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Original Article

Highly expressed DDX10 promotes hepatocellular carcinoma cell proliferation through Wnt/β-catenin signaling

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Abstract: DDX10, a putative DEAD-box RNA helicase gene, is poorly studied in cancer. Our previous study found that DDX10 was significantly up-regulated in hepatocellular carcinoma (HCC). However, the clinical significance of DDX10 and its biological roles and associated mechanisms in HCC tumorigenesis remain elusive. In current study, quantitative real-time PCR, Western bolt were applied to evaluate the expression of DDX10. The roles of DDX10 in cell viability and proliferation were analyzed by cell biological assays *in vitro*. Luciferase reporter assays was employed to investigate the mechanism. And we further confirmed that compared with adjacent tissues, DDX10 is remarkably increased in HCC tissues in fresh tissues. In addition, cellular function assays demonstrated that DDX10 promotes cell viability, proliferation. Furthermore, we validated that up-regulated DDX10 enhances the activity of Wnt/β-catenin signaling to promote cell proliferation.

Keywords: DDX10, Wnt/β-catenin signaling, HCC

Introduction

Hepatocellular carcinoma (HCC) ranks second among the most common cancer-related mortality in the world [1]. Moreover, the median survival for most HCC patients is estimated to be 1 year [2, 3]. Early diagnosis together with prompt surgical removal and other combined therapeutic strategies offer patients the best opportunity for treatment or prolonged survival. Thus, a better understanding of the biochemical pathways involved in HCC, clarifying the mechanisms by which the tumor evades treatment, seems to be the main way forward to improve clinical outcomes [4].

DEAD box proteins (DDX), characterized by the conserved motif Asp-Glu-Ala-Asp (DEAD), are putative RNA helicases [5]. DEAD box proteins comprise a family of putative ATP-dependent RNA helicases implicated in many cellular processes involving alteration of RNA secondary structure, such as translation initiation, nuclear and mitochondrial RNA splicing, ribosome and spliceosome assembly [6]. DDX10 is a novel human DEAD-box RNA helicase gene on chromosome 11q22-q23 [7], although its normal function has not yet been identified, is suggested to be involved in ribosome assembly. Previous studies showed that DDXs plays complex roles in tumor development. DDX1 has been reported could inhibit cancer progression [8]; while DDX3 and DDX18 have been reported play an oncogenic role in cancer [9, 10]; Gai's research demonstrated that epigenetic down-regulated DDX10 promotes cell proliferation through Akt/NF-KB pathway in ovarian cancer [11]. However, the researches about relationship between DDX10 and HCC are poor, not mention to the mechanisms.

In current study, we found that DDX10 was up-regulated in HCC compared to adjacent tissues. Next we focus to determine the biological functions of DDX10 in HCC and found that DDX10 might play a tumor promoter role for it promoting HCC cell viability and proliferation. Taken together, DDX10 obtains a complex role in tumor biology for the dual role of it in hepatocellular carcinoma and other cancers.

Table 1. Sequences of primers and shRNAs

Name	Sequence (5'-3')
DDX10 primer	F: AGGTCGAGCGCGAGAGTAT
	R: CCAAACGGTACTGAGCTTCTTG
GAPDH primer	F: AGCCTCAAGATCATCAGCAATGCC
	R: TGTGGTCATGAGTCCTTCCACGAT
Sh-#1	CCGGCCGATAAAGTAATTGAGCCAACTCGAGTTGGCTCAATTACTTTATCGGTTTTTTG
Sh-#2	CCGGTACTCTTTGCTACTGATATTGCTCGAGCAATATCAGTAGCAAAGAGTATTTTTTGAAT

Materials and methods

Patient tissues and ethic statement

53 fresh tumor tissues and matched adjacent tissues were collected from patients with pathologically and clinically confirmed HCC. All human tumor tissues were obtained with written informed consent from patients. Protocols were approved by the ethical review committee of the World Health Organization Collaborating Center for Research in Human Production.

Cell culture

The hepatocellular carcinoma (HCC) cell lines HepG2, Hep3B were obtained from American Type Culture Collection (ATCC). Huh7 was purchased from Apath, LLC. LO-2 was obtained from Shanghai Cancer Institute. All those cells were maintained under DMEM containing 10% FBS in 37°C, 5% CO_2.

