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Original Paper

LncRNA HANR Promotes Tumorigenesis and Increase of Chemoresistance in Hepatocellular Carcinoma

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Key Words
Hepatocellular carcinoma • Long non-coding RNA • Tumorigenesis • Chemoresistance • GSKIP • GSK3β

Abstract
Background/Aims: Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third leading cause of cancer-related death. Critical roles for long non-coding RNAs (lncRNAs) have recently been demonstrated for a variety of cancers, including hepatocellular carcinoma. However, the effect and mechanism of lncRNAs in HCC tumorigenesis and chemoresistance have not been extensively characterized. ***Methods:*** In the current study, we have identified a HCC-expressed lncRNA termed as *HANR* (HCC associated long non-coding RNA). We identified *HANR* by microarray analysis and validated its up-regulated expression by quantitative PCR. RNA pull-down and pathway analyses were conducted to evaluate physical and functional interactions with *HANR*. *In vivo* experiments were performed to assess tumorigenesis and increase of chemoresistance. In addition, the *HANR* expression in HCC specimens was detected by FISH. Xenograft and orthotopic mice model was constructed to observe the effect of *HANR* on tumorigenesis and chemoresistance *in vivo*. ***Results:*** *HANR* was demonstrated to be up-regulated in HCC patients and HCC cell lines. Increased *HANR* expression in HCC predicted short survival of patients. Knock-down of *HANR* markedly retarded cell proliferation, suppressed HCC xenograft/orthotopic tumor growth, induced apoptosis and enhanced chemosensitivity to doxorubicin, while over-expression of *HANR* showed the opposite effects. It was found that *HANR* bind to GSKIP for regulating the phosphorylation of GSK3β in HCC. ***Conclusion:*** Our results demonstrate that *HANR* contributes to the development of HCC and is a promising therapeutic target for chemosensitization of HCC cells to doxorubicin, which may represent a promising therapeutic target in the future.

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Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world and the third leading cause of cancer-related death [1]. It is primarily induced by long-term liver injury caused by viral hepatitis, toxin exposure, excessive alcohol consumption and inherited metabolic diseases [2]. Although numerous studies have been conducted to investigate the molecular, cellular and environmental mechanisms that drive HCC pathogenesis, limited clinical options are available to retard tumor growth and prolong patient life. In addition, major pathways that induce the high recurrence rate and invasiveness of HCC are still not fully understood [3].

Recent evidence suggests key roles of long noncoding RNAs (lncRNAs), defined as transcripts >200 nt in length with no protein-coding potential, in genomic alteration, diagnosis, prognosis, and therapeutic prediction of a variety of cancer types [4, 5]. To date, lncRNAs have been identified in HCC to have roles in growth [6, 7], chemotherapy sensitivity [8], drug-induced cellular apoptosis [9], metastasis [10, 11], cancer stem cell regulation [12, 13], therapy outcome prediction [14, 15] and viral hepatitis-related progression [16]. Thus, as regulators of HCC pathogenesis, lncRNAs are considered potential targets for cancer therapy.

In hepatocarcinogenesis, suppression of GSK3β phosphorylation and enhanced GSK3β total protein expression regulate glycogen metabolism and cell growth [17]. A recent study also reported that the protein level of GSK3β is significantly lower in HCC tissues than in normal liver tissues and that reduced levels correlate with advanced clinicopathological characteristics and poorer prognosis [18]. In addition, both hepatitis B virus X protein and hepatitis C virus core protein activate the oncogenic Wnt pathway through the inactivation of GSK-3β [19, 20]. As a direct inhibitor, GSK3β interaction protein (GSKIP) negatively regulates GSK3β and downstream Wnt signaling pathway, which is consistent with an oncogenic role of GSKIP in tumor growth [21]. However, direct evidence regarding lncRNAs that interact with GSKIP and regulate its function in cancer, particularly in HCC is lacking.

In the present study, we identify a new lncRNA, which named *HANR* (HCC associated long non-coding RNA) that is crucial for HCC growth and prognosis. We demonstrate that high expression of *HANR* is associated clinically with advanced HCC staging and poorer survival ratios. Activation and inhibition of *HANR* in human hepatoma cells and xenograft/orthotopic tumors in mice significantly modulate growth by regulating proliferation and apoptosis rates. Additionally, modulation of *HANR* expression significantly alters hepatoma cell sensitivity to the chemotherapeutic agent doxorubicin. As a direct binding protein, GSKIP is regulated by *HANR* to inhibit the expression of p-GSK3β which facilitates HCC growth. Therefore, *HANR* may be useful as a prognostic biomarker and therapeutic target against HCC.

Materials and Methods

Cell lines and chemical reagents

Two human HCC cell lines (Hep3B and Huh-7) and one normal human hepatocyte line (LO-2) were provided by the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were maintained in logarithmic growth in a 37 °C, 5% CO_2 incubator. All cell culture consumables and reagents were purchased from either Corning Incorporated (Corning, NY) or Gibco (Carlsbad, CA). Antibodies against PCNA, Bax1, Bcl-2, GSKIP, phosphorylated GSK3β (Ser9), total GSK3β and β-actin were all purchased from Abcam (Cambridge, UK).

Patients and specimens

This study conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Ethics Committee of Shenzhen Third People's Hospital. Informed consent in writing was obtained from each patient. A total of 35 pairs of HCC and corresponding peri-tumor tissues were obtained from patients having surgical resections at Shenzhen Third People's Hospital (Shenzhen, China) from 2009–2010. Another 6 healthy liver specimens (without drug-induced liver injury, viral hepatitis, fatty liver diseases, alcohol abuse, autoimmune liver disease, obvious fibrosis, cirrhosis and HCC) were obtained from patients

receiving liver resection operations and were set as the control group. All 35 HCC patients were observed for overall survival for 60 months. All clinical specimens were stored at −80°C until the time of histological and molecular analysis.

Immunohistochemical analysis

Immunohistochemical staining for GSKIP, GSK3β, in human samples was performed as previously described [7]. Quantification of positive DAB signals was captured by ImageJ software (version 1.51, NIH, Bethesda, MD).

