Oncology and Translational Medicine
DOI 10.1007/s10330-021-0507-7

June 2022, Vol. 8, No. 3, P–P

ORIGINAL ARTICLE

Effects of long non-coding RNA GAS5 on proliferation and apoptosis of hepatocellular carcinoma cells through miR-26a-5p action

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Abstract

Objective Long non-coding RNAs (lncRNAs) regulate tumor development and progression by promoting tumor proliferation, invasion, and metastasis. The aim of the study was to investigate the effects of lncRNA growth arrest-special 5 (GAS5) on proliferation and apoptosis of hepatocellular carcinoma (HCC) cells through miR-26a-5p action.

Methods Expression levels of GAS5 were detected in cancerous and paracancerous tissue of 80 HCC patients by RT-qPCR. The starBase tool predicted that GAS5 had binding sites for the miRNA miR-26a-5p, which was also highly expressed in HCC tissue. The relationship between GAS5 and miR-26a-5p was confirmed using a luciferase reporter assay. The role of these lncRNAs was further explored by transfecting plasmids into SMMC-7721 cells and classifying the cells as follows: NC group, GAS5 group, anti-miR-26a-5p group, and GAS5 + miR-26a-5p group. Cell proliferation, cell cycle, and apoptosis were detected in each group. The relationship between miR-26a-5p and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) was analyzed by TargetScan database prediction and luciferase reporter assay. Western blotting was used to quantify PTEN, phosphatidylinositol 3-kinase (PI3K), phosphorylated protein kinase B (p-Akt), cyclin D1, and human P27 protein (P27).

Results GAS5 was downregulated, while miR-26a-5p was upregulated in HCC tissue compared to in paracancerous tissue. High GAS5 levels and low miR-26a-5p levels inhibited cell proliferation, increased the number of G0/G1 phase cells, promoted cell apoptosis, promoted PTEN and P27 expression, and inhibited PI3K, P-Akt, and cyclin D1 expression at the protein level. Upregulation of miR-26a-5p attenuated the effects of GAS5 upregulation on the proliferation, cell cycle, and apoptosis of HCC cells and on the expression of PTNE/PI3K/Akt signaling pathway-related proteins.

Conclusion Low GAS5 levels regulate the proliferation and apoptosis of HCC cells via the PTNE/PI3K/Akt signaling pathway and are linked to upregulation of miR-26a-5p.

Received: 7 July 2021
Revised: 23 November 2021
Accepted: 2 May 2022

Key words: lncRNA GAS5; miR-26a-5p; hepatocellular carcinoma (HCC); cell proliferation; cell cycle; apoptosis

Primary liver cancer is one of the most common malignant tumors, with an incidence second only to that of lung cancer worldwide [1]. Approximately half of liver cancer patients globally are from China, and approximately 350 000 people die of liver cancer every year, posing a serious threat to the health of Chinese individuals [2]. Hepatocellular carcinoma (HCC) has the highest incidence in primary liver cancers. HCC is characterized by high malignancy and more invasive growth and occurs when cancer cells invade the arteriovenous system and bile ducts. HCC has a low surgical resection rate and frequent recurrence and metastasis after surgery, which seriously affects the survival rate of HCC patients [3]. Recent studies have found that long noncoding RNA (lncRNA) plays an important role in the regulation of the cell cycle, tumor cell invasion and metastasis, dose compensation

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effects, and other biological behaviors and are therefore similar to oncogenes or tumor suppressor genes. The lncRNA small nucleolar RNA growth arrest-special 5 (GAS5) is located at the break point of human 6q15 chromosome translocation. GAS5 plays a role in many cancers, including cervical [4], stomach [5], and prostate cancer [6]. Many tumor suppressor genes, oncopromoter genes, non-coding RNAs, microRNA (miRNA), and protein factors have been implicated in the occurrence and development of HCC. MiR-26a-5p is lowly expressed in HCC cells, and up-regulation of its expression can inhibit the proliferation and invasion of HCC cells and promote cancer cell apoptosis [7]. The expression of miR-21 is up-regulated in HCC, while the expression of PTEN is down-regulated. Up-regualation of miR-21 promotes the proliferation, migration and invation of HCC cells by inhibiting the expression of PTEN [8]. But the role of GAS5 in HCC remains unclear. A variety of microRNA-lncRNA interactions are also involved in the occurrence and development of cancer. GAS5 inhibited the proliferation and invasion ability of HCC cells and promoted HCC cell apoptosis by targeting down-regulation of miR-1323 expression [9]. GAS5 inhibits the proliferation of laryngeal squamous cell carcinoma cells and promotes autophagy in cancer cells by targeting down-regulation of miR-26a-5p expression [10]. Therefore, in the present study, we explored the effects of GAS5 and miR-26a-5p on HCC cell cycle and apoptosis, and analyzed their targeted regulatory roles, may identify novel targets for HCC treatment in future.

