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Pokemon reduces Bcl-2 expression through NF-κBp65: a possible mechanism of hepatocellular carcinoma

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ABSTRACT

Objective: To investigate the relationship among Pokemon, NF-κB p65 and Bcl-2 in hepatoma cells. **Methods:** HCC cell HepG2, SMMC7721 and human fetal liver cell line LO2 cells were used, and expression of Pokemon, NF-κB p65 and Bcl-2 in three cells were detected by real-time PCR and western blot. Then siRNA of Pokemon was applied to inhibit the expression of Pokemon and NF-κB p65 and apoptotic rate was determined by flow cytometric analysis. **Results:** Expressions of Pokemon, NF-κB p65 and Bcl-2 in human hepatoma cell HepG2, SMMC7721 expression were significantly higher than those in human embryonic stem cells LO2. siRNA of Pokemon inhibited the expression of Pokemon, NF-κB p65 and Bcl-2 in liver cancer cells, and significantly increased apoptosis of liver cells. While siRNA of NF-κB p65 inhibited the expression of NF-κB p65 and Bcl-2, but Pokemon expression in hepatoma cells had no significant change. **Conclusions:** The proto-oncogene Pokemon can inhibit P14ARF by specific transcription regulation of cell cycle and can induce tumors. In addition, Pokemon can regulate NF-κB p65 through the expression of apoptosis repressor, and promote the development of liver cancer. It suggests signal network in the liver include the regulation of new non-classical NF-κB regulatory pathway.

1. Introduction

Now it is reported that multi-gene, multi-stage interaction of oncogenes activation or tumor-suppressor gene inactivation constitute the molecular basis of the development of hepatocellular carcinoma. It is also reported that Pokemon (POK family of transcriptional repressors) as a cancer gene may be located upstream, and plays a key role in malignant transformation[1-4]. In some human tumors such as lymphoma, breast, lung, colon, prostate and bladder cancer, Pokemon is highly expressed. Pokemon not only plays a role in the embryonic development and cell differentiation, and cooperates with other classic oncogenes as a proto-oncogene[5-13]. The detailed mechanism and function of Pokemon in hepatocellular carcinoma are still unclear. Maeda *et al*[1,2]reported that pokemon can inhibit transcription of P14ARF, regulate cell cycle and induce tumors, but at the same time pokemon may regulate other target genes to inhibit tumor cell apoptosis.

The occurrence and development of hepatocellular carcinoma are associated with the imbalances of proliferation and apoptosis in hepatocellular carcinoma cell. The changes of nuclear factor-κB (NF-κB) and Bcl-2 gene are more important in this imbalances. NF-κB is a nuclear transcription factor which exists in a variety of cells in the body, and play an important role in carcinogenesis and apoptosis. Current studies have shown that, NF-κB signal transduction pathways is involved in oncogenes and tumor suppressor gene transcription may be involved in apoptosis by inhibiting the development of hepatocellular carcinoma[14-16]. Bcl-2 is a proto-oncogene. The proteins of the Bcl-2 family are important regulators of apoptosis under normal and pathological conditions. The protein encoded by Bcl-2 proto-oncogene is implicated in the prolongation of cell survival by blocking programmed cell death, i.e. apoptosis[17]. Expression of apoptosis related gene Bcl-2 and NF-κB gene are important adjustment factors in the incidence and development of hepatocellular carcinoma[18].

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POK proteins are known to recruit corepressor complexes through the POZ domain, so we hypothesized that Pokemon might exert its oncogenic activity through the direct repression of potent tumor suppressor or apoptotic genes. NF-κB p65 is a very important transcription factor, and can mediate apoptosis process. Concerning the structure of Pokemon POZ protein and that signaling pathways may be involved in NF-κB signaling pathway, we explore the relationship and the signal pathway mechanism of pokemon, NF-κB and Bcl-2 in this study.

