

Decreased expression of long non-coding RNA GAS5 indicates a poor prognosis and promotes cell proliferation and invasion in hepatocellular carcinoma by regulating vimentin

LEI CHANG^{1*}, CUICUI LI^{2*}, TIAN LAN¹, LONG WU¹, YUFENG YUAN¹, QUANYAN LIU¹ and ZHISU LIU¹

¹Department of General Surgery, Research Center of Digestive Diseases, Zhongnan Hospital of Wuhan University, Wuhan, Hubei 430071; ²Zhongnan Hospital of Wuhan University, Institute of Hepatobiliary Diseases of Wuhan University, Transplant Center of Wuhan University, Hubei Key Laboratory of Medical Technology on Transplantation, Wuhan, Hubei 430071, P.R. China

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Abstract. Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide. Recent studies have demonstrated that long non-coding RNAs (lncRNAs) are key in carcinogenesis. The aim of the present study was to investigate the role of lncRNA GAS5 in HCC tissues and to define the role of growth arrest-specific 5 (GAS5) in the regulation of hepatoma cell proliferation, invasion and apoptosis. Quantitative polymerase chain reaction and *in situ* hybridization were performed to investigate the expression of GAS5 in tumor tissues and corresponding adjacent tissues from 50 patients with HCC. Low expression of GAS5 was significantly correlated with differentiation ($P<0.010$) and portal vein tumor thrombosis ($P=0.001$). Multivariate analysis indicated that GAS5 expression was an independent predictor for overall survival ($P=0.017$). Further experiments demonstrated that overexpression of GAS5 significantly suppressed the proliferation and invasion of hepatoma cells *in vitro*. Overexpression of GAS5 significantly promoted the apoptosis of hepatoma cells. In addition, it was demonstrated that GAS5 negatively regulates vimentin expression *in vitro* and *in vivo*. Notably, vimentin knockdown promoted GAS5-pcDNA3.1-inhibition of hepatoma cell proliferation. In conclusion, the present study suggests an important role of GAS5 in the molecular etiology of HCC and suggests the potential application of GAS5 in HCC therapy.

Introduction

Hepatocellular carcinoma (HCC) is the third leading cause of cancer-related mortality worldwide and the principal cause of fatality among patients with cirrhosis (1). Despite recent advances in cancer treatment with respect to surgery, chemotherapy and biologics, the majority of cases of HCC remain incurable once they have become metastatic and are associated with a poor prognosis. Hepatocarcinogenesis has often been described as a multistep process involving a number of genetic alterations, eventually leading to the malignant transformation of hepatocytes (2). The advances in suitable therapies for the purpose of increasing survival rate are limited as the pathophysiological mechanisms causing HCC have not been fully elucidated. Revealing the pathological mechanism underlying HCC development and progression is indispensable for developing effective therapeutic agents.

The human transcriptome comprises not only large numbers of protein-coding messenger RNAs (mRNAs), but also a large set of non-protein coding transcripts that have structural, regulatory or unknown functions (3). Novel evidence shows that lncRNAs (long non-coding RNAs) exhibit important regulatory roles in tumorigenesis and cancer progression (4). For example, HOTAIR levels have been shown to be higher in tumor tissues than the adjacent normal tissue, and overexpression of HOTAIR correlates with the presence of metastasis. In addition, patients with high HOTAIR expression levels have been shown to have a poor prognosis (5). Forced expression of HOTAIR in cancer cells leads to altered histone H3 lysine27 methylation and gene expression, and increased metastasis (6). Maternally expressed gene 3 (MEG3) expression is markedly decreased in several primary human tumors and tumor cell lines by promoter hypermethylation (7). MEG3 is associated with meningioma progression by inducing the accumulation of p53 protein and selectively regulating p53 target gene expression. In addition, overexpression of MEG3 inhibits cancer cell proliferation and colony formation (8).

The long noncoding RNA, small nucleolar RNA host gene-growth arrest-specific 5 (GAS5), was originally identified via subtractive cDNA cloning of genes which are preferentially

Correspondence to: Dr Quanyan Liu or Professor Zhisu Liu, Department of General Surgery, Research Center of Digestive Diseases, Zhongnan Hospital of Wuhan University, 169 Donghu Road, Wuhan, Hubei 430071, P.R. China
E-mail: 390058788@qq.com
E-mail: reniorchang@whu.edu.cn

*Contributed equally

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expressed in growth-arrested cells (9). GAS5 comprises 12 exons and encodes ten box C/D within its introns (10). The open reading frame encoded within GAS5 exons is short and thought not to encode a functional protein. This, together with the presence of a conserved 5'-terminal oligopyrimidine tract (5'-TOP), explains why GAS5-a ncRNA accumulates upon growth arrest in a range of species (11). Several studies have implicated GAS5 ncRNA in other types of cancer (12,13). GAS5 and/or its snoRNAs have been shown to be aberrantly expressed in breast cancer, head and neck squamous cell carcinoma (14-16) and glioblastoma multiforme (17), where tumor expression is reduced, and non-small-cell lung cancer, where overexpression of the snoRNAs U44, U76 and U78 occurs (18). However, the mechanisms underlying GAS5 regulation of cancer cell proliferation in HCC remain unclear.

Based on the above findings, the present study analyzed the expression of GAS5 in HCC tissues and adjacent normal tissues, and its effect on cell proliferation in HCC cells. It was demonstrated that GAS5 levels are significantly decreased in HCC tissues, and decreased expression of GAS5 indicates a poor prognosis in patients with HCC. In addition, GAS5 was shown to suppress hepatoma cell proliferation in a vimentin-dependent manner.

