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Original Article

AF113014 inhibits proliferation and migration of hepatocellular carcinoma cells by binding eEF1A1 protein

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Abstract: Accumulating evidence showed that the aberrant expressions of long non-coding RNAs (lncRNAs) are strongly correlated with tumor occurrence and development. However, the role of lncRNA in HCC remains elusive. In this study, we found lncRNA-AF113014 was aberrantly expressed in HCC cells. Overexpression of AF113014 suppressed HCC cell proliferation and migration. In contrast, AF113014 knockdown promoted HCC cell proliferation and migration. Furthermore, AF113014 bound to the eukaryotic translation elongation factor 1 alpha 1 (eEF1A1) protein and promoted its expression. These findings demonstrate a functional role of AF113014 in HCC development. Our study suggested that AF113014 acted as a tumor suppressor for HCC.

Keywords: AF113014, HCC, binding, eEF1A1

Introduction

Hepatocellular carcinoma (HCC), a high prevalent malignant neoplasm with poor prognosis, is the third leading cause of the lethal cancer systems worldwide [1, 2]. More than estimated 250,000 new HCC cases and approximately 600,000 deaths occur annually. Numerous studies have been done to investigate the underlying mechanism of the pathogenesis of HCC. However, the precise molecular mechanisms underlying liver carcinogenesis and aggressiveness are unclear [3, 4].

Long non-coding RNAs (lncRNAs), tentatively identified as non-protein coding RNA transcripts more than 200nt in length, have been shown to join in diverse molecular functions and pathological processes in epigenetic regulation [5]. To date, lncRNAs are reported to be involved in hepatocarcinogenesis, including cell proliferation, apoptosis and invasion [6-8]. Recent studies have indentified that the aberrant expression of lncRNAs are strongly correlated with tumor occurrence, development and apoptosis in HCC. For example, MEG3 expression was significantly decreased in the majority of HCC samples. MEG3 inhibited proliferation and induced apoptosis through acting on p53 signaling pathway [9]. Wu *et al*. reports that IL-6 transcriptionally promoted the expression of lncTCF7 by activating STAT3 in HCC cells. IL-6-induced EMT and cell invasion were suppressed when lncTCF7 expression was knocked down [10].

Recently, a microarray analysis [11] showed that AF113014 was among the most significant upregulated lncRNAs in malignant human hepatocytes, compared with the normal hepatocytes. AF113014 is a ~1 kb lncRNA from minus strand of chromosome 10. However, whether AF113014 is involved in the development of HCC has not been reported.

In this study, we explored the association between AF113014 and HCC. We further characterized functional role of AF113014 in tumor growth. Our study provided a new perspective in comprehending the connection between

lncRNA and HCC. Our results elucidated that AF113014 might act as a tumor suppressor in HCC.

Materials and methods

Cell lines

Human immortalized normal hepatocytes (LO2) and human HCC cell (SK-Hep1) lines were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone, China), supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 U/ml penicillin and streptomycin. Cells were maintained in a humidified incubator with 5% CO_2 at 37°C.

RNA extraction and quantitative real-time PCR (qRT-PCR)

Total RNAs were extracted with TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with RNase-free DNase (Promega, USA). For AF113014, the first-strand cDNA was generated using the PrimeScript RT reagent kit with gDNA Eraser according to the manufacturer's instructions (Takara). The primers used were 5'-TGGCAACATATCCTTAACTCC-3' (forward) and 5'-TCCTTCCCTTCCTTGAAATCAC-3' (reverse). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was treated as the endogenous control, the primers for it were 5'-CGACCACTTTGTCAAGCTCA-3' (forward), 5'-AGGGGTCTACATGGCAACTG-3' (reverse). All quantitative real-time polymerase chain reaction (qRT-PCR) samples were performed by using UltraSYBR mixture (Cwbio, China) and conducted using the CFX Connet TM real-time PCR system (Bio-Rad). The quantification analysis was analyzed by the $2^{-\Delta\Delta CT}$ method [12].

