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Circular RNA hsa_circ_0010882 promotes the progression of gastric cancer *via* regulation of the PI3K/Akt/mTOR signaling pathway

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Abstract. – OBJECTIVE: Accumulating studies have reported that circular RNAs (circRNAs) can act as novel prognostic biomarkers in multiple malignant tumors. Here, we conducted a study to investigate the potential function and molecular mechanism of action of hsa_circ_0010882 in gastric cancer (GC).

PATIENTS AND METHODS: The expression of hsa_circ_0010882 in the plasma of GC patients and in GC cell lines was verified by qRT-PCR. Its association with overall survival of GC patients was then analyzed by statistical analysis. Gain-of-function and loss-of-function assays were used to investigate the physiological function of hsa_circ_0010882 in GC cells *in vitro* in the context of proliferation, apoptosis, migration, and invasion. Moreover, the molecular mechanism of action of hsa_circ_0010882 was predicted using online databases and a literature review. A Western blot assay was used to detect the levels of proteins in the PI3K/Akt/mTOR signaling pathway.

RESULTS: We found that hsa_circ_0010882 expression was significantly upregulated in the plasma of GC patients and GC cell lines. Increased expression of hsa_circ_0010882 was significantly correlated with tumor size and histological grade. In addition, GC patients with higher expression of hsa_circ_0010882 had significantly lower overall survival than patients with lower expression of hsa_circ_0010882. Multivariate analysis showed that hsa_circ_0010882 expression could be an independent prognostic factor for overall survival. The proliferation, migration, and invasiveness of GC cell lines were inhibited following hsa_circ_0010882 knockdown, while GC cellular apoptosis increased. Further, overexpression of hsa_circ_0010882 leads to increased proliferation, migration, and invasiveness of GC cell lines. While apoptosis was higher in the GC cell line group with low expressing hsa_circ_0010882 than the control group, no significant difference in apoptosis was detected between the hsa_circ_0010882 overexpressing and the control group. Finally, a mechanistic analysis demonstrated that the hsa_circ_0010882 was positively associated with PI3K/Akt/mTOR signaling pathway.

CONCLUSIONS: Hsa_circ_0010882, as an oncogenic molecule, is highly expressed in the plasma of patients with GC and is associated with poor prognosis. It plays an important role in proliferation, migration, and invasive genotypes of GC cell lines via regulation of the PI3K/Akt/mTOR signaling pathway. Additionally, it might be a potential prognostic biomarker for GC patients.

Key Words:
Circular RNA, Hsa_circ_0010882, Gastric cancer, PI3K/Akt/mTOR pathway, Prognosis.

Introduction

Gastric cancer (GC) is one of the most common malignancies worldwide, it is estimated that one million individuals have been diagnosed with GC and 700 thousand deaths have occurred to date[1,2]. In China, the incidence and mortality of GC are 2-3 times higher than the average world rate[3]. Furthermore, the diagnostic rate of early stage GC is less than 10%, and the five-year survival time of GC is less than 30%[2,4]. Consequently, although the diagnosis and prognosis of GC patients have significantly improved, the diagnosis of early stage GC remains a challenge. Unfortunately, the mechanism of tumorigenesis of GC remains unclear and further investigation is required to unveil the potential physiopathological processes.

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CircRNA, as a type of non-coding RNAs, is widely distributed in interior milieu with high abundance and stability[5-7]. Cumulative evidence[8-13] demonstrates that circRNAs are involved in the pathological process of many diseases such as tumors, atherosclerosis, and neurological disorders. Moreover, several studies[14-16] found that circRNAs can act as critical regulators of tumor biology including proliferation, invasion, and metastasis. Recently, a circle RNA molecule named hsa_circ_0010882 from the host gene RPL11 (ribosomal protein L11)[17,18], was shown to be stably overexpressed in the solid tumor, as well as circulating blood of hepatocellular carcinoma (HCC) patients[19]. They also concluded that hsa_circ_0010882 might be regarded as a potential diagnostic biomarker in HCC patients. However, its potential mechanism of tumorigenesis should be explored further.

In our study, we aimed to investigate whether hsa_circ_0010882 could also be considered a prognostic biomarker of GC and whether it acts as a regulator of GC development. Overall, our objective is to elucidate the role of hsa_circ_0010882 in GC by investigating the relationship between the levels of hsa_circ_0010882 circulating in peripheral blood and the patient's clinicopathological features and by exploring the potential tumorigenic mechanism of hsa_circ_0010882 in GC. Finally, we also assessed the prognostic value of hsa_circ_0010882.

