Cancer Cell Research 14 (2017) 351-355

Available at http:// www.cancercellresearch.org ISSN 2161-2609

HBx enhances cell viability, migration and invasion of hepatocellular carcinoma via PD-L1

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Abstract: **Programmed death 1 ligand (PD-L1) is expressed in many cancers. Many studies have confirmed that PD-L1 is involved in multiple behaviors of cancers, such as cell viability, proliferation, migration, invasion, apoptosis and immune escape. In hepatocellular carcinoma (HCC), some studies show that PD-L1 expression is related to HBx, which is encoded by the X gene in Hepatitis B virus (HBV). In our study, HBx gene was transfected into HepG2 cell (HBV negative cell line), expressing HBx protein. Moreover, the HBx gene was down-regulated through small interfering RNA (siRNA). Meanwhile, PD-L1 gene was also transfected into HepG2 cell alone. PD-L1-siRNA can decrease the expression of PD-L1. The results showed that the ability of cell viability, migration, and invasion was promoted when HBx and PD-L1 increased. However, the results were opposite when HBx and PD-L1 were inhibited. Confirmed results indicate that HBx enhances cell proliferation, migration and invasion of hepatocellular carcinoma via PD-L1. These studies suggest that knocking down HBx gene and blocking PD-1 (programmed death 1)/PD-L1 immune detection point may be expected as an immunotherapy for HBV positive HCC patients.**

Keywords: Hepatocellular carcinoma; Programmed death ligand 1; HBx; Immunotherapy; Hepatitis B virus

Received 12 March 2017, Revised 25 April 2017, Accepted 27 April 2017

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1. Introduction

Liver cancer is a complex disease with multiple factors affecting the course and treatment. In our country, the primary hepatic carcinoma is majority and high risk [1]. The prevalence of primary hepatic carcinoma is the fifth in cancer on a world-wide scale and mortality is the third. In primary hepatic carcinoma, hepatocellular carcinoma (HCC) accounts for at least 85 % [2]. The etiology of HCC is unclear and complicated. Some research showed HBV and hepatitis C virus (HCV) infection, aflatoxin, alcohol, nitrosamines were associated with HCC [3]. In previous studies, it is proved that HBV infection is the most main reason of tumorigenesis in HCC [4].

We know HBV genome contains four open coded reading frames, in which the X gene is the smallest one. X gene can encode X protein in vivo and in vitro [5]. Many studies have shown that HBx protein is closely connected with HCC. HBx participates in a variety of functions in HCC, such as cell viability, proliferation, migration, invasion, signal transduction, apoptosis and immune escape and so on [6].

PD-L1 plays an important role in the occurrence, development and prognosis of tumors. PD-L1, a ligand of PD-1, plays a part in negative immune regulation with PD-1 [7]. Some tumor cells can up-regulate the expression of PD-L1, inhibit the T cell-mediated cellular immunity, and then form the tumor microenvironment, and lead to immune escape [8] Some studies have shown that PD-L1 can be expressed in some of tumor cells including HCC cells [9-12].

2. Materials and Methods

2.1. Cell Lines and Cell culture

Over expression of HBx and PD-L1 plasmids were projected and synthesized by GeneChem Company (Shanghai, China). Silencing HBx and PD-L1 gene small interfering RNA were designed and synthesized in chemical technology by RiboBio Company (Guangzhou, China). Human HCC cell HepG2 (HBV−) was nicely given by Dr. Liu Shihai (Central Laboratory of Affiliated Hospital of Qingdao University, Qingdao, China). HepG2 was cultured in RPMI-1640 medium containing 15% fetal bovine serum (FBS, Hyclone, USA) and Penicillin-Streptomycin Solution (100 U/ml Penicillin and 0.1 mg/ml Streptomycin). The cell was cultured in a humid incubator at 37 ℃ with 5 % CO_2.

2.2. RNA Extraction, cDNA Synthesis, and Quantitative Real-time PCR

In order to obtain quantitative expression of HBx and PD-L1 at the genetic level, we extracted the total RNA from different cells disposed with different treatments using Trizol (Ambion, Austin, TX, USA).

