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Effect of hsa_circ_RNA0023397 regulation of miR-106b expression on proliferation and apoptosis of esophageal cancer cells

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Abstract. – **OBJECTIVE:** To explore the potential function of a candidate circular ribonucleic acid (circRNA) [human serum albumin (hsa)_circ_RNA0023397] in esophageal cancer cells.

MATERIALS AND METHODS: Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used to detect the expression level of hsa_circ_RNA0023397 in three esophageal cancer cell lines (KYSE-150, ECA109, and TE-1), which was compared with that in normal human esophageal epithelial cell line (HET-1A). The expression plasmid of hsa_circ_RNA0023397 was constructed, and the effect of overexpression of hsa_circ_RNA0023397 on cell proliferation was determined by cell counting kit-8 (CCK-8) and colony formation assay. The effect of overexpression of hsa_circ_RNA0023397 on cell apoptosis was detected by flow cytometry. Further bioinformatics analysis and Luciferase reporter gene analysis were carried out to explore the role of hsa_circ_RNA0023397 as a sponge of micro RNAs (miRNAs).

RESULTS: Compared with that in normal human esophageal epithelial cell line HET-1A, the expression of hsa_circ_RNA0023397 was down-regulated in three esophageal cancer cell lines *in vitro*. Overexpression of hsa_circ_RNA0023397 overtly inhibited KYSE-150 cell proliferation and promoted its apoptosis. Bioinformatics prediction and Luciferase reporter gene assay confirmed that hsa_circ_RNA0023397 could bind to miR-160b. MiR-106b participated in hsa_circ_RNA0023397-mediated inhibition of proliferation of esophageal cancer KYSE-150 cells.

CONCLUSIONS: Hsa_circ_RNA0023397 is down-regulated in esophageal cancer cells and can act as miR-106b to affect the biological function of esophageal cancer cells.

Key Words:
Esophageal cancer, Hsa_circ_RNA0023397, MiR-106b, Proliferation, Apoptosis.

Introduction

Esophageal carcinoma is one of the most common malignant tumors in the world, with a 5-year survival rate of only 10-15%. In 2018, there are estimated 572,000 new cases and more than 509,000 deaths worldwide[1,2]. Esophageal squamous cell carcinoma (ESCC) is the most popular type in China[3]. Epidemiological evidence[4] shows that the prognosis of patients with early esophageal cancer is better than that of patients with advanced stage esophageal cancer. Hence, early diagnosis is particularly important. However, the poor sensitivity of conventional diagnostic mode and the unclear underlying mechanism of esophageal cancer limit the diagnosis and thus affect the prognosis of patients with early esophageal cancer[5]. Therefore, understanding the molecular mechanisms of the occurrence, development, and metastasis of esophageal cancer cells and screening new, specific, and sensitive targets to prevent and treat esophageal cancer is of vital significance and clinical value.

Non-coding ribonucleic acids (ncRNAs) are a novel type of RNAs, mainly expressed in eukaryotes[6]. With the rapid development of high-throughput sequencing technology and bioinformatics, increasing evidence[7-10] indicates that ncRNAs are involved in the occurrence and development of various diseases, including Alzheimer's disease, cardiovascular disease, and cancer. Abundant and functionally important types of ncRNAs include tRNAs, rRNAs, micro RNAs (miRNAs), siRNAs, long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), etc. NcRNAs play an important role in epigenetic regulation of gene expression. MiRNAs affect the pathogenesis and progress of angiogenesis-related diseases by regulating angiogenesis, cholesterol metabolism,

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and inflammatory response[11]. LncRNAs regulate the proliferation, invasion, metastasis, and apoptosis of various cancer cells[12,13]. Compared with those of miRNAs and lncRNAs, the expression and function of newly discovered circRNAs still need to be clarified.

