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Supplemental information

lncRNA *RP11-147L13.8* suppresses metastasis and chemo-resistance by modulating the phosphorylation of c-Jun protein in GBC

Bohao Zheng, Jiwen Wang, Kun Fan, Wentao Sun, Wenze Wan, Zhihui Gao, Xiaojian Ni, Dexiang Zhang, Xiaoling Ni, Tao Suo, Han Liu, Houbao Liu, and Sheng Shen

Supplementary Figures

Figure S1 The validation of top ten down-regulated long non-coding RNA.
A: The volcano map presented the top ten upregulation (red spot) and downregulation (blue spot) long non-coding RNA in gallbladder cancer. Data represent mean ± s.e.m. (Three biological replicates).
B: The list of the top ten upregulation and downregulation long non-coding RNA in gallbladder cancer.
C: The qPCR results validated that RP-11147L13.8 was significantly downregulated in the 24 pairs of GBC tissues.

Figure S2 LNCipedia and CPAT databases indicate that there is no protein-coding ability for RP11-147L13.8.

Figure S3 The basic information of RP11-147L13.8 and the establishment of the stable knockdown and overexpression GBC cells.
A: The expression level of *RP11-147L13.8* among different kinds of tumors and human tissues.
B: The identification of full-length *RP11-147L13.8* in GBC-SD cells. Representative images and the boundary of the PCR products from 5' RACE and 3' RACE were shown.
C: The expression levels of *RP11-147L13.8* in different GBC cell lines, including GBC-SD, SGC-996, G-415, TGBC2TKB, TGBC24TKB, and NOZ cell lines.
D: The distribution of *RP11-147L13.8* in cyto-plasma (35%) and nucleus (65%) of GBC-SD cell lines. β-actin serves as a cytoplasmic marker, while U6 serves as a nuclear marker. Values are shown as mean ± S.E.M.
E: qPCR results indicated the altered expression level of RP11-147L13.8 in different groups of cell lines (Vector, OE, shNC, shlnc1, shlnc2, shlnc3).

Figure S4 *RP11-147L13.8* could significantly suppress the metastasis of GBC in vivo.
A: The tumor formation was observed 7 days after the injection of the cell lines. No significant differences were observed in the tumor volume. Data represent mean ± s.e.m. Student's t-test. *P<0.05; **P<0.001; ***P<0.0001.
B: No significant differences were observed in the tumor weight. Data represent mean ± s.e.m. Student's t-test. *P<0.05; **P<0.001; ***P<0.0001.
C: Representative images of H&E staining in the metastatic liver loci and statistical data comparing the knockdown group with the control group. (n=6)
D: Representative images of H&E staining in the metastatic lung loci and statistical data comparing the knockdown group with the control group. (n=6)

Figure S5 The correlation between the RP11-147l13.8 and the expression level of c-Jun-ser73 protein and the prognostic significance of c-Jun protein.

A: The typical immunohistochemistry image of the low and high expression of c-Jun-ser73 in gallbladder cancer tissue. (magnification:×200)

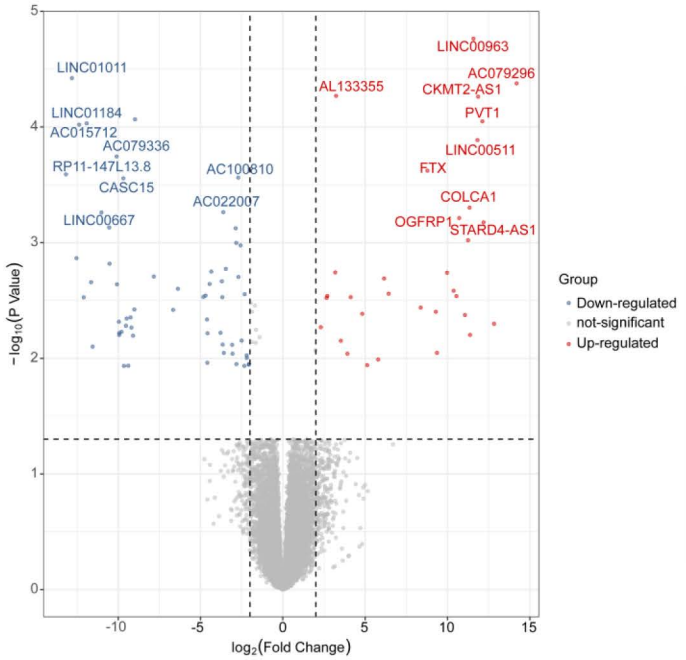
B: Patients with high expression levels of c-Jun-ser73 (red line) were associated with poorer overall survival. (Log-rank test, P=0.03).

C: The expression level of RP11-147L13.8 was negatively correlated with the expression level of c-Jun-ser73(R=; P=0.002). Spearman correlation analysis.

Figure S6 A mechanistic model for the function of RP11-147L13.8 in gallbladder cancer.

Figure S1

A



B

Top ten up-regulation and down-regulation long non coding RNA in gallbladder cancer

Seq Name	Gene name	Log_2(Fc)	P value	Regulation	Significant
ENST00000419300	LINC00963	11.58	0.00	up	yes
ENST00000670061	AC079296	14.19	0.00	up	yes
ENST00000670957	CKMT2-AS1	11.86	0.00	up	yes
ENST00000661988	PVT1	12.12	0.00	up	yes
ENST00000650423	LINC00511	11.82	0.00	up	yes
ENST00000669257	COLCA1	11.34	0.00	up	yes
ENST00000654777	STARD4-AS1	12.19	0.00	up	yes
ENST00000504145	AF117829	11.25	0.00	up	yes
ENST00000609067	AC084018	12.83	0.01	up	yes
ENST00000651019	AC068282	11.37	0.01	up	yes
ENSG00000267731	**RP11-147L13.8**	**-12.82**	**0.00**	**down**	**yes**
ENST00000663841	LINC01184	-11.92	0.00	down	yes
ENST00000560068	AC015712	-12.38	0.00	down	yes
ENST00000655321	AC009242	-13.18	0.00	down	yes
ENST00000654021	LINC00667	-11.02	0.00	down	yes
ENST00000474768	ADAMTS9-AS2	-10.55	0.00	down	yes
ENST00000453136	AC234582	-12.54	0.00	down	yes
ENST00000412639	LNCTAM34A	-11.65	0.00	down	yes
ENST00000403969	RNF216P1	-12.10	0.00	down	yes
ENST00000366437	MIR205HG	-11.56	0.01	down	yes

