# NSUN5 promotes progression and predicts poor prognosis in hepatocellular carcinoma

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**Abstract.** The 5-methylcytosine (m5C) RNA methyltransferase NOP2/Sun RNA methyltransferase 5 (NSUN5) has been reported to serve important roles in numerous diseases. However, the functions and clinical significance of NSUN5 in hepatocellular carcinoma (HCC) remain unknown. Clinical information and NSUN5 mRNA sequencing data for 374 patients with HCC were downloaded from The Cancer Genome Atlas (TCGA) database, and NSUN5 mRNA and protein expression levels in 120 patients with HCC (present study cohorts) were assessed using reverse transcription-quantitative PCR, western blotting or immunohistochemistry. The association between NSUN5 mRNA and protein expression levels and the clinical characteristics (or prognosis) of patients with HCC was analyzed using the $\chi^2$ or log-rank test. The functions of NSUN5 in HCC were evaluated using *in vitro* and *in vivo* experiments, and the mechanism by which NSUN5 affected the progression of HCC was assessed using bioinformatics analysis using LinkedOmics. NSUN5 was significantly upregulated and predicted poor prognosis in HCC according to data from both TCGA database and present study cohorts.

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NSUN5 significantly promoted HCC proliferation and migration *in vitro* and significantly induced HCC tumor growth *in vivo*. Bioinformatics analysis demonstrated that NSUN5 was positively correlated with genes associated with translation in HCC. It was hypothesized that overexpression of NSUN5 strengthened ribosome functions and global protein translation, which may promote the proliferation and migration of HCC. In conclusion, NSUN5 may promote the progression of HCC by enhancing translation, thus making it a potential target for HCC treatment.

## Introduction

Hepatocellular carcinoma (HCC) is a major health problem, which represents the sixth most commonly diagnosed cancer and third most common cause of cancer death worldwide (1). The mortality from HCC is primarily due to its metastasis, complex pathogenesis and postsurgical recurrence (2). Early HCC onset is usually undetected and most patients are in the late stage at diagnosis, which is also an important reason for the poor long-term prognosis (2). Molecularly targeted agents and immune checkpoint inhibitors have been developed; however, the therapeutic effect of such treatments on HCC is still unsatisfactory (3). Drug resistance and limited therapeutic targets are problems to be solved. Therefore, it is critical to study the mechanisms underlying the development and progression of HCC to develop novel therapeutics that may improve the overall survival of patients with HCC across its stages.

Post-transcriptional RNA modifications are crucial regulators of gene expression and are involved in the pathogenesis of numerous diseases, including different types of cancer (4). Notably, 5-methylcytosine (m5C) is an abundant and conserved modification in numerous types of RNA, including transfer RNA, mRNA, ribosomal (r)RNA and other types of noncoding RNA (4). The roles of the RNA m5C modification and its modifying enzymes, defined as writers (proteins upregulating modification level), readers (proteins recognizing modification) and erasers (proteins downregulating modification level), in gene regulation and chromatin organization remain largely unclear (5). NOP2/Sun RNA methyltransferase

5 (NSUN5) is one of the eight evolutionarily conserved m5C RNA methylases (NSUN1-7 and DNA methyltransferase 2) that have been recognized as 'writers' (4,6,7).

Previous studies have reported the role of NSUN5 in human cancer. For example, DNA methylation-associated epigenetic silencing of NSUN5 has been reported to be a hallmark of the long-term survival of patients with glioma (8). In colorectal cancer, NSUN5 has been reported to act as a promotor of tumor development via cell cycle regulation (9). However, to the best of our knowledge, the role of NSUN5 in HCC remains unknown.

In the present study, the clinical significance of NSUN5 expression in patients with HCC was evaluated using The Cancer Genome Atlas (TCGA) database. In addition, the role of NSUN5 in HCC was assessed using *in vitro* and *in vivo* experiments to evaluate whether NSUN5 represented a novel therapeutic target for HCC.

## Materials and methods

*Patients and samples*. Written informed consent was obtained from patients and approval for the present study was obtained from the Ethics Committee of The Eastern Hepatobiliary Surgery Hospital (Shanghai, China; approval no. EHBHKY2 018-02-014). A total of 120 paired HCC and corresponding adjacent noncancerous liver (ANL) tissues were collected from patients undergoing hepatectomy (without preoperative treatment) at The Eastern Hepatobiliary Surgery Hospital. Detailed clinical characteristics are presented in Table SI. Among the samples, 40 pairs (Cohort 1) were collected from April 2021 to July 2021 and assessed using reverse transcription-quantitative (RT-q)PCR and western blotting, and the other 80 pairs (Cohort 2) were collected from February 2014 to November 2015- and assessed using immunohistochemistry (IHC).

*RT-qPCR*. Total RNA was isolated from human HCC tissues, ANL tissues or HCC cell lines using TRIzol® reagent (cat. no. 1559602. Invitrogen; Thermo Fisher Scientific, Inc.). cDNA was synthesized (15 min at 37°C; 5 sec at 85°C and 4°C) using a cDNA Synthesis Kit (cat. no. RR036A; Takara Biotechnology Co., Ltd.) and qPCR was then performed using a StepOnePlus™ Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) and TB Green Fast qPCR Mix (cat. no. RR430A; Takara Biotechnology Co., Ltd.). The quantification of NSUN5 was performed using the $2^{-\Delta\Delta Cq}$ method (10) with normalization to β-actin (ACTB). The thermocycling conditions were as follows: Holding stage (95°C, 3 min), cycling stage (35 cycles; 10 sec at 95°C, 15 sec at 60°C and 20 sec at 72°C), melt curve stage (15 sec at 95°C, 1 min at 60°C and 15 sec at 95°C). The primer sequences used were as follows: NSUN5 forward (F), 5'-CGCTACCATGAGGTC CACTAC-3' and reverse (R), 5'-GCATCTCGCACCACGTCT T-3'; and ACTB F, 5'-CCACCATGTACCCTGGCATTG-3' and R, 5'-TCATCTTGTTTTCTGCGCAAGTTA-3'.