LncRNA microarray analysis

Briefly, RNA from clinical samples were used to synthesize double-stranded complementary DNA and then hybridized to lncRNA microarray V 1.0 (Roche-NimbleGen, Madison, WI) and dual-channel mRNA microarray V 1.0 (RiboBio, Guangzhou, China) according to the manufacturer's instructions. The microarray data were selected by threshold values of > 3 and < −3-fold change under FDR protection ($P < 0.05$) as previously described [8]. Expressional changes of lncRNAs of interest were confirmed by quantitative real-time PCR.

RNA extraction and quantitative real-time PCR

RNA extraction, cDNA synthesis, and quantitative PCR were performed as previously described [22]. Parallel amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control. All real-time PCR procedures including the design of primers, validation of PCR environment and quantification methods were performed according the MIQE guideline [23]. The primer sequences and PCR conditions were listed in (for all online suppl. material, see www.karger.com/doi/10.1159/000484116) Suppl. Table 1.

RNA fluorescent in situ hybridization (FISH) assay

The RNA FISH assay of *HANR* localization and expression in clinical patients were performed as previously described [24]. Fluorescent signals were visualized and quantified using a Zeiss LSM700 confocal microscope (Zeiss, Oberkochen, Germany)

Transient and stable manipulation of HANR in Hep3B and Huh-7

To transiently knock-down or over-express *HANR* in Hep3B and Huh-7 cells, *HANR* siRNA and plasmid (pCDNA3.1) encoding full-length *HANR* were synthesized and validated by Shanghai Integrated Biotech Solutions Co.,Ltd (Shanghai, China). Log-phase Hep3B and Huh-7 cells were transfected with *HANR* siRNA or control siRNA by using lipofectamine RNAiMAX reagent (Thermo Fisher, Waltham, MA) (see online suppl. material, Suppl. Table 2). *HANR*-containing pcDNA3.1 or control pcDNA3.1 vector were transfected with lipofectamine 3000 reagent (Thermo Fisher).

To construct stable *HANR* knock-down (KD) or over-expression (OE) cell lines, Hep3B and Huh-7 cells were transfected with pIBSBIO-U6-GFP-Neo plasmid expressing *HANR* siRNA or pCDNA3.1-*HANR*-OE plasmid. The plasmids were transfected into cells by using lipofectamine 3000. Medium containing 500 μg/ml G418 (Sigma-Aldrich) was added to the cells 24-h post-transfection. Single cell clones were then isolated by three rounds of single cell dilution, and the efficiency of *HANR* KD or OE was screened by quantitative PCR.

Mtt assay

Viable Cell number was evaluated by the conversion of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich) to a purple color product by mitochondria. After treatment, cells from each group were washed by sterile PBS thrice and then incubated with 5 mg/ml MTT in culture medium for 4 hrs, and subsequently dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich). The absorbance of MTT was measured at 570 nm. Pure DMSO was set as the zero point.

Real-time monitoring of cell proliferation

Real-time and label-free impedance-based monitoring of Hep3B and Huh-7 cells were conducted as previously described [25]. Cell proliferation was monitored every 60 minutes for 72-h. Doubling times were calculated with RTCA software 1.2.1 (Roche Applied Science). Changes in cell proliferation were expressed as the "cell index".

Quantification of apoptotic ratio and caspase activity

After the indicated treatment, Hoechst 33342 (5 μg/ml, Sigma-Aldrich) and propidium iodide (5 μg/ml, Sigma-Aldrich) were added to each well to stain live cells. Quantification of the apoptotic ratio and caspase-3/7 activity were conducted as previously described [22]. The apoptotic/necrotic ratio was further confirmed using an Annexin V-FITC/PI detection kit (KeyGEN Biotech, Nanjing, China). After the indicated treatments, cells were collected and washed twice with cold PBS and then incubated with Annexin V-FITC/PI at room temperature for 15 min in the dark. The fluorescence of the cells was detected and quantified by ImageJ software.

Xenograft and orthotopic HCC mice model

All animals received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals". Male nude mice (Athymic NU/NU, 5-6 weeks, 20-25 g) were purchased from Guangdong Medical Laboratory Center (Guangzhou, China) for the establishment of the subcutaneous xenograft HCC model. Mice were injected in the dorsal region with 1×10^6/150 μl (low dose group) or 4×10^6/150 μl (high dose group) viable Hep3B or Huh-7 cells with or without *HANR* manipulation. Three days after cell injection, mice were administered 4 mg/kg doxorubicin by intraperitoneal injection (n = 5 for each group of mice). After a 28-day tumor induction period (25-day doxorubicin treatment), the mice were euthanized by an overdose of anesthesia (150 mg/kg pentobarbital, intraperitoneal injection) according to protocols approved by the Ethical Committee of Shenzhen Third People's Hospital. Tumor volume was assessed with digital calipers at days 8, 13, 18, 23, and 28 post Hep3B/Huh-7 injection. The tumor volume was calculated using the formula $\pi/6 \times$ larger diameter $\times$ (smaller diameter^2) [26].

For the orthotopic HCC model, male NOD/SCID mice (5–6 weeks, 20–25 g, Guangdong Medical Laboratory Center) were used. A single tumor nodule could be observed in the liver after 6 days of injection of 0.5×10^6 (low dose group) or 2×10^6 (high dose group) viable Hep3B or Huh-7 cells with or without *HANR* manipulation into the left liver lobe of nude mice. Then mice received 4 mg/kg doxorubicin by intraperitoneal injection (n = 10 per group). Median survival analysis was conducted using the Kaplan-Meier method and GraphPad Prism v6.0 software (GraphPad Software). The total observation duration was 60 days. Because in some groups all mice died before 60 days, we collected liver tissues from living mice (n = 3) at day 40. Thus, only 7 mice in each group were assessed by survival ratio analysis. Tumor nodules were collected for assessment of intra-tumor expression of key proteins.

Western blotting

Western blot assays for protein expression analysis of cell lysates and liver tissue homogenates were performed as previously described [22]. Parallel blotting of β-actin served as the internal control.

