Online supplementary materials for

The long non-coding RNA LINC00908 facilitates hepatocellular carcinoma progression via interaction with Sox-4

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Supplementary Materials and Methods

Reverse transcription quantitative-PCR (RT-qPCR)

Total RNAs were extracted with a TRIzol kit (Invitrogen) following standard methods provided by the manufacturer. A reverse transcription kit (Promega) was used for cDNA extraction. Primers were designed by and purchased from Takara (Beijing). The details were shown in Table S1. ABI 3000 system was used for examination of the expression. RNA-free DNase was used for treating all samples. *GAPDH* was used as the internal control.

Western blot

The protein extraction was performed by RIPA lysis buffer (Beyotime). Then, samples were separated by 10% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE) and moved onto nitrocellulose membrane (Millipore). Non-fat milk (4%) was used for block and then incubated with specific primary antibodies overnight. HRP-labeled secondary antibody was added at room temperature for 1.5h. We used chemiluminescence ECL reagents (Pierce) to visualize the immunoreactivity. The density analyses were performed using ImageJ (NIH).

RNA immunoprecipitation (RIP)

The RIP experiments were done using Magna RIP™ RNA-Binding Protein Immunoprecipitation Kit (Millipore) following the standard protocols from the manufacturer. The immunoprecipitated RNAs were examined by RT-PCR.

Mouse IgG controls and total input controls were evaluated together. Primers were listed in Table S1.

Fluorescence in situ hybridization (FISH)

The fluorescence-conjugated probes were designed by and purchased from Life Technologies (Beijing). Samples were first treated under a non-denaturing condition followed by covering with specific fluorescence-conjugated probes. After counterstain with DAPI, the results were shown by a confocal microscopy in our institution.

In vivo tumorigenesis

HepG2 cells transfected with genetically silencing or overexpressing vectors were cultured in DMEM for 24 h. Then, ~10^6 resuspended cells were subcutaneously injected into the nude mice (BALB/c female, 5 weeks old, n = 6). Mice were housed at ~20℃ at a strictly controlled 12/12 light/dark cycle with free access to water and food. 28 days later, all mice were sacrificed. Solid tumors were then resected and weighed. Cells were covered with 25 nM FISH probes (Life Technologies) for 15 min for hybridization according to the manufacturer's guidelines and then dehydrated. The animal experiments were performed according to the Institutional Animal Care and Use Committee (IACUC) in Juxian Hospital of Traditional Chinese Medicine.

RNA pulldown and mass spectrometry (MS)

RNAs were labeled by Biotin using the Biotin RNA Labeling Mix by T7 RNA polymerase (Roche) and then cells were incubated with Biotin-labeled RNAs in

cell lysates with 0.5% formaldehyde. Annealed RNAs were then mixed with RIP buffer extracts and incubated for 3 h. Streptavidin agarose beads (ThermoFisher Scientific) were used to extract the biotin-labeled RNAs at room temperature for 2 h and washed by 1× binding washing buffer (5 mM Tris-HCl, 0.5 mM EDTA, 1 M NaCl and 0.005% Tween 20). Specific bands were separated by electrophoresis and sent for mass spectrometry (MS) in Mass Spectrometry Center at Peking University (Beijing).

Northern blot

Totally, 5 μg RNAs were subject to formaldehyde gel electrophoresis and then transferred to Hybond-N^+ membrane (GE Healthcare). A DIG Northern starter kit (Roche) was used to generate labelled RNA. After hybridization for 1 h, the membrane was then hybridized in ULTRAhyb-Oligo buffer (Ambion) surrounded by denatured probe. The bands on membrane were monitored by phosphor-imaging (Molecular Devices).

Luciferase reporter assay

Firefly luciferase activity assay was determined with 1 ng pRL-TK plasmid as the loading control. The Sox-4 promoters were inserted into pGL3 luciferase reporter plasmids. Cells were crashed 24h after transfection using a lysis buffer. Dual-Luciferase Reporter Assay System (Promega) was used with reference to the manufacturer’s instructions.