*Western blotting*. Whole-cell lysates were prepared from HCC tissues and cells (Hep3B and SNU387) using RIPA solution (Beyotime Institute of Biotechnology). The concentration of protein in the lysates was determined by BCA protein assay kit (cat. no. P0012; Beyotime Institute of Biotechnology)

The lysates (50 $\mu$g protein per lane) were then separated by SDS-PAGE on 4-20% gels (SurePAGE™; Bis-Tris; 10x8; 4-20%; gradient, 12 wells; cat. no. M00656; GenScript Biotechnology Co., Ltd.), transferred to a PVDF membrane and blocked using 5% nonfat milk for 1 h at room temperature (~25°C). Primary antibodies were added after blocking and the membranes were incubated overnight at 4°C. The next day, the membranes were incubated for 2 h with the appropriate secondary antibody at room temperature. Finally, the protein bands were visualized using enhanced chemiluminescent solution (Beyotime Institute of Biotechnology) and detected using a chemiluminescence system (Bio-Rad Laboratories, Inc.). The primary antibodies used in the present study were NSUN5 (1:1,000; cat. no, 15449-1-AP; Proteintech Group, Inc.) and ACTB (1:5,000; cat. no, 66009-1-Ig; Proteintech Group, Inc.). The secondary antibodies used were goat anti-mouse IgG H&L (HRP) (1:2,000; cat. no. ab6789; Abcam) and goat anti-rabbit IgG H&L (HRP) (1:2,000; cat. no. ab6721; Abcam). Image J (version 1.8.0; Bharti Airtel, Ltd) was used to semi-quantify western blots.

*IHC and tissue microarray (TMA) analysis*. A TMA was constructed as previously described (11). Briefly, it consisted of collection and selection of tissue blocks (formalin-fixed paraffin-embedded HCC and ANL tissues), design and organization of TMA, punching of TMA and technical cutting of TMA (sections should be ≤5 $\mu$m). IHC was performed on the TMA using a two-step immune-peroxidase technique (12) (https://www.abcam.com/ps/pdf/protocols/ihc_p.pdf). Briefly, it consisted of deparaffinization (xylene and ethanol), antigen retrieval (vegetable steamer, 100°C) and immunohistochemical staining (primary antibody incubated overnight at 4°C; second antibody incubated for 1 h at room temperature; chromogen, 10 min at room temperature). The primary antibody used was NSUN5 (1:100; cat. no, 15449-1-AP; Proteintech Group, Inc.). The blocking reagent was 0.3% $H_2O_2$ in TBS (incubated for 15 min at room temperature). The secondary antibody used was goat anti-rabbit IgG H&L (HRP) (1:1,000; cat. no. ab6721; Abcam). TMA scanning was performed using PANNORAMIC DESK II DW scanner (light microscope; 3DHISTECH, Ltd.). IHC scores were assessed for the IHC stains of NSUN5 (13) by three separate observers who had no knowledge of the patient characteristics. The intensity of specific staining was characterized as follows: Not present, 0; weak but detectable above control, 1+; distinct, 2+; and very strong, 3+. For each observed tissue component, a summary value referred to as the H-Score was calculated. This consisted of a sum of the percentages of positively stained cells multiplied by a weighted intensity of staining; H-Score=$\sum$ Pi (i + 1), where 'Pi' was the percentage of stained cells in each intensity category and 'i' was the intensity.

*Cell culture*. The HCC Hep3B and SNU387 cell lines were purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences and were authenticated using short tandem repeat profiling. Cells were grown in Dulbecco's modified Eagle's medium (DMEM; Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% fetal bovine serum (FBS; Gibco; Thermo Fisher Scientific, Inc.) and maintained in a humidified 37°C incubator containing 5%

$CO_2$. Using the sequence of NSUN5 mRNA (NM_148956) acquired from the Nucleotide database of National Center for Biotechnology Information (https://www.ncbi.nlm.nih.gov/ nuccore/NM_148956) and a lentiviral vector (GV492; Ubi-MCS-3FLAG-CBh-gcGFP-IRES-puromycin; Shanghai GeneChem Co., Ltd.), NSUN5 was overexpressed in Hep3B and SNU387 cell lines using the methods previously described (14,15). The viral titer of NSUN5-overexpression (NSUN5-oe) lentivirus and negative control (NC) lentivirus was $4x10^8$ and $3x10^8$ TU/ml, respectively. A total of 48 h after lentivirus transduction, puromycin (2 $\mu$g/ml) was added into the culture medium to obtain stably transduced cells. After ~10 days of puromycin selection, the stably transduced cells were collected and subsequent experiments were performed.

*In vitro cell behavior assays*. For cell proliferation, a Cell Counting Kit-8 (CCK-8) assay (Dojindo Molecular Technologies, Inc.) was performed according to the manufacturer's protocol. Briefly, ~$2x10^3$ cells/well were seeded on a 96-well plate for 24 h and 10 $\mu$l CCK-8 solution was added to wells at 0, 1, 2 , 3 or 4 days. The 96-well plate was incubated at 37˚C for 2 h after each addition of CCK-8 solution before measuring the absorbance at 450 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

Cell migration was evaluated using a 12-$\mu$m pore polycarbonate membrane Transwell chambers (MilliporeSigma). Cells ($10^5$) were seeded into the upper Transwell chamber in DEEM (cat no. 11995-065; Gibco; Thermo Fisher Scientific) without FBS and 500 $\mu$l DMEM containing 10% FBS was added into the lower chamber. The 24-well plate with Transwell chambers was incubated for 48 h at 37˚C. After incubation for 48 h, the cells were stained using 0.1% crystal violet for 30 min at room temperature (~25˚C). Finally, the upper chambers were observed using a light microscope (Olympus Corporation). These experiments were independently repeated at least three times.