Materials and methods

Tissue samples, cell lines and clinical data collection. A total of 50 patients were analyzed in this study. The patients underwent HCC resection at the Department of General Surgery at the Zhongnan Hospital of Wuhan University (Wuhan, China) between February 2009 and March 2010. The study was approved by the ethics committee of Zhongnan Hospital of Wuhan University (Wuhan, China). Written informed consent was obtained from all patients. The diagnosis of HCC was histopathologically confirmed. No patients received preoperative treatment. Tumor and corresponding adjacent normal tissues were selected for each case. Normal human liver tissues were obtained >5 cm from the edge of the cancerous region. Resected tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction, or fixed in 4% paraformaldehyde for paraffin sectioning. Clinicopathological data was collected for all subjects including details of age, gender and HCC features, such as tumor size, differentiation, hepatitis B virus (HBV) infection, liver cirrhosis, serum α -fetoprotein (AFP) levels, tumor number and the presence of portal vein tumor thrombus (PVT). Patient follow up was performed by telephone interview and questionnaire every 3 months until April 30, 2015. Overall survival (OS) was calculated from the date of the initial surgery to the date of death. Patients who succumbed to diseases not directly related to HCC were excluded from the study.

The following cell lines were used in this study: L02 normal human liver cells, and Huh7, Hep3B, HepG2, QGY-7701, MHCC97L and HCCLM9he human hepatoma cell lines. All cell lines were obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), where they were characterized by mycoplasma detection, DNA fingerprinting, isozyme detection and determination of cell viability. All cells were cultured in the recommended media supplemented with 10% (v/v) fetal bovine serum (FBS; Thermo Fisher Scientific,

Inc., Waltham, MA, USA), and 100 U/ml penicillin (Beyotime Institute of Biotechnology, Shanghai, China) and streptomycin (Beyotime Institute of Biotechnology) at 37°C in an incubator with a 5% CO₂ atmosphere (Thermo Fisher Scientific, Inc.).

Ethics statement. Patient samples were collected and stored in the Zhongnan Hospital of Wuhan University tumor bank. Written informed consent was obtained from all of the patients. All procedures were approved by the Research Ethics Committee of Wuhan University. Patient data and samples were treated according to the ethical and legal standards adopted by the Declaration of Helsinki 2013.

RNA preparation, semiquantitative-reverse transcription and reverse transcription quantitative polymerase chain reaction (RT-qPCR). The expression level of GAS5 was first examined by semiquantitative PCR in tissue from 6 patients with HCC. The expression of GAS5 was then analyzed by RT-qPCR in tissue from all 50 patients with HCC patients. Total RNA was extracted from tissues using TRIzol reagent (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. Quantification of total RNA was performed with a Nanodrop™ spectrophotometer (Thermo Fisher Scientific Inc.) at 260 and 280 nm. Only samples with an A260/A280 ratio between 1.8 and 2.0 were utilized for further analysis. Total RNA (5 μ g) was used to synthesize first-strand cDNA using random primers and SuperScript II reverse transcriptase (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. The 20 μ l RT reactions were performed using a PrimeScript RT reagent kit (Takara Bio Inc., Wuhan, China) and incubated for 30 min at 37°C and 5 sec at 85°C, and then maintained at 4°C for 1% agarose gel electrophoresis. For qPCR, 2 μ l diluted RT products were combined with 10 μ l of 2X SYBR Master mix (Toyobo Co., Ltd. Osaka, Japan), 1 μ l forward and reverse primers (10 μ M; Table I; Beijing View Solid Biotechnology (Beijing, China), and 6 μ l nuclease-free water in a final volume of 20 μ l according to the manufacturer's instructions. Amplification was performed with the iQ5 quantitative PCR system (Bio-Rad Laboratories Inc., Hercules, CA, USA), under the following conditions: 95°C for 30 sec, followed by 40 cycles at 95°C for 15 sec, and 60°C for 30 sec, and 72°C for 30 sec. qPCR was conducted in triplicate, including non-template controls. Amplification of the appropriate product was confirmed by melting curve analysis following amplification. Relative expression of GAS5 and vimentin were calculated using the comparative cycle quantification (Cq) ($2^{-\Delta\Delta Cq}$) method (19) with β -actin as the endogenous control to normalize the data. PCR products were run on agarose gels to determine size, and dissociation curves were subsequently utilized to examine the specificity of the RT-qPCR assay.

In situ hybridization (ISH). ISH was used to detect GAS5 in HCC clinical specimens. A digoxigenin-UTP-labeled antisense RNA probe (Beijing View Solid Biotechnology) was derived from 234 to 478 nt of GAS5 by *in vitro* transcription using the DIG RNA Labeling kit (Roche Diagnostics, Indianapolis, IN, USA). The digoxigenin-UTP labeled sense RNA probe derived from 234 to 478 nt of GAS5 was employed as a negative control. ISH was performed using the ISH kit (Boster Bio-Engineering Company, Wuhan, China). The ISH-staining regions were

Table I. DNA and RNA sequences of primers.

Primer name	Sequence (5'-3')	bp
GAS5-F	CAACTTGCCTGGACCAGCTT	126
GAS5-R	TCAAGCCGACTCTCCATACC	
β -actin-F	AGCGAGCATCCCCCAAAGTT	285
β -actin-R	GGGCACGAAGGCTCATCATT	
Vimentin-F	GCAGGAGGCAGAAGAATGGT	140
Vimentin-R	CCACTTCACAGGTGAGGGAC	
E-cadherin-F	CTTGCGGAAGTCAGTTCAGA	216
E-cadherin-R	CACCGTGAACGTGTAGCTCT	
Vimentin-siRNA-F	UCACGAUGACCUUGAAUAA	
Vimentin-siRNA-R	GAGGGAAACUAAUCUGGAU	

GAS5, growth arrest-specific 5; F, forward; R, reverse; siRNA, small interfering RNA.

reviewed and scored by two pathologists, the score standard for the staining intensity was as follows: 0, negative; 1, weak; 2, medium; and 3, strong. The proportion of GAS5-positive cells was scored as: 0, 0%; 1, 1-25%; 2, 26-50%; 3, 51-75%; and 4, 76-100%. The total scores were calculated by combining two scores and ranged from 0 to 7. Total scores of ≥ 3 were defined as the high-expression group (positive group). This scoring method was simple and reproducible. Results were highly concordant between the two independent pathologists.

