

Original Article

Downregulation of a long noncoding RNA BANCR contributes to proliferation and metastasis of hepatocellular carcinoma cancer cells *in vitro* and *in vivo*

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Received November 28, 2015; Accepted January 26, 2016; Epub March 1, 2016; Published March 15, 2016

Abstract: BANCR, a recently found lncRNA, has been proved to have the ability to regulate proliferation and migration of malignant melanoma cells. An aberrant expression of BANCR was observed in Lung carcinoma, gastric cancer and colorectal cancer. However, the exact effects and molecular mechanisms of BANCR in hepatocellular carcinoma (HCC) progression are still unknown up to now. The present study aimed to determine the expression, roles and functional mechanisms of a long noncoding RNA BANCR in the progression of HCC. In our study, we found that the expression of BANCR was downregulated in HCC tissues or cell lines including Huh7, SMMC-7721 and HepG2 by qRT-PCR analysis. Then, gain- and loss-of-function studies indicated that cell proliferation and migration were greatly suppressed, and the apoptosis was increased when BANCR was ectopically-expressed in HCC cells. Furthermore, ectopic expression of BANCR was demonstrated to inhibit tumor growth and metastasis *in vivo*. Taken together, our study indicates that BANCR is significantly down-regulated in HCC and may be correlated with tumor progression.

Keywords: BANCR, HCC, lncRNA, proliferation, metastasis, progression

Introduction

Hepatocellular carcinoma (HCC) is the second leading cause of cancer death and the fifth most commonly diagnosed malignancy worldwide [1]. To overcome the malignant cancer, research has been performed to detect and validate a variety of molecules associated with HCC cell proliferation, differentiation, invasion and metastasis. However, only a few molecular mechanisms have been revealed and translated into clinical application so far. Therefore, it is crucial to identify novel molecules and novel alternative therapeutic strategies to improve clinical outcome of patients suffering from HCC.

Long noncoding RNAs (lncRNAs) are kinds of transcriptional products of the eukaryotic genome that are composed of more than 200 nucleotides in length with limited protein coding potential, were recently identified as having

functional roles in a variety of biological processes and disease states [2-4]. Previous studies have demonstrated that lncRNAs are involved in various cellular and biological processes such as proliferation, cell-cycle progression, chromosome imprinting and histone modification [5-7]. Notably, several lncRNAs have been shown to play important role in many cancers, and the deregulation has been shown to result in aberrant gene expression that contributes to the progression of cancers, including HCC [8-12]. For example, CCAT1 expression levels are elevated in multiple types of tumor cells and have been associated with poor survival outcomes and high tumor recurrence rates [13]. Enhanced expression of CCAT1 promotes the proliferation and migration of HCC cells, while inhibition of CCAT1 inhibits the proliferation and migration of HCC cells [14].

Similarly, more lncRNAs are being identified and many await functional validation in the context

of HCC. Recent evidence shows that BRAF-activated noncoding RNA (BANCR) acts as a critical role in cell proliferation, migration, and invasion in malignant melanoma, colorectal cancer and lung cancer [15-17]. However, its expression, roles, and function mechanisms in HCC are still unknown and need to be investigated.

To investigate the function of BANCR in HCC, we explored the BANCR expression level in HCC tissues via quantitative RT-PCR. We also identified the function of BANCR in HCC cells by applying gain-of-function and loss-of-function approaches in vitro and in vivo.

Materials and methods

Tissue samples and cell lines

A total of 20 HCC and 20 non-cancerous liver tissue samples were obtained from the Affiliated Hospital of North China University of Science and Technology (Hebei, China). Written informed consent forms were obtained from all subjects according to the Declaration of Helsinki, and the study was approved by the Ethics Committee of North China University of Science and Technology. All the samples were immediately snap frozen in liquid nitrogen after surgery and stored at -80°C before use.

HepG2 and Huh7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/ml). The cells were incubated at 37°C in a humidified incubator under 5% CO₂ condition.