2. Materials and Methods

2.1. Culture

The human HCC cell line HepG2, SMMC7721 and the human fetal liver cell line LO2 were from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences, Shanghai Institutes for Biological Sciences. They were maintained routinely in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) and cultured at 37 ℃ in a humidified incubator containing 5% CO_2. The cells were subcultured once every three days.

2.2. RNA isolation and real time PCR

RNA was prepared with TRIzol ® (Invitrogen, CA, USA). One microgram of total RNA was heated at 70 ℃ for 5 min. and placed on ice for 5 min. A mix of 5 μL M-MLV RT 5× reaction buffer, 10 mM of each of the four dNTPs, 200 U of M-MLV transcriptase enzyme(H-), 25 pg/μL of oligo (dT) primer and 20 U of RNase inhibitor (all from Promega, WI, USA) were added to each sample, followed by incubation at 40 ℃ for 60 min and 70 ℃ for 15 min. Real-time PCR was performed using double stranded DNA dye SYBR Green PCR Master Mix (PE Biosystems, Warrington, UK) on the ABI PRISM 7700 system (Perkin-Elmer, CA,USA). PCRs were performed in triplicate and GAPDH was co-amplified to normalize the amount of RNA. All data were analyzed by the ABI PRISM SDS 2.0 software. Primer sequences were as following, Pokemon: Sense 5′ AGCCCTACGAGTGCAACATC 3′ and antisense 5′ CAGCCGTCTTTCTTGAGGTG 3′, GAPDH: Sense 5′ CACGAAACTACCTTCAACTCC 3′ and antisense 5′ CATACTCCTGCTTGCTGATC 3′; Bcl-2: Sense 5′ CTGGTGGACAACATCGCTCTG 3′ and antisense 5′ GGTCTGCTGACCTCACTTGTG 3′, GAPDH: Sense 5′ GGGAAATCGTGCGTGACAT 3′ and antisense 5′ CAGGAGAGCAATGATCATT 3′. To compare the expression of mRNA in different samples, the relative expression of mRNA was calculated using the comparative delta CT (threshold cycle number) method as described by Yague *et al*[19]. Briefly, the following formula was used:$2^{-\Delta\Delta CT}$, where △CT is the difference in CT between the gene of interest and GAPDH, and △△CT for the sample =△CT for the actual sample−$2^{-\Delta\Delta CT}$ of the lowest expression sample[20].

2.3. Western blot analysis

Nuclear and cytoplasmic proteins were extracted from HepG2, SMMC7721 and LO2 cells using the NE-PER® nuclear and cytoplasmic extraction kit (PIERCE) according to the protocol. For the whole cell extracts, cells were lysed in RIPA extraction buffer (150 mM sodium chloride, 50 mM Tris-HCl, pH 7.4, 1 mM ethylenediaminetetraacetic acid, 1 mM PMSF, 1% Triton ×-100, 1% sodium deoxycholate, 0.1% SDS, 5 mg/mL aprotinin and 5 mg/mL leupeptin) and were then centrifuged. The supernatant was used as the whole cell protein extract.

A total of 40 μg extracts were separated by 10% SDS-PAGE. Proteins were transferred to an Immun-Blot® polyvinylidene difluoride membrane (Bio-Rad) and blocked for 1 h with 5% skim milk. Blotted membranes were incubated with various primary antibodies, which were diluted by 1:2 000, followed by horseradish peroxidase-conjugated mouse or goat IgG. Protein bands were visualized with ECL solution (PerkinElmer Life Sciences).

