Original Paper

Long Non-Coding RNA Linc-USP16 Functions As a Tumour Suppressor in Hepatocellular Carcinoma by Regulating PTEN Expression

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Key Words

Long non-coding RNA • PTEN • miR-21 • AKT • Hepatocellular Carcinoma

Abstract

Background/Aims: Recent evidence has indicated the crucial regulatory roles of long non-coding RNAs (lncRNAs) in tumour biology. In hepatocellular carcinoma (HCC), aberrant expression of lncRNAs plays an essential role in HCC tumorigenesis. However, the potential roles and regulatory mechanisms of the novel human lncRNA, Linc-USP16, in HCC are unclear.

Methods: To investigate the function of Linc-USP16 in HCC, we first analysed the expression levels of Linc-USP16 in HCC patient tissues and cell lines via q-RT-PCR and established overexpressed or knockdown HCC cell lines. **Results:** Here, we found that Linc-USP16 expression was significantly down-regulated in HCC patient tissues and cell lines. Further functional experiments suggested that Linc-USP16 could directly increase PTEN expression by acting as a competing endogenous RNA (ceRNA) for miR-21 and miR-590-5p. These interactions led to repression of AKT pathway and inhibition of HCC cell proliferation and migration. **Conclusion:** Thus, our data showed that Linc-USP16, as a tumour suppressor, plays an important role in HCC pathogenesis and provides a new therapeutic strategy for HCC treatment.

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Introduction

Hepatocellular carcinoma is one of the most common cancers and is the third leading cause of cancer-related deaths in the world [1]. In recent years, specific surgery, radio frequency ablation, and chemotherapy have been common treatments for HCC [2, 3].

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However, owing to disease recurrence and metastasis, the overall survival for HCC patients is still unsatisfactory. To improve the survival rate, an in-depth exploration of the molecular mechanisms of HCC tumorigenesis and further searches for new targets that affect tumour proliferation and metastasis are crucial for HCC treatment.

Long non-coding RNAs (lncRNAs) are a class of transcripts longer than 200 nucleotides with no protein-coding capacity [4-6]. Many reports have shown that lncRNAs are frequently deregulated in various cancers and play important roles in cell migration, proliferation, apoptosis, and tumorigenesis by modulating gene expression at the chromatin organization, transcriptional, and post-transcriptional levels [4, 7, 8]. In hepatocellular carcinoma, several lncRNAs have been reported to take part in the pathogenesis of HCC as oncogenes or tumour suppressor genes. For example, ZFAS1, which encodes a lncRNA that is frequently amplified in HCC, functions as an oncogene and promotes cell growth and metastasis by binding miR-150 [9]. LncRNA-PRAL, a p53 regulation-associated lncRNA on chromosome 17p13.1, has been reported to be down-regulated in HCC tissues and inhibit HCC growth and induce apoptosis *in vivo* and *in vitro* via p53 [6, 10-12]. Although more and more lncRNAs have been demonstrated as important regulators of HCC pathogenesis, the relationship between the majority of lncRNAs and HCC remains to be explored.

In this study, we found that Linc-USP16 was down-regulated in HCC tissues and cell lines when compared with normal tissues, which promoted the proliferation and metastasis of HCC cells. We then investigated the molecular mechanisms of Linc-USP16 during HCC progression and found that Linc-USP16 can directly increase PTEN expression by acting as a competing endogenous RNA (ceRNA) for miR-21 and miR-590-5p. This interaction leads to repression of AKT pathway and inhibition of HCC cell proliferation and migration. Therefore, our data provide a new therapeutic strategy for HCC treatment.

Materials and Methods

Cell culture and Reagents

MHCC97H, MHCC97L, HepG2, SMMC-7721, and LO2 cells were obtained from the China Infrastructure of Cell Line Resources (Beijing, China). MHCC97H, MHCC97L, SMMC-7721, HepG2, and LO2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin. BEL7402 cells were grown in PRMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 50 U/ml penicillin, and 50 µg/ml streptomycin. The cells were maintained at 37°C under 5% CO₂ in humidified air. The following antibodies were used in this study: anti-GAPDH (Santa Cruz Biotechnology, SC-32233, 1:1000), LY294002 (Cell Signaling, 9901), anti-PTEN (Abcam, ab32199, Y184, 1:500 dilution), anti-Akt (Cell Signaling, 9272, 1:1, 000), anti-pThr308-Akt (Cell Signaling, 9275, 1:1, 000), anti-pSer473-Akt (Cell Signaling, 4060, D9E, 1:1, 000), anti-GSK3 (Cell Signaling, 9325, 1:1, 000), and anti-pSer9-GSK3 (Cell Signaling, 9336, 1:1, 000).