CircRNAs are newly discovered RNA molecules formed by reverse splicing of the covalently linked 3'- and 5'-ends, which generally do not encode proteins while can be generated in any genomic region. 85% of circRNAs are arranged with known protein-coding genes in the sense orientation and can span 1-5 exons[14,15]. Zhang et al[16] and Memczak et al[17] have shown that some circRNAs differ greatly in expression in cells, tissues and development stages, and can serve as the cavernous body of competitive endogenous ncRNAs or miRNAs to interact with RNA-binding proteins, thus regulating parental gene expression or encoding proteins. Dysregulation of circRNAs in breast cancer, gastric cancer, and colorectal cancer has been reported, suggesting the possibility of circRNAs becoming a new biomarker. However, the function of circRNAs in esophageal cancer remains to be elucidated.

Materials and Methods

Materials

Dulbecco's Modified Eagle's medium (DMEM) (high glucose), fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO, USA), TRIzol reagent, Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA, USA), Prime Script reverse transcription (RT) Master Mix and SYBR Select Master Mix (TaKaRa, Otsu, Shiga, Japan), Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) apoptosis detection kit (BD Biosciences, Franklin Lakes, NJ, USA), human serum albumin (hsa)_circ_RNA0023397 expression plasmid, negative control (si-NC) and miR-106b mimics designed and synthesized by Guangzhou Geneseed Biotechnology Co., Ltd. (Guangzhou, China), cell counting kit-8 (CCK-8) and Dual-Luciferase reporter gene detection kit (Promega, Madison, WI, USA), real-time fluorescence quantitative polymerase chain reaction (PCR) instrument (Bio-Rad, Hercules, CA, USA), and flow cytometer (BD, Franklin Lakes, NJ, USA).

Cell Culture and Transfection

The human esophageal cancer cell lines (KYSE-150, ECA109, and TE-1) and the normal human esophageal epithelial cell line (HET-1A) were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). The cells were cultured with DMEM supplemented with 10% heat-inactivated FBS, penicillin (100 U/mL) and streptomycin (100 μg/mL) in a humidified incubator containing 5% CO_2 at 37°C. Esophageal cancer cells were seeded on six-well plates and transfected according to instructions of the Lipofectamine 2000 kit.

Total RNA Extraction and Quantitative RT-PCR (qRT-PCR)

Total RNA was extracted from the collected cells using the TRIzol reagent. Then, 500 ng of total RNA was reversely transcribed in a final volume of 10 μL with the RT kit. After that, real-time qRT-PCR was performed using qPCR Mix and 0.5 μL of complementary DNA (cDNA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and snRNA U6 were used as internal controls, and hsa_circ_RNA0023397 and miR-106b expression levels were determined by qRT-PCR. The following primer sequences were chosen: hsa_circ_RNA0023397: F, 5'-CAGACCGACGAGTCTCCTGG-3' and R, 5'-TTGCTGATCCCGCCTATGG-3', GAPDH: F, 5'-CGCTCTCTGCTCCTCCTGTTC-3' and R, 5'-ATCCGTTGACTCCGACCTTCAC-3', miR-106b: F, 5'-TTTTCGCCCTTAGCGTGAAGA-3' and R, 5'-GAGGCAGTCGAAGCTCTCG-3', U6: F, 5'-CTCGCTTCGGCAGCACA-3' and R, 5'-AACGCTTCACGAATTTGCGT-3'. The qRT-PCR procedure was as follows: the initial denaturation was carried out at 95°C for 10 min, followed by 40 cycles at 92°C for 15 s and 60°C for 1 min, respectively. The fold change of expression was obtained in subsequent calculations with the help of the $2^{-\Delta\Delta Ct}$ method.

Cell Proliferation Analysis

Cell proliferation was investigated with the CCK-8. The transfected cells were seeded in 96-well plates (2000 cells/well). Cell proliferation was measured every 24 h in accordance with the experimental protocol. That is to say, each well was added with 10 μL of CCK-8 solution and incubated at 37°C for 2 h. It was then measured by spectrophotometry at the absorbance of 450 nm.

Colony Formation Assay

The cells were trypsinized, and the transfected cells were seeded in 6-well plates (200 cells/well) and cultured at 37°C for 10-14 days. The cells were washed with phosphate-buffered saline (PBS) 2-3 times. Colonies were stained with

a staining solution containing 0.1% crystal violet and 20% methanol. Cell colonies were then counted and analyzed.

