ZNF382 inhibits hepatocellular carcinoma (HCC) cell proliferation and motility and induces apoptosis by up-regulating SOX11

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Abstract

Background and Objective: To detect the mRNA and protein levels of Zinc-finger protein382 (ZNF382) in human hepatocellular carcinoma (HCC) tissues and cell lines and investigate its effects on the cellular processes of HCC cells in vitro.

Methods: Quantitative PCR and Immunoblot assays were respectively conducted to detect the alteration of ZNF382 mRNA and protein levels in HCC human tissues and cell lines. MTT, FCM, wound closure, and transwell assays were performed to detect the effects of ZNF382 on HCC cell proliferation, apoptosis, and motility of HCC cells in vitro. Immunoblot assays were then performed to detect the effects of ZNF382 on the expression of Signal Transducer and Activator of Transcription 3 (STAT3) and SOX11 in HCC cells, and the rescue assays were conducted to confirm the conclusion.

Results: We found ZNF382 was low expression in human HCC tissues and cell lines. ZNF382 suppressed the proliferation and stimulated the apoptosis of HCC cells. Additionally, ZNF382 inhibited the motility of HCC cells in vitro. Mechanically, ZNF382 mediated STAT3/SOX11, and therefore affected the proliferation, apoptosis, and motility of HCC cells.

Conclusion: We showed the abnormal low expression of ZNF382 in human HCC tissues and cell lines, and confirmed the effects of ZNF382 on HCC cellular processes. We thought ZNF382 was a tumor suppressor in HCC.

Keywords

Zinc-finger protein382 (ZNF382); Hepatocellular carcinoma (HCC); Signal transducer and activator of transcription 3 (STAT3); SOX11; Proliferation; Apoptosis

1. Introduction

In China, the vast majority types of primary liver cancer is hepatocellular carcinoma (HCC), accounting for more than 90% [1]. HCC is also one of the most common causes of tumor-related death in the world [2]. HCC is highly malignant and prone to recurrence and metastasis [3]. Therefore, early diagnosis and precise treatment of HCC are crucial [4]. The main causes of HCC include hepatitis B and C virus infection, aflatoxin, liver cirrhosis, and sex hormones [5]. The early clinical symptoms of HCC are not obvious, and the main clinical symptoms are pain and fatigue in the liver area, etc., which are often in the advanced stage, leading to poor prognosis [6]. To improve the prognosis of HCC patients, it is necessary to further explore the pathogenesis of HCC and find out the key regulatory proteins and tumor suppressor genes involved. (ZFPs) are the largest family of transcription factors, in-
cluding approximately 800 members [7]. Zinc-finger protein382 (ZNF382) is a critical member in Krüppel-associated box ZFP proteins, playing multiple important roles in mediating the expression of downstream genes, and serving as a transcription inhibitor [8]. Studies showed that FOS, FZD1, and JUN could be suppressed by ZNF382, activating p53 and suppressing Wnt pathway [9]. Another study showed the wide expression of ZNF382 in early stages of embryonic, suggesting ZNF382 acts as a critical role in embryonic development [8].

Notably, ZNF382 is also known as a tumor suppress protein, in multiple types of tumors, such as esophageal cancer, breast cancer, and gastric cancer [10–12]. ZNF382 could affect multiple types of cancer cell processes, such as proliferation, colony formation, motility, and apoptosis [13]. ZNF382 is often down-regulated in multiple types of cancers due to its capacity of hypermethylation of promoter [14]. In fact, ZNF382 was silenced in multiple tumors due to promoter methylation [12]. A previous study provided the evidence that ZNF382 served as a tumor-suppressor in HBV-related HCC tumorigenesis [9]. However, the possible mechanism needs further study.

Herein, we assessed the expression levels of ZNF382 in HCC, and investigated the effects of ZNF382 on HCC cellular processes. Our data confirmed the abnormal low expression of ZNF382 in HCC, and confirmed the effects of ZNF382 on HCC cell proliferation, apoptosis, and motility via STAT3/SOX11 axis. We thought ZNF382 was a tumor suppressor in HCC.