Plasmids, small interfering (si)RNA and transfection

The full-length AF113014 was PCR-amplified from the genomic DNA of LO2 cells using primers (forward: 5'-CCGGAATTCGATGCCTCTACCTATCAGATGTTG-3', reverse: 5'-CCGCTCGAGTGGGCCCACATTTTTACTCTTGTC-3') based on the start and termination sites of chromosome [11]. The PCR product was purified and subcloned into the the*EcoR I*/*Xho I* sites of pcDNA3.1 (-) expression vector and sequenced. For AF113014 depletion, AF113014 inhibitor (siAF113014) and negative control (NC) were purchased from Invitrogen. Sequences were as follows: sense: 5'-CCUUAACUCCCUCAGUUAUTT-3', antisense: 5'-AUAACUGAGGGAGUUAAGGTT-3'. Transfections were performed with a Lipofectamine 2000 kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The cells were harvested 48 hours after transfection.

Cell proliferation assay

Cells (3900 cells/well) after 24 h transfection were seeded into 96 well plates, and measured at different time points (12, 24, 48, and 72 h) using the MTS kit (Cell Titer 96® AQueous One Solution Cell Proliferation Assay, Promega, USA), followed the manufacturer's protocol. The 490 nm wave-length absorption value was measured. All experiments were performed in triplicate and repeated 3 times.

Colony formation assay

Cells (1×10^5 cells/well) were seeded into 6-well plates after 24 h transfection and cultured for 7 days, 4% paraformaldehyde was used to fix with cells. Then, cells were stained with crystal violet for 20 min.

Wound healing assays

Transfected cells (3.5×10^5 cells/well) were plated in 24-well plates and incubated overnight, and scratched using a 10 ul pipette tip to create the wound. PBS was used to wash the cells and cells were further cultured in DMEM with 2% FBS. Then take photos after wounding at 0 h, 24 h and 48 h.

Transwell assays

After 24 h transfection, 1×10^5 cells suspending in 100 ul of serum-free DMEM were added to the 8 um transwell migration chambers (Costar). 800 ul of DMEM media containing 10% FBS was added to the lower chamber. After 24 h at 37°C in a 5% CO_2 incubator, the membrane was wiped off using PBS. Migrated cells were fixed with 4% paraformaldehyde for 30 min and stained with Giemsa. Then photographed, and counted according to the manufacturer's instructions.

RNA pull-down assay

LncRNA AF113014 and GAPDH RNA were transcribed from vector pcDNA3.1-AF113014 and pcDNA3.1-GAPDH *in vitro*. They were tran-

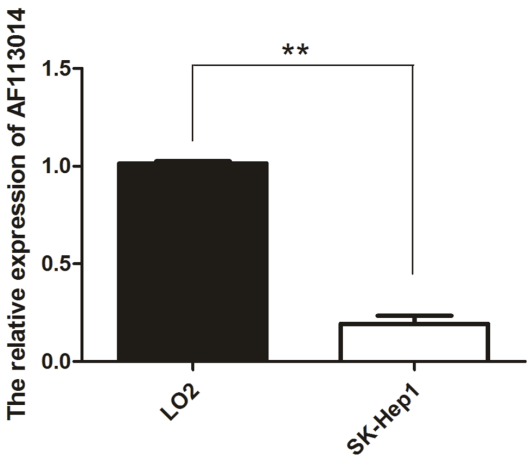

Figure 1. Low expression of AF113014 in HCC cells. Relative expression of AF113014 in LO2 and SK-Hep1 cells. GAPDH was used as the endogenous control.

scribed with the Biotin RNA Labeling Mix (Roche Diagnostics, Indianapolis, IN) and T7/SP6 RNA polymerase (Roche), treated with RQ1 DNase I (Promega), and purified with an RNeasy Mini Kit (Qiagen, Valencia, CA). One milligram of SK-Hep1 cell nuclear extract was mixed with 50 pmol of biotinylated RNA biotin-labeled RNAs, incubated with 40 ul of washed streptavidin agarose beads (Invitrogen, Carlsbad, CA) for 3 hour at 4°C, and then washed 3 times. The retrieved proteins were separated with SDS PAGE and detected with silver-stained. The specific bands were excised and then analyzed by mass spectrometry.

