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ORIGINAL ARTICLE

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# miR-297 acts as an oncogene by targeting GPC5 in lung adenocarcinoma

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## Abstract

**Objectives:** Emerging studies have demonstrated that microRNAs (miRNAs) play crucial roles in carcinogenesis of many developing human tumours. However, the functions and mechanisms of miR-297 in lung cancer have, up to now, been largely undefined.

**Materials and methods:** Here, miR-297 expression was measured in lung adenocarcinoma tissues and cell lines, using qRT-PCR. Lung adenocarcinoma cell line was treated with an miR-297 mimic. MTT and colony analysis were performed to detect cell proliferation and colony formation. The direct target gene of miR-297 was assessed by qRT-PCR, Western blotting and luciferase assays.

**Results:** We demonstrated that miR-297 expression was upregulated in lung adenocarcinomas compared to adjacent normal tissues. Expression of miR-297 was also upregulated in tested lung adenocarcinoma cell lines. Ectopic expression of miR-297 enhanced lung adenocarcinoma cell proliferation and colony formation. Furthermore, overexpression of miR-297 promoted cell migration and invasion. In addition, we identified Glypican-5 (GPC5) as a direct target gene of miR-297 in lung adenocarcinoma cells. Expression of GPC5 was downregulated in both lung adenocarcinoma tissues and cell lines. Moreover, expression of GPC5 was inversely associated with expression of miR-297 in lung adenocarcinoma tissues.

**Conclusions:** These results suggest that miR-297 acted as an oncogenic miRNA, partly by targeting GPC5, adenocarcinoma of the lung.

## 1 | INTRODUCTION

Lung cancer is one of the most common malignancies and the leading cause of cancer-related death worldwide.[1–4] Lung cancer contains four histological types: adenocarcinoma, large cell carcinoma, squamous cell carcinoma, small cell and bronchoalveolar carcinoma.[5–8] Among them, lung adenocarcinoma is the most common type with high metastasis and invasive abilities.[9–11] The 5-year overall survival rate of lung cancer is about 14%, dependent on histology, cancer stage, general health status and other factors.[12–14] However, no biomarker is available for early lung cancer diagnosis and prognosis. Therefore, it is important to find new biomarkers and targets forearly diagnosis of lung cancer.

MicroRNAs (miRNAs) are a new series of endogenous, conserved and small RNAs that inhibit protein translation through binding to the 3′-UTR (3′-untranslated region) of mRNAs (messenger RNAs).[15–18] miRNAs are involved in many processes such as cell proliferation, development, apoptosis, differentiation, inflammation, migration and stress response.[11,13,19–24] Increasing studies have demonstrated that miRNAs participate in the development of various tumours such as gastric cancer, hepatocellular carcinoma, gallbladder carcinoma, bladder cancer, cutaneous squamous cell carcinoma and colorectal cancer.[25–29] Therefore, investigations of miRNAs in lung cancer is important to find new diagnostic biomarker for lung cancer diagnosis.

Recently, increasing studies have proved that miR-297 plays an important role in the tumour development.[30–32] For example, Xu et al.[30] demonstrated that the expression of miR-297 was

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downregulated in MDR (multidrug resistance) colorectal carcinoma cell line HCT116/L-OHP compared with its parental cells. Overexpression of miR-297 suppressed MRP-2 (MDR-associated protein 2) expression and sensitized cells to anti-cancer drugs in MDR colorectal carcinoma cell. Kefas et al.[31] showed that miR-297 was a cytotoxic miRNA with minimal cytotoxicity to normal astrocytes in glioblastoma. However, the role of miR-297 was still unknown in lung cancer. In this study, we demonstrated that miR-297 expression was upregulated in lung adenocarcinoma tissues and cell lines. Ectopic expression of miR-297 promoted the lung adenocarcinoma cell proliferation, migration and invasion. We also identified Glypican-5 (GPC5) as a direct target gene of miR-297 in lung cancer.