Western blot analysis

Total protein extracted from HCC tissues and HCC cells were used for immunoblotting. In brief, cell lysates were clarified by centrifugation at 9,000 g for 10 min, and the supernatant was collected after centrifugation. Protein concentration was determined using the BCA Protein Assay Kit (Pierce, U.S.A.). Total protein (30-60 µg) was separated on an 8% or 10% SDS-PAGE mini-gels followed by transfer to a nitrocellulose (NC) membrane. After blocking with TBST (50 mM Tris-HCl, 0.15 M NaCl, 0.1% Tween-20, pH 7.5) containing 5% fat-free dry milk overnight at 4°C, the membrane was incubated with antibodies, and an enhanced chemiluminescence (ECL) detection system (Amersham) was used to visualize target protein expression. Three samples from each group were analysed, and the results were quantified using Gel-Pro 4.0 analyser software.

HCC tissues were obtained from Shanghai Outdo Biotech (Shanghai, China) and contained 70 HCC tissues and their corresponding adjacent non-malignant normal tissues. Carcinoma tissue samples and the corresponding adjacent tissue samples were obtained in the files of Taizhou Hospital of Zhejiang Province from 2010 to 2015. The characteristics of the patients and their tumours were collected through review of

medical records and pathologic reports. Informed consent with approval of the ethics committee of Taizhou Hospital of Zhejiang Province was obtained. All methods in this study were conducted in accordance with the approved guidelines, and all the experimental protocols were approved by the ethics committee of Taizhou Hospital of Zhejiang Province.

Lentivirus transfection

Linc-USP16 shRNA and miR-21, miR-21 inhibitor, miR-590-5p, and miR-590-5p inhibitor were purchased from GenePharma (Shanghai, China). The lentiviral transduction particles for shRNA-mediated knockdown of PTEN were purchased from Sigma (Shanghai, China). The shRNA sequence targeting PTEN was 5'-GCGCTATGTGTATTATTAT-3'. The shRNA was cloned using the PLKO.1 vector. Stable knockdown cells were established as previously described [13-15].

Human Linc-USP16 cDNA was inserted into the pCDH vector using the following primers: F: 5-TAATAACATCTACCAACAG-3 and R: 5-ACTGGGTCAGAGAATACA-3. Stable Linc-USP16-overexpressing cells were generated as previously described [16, 17].

Dual-luciferase reporter assay.

The assay was performed as previously described [13, 18].

Cell viability assay and colony formation assay

Cells were plated in 96-well plates at a density of 5000 cells in 100 mL medium per well 24 h before the experiment. Cell viability was assessed by the CKK-8 assay.

For the colony formation assay, adherent cells were trypsinised following treatment, and 1000 viable cells were subcultured in six-well plates (in triplicate). The cells were allowed to adhere and colonize for two weeks. To visualize colonies, that media was removed, and the cells were fixed in 96% ethanol for 10 min and then stained with crystal violet staining solution.

RNA extraction and real-time PCR

Total RNA was isolated using Trizol (Invitrogen). One microgram of total RNA was used to synthesize cDNA with the PrimeScript™ RT reagent kit (Takara, DRR037A) according to the manufacturer's instructions. Real-time PCR was performed using SYBR premix EX Taq (TaKaRa) and ROX and analysed with Stratagene Mx3000p (Agilent Technologies). The real-time PCR primer sequences were as follows: Actin 5'-CTCCATCCTGGCCTCGCTGT-3' and 5'-GCTGTACCTTCACCGTTCC-3' and Linc-USP16 5'-AGAGCCAAATAGACTACAAATCGG-3' and 5'-AATTTCCACACTCTTACCAG-3'. The primers for mature miR-21 and miR-590-5p were purchased from Life Technologies.