2. Methods

2.1 Antibodies, plasmids, and siRNAs

Anti-ZNF382 antibody (1 : 2000, ab254918, abcam, USA), anti-STAT3 antibody (1 : 2000, ab68153, abcam, USA), anti-SOX11 antibody (1 : 1500, ab170916, abcam, USA), anti-β-actin antibody (1 : 1000 dilution, ab8226, abcam, USA).

pcDNA3.1-vector, ZNF382, and STAT3 pcDNA3.1 plasmids were all bought from the Addgene, ZNF382 and SOX11 siRNAs, were bought from Riobio.

2.2 Samples

Tumor tissues were obtained from the Affiliated Hospital of North Sichuan Medical College. All the procedures conducted in this study involving human participants were according to the standards upheld by the Ethics Committee of the Affiliated Hospital of North Sichuan Medical College and based on the 1964 Helsinki Declaration and its later amendments for ethical research involving human subjects.

2.3 Cell culture and transfection

The human normal liver cell line, L02, and 4 types of HCC cell lines, including HCCLM3, Huh7, MHCC97-H, and SK-HEP-1 were all bought from ATCC (Maryland, USA), and cultured in the DMEM, added with 10% of fetal bovine serum (FBS) at 37 °C in a 5% CO2 incubator. The plasmids and siRNAs were transfected into HCC cells by the use of lipofectamine 2000 (Invitrogen, USA).

2.4 Quantitative PCR assays

Total RNAs from HCC tissues or cell lines were extracted by Trizol (Invitrogen, USA) reagent. The reverse transcription was performed using reverse transcriptase (M1701, Promega, Wisconsin, USA) to develop cDNA profiles for every sample. Quantitative PCR was conducted using SYBR Ex Taq kit (Takara, Japan), and ZNF382 expression was normalized to the GAPDH mRNA levels. The primers were as follows:

<table>
<thead>
<tr>
<th>Genes</th>
<th>Forward primer (5’-3’)</th>
<th>Reverse primer (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ZNF382</td>
<td>AGGCTGATATGATCGGCAAG</td>
<td>TGAGGGGTCTAGAATGCCTG</td>
</tr>
<tr>
<td>GAPDH</td>
<td>GAGTCAACCAGATTGTCGCTG</td>
<td>TGGATTTGAGGGATCTCG</td>
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2.5 Immunoblot assays

HCC cells or tissues were lysed to isolate the total protein. Protein was then separated through SDS-PAGE method and then transferred onto the PVDF membranes. The membranes were subsequently blocked using 5% BSA in Tris-buffered saline (TBS) buffer for 2 h and incubated with the indicated antibodies overnight at 4 °C. Then the membranes were then incubated with secondary antibodies for 1 h. Signals were visualized with the ECL kit. Quantification in the blots was conducted by the use of ImageJ software.

2.6 MTT assays

HCC cells were seeded into each 96-well plate for 48 h. The adherent cells were fixed and then stained with MTT solution buffer (2.5 mg/mL). Finally, the absorbance at 490 nm wavelength was measured. The cell proliferation degree was calculated in each group.

2.7 Flow cytometry analysis

For flow cytometry detection, HCC cells after the indicated treatment were collected by trypsinization and washed with PBS. Then cells were administrated to annexin V/propidium iodide (PI) staining (Dojindo, Kumamoto, Japan) following the guideline of manufactures.

2.8 Wound healing assays

HCC cells were transfected with the indicated drugs, maintained for a total of 48 h and mechanically wound was made using a 20-μL pipette tip. Then cells were washed for 3 times, and the serum-free medium was re-added to stimulate healing. Photographs were taken at 0 h and 24th time point, and the relative degree of healing was compared.

2.9 Transwell assays

The upper chambers of (8.0 μm pore) transwells added with 20% matrigel were incubated at 37 °C for 30 min. Then HCC cells were all added to the upper of the chambers to stimulate
ZNF382 was significant low expression in HCC.