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Then, according manufacturer specification of First Strand cDNA Synthesis Kit (Roche, Germany), reverse transcription experiment was done. The final result was performed using a LightCycler[96] Real-Time PCR system (Roche). The $2^{-\Delta\Delta Ct}$ was used to display gene expression changes relative to β-Actin.

2.3. Western blot assay

The cells were split with RIPA and PMSF (ratio is 100:1) mixed liquor (Boster; Wuhan, China). After placed on the ice for 1 h, the cells mixture was centrifuged at 14000 rpm for 20 min at 4℃. Detected the content of protein in the supernatant using BCA protein quantitative Kit (Boster). 50 μg protein samples per well were separated by 12% SDS-PAGE and transferred to PVDF membranes (Bio-Rad Laboratories, Hercules, CA). The steps were followed: BSA TBS buffer system (Boster) closure, primary antibodies incubation overnight at 4℃, secondary antibody incubation at room temperature for 2 h, and ultra sensitive light developer usage. β-Actin as a strandard.

2.4. HBx and PD-L1 transfection

The day before transfection, 3×10^5 HepG2 cells per well were inoculated in 6-well plates. Each well contained 2 ml appropriate culture medium and were cultured under usual growth conditions (typically 37℃and 5% CO_2). Plasmids were performed with Attractene Transfection Reagent (Qiagen, Hilden, Germany) according to the manufacturer's instructions. It was about 48 h after transient transfection for subsequent experiments.

2.5. siRNA experiment

Before transfection, 3×10^5 HBx-HepG2 cells per well were seeded in 6-well plates. Each well contained 2 ml appropriate culture medium and were cultured under usual growth conditions (typically 37℃and 5% CO_2). The experiment was implemented with HiPerFect Transfection Reagent (Qiagen) according to the manufacturer's protocols. It was about 48h until the start of subsequent experiments.

2.6. CCK8 assay

The cells of logarithmic growth phase were inoculated to 96-well plates at 1×10^4 cells per well and cultured with 37℃and 5 % CO_2. Added 10μl CCK8 (Boster) per hole after cell attachment, and incubated for 2h in the incubator. The absorbance value of 450nm was measured by enzyme-labeled instrument. Each group of cells was set up with five holes.

2.7. Scratch test

Behind the 6-well plates, several horizontal lines were drawn using a ruler and marker pen. The cells, transfected according to the method described above, were cultured to 95% confluence. The gun head perpendicular to the horizontal line scratches behind as far as possible. The aseptic gun head scratched vertically to those horizontal lines. Washed the cells with PBS for 3 times in order to remove the dropped cells, and then added the serum-free medium. The cells were cultured under normal growth conditions and taken the photos at 0, 12, 24h.

2.8. Transwell invasion experiment

Transwell chambers with Matrigel were inserted into a 24-well plate with 500 μl 15 % FBS of culture medium. Cells were seeded (1.5×10^4/200μl) in serum-free culture medium in the upper of the chamber. The 24-well plate was incubated for 24h in the incubator. After that the cells of the upper side were erased using cotton swabs and the lower chamber cells were treated with 4% paraformaldehyde and stained by crystal violet.

2.9. Statistical analysis

Experimental cartography was made by GraphPad.Prism.v5.0 (La Jolla, CA, USA), and shown as mean ± standard deviation. The differences among the groups were analysed by One-way ANOVA. $P<0.05$ was considered to be statistically significant.