2.4. Down-regulation of Pokemon and NF-κB p65 by siRNA

The small interfering RNA (siRNA) of Pokemon (siRNA: 5′-GCUGGACCUUGUAGAUCAA-3′, from Health Industry Co., Ltd. Shanghai) was used to examine the role of Pokemon in mediating the antiapoptosis. Firstly, cells were transfected in HepG2 or SMMC7721 cells in logarithmic phase of 3.0×10^6 cells/well in 6-well plate shop, until 80%-90% cell fusion. A total of 10 μL of Lipofectamine 2000 and plasmid were added to 250 μL of OPTI-MEM, mixed, and incubated at room temperature after 30 min. After incubation for 4 h, the mixture was added to each hole containing 10% fetal calf serum culture medium, 37 ℃, 5% CO_2. After transfection for 48 h, total Pokemon protein was extracted and detected by Western blot method. The NF-κB p65 siRNA (siRNA; GCCCUAUCCCUUUACGUCA, from Thermo Scientific Dharmacon, Lafayette, CO, U.S.A.) was used to examine the role of NF-κB p65 in mediating the antiapoptosis [21]. Total cellular proteins and relative levels of NF-κB p65 were measured by Western blot analysis as described above after 24 h.

2.5. Flow cytometric analysis

Cells in logarithmic growth phase of 3×10^5/well in 6-well plate were inoculated until 80%-90% cell confluence. Then the trypsin digestion cells were collected. Cells were washed by PBS 3 times, 10 μL of Annexin V from light were added and mixed for 30 min, then 5 μL of PI were added for detection by flow cytometry.

2.6. Statistical analysis

With SPPS 11.0 (SPPS Inc., Chicago, IL) software, statistical comparisons between experimental groups were analyzed by one-way ANOVA. If there was a significant difference among

groups, S-N-K test was used as a post-hoc analysis. Results were expressed as mean±SD. Statistical significance was assumed if $P<0.05$.

3. Results

3.1. Pokemon, NF-κB p65, Bcl-2 expression in three types of cells

Real-time PCR showed that the relative mRNA levels ($2^{-\Delta\Delta CT}$) of Pokemon in HepG2(1.87±0.11), SMMC7721(1.90±0.13) were significantly higher than that of LO2 cells (0.25±0.04) ($P<0.05$). The mRNA levels of Pokemon increased slightly in HepG2 as compared with group SMMC7721, but there was no statistical difference. Western blot showed that the expression of Pokemon protein in HepG2, SMMC7721 were significantly higher than that of LO2 cells ($P<0.05$) (Figure 1).

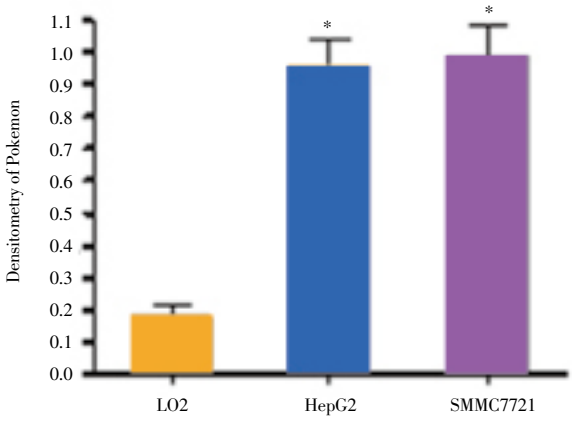

Figure 1. Expression of Pokemon protein in three cells.
* $P<0.05$, LO2 cells *vs.* the other groups.

To determine whether NF-κB p65 was activated, the level of NF-κB p65 in nucleus was measured. Western blot analysis showed that the expression of NF-κB p65 was significantly higher in HepG2 and SMMC7721 than that of LO2 cells ($P<0.05$) (Figure 2).

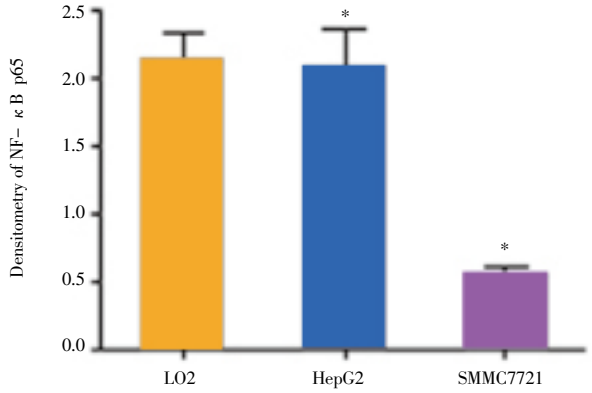

Figure 2. Expression of NF-κB p65 proteins in three cells.
* $P<0.05$, LO2 cells *vs.* the other groups.