Immunohistochemistry. Vimentin and E-cadherin expression in primary tumor tissues and adjacent non-tumor tissues were examined using IHC. Paraffin-embedded blocks containing tumor tissues or non-tumor tissues and $>70\%$ primary tumor tissue were selected for IHC staining. Paraffin sections were cut to 4 μm , mounted on silanized slides and the preserved at 4°C. The paraffin sections were dewaxed, rehydrated and blocked with 0.3% H_2O_2 . Tissue antigens were retrieved with a microwave oven at 95°C for 25 min and cooled to room temperature in 10 mmol sodium citrate buffer (pH 6.0). Each slide was washed with phosphate-buffered saline (PBS) and incubated overnight at 4°C with anti-vimentin (1:100, cat. no. 5741S, Cell Signaling Technologies, Inc.) or anti-E-cadherin (1:100, cat. no. 3195S, Cell Signaling Technologies, Inc.). Primary antibodies were diluted with background-reducing components (Dako, Glostrup, Denmark). Slides were then incubated with goat-anti-mouse IgG-horseradish peroxidase (HRP) (cat. no. BA1050; 1:5,000; Wuhan Boster Biological Technology, Ltd., Wuhan, China) or goat-anti-rabbit IgG-HRP (1:5,000; cat. no. BA1055; Wuhan Boster Biological Technology, Ltd.). Slides were stained for 2 min with diaminobenzidine tetrahydrochloride and then counterstained with hematoxylin. Tissue treated with antibody dilution solution was used as a negative control. All controls yielded satisfactory results. An FV1000 confocal laser-scanning microscope (Olympus, Tokyo, Japan) was used to examine the tissues. The total immunostaining score was calculated as the sum of the positive percentage and the staining intensity of the stained cells, which ranged from 0 to 6. The percent positivity was scored as follows: 0, 0-25%; 1, 26-50%; 2, 51-75%; and 3, $\geq 75\%$. The staining

intensity was scored as: 0, no staining; 1, weakly stained; 2, moderately stained; and 3, strongly stained. The results of the immunohistochemical staining were scored by two experienced pathologists, who were blinded to the clinical data. A negative expression of protein was defined as a total score ≤ 3 , and positive expression was defined as a total score ≥ 4 .

Transfection assays. The GAS5 sequence (Gene-bank: NR_002578.2) was synthesized according to the full length GAS5 sequence (based on the GAS5 sequence) and then cloned into a pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific Inc.). The GAS5-pcDNA3.1 or empty vector was transfected into hepatoma cells. Transfection was conducted using LipJetTM *in vitro* DNA and siRNA Transfection kit (SignaGen Laboratories, Gaithersburg, MD, USA) according to the manufacturer's protocol. All transfections were performed using Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific Inc.) according to the manufacturer's protocol. The empty pcDNA3.1 vector was used as the control. All transfection experiments were performed at least three times.

Fluorescent immunocytochemistry. Hepatoma cells were fixed with 4% paraformaldehyde [China Sinopharm International (Shanghai), Co., Ltd.] for 20 min and then permeabilized with 0.5% Triton X-100 [China Sinopharm International (Shanghai), Co., Ltd.] for 10 min. The fixed cells were blocked with 3% FBS for 30 min. For specific detection of vimentin protein (Cell Signaling Technologies, Inc., Boston, MA, USA), the cells were incubated with polyclonal rabbit anti-rat vimentin antibody (1:100, CST, USA) overnight at 4°C. The samples were incubated with Cy3-labeled goat anti-rabbit IgG (1:100, eBioscience, Inc., San Diego, CA, USA) in PBS for 1 h at 37°C. Then, the cells were washed twice with PBS and incubated with 4', 6-diamidino-2-phenylindole (DAPI; BD Biosciences, La Jolla, CA, USA) for 5 min. The images were analyzed using fluorescence microscopy (Nikon Eclipse 80i, Tokyo, Japan). Identical illumination and camera settings were used within each dataset.

Cell proliferation assay. Cell proliferation assays were performed using Cell Counting kit-8 (Dojindo Molecular

Technologies Inc., Kumamoto, Japan), according to the manufacturer's protocol. Human hepatoma cells were plated in 24-well plates in triplicate at a density of $2-5 \times 10^4$ cells per well and cultured in growth medium. Cells were then treated with pcDNA3.1, empty vector or vimentin-small interfering (si)RNA (Beijing View Solid Biotechnology) and the numbers of cells per well were measured by the (450 nm) at the indicated time points. The plasmid GAS5-pcDNA3.1 (Beijing View Solid Biotechnology) was constructed by introducing a *KpnI*XhoI fragment containing the GAS5 cDNA into the same sites in pcDNA3.1.

Bromodeoxyuridine (BrdU) assay. Hepatoma cells were fixed in 4% paraformaldehyde for 20 min, cells were then washed three times in PBS, dyed with BrdU (BD Biosciences) for 30 min, washed again in PBS three times, and treated with DAPI for 5 min. The images were analyzed using fluorescence microscopy (Nikon Eclipse 80i). Identical illumination and camera settings were used within each dataset.

Flow cytometric analysis. Hepatoma cells ($2-5 \times 10^5$) treated with GAS5-pcDNA3.1 or negative control (NC) were plated in 6-well plates. After 48 h incubation the cells were harvested by trypsinization. The cultures were double stained with incubated with Annexin V and propidium iodide [China Sinopharm International (Shanghai), Co., Ltd.] for 30 min in the dark. Cultures were collected and analyzed for cell apoptosis using a flow cytometer (FACScan; BD Biosciences Franklin Lakes, NJ, USA) equipped with CellQuest 3.3 software. Cells were categorized as early apoptotic cells, late apoptotic cells, dead cells, or viable cells. The ratio of early apoptotic cells was compared with that in the controls from each experiment.

Cell invasion assays. After transfection for 24 h, cells in serum-free media were seeded into the upper chamber of a Transwell apparatus (BD Biosciences). for invasion assays with Matrigel (Sigma-Aldrich, St. Louis, MO, USA). The lower chambers were filled with media containing 10% FBS. After 24 h of incubation at 37°C in a 5% CO₂ atmosphere, the cells which had invaded through the membrane were fixed in 4% paraformaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich). The cells on the lower surface were photographed (Olympus; IX73) and three random fields were counted using a confocal laser-scanning microscope (Olympus FV1000). Three independent experiments were performed.

