

ORIGINAL ARTICLE

PCGEM1 triggers liver cancer progression by positively regulating SNAIL

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Summary

Purpose: To uncover the biological role of long non-coding RNA (lncRNA) PCGEM1 in regulating the progression of liver cancer and the molecular mechanism.

Methods: PCGEM1 levels in clinical specimens of liver cancer and cell lines were detected by quantitative real-time polymerase chain reaction (qRT-PCR). By analyzing the pathological data of recruited patients, the influences of PCGEM1 on tumor node metastasis (TNM) staging, tumor grading, lymphatic metastasis and overall survival of liver cancer patients were assessed. Hep3B and Huh7 cells were transfected with si-PCGEM1, followed by detection of proliferative, migratory and invasive rates through 5-Ethynyl-2'-deoxyuridine (EdU), cell counting kit-8 (CCK-8) and transwell assay. The interaction between PCGEM1 and SNAIL was explored, and the biological functions of SNAIL in liver cancer cell behaviors were finally detected.

Results: PCGEM1 and SNAIL were upregulated in clinical specimens of liver cancer and cell lines. High level of PCGEM1 was detected in liver cancer cases with advanced staging, grading or lymphatic metastasis, which predicted poor prognosis of liver cancer patients. Knockdown of PCGEM1 reduced proliferative, migratory and invasive rates in Hep3B and Huh7 cells. SNAIL was upregulated in liver cancer tissues and positively correlated to PCGEM1. Knockdown of SNAIL also suppressed proliferative and metastatic potentials in Hep3B and Huh7 cells.

Conclusions: LncRNA PCGEM1 triggers liver cancer cells to proliferate, migrate and invade by upregulating SNAIL.

Key words: liver cancer, PCGEM1, SNAIL, proliferation, metastasis

Introduction

Liver cancer is prevalent throughout the world, and its mortality ranks second in malignant tumors. It is estimated that there are about 700,000 new cases of liver cancer each year in the world, and half of them are in China. The incidence and mortality of liver cancer in our country ranks third and second in global liver cancer cases, respectively [1,2]. It seriously affects health and lives of Chinese people. The occurrence and development of liver cancer are complex physiological processes, which are regulated by multiple factors and genes [3]. Lack of diagnostic and therapeutic targets for

early stage liver cancer results in the poor prognosis [4,5].

The human genome contains about 3 billion base pairs. Only fewer than 2% of them are able to encode proteins, and the vast majority are non-coding sequences [6]. Long non-coding RNAs (lncRNAs) are non-coding RNAs exceed 200 nucleotides long, which are functional regulators involved in life activities. LncRNA HULC promotes adipogenesis and proliferation, and inhibits apoptosis of liver cancer cells. Besides, it enhances metastatic risk through strengthening EMT and downregulating

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the anti-cancer gene CDKN2C [7,8]. It is indicated that HULC can be used as a potential diagnostic biomarker for liver cancer. LncRNA PTTG3P is up-regulated in liver cancer cases, and it is positively correlated with the poor prognosis. PTTG3P drives liver cancer cells to proliferate, migrate and invade, while inhibits apoptosis through PTTG1-induced activation of the PI3K/AKT signaling [9]. Through literature review, lncRNAs are extensively involved in the physiological regulation of liver cancer.

LncRNA PCGEM1 was initially found to be overexpressed in human prostate cancer cells LNCaP. PCGEM1 is involved in mediating invasive and proliferative potentials of prostate cancer [10]. PCGEM1 is detected to be highly expressed in colorectal carcinoma specimens than that of paraneoplastic ones [11]. In cancer tissues and serum samples of pancreatic cancer, PCGEM1 level increases, and its level is markedly enhanced during the differentiation of pancreatic cancer cells. Knockdown of PCGEM1 not only weakens proliferative ability of pancreatic cancer cells, but also downregulates proliferation-associated genes [12]. Our research group previously found that PCGEM1 is upregulated in liver cancer specimens. This study aims to explore the role of PCGEM1 in regulating the progression of liver cancer and the molecular mechanism.