## 2 | MATERIALS AND METHODS

### 2.1 | Clinical tissues and cell culture and transfection

Thirty-five lung adenocarcinoma tissues and their adjacent non-tumour tissues were collected from patients who underwent surgery in our hospital. This experiment was approved by the Ethical Committee of Hebei province Cangzhou Hospital of integrated traditional and Western Medicine and was in accordance with the Helsinki Declaration. Tissue was promptly frozen in the liquid nitrogen. No patients underwent chemotherapy or radiotherapy before surgery. Written informed consent from patient was obtained. Lung adenocarcinoma cell lines (H1299, SPC-A1, H23 and A549) and one bronchial epithelial cell line (16HBE) were collected from Cell Resource of CAMS (Chinese Academy of Medical Sciences) (Beijing, China) and cultured in the RPMI 1640 medium. miR-297 mimic and scramble oligoribonucleotides and GPC5 vector was synthesized from RiboBio (Guangzhou, China). Cell transfection was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

### 2.2 | Real-time quantitative PCR

Total RNA from samples or cells was extracted using TRIzol (Invitrogen) according to the manufacturer's protocol. The miR-297 and the GPC5 expression were measured by real-time quantitative PCR (RT-qPCR) according to the previous protocol. The following primers were used in this study: GAPDH Forward: 5′-AATGGGCAGCCGTTAGGAAA-3′; Reverse: 5′-TGAAGGGGTCATTGATGGCA-3′, GPC5 Forward: 5′-AGACCACCACAAGGAACAGTG-3′; Reverse: 5′-AGACTGGGCTTTGATTCCATT-3′. miR-297 expression was normalized to RNU6B. GPC5 expression was normalized to GAPDH.

### 2.3 | Western blot

The protein from cell or tissues was extracted using BCA kit. Total proteins were separated using 12% SDS-PAGE and transferred to the PVDF membranes (Millipore, Boston, MA, USA). The membrane was blocked with non-fat milk and then incubated with primary antibody. The primary antibody was used as following: GPC5, GAPDH (Sigma, St. Louis, MO, USA). Detection of GAPDH was used as the loading control.

### 2.4 | Scratch-wound assays and invasion analysis

Cell migration was measured by the scratch-wound analysis. The scratch was created by a sterile pipette tip. The wound was washed for three times to remove the debris cell. The cells were continuing cultured for another 24 or 48 hours. The wound width was measured by using microscope (Nikon, Tokyo, Japan).

### 2.5 | Cell proliferation and colony formation

Cell proliferation was detected using MMT following the manufacturer's protocol. Cells were cultured into 96-well plate after transfection for 24, 48 and 72 hours. The absorbance was determined at a 450 nm wavelength. For cell colony formation assay, cells were cultured in the 12-well plate after transfection for 2 weeks. The colonies were detected with crystal violet and counted (Sigma-Aldrich).

### 2.6 | Luciferase assays

Cells were cultured in the 24-well plates and were cotransfected with miR-297 mimic or scramble and Renilla vector, wild-type or mutant 3′ UTR of GPC5 luciferase reporter. The luciferase assay was performed as previously described. Luciferase activity was detected 24 hours after transfection.

### 2.7 | Statistical analysis

Results are shown as the means ± SD (standard deviation). ANOVA test was used to measure the difference between more than two groups and Student's *t*-test was used to detect the difference between two groups. Differences were shown significant when $P<.05$.

## 3 | RESULTS

### 3.1 | miR-297 expression was upregulated in lung adenocarcinoma tissues

As shown in Fig. 1a, miR-297 expression was upregulated in lung adenocarcinoma tissues compared with the adjacent normal tissues. Among them, miR-297 expression was higher in 26 lung adenocarcinoma tissues (74%, 26/35) than in normal tissues (Fig. 1b). In addition, miR-297 expression in the metastatic lymph node was higher in lung adenocarcinoma tissues than in the adjacent normal tissues (Fig. 1c).

### 3.2 | miR-297 enhanced lung adenocarcinoma cell proliferation and colony formation

miR-297 expression was upregulated in the lung adenocarcinoma cell lines (H1299, SPC-A1, H23 and A549) compared with one bronchial epithelial cell line (16HBE) (Fig. 2a). miR-297 expression was obviously enhanced in the A549 cells after transfection with