Real-time PCR showed that the relative mRNA levels ($2^{-\Delta\Delta CT}$) of Bcl-2 in HepG2(8.23±0.75), SMMC7721(8.65±0.82) were significantly higher than that of LO2 cells (0.59±0.13) ($P<0.05$). The mRNA levels of Bcl-2 increased slightly in SMMC7721 as compared with HepG2 group, but there was no statistical difference. While the expression of Bcl-2 protein in HepG2, SMMC7721 were significantly higher than that of LO2 cells (Figure 3) ($P<0.05$).

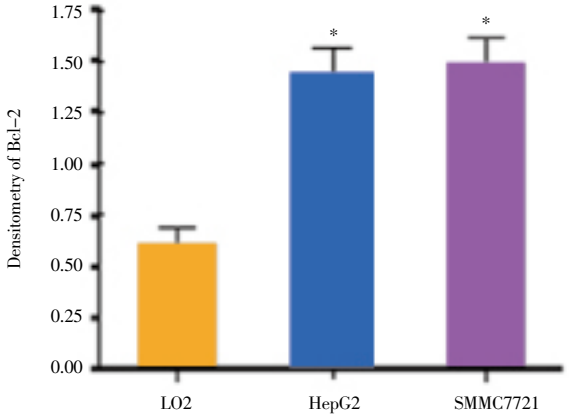

Figure 3. Expression of Bcl-2 proteins in three cells.
* $P<0.05$, LO2 cells *vs.* the other groups.

3.2. Expression of NF-κB p65, Bcl-2 and apoptosis rate after transfection of Pokemon siRNA

The result showed proteins of Pokemon, NF-κB p65 and Bcl-2 were significantly reduced after 48 h transfection in HepG2 and SMMC7721 ($P<0.05$).(Figure 4,5,6). Relative mRNA levels ($2^{-\Delta\Delta CT}$) of Bcl-2 in HepG2 and SMMC7721 were significantly decreased ($P<0.05$)(8.63±0.69 *vs.* 5.21±0.48; 8.27±0.71 *vs.* 5.69±0.60).

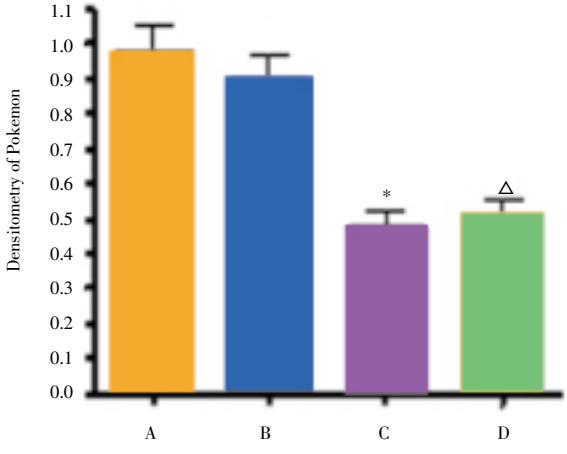

Figure 4. Expression of Pokemon protein after transfection of Pokemon siRNA.
* $P<0.05$, A *vs.* C groups; △$P<0.05$ B *vs.* D groups; A: HepG2 untransfected group; B: SMMC7721 untransfected group; C: HepG2 transfection group; D: SMMC7721 transfected group.

