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Pim-2 Activates API-5 to Inhibit the Apoptosis of Hepatocellular Carcinoma Cells Through NF-κB Pathway

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Abstract Pim-2 is proved to be relevant to the tumorigenesis of hepatocellular carcinoma (HCC), but the mechanism is unclear. We studied the relationship among Pim-2, NF-κB and API-5. In our experiment, expression level of the three factors and phosphorylation level of API-5, as well as NF-κB activity, were detected in HCC tissues and the nontumorous controls. Then Pim-2 gene was transfected into nontumorous liver cells L02, and Pim-2 SiRNA was transfected into hepatoblastoma cell line HepG2. Parthenolide was added as NF-κB inhibitor. The same detections as above were repeated in the cells, along with the apoptosis analysis. We found the levels of Pim-2, NF-κB and API-5, as well as NF-κB activity, were significantly higher in HCC tissues. Pim-2 level was increased in L02 cells after the transfection of Pim-2 gene, but decreased in HepG2 cells after the transfection of Pim-2 SiRNA. The levels of NF-κB and API-5, as well as NF-κB activity and API-5 phosphorylation level, were in accordance with Pim-2 level, but could be reversed by Parthenolide. Cell apoptosis rates were negatively correlated with API-5 phosphorylation level. Therefore, we infer that Pim-2 could activate API-5 to inhibit the apoptosis of liver cells, and NF-κB is the key regulator.

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Keywords Pim-2 · NF-κB · API-5 · Hepatocellular carcinoma cells · Apoptosis

Abbreviations

Pim	Proviral integration of Monoley virus
HCC	hepatocellular carcinoma
NF-κB	nuclear factor kappa B
Bcl-2	B-cell CLL/lymphoma 2
Bad	Bcl2-antagonist of cell death
API-5	apoptosis inhibitor 5
PNL	paired noncancerous liver tissues
NL	normal liver tissues
DEPC	diethyl pyrocarbonate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electropheresis
EMSA	Electrophoretic mobility shift assay
eI4B	Eukaryotic initiation factor 4B
Myc	myelocytomatosis

Introduction

The disorder of cell proliferation and apoptosis induced by the activation of oncogene and the inactivation of antioncogene are two basic molecular biological events of tumorigenesis. Oncogene Pim-2 (Proviral integration of Monoley virus) was firstly discovered in lymphoma by Breuer in 1989 [1], its protein production is a kind of serine/threonine kinase. Pim-2 protein has powerful and extensive anti-apoptotic effect, and has been proved to play an important role in the tumorigenesis of many kinds of tumors such as hematopoietic system tumors and prostate cancer [2–4]. Our previous research has found that the expression level of Pim-2 was significantly higher in hepatocellular carcinoma (HCC) tissues and liver cancer cell line than that in the nontumorous

controls [5,6], and transfection of Pim-2 gene in vitro could induce malignant transformation of nontumorous human liver cell line (under publication). It manifests that there may be some relationship between the anti-apoptotic effect of Pim-2 and the tumorigenesis of HCC. But so far, there is no research reporting the detailed Pim-2 anti-apoptotic signal transduction pathway in HCC.

Researches in lymphoma found that the anti-apoptotic effect of Pim-2 could be totally blocked by NF-κB repressor [7], and a kind of chemotherapeutics called proteasomes inhibitor which targets at NF-κB pathway has been successfully used in tumors which over express Pim-2 [8]. These findings demonstrate that NF-κB may be a key factor in Pim-2 anti-apoptotic pathway. The proapoptotic protein Bad which belongs to Bcl-2 family is the only proved Pim-2 substrate up to now [9]. Pim-2 could phosphorylate different sites of Bad thus exert different effects in the anti-apoptosis mechanism [10]. Lots of previous researches have proved that Bad is just a synergetic factor but not key factor in the tumorigenesis of HCC. Recent research reports that there is a length of conserved sequence between Bad and apoptosis inhibitor 5 (API-5). This conserved sequence is just before a phosphorylating site and has been proved to be the phosphorylating recognition marker of Pim-2 [11]. What's more, API-5 is reported to take part in the tumorigenesis of HCC [12]. Therefore, API-5 is regarded as a possible substrate of Pim-2 in hepatocellular carcinoma cells.

Based on the results above, we infer that API-5 may be the downstream factor of Pim-2 anti-apoptotic pathway in hepatocellular carcinoma cells, and NF-κB may be the key modulator of this pathway. This research aims at illuminating the relationship among the three factors and elucidating the detailed mechanism of Pim-2 anti-apoptotic effect in hepatocellular carcinoma cells.

Materials and Methods

Obtaining of Clinical Specimen

Three groups were set for clinical specimen experiment: A. hepatocellular carcinoma tissues (HCC); B. paired noncancerous liver tissues (PNL); C. normal liver tissues (NL). The obtaining of the clinical specimen was based on the agreement of the patients or their direct relatives. From Jan. 2006 to Dec. 2007, 27 HCC tissues and 27 corresponding PNL tissues were obtained from the surgery operation in the second affiliated hospital of Chongqing medical university (HCC were diagnosed by pathology. PNL were defined as liver tissues 2 cm away from the edge of the carcinoma. All the patients had viral hepatitis type B and liver cirrhosis). There were 18 males and 9 females aged from 41 to 69 years old, with an average age of 57.2 years old. There were 21 cases with single HCC nodule, five cases with two nodules and one case with three nodules. The diameters of the nodules ranged from 3 cm to 15 cm with an averge of 6.5 cm. 11 NL tissues were obtained from surgery operation in the same period in our hospital (eight liver traumatic rupture cases and three hepatic cyst cases. All the NL tissues were verified by pathology). There were nine males and two females aged from 39 to 57 years old with an average age of 46.8 years old. Each tissue was nearly $0.5\times0.5\times0.5$ cm^3 in volume and equally divided into four parts. One part was preserved in formalin for pathological examination, and the others were preserved in liquid nitrogen for mRNA and protein detection. All the procedures were approved by the Ethical Committee of Human Experimentation in our country, and are in accordance with the Helsinki Declaration of 1975.

