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Circ_0001742 promotes tongue squamous cell carcinoma progression *via* miR-431-5p/ATF3 axis

Y.-T. HU[1], X.-X. LI[2], L.-W. ZENG[2]

[1]Department of Stomatology, Zhuji People's Hospital of Zhejiang Province, Zhuji Zhejiang Province, China
[2]Department of Prosthodontics, Affiliated Stomatological Hospital of Nanchang University, Nanchang, Jiangxi Province, China

Dr. Yangtao Hu and *Dr. Xiaoxia Li* contributed equally to the writing of this article

Abstract. – OBJECTIVE: Circular RNAs (circRNAs) have been demonstrated to involve in the development of various cancers. This study aimed to investigate the functions of circ_0001742 on regulating tongue squamous cell carcinoma (TSCC) development and the underlying mechanisms.

PATIENTS AND METHODS: The expression of circ_0001742, miR-431-5p and activating transcription factor 3 (ATF3) mRNA was detected by quantitative real-time polymerase chain reaction (qRT-PCR). The protein levels of epithelial-mesenchymal transition (EMT)-related proteins and ATF3 were measured by Western blot analysis. 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and flow cytometry assay were used to evaluate cell proliferation and apoptosis. Besides, Cell migration and invasion were assessed by transwell assay. The relationships between circ_0001742 and miR-431-5p, miR-431-5p and ATF3 were predicted by online software and confirmed by dual-luciferase reporter assay, RNA immunoprecipitation (RIP), and pull-down assay.

RESULTS: The expression of circ_0001742 was upregulated in TSCC tissues and cells. Knockdown of circ_0001742 inhibited proliferation, migration, invasion and EMT and induced apoptosis in TSCC cells. Then, miR-431-5p was identified as a target of circ_0001742, and knockdown of miR-431-5p reversed the effects of circ_0001742 knockdown on proliferation, apoptosis, migration, invasion and EMT of TSCC cells. Moreover, miR-431-5p could bind to ATF3, and overexpression of ATF3 rescued the effects mediated by miR-431-5p in TSCC cells. In addition, circ_0001742 regulated ATF3 expression through miR-431-5p.

CONCLUSIONS: Our results demonstrated that circ_0001742 plays a tumor-promoting effect in TSCC cells by serving as a competing endogenous RNA (ceRNA) to regulate miR-431-5p/ATF3 axis, which might provide a potential therapeutic target for TSCC.

Key Words:
Circ_0001742, Tongue squamous cell carcinoma, MiR-431-5p, ATF3.

Introduction

Tongue squamous cell carcinoma (TSCC) is one of the fatal malignancies worldwide[1]. TSCC is featured with high invasive capability and recurrence rate and early metastasis, so the patients with TSCC have a high fatality rate since it is difficult to cure. Despite many efforts and advances have been made in the diagnosis and therapy of TSCC, the 5-year survival rate has not been largely improved [2]. Therefore, it is of great significance to understand the regulatory mechanisms underlying its pathogenesis for the treatment of TSCC.

Circular RNAs (circRNAs), kinds of endogenous non-coding RNAs, are very different from line RNAs in structure. They have covalently closed loops without a 3' poly adenylate tail or 5'cap, resulting in a more stable state than line RNAs[3,4]. With the development of sequencing technology, more and more circRNAs have been found and proved to play vital roles in various diseases, including cancers[5-7]. For example, circ_0042666 suppressed laryngeal squamous cell carcinoma cell proliferation and invasion[8]. Knockdown of circABCC2 restrained hepatocel-

Corresponding Author: Liwei Zeng, MS; e-mail: L1573681993@163.com

lular cancer development[9]. Moreover, circOSBPL10 has been demonstrated to exert a tumor-promoting role in gastric cancer, and it can serve as a biomarker for the prognosis of patients with gastric cancer[10]. However, to date, the functions of circRNAs in TSCC have been poorly reported.

Increasing evidence shows that microRNAs (miRNAs) participate in multiple biological processes by repressing the expression of the target gene[11]. It has been reported that miRNAs play vital roles in the development of cancers, including TSCC[12]. Zhang and Zhao[13] indicated that miR-758 suppressed tumor progression in TSCC via sponging metadherin. Further, miR-409-3p has also been proved to play a tumor-suppressing action in TSCC[14]. On the contrary, certain miRNAs have been uncovered to promote TSCC progression, such as miR-19a, miR-424, miR-373-3p and so on[15,16]. In addition, miRNAs can be considered as biomarkers for the diagnosis and prognosis of TSCC[12,17,18].

Activating transcription factor 3 (ATF3), a stress-inducible gene, is a member of ATF/cyclic AMP response element-binding (CREB) family. It contains a bZIP element that can bind to specific DNA, thereby regulating gene expression[19]. ATF3 has been indicated to act as a key regulator of cancer progression. Previous studies[20-23] revealed that ATF3 was overexpressed and played a tumor-promoting role in lung cancer, breast cancer, colon cancer and TSCC. Interestingly, it has been found that AFT3 inhibits tumor progression in colorectal cancer, hepatocellular carcinoma and prostate cancer[24-26]. Thus, the role of ATF3 may depend on the type of cancers.

In this study, we first found the abnormal expression of circ_0001742 in TSCC tissues and then explored the effects of circ_0001742 on proliferation, apoptosis, migration, invasion and epithelial-mesenchymal transition (EMT) in TSCC cells. Subsequently, the downstream target of circ_0001742 was identified, and the underlying mechanism was further explored.