TUNEL assay

Xenograft and orthotopic HCC tumors were dissected, fixed in acetone, and sliced (5-μm sections). TUNEL assays were conducted for all sections by using the *In situ* BrdU-Red DNA Fragmentation (TUNEL) Assay Kit (Abcam).

RNA pull-down and mass spectrometry analysis

RNA pull-down and mass spectrometry analysis were conducted according to a previous report methods developed at the Institute of Biophysics, Chinese Academy of Sciences (Beijing, China) [27].

Data analysis and statistics

Results are reported as mean ± SEM of at least three independent experiments. Comparisons were performed using the two-tailed paired Student's t test. The Fisher's exact test was used for statistical analyses of the correlation between high *HANR* expression (over the average elevated level, which was 5.83-fold) and clinical parameters. Kaplan-Meier survival curves were compared by the Gehan-Breslow Test in Graphpad Prism.

Results

High HANR expression predicts poor prognosis of HCC patients

To identify lncRNAs with important regulatory roles in HCC, we collected HCC specimen and corresponding non-cancerous peri-tumor tissue. Transcriptome microarray analysis

identified an intergenic lncRNA (Gene symbol NR_003932) (Fig. 1a and see online suppl. material, Suppl. Table 3) that was up-regulated in the HCC specimen. To verify its upregulation in HCC, we collected 41 non-cancer specimens (35 HCC specimens with corresponding peri-tumor tissues and 6 healthy liver specimens) and 35 HCC specimens. The expression level of this lncRNA (referred to as *HANR*) was significantly higher in HCC specimens than in non-cancer specimens (Fig. 1b). Meanwhile, to examine the role of *HANR in vitro*, we assessed the endogenous *HANR* expression level in human hepatoma cell lines Hep3B and Huh-7. When compared with normal LO-2 hepatocytes, both hepatoma cell lines showed significantly elevated *HANR* expression (Fig. 1c). Besides, FISH staining of *HANR* in clinical specimens further confirmed its high expression in early and advanced HCC (Fig. 1d). Relative to the expression in peri-tumor specimens, *HANR* expression in HCC specimens from 15 of the 35 HCC patients was more than the average value (5.83-fold), and this high upregulation are correlated with the TNM staging and distant metastasis (Fig. 1e), but not gender, age, tumor size, or cirrhotic history (data not show). Furthermore, Kaplan-Meier analysis demonstrated that high *HANR* expression was closely associated with overall survival (Fig. 1f), which highlights its potential value as a predictive biomarker for the outcome of HCC.

HANR promotes HCC growth and inhibits apoptosis in vitro

To investigate the biological function of *HANR* of HCC growth *in vitro*, we transfected Hep3B and Huh-7 cells with siRNA or over-expression (OE) plasmid to manipulate *HANR* expression (Fig. 2a). The viable cell number of both cell types, as assessed by MTT assay, significantly decreased by *HANR* siRNA and significantly increased by *GNBR* OE plasmid

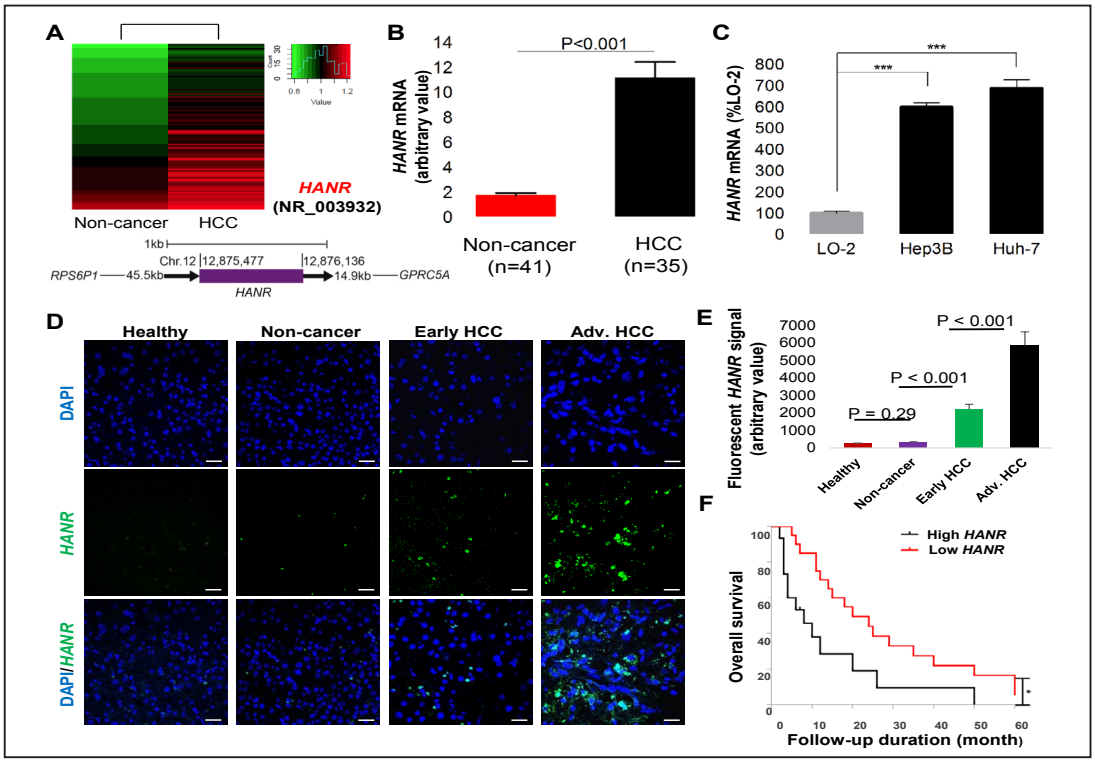

Fig. 1. Up-regulated HANR expression in HCC. (A) Microarray analysis was performed in HCC and corresponding peri-tumor (non-cancer) tissue. The genomic location and genetic map of HANR are indicated. (B) Quantitative PCR analysis of the expression of HANR in non-cancer and HCC samples. (C) Quantitative PCR analysis of the basal expression of HANR in normal LO-2 hepatocytes and two hepatoma cell lines. (D) Representative HANR fluorescent in situ hybridization results for clinical specimens (bar = 50 μM). (E) Corresponding quantification of HANR fluorescent staining. (F) Kaplan-Meier analysis of overall survival for HCC patients with HANR high (cancer/non-cancer ratio > 5.83-fold) or low (cancer/non-cancer ratio < 5.83-fold) expression. Data are presented as means ± SEM. *, P<0.05, **, P<0.01, ***, P<0.001.