Statistical analysis

Data are expressed as the mean ± SD and were analysed using unpaired 2-sided Student's *t* test. Statistical analysis was performed using SPSS 18.0. *P* values <0.05 were considered statistically significant and are indicated as follows: **P* <0.05, ***P* <0.01, ****P* <0.001.

Results

The expression level of Linc-USP16 is significantly decreased in HCC tissues and cells

To determine whether Linc-USP16 plays an important role in the development of HCC, we first measured the expression level of Linc-USP16 in a number of HCC cell lines by q-RT-PCR and discovered a much lower expression of Linc-USP16 in HCC cell lines compared with the human hepatic cell line LO2 (Fig. 1a). Subsequently, we examined Linc-USP16 expression in 70 patients with HCC, and normalized Linc-USP16 expression to that of actin. The expression level of Linc-USP16 was significantly down-regulated in most of the HCC tissues compared with the adjacent normal tissues (Fig. 1b). Therefore, remarkable down-regulation of Linc-USP16 is frequently observed in majority of the HCC tissues and cell lines.

To investigate the correlation between Linc-USP16 expression and clinicopathologic parameters, the 70 HCC patients were classified into a relatively high group or a relatively low

Fig. 1. Analysis of Linc-USP16 expression in both HCC cell lines and tissues. (a) Relative expression levels of Linc-USP16 in five HCC cell lines and the non-tumourigenic human cell line LO2 were detected by q-RT-PCR. * $P < 0.05$, two-sided Student's t-test; $n = 3$. (b) Different Linc-USP16 expression levels in HCC tissues and adjacent normal tissues from 70 patients were assessed by q-RT-PCR. *** $P < 0.001$, two-sided Student's t-test; $n = 3$. (c) Relationship between Linc-USP16 and clinicopathological parameters in HCC patients ($n = 70$).

group using the Mann-Whitney U test and Kruskal-Wallis test. We observed a statistical correlation between Linc-USP16 expression and tumour size ($p = 0.043$), clinical stage ($p = 0.031$), and metastasis ($p = 0.016$). No statistical association was observed between Linc-USP16 expression and the other clinicopathologic variables, including age ($p = 0.398$), gender ($p = 0.302$) and tumour stage ($p = 0.054$) (Fig. 3c).

Ectopic expression or down-regulation of Linc-USP16 affects HCC cell proliferation and migration

To investigate the biological function of Linc-USP16 in HCC, Linc-USP16 was stably expressed in BEL7402 and HepG2 cells (Fig. 2a). Compared with the control cells, elevated Linc-USP16 expression inhibited cell proliferation and colony formation (Fig. 2b-e). Conversely, decreasing Linc-USP16 expression promoted cell growth (Fig. 2f-h). We next assessed the effect of Linc-USP16 on cell migration and found that the expression level of Linc-USP16 was gradually down-regulated in MHCC-97L, MHCC-97H and HCC-LM3 cells with increasing metastatic potential (Fig. 2i). Boyden chamber transwell assays indicated that knockdown of Linc-USP16 in MHCC-97L cells increased cell migration (Fig. 2j-k). In contrast, the ectopic expression of Linc-USP16 impeded cell migration in MHCC-97H cells (Fig. 2l-m).

Linc-USP16 regulates PTEN protein levels and AKT signalling

Due to the pivotal role of the AKT pathway in regulating cell growth and migration of HCC cells [19, 20], we examined whether Linc-USP16 participates in controlling AKT