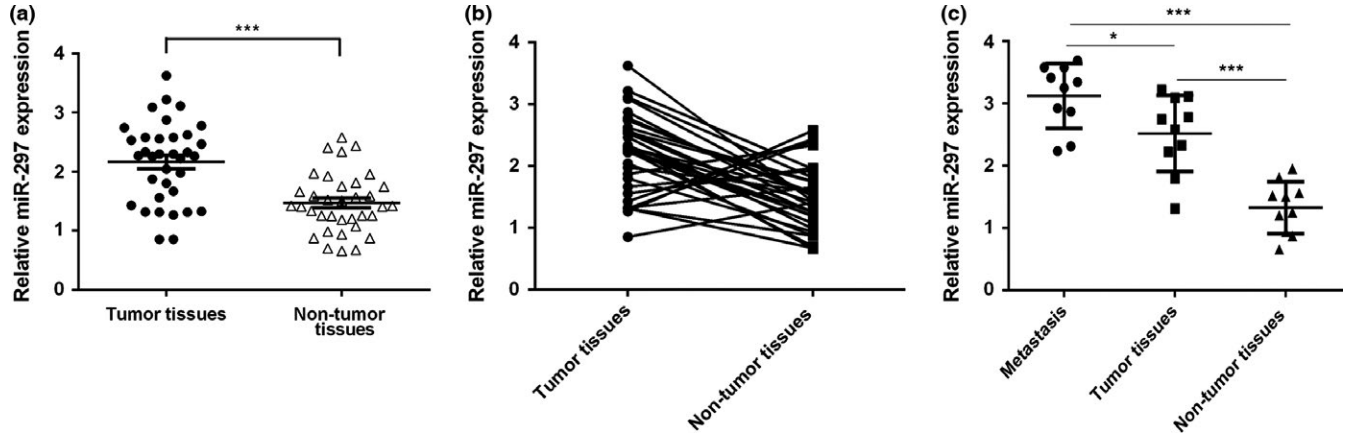


**FIGURE 1** miR-297 expression is upregulated in lung adenocarcinoma tissues. (a) The expression of miR-297 was measured by qRT-PCR. (b) Among them, miR-297 expression was higher in 26 lung adenocarcinoma tissues (74%, 26/35) than in normal tissues. (c) miR-297 expression in the metastatic lymph node was higher compared to lung adenocarcinoma tissues and adjacent normal tissues. *$P$<.05 and ***$P$<.001