Methods

Clinical specimens

Liver cancer (n=50) and adjacent non-tumoral tissues (n=50) were collected from liver cancer patients admitted in our hospital. Clinical specimens were pathologically confirmed, immediately frozen in liquid nitrogen and preserved at -80°C. Each patient was followed up after discharge. This study was approved by the research Ethics Committee of Hunan Provincial People's Hospital and complied with the Helsinki Declaration. Informed consent was obtained from patients.

Cell culture

Liver cancer cell lines (Hep3B, MHCC97-L, HC-CLM3, Huh7, Bel7402) and liver cell line (LO2) were provided by the Institute of Hematology, Chinese Academy of traditional Chinese Medicine (Beijing, China). Cells were cultivated in Roswell Park Memorial Institute 1640 (RPMI 1640) (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (HyClone, South Logan, UT, USA), 100 U/mL penicillin and 100 µg/mL streptomycin at 5%CO₂, 37°C.

Transfection

PCGEM1 siRNA, SNAI1 siRNA and negative control were provided by GenePharma (Shanghai, China). Briefly, cells were implanted in a 6-well plate with 5×10⁵ cells/well. After cell density reached 80%, transfection

was conducted using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA). Transfected cells were used for testing transfection efficacy at 24-48 h.

Quantitative real-time polymerase chain reaction (qRT-PCR)

Cells or tissues were lysed using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) for isolating RNAs. Qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using PrimeScript RT kit (Invitrogen, Carlsbad, CA, USA), followed by qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Tokyo, Japan) at 92°C for 10 min, and 40 cycles at 92°C for 10 s and 60°C for 1 min. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was served as the internal reference. Each sample was performed in triplicate, and relative level was calculated by 2^{-ΔΔCt}. Primer sequences were as follows. PCGEM1 (F: 5'-CTGTGTCTGCAACTTCCTCTAA-3', R: 5'-TCCCAGTGCATCTCGTAGTA-3'); SNAI1 (F: 5'-CCTTCGTCCTTCTCCTTACTT-3', R: 5'-GGCACTGTACTTCTTGACATC-3'); GAPDH (F: 5'-GGAATCCACTGGCGTCTTCA-3', R: 5'-GGTTCACGCCATCACAAAC-3').

Cell counting kit-8 (CCK-8) assay

Cells were inoculated in a 96-well plate with 2×10⁵ cells/well. At 0, 24, 48 and 72 h, optical density at 450 nm of each sample was recorded using the CCK-8 kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Transwell

Transwell chambers (8 µm; Millipore, Billerica, MA, USA) were inserted in each well of a 24-well plate, where 1×10⁴ cells were applied in the upper layer of the chamber, and 600 µL of medium containing 10% FBS was applied in the bottom. After cell culture for 48 h, migratory cells to the bottom were captured following fixation and staining, which were counted in 5 randomly selected fields per sample (magnification 200×). Invasion assay was similarly conducted using Transwell chambers pre-coated with 200 mg/mL Matrigel.

5-Ethynyl-2'- deoxyuridine (EdU) assay

Cells were pre-inoculated in a 12-well plate with 5×10⁴ cells/well. They were incubated in 4% methanol for 30 min, followed by 10-min permeabilization in 0.5% TritonX-100 (Solarbio, Beijing, China), and 30-min reaction in 400 µL of 1×ApollorR. Afterwards, cells were dyed in 4',6-diamidino-2-phenylindole (DAPI) for another 30 min in the dark. Positive EdU-stained cells were calculated.

Statistical analysis

Data processing was conducted using Statistical Product and Service Solutions (SPSS) 17.0 (SPSS Inc., Chicago, IL, USA). Figures were edited using Graph-Pad Prism (Version X; La Jolla, CA, USA). Two-paired independent t-test was performed for comparing differences between groups. Kaplan-Meier method was introduced for survival analysis. Correlation between PCGEM1 and SNAI1 levels in liver cancer tissues was assessed by Pearson correlation test. A significant difference was set at p<0.05.

