

Original Article

Overexpression of DDX3 inhibits cellular activity in hepatocellular carcinoma cells

Ming Jia*, Yadong Luo*, Ying Deng, Ming Liu, Yan Guo, Lufeng Li, Wei Sun, Songtao Zhao, Changlong He, Qin Mao

*Institute of Infectious Diseases of Chinese PLA, Southwest Hospital, Third Military Medical University, Chongqing, China. *Equal contributors.*

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Abstract: Human hepatocellular carcinoma (HCC) is one of the most common and deadly cancers worldwide. DDX3, a highly conserved subfamily of the DEAD-box proteins, has been reported different expression in various tumors. DDX3 has been regarded as a potential target for cancer therapy, but the functional role and the molecular mechanisms of DDX3 in HCC is still unclear. In the present study, we performed western blotting analysis to detect DDX3 expression levels in HCC cells and observed significantly lowering levels compared to normal liver cell lines. Moreover, we overexpressed DDX3 and examine the cellular activity in HCC cells. Lower cell viability and migration capacity were observed after DDX3 overexpression. Furthermore, the relationship between HCV, HIV and DDX3 were evaluated by qRT-PCR and western blotting analysis and we found that HIV-Tat and HIV-Rev may induce the expression of DDX3 and influence the expression level of HCV. Taken together, our results demonstrated that DDX3 plays a crucial role in promoting cellular activity of HCC cell and evaluated the relationship between HCV, HIV and DDX3, which could serve as a valuable therapeutic target for liver disease.

Keywords: DDX3, hepatocellular carcinoma, lentivirus, cancer therapy

Introduction

Hepatocellular carcinoma (HCC) is one of the leading causes of cancer-related deaths worldwide [1]. HCC is the fifth most frequently diagnosed cancer in men and the seventh in women [2]. But it is one of the most deadly cancers, the second most frequent cause of cancer death in men and sixth in women [3]. With improvements of medical standard, chemotherapy, radiotherapy and surgical techniques have been applied for HCC over the past few decades and death rate of HCC was low now [4-7]. Chemotherapy is the most frequently used primary medical treatment for HCC. The highest HCC incidence rates were happened in East and South-East Asia and in Middle and Western Africa [8, 9]. HCC represents the major histological subtype of primary liver cancer, and more than half a million new cases are diagnosed every year [10, 11]. Therefore, the development of new effective therapeutic targets to improve the treatment of HCC is extremely necessary.

DEAD-box RNA helicases play a fundamental role in RNA metabolism, including pre-mRNA splicing, ribosome biogenesis, RNA transport, RNA stability, translation RNA decay, initiation and organelle gene expression [12-14]. The human DDX3, also named as DDX3X, DBX, and CAP-Rf, is located at chromosome Xp11.3-p11.23. DDX3 seems to be one of the most multi-layered helicases with variety of roles in cancer in terms of functionality [15]. In viral manipulation, the function of DDX3 has been comprehensively evaluated, but its vital value in cancer is a more recent promotion in our current medical science [16, 17]. Although the change of DDX3 has been observed in a variety of tumor cells, the role of DDX3 in HCC is yet to be determined. HCC occurs several decades after the early infection with HBV or HCV in the normal population [18]. However, according to suspicion, HIV infection could be a risk factor for HCC, this assumption seems to be excluded in review of consecutive case series. Since HIV/HCV coinfection is widespread and raises the risk of HCV-associated liver disease,

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exploring the pathway of the interaction between HIV and HCV is critical for the survival of group of these patients [19, 20]. Other studies have shown that DDX3, as a RNA helicase, play an important role in HIV and HCV virus replication, which suggests that DDX3 may be involved in interaction between HIV and HCV.

In this study, we aim to explore the role of DDX3 in HCC cell lines and further investigate the effects of DDX3 on the expression of genes associated with HIV and HCV. Taken together, we first explored the connection between DDX3 and human HCC, evaluated the relationship between HCV, HIV and DDX3 and offered some insight into therapeutic method for HCC in future.

Materials and methods

Cell culture

Human HCC cell lines (Huh-7, HepG2, HepG2.2-15, Bel7402, SMMC7721) and normal liver cell line LO2 were provided by the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). Cell lines were cultured in RPMI 1640 medium (Hyclone, USA) supplemented with 10% of fetal bovine serum (Hyclone, USA), 100 U/ml of penicillin and 100 mg/ml of streptomycin. The cells were grown at 37°C in a humidified incubator with 5% CO₂.