Cell Culture, Transfection and RNAi

Eight groups were set for cell experiment: a. nontumorous human liver cell line L02 cells (L02); b. L02 cells transfected with Pim-2 gene (L02/Pim-2); c. L02 cells transfected with empty vector (L02/vector); d. human hepatoblastoma cell line HepG2 cells (HepG2); e. HepG2 cells transfected with Pim-2 SiRNA (HepG2/Pim-2 SiRNA); f. HepG2 cells transfected with scrambled SiRNA (HepG2/scrambled SiRNA); g. L02/Pim-2 cells cultured with parthenolide (a kind of NF-κB specific repressor [13]); h. HepG2 cells cultured with parthenolide.

L02 cells and HepG2 cells were purchased from the Liver Disease Research Institute in Chongqing (China) and cultured in RPMI-1640 complete medium (9 ml RPMI-1640+1 ml calf serum+100 μl 1% penicillin /streptomycin) with 5%CO_2 at 37°C (L02 cell line was isolated from normal human fetal liver tissue. It obtains immortalization ability in vitro but has no malignant biological behaviours. It is commonly used as nontumorous liver cell [14]. HepG2 cell line is well known and widely used as tumorous liver cell). The usage and dosage of parthenolide in cell culture were referred to the reference [15].

L02/Pim-2 cells and the corresponding control L02/vector cells were prepared as previous study [5]. PCI-Pim-2 vector and Lipofectamine TM reagent (Invitrogen) were used in the transfection. The transfection efficiency was verified by PCR and Western blotting (see below).

HepG2/Pim-2 SiRNA cells and the corresponding control HepG2/scrambled SiRNA cells were also prepared as previous study [5]. The RNA interference eukaryotic expression vector pGenesil-1 which is specific to Pim-2 gene was constructed. The Pim-2 SiRNA sequence was designed in a pattern as *Bam HI*—sense DNA—loop (TTCAAGACG)—antisense DNA—stop cod—*Hind III*. The sense DNA was designed as: 5'-CTTGTAGGACTATCTGGAT-3'. The

scrambled SiRNA was randomly designed. The SiRNA was synthesized and sequenced by Shanghai Shenggong Com. Ltd. Lipofectamine TM reagent (Invitrogen) was used in the transfection of SiRNA. The interference efficiency was verified by PCR and Western blotting (see below).

Real Time PCR

Specific primers for target genes were designed using Primer 5.0: hPim-2 sense: 5'-CCAAGTAGTGGCAGCCATCC-3', antisense: 5'-GGGCACCAGAACCAAAATC A-3'; hNF-κB p65 sense: 5'-GGGAATGGTGAGGTCACTCTAA-3', antisense: 5'-AATGAAGGTGGATGATTGCTAAG-3'; hAPI-5 sense: 5'-AGTTGGTCAGCTTG GCTATGGAGT-3', antisense: 5'-TTTGGGCCACTGGCTGATCTATCT-3'; β-actin sense: 5'-T GACGTGGACATCCGCAAAG-3', antisense: 5'-CTGGAAGGTGGACA GCGAGG- 3'.

The tissues were ground and homogenated, and the culturing cells were collected and lysed. Total RNA were extracted using Trizol kit. The reverse transcription reaction system was set as: buffer 10 μl, primer(100 pmol/ul) 1 μl, reverse transcription mixture 1 μl, template RNA 5 μl, DEPC water 13 μl. The reaction were carried out at 25°C for 10 min, then 40°C for 60 min, and finally 70°C for 10 min. fluorescent quantitation PCR reaction system were set as: buffer 25 μl, primer (25 pmol/μl) 0.6 μl, Sybr green I 0.3 μl, cDNA template 1 μl, DEPC water 22.5 μl. The reaction was carried out at 94°C for 4 min, followed by a course of 35 cycles with 94°C for 20 s, 60°C for 25 s and 72°C for 30 s in each cycle. After the last cycle, a final extension step was added at 72°C for 5 min and the fluorescent signals were detected.

Western Blotting

Total protein was extracted from the tissue homogenates and the cell lysates using M-PER Mammalian Protein Extraction Reagent (Pierce). The protein concentration was determined by the Bradford method (Bio-Rad). 10μl total protein in 1×loading buffer of each group was loaded and separated on 10% sodium dodecyl sulfate polyacrylamide gel electropheresis (SDS-PAGE). The protein was transferred to PVDF membrane at 400 mA for 1 h in transfer buffer (25 mM Tris, 0.2M glycine, 20% methanol). Membrane was blocked in Tris-buffered saline -Tween 0.1% (TBST) with 7% skimmed milk powder for 1 h at room temperature. All the primary antibodies were purchased from Santa Cruz Biotechnology and diluted according to protocol (Pim-2 1:800, tAPI-5 1:1000, pAPI-5 1:1000, β-actin 1:400). The proper species and diluted HRP-labeled second antibodies were added. Western blotting results were detected by the SuperSignal West Pico Chemiluminescent Substrate (Pierce) with a 30 s exposure time, the films were developed in developer and the results were scanned by Epson scanner.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA system kit (Viagene) was used for NF-κB p65/p50 activity detection. Neucleoprotein was extracted from the tissue homogenates and the cell lysates. 2 μl nuclear protein extract and 0.5 μl NF-κB probe were added into each combining reaction system. Polyacrylamide gel electrophoresis was carried out at 180 V for 70 min and electrotransfer was carried out in 0.5×TBE at 390 mA for 40 min. The combining membrane was laid 10 cm away from the uviol lamp for cross link for 5 min and then blocked. 15 ml Streptavidin-HRP (1:750) was added and the system was incubated at room temperature for 20 min. Finally, the substrate was added for chemiluminescence reaction. The film was analysed by Labworks4.6.