Fig. 2. Linc-USP16 suppressed cell growth and migration. (a) BEL7402 and HepG2 cells were transfected with or without Linc-USP16. The expression levels of Linc-USP16 were analysed by q-RT-PCR. (b-c) A colony formation assay was performed to measure the proliferation. $**P < 0.01$, Two-sided Student's t-test; $n = 3$. (d-e) Cell viability was detected by CKK8 assay. $*P < 0.05$, $**P < 0.01$, Two-sided Student's t-test; $n = 3$. (f) The BEL7402 and HepG2 cells with or without stable knockdown Linc-USP16 were established using shRNA vectors. The expression levels of Linc-USP16 were analysed by q-RT-PCR. (g-h) Colony formation assay was performed to measure the proliferation. $**P < 0.01$, Two-sided Student's t-test; $n = 3$. (i) The expression levels of Linc-USP16 in MHCC-97L, MHCC-97H, and HCC-LM3 were analysed by q-RT-PCR. (j-k) The effect of Linc-USP16 on migration were examined by Boyden chamber transwell assays in MHCC-97L cells with or without Linc-USP16 knockdown. $**P < 0.01$, Two-sided Student's t-test; $n = 3$. (l-m) Linc-USP16 was overexpressed in MHCC-97H. The effect of Linc-USP16 on the migration was examined by Boyden chamber transwell assays. $***P < 0.001$, Two-sided Student's t-test; $n = 3$.

activation. First, we overexpressed Linc-USP16 in BEL7402 and HepG2 cells. We found that overexpression of Linc-USP16 up-regulated PTEN protein levels and down-regulated AKT and GSK3 β phosphorylation (Fig. 3 a-b). Conversely, inhibition of Linc-USP16 expression by shRNA down-regulated PTEN protein levels and up-regulated AKT and GSK3 β

Fig. 3. Linc-USP16 enhanced PTEN expression and inhibited the AKT pathway. (a-b) Linc-USP16 was over-expressed in BEL7402 and HepG2 cells. Cell lysates were detected by western blot using the indicated antibodies. (c-d) The BEL7402 and HepG2 cells with or without stable Linc-USP16 knockdown were established using shRNA vectors. Cell lysates were detected by western blot using the indicated antibodies. (e) The expression levels of PTEN in HCC tissues and adjacent normal tissues from 30 patients and the correlation between PTEN and Linc-USP16 was analysed.

phosphorylation in BEL7402 and HepG2 cells (Fig. 2c-d). To further confirm the effect of Linc-USP16 on PTEN, we analysed the correlation between Linc-USP16 and PTEN in HCC tissues. We found that the expression of Linc-USP16 was significantly correlated with the expression of PTEN (Fig. 3e).

Linc-USP16 elevates PTEN expression levels by acting as a miR-21 and miR-590-5p sponge

To elucidate the potential molecular mechanism of Linc-USP16, we first detected the subcellular localization of Linc-USP16 using qRT-PCR and found that Linc-USP16 is primarily located in the cytoplasm of HCC cells. This result suggested that Linc-USP16 may exert its regulatory function at the post-transcriptional level (Fig. 4a). Previous reports have shown that lncRNAs can function as ceRNAs [21], and we hypothesized that Linc-USP16 may influence HCC tumorigenesis by inhibiting miRNA function. A bioinformatics analysis revealed that Linc-USP16 harbours binding sequences of the miR-21 and miR-590-5p, which were reported to inhibit PTEN expression in HCC cells [22, 23]. The sequences of Linc-USP16 with the miR-21/miR-590-5p binding site and a mutation (Linc-USP16 MUT) were inserted downstream of the luciferase gene to construct the pSICHECK2 vector for the luciferase assay (Fig. 4b). As shown in figure 4c and 4d, the luciferase activities were significantly decreased in the wild type groups compared with the control vector. However, the reduction in activity was restored when we mutated the binding site of miR-21/miR-590-5p, suggesting sequence-specific binding of miR-21/miR-590-5p to Linc-USP16 (Fig. 4c-f).