Results

Clinical significance of PCGEM1 in liver cancer

QRT-PCR was conducted to detect differential level of PCGEM1 in clinical specimens of liver cancer and normal ones, and PCGEM1 was highly

expressed in the former (Figure 1A). Subsequently, differential level of PCGEM1 in liver cancer tissues classified by tumor grading, TNM staging and lymphatic metastasis was examined. It is shown that PCGEM1 level remained higher in liver cancer cases with grading III, T3-T4 or accompanied

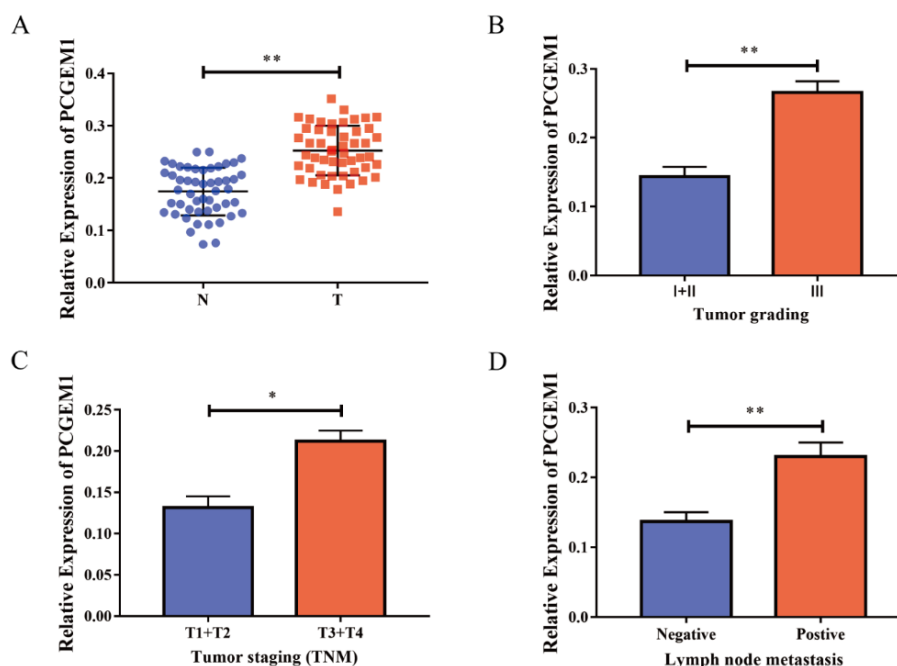


Figure 1. Clinical significance of PCGEM1 in liver cancer. (A) PCGEM1 was upregulated in liver cancer tissues than that of normal ones; (B) PCGEM1 level was linked to tumor grading of liver cancer; (C) PCGEM1 level was linked to tumor staging of liver cancer; (D) PCGEM1 level was linked to lymphatic metastasis of liver cancer. * $p < 0.05$, ** $p < 0.01$.