Cell Apoptosis Assay

Cells at logarithmic growth phase were collected, and the concentration was modulated to 10^6/ml. After centrifugalization at 1000 rpm for 5 min, 100 μl 1×Binding buffer was added for resuspension. 5 μl AnnexinV-FITC and 10 μl PI were added into each reaction system for staining. After laid for 20 min away from light at room temperature, 400 μl 1× Binding buffer was added, and the apoptosis rate was assayed by flow cytometry. The results were analyzed by FACScan.

Statistical Analysis

Measurement data were presented as mean±standard deviation. All the experimental data were statistically analyzed by SPSS13.0. One-Way ANOVA was used in compare of means among multiple groups. S-N-K method was taken for paired comparison. $P<0.05$ was considered as statistically significant.

Results

The mRNA Levels of the Target Factors in the Tissues

The $2^{(deta\text{-}Ct)}$ value of the ratio between target factor and β-actin was regarded as the corresponding mRNA level. The Pim-2 mRNA levels in the three groups were (Fig. 1-A): NL 0.029125±0.002911, PNL 0.048063±0.004113, HCC 0.129676±0.007427 (the difference among the three groups were significant, F-275.879, $p<0.001$). The NF-κB p65 mRNA levels in the three groups were (Fig. 1-B): NL 0.029771±0.002957, PNL 0.062129±0.00486, HCC 0.152134±0.008024 (the difference among the three groups were significant, F=323.562, $p<0.001$). The API-5 mRNA levels in the three groups were (Fig. 1-C): NL 0.0015± 0.00028, PNL 0.005069±0.000764, HCC 0.017701±

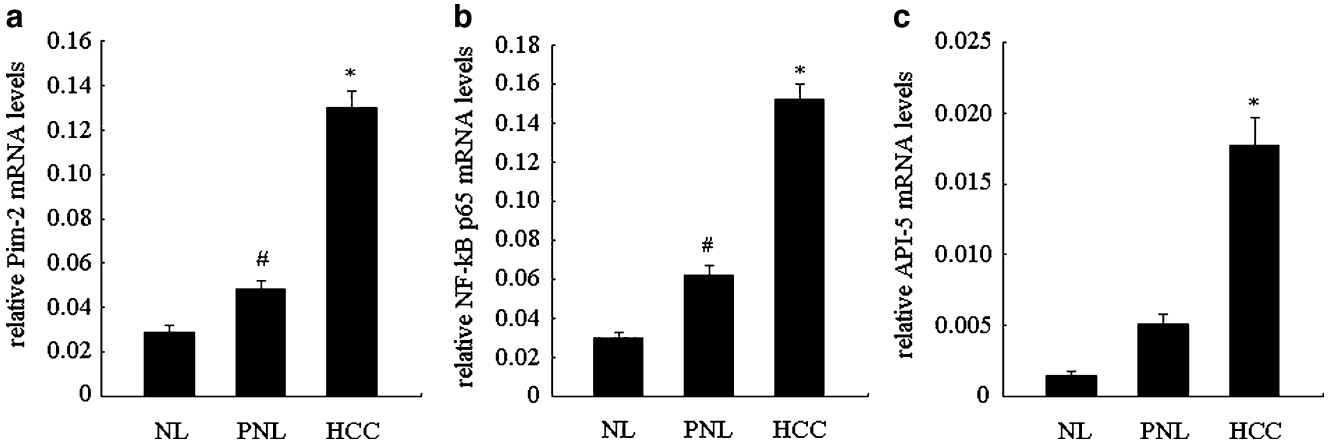

Fig. 1 The mRNA levels of target factors in tissue groups. The mRNA levels of Pim-2 (**A**), NF-κB p65 (**B**) and API-5 (**C**) are all significantly higher in HCC group than those in PNL and NL groups (*p<0.05). The mRNA levels of Pim-2 (**A**) and NF-κB p65 (**B**) are both significantly higher in PNL group than those in NL group (#p< 0.05), but there is no statistical difference in mRNA level of API-5 between PNL and NL group

0.002024 (the difference among the three groups were significant, F=64.550, p<0.001). The mRNA levels of the three factors were all significantly higher in HCC group (Pim-2: 100%, 27/27; NF-κB p65: 100%, 27/27; API-5: 92.59%, 25/27) than those in the other two groups (*p<0.05).

The Protein Levels of the Target Factors in the Tissues

The band gray value ratio between target factor and β-actin was regarded as the corresponding protein level (Fig. 2-A).

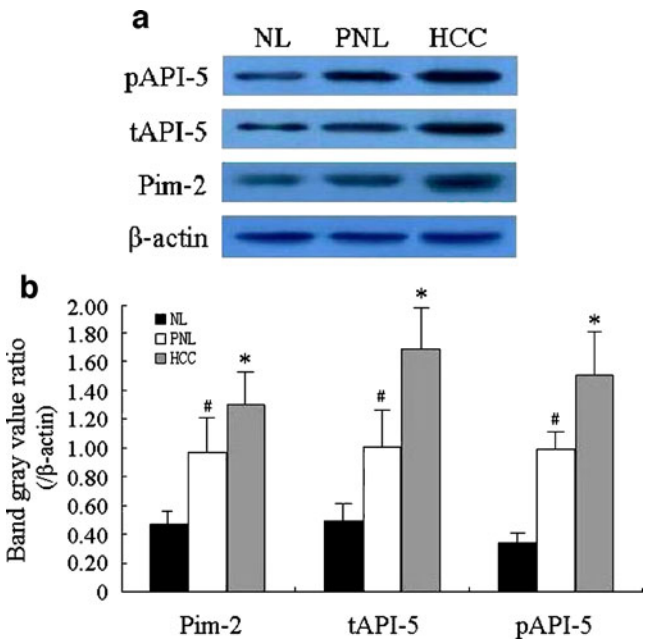

Fig. 2 The protein levels of target factors in tissue groups. The protein levels of Pim-2, tAPI-5 and pAPI-5 are all significantly higher in HCC group than those in PNL and NL groups (*p<0.05), and there is also statistical difference in the protein levels of the three factors between PNL group and NL group (#p<0.05)