Materials and methods

Samples and participants

Cancer and normal tissue samples (more than 2.5 cm away from the cancer tissue) were obtained from 80 patients with HCC who received surgical treatment in our hospital (Yuechi People's Hospital, Guangan, China) from July 2018 to October 2019. All cases were pathologically confirmed to be primary HCC without other primary tumors. This study was approved by the Ethics Committee of our hospital (Yuechi People's Hospital, Guangan, China), with voluntary participation and signed informed consent obtained from the patients and their families. Human hepatocyte *LO2* and human HCC cell lines (*HepG2.2.15, SMMC-7721, Huh7, Bel-7402, MHCC97-H,* and *MHCC97-L*) were purchased from Shanghai Cell Bank at the Chinese Academy of Sciences.

Reagents and instruments

Cell transfection plasmids were purchased from Shanghai Jima Co., Ltd, China. Dulbecco's modification of Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco, USA. The reverse transcription kit was purchased from Sigma, USA. Lipofectamine-2000 was purchased from Vector, Inc., USA. Trizol reagent was purchased from TAKARA, Japan. The EdU cell proliferation kit was purchased from Guangzhou Ruibo, China. The apoptosis detection kit and cell cycle detection kit were purchased from Shanghai Biyuntian Biotechnology, China. The western blotting kit was purchased from BD, USA. Rabbit polyclonal PI3K (AB39670), rabbit monoclonal Bax (AB32503), rabbit monoclonal Bcl-2 (AB32124), rabbit polyclonal P27 (AB137736), and rabbit monoclonal cyclin D1 (AB16663) were purchased from Abcam, USA. Goat anti-rabbit IgG secondary antibody was purchased from Jinqiao, China. Luciferase assay kit was purchased from Promega, USA.

Prediction of the relationship between miR-26a-5p, GAS5, and PTEN

The bioinformatics tools starBase and TargetScan were used to predict the relationship between miR-26a-5p, GAS5, and PTEN.

RT-qPCR of GAS5 and miR-26a-5p in HCC tissue and cells

Total RNA was extracted using the Trizol reagent according to the manufacturer's instructions. Reverse transcription was performed on 20 ng/μL of total RNA according to the manufacturer's instructions. RNA was reverse transcribed into cDNA by Prime Script™ RT Master Mix. And the quality of RNA was determined by nanodrop. Real time quantitative PCR (RT-qPCR) was performed according to the following program: denaturation at 75 °C for 2 min, followed by 40 cycles of denaturation at 90 °C for 5 min, annealing at 60 °C for 60 s, and extension at 72 °C for 30 s. The relative expression of target mRNAs was determined using the $2^{-\Delta\Delta CT}$ method [10–11]. The experiment was repeated independently three times for each sample.

Cell culture and transfection

The human HCC cell line SMMC-7721 was cultured in DMEM containing 10% fetal bovine serum. The medium was changed once every 2 days, until a confluency of about 90% was reached. SMMC-7721 cells in logarithmic growth phase were transfected using Lipofectamine 2000 according to the manufacturer's instructions. The cells were grouped as follows: the NC group was transfected with a negative control plasmid, the GAS5 group was transfected with an overexpression plasmid for GAS5, the anti-miR-26a-5p group was transfected with an siRNA plasmid for miR-26a-5p, and the GAS5 + miR-26a-5p group was transfected simultaneously with a GAS5 overexpression plasmid and a miR-26a-5p overexpression plasmid.

RNA immunoprecipitation (RIP) assay

RIP was performed using a MagnaRNA immunoprecipitation kit according to the manufacturer's instructions. SMMC-7721 cells were transfected with miR-26a-5p mimic or miR-NC and then collected and lysed in cell lysis buffer from RIP kit. The supernatant was collected and incubated in RIP immunoprecipitation buffer containing magnetic beads bound to Ago2 or IgG antibodies, and the expression level of GAS5 enriched on the beads was analyzed by RT-qPCR.

Luciferase reporter assay

Based on the binding sites predicted by TargetScan, the GAS5 and PTEN fragments containing miR-26a-5p binding sites were amplified by PCR, and the amplified fragments were inserted into the psiCHECK luciferase vector. Wild-type plasmid was constructed for both GAS5 and PTEN and denoted as psiCHECK-GAS5-wild and psiCHECK-PTEN-wild, respectively. Simultaneously, nucleotides in the binding sites were mutated, resulting in the construction of mutant plasmids psiCHECK-GAS5-mutant and psiCHECK-PTEN-mutant. Both miR-26a-5p mimic and negative control mimic-NC were co-transfected with no-load plasmid, psiCHECK-GAS5-wild and psiCHECK-PTEN-wild, or psiCHECK-GAS5-mutant and psiCHECK-PTEN-mutant plasmids. The luciferase activity was detected at 24 h after transfection in HEK293 cells according to the manufacturer's instructions.

EdU assay

Cells in each group were digested with trypsin and then inoculated into 96-well plates at a density of 5 × 10^3 cells/well. Cells were cultured for 24 h and then stained and washed according to the manufacturer's instructions. The cells were photographed and analyzed using a fluorescence inverted microscope (Nikon, Japan). Cell proliferation rate = positive edU staining under field / positive Hochest staining under field × 100%.