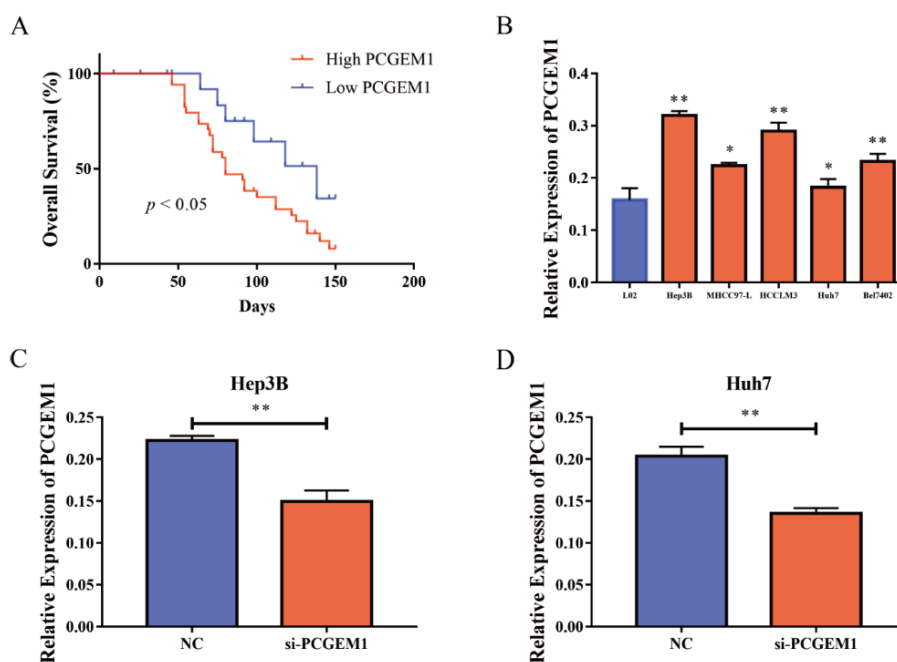


Figure 2. Upregulation of PCGEM1 in liver cancer. (A) Prognostic value of PCGEM1 in liver cancer; (B) PCGEM1 was upregulated in liver cancer cells; (C, D) Transfection of si-PCGEM1 significantly downregulated PCGEM1 in Hep3B and Huh7 cells. * $p < 0.05$, ** $p < 0.01$.