*Animal studies*. The animal experiments performed in the present study conformed to the guidelines of Animal Research: Reporting of *In Vivo* Experiments (http://www.nc3rs.org.uk/arrive-guidelines) and were approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese (Shanghai, China) (approval no. PZ SHUTCM210926007). BALB/c male nude mice (16 mice; age, 5 weeks; weight, ~20 g) used in the present study were purchased from the Laboratory Animal Resources, Chinese Academy of Sciences. All mice were housed in laminar flow cabinets under specific pathogen-free conditions at room temperature (humidity 40-60%) with a 12-h light/dark cycle, and *ad libitum* access to food and water.

The subcutaneous tumor xenograft experiment was performed as previously described (16). A total of eight BALB/C male nude mice were used in the present study. Briefly, for each mouse, NSUN5-oe SNU387 cells ($1.5x10^6$ cells in 150 $\mu$l 0.9% sodium chloride) were injected subcutaneously into the armpit of the right forelimb (site approved by the ethics committee) and the same amount of NC SNU387 cells were injected subcutaneously into the armpit of the left forelimb. Tumor development was assessed weekly using a caliper and the tumor volume was calculated as follows: Larger diameter x

(smaller diameter)$^2$/2. The humane endpoints of the xenograft experiment conformed to the Guidelines for the Welfare and Use of Animals in Cancer Research (17). Briefly, immediate humane termination was required when statistically significant effects could be demonstrated, when the mean diameter of the xenograft tumor exceeded ~1.2 cm or when the mice displayed severe symptoms (persistent hypothermia, hind-limb paralysis or weakness). In the present study, the mice were sacrificed by cervical dislocation 4 weeks after implantation, when NSUN5 showed statistically significant growth promotion effects.

*LinkedOmics*. The 'LinkFinder' module in LinkedOmics (version 4) (18) (http://linkedomics.org/login.php) was used to assess NSUN5-correlated genes in HCC. The 'RNA-seq' platform was chosen for both the 'Search Dataset' and 'Target Dataset', and the 'Spearman Correlation test' was selected as the statistical method. The LinkFinder module of LinkedOmics was used to generate the volcano plots.

NSUN5-correlated genes ('Association Result') were then used to perform enrichment analyses in the 'Link Interpreter-GSEA (Gene Set Enrichment Analysis)' module of LinkedOmics. Biological process, cellular component, molecular function and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways analyses were performed individually. 'Rank Criteria' was chosen as the 'Statistic' ('Spearman correlation' in this situation), 'Minimum Number of Genes' was assigned a value of 3 and 'Simulations' was assigned a value of 500.

*TCGA*. The URL link for TCGA: https://www.cancer.gov/about-nci/organization/ccg/research/structural-genomics/tcga. To evaluate the relationship between the expression of NSUN5 and the clinical characteristics of patients with HCC, the clinical information of 374 patients with HCC was downloaded from TCGA liver HCC database (https://portal.gdc.cancer.gov/) (Table SII). NSUN5 mRNA sequencing data for these 374 HCC tissues and 50 ANL tissues (paired with 50 of the 374 HCC tissues) were also downloaded (19) (https://portal.gdc.cancer.gov/) (columns C and D, Table SII).

*Statistical analysis*. Statistical analyses were performed using SPSS software (version 22.0; IBM, Corp.). Comparisons among continuous variables were performed using paired Student's t-test, unpaired Student's t-test, Mann-Whitney U test or Wilcoxon signed-rank test, whereas comparisons among categorical variables were performed using the Pearson's $\chi^2$ test or Fisher's exact test. The survival curves were generated using the Kaplan-Meier method, and the differences were assessed using a log-rank test. $P<0.05$ (two-tailed) was considered to indicate a statistically significant difference.

## Results

*NSUN5 is upregulated and predicts poor prognosis in HCC according to TCGA database*. The present study demonstrated that the mRNA expression levels of NSUN5 were significantly higher in the 374 HCC tissues compared with those in the 50 ANL tissues (Fig. 1A; $P<0.001$). The mRNA expression levels of NSUN5 in 50 HCC tissues were also significantly higher than those in 50 paired ANL tissues (Fig. 1B; $P<0.001$).
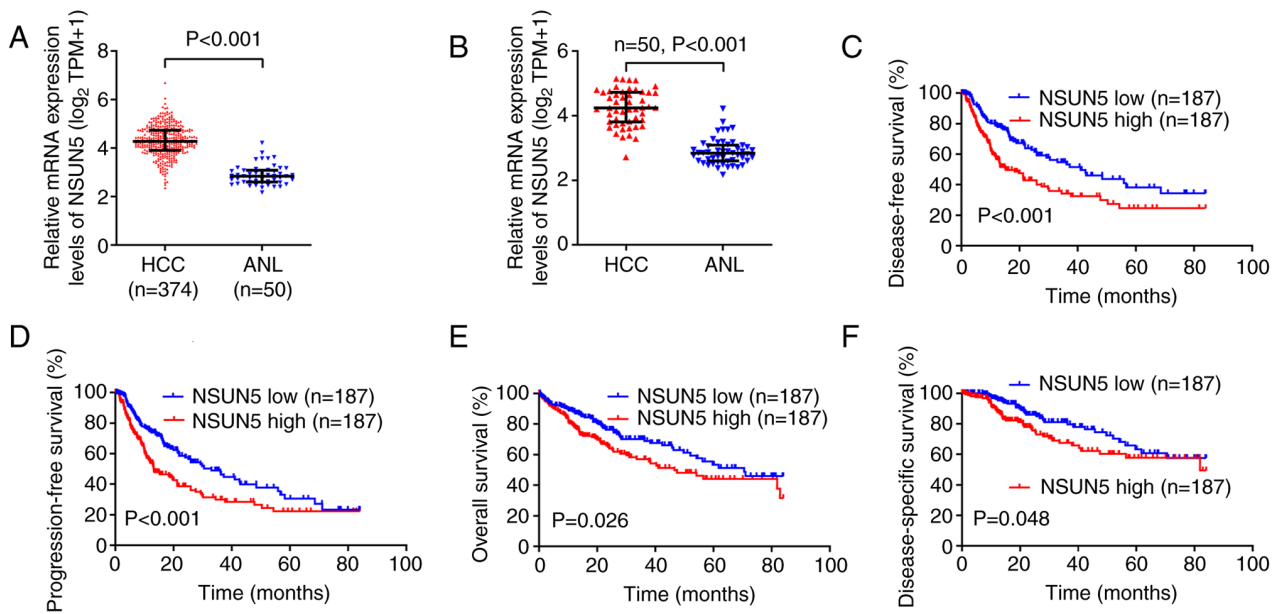
Figure 1. Upregulated NSUN5 predicts poor prognosis in HCC according to The Cancer Genome Atlas database. (A) mRNA expression levels of NSUN5 in 374 HCC and 50 ANL tissues. Mann-Whitney U test was used for statistical comparison. (B) mRNA expression levels of NSUN5 in 50 paired HCC and ANL tissues. The Wilcoxon signed-rank test was used for statistical comparison. Kaplan-Meier survival curves demonstrated the association between NSUN5 expression and (C) disease-free survival, (D) progression-free survival, (E) overall survival and (F) disease-specific survival. The log-rank test was used for statistical comparison. ANL, adjacent noncancerous liver; HCC, hepatocellular carcinoma; NSUN5, NOP2/Sun RNA methyltransferase 5; TPM, transcript per million.