RNA isolation and quantitative real-time PCR

Total RNA was purified from HCC and adjacent tissues or cells using TRIzol (Invitrogen) following the manufacturer's protocol. RNA (1 μg) was reverse transcribed using SuperScript Reverse Transcriptase III (Invitrogen). Quantitative real time PCR was performed using SYBR green Supermix (ABI) in ABI 7300 PCR system. GAPDH was used as a reference gene. Primers using in this study were described in **Table 1**.

Western blots

Tissues and cells were lysed in RIPA lysis buffer (P0013, Beyotime) and nuclear proteins were extracted using lysis buffer (P0028, Beyotime), all the procedures were following the manufacturer's protocol. Subsequently the cell lysates were boiled in 5X SDS-PAGE loading buffer for 10 min and then resolved by 8% SDS-PAGE and transferred to nitrocellulose membrane. The following antibodies were used in this study: DDX10 and β-catenin were purchased from Cell signaling technology. Lamin A/C and GAPDH (Proteintech). Bound antibodies were visualized with the ECL kit (P0018, Beyotime).

shRNA treatment

Two DDX10 shRNAS (Invitrogen) were employed to knockdown endogenous DDX10 in this study and the sequences are described in **Table 1**. Cells were transfected with 100 nM shRNAs or with 100 nM shRNA negative control using Lipofectamine 2000 (Invitrogen, USA).

Construct stable cell lines

To generate stable ectopic expression of DDX10 cell lines, Vectors containing full length of DDX10 was purchased from GeneCopoeia. HCC cells transfected using lipofectamine 2000 (Invitrogen) with those vectors following the manufactures protocols. The supernatant media containing virus was collected by centrifugation to remove cellular contaminant. The resulting viruses were used to infect indicated cells, and then integrated cells were selected by 2 μg/ml puromycin for 2 weeks. The alterations of DDX10 in those cells were confirmed by western blots before further analysis.

CCK8 cell viability assays

Cells were seeded into a 96-well plate at 5×10^3 cells per well with 100 ul cultured medium and cultured at 37°C, 5% CO_2. The cell viability was quantified by addition 10 μl of cell counting kit (CCK8, Dojindo, Japan). After 1.5 hours incubation, the plates were monitored by Power Wave XS microplate reader (BIO-TEK) at an absorbance 450 nm.

Clone formation assay

The indicated cells (2×10^5/well) were seeded in the 6-well plates. Then these cells were collected at 24 h, 48 h and 72 h. Cells were