Flow cytometry

Adherent cells from each group were removed from their culture dish by trypsinization. Cells were resuspended in binding buffer and gently aspirated into single-cell suspensions. After incubating at 25 °C for 15 min, the rate of apoptosis and the cell cycle stage in each group were determined by flow cytometry. Annexin V-FITC (5 μL) and 7-AAD (5 μL) were added to each group, and the number of cells was adjusted to about 1 × 10^5 cells.

Western blotting

Total protein was extracted from sample tissue and cells using a total protein extraction kit (Biyuntian, China) according to the manufacturer's instructions. Protein content was determined by BCA assay (Biyuntian, China). Protein samples were separated using SDS-PAGE and then transferred to PVDF membrane. Membranes were incubated in blocking solution containing 5% BSA at room temperature for 2 h. Antibody diluents of PTEN (1:500), PI3K (1:2000), P-Akt (1:1000), P27 (1:1000), Cyclin D1 (1:1000) and GAPDH (1:5000) were added and incubated at 4 °C overnight. Membranes were washed with buffer solution three times, and then, the diluted secondary antibody was added. After incubation at room temperature for 1 h, the blot was visualized and analyzed using a chemiluminescent substrate reagents (ECL) and ImageJ software.

Statistical analysis

SPSS version 19.0 was used for statistical analysis, and GraphPad version 5.01 was used to generate figures. Measurement data were expressed as the mean ± standard deviation. Pearson's co-efficient was used for correlation analysis. One-way analysis of variance was used for comparison between multiple groups, and independent sample *t* tests were used for comparison between two groups. A $P < 0.05$ was considered to be statistically significant.

Results

Expression of GAS5 and miR-26a-5p in HCC tissue and cell lines

The expression level of GAS5 and miR-26a-5p in HCC and adjacent paracancerous tissue was detected by RT-qPCR. Compared with that in the adjacent tissue, the expression of GAS5 was significantly decreased in HCC tissue, and the expression of miR-26a-5p was significantly increased ($P < 0.05$). The expression levels of GAS5 and miR-26a-5p in HCC cell lines HepG2.2.15, SMMC-7721, HUH7, Bel-7402, MHCC97-H, and MHCC97-L were significantly different from those in the human hepatocyte cell line LO2 ($P < 0.05$; Fig. 1).

Relationship between GAS5 and miR-26a-5p

The starBase database was used to predict several binding sites between GAS5 and miR-26a-5p (Fig. 2a). A dual luciferase reporter assay was performed to confirm these interactions. The luciferase activity of psiCHECK-GAS5-wild was significantly enhanced after transfection of miR-26a-5p, while the luciferase activity of the psiCHECK-GAS5-mutant and no-loading plasmids did not change significantly ($P > 0.05$; Fig. 2b). This suggests that miR-26a-5p has a targeted regulatory relationship with GAS5. The overexpression of miR-26a-5p resulted in a significantly higher level of RIP Ago2-enriched GAS5, while the enrichment effect of the RIP IgG group was significant (Fig. 2c).

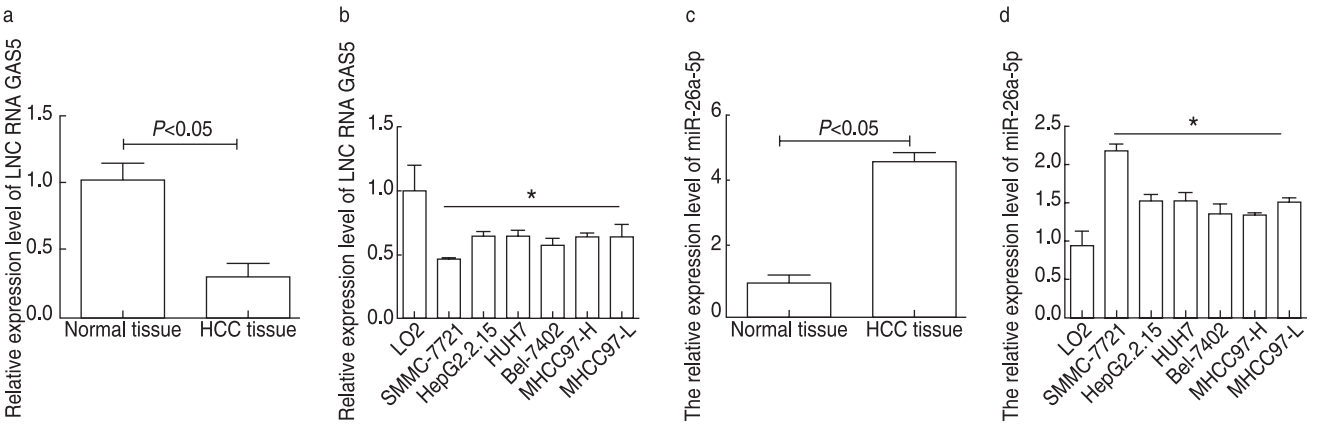

Fig. 1 Quantitation of GAS5 and miR-26a-5p in HCC tissue and cell lines. (a) The expression of GAS5 was detected in HCC and adjacent tissue by RT-qPCR. (b) GAS5 was expressed at low levels in all HCC cell lines but at a high level in a hepatocyte cell line (LO2). (c) The expression of miR-26a-5p was detected in HCC and adjacent tissue by RT-qPCR. (d) miR-26a-5p was expressed at high levels in all HCC cell lines but at a low level in a hepatocyte cell line (LO2). * $P < 0.05$; HCC: hepatocellular carcinoma