(A). Quantitative PCR assays showed mRNA levels of ZNF382 in tumor tissues and normal from 40 HCC patients ($P < 0.001$). (B). Immunoblot assays showed the expression of ZNF382 in representative tumor tissues and normal from 3 HCC patients. (C). Quantitative PCR assays showed the mRNA of ZNF382 in the indicated cell lines, including HCCLM3, Huh7, MHCC97-H, and SK-HEP-1. (D). Immunoblot assays showed the protein levels of ZNF382 in the indicated HCC cell lines. (E). The mRNA levels of ZNF382 in the indicated cell lines. (F). The protein levels of ZNF382 in the indicated cell lines. Results are presented as mean ± SEM, *** $P < 0.001$.

3. Results

3.1 ZNF382 was significant low expression in HCC tissues and cell lines

To investigate the possible role of ZNF382 in HCC progression and development, we first detected its mRNA levels in tissues from a total of 40 HCC patients. Through the analysis in GEPIA database, we found ZNF382 was high...
expression in tumor tissues compared to normal tissues (Fig. 1A). Additionally, ZNF382 was correlated with the survival of patients (Fig. 1B). Through quantitative PCR assays, we found ZNF382 was obvious low expression in tumor tissues (Fig. 1C). Subsequently, we detected the protein levels of ZNF382 in 3 representative tumor tissues and normal tissues through Immunoblot assays. Similar to the previous data, we found ZNF382 protein levels were lower in 3 different tumor tissues than normal (Fig. 1D).

Then we assessed the expression levels of ZNF382 in normal liver cell line, L02, and 4 types of HCC cell lines, including HCCLM3, Huh7, MHCC97-H, and SK-HEP-1. Through quantitative PCR assays, we showed the low ZNF382 mRNA levels in the 4 types of HCC cell lines, compared to normal (Fig. 1E). The results of Immunoblot assays further confirmed the low ZNF382 expression in HCC cell lines, compared to that in normal liver cell line (Fig. 1F). Collectively, these study confirmed ZNF382 was significant low expression in HCC.

3.2 ZNF382 inhibits the proliferation and stimulates apoptosis of HCC cells in vitro

We next investigated the effects of ZNF382 on the proliferation and apoptosis of HCC cells. The ZNF382 overexpression plasmid, pcDNA3.1-ZNF382, and the siRNA of ZNF382, were respectively transfected into a HCC cell line, MHCC97-H, to alter its expression in HCC cells. Through Immunoblot assays, we noticed the protein levels were obvious upregulated upon ZNF382 overexpression in MHCC97-H cells, and were significantly decreased after the transfection of siRNA-ZNF382 (Fig. 2A).

Through MTT assays, we found ZNF382 overexpression suppressed the proliferation of MHCC97-H cells, with the obvious decrease OD value at 490 nm wavelength (Fig. 2B). Reversely, ZNF382 depletion promoted the proliferation of MHCC97-H cells (Fig. 2B). We further performed FCM assays to detect the effects of ZNF382 on HCC cell apoptosis. We found that ZNF382 overexpression stimulated the apoptosis of MHCC97-H cells, and its depletion suppressed the apoptosis (Fig. 2C-E). Therefore, we thought ZNF382 inhibited the proliferation and stimulated apoptosis of HCC cells.

3.3 ZNF382 restraints the motility of HCC cells

We then focused the effects of ZNF382 on HCC cell motility. Through wound closure assays, we noticed ZNF382 overexpression inhibited the migration of MHCC97-H cells, as a comparison, we found knockdown of ZNF382 promoted MHCC97-H cell migration (Fig. 3A). Performing transwell assays, we provided the evidence that ZNF382 overexpression suppression the invasion of MHCC97-H cells, and its depletion promoted cell invasion (Fig. 3B). Therefore, we thought ZNF382 suppressed the motility of HCC cells in vitro.