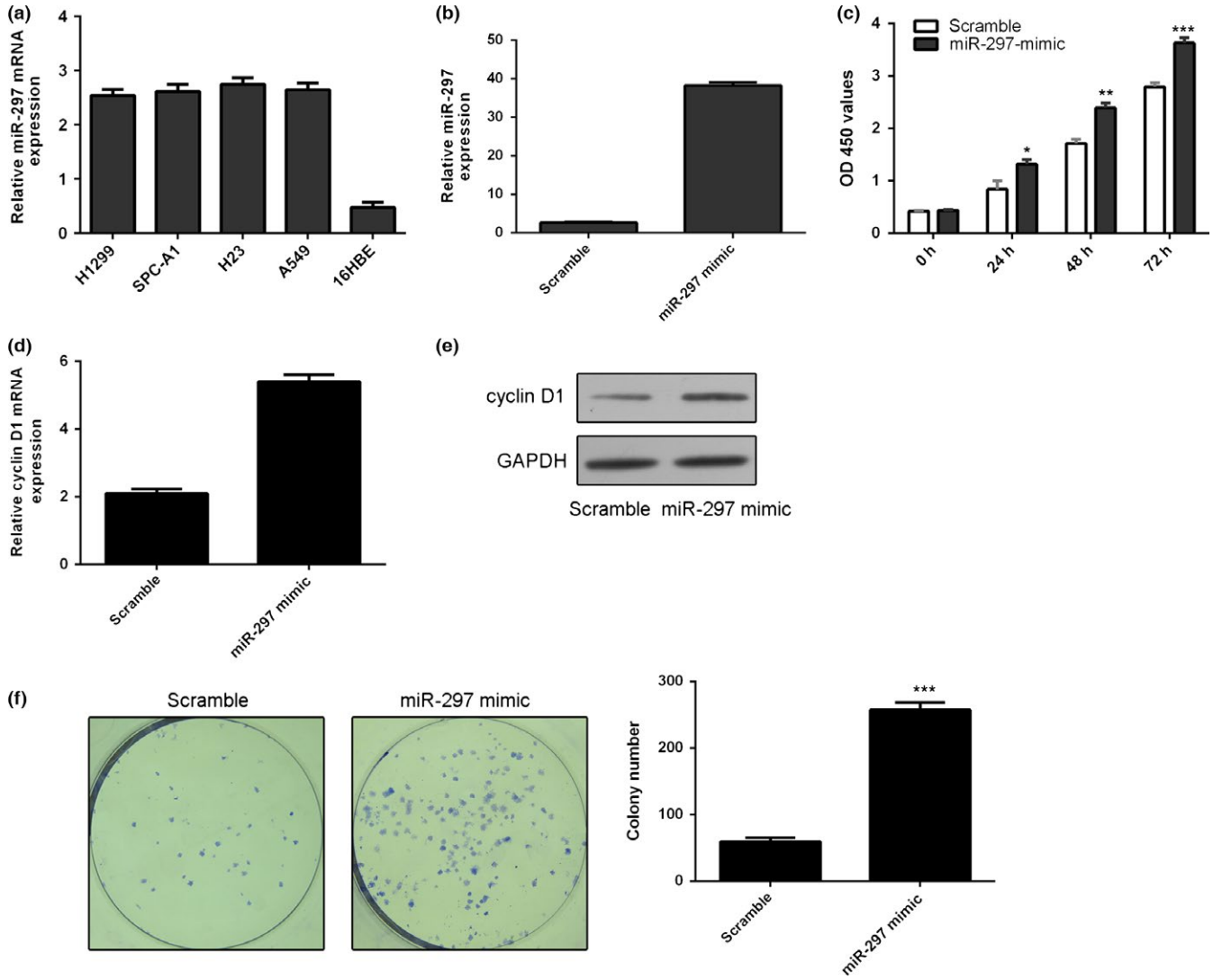


**FIGURE 2** miR-297 enhances lung adenocarcinoma cell proliferation and colony formation. (a) The expression of miR-297 in the lung adenocarcinoma cell lines (H1299, SPC-A1, H23 and A549) and one bronchial epithelial cell line (16HBE) was measured by qRT-PCR. (b) The expression of miR-297 in the A549 cells transfected with miR-297 mimic was detected using qRT-PCR. (c) Ectopic expression of miR-297 enhanced the A549 cells proliferation. (d) Overexpression of miR-297 promoted the cyclin D1 mRNA expression. (e) The protein expression of cyclin D1 was measured by Western blot. (f) Ectopic expression of miR-297 promoted cell colony information in the A549 cells. *$P$<.05, **$P$<.01 and ***$P$<.001

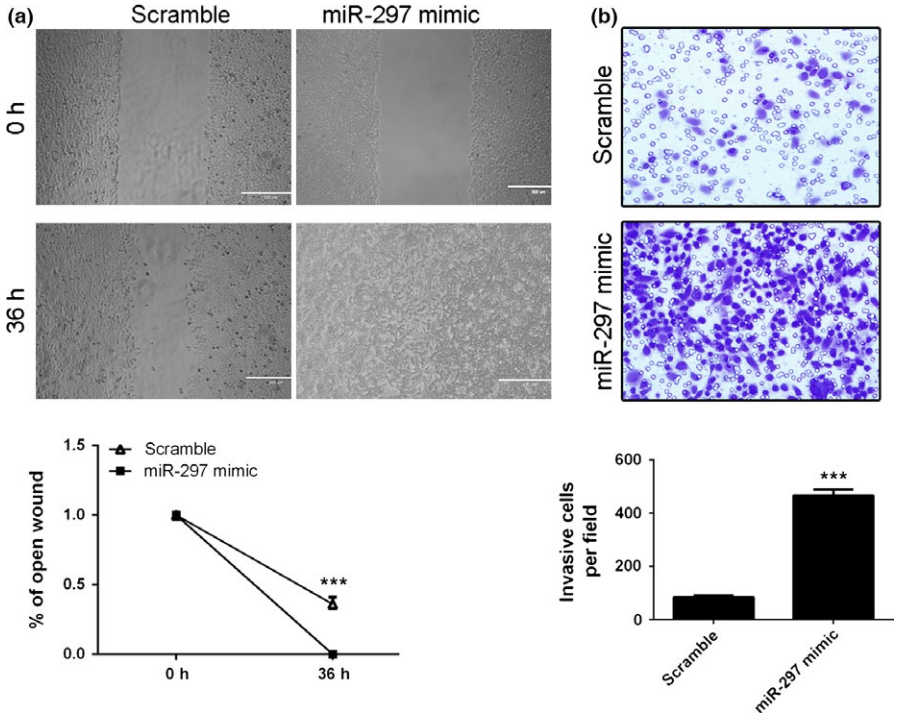


**FIGURE 3** miR-297 promotes lung adenocarcinoma cell migration and invasion. (a) Overexpression of miR-297 promoted the A549 cells invasion. (b) miR-297 overexpression increased the A549 cells migration. ***$P$<.001