Protein extraction and western blotting analysis

Seven days after lentivirus infection, human HCC cell lines (Huh-7, HepG2, HepG2.2-15, Bel7402, SMMC7721) and normal liver cell line LO2 were washed twice with ice-cold PBS and lysed in 2× SDS Sample Buffer which contained 100 mM Tris-HCl (pH 6.8), 10 mM EDTA, 4% SDS and 10% Glycine. Equal amounts (15 µg) of protein were loaded onto a 10% SDS-PAGE and electrophoresis at 80 V for 30 min and 150 V for 1 h. The gel was transferred to polyvinylidene difluoride (PVDF) membranes. Membranes were blocked by 1% bovine serum albumin (BSA) in TBST at room temperature for 1 h and then probed with primary antibodies overnight. Primary antibodies used were as below: anti-DDX3 (Abcam, England); anti-Rev (Abcam, England); anti-Tat (Abcam, England); anti-GAPDH (Abcam, England). After washed by TBST, membranes were incubated with horseradish

peroxidase conjugated secondary antibodies for 2 h at room temperature. The membrane was washed by TBST and signals were detected by enhanced chemiluminescence (ECL).

Plasmid construction, packaging and infection

The overexpression construct for DDX3 gene was developed by subcloning PCR-amplified full-length cDNA into the pCDNA3.1 (Shanghai Hollybio, China). In addition, pCDNA3.1-JFH1, pCDNA3.1-JFH1-Tat and pCDNA3.1-JFH1-Rev vectors were also purchased from Shanghai Hollybio. Infectious lentiviruses were collected at 48 h after transfection and lentiviral particles were purified by ultracentrifugation (4000× g) at 4°C for 10 min and then filtered through 0.45 µm filter. For cell infection, human HCC cell lines Huh-7 cells were incubated in a 6 cm dish at an inoculation density of 1×10⁵ cells/well. The DDX3 overexpression lentiviruses were added into the Huh-7 cells with a multiplicity of infection (MOI) of 60 and 80, respectively. The infection efficiency was observed after seven days through a fluorescence microscope for the green fluorescence protein expression.

CCK-8 assay

Cell Counting Kit-8 (CCK-8) assay was performed to detect the cytotoxicity of gene transfection. After transfected pCDNA3.1-DDX3 lentivirus, human HCC cell lines Huh-7 cells were seeded in 96-well plates, each well of which contained 100 µl RPMI-1640 medium supplemented with 10% FBS (5×10⁴ cells/well). After culturing for 24 h, 36 h and 48 h, 10 µl of CCK-8 reagent was added and incubated at 37°C for 1 h in humid atmosphere containing 5% CO₂. The optical density (OD) was read at 450 nm using Microplate Reader (BioRad, USA).

Transwell invasion assay

Human HCC cell lines Huh-7 cells were cultured with 5% CO₂ at 37°C for 24 hours, trypsinized, and re-suspended with growth medium containing 1% FBS. The cells were added into the top chamber (pore size, 8 µm; Corning), and the growth medium containing 10% FBS was added into the lower chamber. The cells were incubated in the incubator and allowed to migrate to the lower chamber for 48 hours. Cells that did not invade through the membrane were

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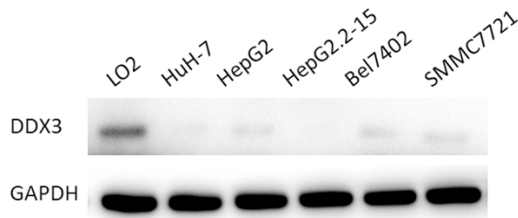


Figure 1. Decreased expression of DDX3 in HCC cell lines. The expression of DDX3 was detected in five HCC cell lines compared with control normal liver cancer cells by western blotting analysis.

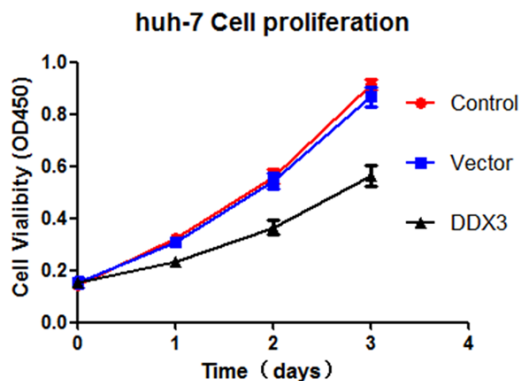


Figure 2. Overexpression of DDX3 inhibits cell viability in Huh-7 cells. The effect of DDX3 overexpression on cell viability of Huh-7 cells transfected with pcDNA3.1-DDX3.

removed by cotton swabs, while cells migrated through the membrane were fixed with 4% paraformaldehyde and stained with crystal violet for observation and counting.