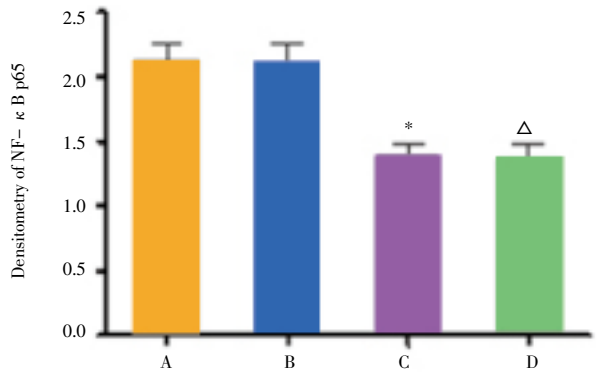

Figure 5. Expression of NF- κ B p65 after transfection of Pokemon siRNA.
* $P<0.05$, A *vs.* C groups; $\triangle P<0.05$ B *vs.* D groups; A: HepG2 untransfected group; B: SMMC7721 untransfected group; C: HepG2 transfection group; D: SMMC7721 transfected group.

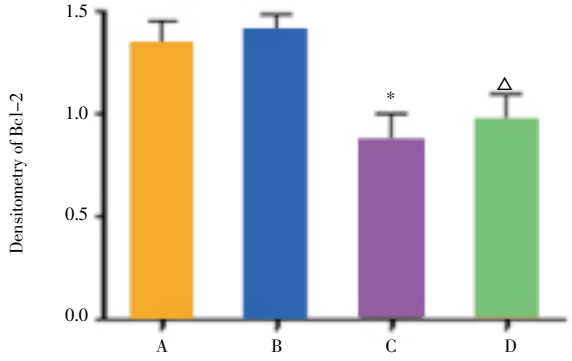

Figure 6. Expression of Bcl-2 protein after transfection of Pokemon siRNA.
* $P<0.05$, A *vs.* C groups; $\triangle P<0.05$ B *vs.* D groups; A: HepG2 untransfected group; B: SMMC7721 untransfected group; C: HepG2 transfection group; D: SMMC7721 transfected group.

The apoptosis rate of siRNA Pokemon transfected cells significantly increased after 48 h [(5.07±0.46)% *vs.* (39.65±3.75)%; (5.71±0.83)% *vs.* (33.21±3.66)%] ($P<0.05$). Between negative control cells, there was no significant difference in apoptosis rate [(39.65±3.75)% *vs.* (33.21±3.66)%] ($P>0.05$).

3.3. Expression of Pokemon, Bcl-2 and the apoptosis rate after transfection of NF- κ B p65 siRNA

In order to further reveal the relationships among Pokemon, NF- κ B p65 and Bcl-2, we apply the method of siRNA to reduced the expression of NF- κ B p65. The result showed NF- κ B p65 protein was significantly reduced after 72 h transfection in HepG2 and SMMC7721 ($P<0.05$).(Figure 7). After application of NF- κ B p65 siRNA, there was no significant difference in relative mRNA levels ($2^{-\Delta\Delta CT}$) of Pokemon in HepG2 and SMMC7721 ($P>0.05$)(1.86±0.12 *vs.* 1.90±0.13;1.91±0.11 *vs.* 1.85±0.11). Result of Western blot showed Pokemon protein also had no significant difference in expression level ($P>0.05$) (Figure 8). Relative mRNA levels ($2^{-\Delta\Delta CT}$) of Bcl-2 in HepG2, SMMC7721 were significantly decreased ($P<0.05$)(8.70±0.76 *vs.* 5.15±0.49; 8.29±0.69 *vs.* 4.96±0.51). And expression of Bcl-2 protein also decreased ($P<0.05$) (Figure 9).

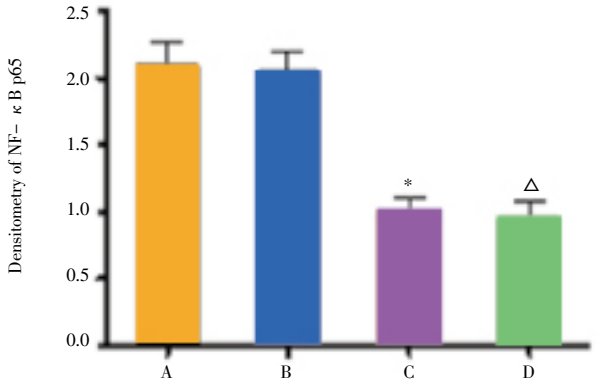

Figure 7. Expression of NF- κ B p65 after transfection of NF- κ B p65 siRNA.
* $P<0.05$, A *vs.* C groups; $\triangle P<0.05$ B *vs.* D groups; A: HepG2 untransfected group; B: SMMC7721 untransfected group; C: HepG2 transfection group; D: SMMC7721 transfected group.