C

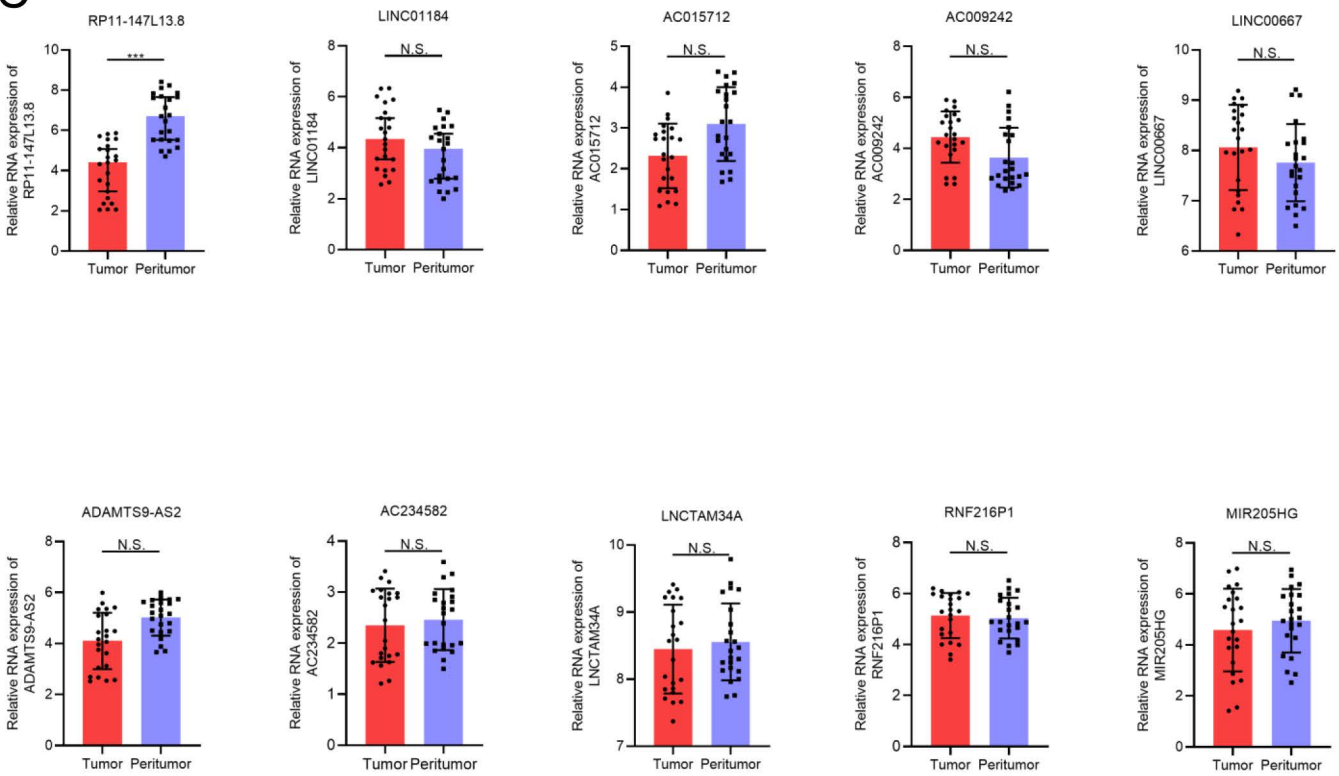


Figure S2

CPAT analysis of RP11-147L13.8

Calculator User Guide Feedback Source Code

Result for species name : hg19 with job ID :1626712581

Data ID	Sequence Name	RNA Size	ORF Size	Ficket Score	Hexamer Score	Coding Probability	Coding Label
0	RP11-147L13.8	2919	336	0.757	-0.150597546964	0.037066854677152	no

This job has been stored with the job ID
Download Table in tab delimetered file (.txt)

For suggestions, comments or queries about this website, please leave your feedback through Feedback.