3.4 ZNF382 mediated the expression of STAT3 and SOX11 in HCC cells

We then explored the possible mechanism underlying ZNF382 affecting HCC progression. It was reported that ZNF382 suppressed the expression of STAT3, which negatively regulated the expression of SOX11 [12, 15]. The effects of SOX11 in HCC progression has been reported [16]. Therefore, we assessed the effects of ZNF382 on STAT3 and SOX11 expression in HCC cells.

We then found the overexpression of ZNF382 decreased the expression of STAT3 in MHCC97-H cells, and increased SOX11 expression (Fig. 4A). We next performed the rescue assays and found that STAT3 overexpression rescued the decrease of STAT3 expression and the upregulation of SOX11 expression caused by ZNF382 overexpression, further confirmed the regulation of ZNF382 on STAT3/SOX11 axis (Fig. 4B).

3.5 ZNF382 mediates HCC cell processes via mediating STAT3/SOX11 axis

We then investigated whether ZNF382 affected HCC cell processes via mediating STAT3/SOX11 axis. Through MTT assays, we found ZNF382 overexpression suppressed the proliferation of MHCC97-H cells, whereas SOX11 depletion rescued the inhibition of proliferation caused by ZNF382 overexpression (Fig. 5A). Through FCM assays, we confirmed SOX11 depletion also reversed the stimulation of cell apoptosis caused by ZNF382 overexpression (Fig. 5B). Through wound closure and migration assays, we further found the knockdown of SOX11 rescued the suppression of cell motility of MHCC97-H cells after the overexpression of ZNF382 (Fig. 5C,D). Collectively, these data confirmed ZNF382 mediated HCC cell processes via mediating STAT3/SOX11 axis.

4. Discussion

The number of new liver cancer patients in China is 370,000 every year, among which 90% are HCC and 70% are at advanced stage [17]. Nowadays, liver cancer is mainly treated with chemotherapy [18]. Despite the rise of targeted therapy and immunotherapy, the total survival rate of 5 years is still low [19]. The reason of poor prognosis of liver cancer is high recurrence rate and metastasis rate [3]. To further improve the survival rate of patients with advanced HCC, it is still necessary to further explore the pathogenesis of HCC, find out the key tumor suppressor genes, and further develop effective targeted therapies for HCC [20]. Interestingly, we here found a transcription factor, ZNF382, had the potential to play as a tumor suppressor in HCC progression. We found ZNF382 was low expression in human HCC tissues and cell lines. Moreover, ZNF382 depletion promoted the proliferation and motility, and suppressed the apoptosis of HCC cells. Therefore, we identified a promising tumor suppressor in HCC.
FIG. 2. ZNF382 inhibits the proliferation and stimulates apoptosis of HCC cells in vitro. (A) Immunoblot assays showed ZNF382 expression in MHCC97-H cells upon the transfection of the indicated plasmids or siRNAs. (B) MTT assays showed the OD value at 490 nm wavelength of MHCC97-H cells upon the indicated treatment. (C) FCM assays showed the apoptosis degree of MHCC97-H cells upon the transfection of the indicated plasmids or siRNAs. (D) The percentage of apoptosis MHCC97-H cells in the indicated group. (E) The percentage of apoptosis HCCLM3 cells in the indicated group. The percentage of apoptosis cells was calculated and compared. Results are presented as mean ± SEM, pcDNA3.1-ZNF382 verus pcDNA3.1, * P < 0.05, ** P < 0.01, *** P < 0.001. siRNA-ZNF382 verus control siRNA, # P < 0.05, ## P < 0.01, ### P < 0.001.
FIG. 3. ZNF382 inhibits the motility of HCC cells in vitro. (A). The migration capacity of MHCC97-H cells upon the transfection of the indicated plasmids or siRNAs. (B). The invasion capacity of MHCC97-H cells upon the indicated treatment, and the number of invasive cells were counted. Results are presented as mean ± SEM, pcDNA3.1-ZNF382 verus pcDNA3.1, **P < 0.01, ***P < 0.001. siRNA-ZNF382 verus control siRNA, ###P < 0.001.