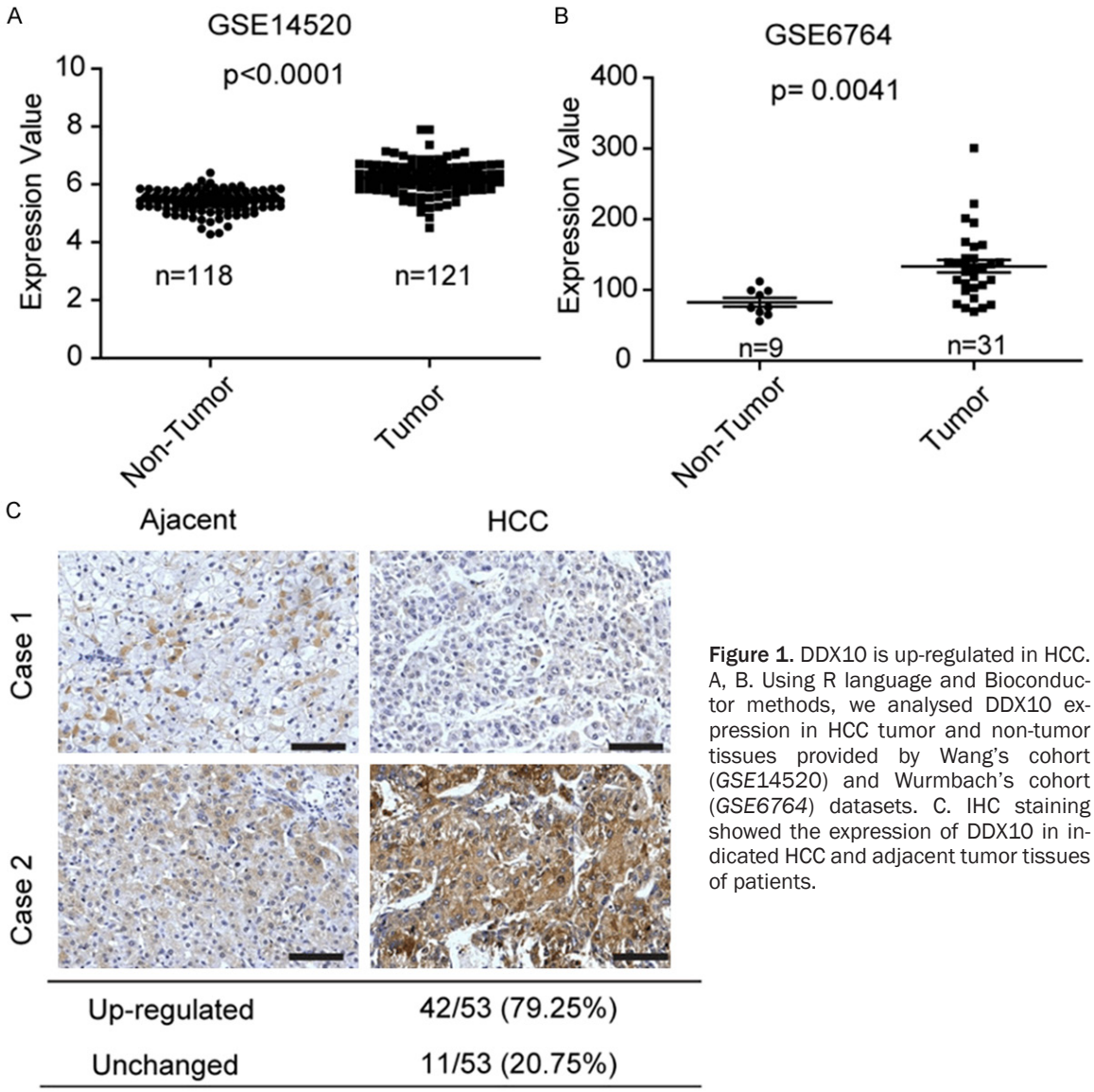

Figure 1. DDX10 is up-regulated in HCC. A, B. Using R language and Bioconductor methods, we analysed DDX10 expression in HCC tumor and non-tumor tissues provided by Wang's cohort (*GSE*14520) and Wurmbach's cohort (*GSE6764*) datasets. C. IHC staining showed the expression of DDX10 in indicated HCC and adjacent tumor tissues of patients.

washed twice with 1×PBS, then resuspended and fixed in 2 ml 70% ethanol at -20°C. Cells were then stained with PI (BD) and followed as the manufacture's protocol.

Luciferase reporter assays

Indicated cells were seeded in 96-well plates and transfected with β-catenin reporter plasmid and 10 ng Renilla following the recommended protocol for the Lipofectamine 2000 transfection system. After 48 hours incubation, firefly and Renilla luciferase activities were measured using the dual-luciferase reporter assay system (Promega, Madison, WI) from the cell lysates.

Statistics analysis

Data are expressed as the mean ± standard deviation. The correlation between DDX10 expression and the clinicopathological parameters was evaluated using the χ^2 test. Student's t-test was used for comparisons between groups and $P<0.05$ was considered statistically significant difference.

Results

Up-regulation of DDX10 in hepatocellular carcinoma

To search for driver genes in the oncogenesis of HCC, we performed genome-wide analyses