Patients and Methods

Patients and Plasma Samples

Sixty-six patients were selected from a group of patients who underwent surgical resection with informed consent in the First Affiliated Hospital of Lanzhou University (Gansu, China). Plasma specimens were collected from each patient following the diagnosis of gastric cancer, which was validated by pathological biopsy. Our investigation was approved by the Ethics Committee of the First Affiliated Hospital of Lanzhou University. All patients were followed up for a mean observation period of 30 months.

Cell Line

Gastric cancer cell lines (HGC-27, MKN-45, SGC-7901, and BGC-823), as well as a normal gastric cell line (GES-1) were provided by the cell bank department of Xiangya School of Medicine, Central South University (Hunan, China). Cell lines were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) complete medium containing fetal bovine serum (FBS). Cell lines were kept at 95% humidity, 5% CO_2 and 37°C. Cells in exponential phase were collected and passaged.

Cell Transfection

MKN-45 and SGC-7901 cells were seeded into 6-well plates (3 wells per line) at a cell concentration of 5×10^5/ml. A lentivirus (Hanyin, Shanghai, China) was then used to transfect the negative control (NC), overexpression (OE), and knock-down (KD) groups. The same volume of phosphate-buffered saline (PBS) was added to the control group. Cells in each group were cultured and passaged and after 8 to 12 h fresh cell media was added. Puromycin was used to select infected cells. QRT-PCR was used to quantitatively detect the levels of hsa_circ_0010882.

Real Time Quantitative PCR

RNA was isolated using TRIzol (Invitrogen, Carlsbad, CA, USA), followed by reverse transcription into cDNA according to manufacturer's (TaKaRa, Otsu, Shiga, Japan) protocol, and under the following conditions: 37°C for 15 min, then 85°C for 5 s. QRT-PCR was performed using SYBR TB green Taq (TaKaRa, Otsu, Shiga, Japan), in triplicate, using a Roche Light Cycler 480 and with β-Actin as a reference gene. The relative expression of the genes was calculated using the $2^{-\Delta\Delta Ct}$ method. Primer sequences were as follows: hsa_circ_0010882: 5'-AATGCTGAAAACTGCTGAGACACA-3' (forward) and 5'-TGACAGAAAACGAGTGCTTTGG-3' (reverse); and β-Actin: 5'-AGAGCCTC-GCCTTTGCCGATCC-3' (forward) and 5'-CTGGGCCTCGTCGCCCACATA-3' (reverse).

Cell Counting Kit-8 (CCK8) Assay

After transfection, cells were seeded at 3×10^3 per well in 96-well plates and incubated for 0, 24, 48, and 72 h. After incubation, 10 μL of WST-8 (Dojindo Laboratories, Kumamoto, Japan) was added to each well. A plate reader (Molecular Devices LLC, San Jose, CA, USA) was used to measure the absorbance at 450 nm after a 1-h incubation at 37°C.

Flow Cytometry Cell Cycle Analysis

The apoptosis of SGC-7901 and MKN-45 cells after treatment with hsa_circ_0010882 or control shRNAs were detected by an Attune NxT flow cytometer (Thermo Fisher Scientific,

Pudong, Shanghai, China), using a MultiSciences Annexin V Apoptosis assay kit (Hangzhou, Zhejiang, China). In brief, the treated cells were harvested, washed with ice-cold PBS, and incubated with annexin V-FITC and propidium iodide (PI) for 20 min, after being analyzed by the flow cytometer.

Transwell Invasion and Migration Assay

Transwell system and Matrigel (BD Biosciences, Franklin Lakes, NJ, USA) were conducted to assess the invasiveness and migration of cancer cells. Transwell chambers were added to a 24-well-plate containing 0.1% bovine serum albumin-Roswell Park Memorial Institute-1640 (BSA-RPMI-1640), followed by the addition of 100 μL cell suspension (1×10^5cells per well) into the chambers. Chambers were taken out after 12 h, fixed in formalin, and stained. Subsequently, cells on the surface of the permeable membrane were removed, and samples were processed in neutral balsam. The number of cells infiltrating from the chambers was counted at five different randomly selected fields. Each group was counted three times. To evaluate the invasiveness of cancer cells, 5 μg Matrigel was added to the surface of the membrane, forming a basement membrane, and subsequently, the transwell assay was performed as described above.

Western Blotting

Proteins were extracted using Radio Immunoprecipitation Assay (RIPA) lysis buffer. Total protein concentration was then measured by bicinchoninic acid (BCA) assay (Boster, Wuhan, China). Lysates with 30 μg of protein per well were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The membrane was blocked with 5% nonfat milk in Tris-Buffered Saline and Tween-20 (TBST) for 1 h. Membranes were then incubated with primary antibodies, followed by secondary antibodies. Membranes were washed three times before protein detection using enhanced chemiluminescence (ECL) substrate on a Tanon 5200 system. Primary antibodies against β-Actin, PI3K, Akt, and mTOR were purchased from Wuhan Boster Biotechnology Co., Ltd. (Wuhan, Hubei, China). Primary antibodies against p-PI3K (phosphorylated-PI3K), p-AKT (phosphorylated-PI3K), and p-mTOR (phosphorylated-mTOR) were obtained from Cell Signaling Technology Co., Ltd. (Beverly, MA, USA).