BCM Baylor College of Medicine MAYO CLINIC

RNA sequence data of RP11-147L13.8

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AAACAGATATATGTATATGCCAA

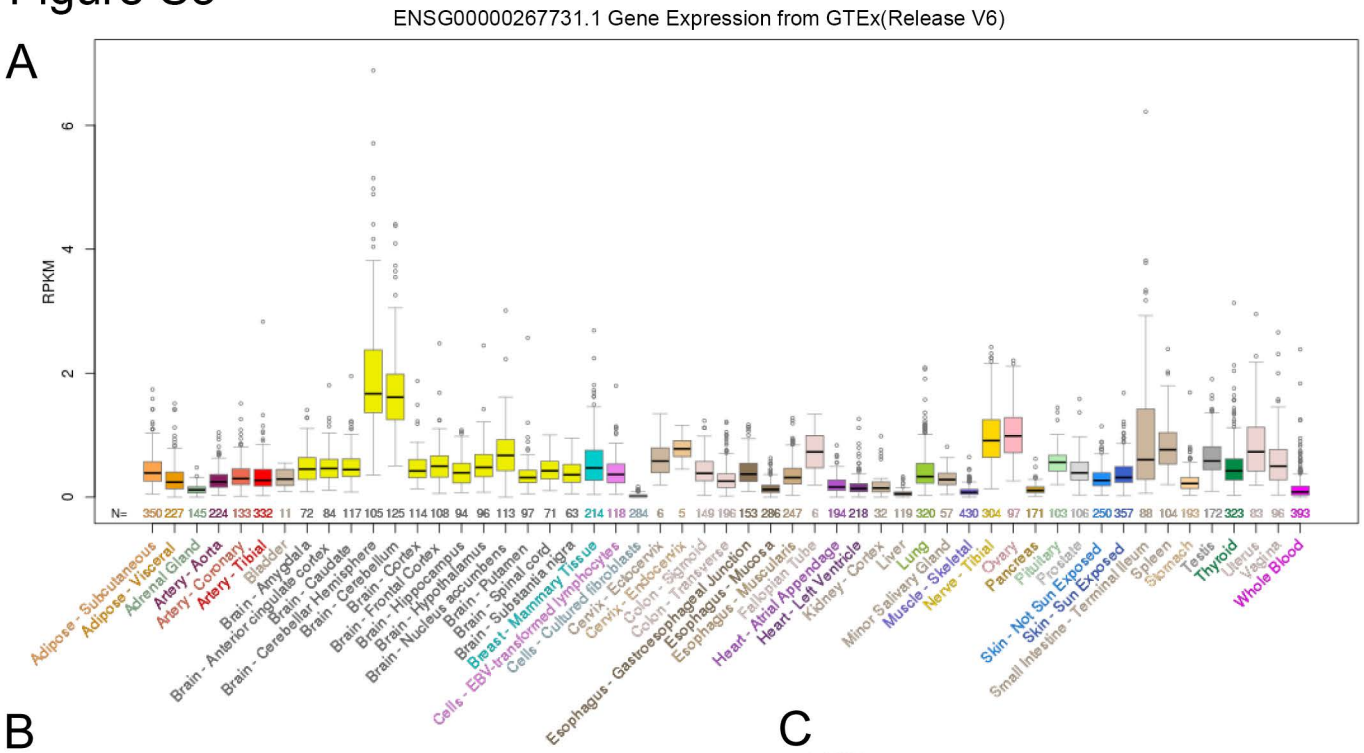
Lncipedia data of RP11-147L13.8

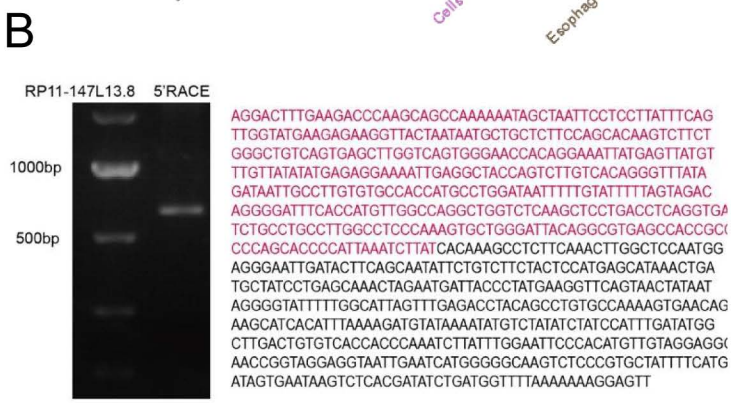
Protein coding potential

Metric	Raw result	Interpretation
PRIDE reprocessing 2.0	0	non-coding
Lee translation initiation sites	0	non-coding
PhyloCSF score	9.343	non-coding
CPAT coding probability	7.35%	non-coding
Bazzini small ORFs	0	non-coding

In stringent set: **yes**

Figure S3


ENSG00000267731.1 Gene Expression from GTEx(Release V6)
A
RPKM
N=
Adipose - Subcutaneous
Adipose - Visceral
Adrenal Gland
Artery - Aorta
Artery - Coronary
Artery - Tibial
Bladder
Brain - Amygdala
Brain - Anterior cingulate cortex
Brain - Caudate
Brain - Cerebellar Hemisphere
Brain - Cerebellum
Brain - Cortex
Brain - Frontal Cortex
Brain - Hippocampus
Brain - Hypothalamus
Brain - Nucleus accumbens
Brain - Putamen
Brain - Spinal cord
Brain - Substantia nigra
Breast - Mammary Tissue
Cells - EBV-transformed lymphocytes
Cells - Cultured fibroblasts
Cervix - Ectocervix
Cervix - Endocervix
Colon - Sigmoid
Colon - Transverse
Esophagus - Gastroesophageal Junction
Esophagus - Mucosa
Esophagus - Muscularis
Fallopian Tube
Heart - Atrial Appendage
Heart - Left Ventricle
Kidney - Cortex
Liver
Lung
Minor Salivary Gland
Muscle - Skeletal
Nerve - Tibial
Ovary
Pancreas
Pituitary
Prostate
Skin - Not Sun Exposed
Skin - Sun Exposed
Small Intestine - Terminal Ileum
Spleen
Stomach
Testis
Thyroid
Uterus
Vagina
Whole Blood


B
RP11-147L13.8 5'RACE
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500bp
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AGGGGATTTCACCATGTTGGCCAGGCTGGTCTCAAGCTCCTGACCTCAGGTGA
TCTGCCTGCCTTGGCCTCCCAAAGTGCTGGGATTACAGGCGTGAGCCACCGC
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AACCGGTAGGAGGTAATTGAATCATGGGGGCAAGTCTCCCGTGCTATTTTCATG
ATAGTGAATAAGTCTCACGATATCTGATGGTTTTAAAAAAAGGAGTT
RP11-147L13.8 3'RACE
1000bp
500bp
CCCTATCTCTAACCCCCTGCCATGTAGAGAATTGAACTAGCCTTCGCTCTCATA
AGTGGAATTTTAAAAAATAATGAGTTACATGAGCTGATAAGCACACATGCATAG
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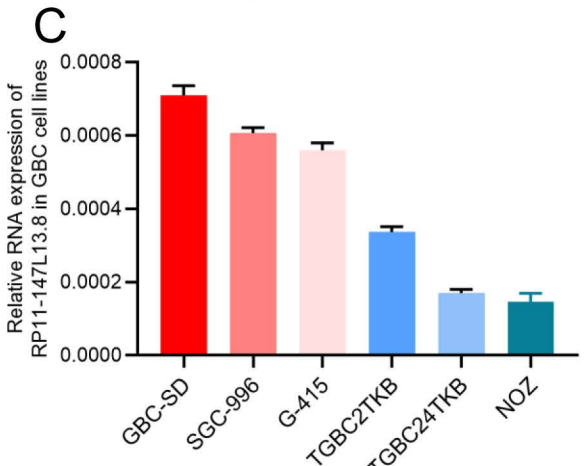
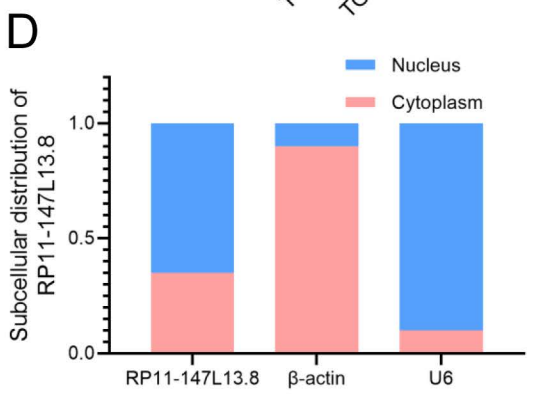