(Fig. 2b). The effect of *GNBR* on cell growth was verified by corresponding changes in cell proliferation, as characterized by PCNA protein expression (Fig. 2c), the cell index (Fig. 2d), and the doubling times (Fig. 2e) over a 72-h observation period.

Given our evidence that *HANR* promotes tumor growth, we speculated that *HANR* may also exert an effect on HCC apoptosis. Indeed, *in vitro* flow cytometry assay of Hep3B and Huh-7 demonstrated that *HANR* siRNA significantly increased the apoptotic and necrotic ratios of both cell types (Fig. 3a), which was consistent with the Hoechst/PI live cell staining results (Fig. 3b). The induction of caspase-3/7 activity and Bax1 mRNA/protein expression and the inhibition of Bcl-2 mRNA/protein expression in *GNBR* siRNA cells further confirmed these observations (Fig. 3c-e and see online suppl. material, Suppl. Fig. S1A). Although no obvious effect on the apoptotic ratio was observed in *HANR* OE cells, the basal caspase-3/7 activity and Bax1 mRNA expression were inhibited in *HANR* OE cells, while Bcl-2 mRNA/protein expression was elevated (Fig. 3c-e). These results suggested that LncRNA *HANR* can promotes HCC growth and inhibits apoptosis *in vitro.*

Overexpression of HANR can promotes HCC chemoresistance in vitro

Chemoresistance is a very thorny problem in tumor treatment, doxorubicin is one of major agents for anti-HCC [28], we investigated the role of *HANR* in 1μg/ml doxorubicin treated 24h to induced cell growth retardation. As expected, doxorubicin incubation significantly reduced viable cell number and the proliferative rate of both Hep3B and Huh-7 cells, which were assessed by MTT, immunoblotting and the doubling times observe assay. Furthermore, the effects of doxorubicin on cell growth were strengthened by *HANR* knock-down and impaired by *HANR* OE (Fig. 4a-c).

To determine whether the effects of *GNBR* modulation on apoptosis are also observed after chemotherapeutic treatment, we repeated these assays in the presence of doxorubicin. In agreement with the growth studies, apoptosis of hepatoma cells induced by doxorubicin was further exacerbated by *HANR* KD and was impaired by *HANR* OE, as verified by flow cytometry Annexin/PI staining (Fig. 4d), Hoechst/PI live cell staining results (Fig. 4e) and changes in the expression of apoptotic enzyme, genes and proteins (Fig. 4f-h and see online suppl. material, Suppl. Fig. S1B). It should be noted that vehicle treatment with ctrl siRNA

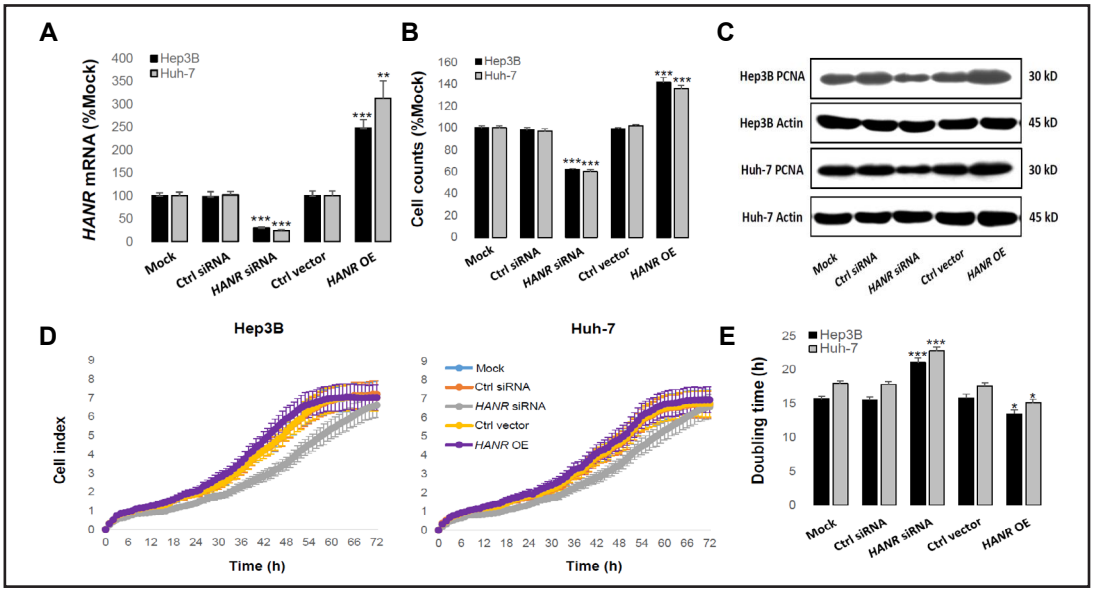

Fig. 2. HANR promotes HCC growth in vitro. (A) Effects of HANR knock-down or overexpression on basal HANR expression in Hep3B and Huh-7 cells. (B) The viable cell number of Hep3B and Huh-7 cells with HANR siRNA or HANR OE. (C) Western blotting of PCNA protein expression in HANR knock-down and OE cells. (D and E) Cell index and doubling times of HANR knock-down and OE cells over a 72-h observation period. Data are presented as means ± SEM. *, P<0.05, ***, P < 0.001