Western blot analysis. Western blot analysis to assess vimentin and E-cadherin protein expression was performed as previously described (20). Glyceraldehyde 3-phosphate dehydrogenase served as a control. Mouse monoclonal anti-GAPDH (cat. no. KM9002; 1:5,000; Sungene, Tianjin, China), rabbit monoclonal anti-vimentin (cat. no. 5741S; 1:1,000; Cell Signaling Technology, Inc.) and rabbit monoclonal E-cadherin (cat. no. 3195S; 1:1,000; Cell Signaling Technology, Inc.) primary antibodies were used. Goat anti-mouse IgG-HRP (cat. no. BA1050; 1:5,000; Boster Bio-Engineering Company) and goat anti-rabbit IgG-HRP (cat. no. BA1055; 1:5,000, Boster Bio-Engineering Company).

Statistical analysis. All statistical analyses were performed using SPSS software (SPSS, Inc., Chicago, IL, USA). The differences between groups were tested using Student's t-test. Survival curves were calculated using Kaplan-Meier and log-rank tests. The effects of variables on survival were determined by univariate and multivariate Cox proportional hazards modeling. The correlation between GAS5 and vimentin was analyzed by Spearman's rank correlation. Two-sided P-values were calculated. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

GAS5 is significantly downregulated in HCC tissues and hepatoma cells. To investigate whether GAS5 regulates hepatocarcinogenesis, the expression level of GAS5 was examined in HCC and adjacent normal tissues (Fig. 1). The expression level of GAS5 was first examined by semiquantitative RT-PCR in tissue from 6 patients with HCC. GAS5 was downregulated in HCC tissues compared with adjacent non-tumor tissues (Fig. 1C). The expression of GAS5 was then investigated by RT-qPCR in tissue from all 50 patients with HCC. Fig. 1A showed significantly lower GAS5 expression in HCC tissues. Fig. 1A and C show that GAS5 is significantly downregulated in HCC tissues compared with adjacent normal tissues. The mean value of GAS5 expression in normal tissues was 40.74 but the mean value of GAS5 expression in HCC tissues is 17.35. GAS5 expression levels were also assessed in hepatoma cells, as shown in Fig. 1B, GAS5 was observed to be downregulated in hepatoma cells compared with normal liver cells. An ISH assay (Fig. 1D) further confirmed that GAS5 was significantly downregulated in HCC tissues compared with adjacent normal tissues.

GAS5 expression and clinicopathological factors in HCC. As shown in Table II, in order to assess the correlation between GAS5 expression and clinicopathological data, tumor tissues were divided into the low-expression group (mean expression value 1.24, $n=25$) and the high-expression group (mean expression value 33.45, $n=25$), based on the mean expression level of all tumor tissues (mean expression value, 2.98). Clinicopathological factors were analyzed in the high and low GAS5 expression groups. The patients with low GAS5 ($n=25$) exhibited a poorer histologic grade ($P < 0.010$) and increased presence of PVT (P=0.001) than the high GAS5 expression group ($n=25$). However, there was no significant correlation between GAS5 expression and other clinicopathologic features, such as age, gender, HBV infection, liver cirrhosis, serum AFP and tumor number ($P > 0.05$).

Low expression of GAS5 indicates a poor prognosis in HCC. To determine the factors responsible for patient survival, univariate and multivariate analysis were performed. GAS5 expression levels were obtained from the RT-qPCR data of the cohort of 50 patients mentioned above. Univariate analysis of overall survival revealed that GAS5 expression ($P=0.000$), PVT (P=0.008), and differentiation ($P=0.003$) were prognostic indicators (Table III). Multivariate analysis showed that GAS5 expression was an independent prognostic indicator for overall survival ($P < 0.001$; Fig. 2A). No significant associations