Fig. 4. Linc-USP16 functioned as a miR-21/miR-590-5p decoy. (a) Fractionation of BEL7402 cells followed by q-RT-PCR (left panel) and fractionation controls by western blot (right panel). U1 RNA served as a positive control for nuclear gene expression. N, Nuclear fraction; C, Cytoplasmic fraction. Data are representative of at least three independent experiments. (b) Schematic diagrams of the sequence containing the wild type binding site psiCHECK2-Linc-USP16-WT or mutant binding site psiCHECK2-Linc-USP16-MUT. (c-d) miR-21 or miR-590-5p mimics or control (CTR) were cotransfected with Linc-USP16-WT or MUT in BEL7402 cells. Luciferase activity was measured. The data represent mean $\pm$ SD of three independent experiments. $**p < 0.01$ vs. CTR. (e-f) The expression levels of miR-21 or miR-590-5p were detected by q-RT-PCR. (g-h) Linc-USP16 WT or MUT were cotransfected with or without PTEN 3'UTR in BEL7402 cells. Luciferase activity was measured. The data represent mean $\pm$ SD of three independent experiments, $***p < 0.001$ vs. CTR (g). The protein levels of PTEN and pAKT (S473) were detected by western blot (h). (i) PTEN 3'UTR WT or MUT were transfected into the BEL7402 cells with or without stably expression of Linc-USP16. Luciferase activity was measured. The data represent mean $\pm$ SD of three independent experiments. $***p < 0.001$ vs. CTR. (j) The PTEN 3'UTR was transfected into the BEL7402 cells with or without stable knockdown of Linc-USP16. Luciferase activity was measured. The data represent mean $\pm$ SD of three independent experiments, $*p < 0.05$ vs. NC. (k) miR-21 or miR-590-5p mimics or control (CTR) were cotransfected with PTEN 3'UTR into BEL7402 cells with or without stable expression of Linc-USP16. Luciferase activity was measured. The data represent mean $\pm$ SD of three independent experiments, $*p < 0.05$ vs. CTR.

To assess the relationship between Linc-USP16 and PTEN, the PTEN 3'UTR was cotransfected with pCDH-Linc-USP16WT and Linc-USP16 MUT into BEL7402 cells for a luciferase assay. Compared with the control cells, overexpression of Linc-USP16 WT, but not that of the Linc-USP16 MUT, could significantly enhance the luciferase activity of PTEN 3'UTR and increase PTEN protein levels following inhibition of AKT phosphorylation (Fig. 4g-h). Subsequently, the PTEN 3'UTR WT or MUT were co-transfected with or without Linc-USP16, and the activities were detected by luciferase assay. As shown in figure 4i, the

Fig. 5. Linc-USP16 regulated cell growth and migration via the miR-21/miR-590-5p axis. (a-e) miR-21 or miR-590-5p inhibitor or negative control was transfected into BEL7402 cells with or without Linc-USP16 knockdown. Colony formation assay and Boyden chamber transwell assay were performed to measure the proliferation and migration. The data are presented as the mean $\pm$ SD from three experiments, ** P <0.01, *** P <0.001 compared with the control, Two-sided Student's t -test; n = 3. The PTEN protein level was detected by western blot (e). (f-j) Linc-USP16 was overexpressed in BEL7402 cells with or without stable PTEN knockdown. The colony formation assay and Boyden chamber transwell assay were performed to measure proliferation and migration. The data are presented as the mean $\pm$ SD from three experiments, * P <0.05, ** P <0.01, *** P <0.001 compared with the control, Two-sided Student's t -test; n = 3. The protein level of PTEN was detected by western blot (j). (k-l) BEL7402 cells with or without knockdown Linc-USP16 or PTEN were treated with LY294002. A colony formation assay was performed to measure the proliferation. The data are presented as the mean $\pm$ SD from three experiments, ** P <0.01 compared with the control, Two-sided Student's t -test; n = 3.

luciferase activity of the PTEN 3'UTR was elevated by Linc-USP16, but this elevation was abolished when the sequence of the PTEN 3'UTR, which was bound by miR-21/miR-590-5p, was mutated. Similarly, the luciferase activity was suppressed in Linc-USP16 knockdown cells (Fig. 4j).

To further illuminate the effect of Linc-USP16 on PTEN via miR-21/miR-590-5p, miR-21 or miR-590-5p were co-transfected with the PTEN 3'UTR into BEL7402 cells with or without overexpressing Linc-USP16, and the luciferase activity was analysed. The data indicated that miR-21/miR-590-5p could inhibit the luciferase activity of PTEN3'UTR, which was restored in Linc-USP16 overexpressed cells (Fig. 4k).