Extraction of RNA and quantitative reverse transcription polymerase chain reaction

Total RNA was extracted using the TRIzol reagent (Applied Biosystems, Foster City, CA). cDNA was synthesized using Reverse Transcription Kit (Applied Biosystems). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses for genes were performed with the SYBRGreen PCR Master mix (Applied Biosystems) on an ABI 7900 System (Bio-Rad). GAPDH was used as an internal control. The 2^{-ΔΔCt} method was employed to calculate the relative expression levels. The primers (Invitrogen) were designed as follows: for human lncRNA BANCR, the forward primer was

5'-ACAGGACTCCATGGCAAACG-3' and the reverse primer was 5'-ATGAAGAAAGCCTGGTGCAGT-3'. For human GAPDH, the forward primer was 5'-ACCACAGTCCATGCCATCAC-3' and the reverse primer was 5'-TCCACCACCCTGTTGCTGTA-3'.

Plasmid construction and cell transfection

The BANCR sequence was subcloned into the pcDNA3.1 vector (Invitrogen, USA). BANCR Ectopic expression was achieved through pcDNA3.1-BANCR transfection using lipofectamine2000 (Invitrogen, USA), with an empty pcDNA3.1 vector used as a control. The expression levels of BANCR were measured by quantitative PCR. Plasmid vectors (pcDNA3.1-BANCR and pcDNA3.1) for transfection were extracted using Midiprep kits (Qiagen, Germany), and respectively transfected into HepG2 or Huh7 cells. The siRNAs (si-BANCR and si-NC) were respectively transfected into HepG2 or Huh7 cells. According to the manufacturer's instructions, fusion and transfection of HepG2 or Huh7 cells by lipofectamine 2000 (Invitrogen, USA) were performed when the cells were cultivated on six-well plates. After transfection for 48 h, cells were collected for cell proliferation, apoptosis and migration assays, and lysed for quantitative PCR.

Cell viability assay

Cells were seeded in the 96 well plates 24 h after transfection at a density of 1500 cells per well. The cell viability assay was performed using Cell Counting Kit-8 (CCK8; Dojindo) according to the manufacturer's protocol. The absorbance at 450 nm was measured. Experiments were performed at three times.

Migration and invasion assays

Briefly, 5×10⁴ transfected or non-transfected cells were seeded on a fibronectin-coated polycarbonate membrane insert in a Transwell apparatus (Corning, Corning, USA). After the cells were incubated for 12 h, Giemsa-stained cells adhering to the lower surface were counted under a microscope in five predetermined fields (×100). For the cell invasion assay, the procedure was similar Tumor Biol. To the cell migration assay, except that the Transwell membranes were pre-coated with 24 mg/ml

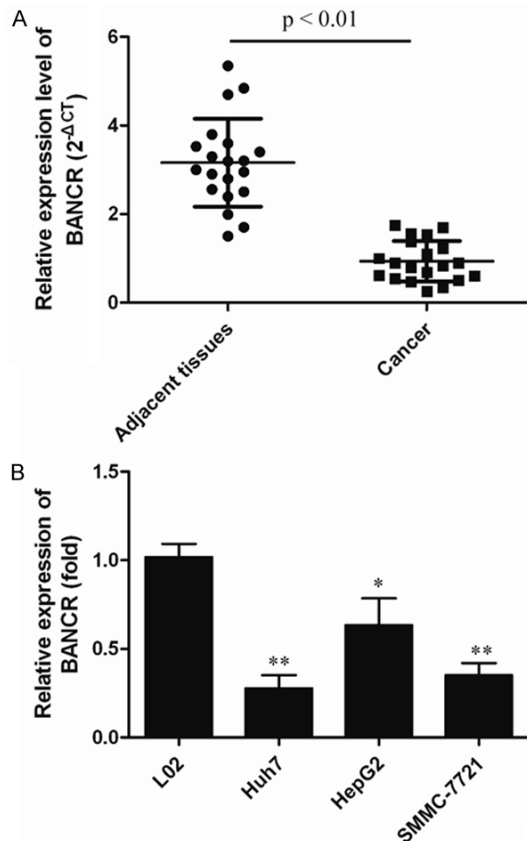


Figure 1. BANCR expressions in HCC tissues and cell lines. A. Relative BANCR levels in tumor tissues from HCC patients before surgery compared to those in their adjacent non-tumor lung tissues. B. Relative BANCR levels in HCC cell lines (Huh7, HepG2 and SMMC-7721). Data are presented as mean \pm standard error based on at least three independent experiments. ** $P < 0.01$ vs. L02 cells.