Detection of Cell Apoptosis by Flow Cytometry

Transfected cells were collected after 24 h, and stained with Annexin V-FITC and PI using the Annexin V-FITC/PI apoptosis detection kit prior to the analysis *via* the flow cytometry. The FACScan analyzer was then employed for the on-machine analysis.

Detection of Luciferase Activity

The Luciferase reporter gene assay was chosen to investigate the interaction between hsa_circ_RNA0023397 and miRNAs. The 3′-untranslated region (3′-UTR) sequence of hsa_circ_RNA0023397 with the predicted miRNAs binding site was amplified by PCR and inserted into the psiCHECK-2 luciferase vector. Plasmids with wild-type or mutant hsa_circ_RNA0023397 and miR-106b mimics were transfected into esophageal cancer cell lines with the Lipofectamine 2000. After cotransfection for 48 h, the relative luciferase reporter gene activity was detected with the Dual-Luciferase.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) 20.0 (IBM, Armonk, NY, USA) and GraphPad Prism (La Jolla, CA, USA). Student's *t*-test or one-way analysis of variance (ANOVA) was adopted to evaluate the statistical significance of differences between two groups. $p<0.05$ indicated that the difference was statistically significant.

Results

Down-Regulation of Hsa_circ_RNA0023397 Expression in Human Esophageal Cancer Cell Lines

First, it was explored whether hsa_circ_RNA0023397 plays a role in the development of esophageal cancer. QRT-PCR was employed to determine the expression level of hsa_circ_RNA0023397 in three esophageal cancer cell lines: KYSE-150, ECA109, and TE-1. These data were compared with those of normal human esophageal epithelial cell line HET-1A. The results revealed that hsa_circ_RNA0023397 expression was down-regulated in three esophageal cancer cell lines compared with that in HET-1A and the difference was statistically significant ($p<0.05$, Figure 1).

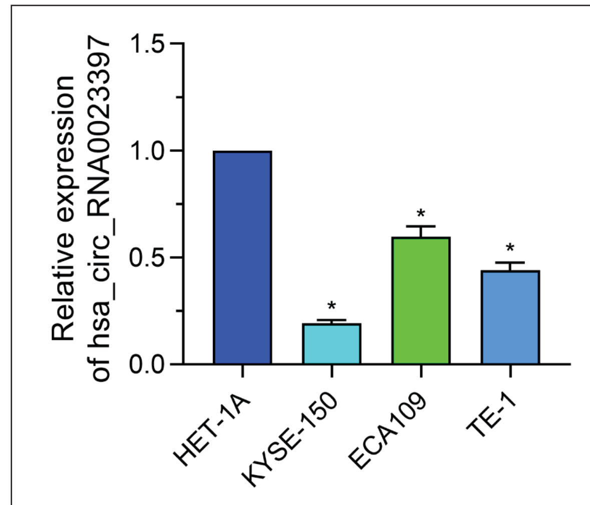

Figure 1. The expression level of hsa_circ_RNA0023397 in three esophageal cancer cell lines KYSE-150, ECA109, and TE-1 compared with that in normal human esophageal epithelial cell line HET-1A via qRT-PCR (*$p<0.05$).

The Effect of Overexpressed Hsa_circ_RNA0023397 on the Proliferation of Esophageal Cancer KYSE-150 Cells

To explore the effect of hsa_circ_RNA0023397 on the proliferation of esophageal cancer cells, KYSE-150 cells with the most evident down-regulation were selected. Hsa_circ_RNA0023397 expression plasmids were transfected and CCK-8 was applied to detect the effect of hsa_circ_RNA0023397 overexpression on the proliferation of esophageal cancer KYSE-150 cells. The overexpression efficiency of hsa_circ_RNA0023397 was verified via qRT-PCR (Figure 2A). CCK-8 assay denoted that the overexpression of hsa_circ_RNA0023397 significantly reduced the proliferation of KYSE-150 cells ($p<0.05$, Figure 2B).

The Effect of Overexpressed Hsa_circ_RNA0023397 on Colony Formation of Esophageal Cancer KYSE-150 Cells

The effect of overexpressed hsa_circ_RNA0023397 on the proliferation of esophageal cancer KYSE-150 cells was further confirmed by colony formation assay. The results indicated that the transfected hsa_circ_RNA0023397 expression plasmid group had remarkably inhibited formation of cell colony in comparison with the control group ($p<0.05$, Figure 3).