The 374 patients with HCC were divided into NSUN5-high and NSUN5-low groups according to the median mRNA expression (transcript per million, 18.497) levels of NSUN5 in the 374 HCC tissues. The longest follow-up time assessed in the present study was 7 years (84 months). Kaplan-Meier survival curves demonstrated that higher NSUN5 mRNA expression levels in HCC were associated with significantly lower disease-free survival (Fig. 1C; P<0.001), lower progression-free survival (Fig. 1D; P<0.001), lower overall survival (Fig. 1E; P=0.026) and lower disease-specific survival (Fig. 1F; P=0.048) compared with the NSUN5-low group.

Furthermore, analysis of the association between NSUN5 expression in HCC tissues and the clinical characteristics of patients with HCC demonstrated that higher NSUN5 mRNA expression levels were significantly positively associated with lower weight (P=0.003), lower body mass index (P=0.001), higher serum α-fetoprotein levels (P=0.003), worse tumor node metastasis (TNM) stage (20) (P=0.043), worse histological grade (P=0.004) and vascular invasion (P=0.002) compared with the NSUN5-low group (Table I). These results indicated that NSUN5 may be associated with HCC tumor growth and migration.

Collectively, the bioinformatics analysis of TCGA data demonstrated that NSUN5 was upregulated, associated with worse clinical characteristics and predicted poor prognosis in HCC.

*NSUN5 is upregulated and predicts poor prognosis in HCC in cohorts of the present study.* To further evaluate the relationship between the expression of NSUN5 and the clinical characteristics of patients with HCC, the mRNA and protein expression levels of NSUN5 were evaluated in the cohorts of the present study (patients with HCC from The Eastern

Hepatobiliary Surgery Hospital). RT-qPCR performed in 40 paired HCC and ANL tissues (Cohort 1) demonstrated that the mRNA expression levels of NSUN5 were significantly upregulated in HCC tissues compared with those in the ANL tissues (Fig. 2A; P<0.001). Western blotting in 12 paired HCC and ANL tissues randomly selected from Cohort 1 demonstrated that the protein expression levels of NSUN5 were significantly upregulated in HCC tissues compared with those in the ANL tissues (Fig. 2B and C; P=0.003). Moreover, IHC in Cohort 2 demonstrated that NSUN5 was mainly expressed in the nucleus and indicated that the protein expression levels [indicated by the IHC score (21)] of NSUN5 in 80 HCC tissues was significantly higher than those in the paired ANL tissues (Fig. 2D; P<0.001). The 80 patients with HCC from Cohort 2 were then divided into NSUN5-high and NSUN5-low groups according to the median IHC score (159.029) of NSUN5. Kaplan-Meier survival curves demonstrated that higher NSUN5 expression in HCC was associated with significantly lower recurrence-free survival (Fig. 2E; P=0.002) and lower overall survival (Fig. 2F; P<0.001) compared with the NSUN5-low group.

Furthermore, analysis of the association between the expression of NSUN5 in HCC tissues and the clinical characteristics of patients with HCC demonstrated that higher NSUN5 protein expression levels were positively associated with multiple tumor number (P<0.001), large tumor size (P=0.003), presence of microvascular invasion (P=0.001), worse TNM stage (P<0.001) and worse Barcelona Clinic Liver Cancer stage (22) (P=0.001) compared with the NSUN5-low group (Table II).

In summary, NSUN5 mRNA and protein expression levels were significantly upregulated in HCC, associated with worse clinical characteristics and predictive of poor prognosis in the present study cohorts.

Table I. Clinical characteristics of 374 patients with hepatocellular carcinoma from The Cancer Genome Atlas according to the mRNA expression levels of NSUN5.