miR-297 mimics compared with transfection with scramble (Fig. 2b). Ectopic expression of miR-297 enhanced the cell proliferation of A549 cells (Fig. 2c). Meanwhile, overexpression of miR-297 promoted the cyclin D1 expression in the A549 cells (Fig. 2d,e). Ectopic expression of miR-297 promoted cell colony information in the A549 cells (Fig. 2f).

### 3.3 | miR-297 promoted lung adenocarcinoma cell migration and invasion

Cell invasion and migration are two important processes of tumour metastasis. Therefore, we measured the invasive and migrative ability of lung adenocarcinoma cells transfected with miR-297 mimic and found that the A549 cells transfected with miR-297 mimic presented less migrative and invasion ability than those with control miRNA. As shown in the Fig. 3a, invasion assays showed that ectopic expression of miR-297 promoted the A549 cells invasion. In addition, overexpression of miR-297 increased the A549 cells migration (Fig. 3b).

### 3.4 | GPC5 was a direct target of miR-297

We found the potential targets of miR-297 using TargetScan. The 3′UTR of GPC5 mRNA contained a potential site for the seed region of miR-297 (Fig. 4a). As shown in Fig. 4b, ectopic expression of miR-297 inhibited the luciferase activity of GPC5 3′UTR by about 60% in the A549 cells (Fig. 4b). Overexpression of miR-297 suppressed the expression of GPC5 in the A549 cells (Fig. 4c,d). The expression of GPC5 was lower than in the lung adenocarcinoma cell lines (H1299, SPC-A1, H23 and A549) compared with 16HBE (Fig. 4e,f).

### 3.5 | The expression of GPC5 was inversely correlated with the expression of miR-297 in the lung adenocarcinoma tissues

GPC5 expression was downregulated in the lung adenocarcinoma tissues compared with the adjacent normal tissues (Fig. 5a). Among them, GPC5 expression was lower in 26 lung adenocarcinoma tissues (77%, 27/35) than in the normal tissues (Fig. 5b). Furthermore, the expression level of GPC5 was inversely associated with that in the lung adenocarcinoma tissues (Fig. 5c).

### 3.6 | Overexpression of GPC5 abrogated miR-297-induced cell proliferation and invasion

The protein expression of GPC5 was overexpressed after treatment with pcDNA-GPC5 in the A549 cells (Fig. 6a). We next treated the miR-297-overexpression A549 cells with pcDNA-GPC5 or control. Overexpression of GPC5 abrogated miR-297-induced cell growth (Fig. 6b). Moreover, ectopic expression of GPC5 inhibited the miR-297-induced cell invasion (Fig. 6c,d).

## 4 | DISCUSSION

In this study, we demonstrated that miR-297 expression was upregulated in lung adenocarcinoma tissues compared with the adjacent normal tissues. In addition, miR-297 expression was also upregulated in lung adenocarcinoma cell lines. Ectopic expression of miR-297 enhanced cell proliferation and colony formation in lung adenocarcinoma. Furthermore, overexpression of miR-297 promoted lung adenocarcinoma cell migration and invasion. We identified GPC5 as