Statistical Analysis

The statistical analysis was carried out using STATA12.0. An independent two-tailed *t*-test was used for analysis of continuous data. Categorical data were analyzed using a two-side Chi-square test. Overall survival was estimated using the Kaplan-Meier method, and univariate analysis was conducted using a log-rank test. The Cox proportional hazards model was used for multivariate analysis. Values of $p<0.05$ were considered statistically significant.

Results

Hsa_circ_0010882 Was Up-Regulated in GC Patients

Using qRT-PCR, we aimed to measure the levels of hsa_circ_0010882 in the circulating blood of GC patients. Thus, we matched 66 GC patients with 66 healthy controls and used β-Actin as a reference gene. As shown in Figure 1A, the expression levels of hsa_circ_0010882 were significantly increased in GC patients, compared to healthy people ($p<0.05$). In addition, we compared the expression levels of hsa_circ_0010882 between the human gastric epithelial cell GES-1 and the gastric cancer cell lines (HGC-27, MKN-45, SGC-7901, and BGC-823). Results from qRT-PCR showed that the levels of hsa_circ_0010882 in SGC-7901 and MKN-45 cell lines were significantly higher compared to GES-1 cells (Figure 1B). In summary, the expression of hsa_circ_0010882 was up-regulated in both the circulating blood of GC patients and in GC cell lines.

Regarding clinical analysis, a total of 49 patients were investigated, while 17 patients failed to follow-up. Using the data from the 49 patients, we used the median level of hsa_circ_0010882 expression (1.67) to divide the 49 patients into either a high-expression group or a low-expression group. A Chi-square test was used to explore the clinical significance of hsa_circ_0010882 expression. As shown in Table I, we found that high hsa_circ_0010882 expression was significantly correlated with an advanced TNM stage ($p=0.044$) and tumor size ($p=0.005$), suggesting that hsa_circ_0010882 may influence the clinical prognosis of GC patients. Moreover, survival analysis shows that the overall survival of patients in the high expression group is significantly lower than that in the low expression group (Figure 1C). Univariate and multivariate analyses showed

Table I. Correlation between Hsa_circ_0010882 expression with clinicopathological characteristics in GC.

Variable	No.	Hsa_circ_0010882 expression		*p*-value
		Low	High	
Age (years)				0.804
<60	37	17	20	
≥60	29	16	13	
Gender				0.523
Male	54	28	26	
Female	12	7	5	
Tumor size (cm)				0.006
<4	27	19	8	
≥4	39	14	25	
TNM stage				0.044
I+II	26	17	9	
III+IV	40	16	24	