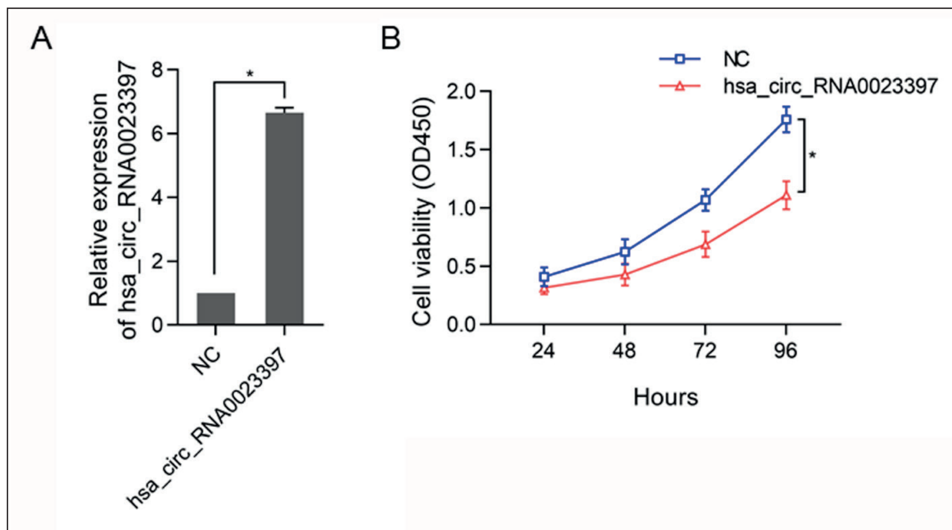

Figure 2. The effect of overexpressed hsa_circ_RNA0023397 on the proliferation of esophageal cancer KYSE-150 cells. **A**, Overexpression efficiency of hsa_circ_RNA0023397 verified by QRT-PCR. **B**, Effect of overexpressed hsa_circ_RNA0023397 on the proliferation of esophageal cancer KYSE-150 cells detected through CCK-8 (*p<0.05).

The Effect of Overexpressed Hsa_circ_RNA0023397 on the Apoptotic Rate of Esophageal Cancer KYSE-150 Cells

The effect of overexpressed hsa_circ_RNA0023397 on the apoptotic rate of esophageal cancer KYSE-150 cells was analyzed with the help of the flow cytometry using the Annexin V-FITC/PI apoptosis assay kit. It was suggested that the apoptotic rate in the transfected hsa_circ_RNA0023397 expression plasmid group was distinctly higher than that in the control group ($p<0.05$, Figure 4).

Hsa_Circ_RNA0023397 Functioned as a Sponge of MiR-106b

Previously, it was pointed out that circRNAs can serve as the cavernous body of competitive endogenous ncRNAs or miRNAs, and the regulatory mechanism of miRNAs was explored in this study. MiRNAs that were predicted by a bioinformatics online website to interact with hsa_circ_RNA0023397 and have increased expression in esophageal cancer cells were focused in this study. Among these miRNAs, miR-106b was chosen, which is up-regulated in esopha-