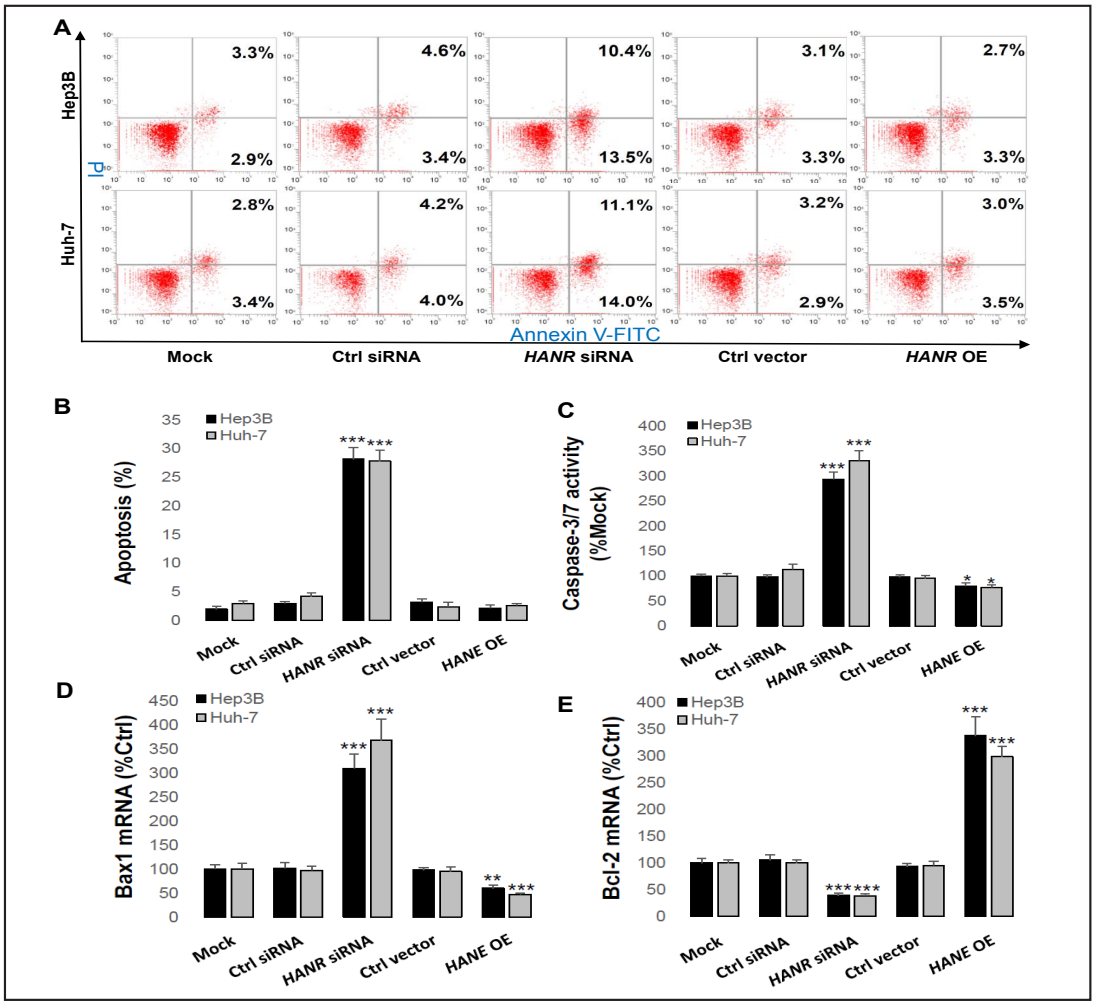

Fig. 3. HANR inhibits HCC apoptosis in vitro. (A) Annexin V-FITC/PI double-staining analysis by flow cytometry to assess basal apoptosis of Hep3B and Huh-7 cells after transfection with HANR siRNA or over-expression (OE) plasmid, (each group 10,000 cells to analysis). Untransfected cells (Mock) and control transfected cells (Ctrl siRNA and Ctrl vector) are shown for comparison. (B) Assessment of apoptosis by Hoechst/PI live cell staining. (C-E) Caspase-3/7 activity and mRNA expression of Bax-1 and Bcl-2 in Hep3B and Huh-7 cells after transfection with HANR siRNA or OE plasmid. Data are presented as means ± SEM. *, P<0.05, ***, P < 0.001.

or vector did not influence the effects of doxorubicin on cell viability, doubling time and apoptotic ratio (see online suppl. material, Suppl. Fig. S2). The above data indicated that *HANR* can promotes chemoresistance *in vitro*

HANR also promotes HCC growth, chemoresistance and inhibits apoptosis in vivo

To further confirm the ability of *HANR* to promote HCC growth *in vivo*, both a nude mouse-based xenograft model and a NOD/SCID mouse-based orthotopic HCC model were applied (Fig. 5a). HCC tumors were induced using stable *HANR* knock-down or OE in Hep3B and Huh-7 cells in the absence or presence of doxorubicin administration. In the xenograft model, HCC tumors derived from *HANR* OE cells (low dose and high dose) grew most rapidly. While co-treatment with doxorubicin restricted tumor sizes at all time-points, *HANR* KD + Doxo co-treatment provided the most obvious tumor-suppressive effects. Changes in the patterns of growth in the orthotopic model were consistent with those in the xenograft model (Fig. 5b). Additionally, both xenogragt and orthotopic models survival rates illustrated that