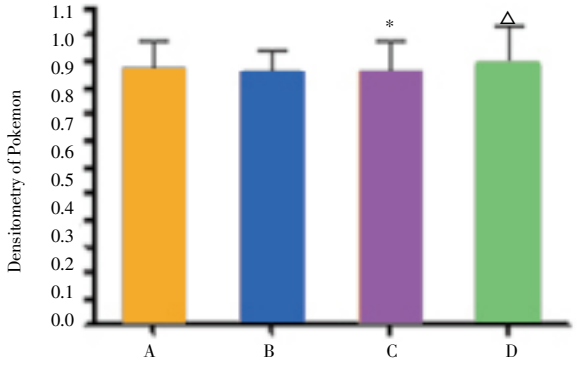

Figure 8. Expression of Pokemon protein after transfection of NF- κ B p65 siRNA.
* $P<0.05$, A *vs.* C groups; $\triangle P<0.05$ B *vs.* D groups; A: HepG2 untransfected group; B: SMMC7721 untransfected group; C: HepG2 transfection group; D: SMMC7721 transfected group.

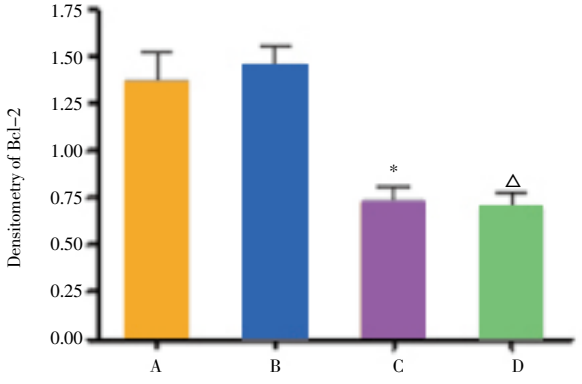

Figure 9. Expression of Bcl-2 protein after transfection of NF- κ B p65 siRNA.
* $P<0.05$, A *vs.* C groups; $\triangle P<0.05$ B *vs.* D groups; A: HepG2 untransfected group; B: SMMC7721 untransfected group; C: HepG2 transfection group; D: SMMC7721 transfected group.

The apoptosis rate of NF-κB p65 siRNA transfected cells significantly increased apoptosis after 72 h ($P<0.05$) [(5.12±0.61)% *vs.* (37.87±4.10)%; (5.80±0.71)% *vs.* (40.19±3.78)%]. While there was no significant difference in apoptosis rate of negative control cells ($P>0.05$) [(37.87±4.10)% *vs.* (40.19±3.78)%].

4. Discussion

Hepatocellular carcinoma (HCC) is one of the world's top ten malignant tumors, ranking fourth in the incidence of malignant tumors, primary liver cancer in China ranks second in cancer mortality. Present study suggests that: the occurrence and development of HCC more genes involved in vivo, the complex multi-step collaborative process, and its pathogenesis is still not fully understood.

We know some of hepatitis virus infection and liver cancer-specific carcinogens are the main external factor; but, like liver cancer and other malignancies, the occurrence and development is also involved in multiple genes in vivo, the complex process of synergy.

So far, studies have shown that liver cancer cells in more than 20 abnormal expression of genes such as ras, c-myc, c-fos, c-jun, rho, TGFa and so on, these gene mutations, loss of heterozygosity, methylation the change of status or the abnormal expression of their protein products involved in tumor cell signal transduction and cell cycle progression abnormal, leading to malignant proliferation of tumor cells. So they change at the genetic level and the cumulative effects of the interaction between them possible synergy, and jointly promote tumor development. However, these genetic changes are common in addition to other tumors outside the liver, therefore, to find in the liver-specific expression of genes and their function in cancer has always been our unremitting efforts.