The Pim-2 protein levels in the three groups were: NL 0.47000±0.10060, PNL 0.97000±0.22312, HCC 1.30000± 0.23289 (the difference among the three groups were significant, F=60.893, p<0.001). The total API-5 (tAPI-5) protein levels in the three groups were: NL 0.49000± 0.12256, PNL 1.0063±0.23321, HCC 1.68000±0.29844 (the difference among the three groups were significant, F=102.098, p<0.001). The phosphorylated API-5 (pAPI-5) protein levels in the three groups were: NL 0.34270± 0.07129, PNL 0.99000±0.17508, HCC 1.51±0.29983 (the difference among the three groups were significant, F=108.738, p<0.001).The protein levels of the three factors were all significantly higher in HCC group (Pim-2: 100%, 27/27; tAPI-5: 85.19%, 23/27; pAPI-5: 100%, 27/27) than those in the other two groups (Fig. 2-B, *p<0.05).

NF-κB Activity in the Tissues

The band gray value of NF-κB p65/p50 protein was regarded as the corresponding NF-κB activity (Fig. 3-A). The NF-κB activity in the three groups were: NL 49.50±2.68, PNL 185.57±9.42, HCC 216.73±12.36 (the difference among the three groups were significant, F=1089.581, p<0.001). NF-κB activity was significantly higher in HCC group (100%, 27/27) than those in the other two groups (Fig. 3-B, *p<0.001).

The mRNA Levels of the Target Factors in the Cell Groups

The $2^{(\text{deta-Ct})}$ value of the ratio between target factor and β-actin was regarded as the corresponding mRNA level. The experiment was repeated three times for each group and the mean was taken. The expression levels of Pim-2 mRNA were significantly different among the cell groups (Fig. 4-A: F=224.033, p<0.001). In L02 cell line,

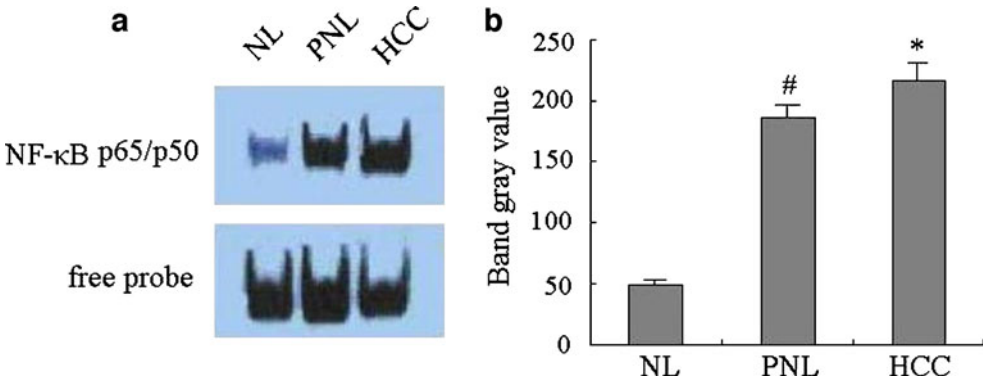

Fig. 3 NF-κB p65/p50 levels in neucleoprotein in the tissue groups. The NF-κB p65/p50 level is significantly higher in HCC group than that in PNL and NL groups (*$p<0.05$), and there is also statistical difference in the NF-κB p65/p50 level between PNL group and NL group (#$p<0.05$)

compared with the control groups (a. L02: 0.031788±0.0035; c. L02/Vector: 0.046571±0.0031), Pim-2 mRNA level in the transfection group (b. L02/Pim-2: 0.207296±0.0128) was significantly higher (Fig. 4-A: *$p<0.05$ vs. a and c). It manifest that the transfection of Pim-2 gene was successful. In HepG2 cell line, compared with the control groups (d. HepG2: 0.161235±0.0039; f. HepG2/scrambled SiRNA: 0.247014±0.0176), Pim-2 mRNA level in the RNAi group (e. HepG2/Pim-2 SiRNA: 0.083270±0.0045) was significantly lower (Fig. 4-A: #$p<0.05$ vs. d and f). It manifest that the SiRNA we constructed can efficiently inhibit the Pim-2 expression. The expression levels of NF-κB p65 mRNA were significantly different among the groups (Fig. 4-B: $F=1793.018$, $p<0.001$). The tendency of NF-κB p65 mRNA levels were parallel to those of Pim-2 from group a to group f (Fig. 4-B: *$p<0.05$ vs. a and c; #$p<0.05$ vs. d and f). When parthenolide were added into L02/Pim-2 and HepG2 groups (g. L02/Pim-2+parthenolide: 0.048863±0.0013; h. HepG2+parthenolide: 0.045198±0.00320), the NF-κB p65 mRNA levels significantly decreased (Fig. 4-B: ※$p<0.05$ vs. b and d). It verified the inhibitory effect of parthenolide to NF-κB. The expression levels of API-5 mRNA were significantly different among the groups (Fig. 4-C: $F=113.937$, $p<0.001$). The tendency of API-5 mRNA levels were totally parallel to those of NF-κB p65 in all the groups (Fig. 4-C: *$p<0.05$ vs. a and c; #$p<0.05$ vs. d and f; ※$p<0.05$ vs. b and d).