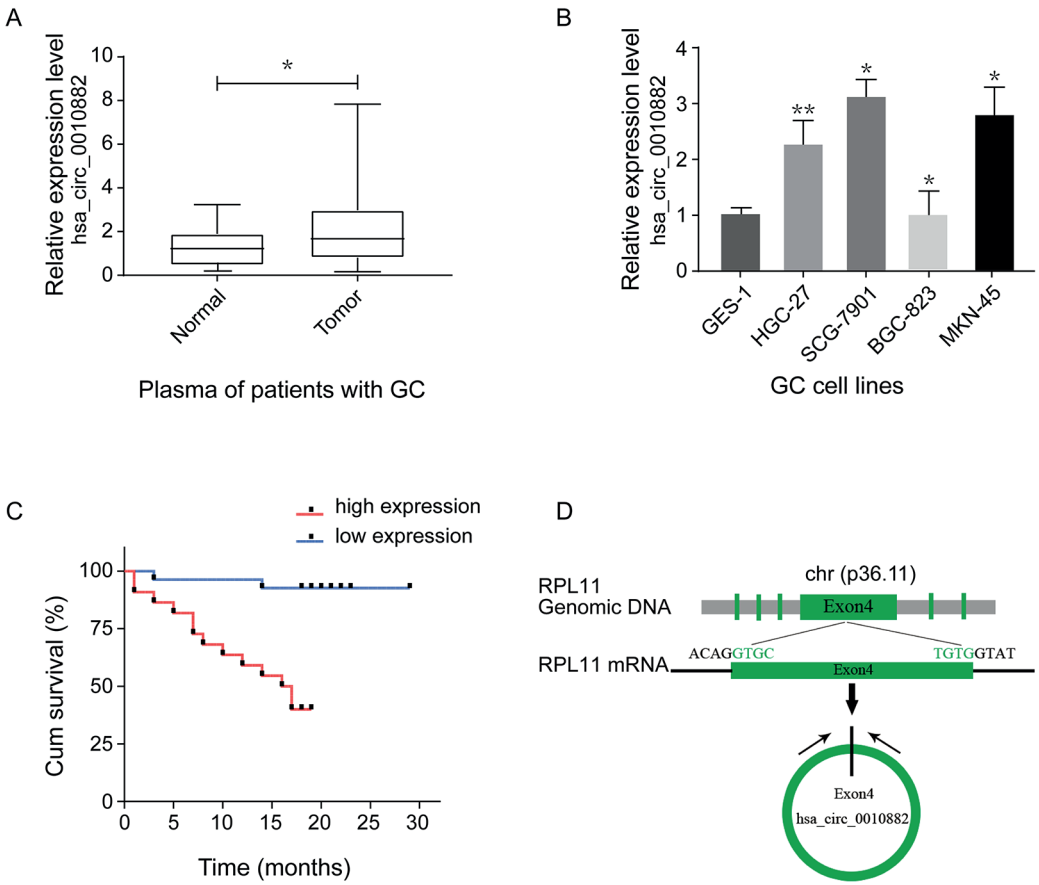

Figure 1. Hsa_circ_0010882 is overexpressed in the plasma of GC patients and is related to a poor prognosis for GC patients. **A**, Hsa_circ_0010882 expression is significantly increased in GC patients compared to healthy controls. **B**, Hsa_circ_0010882 expression is increased in GC cell lines compared to normal gastric epithelium cells (GES-1). **C**, Kaplan-Meier curve indicating that GC patients with high expression of hsa_circ_0010882 had a lower survival. **D**, Schematic illustrating the genomic region of RPL11 that validates back-splicing of circular exon 4. A divergent primer was designed to detect hsa_circ_0010882 (132 bp).

that the expression level of hsa_circ_0010882 is an independent predictor of prognosis (p=0.042) in patients with GC (Table II).

Hsa_circ_0010882 Promotes GC Cell Proliferation While Inhibiting Apoptosis

Since hsa_circ_0010882 is up-regulated in GC cell lines and in the blood of GC patients, we hypothesized that this circular molecule might play a role in the tumorigenesis and development of GC. To confirm this, we designed a viral vector of overexpressed hsa_circ_0010882 and used it for transfection into both SGC-7901 and MKN-45 cell lines. Moreover, hsa_circ_0010882 shRNAs were transfected to achieve low expression of hsa_circ_0010882 in GC cell lines.

We investigated the proliferation of transfected SGC-7901 and MKN-45 cells using CCK-8. Results show that, compared to the negative control group , the proliferative capacity of both cell lines were promoted in the hsa_circ_0010882 overexpression group (Figure 2A), while transfection of hsa_circ_0010882 shRNAs led to a significant decrease in proliferative rates of both GC cell lines at 24 h, 48 h, and 72 h (Figure 2B). Overall, results indicate that hsa_circ_0010882 expression is linked to GC cell viability.

We used flow cytometry to investigate the relationship between expression levels of hsa_circ_0010882 and apoptosis. Results showed that the difference in levels of apoptosis between the hsa_circ_0010882 overexpression group and the NC group was not statistically significant (Figure 2C). However, the apoptosis rate was significantly higher in the GC cell lines with low expression of hsa_circ_0010882, compared to the control group (Figure 2D).

Hsa_circ_0010882 Significantly Promotes Invasion and Migration of GC Cells

To explore the effect of hsa_circ_0010882 on GC cell invasion and migration, a transwell assay was carried out. Results showed that overexpression of hsa_circ_0010882 promoted both the invasiveness and migration abilities of SGC-7901 and MKN-45 cells. In contrast, following transfection of hsa_circ_0010882 shRNAs, the invasiveness and migration abilities of SGC-7901 and MKN-45 cells were inhibited (Figure 3A and B). These results indicate that hsa_circ_0010882 expression is positively correlated with the invasiveness and migration ability of GC cells.

Hsa_circ_0010882 Promotes GC Cells Progression Through PI3K/Akt/mTOR Signaling Pathway

Our next objective was to discover the molecular mechanism by which hsa_circ_0010882 regulates growth, apoptosis, and metastasis potential in GC cells. To that end, we used bioinformatics to investigate potential signaling pathways that could be influenced by hsa_circ_0010882. The PI3K/Akt/mTOR pathway was selected from the candidate list. As shown in Figure 4, the levels of p-PI3K, p-Akt, and p-mTOR expression were up-regulated following an increased hsa_circ_0010882 expression. In contrast, decreased expression of hsa_circ_0010882, led to a down-regulation in the levels of p-PI3K, p-Akt, and p-mTOR expression. In contrast, the protein lev-

Table II. Univariate and multivariate analysis of overall survival in GC patients.