Matrigel (Corning, USA). All experiments were performed in triplicate.

Flow cytometry

For apoptosis assay, the cells were cultured in low-serum medium and collected after 48 h transfection. Cells were subsequently stained with Annexin V-FITC (eBioscience, USA) and PI for 30 min as described by the manufacturer. Apoptosis cells were analyzed by FACS.

In vivo tumorigenesis and metastasis

Forty-five-week-old male BALB/c nude mice (Institute of Zoology, Chinese Academy of Sciences, Shanghai, China) were randomly divided into 3 groups and grew under specific-pathogen-free conditions in the Animal Care Facility Service (the Affiliated Hospital of North China

University of Science and Technology). The mice were injected subcutaneously with 5×10^6 cells (HepG2 or Huh7 cells transfected with empty vector pcDNA3.1/plasmid pcDNA3.1-BANCR/si-NC/si-BANCR) into right flanks. Five weeks later, all the mice were sacrificed by 1% overdosed pentobarbital and exsanguination according to American Veterinary Medical Association guidelines on euthanasia, and the tumor mass was weighed.

Statistical analysis

All values were expressed as mean \pm SD and processed by the DPS software (version 6.55). Differences among the groups were assessed by Student's t-test, and they were considered statistical significance if $P < 0.05$.

Results

LncRNABANCR expression was downregulated in both HCC tissues and cell lines

To evaluate the expression of BANCR in clinical specimens, qRT-PCR was used to detect between 20 pairs of HCC tissues and normal tissue. As shown in **Figure 1A**, BANCR expression was significantly downregulated in HCC tissues compared to the normal tissue in all the detected specimens. It implied that BANCR might be involved in the progression of HCC. In parallel, BANCR was expressed at lower levels in three HCC cell lines than that in normal HCC line (**Figure 1B**, $P < 0.01$).

Overexpression of BANCR inhibits the proliferation and migration and promotes apoptosis in HCC cells lines

To investigate the biological functions of BANCR on HCC, we stably enhanced BANCR expression by transfecting BANCR expression vector (pcDNA3.1-BANCR) into the HCC cell lines Huh7 and HepG2, employing the pcDNA3.1 vector as a negative control (**Figure 2A**). After Huh7 and HepG2 cells were transfected, cells proliferation was measured by a CCK-8 assay. As shown in **Figure 2B**, the proliferation rate of HCC cells was remarkably decreased after BANCR expression vector transfection on the 4th and 5th day ($P < 0.01$).

To evaluate the influence of apoptosis caused by overexpression of BANCR, HCC cells apoptosis was measured by Annexin-V FITC/PI double