The Protein Levels of the Target Factors in the Cell Groups

The band gray value ratio between target factor and β-actin was regarded as the corresponding protein level (Fig. 5-A). The experiment was repeated three times for each group and the mean was taken. The expression levels of Pim-2 protein were significantly different among the groups (Fig. 5-B: $F=94.630$, $p<0.001$). In L02 cell line, compared with the control groups (a. L02: 1.05±0.07; c. L02/Vector: 0.93±0.01), Pim-2 protein level in the transfection group (b. L02/Pim-2: 2.07±0.02) was significantly higher (Fig. 5-B: #$p<0.05$ vs. a and c). It manifest that Pim-2 protein could be stably expressed in the L02/Pim-2 transfected cells. In HepG2 cell line, compared with the control groups (d. HepG2: 1.74±0.11; f. HepG2/scrambled SiRNA: 2.15±0.14), Pim-2 protein level in the RNAi group (e. HepG2/Pim-2 SiRNA: 1.47±0.07) was significantly lower (Fig. 5-B: *$p<0.05$ vs. d and f). It manifest that the Pim-2 SiRNA we constructed can efficiently inhibit the Pim-2 expression. The expression levels of tAPI-5 proteins were significantly different among the groups (Fig. 5-C: $F=200.162$, $p<0.001$). The tendency of tAPI-5 protein levels were parallel to those of Pim-2 from group a to group f (Fig. 5-C: *$p<0.05$ vs. a and c ; #$p<0.05$ vs. d and f). When parthenolide were added into L02/Pim-2 and HepG2 groups (g. L02/Pim-2+parthenolide: 0.10±0.01; h. HepG2+parthenolide: 0.13±0.02), the tAPI-5 protein levels significantly decreased (Fig. 5-C: ※$p<0.05$ vs. b and d). It manifest that the relationship between Pim-2 and API-5 could be broken by NF-κB repressor. The expression levels of pAPI-5 proteins were significantly different among the groups (Fig. 5-D: $F=53.940$, $p<0.001$). The tendency of pAPI-5 protein levels were totally parallel to those of tAPI-5 in all the groups (Fig. 5-D: *$p<0.05$ vs. a and c ; #$p<0.05$ vs. d and f; ※$p<0.05$ vs. b and d).

NF-κB Activity in the Cell Groups

The band gray value of NF-κB p65/p50 protein was regarded as corresponding NF-κB activity (Fig. 6-A). The experiment was repeated three times for each group and the mean was taken. The expression levels of NF-κB p65/50 were significantly different among the groups (Fig. 6-B: $F=620.425$, $p<0.001$). In L02 cell line, compared with the control groups (a. L02: 58.21±3.52; c. L02/Vector: 49.56±6.76), NF-κB p65/50 level in the transfection group (b. L02/Pim-2: 164±5.44) was significantly higher (*$p<0.05$ vs. a and c). In HepG2 cell line, compared with the control groups (d. HepG2: 169.30±2.02; f. HepG2/scrambled SiRNA: 208.11±4.06), NF-κB p65/50 level in the RNAi group (e. HepG2/Pim-2 SiRNA: 98.82±3.37) was significantly lower (#$p<0.05$ vs. d and f). When parthenolide were added into L02/Pim-2 and HepG2 groups (g. L02/

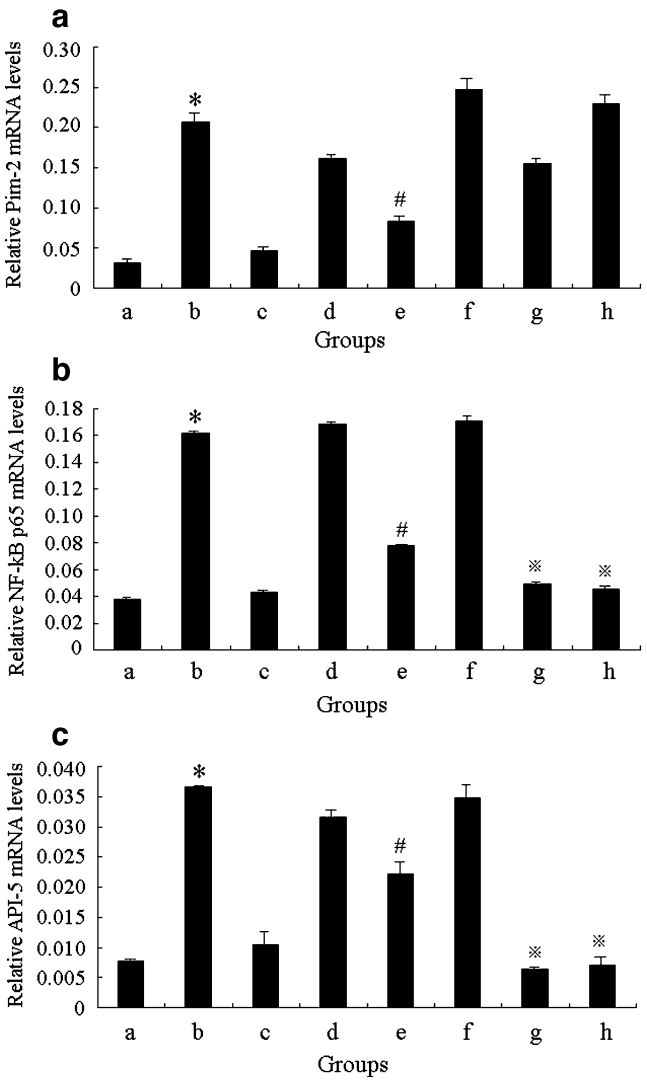

Fig. 4 The mRNA levels of the target factors in cell groups. (*a*. L02; *b*. L02/Pim-2; *c*. L02/Vector; *d*. HepG2; *e*. HepG2/Pim-2 SiRNA; *f*. HepG2/scrambled SiRNA; *g*. L02/Pim-2+parthenolide; *h*. HepG2+parthenolide) (**A**): compared with the control groups, Pim-2 mRNA level in group b is significantly higher (*$p<0.05$ vs. *a* and *c*), Pim-2 mRNA level in group e is significantly lower (#$p<0.05$ vs. *d* and *f*). (**B**): The tendency of NF-κB p65 mRNA levels are parallel to those of Pim-2 from group a to group f (*$p<0.05$ vs. *a* and *c*; #$p<0.05$ vs. *d* and *f*). When parthenolide are added in, the NF-κB p65 mRNA levels significantly decreased (※$p<0.05$ vs. *b* and *d*). (**C**): The tendency of API-5 mRNA levels are totally parallel to those of NF-κB p65 in all the groups (*$p<0.05$ vs. *a* and *c*; #$p<0.05$ vs. *d* and *f*; ※$p<0.05$ vs. *b* and *d*)

Pim-2+parthenolide: 38.53±2.61; h. HepG2+parthenolide: 37.43±7.31), the NF-κB p65/50 levels significantly decreased (※$p<0.05$ vs. b and d). It verified the inhibitory effect of parthenolide to NF-κB.