	Univariate analysis			Multivariate analysis		
Variable	**HR**	**95% CI**	***p*-value**	**HR**	**95% CI**	***p*-value**
Age (years) ≥ 60 *vs.* < 60	1.012	0.367-2.793	0.981	-	-	-
Gender Male *vs.* Female	0.48	0.135-1.706	0.257	-	-	-
Chemotherapy Yes vs. No	0.526	0.148-1.867	0.321	-	-	-
TNM stage I +II vs. III+IV	4.572	1.449-14.423	0.01	1.346	0.390-4.646	0.638
Tumor size (cm) <4 *vs.* ≥4	12.356	2.769-55.140	0.001	5.67	1.089-29.506	0.039
Hsa_circ_0010882 high *vs.* low	11.055	2.482- 49.233	0.002	5.132	1.064-24.750	0.042

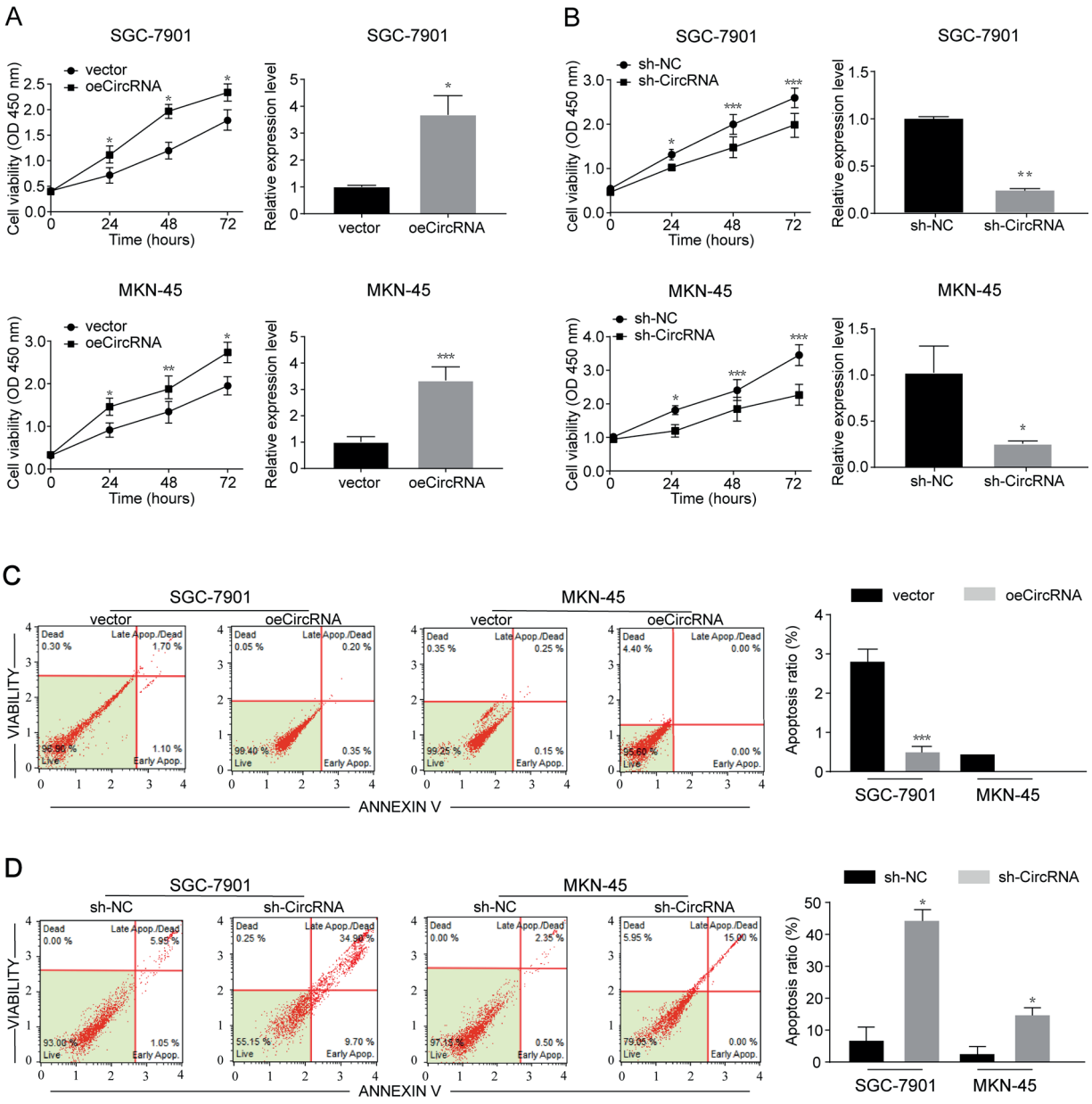

Figure 2. Hsa_circ_0010882 promotes GC cell proliferation and inhibits GC cell apoptosis. **A-B**, Cell counting assay 8 showed that, compared to the NC group, overexpression of hsa_circ_0010882 promotes proliferative capacity, while hsa_circ_0010882 shRNAs transfection leads to reduced cell proliferation at 24 h, 48 h, and 72 h. Transfection efficiency was detected by qRT-PCR. **C-D**, Flow cytometry analysis was used to examine the apoptotic rates of SGC-7901 and MKN-45 cells after transfection. All experiments were performed in triplicate. *p<0.05, **p<0.01, ***p<0.001.

els of PI3K, Akt, and mTOR remained unchanged regardless of the levels of hsa_circ_0010882. Overall, results indicate that hsa_circ_0010882 might influence the behavior of GC cells via activation of the PI3K/Akt/mTOR signaling pathway.

Discussion

With the development of RNA deep sequencing technology and bioinformatics analysis, it has been reported that some circular RNAs are

abundantly expressed in cells and exist stably due to their cyclic structure[7,20-22]. In recent years, the role of circular RNA in tumors has become a hot topic. In this study, we explored the role of hsa_circ_0010882 in gastric cancer cells and whether this molecule could be an independent prognostic biomarker for GC patients. Weng et al[19] showed hsa_circ_0010882 to