Viability assay

The Cell Counting Kit-8 (CCK-8, Dojindo, Japan) was purchased for quantifying viability. Cells were first re-suspended and then moved into 24-well

plates (~10^5 cells/well) and for 5 days. 25 □l CCK-8 solution was added into the culture. Optical density at 450 nm was monitored using Spectramax M5 microplate monitor (Molecular Devices).

5’ and 3’ Rapid amplification of cDNA ends (RACE)

2 μg RNAs (poly A^- or poly A^+) were used and subject to SMARTer RACE 5’/3’ kit (Clonetech) analysis in accordance with manufacturer’s instructions. Specific primers were designed by Invitrogen and listed in Table S1.

Immunohistochemistry

Sections (5 μm) were resected and stained with H&E. Deparaffinized sections were hydrated by peroxidase and blocked with 2% H_2O_2 for 20 min. Then, citrate buffer (pH 6.5, 30 mmol/L) was added for antigen retrieval at duration of 30 min. Cooldown was done by TBS with Tween 20 washing at triplicates. After treatment with blocking agents (Biocare Medical) for 20 min, specific primary antibodies were added for 1.5 h and washed by TTBS twice. For *in situ* hybridization (ISH), we used HRP-conjugated secondary antibodies. Slides were covered with 3,3’-diaminobenzidine (Sigma) and detected under a microscopy. Ki-67 staining was performed using a Ki-67 ELISA kit (MyBioSource).

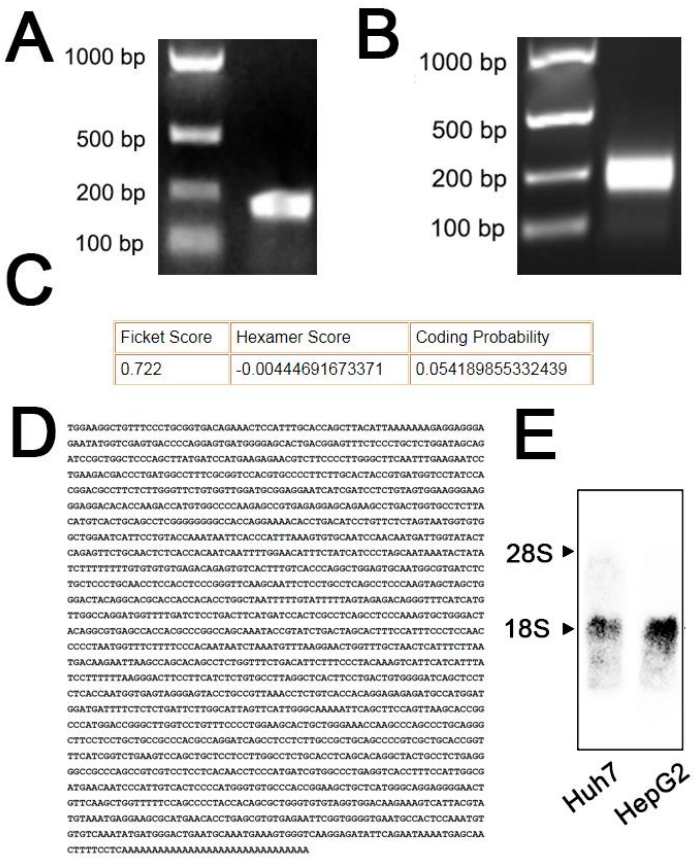

Figure S1. Features of LINC00908 in HCC. (A-B) Images of PCR products from the 5' RACE (**A**) and 3' RACE (B). (C) Evaluation of the coding potential for lncRNA LINC00908 using Coding Potential Assessment Tool (CPAT, http://lilab.research.bcm.edu/cpat/index.php). (D) The sequences of full-length LINC00908 in HepG2 cells. (E) Northern blot for LINC00908 in Huh7 and HepG2 cells. The bands showed LINC00908.