Apoptosis Rate in the Cell Groups

The ratio between apoptotic cell count and total cell count was regarded as the apoptosis rate of the corresponding

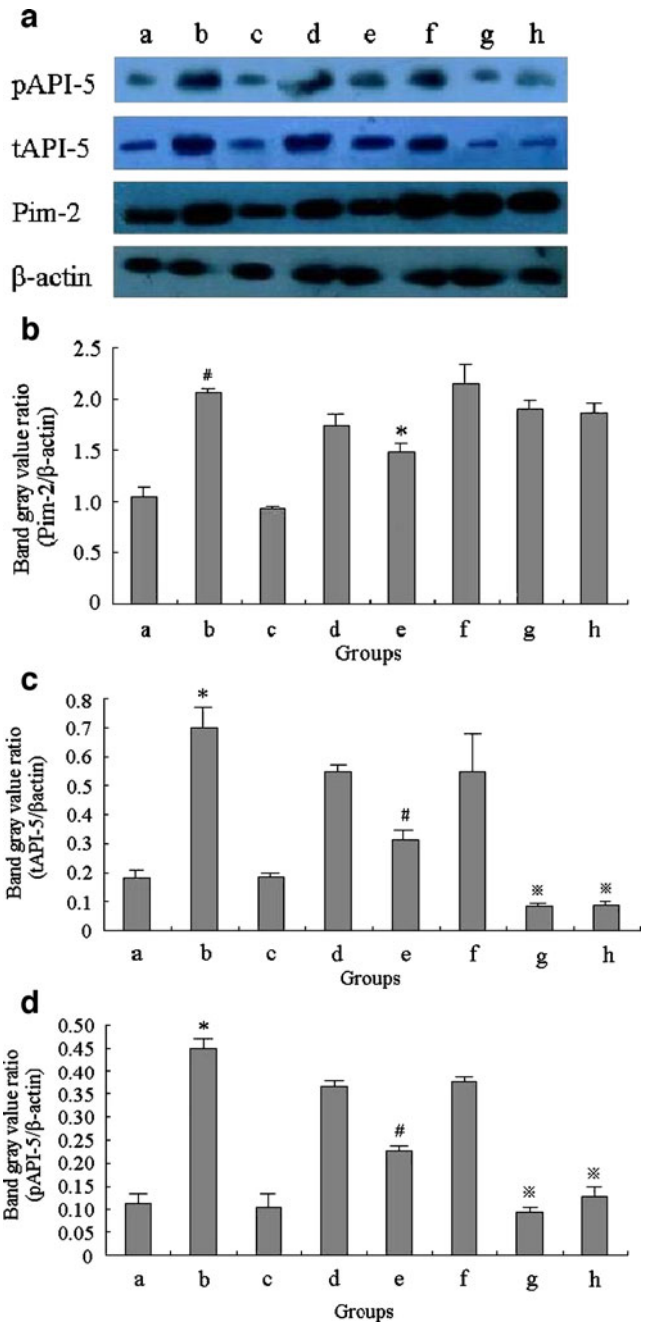

Fig. 5 The protein levels of the target factors in cell groups. (*a*. L02; *b*. L02/Pim-2; *c*. L02/Vector; *d*. HepG2; *e*. HepG2/Pim-2 SiRNA; *f*. HepG2/scrambled SiRNA; *g*. L02/Pim-2+parthenolide; *h*. HepG2+parthenolide) (**B**): compared with the control groups, Pim-2 protein level in group b is significantly higher (#$p<0.05$ vs. *a* and *c*), Pim-2 protein level in group e is significantly lower (*$p<0.05$ vs. *d* and *f*). (**C**): The tendency of tAPI-5 protein levels are parallel to those of Pim-2 from group *a* to group *f* (*$p<0.05$ vs. *a* and *c* ; #$p<0.05$ vs. *d* and *f*). When parthenolide are added in, the tAPI-5 protein levels significantly decreased (※$p<0.05$ vs. *b* and *d*). (**D**): The tendency of pAPI-5 protein levels are totally parallel to those of tAPI-5 in all the groups (*$p<0.05$ vs. *a* and *c* ; #$p<0.05$ vs. *d* and *f*; ※$p<0.05$ vs. *b* and *d*)

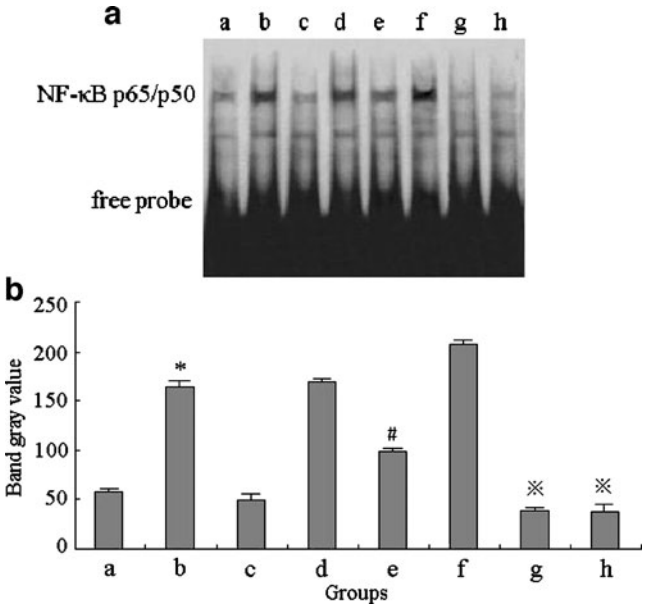

Fig. 6 NF-κB p65/p50 levels in neucleoprotein in the cell groups. (*a*. L02; *b*. L02/Pim-2; *c*. L02/Vector; *d*. HepG2; *e*. HepG2/Pim-2 SiRNA; *f*. HepG2/scrambled SiRNA; *g*. L02/Pim-2+parthenolide; *h*. HepG2+parthenolide) Compared with the control groups, NF-κB activity in group b is significantly higher (*$p<0.05$ vs. *a* and *c*), NF-κB activity in group e is significantly lower (#$p<0.05$ vs. *d* and *f*). When parthenolide are added in, NF-κB activity significantly decreased (※$p<0.05$ vs. *b* and *d*)