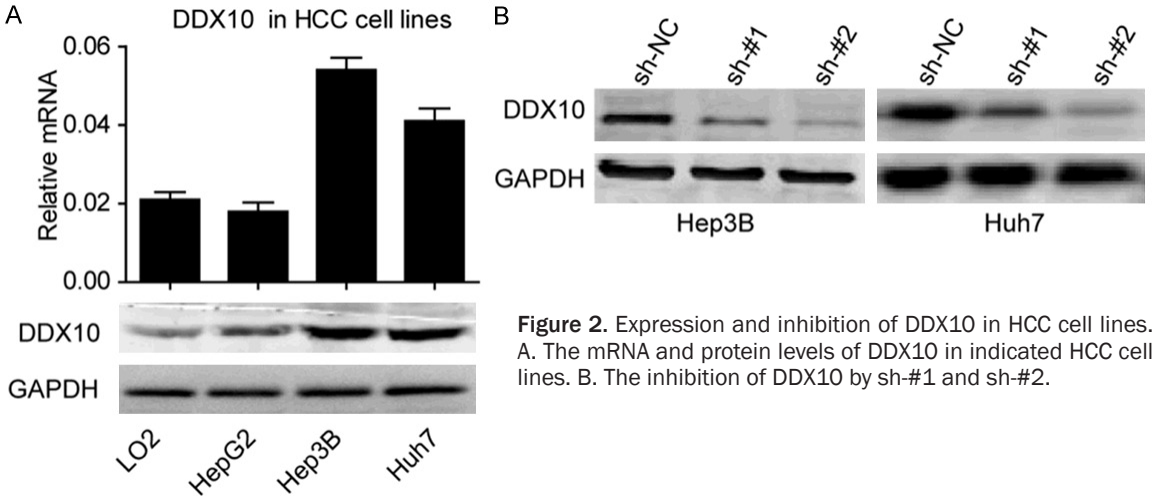

Figure 2. Expression and inhibition of DDX10 in HCC cell lines. A. The mRNA and protein levels of DDX10 in indicated HCC cell lines. B. The inhibition of DDX10 by sh-#1 and sh-#2.

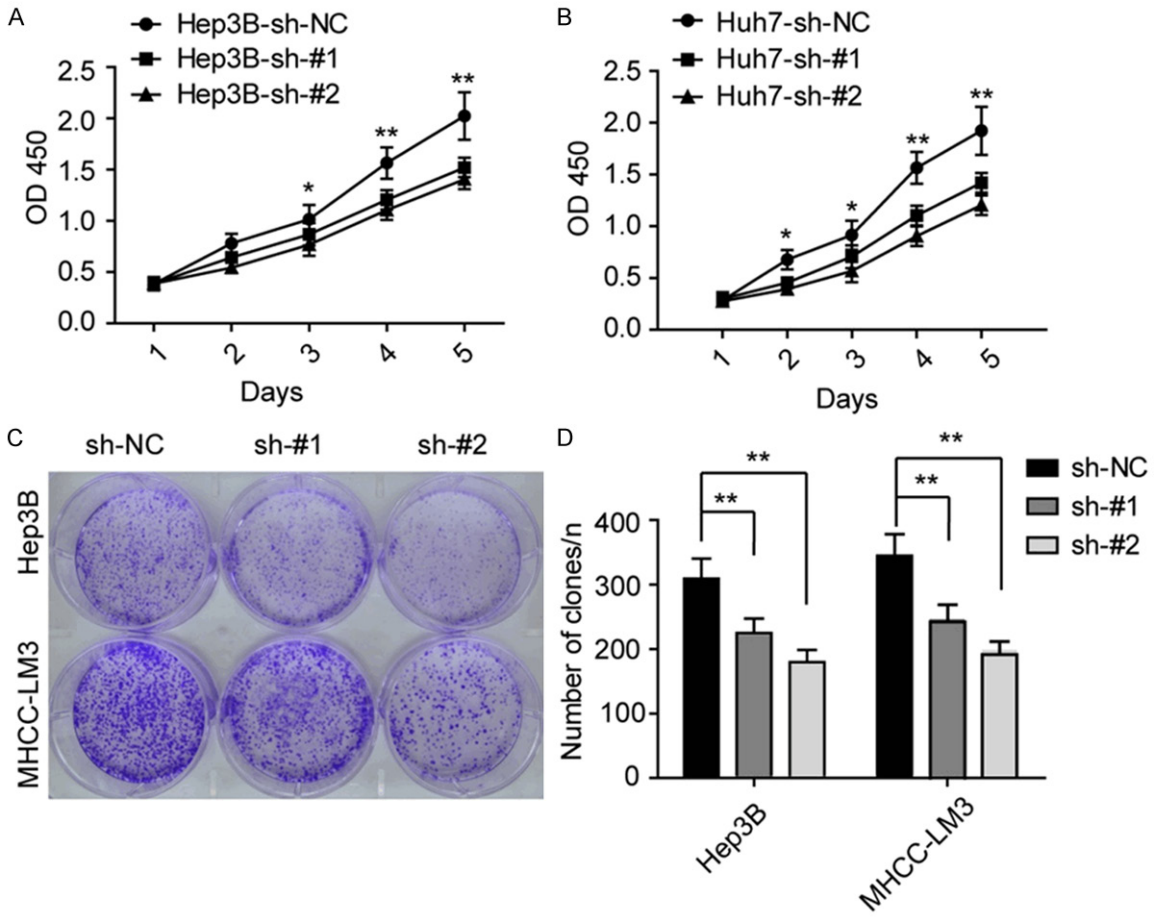

Figure 3. DDX10 promotes cell viability and proliferation. A, B. CCK-8 cell viability assays revealed that expression of DDX10 remarkably promotes Hep3B and Huh7 cell viability. C, D. The inhibition of DDX10 by sh-#1 and sh-#2 in Hep3B and MHCC-LM3 dramatically inhibited cell proliferation. *, P<0.05; **, P<0.01. All these cell biological function assays were triplicate.