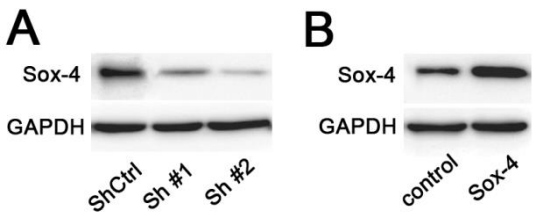

Figure S2. Efficiency of Sox-4 silence and overexpression. (A) The efficiency of Sox-4 knockdown in HepG2 cells. Consistently, ShSox-4 #2 (Sh #2) revealed higher efficiency and was chosen as ShSox-4. (B) HepG2 cells were transfected with control lentiviral vector (control) or lentiviral vectors carrying Sox-4 sequences (Sox-4).

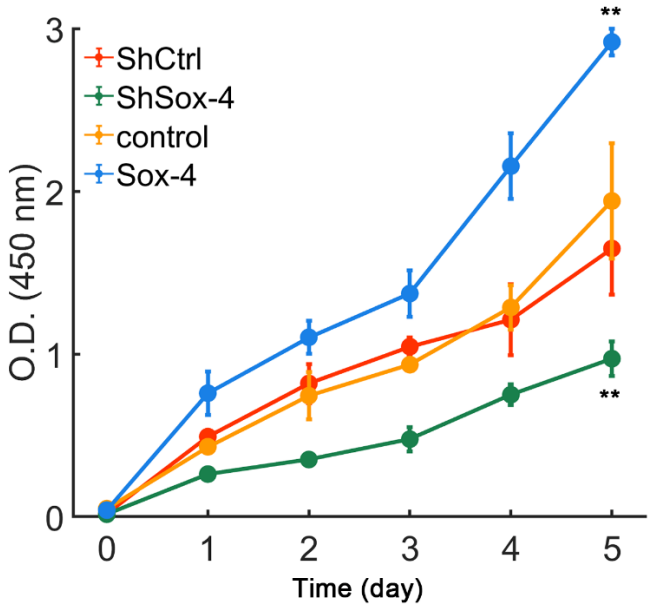

Figure S3. The effect of Sox-4 on HCC viability. HepG2 cells transfected with ShCtrl, ShSox-4, lentiviral control (control) or lentivirus containing Sox-4 (Sox-4). **: $P < 0.01$.

Table S1. Primers and antibodies

Name	Sequences
LINC00908-F	GAGAAATTGGTCAAAGGCGCGAC
LINC00908-R	TACACGCTTCACTTGACGTG
U1-F	CGGTTATGCCATGACTCG
U1-R	ACCGCCCCTGTTAGCGTA
GAPDH-F	CTGAGCACGCGTCAAGGAT
GAPDH-R	CCAGTGGGTGAAGACGGA
Sox-4-F	TCATCACCTCCCGGGTTCAAGTGTC
Sox-4-R	CTCTGATACTGCATTATTG
plasmid primer name	**Primer sequences**
5’RACE-GSP	CTCCAACTCCCCCGAATAACTCGTCC
5’RACE-NGSP	AGGAACACGTTGATGGAGCGTG
3’RACE-GSP	CTGGGAGGATAGAAAGGG
3’RACE-NGSP	TTAGTGCCTTCTTGCGACTGC
Northern blot-F	GGAGCAACAGCGAAGGTCACTATAGG
Northern blot-R	TAATACCGCAGTCTGGAGAGAAAGAGCTAAC
shLINC00908 #1	AGAATAAACAGTTTTATTCAGAGAATTTTAAGC TAACCATTATAACCACATTG
shLINC00908 #2	GAAAACTTTCCTCGTTGTAACTTAGCTCGAGC TAAATCCCAGGAGG
shSox-4 #1	CCGGCCGTTGAGCTCAGCTCGAGTCCGGTTT CGCCTTGCAGCCAAGTGA
shSox-4 #2	AATGTAATGGAAAAGCTCCAGACCTCTAGGCT TGACTGTGCTAGGTGGGGATTACCCGG
LINC00908 RIP-F	GATCTAATGTTTGCACGGTCTCT
LINC00908 RIP-R	AGCTCTCACCTTAGTTCCGC
LINC00908 1-389-F	GGAGATGTAAGGAGAGTGGA
LINC00908 1-389-R	GCGCGGAGATTAGTTAATCGC
390-1100-F	CGTCATCAGGGGCTG
390-1100-R	TCTGAGTTGTCTTCCGGAC
1101-1390-F	AGAAGCAAAAGTGATTGGAA
1101-1390-R	TGGTCTGCTCCCTACA
1391-1903-F	ATTAGTTTGAGCTTGACCCTT
1391-1903-R	TCCAGGCACGGTGCTTACGT
antisense-F	CTTGCTCATZGGGCCGATTTGTT
antisense-R	ACACCTGGCGAAGAAGT
Sox-4 1-150-F	TCGATCGGCGCGTGCCAATG