C
Relative RNA expression of RP11-147L13.8 in GBC cell lines
0.0008
0.0006
0.0004
0.0002
0.0000
GBC-SD
SGC-996
G-415
TGBC2TKB
TGBC24TKB
NOZ


D
Subcellular distribution of RP11-147L13.8
Nucleus
Cytoplasm
1.0
0.5
0.0
RP11-147L13.8
β-actin
U6

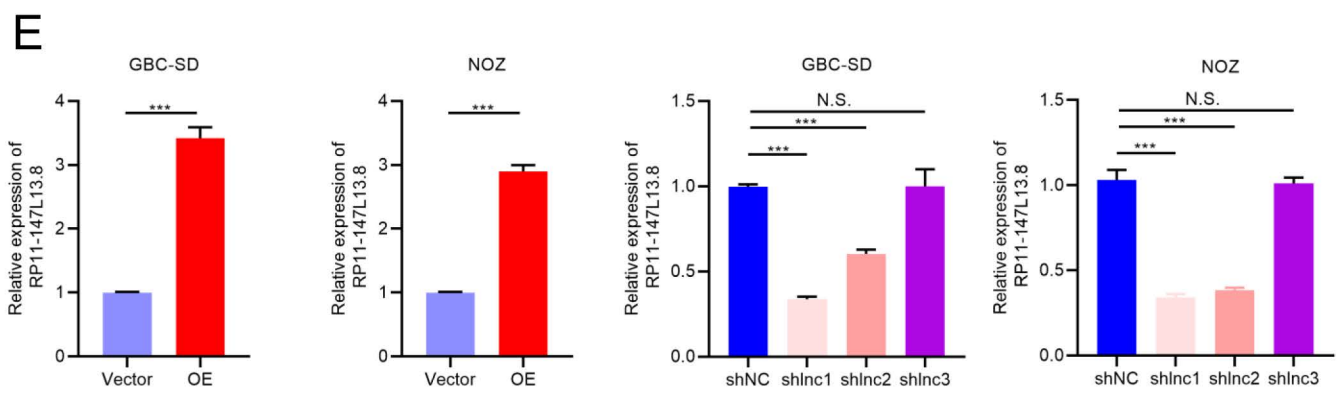

E
GBC-SD
NOZ
GBC-SD
NOZ
Relative expression of RP11-147L13.8
***
N.S.
Vector
OE
shNC
shlnc1
shlnc2
shlnc3