using several online-available HCC transcriptome datasets by R language and Bioconductor approaches. After analysing gene expression profiles of HCC tumor and non-tumor tissues,

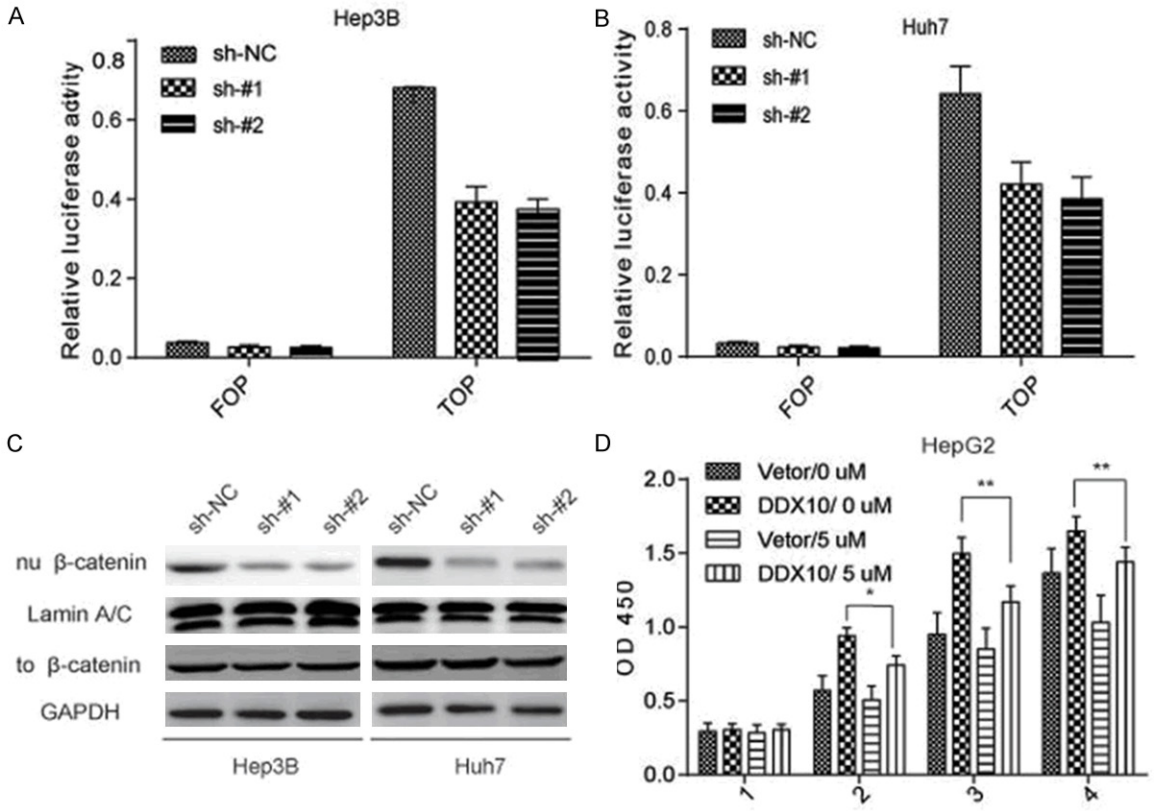

Figure 4. DDX10 promotes the activity of Wnt/β-catenin signaling in HCC cell lines. A, B. Dual luciferase reporter assays revealed that DDX10 promotes the activity of Wnt signaling. C. Inhibition of DDX10 partly impedes the translocation of β-catenin to nuclear in Hep3B and MHCC-LM3 cell lines. D. Inhibition of β-catenin can partly inhibit cell viability in LO2 cell lines. *, P<0.05; **, P<0.01. All these cell biological function assays were triplicate.

we identified >300 differentially expressed genes from both Wang's cohort (GSE36376) [12] and Wurmbach's cohort (GSE14520) [13]. Of these changed genes, we focused on DDX10, which was highly expressed in HCC tumors derived from both Wang's cohort (GSE36376) and Wurmbach's cohort (GSE14520), as shown in **Figure 1A** and **1B**; in addition, these observations were further validated by immunohistochemical (IHC) staining that DDX10 was up-regulated in large number of patients' tissues (**Figure 1C**). These data indicate that DDX10 is highly expressed in HCC tumor tissues.