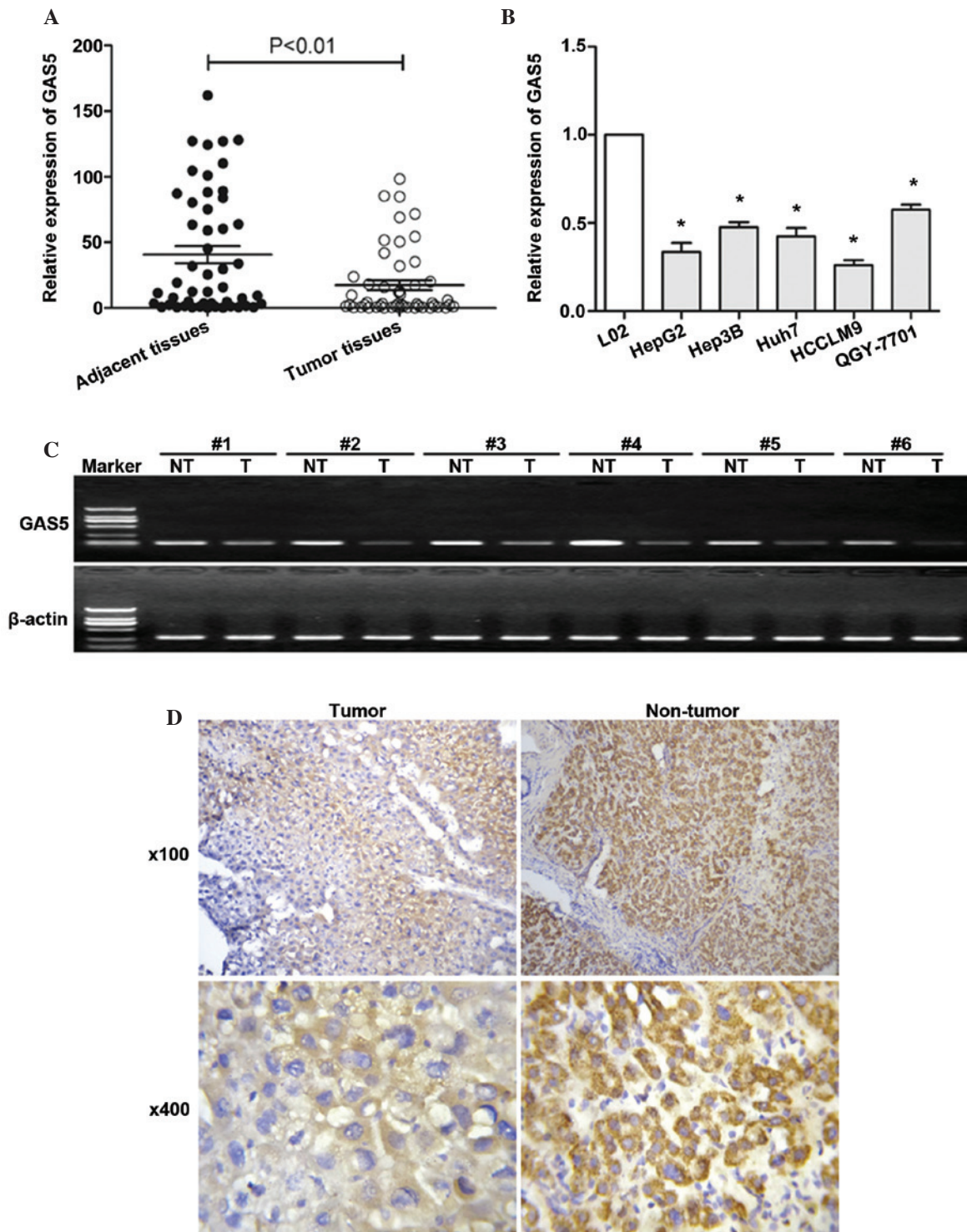


Figure 1. Analysis of GAS5 expression in HCC tissues and hepatoma cells. (A) Relative expression of GAS5 in HCC tissues (n=50) compared with adjacent normal tissues (n=50). GAS5 expression was examined by RT-qPCR and normalized to β -actin expression. (B) GAS5 expression levels were determined by RT-qPCR in human hepatoma cell lines (Huh7, Hep3B, HepG2, QGY-7701 and HCCLM9) and the normal human liver cell line (L02). Data are presented as the mean \pm standard error of the mean (n=3). *P<0.05, compared with L02. (C) Semiquantitative RT-PCR of GAS5 expression in six representative human HCC tissues and adjacent normal tissues. The expression of β -actin is also shown as a control. (D) Representative images of GAS5 expression in human HCC tissues and adjacent normal by *in situ* hybridization assays. GAS5, growth arrest-specific 5; HCC, hepatocellular carcinoma; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; NT, non-tumor; T, tumor.

were found with age, gender, HBV infection, liver cirrhosis, serum AFP and tumor number (Table III). Furthermore, high expression of GAS5 was found to be associated with lower rates of OS (P=0.017; log-rank test; Fig. 2B). However, no significant associations were found between OS and age,

gender, HBV infection, liver cirrhosis, serum AFP and tumor number.

GAS5 regulates cell apoptosis and cell proliferation in vitro. To investigate the role of GAS5 in the regulation of cell

Table II. Correlation between GAS5 expression and clinicopathologic parameters of HCC.

Parameter	Number of cases	GAS5 expression		P-value
		Low (n=25)	High (n=25)	
Age (years)				0.869
≥65	32	17	15	
<65	18	8	10	
Gender				0.747
Male	37	18	19	
Female	13	7	6	
Tumor size (cm)				0.774
≥5	29	15	14	
<5	21	10	11	
Tumor differentiation				<0.010
Well or moderate	24	5	19	
Poor	26	20	6	
HBV infection				0.239
Yes	32	14	18	
No	18	11	7	
Liver cirrhosis				0.569
Yes	22	10	12	
No	28	15	13	
Serum AFP (μg/l)				0.544
≥400	34	16	18	
<400	16	9	7	
Tumor number				0.157
Singular	40	22	18	
Multifocal	10	3	7	
PVTT				0.001
Yes	15	13	2	
No	35	12	23	

GAS5, growth arrest-specific 5; HBV, hepatitis B virus; AFP, α-fetoprotein; PVTT, portal vein tumor thrombus.

proliferation, cell apoptosis and the ability of cell invasion, the hepatoma cells were treated with GAS5-pcDNA3.1. RT-qPCR was used to observe the expression of GAS5 (Fig. 3A). Overexpression of GAS5 inhibited HCCLM9 proliferation (Fig. 3B and C) and promoted HCCLM9 apoptosis (Fig. 3D and E). In addition, overexpression of GAS5 suppressed the invasion ability of HCCLM9 (Fig. 3F and G). The above data demonstrated that GAS5 is key in hepatocarcinogenesis.

Expression of vimentin and E-cadherin in HCC. Epithelial-mesenchymal transition (EMT) has been shown to be of critical importance in the early events of tumor cell metastatic dissemination as cells become more motile and acquire invasive potential (21). It was previously found that GAS5 suppressed the invasion ability of tumor cells. Thus, the present study determined the expression of vimentin and E-cadherin (marker protein of EMT) (22). As shown in

Fig. 4A, immunohistochemistry indicated that vimentin was significantly upregulated in tumor tissues, while E-cadherin was downregulated in tumor tissues. In addition, RT-qPCR confirmed the above phenomenon (Fig. 4B).

GAS5 negatively regulates vimentin expression in vitro. To investigate the correlation between GAS5 and vimentin, Spearman's rank correlation analysis was used. As shown in Fig. 4C a significant negative correlation is observed between the GAS5 levels and the vimentin levels in HCC tissues ($r^2=0.6590$, $P<0.01$). In addition, fluorescent immunocytochemistry indicated that overexpression of GAS5 decreased vimentin expression and increased E-cadherin expression (Fig. 4D). Western blot analysis demonstrated that the vimentin protein level is downregulated and E-cadherin is upregulated in Huh7 and HCCLM9 after GAS5 overexpression (Fig. 4E). Figure 4F shows that overexpression of GAS5 significantly decreased the vimentin mRNA levels and increased E-cadherin