Figure S4

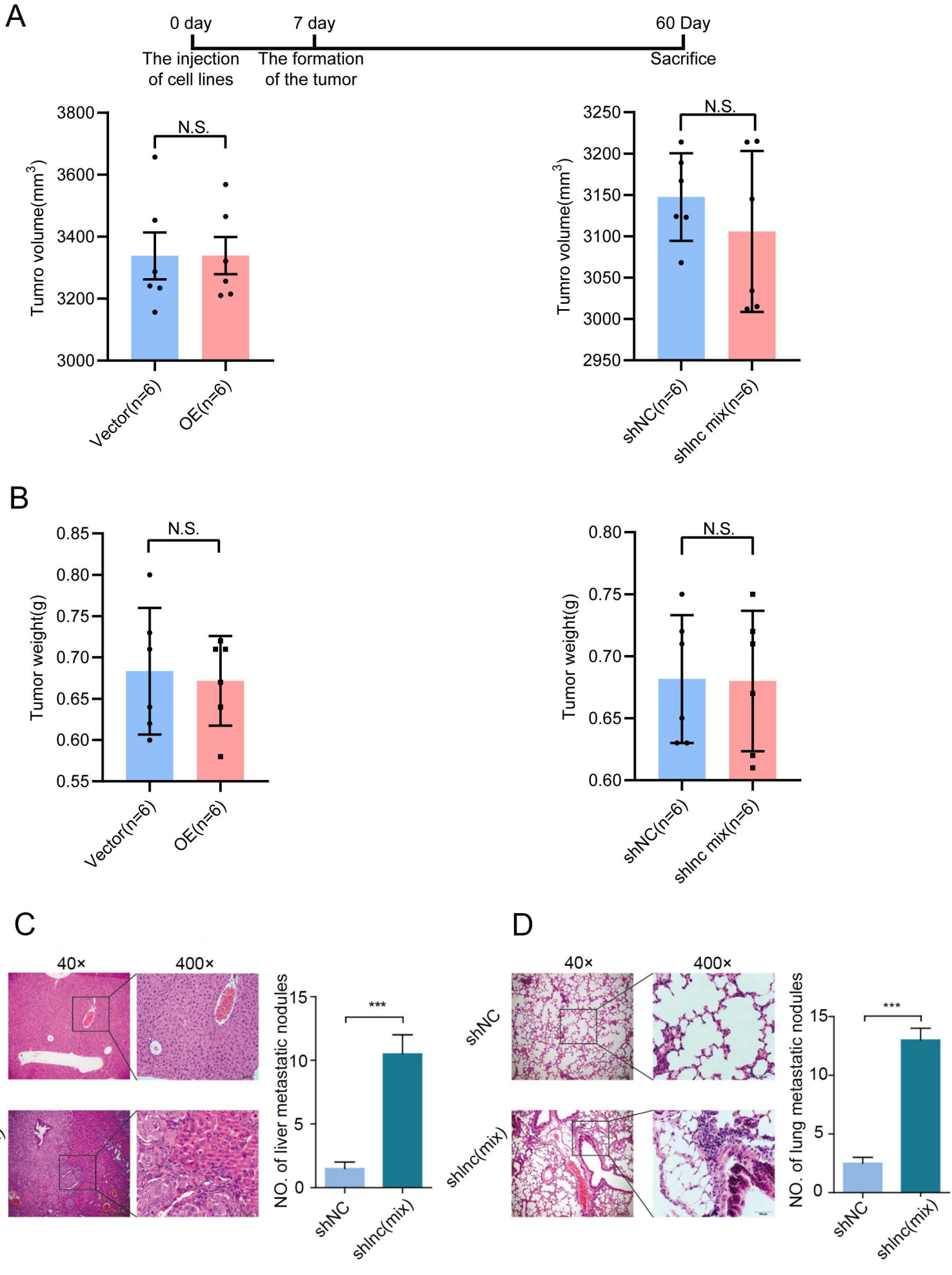

A
0 day
7 day
60 Day
The injection of cell lines
The formation of the tumor
Sacrifice
Tumro volume(mm3)
N.S.
Vector(n=6)
OE(n=6)
shNC(n=6)
shlnc mix(n=6)
B
Tumor weight(g)
C
40×
400×
shNC
shlnc(mix)
NO. of liver metastatic nodules
***
D
NO. of lung metastatic nodules

Figure S5

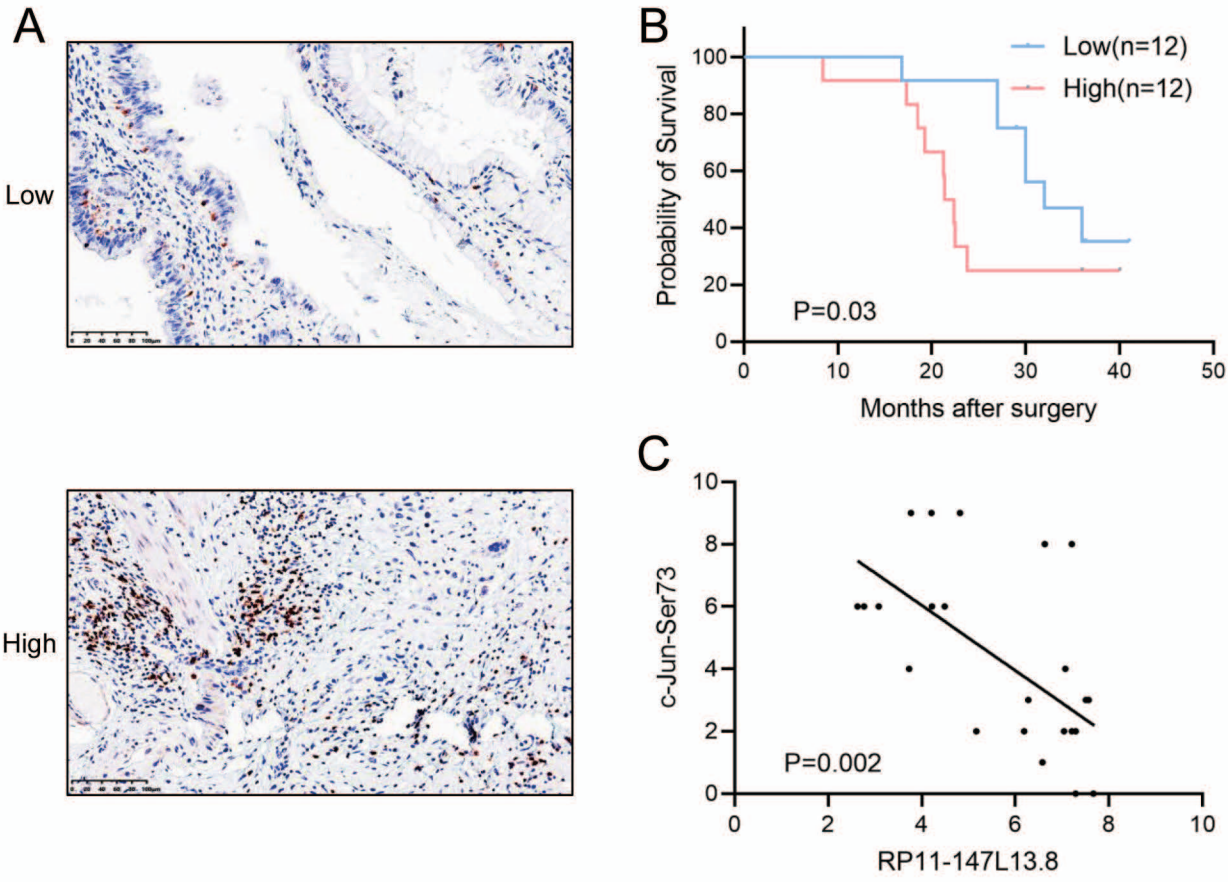

A
Low
High
B
Low(n=12)
High(n=12)
Probability of Survival
100
80
60
40
20
0
P=0.03
0
10
20
30
40
50
Months after surgery
C
c-Jun-Ser73
10
8
6
4
2
0
P=0.002
0
2
4
6
8
10
RP11-147L13.8