| Characteristic | NSUN5 Low | NSUN5 High | P-value |
|---|---|---|---|
| n | 187 | 187 | |
| Median age, years (IQR) | 61 (52-68) | 61 (51-69) | 0.956[a] |
| Median height, cm (IQR) | 168 (161-175) | 167 (161-172) | 0.289[a] |
| Median weight, kg (IQR) | 73 (61.5-85.5) | 68 (58.0-77.5) | 0.003[a] |
| Median BMI, kg/m$^2$ (IQR) | 25.65 (22.41-29.85) | 23.86 (20.80-27.44) | 0.001[a] |
| Median AFP, ng/ml (IQR) | 9.5 (4.00-52.25) | 26 (4.00-1772.25) | 0.003[a] |
| Median albumin, g/dl (IQR) | 4 (3.5-4.3) | 4 (3.5-4.3) | 0.895[a] |
| Median prothrombin time, sec (IQR) | 1.1 (1.00-9.45) | 1.1 (1.00-8.75) | 0.302[a] |
| TNM stage, n (%) | | | 0.043[c] |
| Stage I | 100 (53.5%) | 73 (39.0%) | |
| Stage II | 37 (19.8%) | 50 (26.7%) | |
| Stage III | 37 (19.8%) | 48 (25.7%) | |
| Stage IV | 2 (1.1%) | 3 (1.6%) | |
| Missing | 11 (5.9%) | 13 (7.0%) | |
| Sex, n (%) | | | 0.320[b] |
| Female | 65 (34.8%) | 56 (29.9%) | |
| Male | 122 (65.2%) | 131 (70.1%) | |
| Residual tumor, n (%) | | | 0.713[c] |
| R0 | 166 (88.8%) | 161 (86.1%) | |
| R1 | 8 (4.3%) | 9 (4.8%) | |
| R2 | 0 (0.0%) | 1 (0.5%) | |
| Missing | 13 (7.0%) | 16 (8.6%) | |
| Histologic grade, n (%) | | | 0.004[b] |
| G1 | 34 (18.2%) | 21 (11.2%) | |
| G2 | 98 (52.4%) | 80 (42.8%) | |
| G3 | 46 (24.6%) | 78 (41.7%) | |
| G4 | 6 (3.2%) | 6 (3.2%) | |
| Missing | 3 (1.6%) | 2 (1.1%) | |
| Adjacent hepatic tissue inflammation, n (%) | | | 0.835[b] |
| None | 65 (34.8%) | 53 (28.3%) | |
| Mild | 52 (27.8%) | 49 (26.2%) | |
| Severe | 9 (4.8%) | 9 (4.8%) | |
| Missing | 61 (32.6%) | 76 (40.6%) | |
| Child-Pugh grade, n (%) | | | 1.000[c] |
| A | 115 (61.5%) | 104 (55.6%) | |
| B | 11 (5.9%) | 10 (5.3%) | |
| C | 1 (0.5%) | 0 (0.0%) | |
| Missing | 60 (32.1%) | 73 (39.0%) | |
| Fibrosis Ishak score, n (%) | | | 0.088[b] |
| 0 | 45 (24.1%) | 30 (16.0%) | |
| 1/2 | 13 (7.0%) | 18 (9.6%) | |
| 3/4 | 10 (5.3%) | 18 (9.6%) | |
| 5/6 | 45 (24.1%) | 36 (19.3%) | |
| Missing | 74 (39.6%) | 85 (45.5%) | |
| Vascular invasion, n (%) | | | 0.002[b] |
| No | 122 (65.2%) | 86 (46.0%) | |
| Yes | 44 (23.5%) | 66 (35.3%) | |
| Missing | 21 (11.2%) | 35 (18.7%) | |