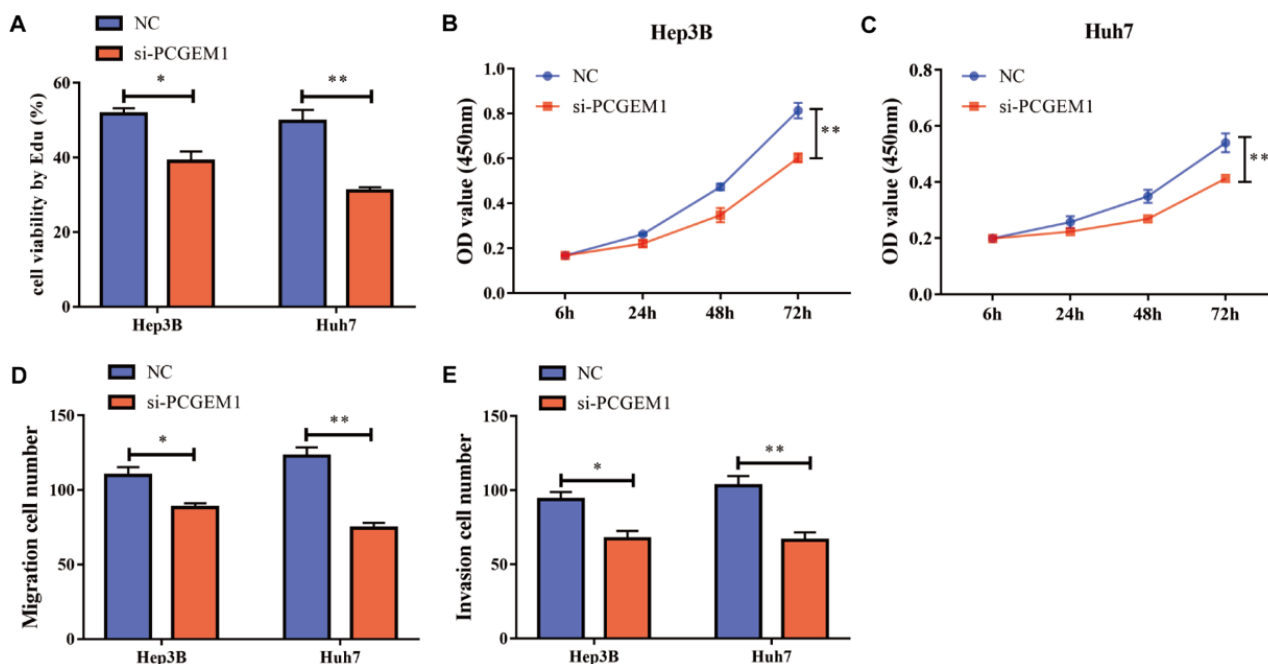


Figure 3. Knockdown of PCGEM1 reduced proliferative, migratory and invasive rates in liver cancer. **(A)** Transfection of si-PCGEM1 significantly decreased EdU-positive rate in Hep3B and Huh7 cells; **(B, C)** Transfection of si-PCGEM1 significantly decreased viability in Hep3B and Huh7 cells; **(D)** Transfection of si-PCGEM1 significantly suppressed migration in Hep3B and Huh7 cells; **(E)** Transfection of si-PCGEM1 significantly suppressed invasion in Hep3B and Huh7 cells. * $p < 0.05$, ** $p < 0.01$.

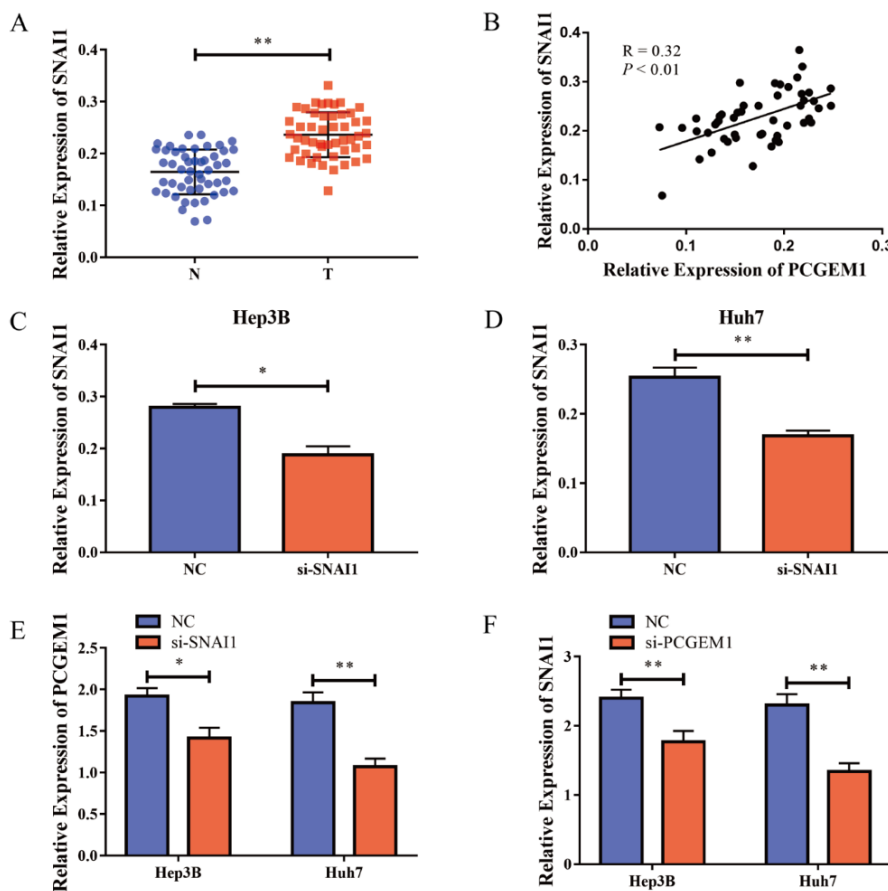


Figure 4. PCGEM1 positively regulated SNAI1 expression. **(A)** SNAI1 was upregulated in liver cancer tissues than that of normal ones; **(B)** SNAI1 was positively correlated to PCGEM1 in liver cancer tissues; **(C, D)** Transfection of si-SNAI1 significantly downregulated SNAI1 in Hep3B and Huh7 cells; **(E)** Transfection of si-SNAI1 significantly downregulated PCGEM1 in Hep3B and Huh7 cells; **(F)** Transfection of si-PCGEM1 significantly downregulated SNAI1 in Hep3B and Huh7 cells. * $p < 0.05$, ** $p < 0.01$.

lymphatic metastasis than those of controls (Figure 1B-D). Therefore, PCGEM1 was suggested to have a close relation to tumor grading, staging and lymphatic metastasis of liver cancer as a potential biomarker.