LncRNA BANCER suppress proliferation and metastasis of HCC

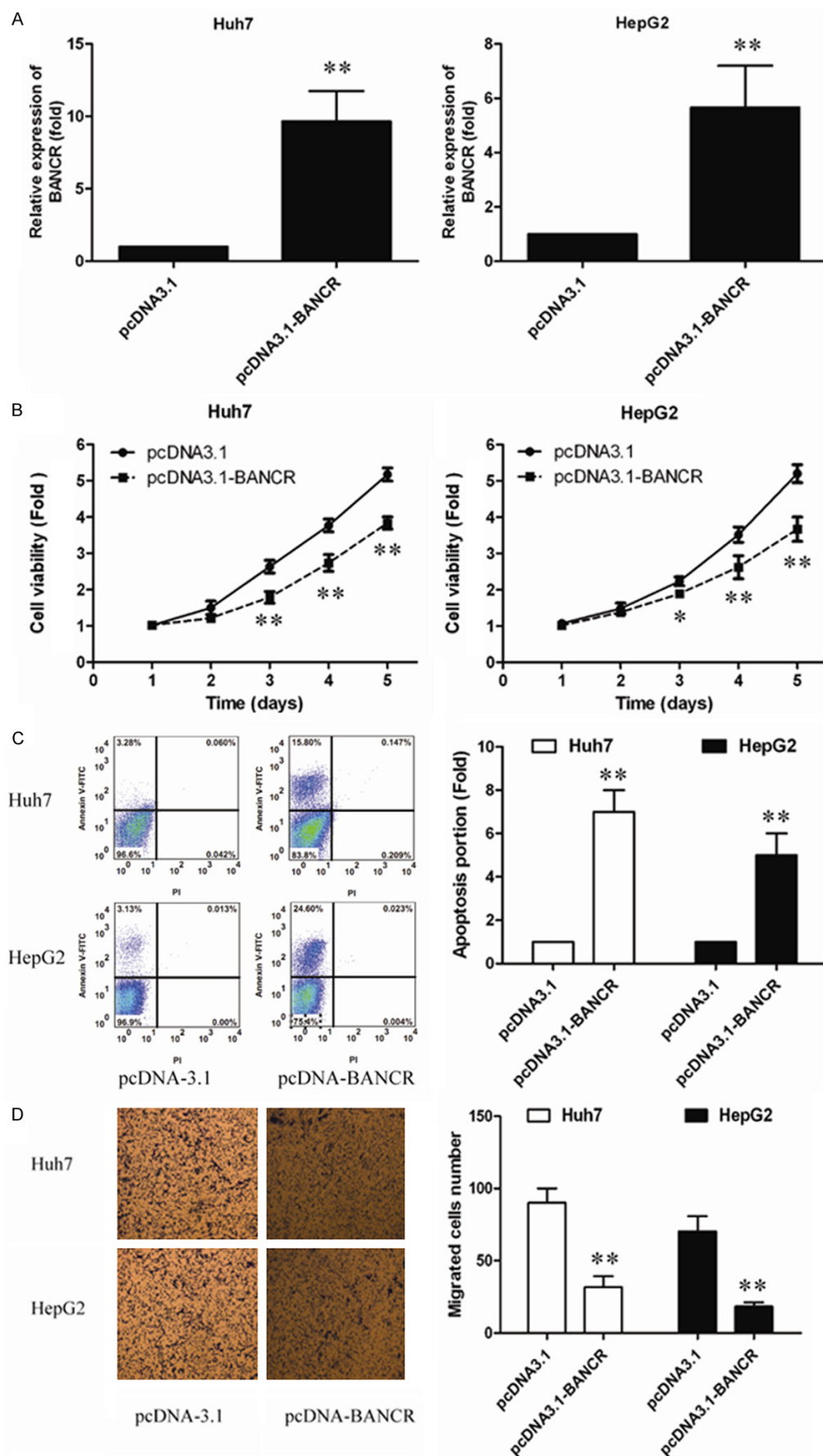


Figure 2. Effects of BANCR overexpression on HCC cell proliferation, cell apoptosis and migration in vitro. A. Over-expressed BANCR after the transfection of Huh7 and HepG2 cells with pcDNA3.1-BANCR. B. BANCR inhibited the proliferation of Huh7 and HepG2 cells. Cell number was determined by the CCK-8 assay, and the relative number of cells to 1 day is presented. C. Cells apoptosis were detected by flow cytometry after pcDNA3.1-BANCR or pcDNA3.1 vector transfection. D. BANCR decreased cell migration by transwell assays after pcDNA3.1-BANCR transfection. All values are presented as mean \pm standard error based on at least three independent experiments. * $P < 0.05$, ** $P < 0.01$.

staining assay. As shown in **Figure 2C**, the percentage of apoptotic cells was remarkably increased after BANCR expression vector transfection compared with negative control.

To determine the effect of BANCR overexpression on HCC cells migration, transwell assays were performed. As presented in **Figure 2D**, enhanced expression of BANCR significantly decreased cell migration in Huh7 and HepG2 cells.