be up-regulated in both the blood and cancer tissue of HCC patients and to have a role in HCC prognosis. In addition, data from NCBI and CircBase show hsa_circ_0010882 to have a length of 132 bp and to originate from the fourth exon of RPL11, which is located in chr1 (p36.11; Figure 1D).

To investigate the role of hsa_circ_0010882 in GC, which is also a digestive malignancy, we examined its expression in the circulating blood of GC patients and further investigated its impact on the behavior of GC cells. Results from qRT-PCR indicated that hsa_circ_0010882 is up-regulated in the circulating blood of GC patients. In addition, a Chi-square analysis showed hsa_circ_0010882 expression to be positively correlated with advanced TNM stage and bigger tumor size, suggesting it plays an oncogenic role in the clinical progression of GC. Furthermore, Kaplan-Meier assays revealed that high hsa_circ_0010882 expression was closely related to shorter overall survival, indicating a negative influence in long-term survival. Of note, a Cox regression model showed that hsa_circ_0010882 might be an independent poor prognostic factor for overall survival of GC patients. Overall, our current data suggest that high hsa_circ_0010882 could be a prognostic predictor for GC patients.

Increasing studies have shown that cirRNAs play a key regulatory role in tumor progression. Several circRNAs have been identified as functional regulators of GC as either tumor suppressor genes or oncogenes. Particularly, Shen et al[11] reported that CircRNA_001569 is overexpressed in tissues and cells of GC patients and promotes cell proliferation through absorption of miR-145 in gastric cancer. Rong et al[23] showed CircPSMC3 expression to be down-regulated in both tissues and cell lines of GC patients. The study also showed that CircPSMC3 could inhibit the tumorigenesis of gastric cancer cells *in vivo* and *vitro*