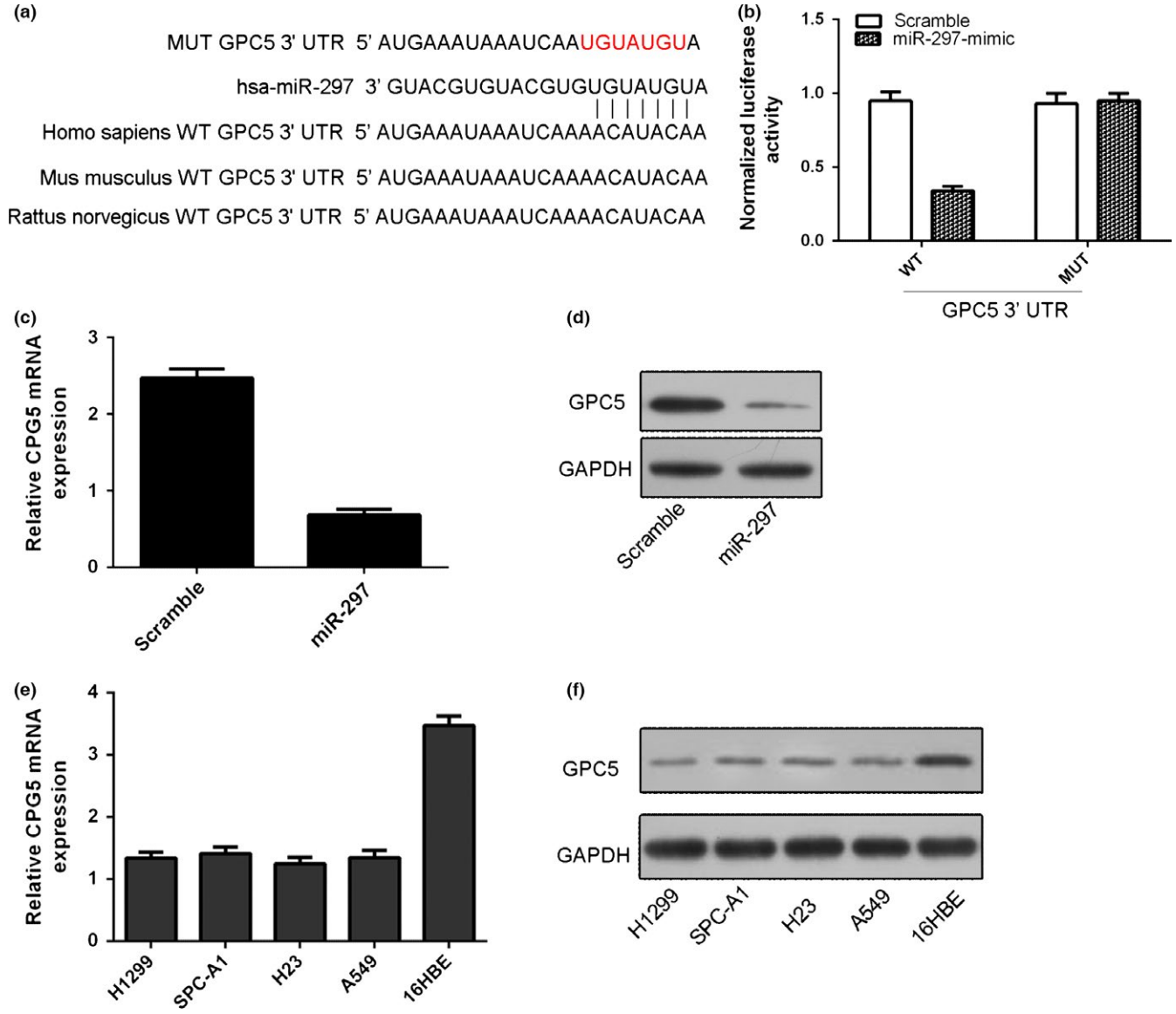


**FIGURE 4** GPC5 is a direct target of miR-297. (a) The 3′UTR of GPC5 mRNA contained a potential site for the seed region of miR-297 using TargetScan. (b) Ectopic expression of miR-297 inhibited the luciferase activity of GPC5 3′UTR by about 60% in the A549 cells by using Luciferase reporter analysis. (c) Overexpression of miR-297 inhibited the GPC5 mRNA in the A549 cells. (d) Overexpression of miR-297 inhibited the GPC5 protein expression in the A549 cells. (e) The mRNA expression of GPC5 in the lung adenocarcinoma cell lines (H1299, SPC-A1, H23 and A549) and one bronchial epithelial cell line (16HBE) was measured by qRT-PCR. (f) The protein expression of GPC5 in the lung adenocarcinoma cell lines (H1299, SPC-A1, H23 and A549) and one bronchial epithelial cell line (16HBE) was measured by Western blot

a direct target gene of miR-297 in the lung adenocarcinoma cell. The expression of GPC5 was downregulated in the lung adenocarcinoma tissues compared with the adjacent normal tissues. Moreover, the expression of GPC5 was inversely associated with the expression of miR-297 in the lung adenocarcinoma tissues. These results suggested that miR-297 acted as an oncogenic miRNA partly by targeting GPC5 expression in lung adenocarcinoma.