Knockdown of BANCR promotes the proliferation and migration and suppresses apoptosis in HCC cell lines

To further confirm the functional role of BANCR in HCC, siRNA experiment was used to silence BANCR in both Huh7 and HepG2 cells. As shown in **Figure 3A**, the efficiency of si-BANCR was confirmed by RT-PCR in HCC cells. Subsequently, to determine the effect of BANCR in HCC cell growth, siRNA was transfected into HCC cells and cells proliferation was measured by a CCK-8 assay. As shown in **Figure 3B**, the proliferation rate of HCC cells was remarkably increased on the 4th and 5th day ($P < 0.01$). As demonstrated by Annexin-V FITC/PI double staining assay, knockdown of BANCR significantly suppressed the percentage of apoptotic cells (**Figure 3C**). This result revealed that BANCR might impact the proliferation of HCC cells by affecting apoptosis. In addition, as demonstrated by transwell assays, repression of BANCR increased cell migration (**Figure 3D**). These data proved that BANCR played important roles in HCC progression.

BANCR inhibits tumor growth of HCC in vivo

Finally, we investigated whether BANCR could affect tumorigenesis of HCC *in vivo*. We established nude mouse xenograft models in which pcDNA3.1-BANCR cells, siRNA-BANCR cells and control cells were subcutaneously injected in the right inguina, respectively. Five weeks after injection, autopsy analysis showed that

the xenografts derived from pcDNA3.1-BANCR cells were grown smaller than those developed from control cells as measured by tumor weight and size (**Figure 4A**). In contrast, the subcutaneous tumors developed from siRNA-BANCR cells were grown distinctly larger than those in the control group (**Figure 4B**). These results suggested that BANCR could inhibit tumor proliferation.

Discussion

In the present study, we found that BANCR expression is down-regulated in HCC tissues and cells. Furthermore, we have shown that knockdown of BANCR can promote cancer cell proliferation and metastasis.

Non-coding RNAs (ncRNAs) were once neglected and considered non-function RNA in for a long time because they do not encode any proteins. Recently, more and more evidence has been demonstrated that the LncRNAs played important roles in complicated diseases such as cancer [5]. For example, LncRNA HOTAIR is a strong prognosis marker of patient outcomes and survival in several human cancers [18-20]. LncRNA MEG3 transcribes from maternally expressed gene3, a tumor suppressor gene. Re-expression MEG3 could induce cell growth arrest and promote cell apoptosis [21, 22]. In HCC, several LncRNAs have been identified to involve in the development and progression of HCC, such as MALT1 [23], TUC388 [24], Dreh [25], LET [26], and H19 [27]. Here we found another LncRNA, BANCR, which is implicated in HCC progression.

BRAF-activated non-coding RNA (BANCR) was first found via an RNA-seq screen for transcripts affected by the expression of the oncogene BRAFV600E [15, 17]. A recent study found that downregulation of BANCR obviously promoted growth, migration and invasion and upregulation of BANCR significantly inhibited growth, migration and invasion in lung cancer cell lines [17, 28]. In addition, many studies demonstrat-