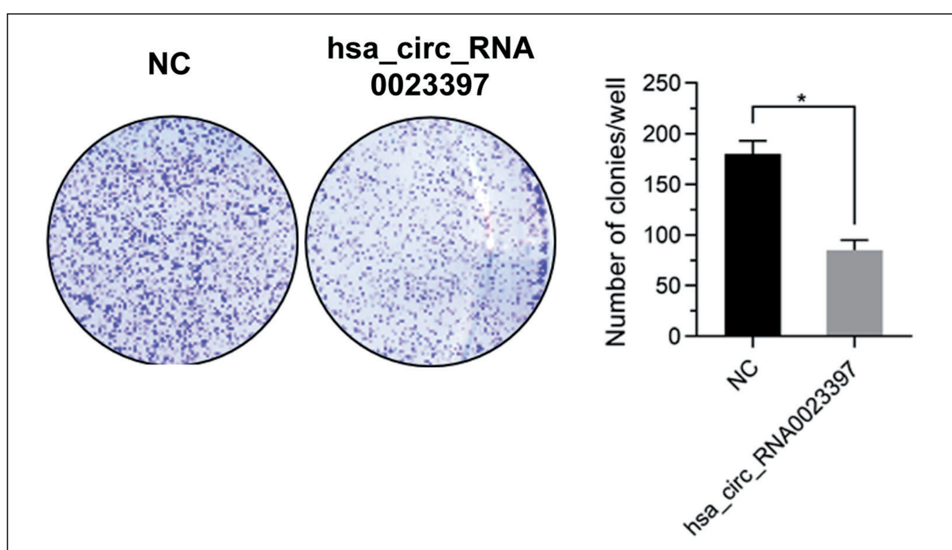

Figure 3. The effect of overexpressed hsa_circ_RNA0023397 on the colony formation of esophageal cancer KYSE-150 cells detected by colony formation assay (magnification: 10×) (*p<0.05).

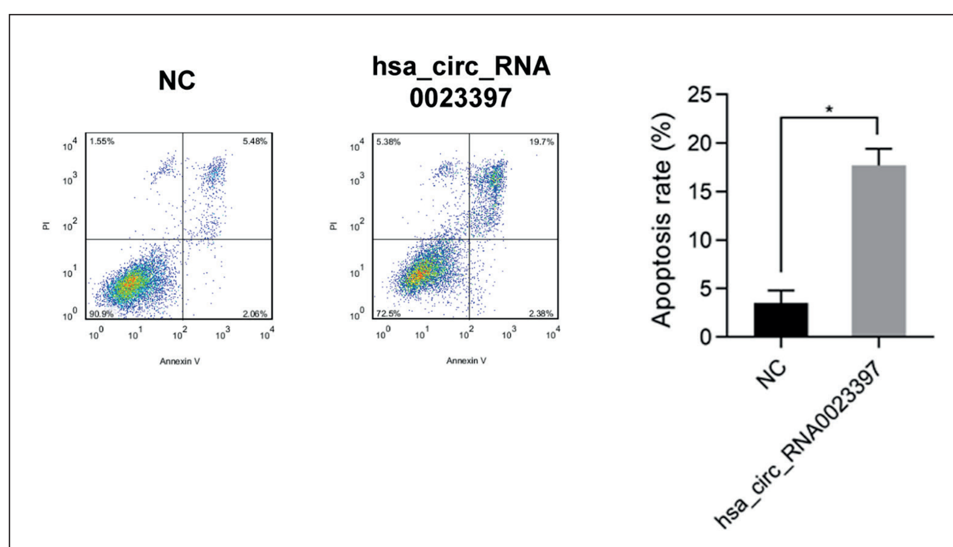

Figure 4. The effect of overexpressed hsa_circ_RNA0023397 on the apoptotic rate of esophageal cancer KYSE-150 cells by the flow cytometry (* $p<0.05$).

geal cancer[18] and has a binding site with hsa_circ_RNA0023397 (Figure 5A). The two were supposed to interact with each other. To verify whether hsa_circ_RNA0023397 can regulate miR-106b, Luciferase reporter gene plasmids were constructed, including wild type (WT) and mutant type (MUT) of hsa_circ_RNA0023397 (Figure 5A). The results of Dual-Luciferase reporter gene assay revealed that miR-106b mimic distinctly decreased the Luciferase intensity in hsa_circ_RNA0023397-WT compared with that in hsa_circ_RNA0023397-MUT ($p<0.05$, Figure 5B). It was also discovered that after overexpression of hsa_circ_RNA0023397-WT in KYSE-150 cells, the expression of miR-106b was markedly lowered ($p<0.05$, Figure 5C). Similarly, the knockdown or overexpression of miR-106b also affected the expression of hsa_circ_RNA0023397 ($p<0.05$, Figure 5D).