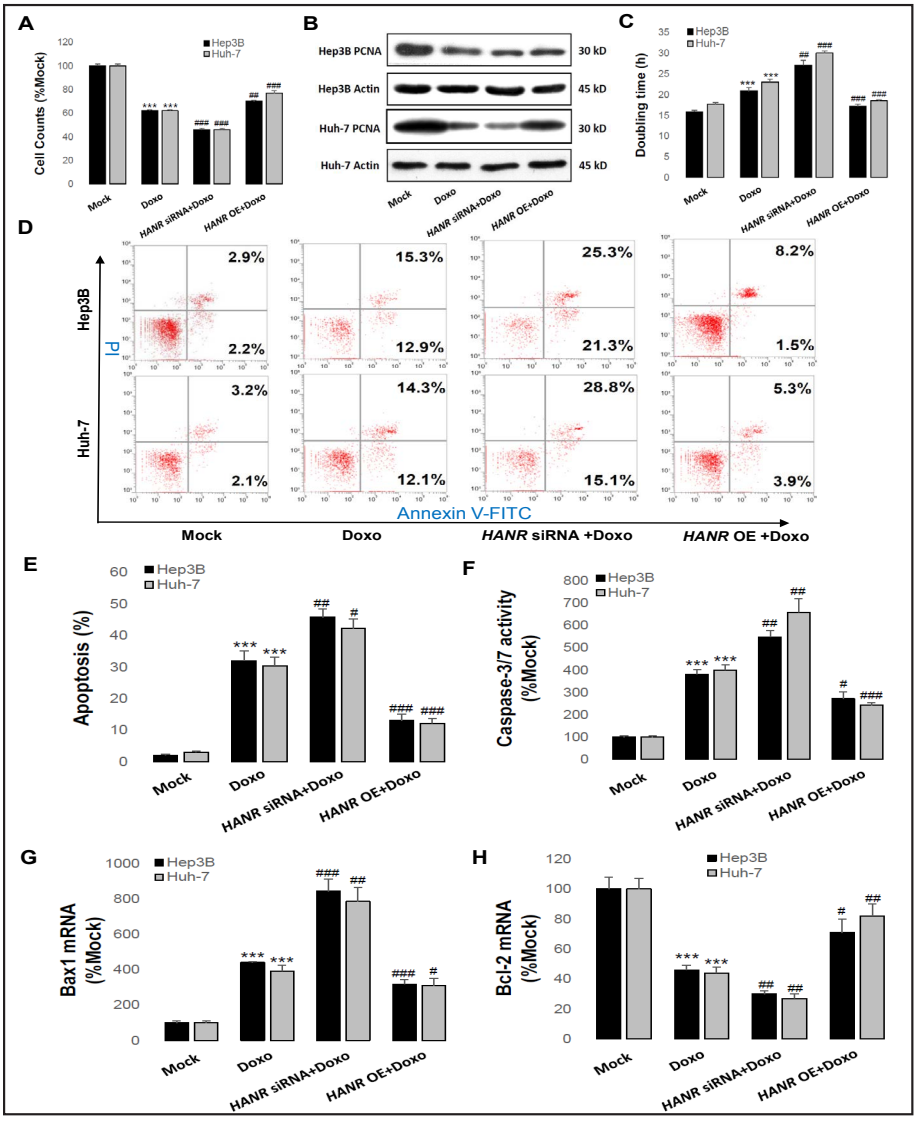

Fig. 4. HANR promotes HCC chemoresistance in vitro. (A) The viabilities of HANR knock-down and OE Hep3B or Huh-7 cells were assessed by MTT assay after treatment with doxorubicin. (B) Western blotting of PCNA protein expression after treatment with doxorubicin. (C)Doubling times after treatment with doxorubicin. (D-H) Analysis of apoptosis in Hep3B and Huh-7 HANR siRNA or OE cells after the incubation with doxorubicin. Apoptosis was assessed by AnnexinV/PI staining and flow cytometry (each group 10,000 cells to analysis), live cell staining, caspase-3/7 activity and Bax-1/Bcl-2 expression. Data are presented as means ± SEM. "*" indicates comparison with control, *, P<0.05, ***, P<0.001; "#" indicates comparison with doxorubicin group, #, P<0.05, ##, P<0.01, ###, P<0.001.

HANR OE decreased survival rates and *HANR* KD increased survival rates in both vehicle- and doxorubicin-treated mice (Fig. 5c). Collectively, these results support the ability of *HANR* to promote tumor growth and chemoresistence *in vivo*.

We also performed TUNEL assays of tumors from both the xenograft and orthotopic HCC models to analysis of apoptosis *in vivo*. In both high dose and low dose experiments, *HANR* KD further enhanced the effects of doxorubicin, while *HANR* OE suppressed the effects of doxorubicin (Fig. 5d). These results provide support for the role of *HANR* in promoting HCC growth by suppressing apoptosis.

HANR is physically associated with GSKIP to regulate the GSKIP/GSK3β pathway

To identify a direct target of *HANR*, we first determined its cytoplasmic/nuclear ratio in HCC patients. FISH results demonstrated that *HANR* predominantly is distributed in the cytoplasmic region of tumors from patients with HCC (early and advanced) with an approximate cytoplasm/nucleus ratio of 76%/24% (Fig. 6a). Next, we performed