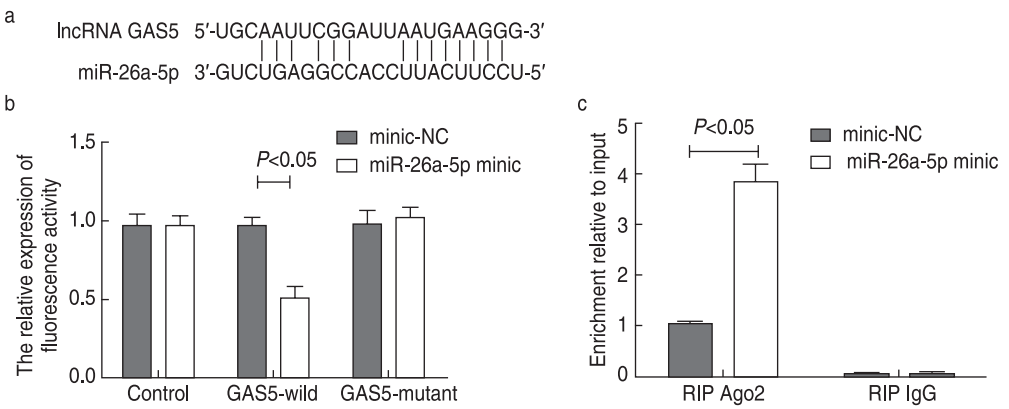

Fig. 2 Relationship between GAS5 and miR-26a-5p. (a) The predicted binding sites of GAS5 and miR-26a-5p. (b) Double luciferase reporter assay to confirm the relationship between GAS5 and miR-26a-5p. (c) RT-qPCR was used to detect RIP Ago2 and RIP IgG-enriched GAS5 levels in SMMC-7721 cells transfected with miR-26a-5p or miR-NC

Effects of GAS5 and miR-26a-5p on the proliferation of SMMC-7721 cells

An EdU assay was used to detect the proliferative ability of cells in each group. There was a statistically significant difference in the number of EdU positive cells between all groups ($P < 0.05$). Pairwise comparison revealed that the GAS5 and anti-miR-26a-5p groups had significantly fewer EdU-positive cells compared to the NC group ($P < 0.05$). The number of EdU positive cells in the lncRNA GAS5 + miR-26a-5p group was significantly higher than that of the GAS5 group ($P < 0.05$; Fig. 3).

Effect of GAS5 and miR-26a-5p on the cell cycle of SMMC-7721 cells

Flow cytometry was used to detect the cell cycle of cells in each group. There was a statistically significant difference in cell cycle phases among all groups ($P < 0.05$). Pairwise comparisons revealed that the number of cells in the G0/G1 phase was significantly higher in the GAS5 and anti-miR-26a-5p groups compared to that in the NC group, while the number of cells in the S-phase was significantly decreased ($P < 0.05$). The number of cells in the G0/G1 phase was significantly lower in the lncRNA GAS5 + miR-26a-5p group compared to that in the GAS5 group, while the number of cells in the S phase was significantly higher ($P < 0.05$; Fig. 4).

Effects of GAS5 and miR-26a-5p on apoptosis in SMMC-7721 cells

There was a statistically significant difference in apoptosis rates among all groups ($P < 0.05$). Pairwise comparisons revealed that the number of apoptotic cells