1-150-R	CCTCTGCCCATGTTATCTGAG
151-316-F	CCCATCCTGGCTTCAACCCTGGATCT
151-316-R	GCCTCCAGGAGGGCAGCGG
317-474-F	TGATTGCTCTGGCTTCGAGGTT
317-474-R	AGGGGTTCGACCGTACAGTCGC

Antibodies or reagents (Catalog NO.)	**Company**
Sox-4 (#AV38234)	Sigma
GAPDH（G8795）	Sigma
Flag-Tag (#14793)	Cell Signaling
HRP-conjugated secondary antibody (SAB5300168)	Sigma
DAPI (#32670)	Sigma
anti-HA (#5017)	Cell Signaling
anti-GST (#2624)	Cell Signaling
anti-Ub (#AB137025)	Abcam
MG132 (#M8699)	Sigma
Biotin RNA Labeling Mix (#11685597910)	Roche
Rabbit IgG Alexa Fluor® 488 Conjugate (#2975)	Cell Signaling

Table S2: Consistently upregulated novel lncRNAs

LncRNA names	Log_2FC	*p* value
RP11-443B7.1	6.1114	0.0046
RP5-1057J7.6	4.0359	0.0054
LINC00908	7.8275	0.0006
GABPB1-AS1	5.6893	0.0087
RP11-410F14.1	6.6651	0.0025
RP11-77B22.2	5.5659	0.0098

Table S3: Correlation between clinicopathological characteristics and LINC00908 expression.

Features	NO.	LINC00908 levels		*P*
		low (60)	high (60)	
Age				
< 60	55	30 (54.5%)	25 (45.5%)	0.232
≥ 60	65	30 (46.2%)	35 (53.8%)	
Gender				
Female	53	24 (45.3%)	29 (51.0%)	0.231
Male	67	36 (53.7%)	31 (49.1%)	
TNM stage				
I-II	54	37 (64.6%)	17 (35.4%)	<0.001**
III-IV	66	23 (37.9%)	43 (62.1%)	
Tumor size				
< 3 cm	49	33 (67.3%)	16 (32.7%)	0.003**
≥ 3 cm	71	27 (38.0%)	44 (62.0%)	
Metastasis				
Present	58	21 (36.2%)	37 (63.8%)	0.006**
Absent	62	39 (62.9%)	23 (27.1%)	

TNM: tumor (T), the extent of spread to the lymph nodes (N), and the presence of metastasis (M) (* P < 0.05, ** *P* <0.01). The median value was used as the cut-off. The number in brackets denotes the number of cases.

Table S4: Potential binding proteins using mass spectrometry (MS)

Accession	Name	Species	Genes	Scores
Q14103	Heterogeneous nuclear ribonucleoprotein D	Homo sapiens	*HNRNPD*	1080.1112
Q4G0N4	NAD kinase 2	Homo sapiens	*NADK2*	1324.3534
Q9UKA9	Polypyrimidine tract-binding protein 2	Homo sapiens	*PTBP2*	1632.7728
P38159	RNA-binding motif protein, X chromosome	Homo sapiens	*RBMX*	1012.3480
Q06945	Transcription factor SOX-4	Homo sapiens	*SOX-4*	1841.1254
P08865	Ribosomal protein SA	Homo sapiens	*RPSA*	1874.1532
Q8NHM5	Lysine-specific demethylase 2B	Homo sapiens	*KDM2B*	1224.4458

See UniProt for details, http://www.uniprot.org/