Recently, increasing evidences have showed that miR-297 plays an important role in the development of tumour.[30–32] For example, Xu et al.[30] demonstrated that the expression of miR-297 was downregulated in MDR (multidrug resistance) colorectal carcinoma cell line HCT116/L-OHP compared with its parental cells. Overexpression of miR-297 suppressed MRP-2 (MDR-associated protein 2) expression and sensitized cells to anti-cancer drugs in MDR colorectal carcinoma cell. Kefas et al.[31] showed that miR-297 was a cytotoxic miRNA with minimal cytotoxicity to normal astrocytes in glioblastoma. Overexpression of miR-297 suppressed the glioblastoma cell invasion and tumour formation by targeting DGK-α. However, the role of miR-297 was still unknown in lung cancer. In this study, we found that miR-297 expression was upregulated in lung adenocarcinoma tissues compared with the adjacent normal tissues. Among them, miR-297 expression was higher in 26 lung adenocarcinoma tissues (74%, 26/35) than in normal tissues. Moreover, the expression of mir-297 was higher in the metastatic lymph node than that in lung adenocarcinoma

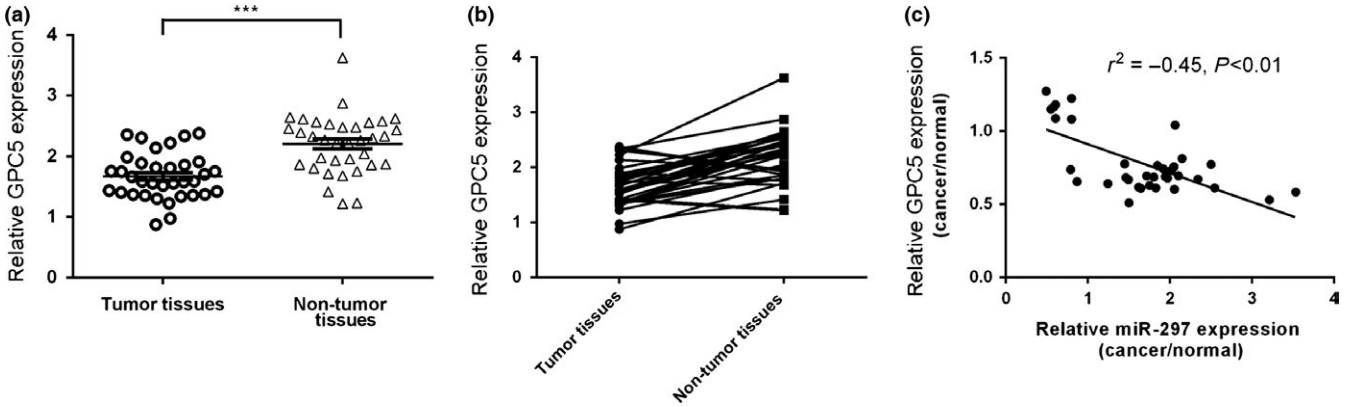


**FIGURE 5** The expression of GPC5 was inversely correlated with the expression of miR-297 in the lung adenocarcinoma tissues. (a) The expression of GPC5 was measured by qRT-PCR in the lung adenocarcinoma tissues and adjacent normal tissues. (b) Among them, GPC5 expression was lower in 26 lung adenocarcinoma tissues (77%, 27/35) than normal tissues. (c) The expression of GPC5 was inversely associated with the expression of miR-297 in the lung adenocarcinoma tissues. ***$P$<.001