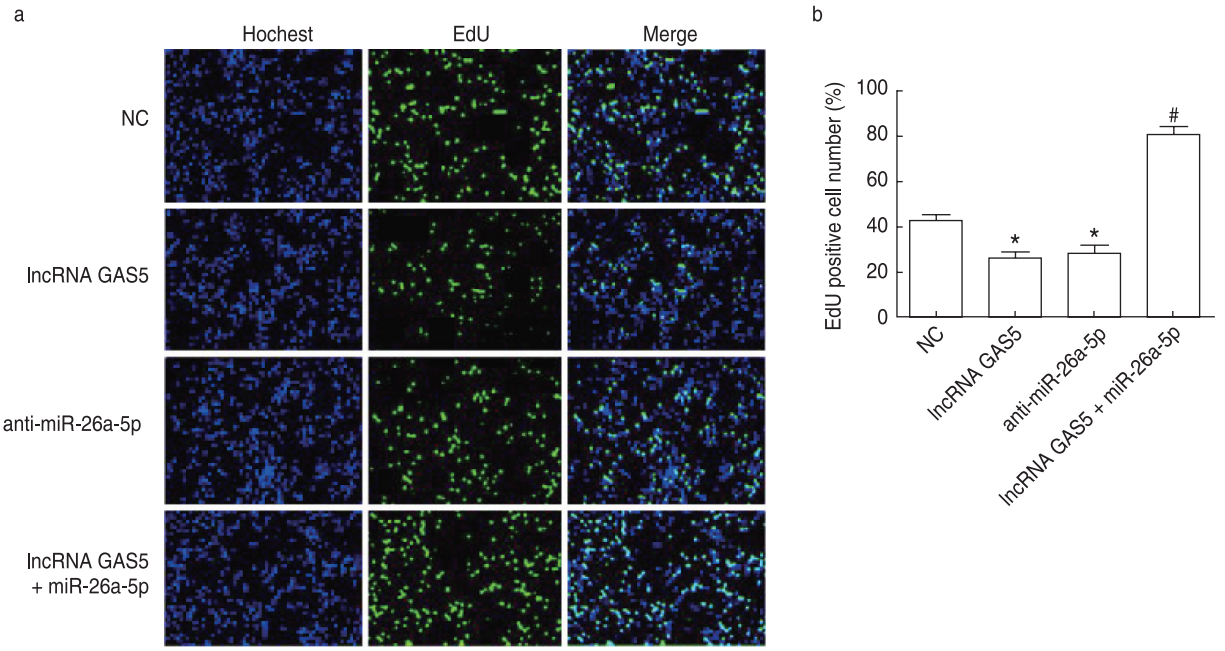

Fig. 3 Proliferative ability of transfected SMMC-7721 cells in each group detected by EdU assay (100×). (a) An EdU assay was used to detect cell proliferation in each group. (b) The number of EdU- positive cells in each group. * $P < 0.05$ compared to the NC group, # $P < 0.05$ compared with the GAS5 group

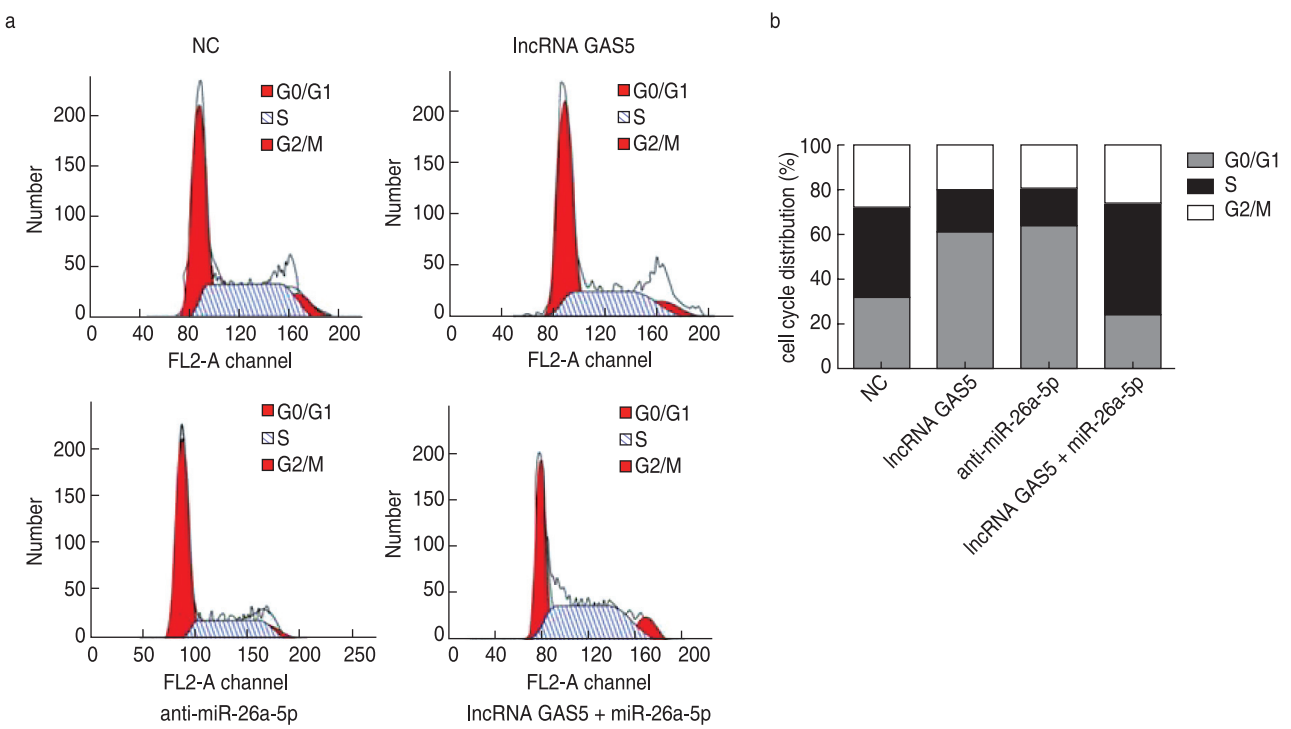

Fig. 4 Cell cycle phase differences in transfected SMMC-7721 cells in each group according to flow cytometry. (a) Flow cytometry results for each group. (b) Cell cycle stage distribution in each group

in the GAS5 and anti-miR-26a-5p groups was significantly higher than in the NC group ($P < 0.05$). The number of apoptotic cells in the lncRNA GAS5 + miR-26a-5p group was significantly reduced compared to in the GAS5 group ($P < 0.05$; Fig. 5),