Upregulation of PCGEM1 in liver cancer

Through analyzing follow-up data of recruited liver cancer patients, it is found that upregulation of PCGEM1 predicted the worsening of overall survival in liver cancer (Figure 2A). Compared with normal liver cells, PCGEM1 was upregulated in liver cancer cells (Figure 2B). Hep3B and Huh7 cells were selected to generate *in vitro* knockdown model of PCGEM1 by transfection of si-PCGEM1 (Figure 2C, 2D).

Knockdown of PCGEM1 reduced proliferative, migratory and invasive rates in liver cancer

A series of functional experiments were carried out to explore the role of PCGEM1 in regulating liver cancer cells *in vitro*. Firstly, EdU assay showed a decline in EdU-positive rate after transfection of si-PCGEM1 in Hep3B and Huh7 cells (Figure 3A). Cell viability was also reduced in liver cancer cells with PCGEM1 knockdown (Figure 3B, 3C). Moreover, Transwell assay obtained the results that knockdown of PCGEM1 reduced migratory (Figure 3D) and invasive rates in liver cancer cells (Figure 3E).

PCGEM1 positively regulated SNAIL expression

SNAIL was detected to be highly expressed in liver cancer tissues than that of normal ones (Figure 4A). Its level was positively correlated to that of PCGEM1 in liver cancer (Figure 4B). Subsequently, we examined transfection efficacy of si-SNAIL in Hep3B and Huh7 cells by qRT-PCR (Figure 4C, 4D). As expected, knockdown of SNAIL downregulated PCGEM1 in liver cancer cells, and knockdown of PCGEM1 downregulated SNAIL (Figure 4E, 4F).

Knockdown of SNAIL reduced proliferative, migratory and invasive rates in liver cancer

We thereafter explored the biological function of SNAIL in liver cancer cells. Both EdU and CCK-8 assay demonstrated that knockdown of SNAIL attenuated proliferative capacity in Hep3B and Huh7 cells (Figure 5A-C). Besides, migratory and invasive capacities in liver cancer cells were suppressed by transfection of si-SNAIL (Figure 5D-E).

Discussion

LncRNAs are becoming promising targets for tumor diagnosis, treatment and prognosis [13,14]. A growing number of lncRNAs have been identified in tumor profile, which are specifically expressed and provide novel ideas for developing diagnostic and therapeutic strategies [15,16]. Excessive proliferative