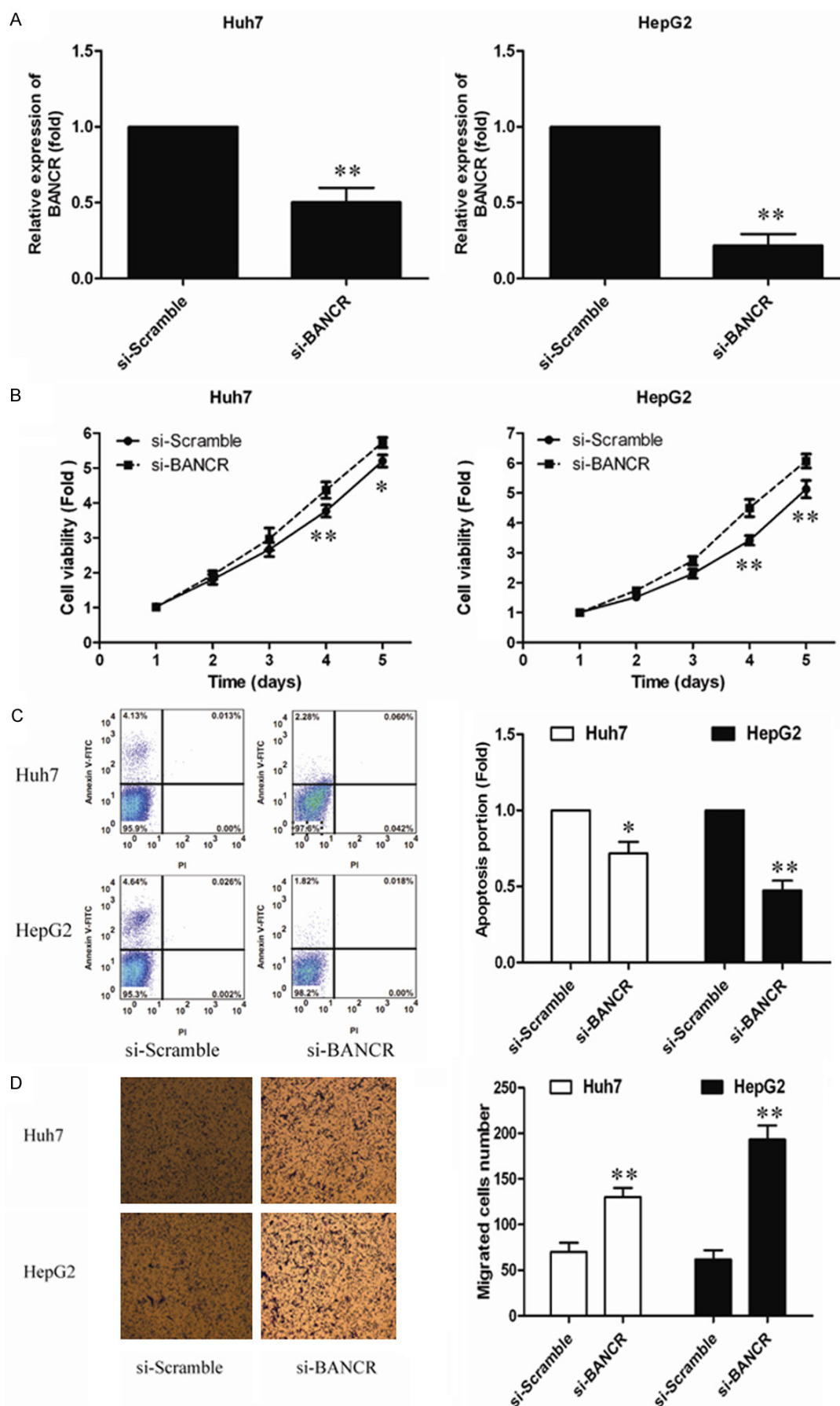


Figure 3. Effects of BANCR knockdown on HCC cell proliferation, cell apoptosis, migration and invasion in vitro. A. BANCR levels were determined by quantitative PCR after Huh7 and HepG2 cells treated with si-BANCR. B. BANCR promoted the proliferation of Huh7 and HepG2 cells. Cell number was determined by the CCK-8 assay, and the relative number of cells to 1 d was presented. C. Cells apoptosis were detected by flow cytometry after si-BANCR or si-Scramble transfection. D. BANCR promotes cell migration by transwell assays after si-BANCR transfection. All values are presented as mean \pm standard error based on at least three independent experiments. * $P < 0.05$, ** $P < 0.01$.