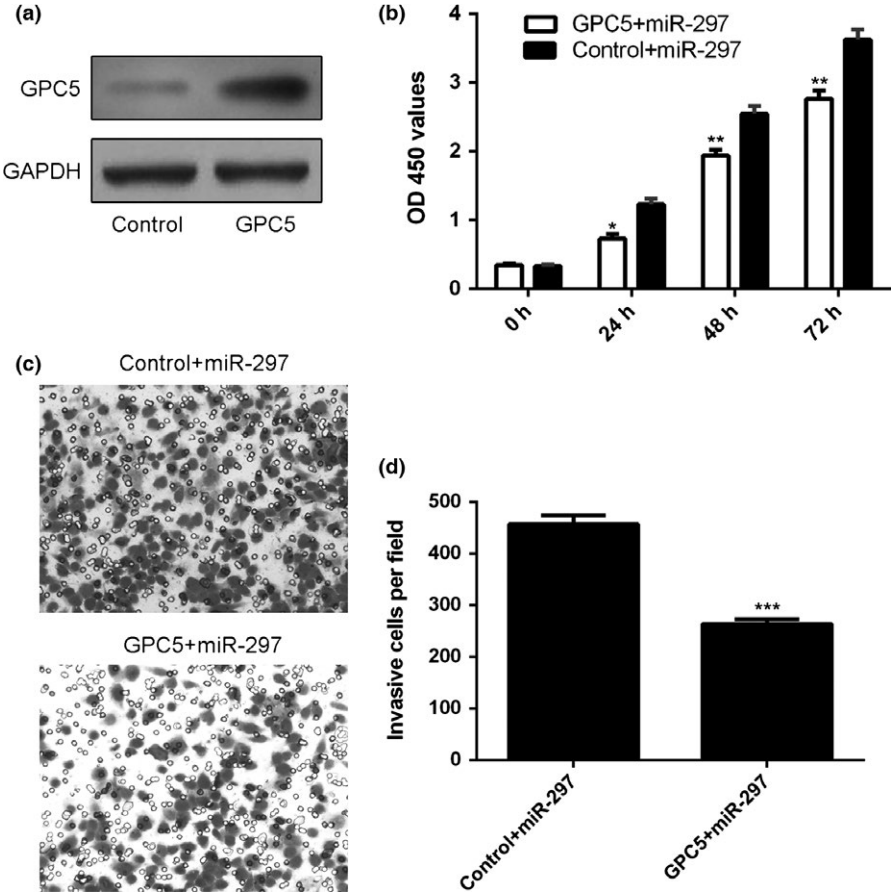


**FIGURE 6** Overexpression of GPC5 abrogated miR-297-induced cell proliferation and invasion. (a) The protein expression of GPC5 in the A549 cells was detected by Western blot. (b) The A549 cells proliferation was measured by MMT analysis. (c) Ecotpic expression of GPC5 inhibited the miR-297-induced cell invasion. (d) Relative ratio of invasive cells per field is shown. *$P$<.05, **$P$<.01 and ***$P$<.001

tissues and adjacent normal tissues. In addition, ectopic expression of miR-297 promoted the lung adenocarcinoma cell proliferation, migration and invasion.

Glypicans (GPCs) are a class of HSPGs (heparan sulphate proteoglycans) that bind to the plasma membrane by a GPI (glycosylphosphatidylinositol) anchor.[33,34] Increasing evidences have demonstrated that GPCs, especially GPC5 and GPC3, act a crucial role in the development of various tumours.[35–37] Recent study has demonstrated that GPC5 plays important roles in the progression of lung cancer.[38–40] For example, Li et al.[41] found that GPC5 expression was upregulated in non-small cell lung cancer (NSCLC) cell lines and overexpression of GPC5 promoted the NSCLC cell migration and invasion. Moreover, GPC5 expression was upregulated in NSCLC tissues and the GPC5 expression level was correlated with poor differentiation, metastasis, vascular invasion and TNM stage. However, Zhao et al.[40] demonstrated that GPC5 expression was downregulated

in lung adenocarcinoma cell and inhibition of miR-620 suppressed the lung adenocarcinoma cell migration, proliferation and invasion by inhibiting the expression of GPC5. Furthermore, Zhang et al.[39] showed that GPC5 expression was upregulated in SACC-M (high lung-metastatic cell line) compared with SACC-2 and SACC-83 (low lung-metastatic cell lines) cells. In line with these results, we also found that GPC5 expression was downregulated in the lung adenocarcinoma tissues compared with the adjacent normal tissues. We identified GPC5 was a direct target gene of miR-297 in lung adenocarcinoma cell. Moreover, the expression of GPC5 was inversely associated with the expression of miR-297 in the lung adenocarcinoma tissues.

In conclusion, our results demonstrated that miR-297 expression was upregulated in lung adenocarcinoma tissues and cell lines. Ectopic expression of miR-297 promoted the lung adenocarcinoma cell proliferation, migration and invasion. We also identified that GPC5 was a direct target gene of miR-297 in lung cancer. These data suggested that miR-297 played an oncogenic role in the development and progression of lung adenocarcinoma.

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