Inhibition of DDX10 in HCC cell lines

To confirm the phenomenon, we further detected mRNA and protein levels of DDX10 in 3 HCC cells line, LO2 is normal liver cell lines as a control. We found no change of DDX10 in HepG2 cell lines but prominently up-regulated in Hep3B and Huh7 cell lines (**Figure 2A**), which selected for investigating the biological functions of DDX10 in HCC. Specific shRNAs were employed to silence DDX10. The result showed sh-#1 and sh-#2 both inhibit DDX10 in Hep3B and Huh7. Sh-#2 exhibited more effective to knock-down DDX10 compared with sh-#1 (**Figure 2B**).

Expression of DDX10 promotes cell viability and proliferation in HCC cell lines

To clarify the cellular functions of DDX10, we then performed CCK8 viability assay for HCC cell lines, Hep3B, Huh7, which treated with Sh-#1 and Sh-#2. As illustrated in **Figure 3A** and **3B**, cell viability was significantly decreased after inhibition of DDX10 by sh-#1 and sh-#2 in Hep3B and Huh7 cell lines. Clone formation assay showed that the cell proliferation was remarkably inhibited by the inhibition of DDX10 (**Figure 3C**, **3D**). Taken together, we confirmed that DDX10 plays a positive role in cell viability and proliferation.

Expression of DDX10 prominently promotes Wnt/β-catenin signaling in HCC cells

As Wnt/β-catenin pathway plays a key role in HCC cell growth, this promoted us to explore the correlation between DDX10 and Wnt/β-catenin signaling. We further performed dual luciferase reporter assays in two stable cell lines, Hep3B and MHCC-LM3, as illustrated in **Figure 4A** and **4B**, the absence of DDX10 significantly inhibited the activity of Wnt/β-catenin signaling. In addition, we found that inhibition of DDX10 results in the robustly decreased β-catenin in nuclear (**Figure 4C**). These results reminded us that DDX10 promotes the nuclear localization of β-catenin. To further confirm DDX10 function, Wnt/β-catenin signaling was inhibited in MHHC-LM3 stable cell line through IWR-endo, as shown in **Figure 4D**, we found cell proliferation is partly inhibited after ectopic expression of DDX10. Taken together, these findings suggested that DDX10 promotes the nuclear location of β-catenin to promote cell proliferation and viability.

Discussion

Hepatocellular carcinoma (HCC) is the most common tumor of liver parenchyma and the fifth most common cancer in the world [14]. The epidemiological distribution of HCC varies across the globe being the highest in south East Asia and sub-Saharan Africa [15]. Previously, we found that DDX10 is up-regulated in HCC tissues, but the underlying mechanisms remain to be illuminated.

This is the first report focus on the biological functions and associated mechanisms of DDX10 in HCC. DDX10, previously reported as a tumor suppressive gene in ovarian cancer [11], is highly expressed in malignant tissues compare to adjacent tissues. This result reminded us that DDX10 may play a role in HCC tumorigenesis. Furthermore, we elucidated that silencing DDX10 significantly inhibited cell viability and proliferation. This finding indicates that DDX10 promotes HCC cell proliferation in the tumor microenvironment.

Considering that abnormal activation of Wnt/β-catenin signaling is observed in numerous solid tumors and it plays a central role in regulating cancer cell proliferation, growth, survival and angiogenesis [16, 17], we hypothesized that there might be some significance between DDX10 and Wnt/β-catenin signaling. We then find that the activity of Wnt/β-catenin signaling is positively correlated with the level change of DDX10. Additionally, we found that the level of nucleus β-catenin, which functions as a transcription factor to target downstream genes in nucleus, was subsequently decreased when the level of DDX10 is inhibited. These findings suggested that DDX10 could in some way promote β-catenin enter nucleus, and finally promotes cell proliferation and growth, while the detailed mechanisms remain to be elucidated in further study.

Taken together, in current study we reveal that the DDX10 is commonly up-regulated in HCC tissues, and this expression pattern plays an oncogenic role. And we further find that DDX10 might regulate HCC cell proliferation through regulating Wnt/β-catenin pathway. Revealing a dual role of PLAC8 in tumorgenesis and giving a novel view to investigate the molecular pathogenesis of HCC.

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Disclosure of conflict of interest

None.

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