[a]Mann-Whitney U test; [b]Pearson's $\chi^2$ test; [c]Fisher's exact test. AFP, α-fetoprotein; BMI, body mass index; IQR, interquartile range; NSUN5, NOP2/Sun RNA methyltransferase 5; TNM, tumor node metastasis. The missing data in this table were not included in the statistical analyses.
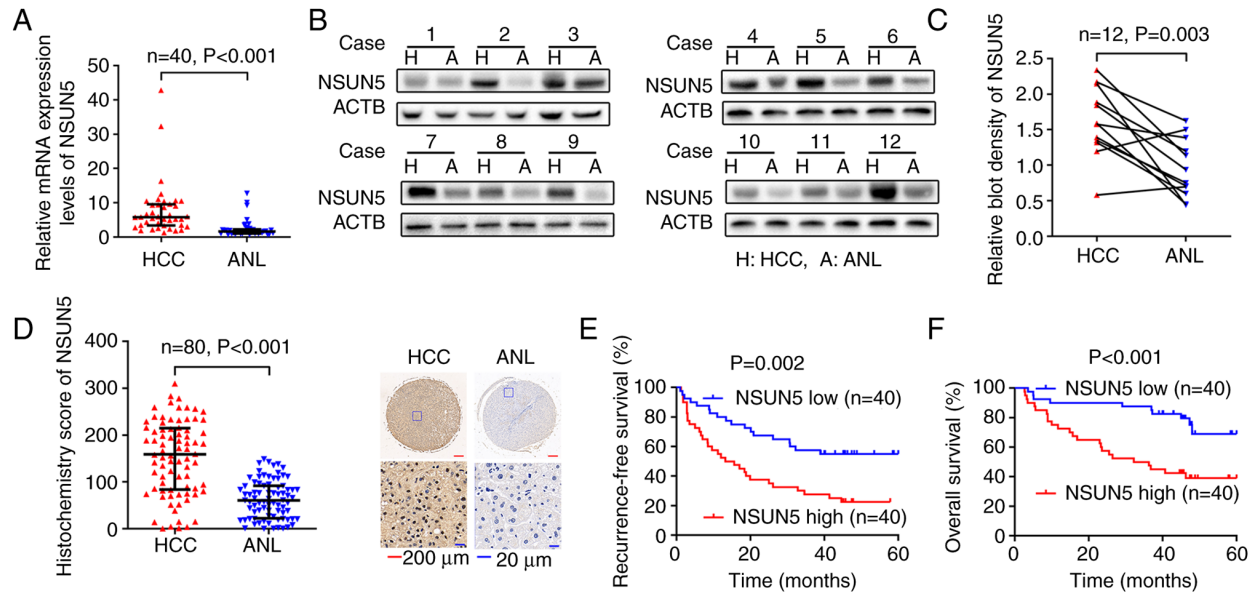
Figure 2. Upregulated NSUN5 mRNA and protein expression levels predict poor prognosis in HCC. (A) mRNA expression levels of NSUN5 were assessed using reverse transcription-quantitative PCR. The Wilcoxon signed-rank test was used for statistical comparison. (B and C) Protein expression levels of NSUN5 were semi-quantified using western blotting in 12 paired HCC and ANL tissues relative to ACTB. Paired Student's t-test was used for statistical comparison. (D) Immunohistochemistry staining of NSUN5, (left) histochemistry score of NSUN5 in 80 paired HCC tissues and ANL tissues, Wilcoxon signed-rank test was used for statistical comparison and (right) representative samples. Kaplan-Meier survival curves presented the association between NSUN5 protein expression levels and (E) recurrence-free survival or (F) overall survival. The log-rank test was used for statistical comparison. ANL, adjacent noncancerous liver; HCC, hepatocellular carcinoma; NSUN5, NOP2/Sun RNA methyltransferase 5; ACTB, β-actin.
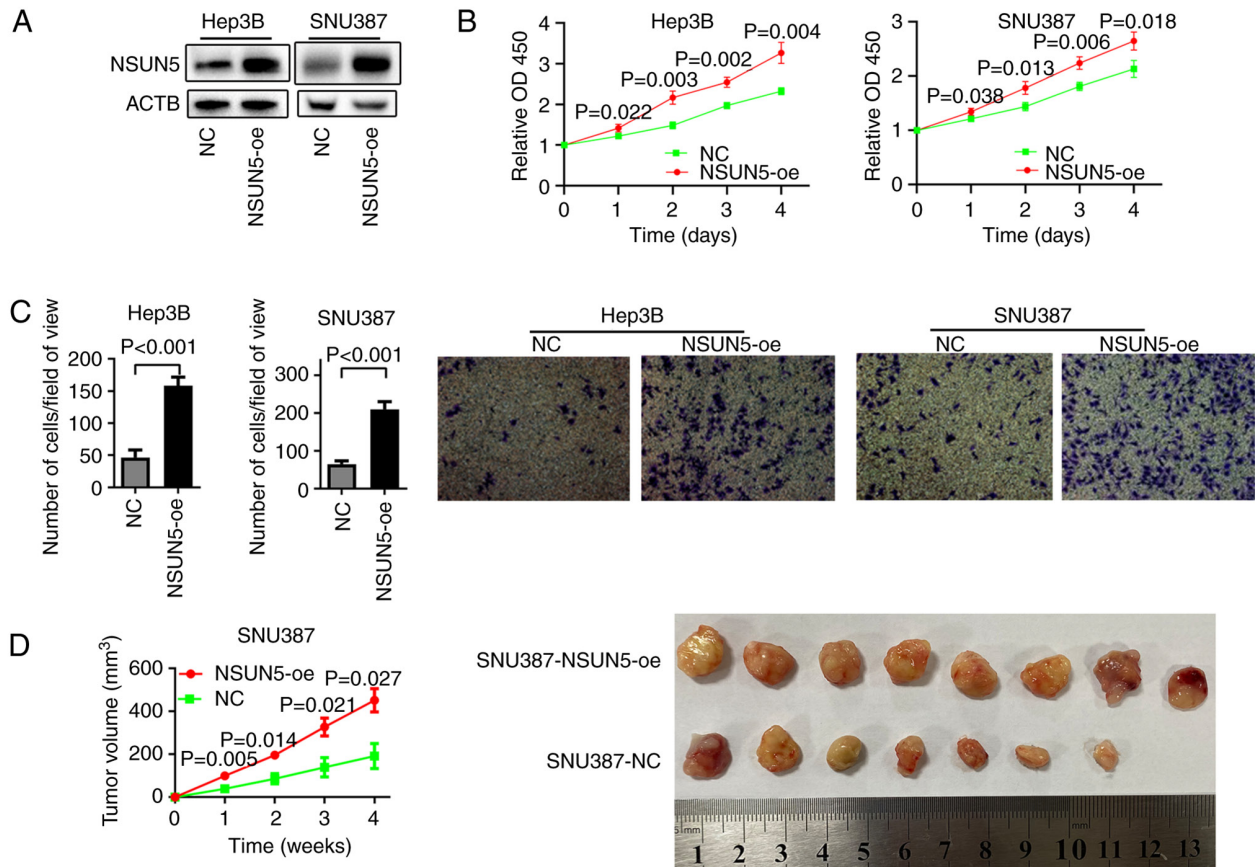


Figure 3. NSUN5 promotes HCC cell proliferation and migration. (A) Protein expression levels of NSUN5 were assessed using western blotting in NSUN5-oe and NC HCC cells. (B) Cell Counting Kit-8 assay results demonstrated that NSUN5-oe promoted the proliferation of HCC cells. (C) Transwell migration assays demonstrated that NSUN5-oe promoted the migration of HCC cells. (B and C) Data are presented as the mean ± SD, n=3; unpaired Student's t-test was used for statistical comparison. (D) Subcutaneous xenografts of HCC SNU387 cells excised from nude mice and tumor volume (n=8). Data are presented as the mean ± SEM and the Mann-Whitney U test was used for statistical comparison. NSUN5, NOP2/Sun RNA methyltransferase 5; HCC, hepatocellular carcinoma; NC, negative control; oe, overexpression.

Table II. Clinical characteristics of 80 patients with hepatocellular carcinoma according to the protein expression levels of NSUN5.

| Variable | NSUN5 Low | NSUN5 High | P-value |
|---|---|---|---|
| n | 40 | 40 | |
| Age, years | | | 0.653[a] |
| ≤50 | 23 | 21 | |
| >50 | 17 | 19 | |
| Sex | | | 0.793[a] |
| Female | 9 | 10 | |
| Male | 31 | 30 | |
| HBsAg | | | 1.000[b] |
| Negative | 3 | 2 | |
| Positive | 37 | 38 | |
| Liver cirrhosis | | | 0.633[a] |
| Without | 12 | 14 | |
| With | 28 | 26 | |
| AFP, $\mu$g/l | | | 0.143[a] |
| ≤20 | 15 | 9 | |
| >20 | 25 | 31 | |
| Pathological satellite | | | 0.179[a] |
| Absent | 24 | 18 | |
| Present | 16 | 22 | |
| Tumor number | | | <0.001[a] |
| Solitary | 39 | 27 | |
| Multiple | 1 | 13 | |
| Edmondson's grade | | | 0.363[a] |
| I + II | 8 | 5 | |
| III + IV | 32 | 35 | |
| Tumor size, cm | | | 0.003[a] |
| ≤5 | 24 | 11 | |
| >5 | 16 | 29 | |
| Microvascular invasion | | | 0.001[a] |
| Absent | 31 | 16 | |
| Present | 9 | 24 | |
| Encapsulation | | | 1.000[a] |
| Complete | 14 | 14 | |
| Incomplete | 26 | 26 | |
| TNM stage | | | <0.001[a] |
| I | 39 | 25 | |
| I + II | 1 | 15 | |
| BCLC stage | | | 0.001[a] |
| 0 + A | 39 | 28 | |
| B + C | 1 | 12 | |

Statistical comparisons were performed using [a]Pearson's $\chi^2$ test or [b]Fisher's exact test. HBsAg, hepatitis B surface antigen; AFP, α-fetoprotein; TNM, Tumor Node Metastasis; BCLC, Barcelona Clinic Liver Cancer.