RNA extraction and quantitative PCR analysis

Total RNAs was extracted from human HCC cell lines Huh-7 cells using Trizol reagent (Invitrogen, CA, USA). Then, single strand cDNA was obtained using M-MLV reverse transcriptase kit (Promega, USA) for quantitative real-time PCR (qRT-PCR) analysis to examine protein expression levels. The HCV primers were as follows: forward 5'-CAAGAGTCAGCAACTGCAG-AAG-3' and reverse 5'-TAAGGTCGCAATCCAA-GTCAGT-3'. β -actin was used as internal control and primers were as follows: forward 5'-GTGGACATGCGCTAAGAC-3', and reverse 5'-AAGGGTGTGAGCCAACTA-3'. PCR was carried out by PCR reaction mixture containing 10 μ l of 2 \times SYBR premix EX Taq, 0.8 μ l of forward and reverse primers (2.5 μ M), 5 μ l of cDNA (2 ng), 4.2 μ l of ddH₂O. Cycling conditions were a de-

naturation at 95°C for 1 min, 40 cycles of annealing at 60°C for 20 s and extension at 72°C for 10 min using BioRad Real-time PCR platform. The expression levels were calculated using $2^{-\Delta\Delta CT}$ formula.

Statistical analysis

All data were expressed as mean \pm SD of at least three independent experiments. Statistical comparisons were assessed using the Student's t-test on SPSS 22.0 software. Values of $P < 0.01$ were considered as statistically significant difference.

Results

Differential expression level of DDX3 in various human HCC cell lines

To investigate whether the level of DDX3 expression were downregulated in human HCC cells, we performed western blotting analysis to detect DDX3 expression levels in various cell lines. As shown in **Figure 1**, DDX3 was remarkably decreased in multiple HCC cells including Huh-7, HepG2, HepG2.2-15, Bel7402 and SMMC7721, compared with normal liver cell line LO2, especially in Huh-7 cells. These results indicate that DDX3 is a biomarker for differentiating HCC cells and normal epithelial cells.

Overexpression of DDX3 inhibits cell viability in Huh-7 cells

To carry out the physiological function of DDX3, in the first place, we overexpressed DDX3 in human HCC cell lines Huh-7 cells infected with the lentivirus carrying DDX3. Then, we tested the effect of DDX3 overexpression on cytotoxicity in human HCC cell lines Huh-7 cells. After transfection, CCK-8 assay was utilized to examine the cellular activity in human HCC cell lines Huh-7 cells. As shown in **Figure 2**, after transfected pcDNA-DDX3 lentivirus, cells had remarkably lower cell viability than that in Control and Vector group ($P < 0.01$).

Overexpression of DDX3 inhibits invasion and migration in Huh-7 cells

Based on the above results, we tested the effect of DDX3 overexpression on activity in human HCC cell lines Huh-7 cells. Transwell migra-

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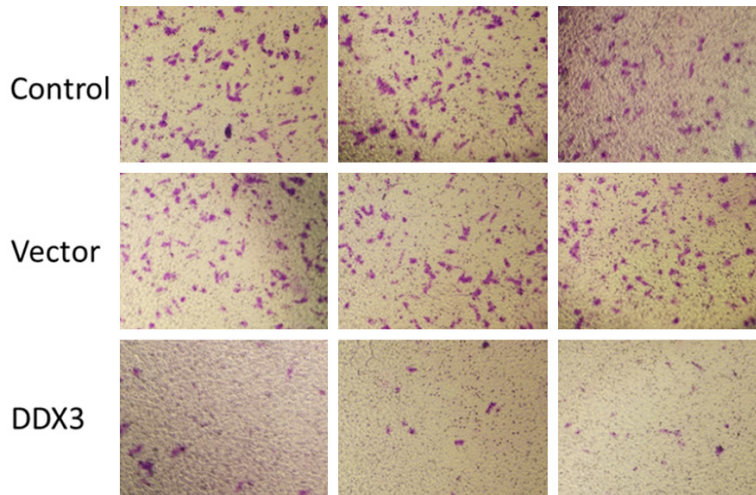


Figure 3. HCC cell migration was effected by DDX3 overexpression. Huh-7 cells were subjected to oeDDX3 and then seeded onto the upper chamber of the Transwell plate. Migrated cells after DDX3 overexpression were stained with crystal violet. OD: optical density.

tion assay was carried out to determine the invasion and migration of human HCC cell lines Huh-7 cells after DDX3 overexpression. As shown in **Figure 3**, the number of Huh-7 cells penetrating the basement membrane was remarkably reduced after DDX3 overexpression than that in Control and Vector group. These results demonstrate that overexpression of DDX3 markedly decreased the cell invasion and migration capacity in Huh-7 cells.