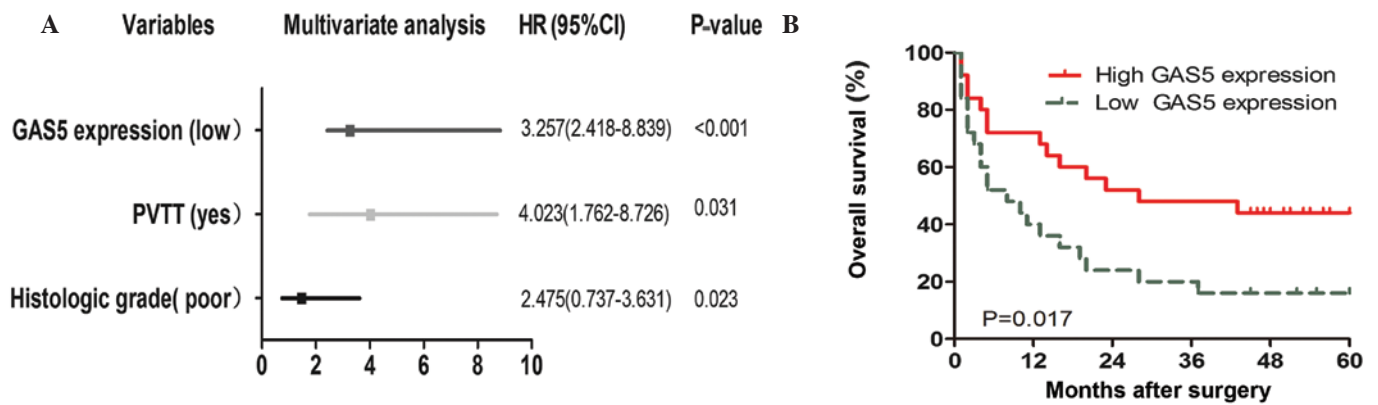


Figure 2. lncRNA GAS5 is an independent prognostic factor to predict overall survival. (A) Multivariate analysis of HRs for overall survival is shown. HRs are presented as the means (95% CI). (B) Kaplan-Meier curves displaying overall survival of patients with high GAS5 expression (n=25) vs. low GAS5 expression (n=25) (P=0.017), using a long-rank test. Variables included in the multivariate analysis were selected using a univariate analysis. lncRNA, long non-coding RNA; GAS5, growth arrest-specific 5; HR, hazard ratio; CI, confidence interval; PVTT, portal vein tumor thrombus.

mRNA in Huh7 and HCCLM9 cells. These data indicated that GAS5 regulates hepatoma cell proliferation and invasion by the regulation of vimentin.

GAS5 inhibits cell proliferation by regulating vimentin. GAS5 overexpression increased hepatoma cell proliferation and a significant negative correlation was observed between GAS5 and vimentin. Thus, it was hypothesized that the role of GAS5 in regulating hepatoma cell proliferation is mediated by vimentin. Figure 5A shows that vimentin knockdown partially strengthened the ability of GAS5-pcDNA3.1-inhibited cell proliferation in HCCLM9 cells. Vimentin inhibition also strengthened the ability of GAS5-pcDNA3.1-inhibited cell proliferation in Huh7 cells (Fig. 5B). These data confirmed that GAS5 suppressed hepatoma cell progression, at least in part, by regulating vimentin expression.

Discussion

In recent years, genome-wide surveys have revealed that 98% of the total human genome can be transcribed, including certain short or lncRNAs with numbered or no protein-coding capacity (23-25). Up to now, studies have identified a huge number of lncRNAs involved in the development and progress of human diseases, including cancer (21,26). lncRNAs can function as regulators of tumor suppressor or oncogene expression and may be one of the 'cutt' that lead to oncogenesis (27).

To date, there is increasing evidence that suggests that certain lncRNAs can be identified as biomarkers for the prognosis of tumor therapeutic targets in human cancer (28). GAS5 is an lncRNA involved in the regulation of cell cycles. GAS5 is key in normal growth arrest in T-cell and non-transformed lymphocytes. GAS5 inhibition suppresses cell apoptosis and maintains a more rapid cell cycle, whereas GAS5 overexpression results in an increase in cell apoptosis and a reduction in the rate of progression through the cell cycle, revealing that GAS5 is required for normal growth arrest (14). Recently, studies have identified a novel role of GAS5 in the regulation of tumorigenesis (29). GAS5 was downregulated in a number of tumor types, such as breast cancer, head and renal cell carcinoma, prostate cancer, and glioblastoma (30-32).

Table III. Univariate and multivariate analysis using the Cox proportional hazard regression model for overall survival.

Variable	Univariate (P-value)	Multivariate (P-value)
Age	0.943	
Gender	0.574	
Tumor size	0.891	
Histologic grade	0.003	0.023
HBV infection	0.121	
Liver cirrhosis	0.385	
Serum AFP	0.270	
Tumor number	0.476	
PVTT	0.008	0.031
GAS5 expression	0.000	<0.001

HBV, hepatitis B virus; AFP, α -fetoprotein; PVTT, portal vein tumor thrombus; GAS5, growth arrest-specific 5.

These observations indicate that GAS5 may function as a tumor suppressor in human tumor progression. However, the molecular mechanisms underlying GAS5 regulation of cancer cell proliferation in HCC remain unclear.

EMT has been shown to be of critical importance in the early events of tumor cell metastatic dissemination during which cells become more motile and exhibit invasive potential (33). EMT is not just a mechanism that forms fibroblast-like cells; it is a process that results in cancer cell migration, invasion, and metastasis (34,35). Various biomarkers have been screened to indicate the EMT process, including the loss of E-cadherin, zona occludens-1 and cytokeratin, and the upregulation of matrix metalloproteinase, fibronectin, α -smooth muscle actin, vimentin, snail and slug (35). Vimentin is a 57 kDa, type III intermediate filament that is found in mesenchymal cells of various types of tissues during their developmental stages. Its function is to maintain cell and tissue integrity. Vimentin is associated with tumor invasion and a poor prognosis in a number of types of cancers, including breast cancer, prostate