*NSUN5 promotes the proliferation and migration of HCC cells.* To assess the role of NSUN5 in HCC progression, NSUN5 was overexpressed in Hep3B and SNU387 HCC cells (Fig. 3A). CCK-8 and Transwell migration assay results demonstrated that overexpression of NSUN5 significantly promoted the proliferation (Fig. 3B) and migration (Fig. 3C) of HCC cells compared with in the NC group. To assess the function of NSUN5 *in vivo*, a subcutaneous tumor xenograft experiment was performed, which demonstrated that the NSUN5-oe SNU387 xenograft grew significantly faster than the negative control (Fig. 3D). Notably, the NSUN5-NC cells injected into one of the mice did not develop into a tumor.

These results indicated that NSUN5 could promote the proliferation and migration of HCC *in vitro*, and induce the tumor growth of HCC *in vivo*.

*NSUN5 is positively correlated with translation in HCC.* To evaluate the mechanism by which NSUN5 promoted the progression of HCC, a bioinformatics analysis of NSUN5-correlated genes was performed using LinkedOmics. Among the 19,922 genes analyzed in Table SIII, 4,212 genes were positively correlated and 5,981 genes were negatively correlated with NSUN5 in HCC (P<0.01; Table SIII). The volcano plot showing the correlated genes is presented in Fig. 4A. The top 50 NSUN5 positively and negatively correlated genes are presented in Fig. 4B. Furthermore, Gene Ontology (biological process, cellular component, molecular function) and KEGG pathway analyses of the NSUN5-correlated genes were performed (Fig. 4C-F). Among the 10 biological processes in which NSUN5-positively correlated genes were enriched, five ('protein localization to endoplasmic reticulum', 'ribonucleoprotein complex biogenesis', 'translational elongation', 'protein transmembrane transport' and 'protein folding') were associated with translation (Fig. 4C). Among the seven cellular components in which NSUN5-positively correlated genes were enriched, two ('spliceosomal complex' and 'small nucleolar ribonucleoprotein complex') were associated with translation (Fig. 4D). Among the nine molecular functions in which NSUN5-positively correlated genes were enriched, three ('rRNA binding', 'unfolded protein binding' and 'translation factor activity, RNA binding') were associated with translation (Fig. 4E). Among the 10 KEGG pathways in which NSUN5-positively correlated genes were enriched, three ('ribosome', 'spliceosome' and 'ribosome biogenesis in eukaryotes') were associated with translation (Fig. 4F).

Taken together, these results indicated that NSUN5 was positively correlated with translation in HCC.

**Discussion**

HCC has a high mortality-to-incidence ratio (23) and the treatment options are severely limited (24); therefore, new treatment approaches are urgently needed. Although emerging immunotherapies (such as immune checkpoint inhibitors) can prolong the survival time of certain patients, the therapeutic effect is limited (25,26). Identifying novel mechanisms may aid the design of new targeted therapies. To the best of our knowledge, the present study is the first to report NSUN5 as a promising molecular target for HCC treatment.