The role of DDX3 in HIV and HCV virus replication

To evaluate the relationship between HCV, HIV and DDX3, human HCC cell lines Huh-7 cells had been successfully transfected with pCDNA3.1-JFH1, pCDNA3.1-JFH1-Tat and pCDNA3.1-JFH1-Rev vectors (**Figure 4A**). Moreover, we detected the change of DDX3 protein levels after various plasmids transfection, which performed by western blotting analysis. As shown in **Figure 4B**, pCDNA3.1-JFH1 transfection failed to induce DDX3 expression. pCDNA3.1-JFH1-Tat and pCDNA3.1-JFH1-Rev transfection could induce the expression of DDX3. After HIV-Tat and HIV-Rev co-transfection, the protein expression levels were remarkably increased. Furthermore, after various plasmids transfection, we also detected the change of HCV mRNA levels by qRT-PCR analysis. As shown in **Figure 4C**, after HIV-Tat or HIV-Rev overexpression, the mRNA levels of

HCV downregulated in Huh-7 cells. While the protein expression levels were remarkably increased after HIV-Tat and HIV-Rev co-transfection. Taken together, these results indicate that HIV-Tat and HIV-Rev may induce the expression of DDX3 and influence the expression level of HCV.

Discussion

HCC is one of the most common human malignancies, with high recurrence rate and poor long-term survival [21, 22]. There have been significant advances in clinical diagnosis and bring the HCC under control and lots of therapeutic strategies including chemotherapy, radiotherapy and surgical techniques have been improved. The treatment prospects of HCC is not optimistic, however, the 5-year overall survival rate remains very poor [23]. The dilemma makes an urgent necessity to identify novel treatment strategies. Previous studies of the cellular and molecular biology of HCC cell lines, which have produced a group of agents called targeted therapeutics for their interfering with the specific molecules rather than by simply interfering with all rapidly dividing cells. Gene target therapy is a new way for cancer therapy and the key point is to searching for specific and efficient targets.

Understanding the molecular alterations behind the initiation and progression of HCC is crucial in finding novel markers for early diagnosis, targeted treatment and prognosis evaluation. The different expression of DDX3 has been reported in various cancer clinical cases. In lung cancer patients, it was found that DDX3 acted as an important prognostic factor on total survival. In oral squamous cell carcinoma (OSCC) patients, DDX3's pooling nuclear and cytoplasmic expression was evaluated and the scores were the highest [24]. In breast cancer patients, DDX3 was increased in cell cytoplasm and had a total worse survival [25]. Moreover, in colon cancer patients, the expression of DDX3, both at the RNA and the protein levels, was shown to be a significant predictor for survival [26, 27]. However, the functional