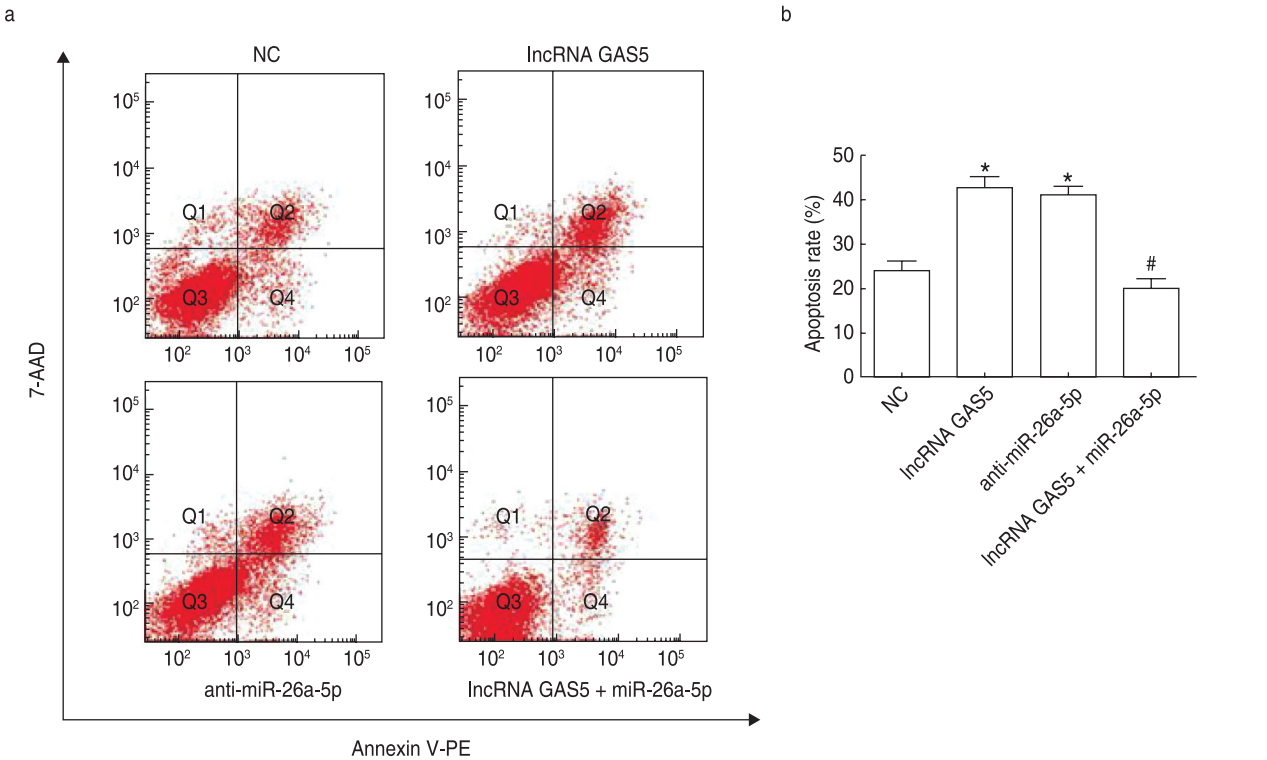

Fig. 5 Apoptosis of SMMC-7721 cells in each group according to flow cytometry. (a) Flow cytometry was used to detect apoptosis in each group. (b) Apoptosis rate in each group. * $P < 0.05$ compared with the NC group, # $P < 0.05$ compared with the GAS5 group