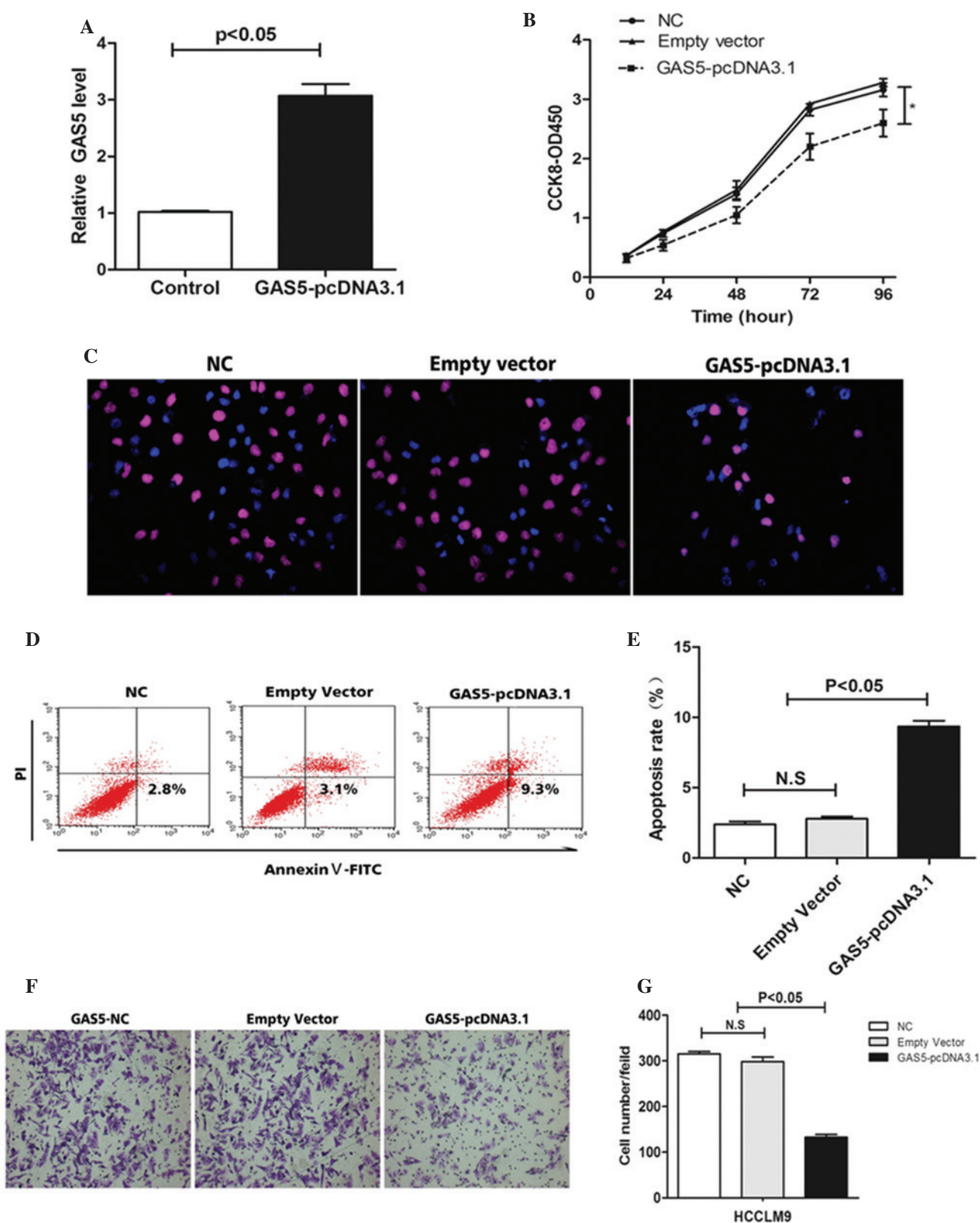


Figure 3. Effect of GAS5 on hepatoma cell growth, invasion and apoptosis *in vitro*. (A) The relative expression level of GAS5 in HCCLM9 cells, transfected with empty vector or GAS5-pcDNA3.1, was tested by reverse transcription-quantitative polymerase chain reaction. (B) At 48 h after transfection, a cell counting kit-8 assay was performed to determine the proliferation of HCCLM9 cells. (C) At 48 h after transfection, a BrdU assay indicated that overexpression of GAS5 inhibited HCCLM9 proliferation (magnification, $\times 400$). (D and E) At 48 h after transfection, cell apoptosis of HCCLM9 was analyzed by flow cytometry. (F and G) At 48 h after transfection a cell invasion assay was performed to investigate the ability of invasion of HCCLM9. The results show data from at least three independent experiments, expressed as the mean \pm standard deviation. $^*P < 0.05$. NC, negative control; GAS5, growth-arrest specific 5; BrdU, bromodeoxyuridine; N.S. not significant.

cancer, melanoma and lung cancer, and serves as a potential target for cancer therapy (36,37).

The current study verified that the expression level of GAS5 is significantly downregulated in HCC tissues compared