MiR-106b Related to Hsa_circ_RNA0023397-Mediated Inhibition of Proliferation of Esophageal Cancer KYSE-150 Cells

To confirm whether miR-106b has an effect on hsa_circ_RNA0023397-mediated inhibition of esophageal cancer cell proliferation, a rescue experiment was conducted in KYSE-150 cells. Cell proliferation activity was detected by CCK-8. Results shown in Figure 6 manifested that the overexpression of miR-106b restored the inhibitory effect of hsa-circ-001193 on proliferation of KYSE-150 cells (* $p<0.05$), indicating that miR-106b is correlated with hsa_circ_RNA0023397-mediated inhibition of proliferation of esophageal cancer KYSE-150 cells.

Discussion

Esophageal cancer is an important public health problem worldwide, ranking 8th in cancer-related mortality rate and 6th in common causes of cancer deaths[19]. According to its pathological characteristics, esophageal cancer is mainly divided into ESCC and esophageal adenocarcinoma (EA), of which ESCC accounts for more than 90%. The main treatment methods for esophageal cancer include surgery, chemotherapy, radiotherapy, and combination therapy. Despite great progress in treatment, the 5-year survival rate of esophageal cancer patients is still below 15%[1]. Recurrence and metastasis are the fundamental causes affecting long-term survival. Therefore, exploring the potential pathogenesis of esophageal cancer is helpful to develop new diagnostic and therapeutic targets.

Most mature mRNAs are linear molecules with 5'- and 3'-ends, which reflect the initiation and termination of RNA polymerase on DNA templates. Various RNA molecules can be combined through splicing reactions (trans-splicing) in cells. However, circRNAs formed by covalent attachment of the ends of individual RNA molecules are rare[16]. CircRNAs were originally found in plants and proved to encode subviral agents 30 years ago. Accumu-

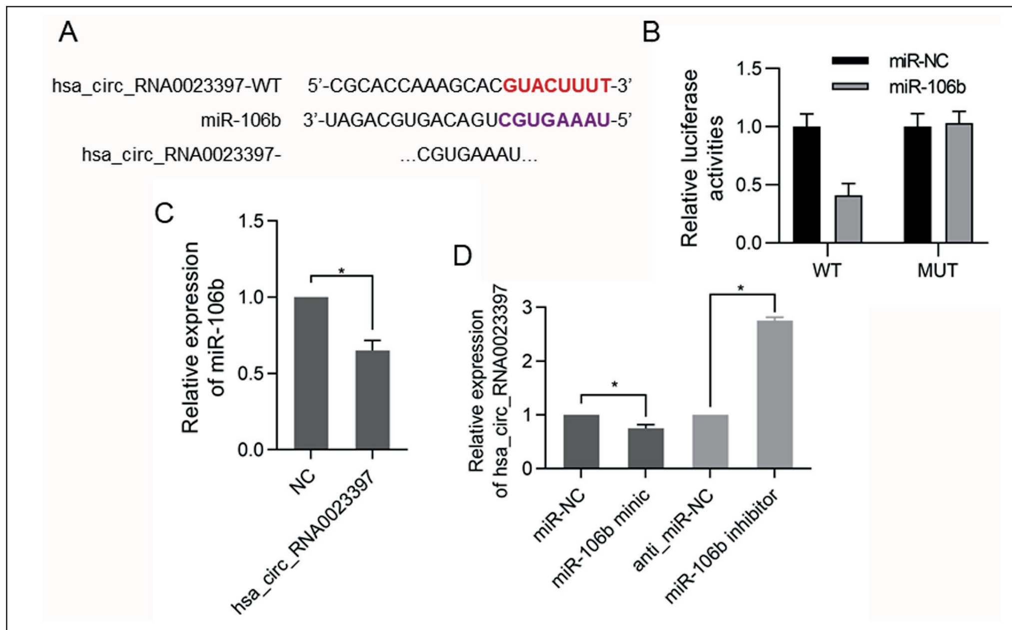

Figure 5. ***A***, Bioinformatics prediction results of hsa_circ_RNA0023397 and miR-106b (*p<0.05). **B**, Dual-Luciferase gene reporter assay for the interaction between hsa_circ_RNA0023397 and miR-106b (*p<0.05). C-D, The detection of the regulation of hsa_circ_RNA0023397 and miR-106b at mRNA level via qRT-PCR (*p<0.05).