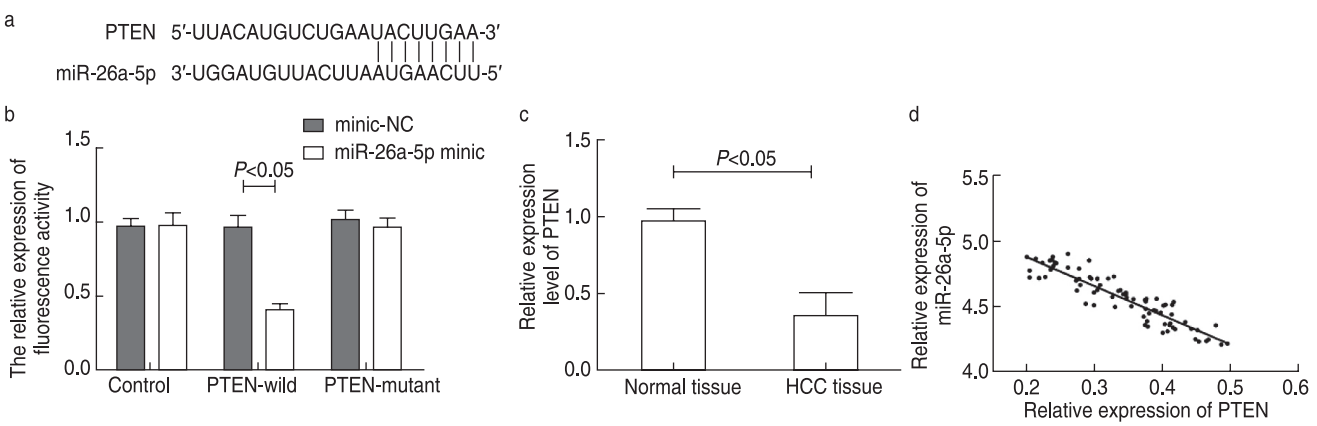

Fig. 6 Relationship between PTEN and miR-26a-5p. (a) Predicted binding site between miR-26a-5p and PTEN. (b) Binding was confirmed by a double luciferase reporter assay. (c) Western blotting was used to detect the expression levels of PTEN in HCC and normal tissue. (d) The level of mir-26a-5p was negatively correlated with the level of PTEN

Relationship between PTEN and miR-26a-5p

TargetScan analysis predicted that miR-26a-5p and PTEN had binding sites (Fig. 6a). A dual luciferase reporter assay showed that the luciferase activity of psiCHECK-PTEN-wild was significantly reduced after transfection with an miR-26a-5p mimic, while the luciferase activity of psiCHECK-PTEN-mutant and no-plasmid did not change significantly ($P > 0.05$; Fig. 6b). This suggests that miR-26a-5p has a regulatory relationship with PTEN protein. The expression of PTEN in HCC tissue was significantly lower than that in the adjacent normal tissue ($P > 0.05$; Fig. 6c). Interestingly, mir-26a-5p levels were negatively correlated with PTEN levels ($r = –0.915$, $P < 0.05$; Fig. 6d).

Western blotting

Western blotting was used to quantify the expression levels of PTEN, PI3K, p-Akt, P27, and cyclin D1 protein in each group. The differences between groups were

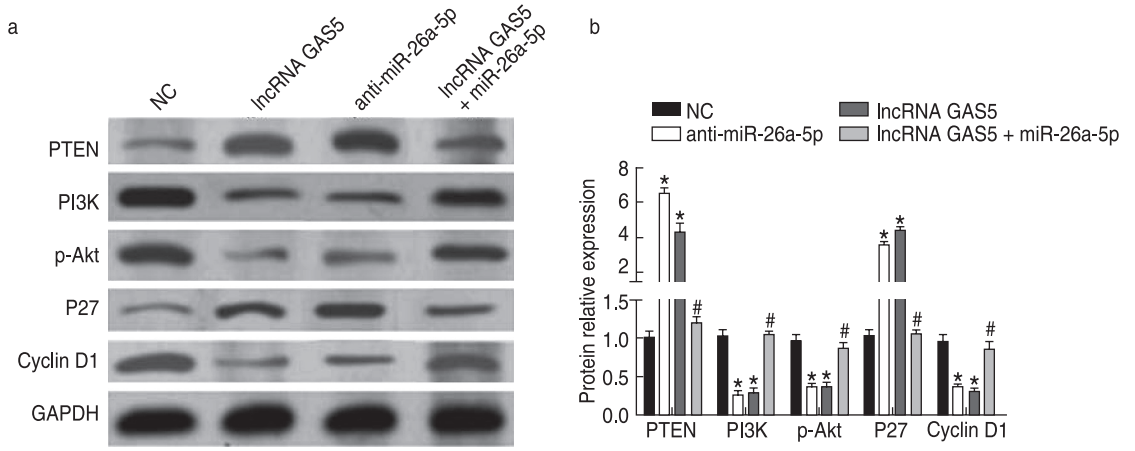

Fig. 7 Apoptosis and cyclin-related protein expression in transfected SMMC-7721 cells in each group according to western blot analysis. (a) Protein bands were detected in each group, (b) Relative protein expression based on densitometry of (a). * $P < 0.05$ compared with the NC group, # $P < 0.05$ compared with the GAS5 group

statistically significant (all $P < 0.05$). The expression of PTEN and P27 was significantly higher in the GAS5 and anti-miR-26a-5p groups than in the NC group, while the expression of PI3K, p-Akt, and cyclin D1 was significantly lower ($P < 0.05$). The expression of PTEN and P27 was significantly lower in the lncRNA GAS5 + miR-26a-5p group than in the GAS5 group, while the expression of PI3K, p-Akt, and cyclin D1 was higher ($P < 0.05$; Fig. 7).