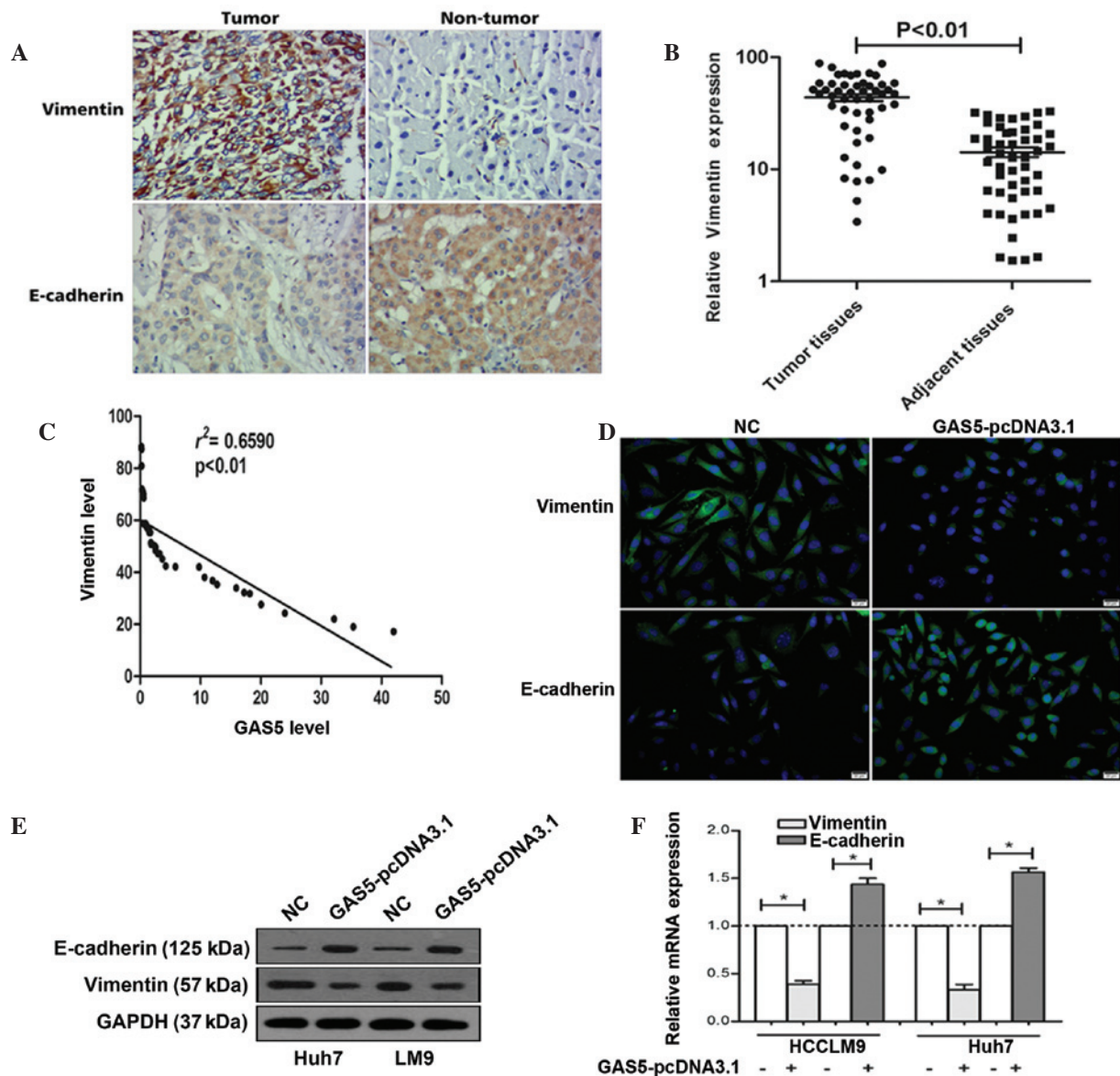


Figure 4. GAS5 negatively regulates vimentin expression. (A) Analysis of the vimentin and E-cadherin levels was conducted by immunohistochemistry in HCC tissues and adjacent normal tissues (magnification, x200). (B) Analysis of the vimentin mRNA level was performed in HCC tissues and adjacent normal tissues by RT-qPCR. (C) A negative correlation was observed between the GAS5 level and the vimentin mRNA level in HCC tissues ($r^2=0.6590$, $P<0.010$). (D) Immunofluorescence showed the expression changes of vimentin and E-cadherin following GAS5 overexpression (Scale bar, 20 μ m). (E) Western blot analysis and (F) RT-qPCR analysis of vimentin protein levels and mRNA levels in Huh7 and HCCLM9 cells following GAS5 overexpression. * $P<0.05$. GAS5, growth arrest-specific 5; NC, negative control; RT-qPCR, reverse transcription-quantitative polymerase chain reaction.

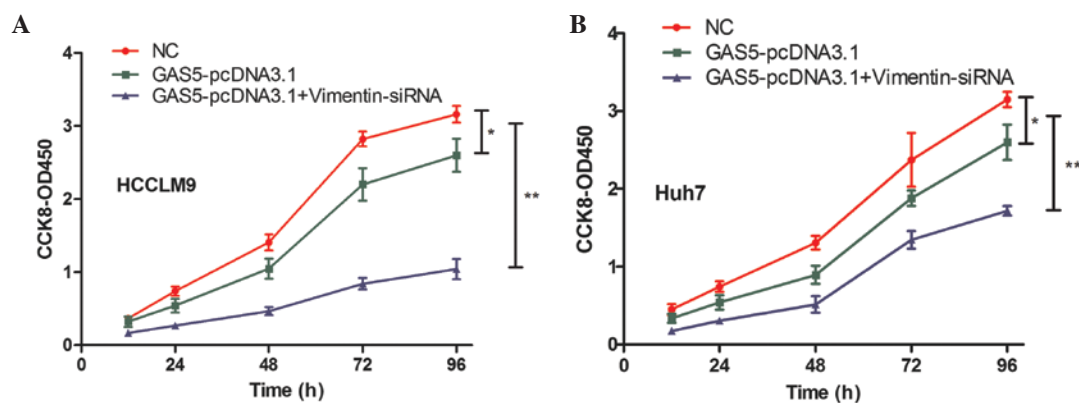


Figure 5. GAS5 overexpression inhibited hepatoma cell proliferation by regulating vimentin. (A) HCCLM9 cells and (B) Huh7 cells were treated with GAS5-pcDNA3.1 and vimentin-siRNA and the numbers of cells per well were measured using CCK8. * $P<0.05$ and ** $P<0.01$. CCK8, cell counting kit-8; OD, optical density; NC, negative control; GAS5, growth arrest-specific 5; siRNA, small interfering RNA.

with adjacent normal controls. Decreased expression of GAS5 was associated closely with the tumor differentiation ($P < 0.010$) and PVT (P=0.001). The downregulated expression of GAS5 was associated with poor prognosis of HCC. GAS5 overexpression decreases hepatoma cell proliferation and invasion. In addition, GAS5 overexpression promotes the apoptosis of hepatoma cells. It was further demonstrated that GAS5 is involved in the EMT of HCC cells. Overexpression of GAS5 downregulated the vimentin level and the upregulated E-cadherin level in hepatoma cells. A significant negative correlation was observed between GAS5 and the vimentin level *in vivo*. Notably, knockdown of vimentin partially increased GAS5-pcDNA3.1-induced inhibition of cell proliferation.

These data suggest an important role of GAS5 in the molecular etiology of HCC and implicate the potential application of GAS5 in HCC therapy.

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