Figure S6

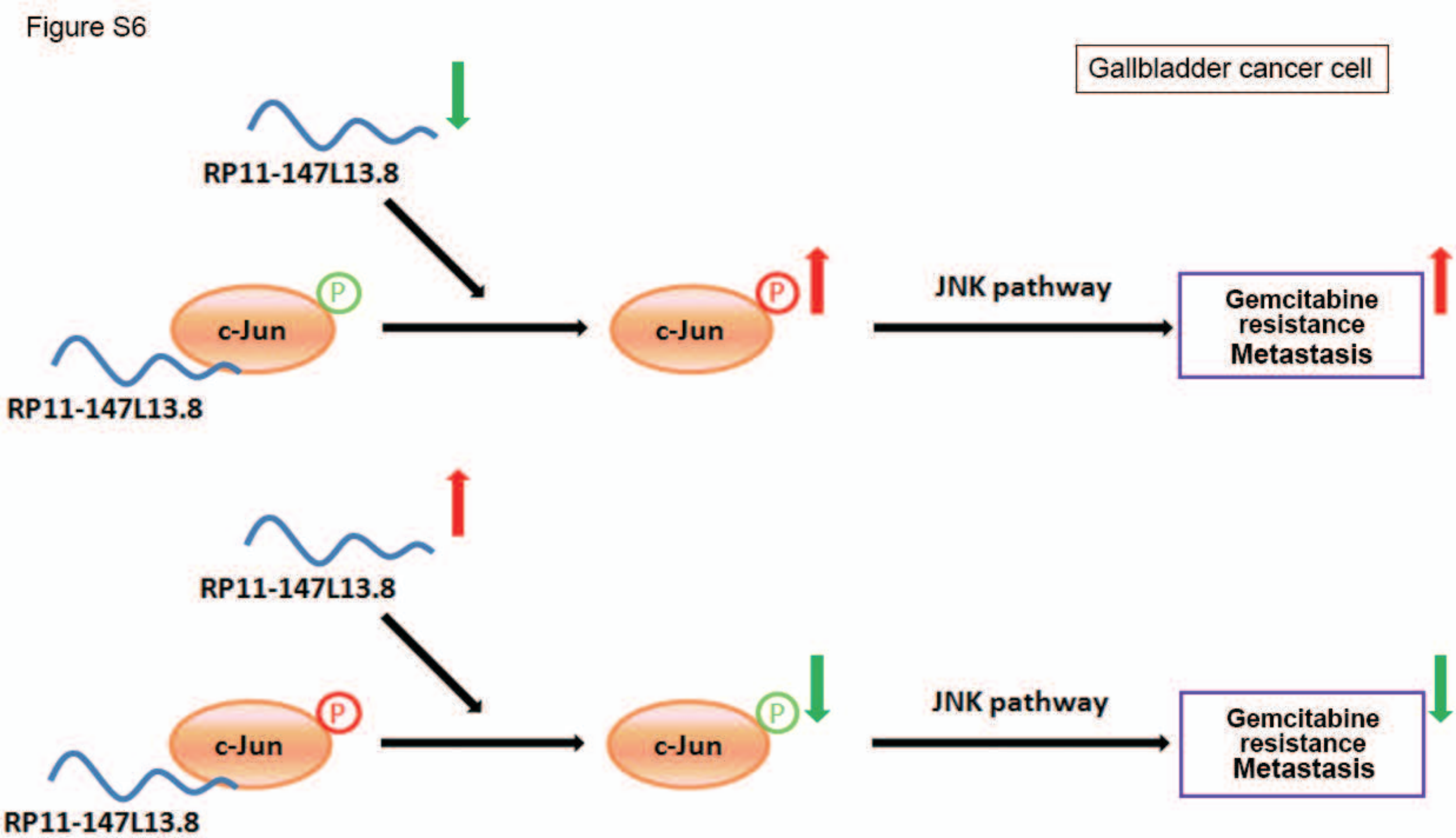

Gallbladder cancer cell
RP11-147L13.8
c-Jun
P
RP11-147L13.8
c-Jun
P
JNK pathway
Gemcitabine resistance Metastasis
RP11-147L13.8
c-Jun
P
RP11-147L13.8
c-Jun
P
JNK pathway
Gemcitabine resistance Metastasis

Table S1 Primers used for reverse transcription-quantitative polymerase chain reaction.

	Forward primer (5’-3’)	Reverse primer (5’-3’)
GAPDH	5’- AGCCACATCGCTCAGACAC-3’	5’- GAATTTGCCATGGGTGGA-3’
RP11-147L13.8	5’- CGATCCTCCCCTCCAACACT-3’	5’-ACTCCAAGGAAGGAACCCCC-3’
LINC01184	5’-AAAGAAGCTGAAAGGGCTCGG-3’	5’-AGCCATCCTCCACTGCAAAC-3’
AC015712	5’-CTGTGGTTCTGCCTCACAGG-3’	5’-TGCCAGATGAGGTTGAGCTG-3’
AC009242	5’-AGACCACCACCACCAATTCC-3’	5’-TGGAGGATGTGCCAGAGGTA-3’
LINC00667	5’-CCGCAGCTGACACCATGAAT-3’	5’-ACCCACCTCTATCTGACGGC-3’
ADAMTS9-AS2	5’-GAGACACAGCTGGGCTGAAC-3’	5’-GCTGCTAGCTCTTCTGGTGC-3’
AC234582	5’-AAAGAAGCTGAAAGGGCTCGG-3’	5’-AGCCATCCTCCACTGCAAAC-3’
LNCTAM34A	5’-AGCGGCATCTCCTCCACCTGAAA-3’	5’-TTGCCTCGTGAGTCCAAGGAGAAT-3’
RNF216P1	5’-AGTGTGGCTGGTATCGGTGT-3’	5’-TGGAGTCACAGGCAGTCGTA-3’
MIR205HG	5’- GGAGTGCAGTGGCTCAATCT-3’	5’- TGGATTGCTTAAGCTCAGGA -3’

Table S2 Primary antibodies used for western blot

Antibodies	Concentration	Specificity	Company	
GAPDH	1:1000 WB	Rabbit mAb	Abcam	EPR13796
c-Jun	1:1000 WB	Rabbit mAb	Cell Signaling Technology	60A8
Phospho-c-Jun (Ser73)	1:1000 WB 1:100 immunohistochemistry	Rabbit mAb	Cell Signaling Technology	D47G9