Western blot analysis

Cells were lysed in RIPA Buffer (Beyotime, China), supplementing with 1 mmol/L PMSF, and protein concentration was measured by the BCA Assay Kit (Beyotime, China). 50 ug of proteins were separated on a 12% polyacrylamide gel and transferred to a PVDF membrane. Proteins were probed with EEF1A1 primary antibody (Cat # 11402-1-AP, 1:1000, Proteintech). Then the blots were incubated with a goat anti-rabbit or anti-mouse HRP secondary antibody. Finally, ECL Detection Reagent (Millipore, Billerica, MA) was used to detect the signal. The data were normalized to GAPDH (Cat # 60004-1-Ig, 1:5000, Proteintech).

Statistical analysis

Data are expressed as means ± standard deviation (SD). Statistical analysis was performed by X^2 analysis and Student's t test. $P < 0.05$ was considered statistically significant.

Results

Low expression of AF113014 in HCC cells

Firstly, we examined AF113014 expression by using qRT-PCR. We found that the expression of AF113014 was significantly lower in SK-Hep1 cells than in the hepatic immortal cell line LO2 (**Figure 1**).

AF113014 inhibits HCC cell proliferation in vitro

To further investigate the biological effect of AF113014 in HCC cells, SK-Hep1 cells were transiently transfected with pcDNA3.1-AF1130-14, and pcDNA3.1 and treated withAF113014 inhibitor. As shown in **Figure 2A**, AF113014 level was increased approximately 370 times in SK-Hep-1 cells transfected with pcDNA3.1-AF113014. In contrast, AF113014 inhibitor resulted in decreased AF113014 level in SK-Hep-1 cells ($P < 0.01$). MTS and colony formation assay were then performed to analyze the function of AF113014 in HCC cells. Overexpression of AF113014 repressed cell proliferation, whereas AF113014 inhibitor promoted cell proliferation in SK-Hep1 cells, over a course of 3 days ($P < 0.05$) (**Figure 2B-D**). These results suggested that AF113014 could inhibit HCC cell proliferation.

Up-regulation of AF113014 represses migration of hepatoma cells

To further determine whether AF113014 could affect the migration capacity of hepatoma cells, transwell assay and wound healing assay were performed. We found that AF113014 overexpression inhibited the migration of SK-Hep1 cells while AF113014 knockdown promoted the migration of cells (**Figure 3A-C**). These findings indicated that AF113014 played an important role in themigration of HCC cells.

AF113014 binds to eEF1A1 protein and promotes eEF1A1 expression

Recent studies show that lncRNAs could affect activity and localization of target protein by binding to specific protein partners [13, 14]. Therefore, we performed an RNA pull-down experiment to identify proteins that associated with AF113014. As showed in **Figure 4A**, there were several candidate proteins found from the SDS PAGE and identified by using the mass spectrometry analysis (**Table 1**). Western blot-