group (Fig. 7-A). The experiment was repeated three times for each group and the mean was taken. The apoptosis rate of the cells were significantly different among the groups (Fig. 7-B: $F=54.203$, $p<0.001$). In L02 cell line, compared with the control groups (a. L02: 5.59±1.75%; c. L02/Vector: 6.05±1.71%), cell apoptosis rate in the transfection group (b. L02/Pim-2: 0.77±0.20%) was significantly lower (*$p<0.05$ vs. a and c). In HepG2 cell line, compared with the control groups (d. HepG2: 1.01±0.32%; f. HepG2/scrambled SiRNA: 1.04±0.29%), cell apoptosis rate in the RNAi group (e. HepG2/Pim-2 SiRNA: 6.28±0.61%) was significantly higher (#$p<0.05$ vs. d and f). When parthenolide were added into L02/Pim-2 and HepG2 groups (g. L02/Pim-2+parthenolide: 12.83±1.79%; h. HepG2+parthenolide: 13.27±1.17%), the cell apoptosis rate significantly increased (※$p<0.05$ vs. b and d).

Discussion

From the results of the tissue experiments we could find that Pim-2 mRNA and protein levels were significantly higher in HCC tissues than those in the PNL and NL tissues. This is in accordance with our previous results [5,6]. Both the mRNA level and activity of NF-κB p65 were parallel to the levels of Pim-2 in the tissue groups, and so it is with the mRNA and protein level of tAPI-5. It shows a same tendency of the three factors in the tissue groups. We could preliminarily infer that the expression level and function of NF-κB p65 and API-5 may be regulated by Pim-2, in other words, NF-κB and API-5 may be the downstream factors of Pim-2 pathway in liver cells. Further evidences could be found next. After the factitious intervention in the cell experiments, Pim-2 mRNA and protein levels were both significantly increased in L02 cells after the transfection of Pim-2 gene, while both significantly decreased in HepG2 cells after the transfection of Pim-2 SiRNA. At the same time, the mRNA level and activity of NF-κB p65, as well as the mRNA and protein level of tAPI-5, were all changed in the same tendency with Pim-2 levels among the cell groups. However, after the adding of parthenolide, the mRNA level and activity of NF-κB p65, as well as the mRNA and protein level of tAPI-5 were all decreased, while the levels of Pim-2 were not affected at all. From these results we could draw the conclusion that Pim-2 is the upstream factor of NF-κB p65 while API-5 is its downstream factor. Pim-2 could activate the transcription activity of NF-κB p65, thus promote the expression of API-5, but the final API-5 level is directly determined by NF-κB p65 activity. We also found that the pAPI-5 levels were generally in accordance with the Pim-2 levels in both the tissue experiment and cell experiment. It proves that API-5 is the phosphorylating substrate of Pim-2. But when NF-κB activity was inhibited and the tAPI-5 level was accordingly down regulated, the pAPI-5 level was decreased even if Pim-2 was still highly expressed. After all, tAPI-5 level is the base of pAPI-5 level. The apoptosis results demonstrate that the higher Pim-2 level is, the lower cell apoptosis rate is, but this could be reversed by NF-κB repressor. The cell apoptosis rate is finally determined by the level of pAPI-5. To sum up, we draw the conclusion that Pim-2 could phosphorylate API-5 to inhibit the apoptosis rate of hepatocellular carcinoma cells, and NF-κB is the key modulator of this pathway.

The key role of NF-κB in Pim-2 pathway demonstrated in our experiment is consistent with the results in lymphoma [7]. As a nuclear factor, NF-κB activated by different upstream factors could transcript different downstream products, and thus plays different roles in inflammatory response, immunological regulation, cell proliferation and tumorigenesis, etc [16–18]. Thus, there are some problems we should pay attention to. Besides anti-apoptosis, whether NF-κB activated by Pim-2 has other biological effects? Have these effects got any relationship with tumorigenesis? Whether the normal physiological functions are influenced by the inhibition of NF-κB? What's more, the course in endochylema and nuclear of NF-κB pathway have been illuminated clearly, but how the signal transduct from endochylema to nuclear is still unknown. To clarify these problems will be benefit to illuminating Pim-2 pathway.