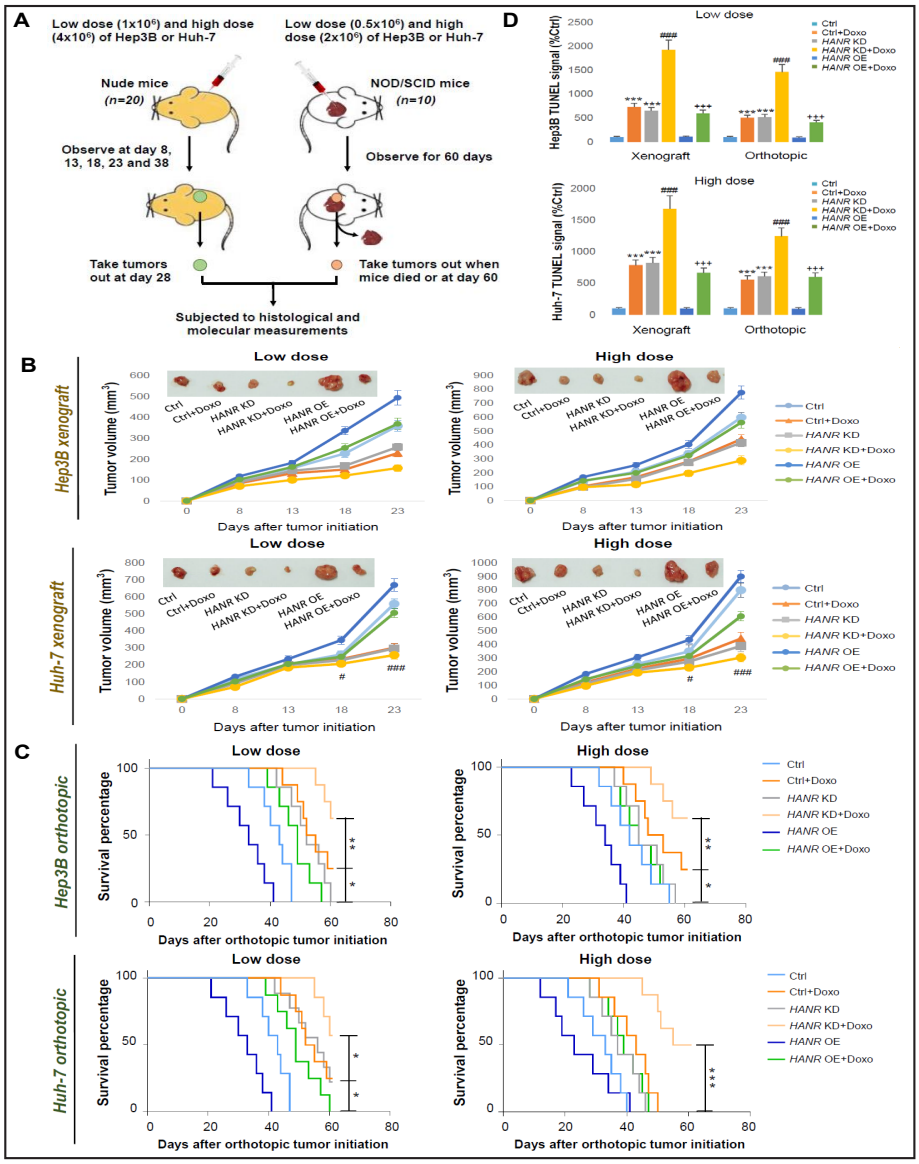

Fig. 5. HANR promotes HCC growth, chemoresistance and inhibits apoptosis *in vivo*. (A) Schematic of hepatoma cell-induced xenograft and orthotopic HCC mouse models in the current study. (B) Tumor volume of Hep3B (top) and Huh-7 (bottom) tumors in xenograft model mice treated injected with low and high cell doses and then treated with doxorubicin. (C) The survival analysis of orthotopic model mice for 60 days following tumor initiation. (D) TUNEL analysis of apoptotic cells in xenograft and orthotopic tumor samples from mice injected with a low dose and a high dose of Hep3B or Huh-7 HANR siRNA or OE cells and treated with doxorubicin. Data are presented as means ± SEM. "*" indicates comparison with control, *, P<0.05, **, P<0.01, ***, P<0.001; "#" indicates comparison with doxorubicin group, #, P<0.05, ##, P<0.01, ###, P<0.001; indicates comparison with HANR OE group, +, P<0.05, ++, P<0.01, +++, P<0.001.

RNA pulldown assays to identify *GNBR*-binding proteins (Fig. 6b). Mass spectrometry and RNA pulldown confirmed the interaction between *HANR* and GSKIP (Fig. 6c). RNA immunoprecipitation assays in both Hep3B and Huh-7 cells provided further verification that *HANR* interacts with GSKIP (Fig. 6d). To explore the influencing mechanism, we firstly measured the change of GSKIP expression, at both transcriptional and translational levels after *HANR* manipulation or doxorubicin incubation. We found that *HANR* KD or OE, as well as the incubation with doxorubicin, had no significant effect on GSKIP mRNA and protein expressions, but altered the phosphorylated GSK3β level instead of total GSK3β protein expression in both Hep3B and Huh-7 cells (Fig. 6e, f). Thus, it seems that the main regulating target of *HANR* is the phosphorylation level of GSK3β, but not the GSKIP expression.