Table S3 The pull down protein list of the RP11-147L13.8

Accession	Gene Symbol	Description	Coverage [%]	# Peptides	# PSMs	# Unique Peptides
P60709	ACTB	Actin, cytoplasmic 1 OS Homo sapiens GN=ACTB PE=1 SV=1	12	9	8	4
Q86U42	PABP2	Polyadenylate-binding protein 2 OS Homo sapiens GN=PABP2 PE=1 SV=2	30	11	12	5
O95361	TRIM16	Tripartite motif-containing protein 16 OS Homo sapiens GN=TRIM16 PE=1 SV=2	27	6	6	5
Q9NRW3	APOBEC3C	DNA dC->dU-editing enzyme APOBEC-3C OS Homo sapiens GN=APOBEC3C PE=1 SV=2	18	5	5	5
P05412	JUN	Transcription factor AP-1 OS Homo sapiens GN=JUN PE=1 SV=1	35	15	15	14
P67809	YBOX1	Y-box-binding protein 1 OS Homo sapiens GN=YBOX1 PE=1 SV=1	11	7	5	3
E7EX29	E7EX29	14-3-3 protein zeta/delta OS Homo sapiens GN=E7EX29 PE=1 SV=1	9	3	2	1
Q9BQ67	GRWD1	Glutamate-rich WD repeat-containing protein 1 OS Homo sapiens GN=PGRWD1 PE=1 SV=2	10	1	1	1
O43395	PRPF3	U4/U6 small nuclear ribonucleoprotein Prp3 OS Homo sapiens GN=PRPF3 PE=1 SV=2	13	1	1	1

Supplementary Method

RNA sequencing

Total RNAs were collected by TRIzol reagent (Invitrogen), then rRNAs were removed by using the RiboMinus Eukaryote kit (Qiagen, Valencia, CA, USA). We next prepared the RNA-seq libraries using NEBNext Ultra Directional RNA Library Prep Kit (New England Biolabs, Beverly, MA, USA) according to the manufacturer's instructions. The ribosome-depleted RNA libraries were subjected to Illumina HiSeq3000 (Illumina, San Diego, CA, USA) for sequencing. The raw sequencing reads were first processed to clip adapter sequences and low-quality bases by software Trimmomatic. Afterward, all filtered reads were aligned to the human reference genome (hg38) using the splice-aware aligner HISAT2. The Cufflinks program was used to calculate the gene expression level in FPKM units (FPKM = Fragments Per Kilobase of transcript per Million mapped reads).

Survival analysis

Survival analysis was performed as described in a previous study [39]. Details were listed in the supplementary materials. Briefly, the expression levels of each lncRNA across all tumor samples in each sample group were used to explore whether they were associated with the prognosis status of tumor patients. For each lncRNA, GBC tumor samples were divided into two groups according to the median value of the expression. Then, the log-rank test was used to compare the difference between the two groups.

Northern blot assays and western blot assays

The Ambion Northern Max-Gly Kit (Austin, Texas, USA) was employed to detect *RP11-147L13.8* RNA levels in tissue and cells. Briefly, the nylon membrane (NC) with a positive charge was utilized to electrophorese and siphon the extracted total RNA samples. UV cross-linking was conducted to fix the RNA on the NC membrane. Then, the DIG Northern Starter Kit (Roche, Indianapolis, Indiana, USA) with a Digoxin-labelled RP11-147L13.8-specific oligonucleotide probe was employed to detect *RP11-147L13.8*.

The SDS-PAGE was employed to separate proteins and transfer proteins to nitrocellulose membranes (Bio-Rad, Hercules, California, USA), which were blocked with 5% non-fat milk and incubated with corresponding primary antibodies followed by horseradish peroxidase-conjugated secondary antibodies. Then, the chemiluminescence ECL reagents (Pierce Biotechnology, Illinois, USA) were utilized for visualization of the immunoreactivity. Additionally, the densitometry was measured by Image-Pro Plus 6.0 (Media Cybernetics, Maryland, USA).The antibody used for western blot assays were listed is **Table S2**.

RNA interference and lentivirus construction

The short hairpin RNA (shRNAs) oligonucleotides used for RP11-147L13.8 in the current study were purchased from RiboBio (Guangzhou, China). The human RP11-147L13.8 sequence was amplified from cDNAs. The pCDH-RP11-147L13.8 was generated by cloning the RP11-147L13.8 sequence into the BamHI and EcoRI sites of pCDH lentiviral vectors. The pCDH, pCDH-RP11-147L13.8 were transfected along with the packaging plasmid psPAX2 and the envelope plasmid pMD2G into the GBC cell lines by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The virus particles were collected 48h after transfection then were infected into GBC cells with recombinant lentivirus-transducing units using 1 μg/ml polybrene (Sigma-Aldrich, Missouri, USA).

***In vitro* cell migration and invasion assays and *in vivo* metastasis assays**

The invasion assays were performed in Millicell chambers that were coated with 30μg of Matrigel (BD Biosciences, Franklin Lakes, New Jersey, USA). Similar operations were conducted in the migration assays but without a coated membrane. The cells (5×10^4 and 1×10^5 for migration and invasion assays, respectively) were added to the upper chambers. DMEM containing 10% FBS was placed into the lower chambers as a chemoattractant. The cells were then incubated at 37℃ with 5% CO_2 for 24h. After the incubation, we fixed the cells that migrated or invaded through the filters with 20% methanol. Then fixed cells were then stained with 0.1% crystal violet. We randomly selected five fields to count the cell numbers by using an inverted microscope (Olympus, Tokyo, Japan).

Nude mice (female BALB/c-nu/nu mice) were purchased from the Experimental Animal Center of Shanghai Cancer Institute (Shanghai, China) for our *in vivo* metastasis assays. GBC-SD cells (1×10^6 sh vector or stable sh*RP11-147L13.8* GBC-SD cells) that were suspended in 0.2 ml serum-free DMEM were injected subcutaneously into each mouse (10 mice for each group) through the right axilla. The tumor growth was monitored. The mice were sacrificed after 60 days, then livers and lungs were dissected. The liver and lung tissue derived from the mice were fixed with phosphate-buffered neutral formalin and prepared for the following histological examination. The hematoxylin-eosin (H&E) staining was utilized to determine the number of metastatic foci in the liver or lung tissue under a binocular microscope (Leica, Wetzlar Lottehaus, Germany). The tumor volume was measured as length × width^2 × 0.5. Experiments performed in this part were all under the regulations of the Shanghai Medical Experimental Animal Care Commission.