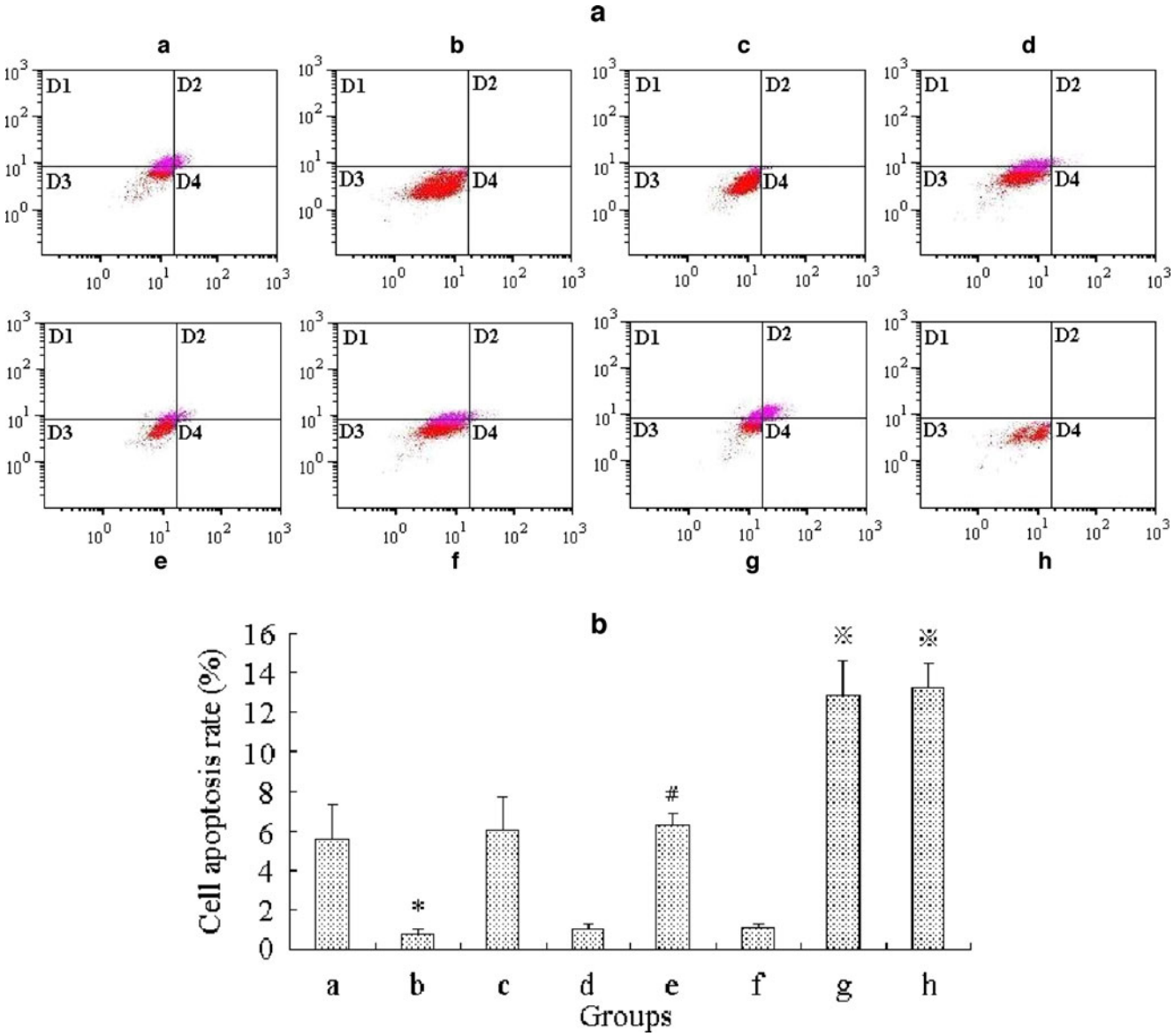

Fig. 7 Apoptosis rate in cell groups. (*a*. L02; *b*. L02/Pim-2; *c*. L02/Vector; *d*. HepG2; *e*. HepG2/Pim-2 SiRNA; *f*. HepG2/scrambled SiRNA; *g*. L02/Pim-2+parthenolide; *h*. HepG2+parthenolide) Compared with the control groups, cell apoptosis rate in group b is significantly lower (*$p<0.05$ vs. *a* and *c*), cell apoptosis rate in group e is significantly higher (#$p<0.05$ vs. *d* and *f*). When parthenolide are added in, cell apoptosis rate significantly increased (※$p<0.05$ vs. *b* and *d*)

API-5 is an anti-apoptotic factor which receives more and more focuses recently. Its promoting role in the tumorigenesis of liver cancer has been initially proved [11], and it is regarded as a newly found Pim-2 substrate because it has homologous phosphorylation sequence with the known Pim-2 substrate Bad [12]. Our research proves this inference. But the detailed anti-apoptotic mechanism of API-5 in liver cell is still unknown nowadays. It is reported that the expression of API-5 in hepatocellular carcinoma cells could be inhibit by miR-224, and the expression of miR-224 is conversely correlated with the expression of API-5 [11]. It seems as if there is a negative feedback balance between API-5 and miR-224, and the normal cell apoptosis would be out of control if the balance is broken. Enhanced API-5 expression induced by Pim-2 may be one of the stimuli which break the balance. These inferences still need further study. Eukaryotic initiation factor 4B (eI4B) is another possible Pim-2 substrate which is found in the same research as API-5 [12], but the role of eI4B in the tumorigenesis of liver cancer still need further study.

Activating API-5 through NF-κB pathway is not the only mechanism of Pim-2 anti-apoptotic effect. C-Myc has long been regarded as a coordinating factor with Pim family in tumorigenesis [19,20]. Recent research found that Pim-2 could stabilize c-Myc and prevent its degradation by phosphorylating its Ser329 site, and this mechanism could

accelerate the development of murine lymphoma [21]. Whether this mechanism exists in hepatocellular carcinoma cells is still unknown. The interaction among Pim family members is another focus. Pim-1 has long been regarded as an isoenzyme to Pim-2 because of their same kinase domain [22]. Pim-3 has been reported to abnormally over express in murine HCC, and the apoptosis rate of the murine hepatocellular carcinoma cells could increase if Pim-3 is blocked using RNAi [23]. The detailed mechanisms of the interaction among Pim family members in HCC also need further study. In view of the important roles played by Pim-2 in tumorigenesis, researches about its inhibitors grow hotter [24]. Thiazolidine congeners and isoxazole homologues are the most popular ones [25,26]. Although NF-κB repressor could block Pim-2 pathway, it may influence the normal physiologic function of the cells. So Pim-2 specific inhibitor may be more practical.

This research is an important supplement to our previous study. Based on these results, we are more affirmative to say that Pim-2 anti-apoptotic effect may be an important mechanism in the tumorigenesis of liver cancer. We believe that the deep study of Pim-2 pathway will be benefit to illuminating the pathogenesis of liver cancer and to listing Pim-2 as one of the targets in the gene therapy of liver cancer.

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