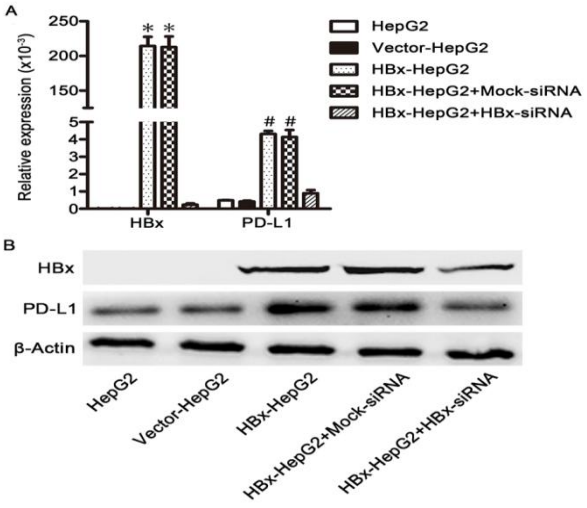


Figure 1. HBx induced PD-L1 expression in HepG2 cells. (A) HBx and PD-L1 gene relative expression in HepG2, Vector-HepG2, HBx-HepG2, HBx-HepG2+Mock-siRNA and HBx-HepG2+HBx-siRNA. (B) HBx and PD-L1 protein changes in HepG2, Vector-HepG2, HBx-HepG2, HBx-HepG2+Mock-siRNA and HBx-HepG2+HBx-siRNA.

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3. Results

3.1. HBx promoted PD-L1 expression in HepG2

HBx plasmid with green fluorescence was transfected into HepG2 cell (no HBx expression). By analyzing the data, obviously, we obtained that the expression of PD-L1 was increased in HBx-HepG2 cell compared with HepG2 and Vector-HepG2 cell (Figure 1A, 1B, $^{*}P<0.05$, $^{\#}P<0.05$). Meanwhile, we silenced HBx gene by siRNA, and displayed that PD-L1 decreased when HBx was inhibited (Figure 1A, 1B, $^{*}P<0.05$, $^{\#}P<0.05$). The results showed that HBx up-regulated PD-L1 expression.

3.2. HBx promoted cell viability via PD-L1

CCK8 assay was used to investigate the effect of HBx on cell viability via PD-L1. The results showed that the cell viability increased after transfection with HBx or PD-L1 gene. But the viability decreased with the inhibition of HBx or PD-L1 (Figure 2, $^{*}P < 0.05$).

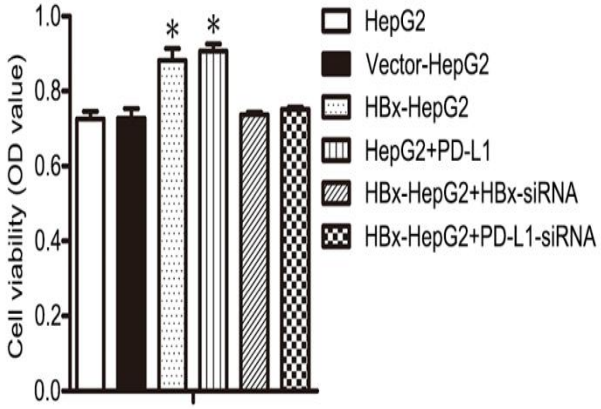


Figure 2. HBx promoted cell viability via PD-L1. Non transfected cell and no-load plasmid transfected cell as a control.

3.3. HBx promoted cell migration via PD-L1

To determine the migratory ability of the cell lines, we performed scratch test as described above. The result showed that migratory ability of HBx-HepG2 and HepG2+PD-L1 was stronger than HepG2 and Vector-HepG2. However, the cell migration was weakened when HBx or PD-L1 was inhibited (Figure 3).

3.4. HBx promoted cell invasion via PD-L1

Transwell experiments could reflect the invasive ability of the cell lines. We counted the number of invasive cells and found that the invasive ability with HBx or PD-L1 transfection was raised compared with non transfected cell and no-load plasmid transfected cell. Nevertheless, the invasive ability was restrained by si-HBx or si-PD-L1 (Figurea 4A, 4B). These suggested HBx may promote cell invasion via PD-L1. $^{*}P < 0.05$.

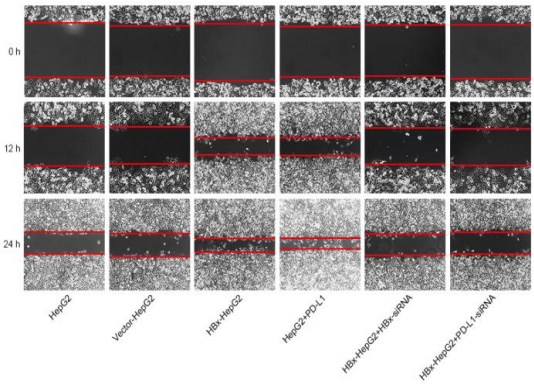


Figure 3. HBx promoted cell migration in HepG2. The photos were magnified 200 times.