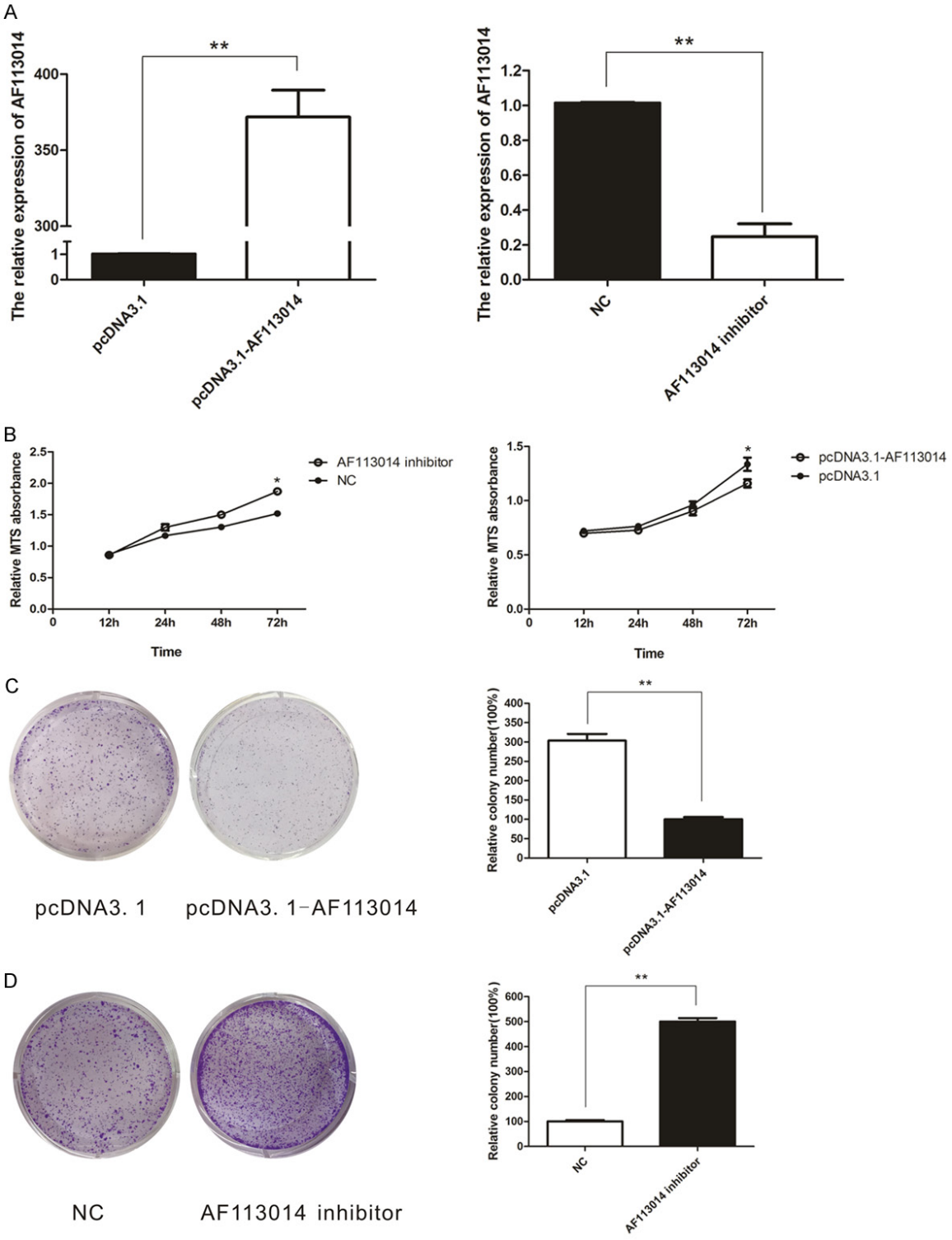

Figure 2. AF113014 inhibits HCC cell proliferation *in vitro*. A. SK-Hep1 cells were transfected with pcDNA3.1-AF113014, AF113014 inhibitor and their negative controls. 48 h after transfection, expression of AF113014 was measured by qRT-PCR. B. Cell growth was measured at different times (12, 24, 48, and 72 h, respectively) by MTS assay in SK-Hep1 cells in pcDNA3.1-AF113014 or AF113014 inhibitor, compared with the controls. C, D. Representative results of colony formation of pcDNA3.1-AF113014 or AF113014 inhibitor transfected SK-Hep1 cells, compared with the controls. *P < 0.05, **P < 0.01.