RNA pull-down assays, mass spectrometry analyses, and RNA immunoprecipitation (RIP) assays

First, *RP11-147L13.8* and antisense *RP11-147L13.8* were transcribed *in vitro* and labeled with the Biotin RNA Labelling Mix (Roche, USA). The RNA samples were treated with RNase-free DNase I (Takara, Japan) then purified with an RNeasy Mini Kit (QIAGEN, USA). Second, to format an appropriate secondary structure, an RNA structure buffer was used to pre-treat the biotinylated RNAs. Then the pre-treated biotinylated RNAs were incubated with 1 mg protein extracts at 4℃ for 1 h. After the incubation, 40 μl streptavidin-linked magnetic beads (ThermoFisher, USA) were utilized to perform the pull-down at room temperature for 2h. Next, the beads-RNA-proteins mixture was washed in 1 × washing buffer (5mM Tris-HCl, 1 M NaCl, 0.5 mM EDTA, and 0.005% Tween 20) five times. The precipitation and dilution were conducted in 60 μl protein lysis buffer, then the proteins were separated by using gel electrophoresis. And the visualization was shown after silver staining according to the manufacturer's instructions. Finally, the retrieved proteins were measured on SDS-PAGE gels for mass spectrometry analysis (Shanghai Applied Protein Technology, Shanghai, China) or Western blot.

The Magna RIP RNA-binding protein immunoprecipitation kit (Millipore, Massachusetts, USA) was employed to conduct the RIP assays in the current study. In brief, lysis buffer (0.5 ml) were utilized to lyse cells in 10 cm dishes with protease inhibitors and RNase Inhibitor (Thermo Fisher Scientific, Illinois, USA) according to the manufacturer's instructions. The lysed cells were then subjected to centrifuge at 12,000 r.p.m. for 30 min. Then the supernatants were incubated with Protein G Dynabeads (Thermo Fisher Scientific, California, USA) and indicated antibodies. After incubation at 4℃ for 12 h, the beads were washed thrice with wash buffer then twice with PBS. Both the wash buffer and PBS contained RNase inhibitor. The Total RNA isolation kit (Thermo Fisher Scientific) was employed to extract co-precipitated RNAs, which were then subjected to qRT-PCR assays.

Immunoblotting analysis and co-immunoprecipitation (Co-IP).

The immunoblotting analysis was The lysis buffer (Beyotime Biotechnology, Shanghai, China) with protease inhibitors (Roche, Indiana, USA) was employed to lyse cells (5×10^6). The BCA method was used to determine protein concentrations (Pierce, Thermo Fisher Scientific, Illinois, USA). SDS-PAGE was utilized to analyze the samples after centrifugation at 4℃ for 15 min. The samples then were transferred to PVDF membranes (Immobilon-P membrane, Millipore, Massachusetts, USA) and HRP-conjugated secondary antibodies were employed in the following immune blotting analysis. Specifically, TBS plus Tween 20 with skimmed milk (5%) were used to block the membranes at 4℃ before the probing was conducted. Finally, the assay results were visualized by employing the Enhanced Chemiluminescence Plus Westen Blotting Detection Systems (GE Healthcare, Connecticut, USA) and LAS-4000EPUV mini Luminescent Image Analyzer with enhanced chemiluminescent (ECL) chromogenic substrates.

The details of the Co-IP were listed below. Briefly, plasmids were transiently transfected to cell lines at the 60% confluence for 48 h, and then cells in a 10 cm dish were lysed by 0.3% NP40 (with protease inhibitor, 1 mM PMSF, and phosphatase inhibitor, 1 mM Na_3VO_4) on ice. Cell lysates were incubated with GFP, Flag, or HA

beads overnight at 4 °C. Protein precipitate was harvested through centrifugation at 12,000 rpm for 10 min, washed with PBS, added with 2 × SDS sample buffer for immunoblotting samples preparation. Protein samples were separated by SDS-PAGE electrophoresis, transferred onto PVDF membrane, blocked with 5% non-fat milk for 1 h at room temperature, incubated with primary antibody overnight at 4°C, washed with PBST three times for 10 min each time, then incubated with HRP-conjugated secondary antibody for 1h at room temperature, and washed with PBST three times for 10 min each time. Finally, the target proteins were visualized.

5′ and 3′ rapid amplification of cDNA ends (RACE) analysis

Total RNA was isolated using TRIzol Plus RNA Purification Kit (Invitrogen), according to the manufacturer's instructions. 5′ RACE and 3′ RACE were performed using GeneRacer™ Kit (Invitrogen) according to the manufacturer's instructions. The following gene-specific primers (GSP) are used for PCR: 5′-ATCGATTAAGGAGGAATAAAGTCAACC-3′ (5′ RACE GSP1), 5′-CCCGACAGTCACTCGAACCAGTCGAACC-3′ (5′ RACE GSP2), 5′-CCAGGTTTTGATCTCCTTGAAGCTCCACCTC-3′ (3′ RACE GSP1), 5′-CACCCGCTATATTGGCGCCACTGTAC-3′ (3′ RACE GSP2).

The isolation of cytoplasmic and nuclear RNA

According to the manufacturer's instructions Cytoplasmic and nuclear RNAs of NOZ cells were extracted and purified using PARIS™ Kit (Invitrogen).

The establishment of gemcitabine-resistant GBC-SD cell line

The GBC cell line, GBC-SD, was cultured in DMEM (Sigma–Aldrich-Merk, Darmstadt, Germany) complete medium containing 10% fetal bovine serum (FBS) (Sigma–Aldrich-Merk, Darmstadt, Germany), 100 U/mL penicillin, and 100 μg/mL streptomycin (Life Technologies Gathersburg, MD, USA). The gemcitabine resistant

clone, named GBC-R, was obtained by exposing parental cells intermittently to escalating doses of the drug, starting from 10 nM of GEM to 1.5 μM for 9 months. After another 3 months of being continuously cultured in the presence of 1.5 μM, the GBC-R1.5 cells were considered stable.