FIG. 4. ZNF382 mediated the expression of STAT3 and SOX11 in HCC cells. (A). The expression of STAT3 and SOX11 in MHCC97-H cells upon the transfection of pcDNA3.1-ZNF382 plasmids. (B). The expression of STAT3 and SOX11 in MHCC97-H cells upon the transfection of indicated plasmids. Results are presented as mean ± SEM, pcDNA3.1-ZNF382 verus pcDNA3.1, **P < 0.01, ***P < 0.001. pcDNA3.1-ZNF382 + STAT3 verus pcDNA3.1-ZNF382, ###P < 0.001.

and esophageal cancer [14]. ZNF382 regulated EMT and played as a tumor suppressor in gastric cancer [21]. Additionally, ZNF382 suppressed NF-κB and AP-1 reporter activities in colon cancer, and therefore suppressed the cancer progression [12]. Notably, ZNF382 was frequently downregulated in several cancer cell lines and primary tumors, such as gastric cancer and esophageal cancer [14]. These studies confirmed ZNF382 acted as a critical tumor suppressor in different types of cancers.

Previous studies provided the evidence that ZNF382 was downregulated in multiple types of tumors due to the promoter hypermethylation [14]. It was noticed that the methylation of promoter CpG island had the potential to promote the suppression of gene expression [21]. In this study, we confirmed the low expression of ZNF382 in human HCC tissues, and its depletion promoted HCC progression in vitro. ZNF382 had the potential to affect the methylation of downstream genes, and therefore affected HCC progression. Previous studies showed the expression ZNF382 was lower in ESCC than normal tissues [10]. The previous study also showed ZNF382 affected multiple cell biological processes, including cell growth (VEGFA and CSF1), metastasis (SLUG and SNAIL), and regulation of stem cell differentiation (SOX2) through the ectopic expression ZNF382 in ESCC.
FIG. 5. ZNF382 mediates HCC cell processes via mediating STAT3/SOX11 axis. (A). MTT assays showed the OD value at 490 nm wavelength of MHCC97-H cells upon the indicated treatment. (B). FCM assays showed the apoptosis degree of MHCC97-H cells upon the transfection of the indicated plasmids or siRNAs. The percentage of apoptosis cells was calculated and compared. (C). (A). Wound closure assays showed the migration capacity of MHCC97-H cells upon the transfection of the indicated plasmids or siRNAs. (B). Transwell assays showed the invasion capacity of MHCC97-H cells upon the indicated treatment, and the number of invasive cells were counted. (D). Transwell assays showed the migration capacity of MHCC97-H cells in the indicated group. (E). ZNF382 affected HCC progression via affecting proliferation, apoptosis, and motility through STAT3/SOX11 axis. Results are presented as mean ± SEM, pcDNA3.1-ZNF382 verus pcDNA3.1, **P < 0.01, ***P < 0.001. pcDNA3.1-ZNF382 + SOX11 siRNA verus pcDNA3.1-ZNF382, ###P < 0.001.

In conclusion, our data confirmed the abnormal low expression of ZNF382 in human HCC tissues and cell lines, and confirmed the effects of ZNF382 on HCC cell proliferation, apoptosis, and motility via STAT3/SOX11 axis (Fig. 5E). We thought ZNF382 was a tumor suppressor in HCC.

Author contributions
ZL and YZ designed the study, supervised the data collection, JC analyzed the data, interpreted the data, CL, JL and SH prepare the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

Ethics approval and consent to participate
Ethical approval was obtained from the Ethics Committee of Affiliated Hospital of North Sichuan Medical College.
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Conflict of interest

The authors state that there are no conflicts of interest to disclose.

Statement of informed consent

Written informed consent was obtained from a legally authorized representative(s) for anonymized patient information to be published in this article.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

References