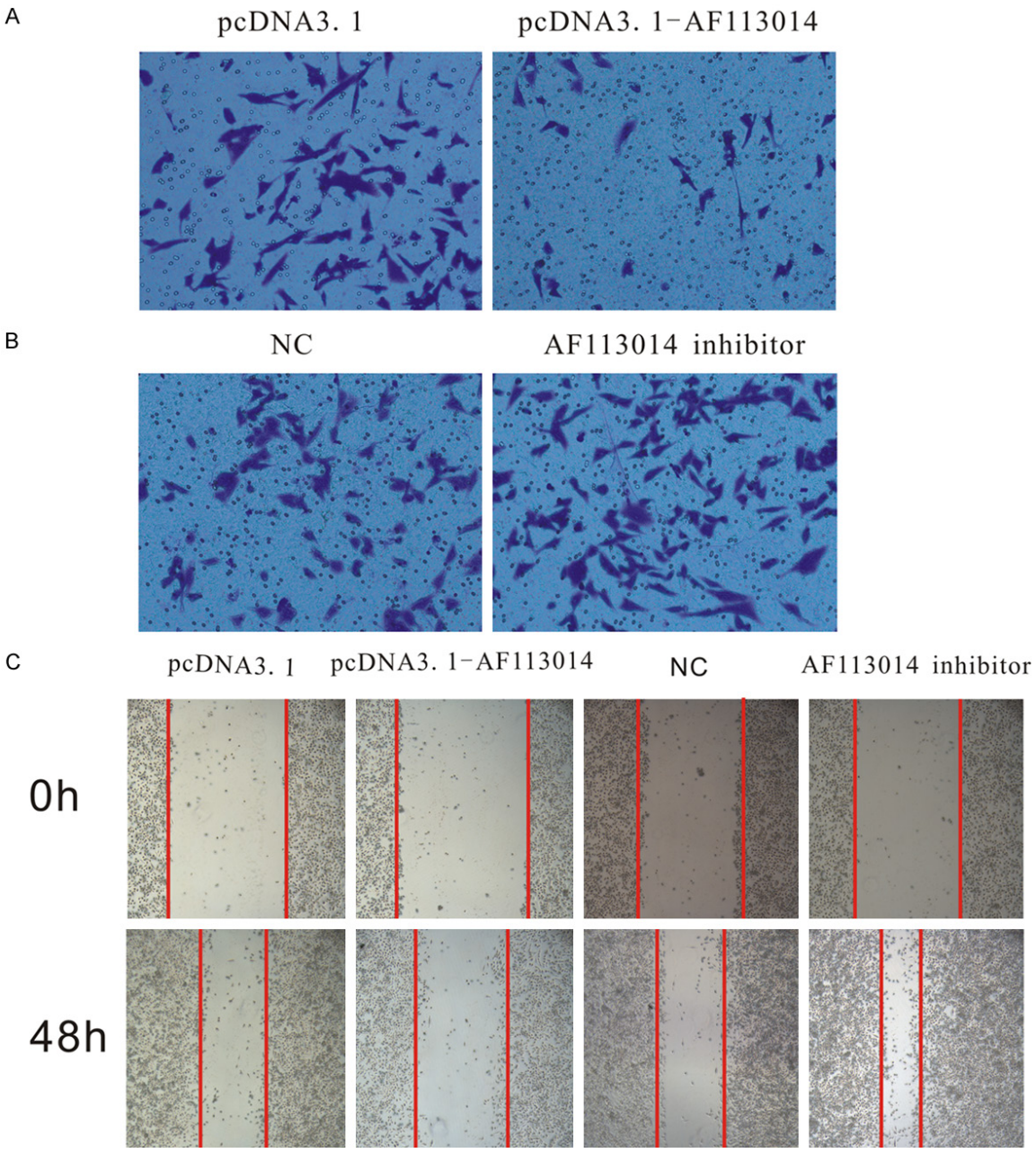

Figure 3. Up-regulation of AF113014 repressed migration of hepatoma cells. A, B. Cell migration was evaluated in SK-Hep1 cells expressing AF113014, AF113014 inhibitor and their control by using Matrigel migration chamber. Cells that migrated through the pores were fixed and stained with crystal violet after incubation(magnification, × 400). C, D. Wound healing for SK-Hep1 cells transfected as described. *P < 0.05, **P < 0.01.

ting analysis confirmed only eukaryotic translation elongation factor 1 alpha 1 (eEF1A1) was specifically associated with AF113014 (**Figure 4B**). The protein expression level of eEF1A1 was further measured by Western blot analysis in SK-Hep1 cells with AF113014 overexpression or knockdown. Our results showed that AF113014 overexpression promoted eEF1A1 protein level, whereas AF113014 knockdown inhibited eEF1A1 expression (**Figure 4C**, **4D**). These results imply that AF113014 regulating eEF1A1 expression.