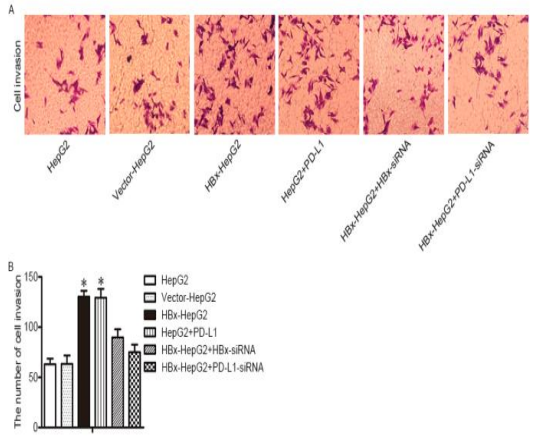


Figure 4. HBx transfection enhanced the invasion ability in HepG2. The photos were shown by microscope (200 ×). (A) The photos showed the invasion activity of lower compartment cells in treated cell lines. (B) Image J software was used to count and analyze in Figure 4A.

4. Discussion

HCC is a global disease with an annual increase of more than 700 thousand [13]. In China, the tumor related mortality is the second in malignant tumors [14]. HBV is the most common cause of HCC. It can encrypt HBx protein, which plays an important role in various biological behaviors of tumor cells [15]. Strangely, some studies have shown that when HBV infection is cured, HBx protein can still be created in the body. Just because of this, HCC is easy recurrence, prognosis is poor, and the survival rate is extremely low [16]. Therefore, the treatments of HCC are also crucial. Surgical treatment and

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orthotropic liver transplantation are limited by a variety of conditions, so the treatments of HCC patients have limited impact. Palliative care can only control the state of the illness as an auxiliary treatment. Meanwhile, curative effect is also limited and side effects are great [17]. Looking for a new effective treatment is particularly important. In recent years, we have made great progress in molecular mechanism of tumor. This gives us a chance to imagine therapeutic direction [18].

HCC tumor cells can express PD-L1 [19]. PD-1, as the receptor of PD-L1, has the main expression in T cells, macrophages, dendritic cells [20]. After the PD-1 and PD-L1 combination, it can inhibit the activity of T cells, negative regulate immune response process of organism, and the tumor cells avoid being killed [21]. At present, PD-1 antibody in phase I and II clinical trial is under way [22]. This study has confirmed that PD-L1 can affect the cell vitality, the ability of invasion and migration (Figure 2, 3, 4). Therefore, blocking PD-1/PD-L1 pathway can inhibit these functions of HCC cells, which provides a new method for HCC treatment.

HBV infection is the basic cause of leading to HCC. It is reported that HBx participates in a variety of cell activities, for example, cell viability, migration, invasion, apoptosis, immunity and prognosis [15]. These cellular activities in which HBx involved are similar to PD-L1. Some studies have shown that HBx and PD-L1 have a close relationship [23]. Our studies have demonstrated HBx can raise PD-L1 expression (Figure 1). And HBx promotes cell viability, migration and invasion of HCC. Therefore, when we increased the expression of HBx, PD-L1 expression, cell vitality, migration, and invasion of HCC cells were increased as well. The same results can be obtained by increasing the expression of PD-L1 alone. On the contrary, when HBx or PD-L1 expression was inhibited, cell vitality, migration, and invasion of HCC cells were dropped (Figure 2, 3, 4). This confirms that HBx may play a role in HCC through PD-L1. It may also suggest that preventing HBV infection improves the prognosis of HCC.

This experimental study is still some shortcomings. We have no further confirmation on this conclusion in HCC patients. We will conduct the experiment in the next step. However, our study confirms that HBx influences cell viability, migration and invasion of HCC via PD-L1. This implies that preventing HBV infection and blocking PD-1/PD-L1 pathway will have an impact on the occurrence and development of HCC. At the same time, it also provides some ways for the immunotherapy of HCC.

Acknowledgement

This study was supported by postdoctoral research project of Qingdao (Grant No. 2015163). There is no conflict of interest.

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