Previous studies on NSUN5 have mostly focused on nervous system diseases. For example, NSUN5 has been reported to contribute to the pathology of Williams-Beuren syndrome (WBS) (27), a neurodevelopmental disorder caused
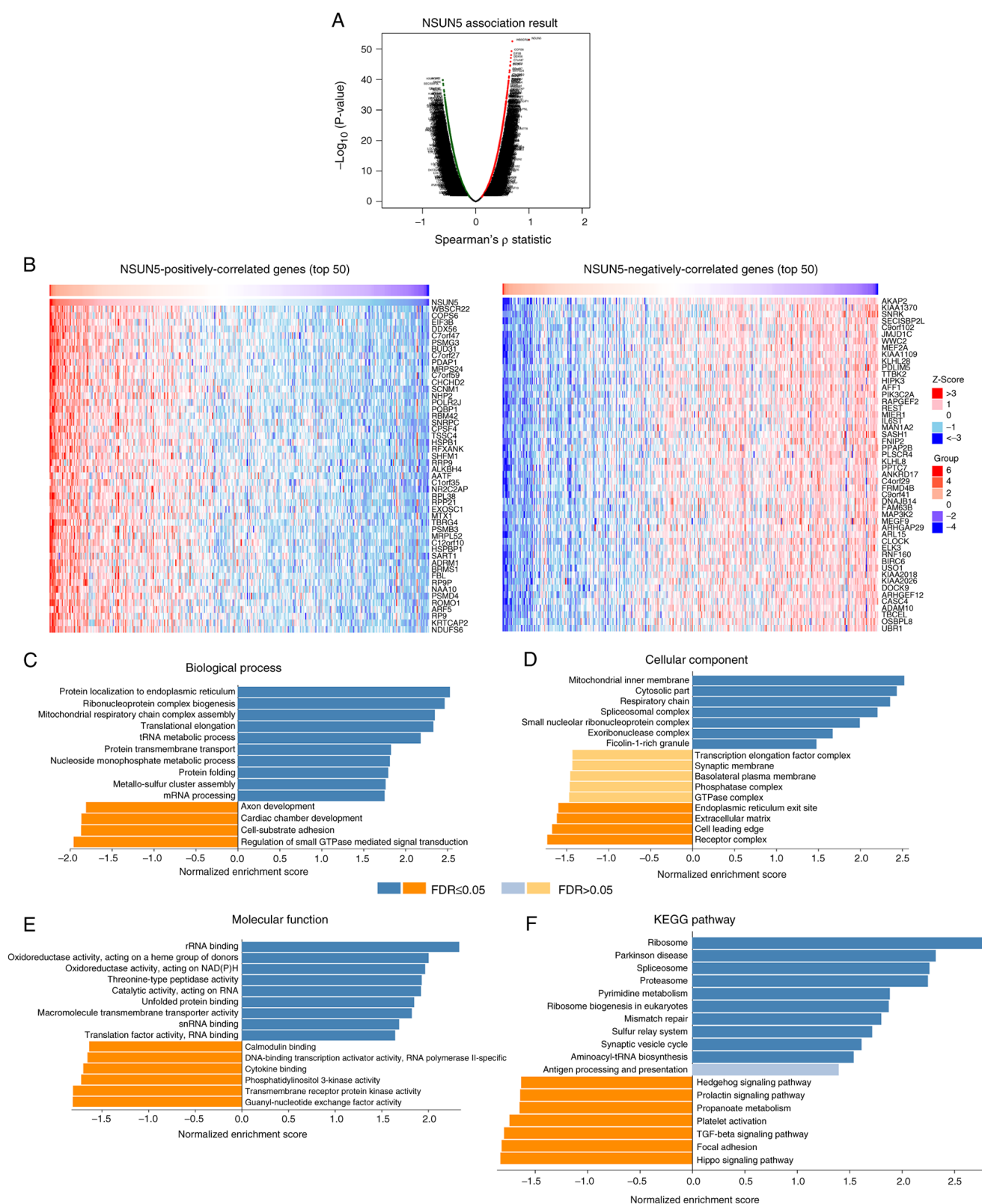
Figure 4. Bioinformatics analysis of NSUN5-correlated genes using LinkedOmics. (A) Spearman's correlation test was performed to assess the correlations between NSUN5 and differentially expressed genes in HCC. (B) Heatmaps presented the top 50 genes that were positively and negatively correlated with NSUN5 in HCC. (C) Biological process, (D) cellular component, (E) molecular function and (F) KEGG pathway analyses of NSUN5-correlated genes in HCC. Blue indicated a positive correlation and orange indicated a negative correlation. NSUN5, NOP2/Sun RNA methyltransferase 5; HCC, hepatocellular carcinoma; FDR, false discovery rate; KEGG, Kyoto Encyclopedia of Genes and Genomes.

by microdeletions of 28 genes, and characterized by cognitive disorders and hypertrophy of the corpus callosum (28,29). The NSUN5 gene is deleted in ~95% of patients with WBS (28).

NSUN5-knockout mice have been reported to exhibit spatial cognition deficits, impaired cerebral cortex development and corpus callosum agenesis (5,28,29).

In recent years, the role of NSUN5 in human cancer has been reported. For example, Jiang *et al* (9) reported that NSUN5 promoted the proliferation of colorectal cancer via regulation of the cell cycle. In addition, Janin *et al* (8) reported that NSUN5 inhibited the growth of glioma tumors *in vivo*. In the present study, it was demonstrated that NSUN5, which is mainly expressed in the nucleus, was upregulated in HCC, and this was associated with worse clinical characteristics and was predictive of poor prognosis in HCC, according to TCGA database and the present study cohorts. The results of the present study also indicated that NSUN5 could promote the proliferation and migration of HCC *in vitro* and induce the growth of HCC tumors *in vivo*. As NSUN5 may serve contradictory roles in different organs, potential drugs targeting NSUN5 in HCC should be hepatocyte specific. It has been reported that N-acetylgalactosamine or apolipoprotein-modified nanoparticles can selectively deliver small interfering (si)RNAs to HCC cells (30). Therefore, siRNAs against NSUN5 could be developed as a drug to treat HCC using this method in the future.

Heissenberger *et al* (27) reported that NSUN5 is an rRNA methyltransferase that can mediate the m5C modification of the C3782 position of 28S rRNA in humans and the C3438 position of 28S rRNA in mice. Notably, in this previous study, NSUN5 knockout inhibited the proliferation of human cervical carcinoma HeLa cells and knockout of Nsun5 in the whole mouse body reduced mouse body weight (27). Mechanistically, the loss of NSUN5 was reported to have generated a lower m5C level of 28S rRNA, which impaired the functions of ribosomes and translation of global proteins, thereby decreasing cell proliferation and size (27). In the present study, the bioinformatics analysis demonstrated that NSUN5 was significantly positively correlated with genes associated with ribosomes and translation in HCC. Therefore, it could be hypothesized that overexpression of NSUN5 in HCC would upregulate the m5C level of 28S rRNA; therefore, strengthening the functions of ribosomes and translation of global proteins, and promoting the growth and migration of HCC. Further experiments need to be performed to evaluate this hypothesis in the future.

In conclusion, to the best of our knowledge, the present study was the first to demonstrate that NSUN5 promoted the development of HCC. Therefore, it could be hypothesized that NSUN5 may represent a potential therapeutic target in HCC.

## Acknowledgements

## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

XWZ, HRL, YH and LYW performed the experiments. LYW, HRL and CFG designed the experiments. QQ, CFG and LiZ collected the clinical data. LuZ and RZ performed the statistical analysis. LYW and JY wrote the manuscript. JY, LiZ and HGW conceived the project. XWZ and JY confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

## Ethics approval and consent to participate

The present study using human tissues was approved by the Ethics Committee of Eastern Hepatobiliary Surgery Hospital (approval no. EHBHKY2018-02-014). Written informed consent was obtained from each patient according to the policies of the committee. The animal experiments in this study conformed to the Animal Research: Reporting of In Vivo Experiments guidelines (http://www.nc3rs.org.uk/arrive-guidelines) and were approved by the Institutional Animal Care and Use Committee of Shanghai University of Traditional Chinese Medicine (approval no. PZSHUTCM210926007).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no competing interests.

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