Discussion

The role of lncRNA in cancer is expanding rapidly, with increased research interest in recent years driving important new discoveries. The lncRNA GAS5 can exert pro-cancer or anti-cancer roles in different tumors. For example, Liu *et al* [12] found that downregulation of GAS5 could inhibit the invasion and metastasis of gastric cancer cells by altering the levels of serine-rich spermatogenesis-related protein 2 (SPATS2). However, Song *et al* [13] found that GAS5 was expressed at low levels in colorectal cancer tissue and that overexpression of GAS5 could regulate enhancer of zeste homolog 2 (EZH2) by promoting and regulating enhancer of histone methyltransferase 2 (EHMT2). EZH2 inhibits the invasion and metastasis of breast cancer. However, the role of GAS5 in HCC had not yet been established. Therefore, in the present study, we determined that GAS5 expression was decreased in HCC tissue compared with in the surrounding paracancerous tissue. GAS5 may, therefore, play a tumor suppressive role in HCC. The occurrence of tumors is often accompanied by changes in cell proliferation and growth patterns [14]. To verify the biological role of GAS5 in the development of HCC, we overexpressed GAS5 in the HCC cell line SMMC-7721 and found that overexpression of GAS5 reduced the proliferation of SMMC-7721 cells. Moreover, the number of cells in the G0/G1 phase increased, and the number of apoptotic cells increased. Overexpression of GAS5 could therefore inhibit the proliferation of cancer cells, block the cycle of cancer cells in the G0/G1 phase, and promote the apoptosis of cancer cells.

Previous studies have shown that miRNA binds to target genes through incomplete base complementarity, thus affecting the apoptosis, migration, and metastasis of tumor cells and triggering the necrosis and apoptosis of the surrounding cells [15]. Due to this ability, miRNA is also an important link in the role of lncRNA. In the present study, we used cluster analysis and starBase to predict that miR-26a-5p had binding sites with lncRNA GAS5 and then confirmed that miR-26a-5p was highly expressed in HCC tissue. Previous studies have shown that high levels of miR-26a-5p can promote gastric cancer and that the downregulation of miR-26a-5p plays an important role in blocking the cell cycle in gastric cancer and inducing apoptosis [16]. In the present study, inhibition of miR-26a-5p reduced cell proliferation, arrested cells in the G0/G1 phase, and promoted apoptosis in an HCC cell line. When GAS5 is overexpressed, simultaneous overexpression of miR-26a-5p can reverse the anti-cancer effects of GAS5. These results suggest that GAS5 regulates cell proliferation, cell cycle, and apoptosis by inhibiting the expression of miR-26a-5p, thereby inhibiting the progression of HCC.

A binding site for miR-26a-5p was predicted on PTEN protein using the TargetScan database. Abnormal expression of PTEN protein activates the PTEN/PI3K/Akt signaling pathway, which is one of the most important signal transduction pathways and can regulate the occurrence and development of autoimmune diseases, diabetes, and other diseases. PTEN is an important tumor suppressor gene with abnormal expression in gastric

cancer, colorectal cancer, and other cancers [17]. In normal cells, PTEN and its downstream target PI3K maintain the dynamic balance of PIP2 and PIP3 by regulating their phosphorylation levels. When the expression of PTEN is reduced or absent, the dephosphorylation of PIP3 is limited, and it cannot be transformed into PIP2. The accumulation of PIP3 stimulates the activation of Akt, an important central link in the PTEN/PI3K/Akt signaling pathway, thereby stimulating the expression of downstream molecules [18]. Among these, P27 is a negative regulator of the cell cycle, and cyclin D1 is a key protein that regulates cell proliferation in the G1 phase. P27 binds to cyclin D1 and inhibits its activity, thus preventing cell cycle progression from G1 to S phase and thereby inhibiting cell proliferation [19]. Lv *et al* [20] found that miR-26a-5p was overexpressed in liver cancer, which caused a decrease in P27 expression and an increase in cyclin Dl expression, thus promoting cell cycle progression from the G1 to the S phase and promoting the proliferation of liver cancer cells. The PTEN/PI3K/Akt signaling pathway plays an important regulatory role in tumor cell cycle progression and arrest. In the present study, both the overexpression of GAS5 and the inhibition of miR-26a-5p could promote the expression of PTEN and thus reduce the levels of PI3K, p-Akt, and cyclin D1 downstream, while the expression level of P27 was increased. The expression of PTEN in the lncRNA GAS5 + miR-26a-5p group decreased, while the levels of PI3K, p-Akt, and cyclin D1 increased compared to in the GAS5 group, and the expression level of P27 decreased. This suggests that overexpression of GAS5 could regulate the expression of cyclin D1 and P27 by downregulating the expression of miR-26a-5p, thus activating the PTEN/PI3K/Akt signaling pathway, and arrest the cell cycle of HCC cells, which would inhibit proliferation and promote apoptosis.

In conclusion, GAS5 is expressed at low levels in HCC tissue, which may inhibit the activation of the PTEN/PI3K/Akt signaling pathway by upregulating the expression of miR-26a-5p, thereby affecting the expression of downstream cyclin D1 and regulating cancer cell proliferation and apoptosis.

Acknowledgments
Not applicable.

Funding
Not applicable.

Conflicts of interest
The authors indicated no potential conflicts of interest.

Author contributions
All authors contributed to data acquisition, data interpretation, and reviewed and approved the final version of this manuscript.

Data availability statement
Not applicable.

Ethical approval
Not applicable.

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DOI 10.1007/s10330-021-0507-7
Cite this article as: Yi ZL, Guo XG, Jiang XX, et al. Effects of long non-coding RNA GAS5 on proliferation and apoptosis of hepatocellular carcinoma cells through miR-26a-5p action. Oncol Transl Med. 2022;8(3): –.