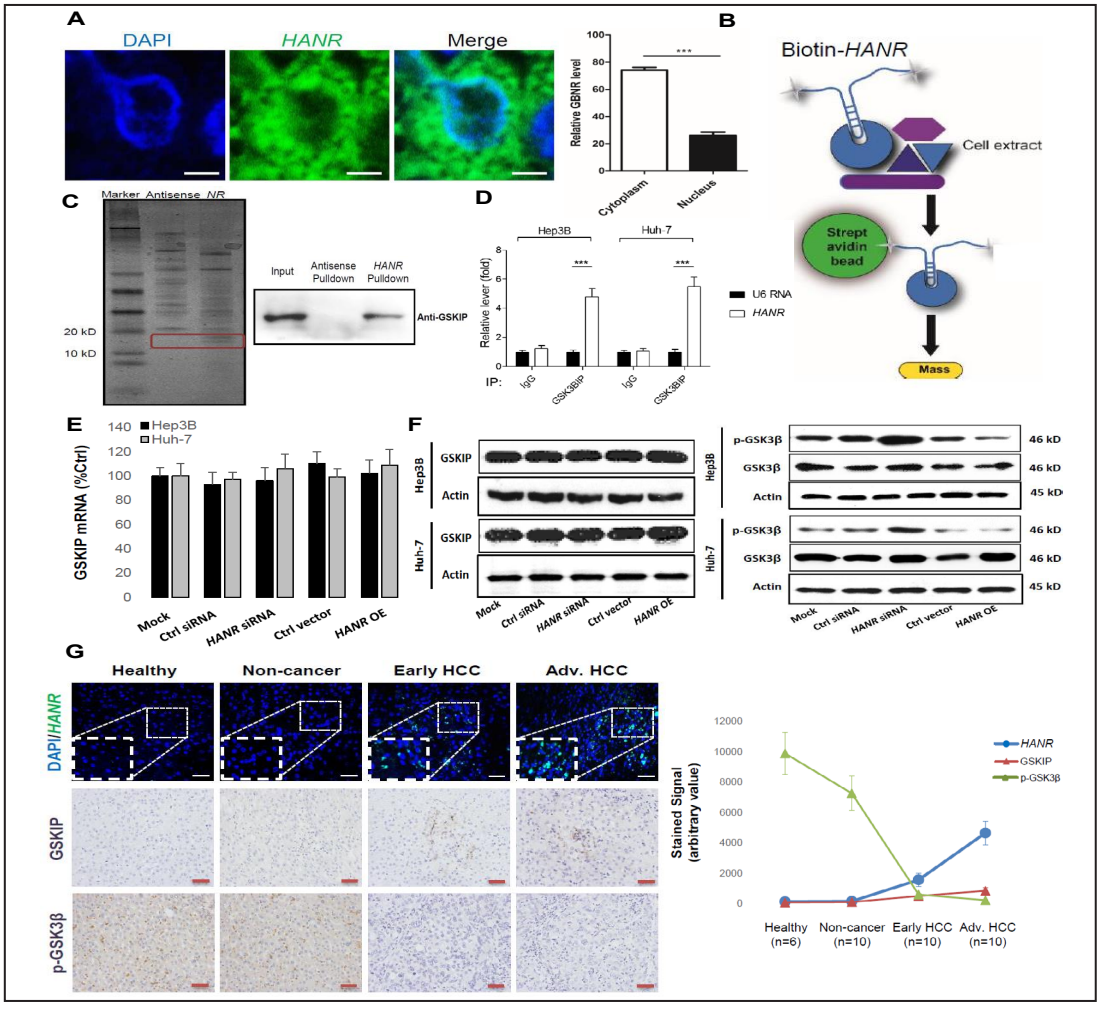

Fig. 6. GSKIP is the direct target of HANR to influence GSK3β phosphorylation. (A) Fluorescent in situ hybridization was performed in a human HCC sample to determine the HANR sub-cellar distribution (630X, bar = 10 μM) and corresponding quantification of the HANR cytoplasmic/nucleus ratio. (B) The flow chart of HANR pull-down experiment. (C) Proteins recovered from a HANR pull-down assay were analyzed by silver staining. Western blotting validated the identity of GSKIP. (D) GSKIP was immunoprecipitated from cell extracts and associated RNAs after formaldehyde crosslinking were determined by quantitative PCR. IgG was tested as a control. (E) Changes of GSKIP mRNA expressions after the HANR manipulation in Hep3B and Huh-7 cells. (F)The effects of HANR manipulation on GSKIP, phosphorylated and total GSK3β protein levels in Hep3B and Huh-7 cells. (G) The expression of HANR (with FISH assay) and protein expression of GSKIP, phosphorylated level of GSK3β (with IHC assay) were visualized and quantified in clinical liver samples from healthy, non-cancerous, early and advanced HCC patients .Data are presented in means ± SEM. "*" indicates comparison with control, ***, P<0.001.

Lastly, we measured the correlation between *HANR* expression and the activation of the GSKIP/GSK3β pathway in human healthy and HCC liver specimens. *HANR* was highly positive correlated with HCC TNM staging, it was highest in advanced HCC samples. In contrast, the phosphorylated level of GSK3β was quite obvious in both healthy and peri-HCC liver samples but diminished in HCC samples (Fig. 6h). Collectively, these results suggest that *HANR* promotes HCC growth, inhibits apoptosis, and enhances doxorubicin chemoresistance partly through direct interaction with GSKIP and subsequent GSK3β pathway modulation.

Discussion

Emerging evidence indicates key regulatory roles of lncRNAs in HCC [29, 30]. However, evidence of lncRNAs with clinical prognostic value is still limited. Ideally, in addition to exhibiting HCC-specific expression patterns, lncRNAs should be demonstrated to regulate clear mechanistic pathways that drive the growth of HCC to support their potential use as therapeutic target during clinical treatment [4]. By using clinical specimen, *in vitro* hepatoma cell lines, and xenograft/orthotopic mice models, we demonstrated that a novel lncRNA, *HANR,* plays a key role in HCC growth. Our data showed that over-expression of *HANR* could be used to predicts the prognostic outcome of HCC patients overall survival ratios. Alteration of endogenous cellular *HANR* expression influenced the sensitivity of both hepatoma cells and HCC tumors to doxorubicin-mediated chemotherapy. In addition, *HANR* promoted HCC growth partly via binding to GSKIP to suppress phosphorylation of GSK3β. Therefore, our study provides clinical and mechanistic data to support the role of this lncRNA in HCC.

Previously defined mechanisms of lncRNAs in cancer growth include regulation of viability, proliferation, immortality, mobility and angiogenesis [5, 31, 32]. Additionally, HCC-related lncRNAs that have previously been characterized as target mRNAs (e.g. IL-11) [11], promoter regions (e.g. TCF7 and IL-6) [12, 13], or proteins (e.g. HuR and IGF2BP1) [6, 33] to exert their regulatory effects on HCC cells. Our study provides a noval lncRNA *HANR* to regulate cancer growth and to serve as a potential prognostic marker.

The regulatory functions of the GSK3β and Wnt/β-catenin pathways in cancer phenotypes were studied in several studies. For example, GSK3β and Wnt/Axin2 coordinately regulate β-catenin-TCF to control tumor proliferation and metastasis in breast cancer [34]. Wnt pathway is reported as downstream target of GSK3β in controlling neuroblastoma growth [35]. In addition, although not investigated for cancer, GSK3β directly induces the expression of Bax1 in an ischemic injury model [36]. By using RNA pull-down and RIP assays, we confirmed that GSKIP is a direct interacting partner of *HANR* in hepatoma cells. High expression of *HANR* inhibited the activity of GSK3β, which impaired the therapeutic outcome of doxorubicin. Moreover, Bax1 was also suppressed by *HANR* over-expression, which is likely to occur indirectly. Conversely, knock-down of *HANR* in hepatoma cells, reversed these phenotypes. These data are consistent with the well-defined oncogenic function of the Wnt/β-catenin pathway in HCC progression [37] and the anti-cancer properties of GSK3β in other cancer types [38].

One of the major reasons for the low efficacy of clinical HCC chemotherapy is the resistance to anticancer agents, such as doxorubicin [28]. In addition to its function in controlling HCC growth and apoptosis, modulation of *HANR* expression also significantly influenced the sensitivity of both hepatoma cells and *in vivo* tumors to doxorubicin. When applied in the context of doxorubicin treatment, inhibition of *HANR* potently enhanced HCC growth/proliferation retardation and apoptosis induction.

Taken together, *HANR* is thus serves as a crucial regulatory lncRNA in mediating the growth and chemoresistance of HCC, which occurs, at least partially, through modulation of the GSKIP/GSK3β axis. Targeting of *HANR* may have therapeutic implications in HCC treatment.

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Disclosure Statement

The authors declared no conflict of interest.

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