Pokemon is overexpressed in many human cancers and cells lacking Pokemon are refractory to oncogenic transformation. Pokemon mediates the development of tumors through the ARF-p53 pathway[1,2]. Maeda[2] discovered that Pokemon directly binds the p14 AFR promoter *in vivo* and is able to repress its activity. In the absence of Pokemon, p14AFR expression was found markedly elevated upon both culture shock and oncogenic transformation. Pokemon has multiple additional target genes by which it can exert its oncogenic activity. NF-κB is a multi-directional, multi-functional nuclear transcription factor, and can regulate cell apoptosis and proliferation related genes in the cells, which play an important role in carcinogenesis[22,23]. NF-κB protein family consists of 5 different subunits, of which the most common heterodimer is p50/p65[24]. NF-κB is abnormally activated in hepatocellular carcinoma. It can inhibit apoptosis, and promote liver cell survival, which is closely related to the development of hepatocellular carcinoma[25]. Bcl-2 is an apoptosis suppressor gene in human tumors. It inhibits apoptosis by inhibition of apoptotic stimuli, which has an important role in the occurrence of hepatocellular carcinoma.

This study is to investigate the relationship among Pokemon, NF-κB and Bcl-2.

First, we examined Pokemon, NF-κB and Bcl-2 in the hepatoma cells or human fetal liver cells, and found they were expressed significantly higher in hepatocellular carcinoma cell lines (HepG2, SMMC 7721) than in human fetal liver cells. This is consistent with the findings at home and abroad. In order to further reveal the relationships among Pokemon, NF-κB p65 and Bcl-2, we apply the method of siRNA to reduced the expression of Pokemon. We found reduced expression of Pokemon can significantly increased apoptosis of hepatoma cells. We also found that NF-κB p65, BCL -2 expressions significantly decreased in HCC cells, which suggested Pokemon affect NF-κB p65 and Bcl-2, and there may be a regulatory mechanism.

Next, we applied siRNA to reduce the expression of NF-κB p65, the results showed that transfection p65siRNA blocked NF-κB signaling pathway, and significantly increased apoptosis rate of hepatoma cells. It can not affect the expression of Pokemon, but Bcl-2 expression was significantly reduced in the experimental group. These results showed that in the development of hepatocellular carcinoma, oncogene Pokemon regulates NF-κB, then NF-κB regulates Bcl-2 and inhibits apoptosis, which plays an important role in the occurrence and development of hepatocellular carcinoma.

Our study reveals Pokemon is the upstream of NF-κB gene and NF-κB is the upstream regulator of Bcl-2.

Lee[26] found that the POZ domain of Pokemon interacted with the Rel homology domain of the p65 subunit of NF-κB in both *in vivo* and *in vitro* protein-protein interaction assays. Our results show Pokemon can affect the expression of NF-κB. Therefore, we resume that Pokemon may be an important regulatory factor of NF-κB p65. Pokemon can participate in the occurrence and development of hepatocellular carcinoma by regulating the expression of NF-κB.

A large number of studies have suggested NF-κB and Bcl-2 may have a synergistic effect in promoting the formation of hepatocellular carcinoma[27,28]. Our experiments showed that when p65siRNA blocks NF-κB signaling pathway in hepatocellular carcinoma cells, Bcl-2 expression is decreased and apoptosis rate is increased. Therefore, we conclude that Bcl-2 gene is downstream gene of NF-κB gene.

In conclusion, this study suggests that there is a new non-classical NF-κB regulatory pathway in the regulation of hepatocellular carcinoma signaling networks. We think pokemon is a new regulatory factor of NF-κB, and plays a unique role during the development of hepatocellular carcinoma. This is a new discovery and supplement of the regulatory mechanism in NF-κB pathway. Also this

is helpful to further elucidate molecular mechanism of hepatocellular carcinoma.

Conflict of interest statement

We declare that we have no conflict of interest.

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