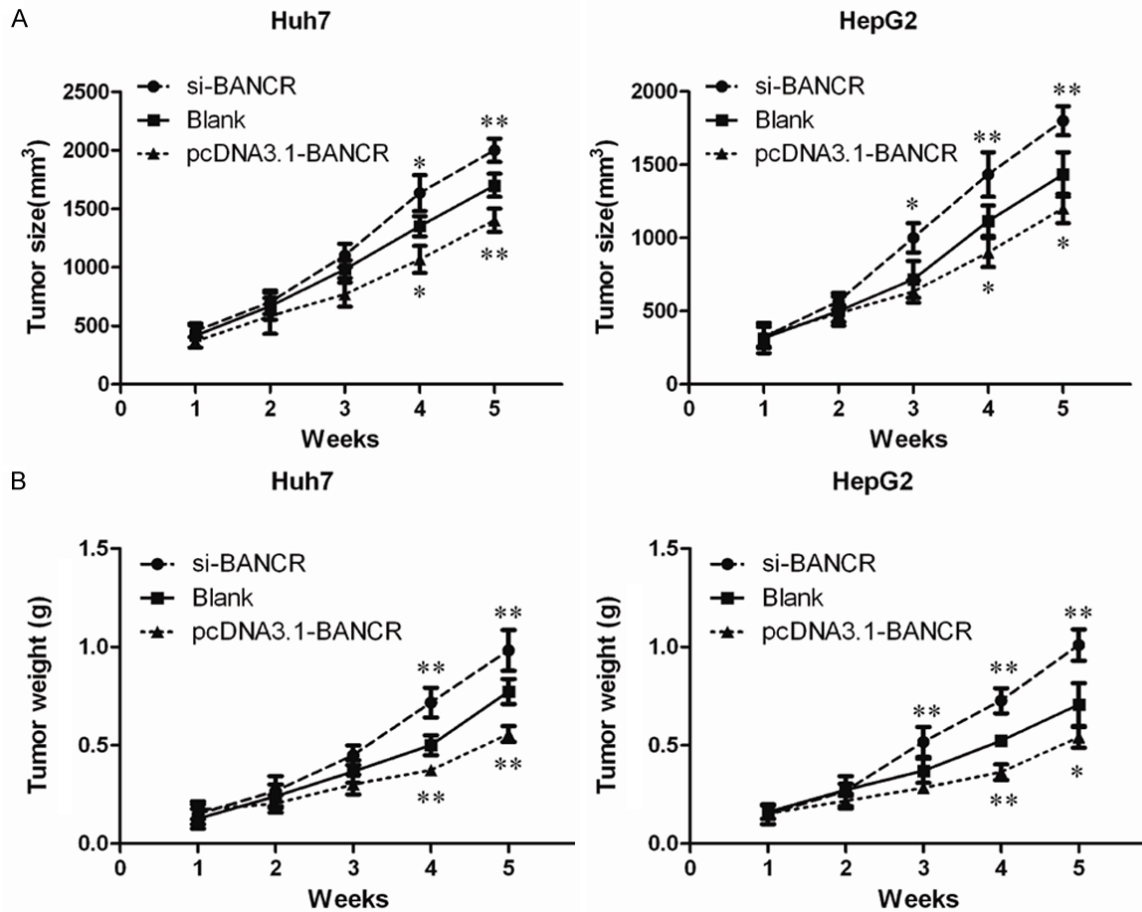


Figure 4. Effects of BANCR on tumor growth in vivo. Huh7 and HepG2 cells transfected with pcDNA-BANCR or si-BANCR were injected into the back region of nude mice at a single site. After the transplantation, the volumes of the xenografts were measured every week. Tumor-bored mice were sacrificed and the xenografts were harvested and weighted (in grams, recorded every week). A. Growth curve of tumors in nude mice. B. Average weight of tumors in nude mice. ** $P < 0.05$ vs. Blank.

ed that BANCR knockdown induced by shRNA transfection markedly suppressed tumor growth in vitro and vivo [29]. Later similar studies were respectively reported in colorectal cancer and papillary thyroid carcinoma [16, 30]. However, the prevalence and influence of BANCR expression in HCC is currently unknown, and the underlying mechanism remains to be elucidated. In this study, we found another lncRNA, BANCR, whose expression is significantly downregulated in HCC tissues from the patients or the cell lines, consistently with

previous reports [17, 28, 31]. Importantly, we demonstrated knockdown of BANCR expression promoted cell proliferation, migration, and invasion and suppressed apoptosis in vitro. Additionally, our in vivo experiment showed that knockdown of BANCR expression significantly increased xenografts growth and enhanced tumor weight in nude mice. These findings suggest that BANCR plays a direct role in the modulation of cell metastasis and HCC progression, and may be useful as a novel prognostic or progression marker for HCC.

In conclusion, we found that BANCR was significantly downregulated in HCC tissues and HCC cells. Knockdown of BANCR could promote HCC cell proliferation and metastasis both in vitro and in vivo. These findings would provide us important basic information and a wider perspective on HCC intervention/prevention and treatment. Due to the limited sample size in our study, more studies would be needed to further verify the clinical significance of BANCR in HCC patients.

Disclosure of conflict of interest

None.

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