Discussion

HCC is one of the most common causes of cancer-related death with high incidence and mortality rates, affecting certain of carcinogenic-related genes by genetic or epigenetic changes

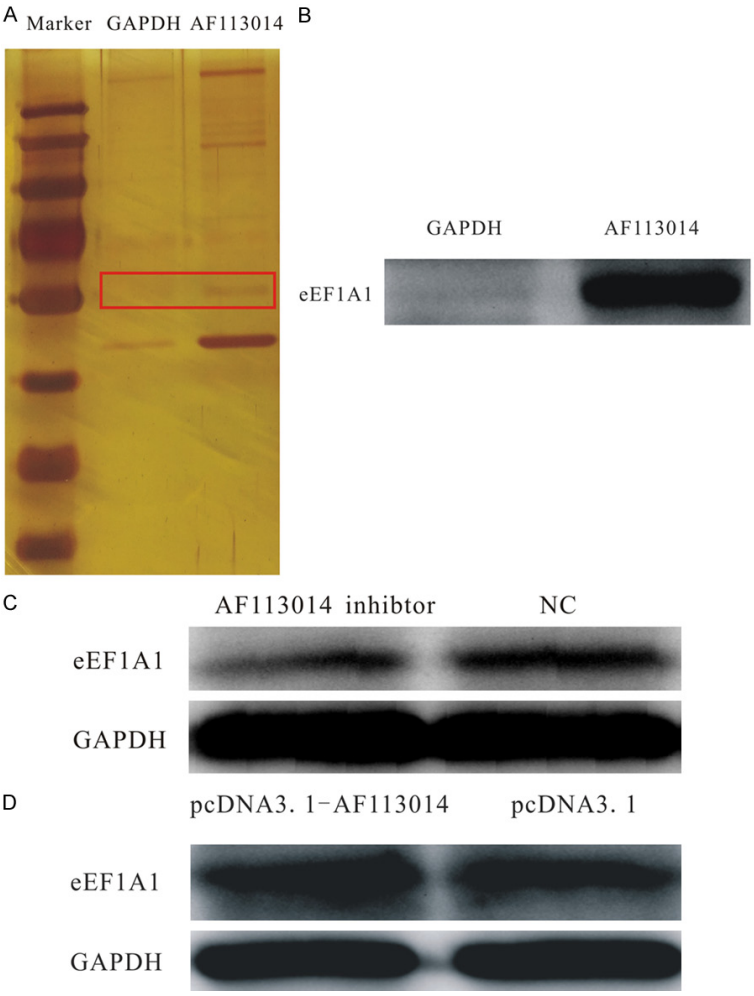

Figure 4. AF113014 binds to eEF1A1 protein and promotes eEF1A1 expression. A. Silver staining of the SDS-PAGE gel. Samples derived from proteins pulled down by AF113014 and its control RNA. The arrow indicates the gel cutting for proteomic analysis by LC-MS/MS. B. Western blotting analysis of RNA pull-down assay from SK-Hep1 cellular extracts was performed. An antibody against the eEF1A1 protein was used as the target protein. C, D. Relative protein expression levels of eEF1A1 in SK-Hep1 transfected with pcDNA3.1-AF113014 or AF113014 siRNA were examined by Western blotting, compared to GAPDH.

[15]. In recent years, roles for lncRNAs as tumorigenic or tumor suppressive functions have been found in diverse cancers, including HCC [16]. For instance, lncRNA-HEIH arrested cell cycle by repressing the expression of p16 gene, through interacting with EZH2 [17]. LncRNA-HULC promoted hepatocyte proliferation by inhibiting p18 [18]. LncRNA H19 associated with the protein complex hnRNP U/PCAF/RNAPol II and activated miR-200 family through histone acetylation, which resulting in HCC progression suppression [19]. Much more evidences have shown that HCC-related lncRNAs affects many genes related to the life cycle, such as lncRNA-miRNA interaction, lncRNA-protein interaction, splicing regulation, epigenetic silencing and genetic variation [20]. Therefore, we hypothesized that the amount of lncRNAs acted as biomarkers remain to be elucidated.

In this study, we found a novel AF113014, which was aberrantly expressed in HCC cells. And we explored the biological functions of AF113014 in HCC cells. We revealed that AF113014 could suppress cell proliferation *in vitro* by doing gain-of-function and loss-of-function experiments. Furthermore, AF113014 repressed the migration of hepatoma cells. These results suggested that AF113014 may act as a tumor suppressor gene in HCC.