Linc-USP16 inhibits cell proliferation and migration via regulation of the miR-21/miR-590-5p-PTEN axis

To validate the effect of Linc-USP16 on cell proliferation and migration via the miR-21/miR-590-5p-PTEN axis, we first introduced a miR-21 or miR-590-5p inhibitor into Linc-USP16 stable knockdown cells or not. Compared with the control group, inhibition of Linc-USP16 expression promoted cell growth and migration. However, the phenotype could be reversed by miR-21 or miR-590-5p inhibitor treatment (Fig. 5a-d). Similarly, the protein level of PTEN was suppressed by Linc-USP16, and this effect was reversed by miR-21 or miR-590-5p inhibitor treatment (Fig. 5e). Subsequently, we inhibited PTEN expression in Linc-USP16 overexpressed cells or not. We found that elevated Linc-USP16 decreased cell proliferation and migration. The effect of Linc-USP16 was eliminated by PTEN inhibition (Fig. 5f-j). To further confirm the influence of Linc-USP16 on cell growth via regulation of the PI3K/AKT pathway, BEL7402 cells with or without stable knockdown Linc-USP16 or PTEN were treated with LY294002, which inhibits the PI3K/AKT pathway. As shown in figure 5k-l, LY294002 treatment prevented cell growth, which was induced by Linc-USP16 or PTEN knockdown (Fig. 5k-l).

Discussion

The noncoding portion of the genome accounts for greater than 90% of the total mammalian genome. Many studies have demonstrated that among these ncRNAs, 18% of lncRNAs are associated with human cancers, compared with only 9% of human protein-coding genes, suggesting that lncRNAs could act as major contributors to carcinogenesis and cancer progression [24-30]. Thus, the roles of dysregulated lncRNAs in the pathogenesis of most cancers have garnered increased scientific interest in recent years.

LncRNAs exert their functions via diverse mechanisms, including co-transcriptional regulation, modulation of gene expression, and scaffolding of nuclear or cytoplasmic complexes [31]. Moreover, many experiments have demonstrated that some lncRNAs can serve as miRNA “sponges” by sharing common MREs [32]. These interactions influence post-transcriptional regulation by inhibiting available miRNA activity. Here, we found Linc-USP16 acted as a competing endogenous RNA (ceRNA) for miR-21 and miR-590-5p, thus increasing PTEN expression.

Recently, several studies have revealed the contributions of some lncRNAs to the development of HCC [21]. However, the role of Linc-USP16 was unknown. To investigate Linc-USP16, we first analysed the expression levels of Linc-USP16 between HCC tissues and normal tissues. We found that Linc-USP16 was decreased in HCC tissues and cell lines. Then, we observed a statistical correlation between Linc-USP16 expression and tumour size ($p=0.043$), clinical stage ($p=0.031$), and metastasis ($p=0.016$). Overexpression of Linc-USP16 in HCC cells inhibited cell growth and migration. These data suggested that Linc-USP16 played an important role in the modulation of HCC progress.

To fully uncover its molecular mechanism, we investigated the effect of Linc-USP16 on the AKT pathway and found that Linc-USP16 suppressed AKT pathway activation via regulation of PTEN expression in a transcription-independent manner. A previous study indicated that lncRNAs can act as miRNA “sponges”. Therefore, we focused on the mechanism of Linc-USP16 as a ceRNA. Some microRNAs have been reported to inhibit PTEN expression in HCC, such as miR-21, miR-590-5p, miR-32, and miR-494. Subsequently, bioinformatics prediction combined with experimental analysis indicated the interaction between Linc-USP16 and miR-21/miR-590-5p. Consistent with previous reports, the expression levels of miR-21/miR-590-5p were inversely correlated with Linc-USP16 in HCC tissues. Further studies indicated that Linc-USP16 regulated HCC tumourigenesis via the miR-21/miR-590-5p-PTEN axis.

Thus, our data demonstrated that Linc-USP16 as a tumour suppressor plays an important role in HCC pathogenesis, and our data provide a new therapeutic strategy for HCC treatment.

Disclosure Statement

The authors declare that they have no competing interests.

Acknowledgements

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Deguang Sun, Zhenming Gao, and Liming Wang designed the project. Jidong Sui, Xuejun Yang, Wenjing Qi, and Kun Guo performed the experiments and analysed the data. Deguang Sun wrote the manuscript. All authors reviewed the manuscript.

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