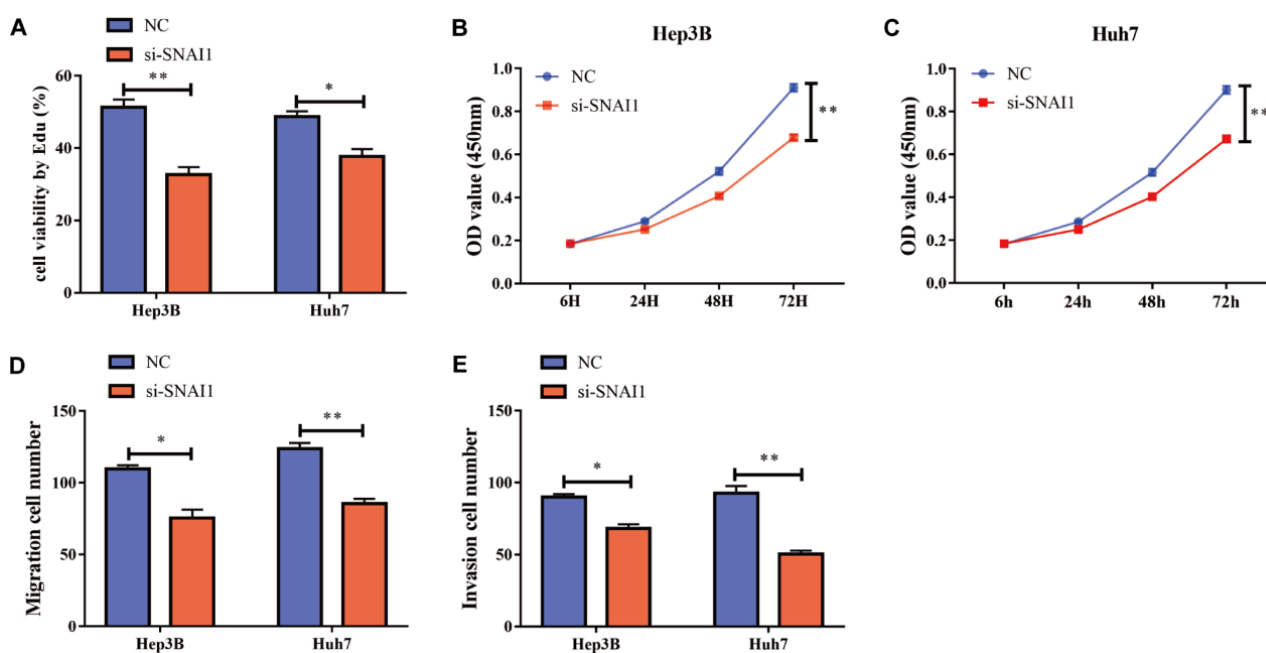


Figure 5. Knockdown of SNAIL reduced proliferative, migratory and invasive rates in liver cancer. (A) Transfection of si-SNAIL significantly decreased EdU-positive rate in Hep3B and Huh7 cells; (B, C) Transfection of si-SNAIL significantly decreased viability in Hep3B and Huh7 cells; (D) Transfection of si-SNAIL significantly suppressed migration in Hep3B and Huh7 cells; (E) Transfection of si-SNAIL significantly suppressed invasion in Hep3B and Huh7 cells. * $p < 0.05$, ** $p < 0.01$.

eration of normal cells leads to carcinogenesis, that is, uncontrolled cell cycle progression. Abnormal division and proliferation that are not controlled by the normal growth system trigger the malignant transformation of normal cells to tumor ones [17,18]. Experimental data have verified that lncRNAs are capable of regulating tumor development through mediating cell cycle progression. In the present study, lncRNA PCGEM1 was upregulated in clinical specimens of liver cancer and cell lines. High level of PCGEM1 was detected in liver cancer cases with advanced staging, grading or lymphatic metastasis, which predicted poor prognosis of liver cancer patients. *In vitro* experiments showed that knockdown of PCGEM1 reduced proliferative, migratory and invasive rates in Hep3B and Huh7 cells. It is concluded that PCGEM1 was an oncogene involved in liver cancer.

Drosophila embryonic protein SNAI1 is a zinc finger transcription factor gene that suppresses ectoderm and endoderm development. The nuclear protein encoded by SNAI1 is similar in structure to the Drosophila SNAI1 protein. SNAI1 is also considered to be the key gene to the formation of mesoderm during embryonic development [19]. SNAI1 participates in the negative expressions of EMT-associated transcription factors, which further inhibits tumor metastasis [20]. It is reported

that the positive expression of SNAI1 is detected in epithelial and endothelial cells of aggressive breast cancer, while it is not expressed in normal mammary glands [21]. SNAI1 is abundantly expressed in cadherin-negative clones of the epithelium of highly invasive oral squamous cell carcinoma (OSCC). However, it can be hardly detected in cadherin-positive clones of the epithelium of weakly invasive OSCC [22]. As a vital regulator of E-cadherin, SNAI1 is detected in colorectal adenoma, which is responsible for the downregulation of E-cadherin [23]. Our findings showed that SNAI1 was upregulated in liver cancer specimens, and displayed a positive correlation to PCGEM1. Knockdown of SNAI1 in Hep3B and Huh7 cells similarly weakened proliferative, migratory and invasive abilities. To sum up, PCGEM1 exerted its carcinogenic role in liver cancer by positively regulating SNAI1.

Conclusions

LncRNA PCGEM1 triggers liver cancer cells to proliferate, migrate and invade by upregulating SNAI1.

Conflict of interests

The authors declare no conflict of interests.

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