It is reported that eEF1A1 is abundant in heart, brain and muscle tissue during embryonic development. However, eEF1A1 expression is decreased and replaced by expression of eEF1A, eEF1A2 during early postnatal development [21]. Furthermore, eEF1A is reported to directly bind mammalian mRNAs and mediates stability of RNAs through binding to their 3' regions [22]. In this study, we revealed that AF113014 specific bound to eEF1A1 protein in SK-Hep1 cells. Overexpression of AF113014 promoted the expression of eEF1A1. We hypothesized that the biological function of AF113014 in HCC might be associated with eEF1A1. However the mechanism underlying this needs our further investigation.

In summary, our study provides novel understanding of the relationship between lncRNA and HCC. We have showed that AF113014 suppressed cell proliferation and migration in HCC cells. Meanwhile, we found that eEF1A1 is one

Table 1. AF113014 binds to eEF1A1 protein and promotes eEF1A1 expression

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
P04264	Keratin, type II cytoskeletal 1 OS=Homo sapiens GN=KRT1 PE=1 SV=6 - [K2C1_HUMAN]	331.14	18.17	1	9	12	15	644	66.0	8.12
P13645	Keratin, type I cytoskeletal 10 OS=Homo sapiens GN=KRT10 PE=1 SV=6 - [K1C10_HUMAN]	301.79	18.66	15	8	11	12	584	58.8	5.21
P35908	Keratin, type II cytoskeletal 2 epidermal OS=Homo sapiens GN=KRT2 PE=1 SV=2 - [K22E_HUMAN]	201.17	9.55	11	2	6	7	639	65.4	8.00
P02533	Keratin, type I cytoskeletal 14 OS=Homo sapiens GN=KRT14 PE=1 SV=4 - [K1C14_HUMAN]	170.94	22.03	17	7	10	10	472	51.5	5.16
P35527	Keratin, type I cytoskeletal 9 OS=Homo sapiens GN=KRT9 PE=1 SV=3 - [K1C9_HUMAN]	153.90	9.79	2	6	7	9	623	62.0	5.24
P68104	Elongation factor 1-alpha 1 OS=Homo sapiens GN=EEF1A1 PE=1 SV=1 - [EF1A1_HUMAN]	120.60	7.79	3	4	4	5	462	50.1	9.01
P02538	Keratin, type II cytoskeletal 6A OS=Homo sapiens GN=KRT6A PE=1 SV=3 - [K2C6A_HUMAN]	95.94	12.94	14	3	7	8	564	60.0	8.00
P13647	Keratin, type II cytoskeletal 5 OS=Homo sapiens GN=KRT5 PE=1 SV=3 - [K2C5_HUMAN]	60.61	8.31	10	2	5	6	590	62.3	7.74
P06733	Alpha-enolase OS=Homo sapiens GN=ENO1 PE=1 SV=2 - [ENOA_HUMAN]	53.09	1.38	1	1	1	1	434	47.1	7.39
P80723	Brain acid soluble protein 1 OS=Homo sapiens GN=BASP1 PE=1 SV=2 - [BASP1_HUMAN]	50.93	6.17	1	1	1	1	227	22.7	4.63
P02768	Serum albumin OS=Homo sapiens GN=ALB PE=1 SV=2 - [ALBU_HUMAN]	48.80	2.63	1	2	2	2	609	69.3	6.28
A6NNZ2	Tubulin beta-8 chain-like protein LOC260334 OS=Homo sapiens PE=1 SV=1 - [TBB8L_HUMAN]	45.08	2.03	7	1	1	1	444	49.5	4.86
O00148	ATP-dependent RNA helicase DDX39A OS=Homo sapiens GN=DDX39A PE=1 SV=2 - [DX39A_HUMAN]	36.42	1.64	2	1	1	1	427	49.1	5.68
Q9P2E9	Ribosome-binding protein 1 OS=Homo sapiens GN=RRBP1 PE=1 SV=4 - [RRBP1_HUMAN]	31.98	0.43	1	1	1	1	1410	152.4	8.60

of the proteins that bind to AF113014 by using RNA pull down assay. Our results demonstrate that AF113014 plays an important role in HCC pathogenesis.

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Disclosure of conflict of interest

None.

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