue than in normal liver tissue.

After the eukaryotic expression vector pCI-neo-Pim2 was transfected into the SMMC7721 cells, we detected the variation of several apoptosis related proteins. The fact that Pim-2 expression level was obviously enhanced ($P < 0.01$) demonstrated that Pim-2 was indeed expressed in SMMC7721 cells. Other apoptosis related proteins such as Akt-1, SOCS-1, 4E-BP1, Bad, Bim and Puma were also found in the transfected MMC7721 cells, but only the decrease of Bad expression level was of significance according to the statistical analysis.

It implies that the increasingly expressed Pim-2 in SMMC7721 cells mainly inhibit the expression of Bad thus inhibit its role in promoting apoptosis. And Bad may be one of the downstream substrates of Pim-2 pathway. What is interesting is that the expression of Akt-1 is also found increased to some degree. It suggests that Pim-2 may not be the only gene in maintaining the growth and proliferation of SMMC7721 cells, and Pim-2 and Akt-1 may be redundant backup in function. We also find that the over-expressed Pim-2 has no obvious impact on the cell cycle of SMMC7721 cells according to the result of FCM. It suggests that Pim-2 may be an important but not the only gene in maintaining the growth and proliferation of SMMC7721 cells, and Pim-2 may be one of the redundant pathways in inhibiting the apoptosis of HCC.

In brief, through the research about the expression of Pim-2 in HCC and the relationship between Pim-2 and other apoptosis related proteins in the signal transduction pathway, we identify that Pim-2 gene and protein are expressed in both normal liver tissue and HCC tissue. Pim-2 plays an important role in the development of HCC by phosphorylating Bad or API-5 and inhibiting the apoptosis of the tumor cells.¹⁴ Pim-2 is not the only gene in inhibiting the apoptosis of tumor cells but one of the redundant pathways in cell signal transduction. What's more, inhibiting the expression of Pim-2 may be a new method in the gene therapy of HCC.

ACKNOWLEDGEMENT

This study is supported by the China National Natural Scientific Foundation (No. 30400424).

Funding: No funding sources

Conflict of interest: None declared

Ethical approval: The study was approved by the institutional ethics committee

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Cite this article as: Chen J, Yang X. The role of Pim-2 in apoptotic signal transduction pathway of hepatocellular carcinoma. *Int Surg J* 2017;4:1380-4.