lated evidence has demonstrated that circRNAs are essential in disease development. Zhang et al[20] analyzed the associations of large tumor suppressor homolog 1 (LATS1) and has-miR-424-5p expressions with clinicopathological features and prognosis of gastric cancer patients through the Cancer Genome Atlas (TCGA) RNA sequencing data in the study of the potential molecular mechanism of ncRNA in regulating the expression of LATS1 in gastric cancer. It was discovered that either increased miR-424 expression or decreased LATS1 expression is related to the pathological stage of gastric cancer and is not conducive to the relevant prognosis of patients. Besides, the ectopic expression of miR-424 stimulates the proliferation and invasion of gastric cancer cells by means of targeting gene LATS1. In this study, the biological function and potential mechanism of hsa_circ_RNA0023397 in esophageal cancer cells were mainly explored. First of all, whether hsa_circ_RNA0023397 plays a role in the development of esophageal cancer was discussed, and the expression of hsa_circ_RNA0023397 in esophageal cancer cells was examined. It was revealed that the expression of hsa_circ_RNA0023397 was down-regulated in the human esophageal cancer cell line. By constructing hsa_circ_RNA0023397 overexpression plasmid, the effects of overexpression on proliferation and apoptosis of esophageal cancer cells were explored. It was indicated that overexpression of hsa_circ_RNA0023397 could inhibit the proliferation of esophageal cancer KYSE-150 cells and promote their apoptosis. It was speculated that hsa_circ_RNA0023397 may play the role of the tumor suppressor gene, and the down-regulation of expression in esophageal cancer cells may be related to the occurrence and development of the tumor.

There are abundant miRNA binding sites in circRNAs, which can competitively bind to miR-

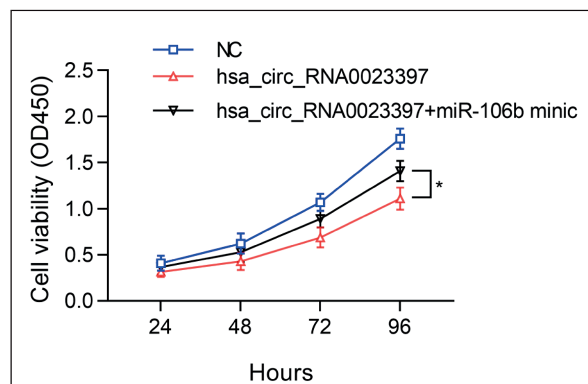

Figure 6. A rescue experiment to see if miR-106b is involved in hsa_circ_RNA0023397-mediated inhibition of proliferation of esophageal cancer KYSE-150 cells (*p<0.05).

NAs to regulate the expression of target genes, also known as competitive endogenous RNA (ceRNA) mechanism. CircRNAs can promote or inhibit tumor development through interaction with miRNAs. Han et al[21] analyzed the expression profile of human circRNAs in hepatocellular carcinoma tissues, identified the circMTO1 with down-regulated expression in hepatocellular carcinoma tissues and found that circMTO1 inhibits the progression of hepatocellular carcinoma by acting as a sponge of miR-9 to promote p21 expression, indicating that circMTO1 is a potential target in the treatment of hepatocellular carcinoma. Previous evidence has shown that miR-106b can promote the progress of esophageal cancer. Our bioinformatics suggested that hsa_Circ_RNA0023397 can function as a sponge of miR-106b, thus inhibiting the development of esophageal cancer. In this study, it was also started with miRNA to explore the regulatory mechanism of hsa_circ_RNA0023397. The miRNA, miR-106b, which is up-regulated in esophageal cancer, was confirmed to participate in hsa_circ_RNA0023397-mediated inhibition of proliferation of esophageal cancer KYSE-150 cells.

Conclusions

All together, these results reveal that hsa_circ_RNA0023397 is found to be down-regulated in esophageal cancer cells, which can serve as miR-106b to affect the biological function of esophageal cancer cells. This is helpful for people to understand the molecular mechanism of occurrence and development of esophageal cancer cells and lays a foundation for screening new specific targets.

Conflict of Interests

The authors declare that they have no conflict of interests.

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