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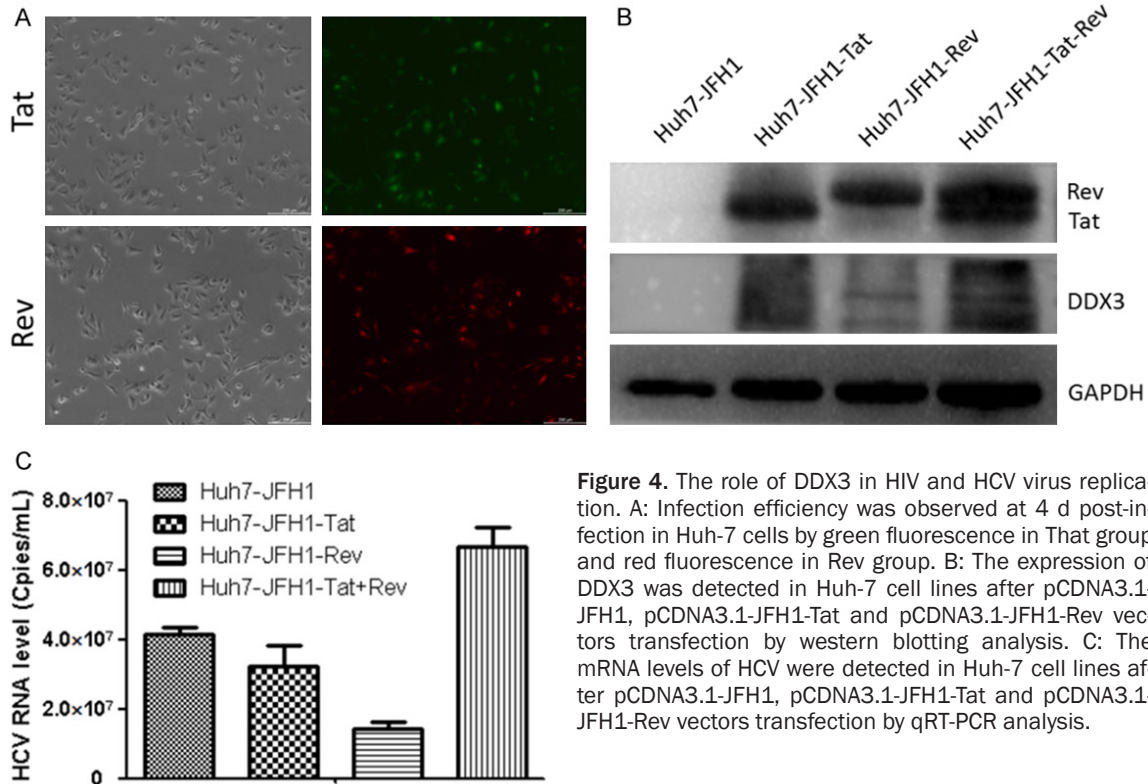


Figure 4. The role of DDX3 in HIV and HCV virus replication. A: Infection efficiency was observed at 4 d post-infection in Huh-7 cells by green fluorescence in Tat group and red fluorescence in Rev group. B: The expression of DDX3 was detected in Huh-7 cell lines after pCDNA3.1-JFH1, pCDNA3.1-JFH1-Tat and pCDNA3.1-JFH1-Rev vectors transfection by western blotting analysis. C: The mRNA levels of HCV were detected in Huh-7 cell lines after pCDNA3.1-JFH1, pCDNA3.1-JFH1-Tat and pCDNA3.1-JFH1-Rev vectors transfection by qRT-PCR analysis.

role of DDX3 in HCC is poorly understood. In this study, we firstly used lentivirus-mediated overexpression vector that potently increased the expression of DDX3 in human HCC cell lines Huh-7 cells. DDX3 overexpression significantly inhibits the cell viability and migration capacity of Huh-7 cells.

Previous study has reported that the nuclear export of single-spliced or un-spliced HIV-1 RNAs is regulated by a direct correlation between the Rev and cellular co-factors like DDX3. DDX3, an RNA-dependent ATPase/helicase, play an important role in function for partially spliced HIV-1 RNAs' export [28, 29]. Moreover, 90 percent of all people with HIV in China tested positive for HCV infection, which suggest that HIV infection can be associated with HCV through some pathway [30]. Also in consideration of DDX3's fundamental role in RNA replication, which is critical for HIV and HCV virus spread, we evaluate the relationship between HCV, HIV and DDX3 in our study. We found that HIV-Tat or HIV-Rev transfection could induce the expression of DDX3 and their co-transfection could remarkably increase the DDX3 expression levels. After HIV-Tat or HIV-

Rev overexpression, the mRNA levels of HCV downregulated and their co-transfection could noticeably increase the mRNA levels of HCV. We thought the cause might be that HIV-Tat/Rev is in combination with DDX3 form compound so that reduce DDX3 levels within cells and have lowered the RNA replication of HCV. When HIV-Tat or HIV-Rev co-transfection, a large amount of DDX3 were induced and sped up the copying of HCV in Huh-7 cells.

In conclusion, these results reveal that lentivirus-mediated overexpression of DDX3 in HCC cells decreased the cell viability and migration capacity and evaluated the relationship between HCV, HIV and DDX3. Further in-depth studies are recommended to fully understand the molecular mechanisms of DDX3 and liver disease. Such breaking-through discovery offers a platform for researchers to widen therapeutic method in HCC treatments.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Qin Mao, Institute of Infectious Diseases of Chinese PLA, Southwest Hospital, Third Military Medical University, 30 Gaotanyan Centre Street, Shapingba District, Chongqing 400038, China. Tel: +86-13594130253; Fax: +86-23-68754141; E-mail: jiaming7715@163.com

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