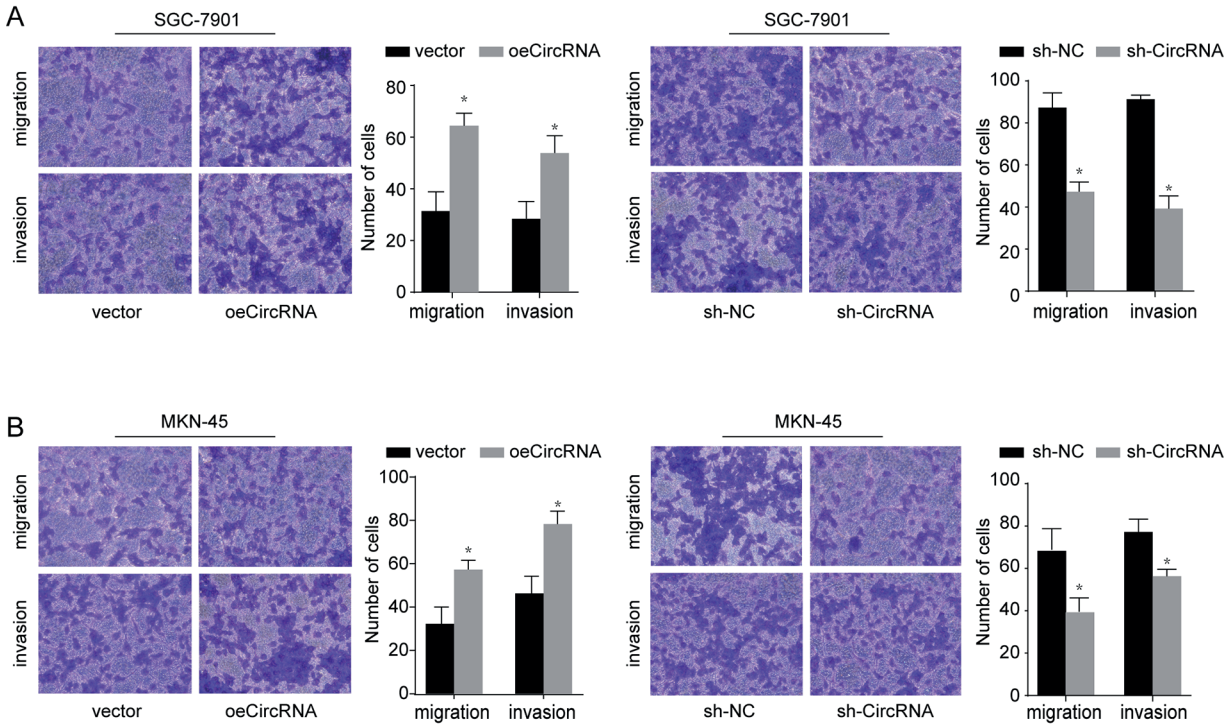

Figure 3. Hsa_circ_0010882 promotes GC cell migration and invasion. **A**, Overexpression of hsa_circ_0010882 leads to increased invasion and migration in SGC-7901 cells. In contrast, after transfection of hsa_circ_0010882 shRNAs, the numbers of invasive and migratory cells were decreased (magnification: 40×). **B**, Transwell invasion and migration assays were used to test the invasion and migration abilities of MKN-45 cells after transfection (magnification: 40×). All experiments were performed in triplicate. **p*<0.05, ***p*<0.01, ****p*<0.001.

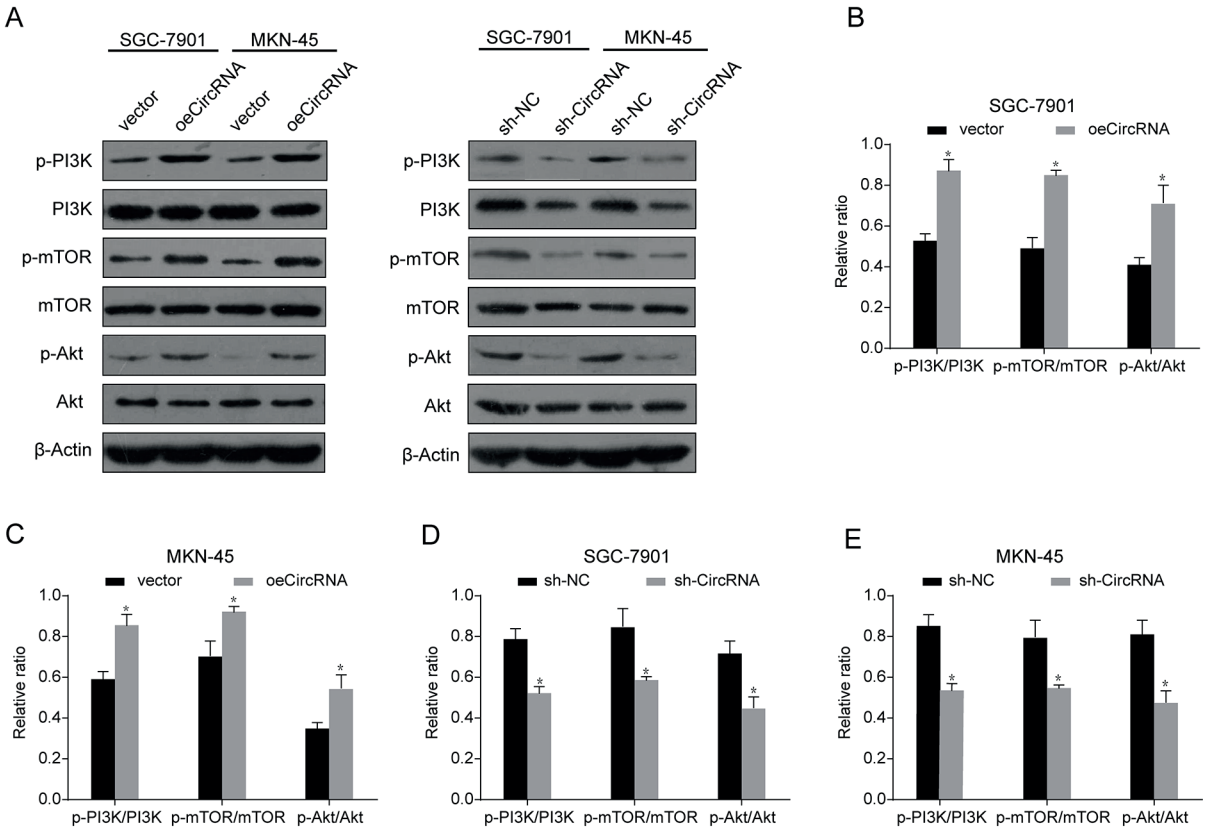

Figure 4. PI3K/Akt/mTOR signaling pathway activity in SGC-7901 and MKN-45 cells is to hsa_circ_0010882 expression. **A**, Western blot shows that the protein levels of p-PI3K, p-Akt, and p-mTOR changed with the expression of hsa_circ_0010882, while total PI3K, Akt, mTOR protein expression had no change following transfection. **B-E**, Phospho-PI3K/total PI3K, phospho-Akt/total Akt, and phospho-mTOR/total mTOR are shown. All experiments were performed in triplicate. *p<0.05, **p<0.01, ***p<0.001.

by sponging of miR-296-5p. These findings have greatly improved our understanding of the progression of gastric cancer. However, the expression and potential function of hsa_circ_0010882 in GC patients has not been investigated. Results of this study show that expression of hsa_circ_0010882 influences the biological behavior of GC cells. Increased levels of hsa_circ_0010882 were associated with the promotion of growth, invasion, and migration of cancer cells while decreased levels led to the reverse effect. Regarding apoptosis, although there was no change in apoptosis when the expression of hsa_circ_0010882 was up-regulated, its down-regulation significantly promoted apoptosis. The above results indicate that hsa_circ_0010882 expression is an independent prognostic factor for GC patients and that it plays an important role in the tumorigenesis and progression of GC.

To further elucidate the molecular mechanism of hsa_circ_0010882's effects on GC, we used bioinformatics to investigate signaling pathways potentially influenced by hsa_circ_0010882. The PI3K/Akt/mTOR pathway, as a well-studied signaling pathway, was selected from the candidate list. We carried out qRT-PCR and Western blot assays to evaluate the gene expression and protein levels of the PI3K/Akt/mTOR signaling pathway. As shown in Figure 4, p-PI3K, p-Akt, and p-mTOR protein levels were increased following hsa_circ_0010882 overexpression. In contrast, p-PI3K, p-Akt, and p-mTOR protein levels were decreased following hsa_circ_0010882 down-regulation. Overall, our current data shows that hsa_circ_0010882 acts as a tumor promoter in GC patients by promoting GC cell proliferation, metastasis, invasion via activation of the PI3K/Akt/mTOR pathway. Phosphatidylinositol3-kinase (PI3K) signaling pathway is the most common pathway in cell metabolism and proliferation processes. PI3K is a kinase which contains a p110 catalytic subunit and a p85 regulatory sub-

unit. Activated p85 phosphorylates Akt, which in turn activates mTOR. mTOR is a member of the phosphatidylinositol 3-kinase-related kinase family and according to Zarogoulidis et al[24] and Yu et al[25], it is related to cell growth, proliferation, and differentiation. It is involved in the regulation of various signaling pathways such as MAPK pathway. Inhibiting of mTOR induces cell apoptosis through cell cycle arrest.

In GC cell lines with down-regulated expression of hsa_circ_0010882, expression of the PI3K/Akt/mTOR pathway's phosphorylated proteins was decreased. In a functional assay, down-regulated hsa_circ_0010882 inhibited proliferation, migration, and invasion and promoted apoptosis of GC cells. On the contrary, overexpression of hsa_circ_0010882 in GC lines increased the levels of phosphorylated proteins of the PI3K/Akt/mTOR pathway and promoted the proliferation, migration, and invasion of GC cells. Therefore, it is possible that hsa_circ_0010882 influences the biological behavior of GC cells via activation of the PI3K/Akt/mTOR pathway.

Conclusions

In summary, this study demonstrates that hsa_circ_0010882 is highly expressed in the plasma of GC patients, which is associated with a lower overall survival. We also showed that hsa_circ_0010882 plays a role in regulating the PI3K/Akt/mTOR pathway and thus affecting the proliferation, migration, invasion, and apoptosis of GC cells. Finally, we revealed that hsa_circ_0010882 may be a potential therapeutic target for the treatment of GC, as well as a potential independent prognostic factor for GC patients.

Conflicts of interest

The authors declare no conflicts of interest.

Acknowledgments

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