Patients and Methods

Tissue Samples

TSCC and adjacent normal tissues were obtained from 30 patients with TSCC through surgical dissection at Zhuji People's Hospital of Zhejiang Province. Patients didn't undergo any other therapies before surgery. This study was approved from the Ethics Committee of Zhuji People's Hospital of Zhejiang Province (Zhuji, China). All patients read and signed informed consent.

Cell culture

Human oral keratinocytes cell line (HOK) and human TSCC cell lines (CAL27 and SCC4) were bought from BeNa Culture Collection (Beijing, China). Cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA) at 37°C with 5% CO_2.

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted according to the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) instructions. PrimeScript RT Reagent Kit (TaKaRa, Dalian, China) and microRNA First-Strand cDNA Synthesis Kit (Sangon Biotech, Shanghai, China) were used for reverse-transcription. The quantification was performed using SYBR Green Master Mix (TaKaRa, Dalian, China) and normalized by glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6. The data was calculated by $2^{-\Delta\Delta Ct}$ method. The primers were used as follows:

circ_0001742-F:GGGATTTGTTTTGTGGGCTA
circ_0001742-R:CACTGGCCTGAACTGTTGAA
ATF3-F: TGGCAACACGGAGTAAACGA
ATF3-R: GCATCATTTTGCTCCAGGCTC
GAPDH-F: CGGAGTCAACGGATTTGGTCGTAT
GAPDH-R: AGCCTTCTCCATGGTGGTGAAGAC
miR-431-5p-F: TGTCTTGCAGGCCGTCATG
miR-431-5p-R: GCTGTCAACGAT ACGCTACCTA
U6-F: CTTCGGCAGCACATATACT
U6-R: AAAATATGGAACGCTTCACG

Cell Transfection

Small interfering RNA (siRNA) of circ_0001742 (si-circ_0001742#1, si-circ_0001742#2 and si-circ_0001742#3), miR-431-5p mimic (miR-431-5p), miR-431-5p inhibitor and their control fragments (si-NC, miR-NC and inhibitor-NC) were obtained from GenePharma (Shanghai, China). For overexpression of ATF3, pcDNA-ATF3 was constructed by inserting the full-length sequences of ATF3 into pcDNA3.1 (Invitrogen, Carlsbad, CA, USA). Cell transfection was conducted by Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

3-(4,5-Dimethyl-2-Thiazolyl)-2,5-Diphenyl-2-H-Tetrazolium Bromide (MTT) Assay

Cell proliferation was assessed by an MTT kit (Solarbio, Beijing, China). Transfected cells were cultured in a 96-well plate for 24 h. Each well was

added with 10 μL of MTT to incubate the cells for 4 h at 37°C. Then, the supernatants were removed. The cells were added with 110 μL dimethyl sulfoxide (DMSO) to dissolve the formazan crystals. Finally, the absorbance at 490 nm wavelengths was measured with a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA).

Flow Cytometry Assay

The annexin V-fluorescein isothiocyanate (V-FITC)/ propidium iodide (PI) apoptosis kit (BD Bioscience, Franklin Lakes, NJ, USA) was used for the detection of apoptosis. After transfection, cells were collected and resuspended with binding buffer. Next, FITC and PI were added to incubate the cells for 20 min in the dark condition, and cell apoptosis was monitored by flow cytometry (BD Bioscience, Franklin Lakes, NJ, USA).

Transwell Assay

For the detection of cell migratory and invasive capability, a transwell assay was performed. For the migration detection, cells in serum-free medium were added into the upper chamber. While for cell invasion detection, Matrigel (BD Bioscience, Franklin Lakes, NJ, USA) was added to cover the upper chamber. The basolateral chamber was added to the cell medium with 10% serum. After incubation for 48 h, cells that stuck to the lower surface of the membrane were fixed by 4% paraformaldehyde and then dyed with 0.5% crystal violet solution. The dyed cells were imaged by a microscope (Thermo Fisher Scientific, Waltham, MA, USA).

Western Blot Analysis

RIPA solution (Thermo Fisher Scientific, Waltham, MA, USA) was used for the extraction of total proteins. Equal amounts of proteins were added to each well and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE); then, they were transferred into a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The membrane was blocked in 5% non-fat milk at room temperature for 1 h and incubated with the primary antibodies overnight at 4°C. Subsequently, the secondary antibody was used to incubate the membrane for 1 h at room temperature. The signals were tested by an enhanced chemiluminescence (ECL) system (Millipore, Billerica, MA, USA). The primary antibodies against MMP9 (1:1000; ab38898), vimentin (1:1000; ab92547), N-cadherin (1:1000; ab18203), E-cadherin (1:5000; ab40772), ATF3 (1:1000; ab207434), β-actin (1:5000; ab179467) and secondary antibody (1:5000; ab205718) were bought from Abcam (Cambridge, UK).

Target Prediction and Dual-Luciferase Reporter Assay

The putative target sites between circ_0001742 and miR-431-5p, miR-431-5p and ATF3 were predicted by Circular RNA Interactome and Starbase3.0, respectively. To confirm the relationships between circ_0001742 and miR-431-5p, miR-431-5p and ATF3, the wild type circ_0001742 containing the predicated target sites for miR-431-5p (WT-circ_0001742) and the mutant sequences (MUT-circ_0001742), the 3'-untranslated region (3'UTR) of ATF3 containing the predicted binding site for miR-431-5p (ATF3 3'UTR-WT) and the mutant sequences (ATF3 3'UTR-MUT) were inserted pGL3 luciferase reporter vectors (Promega, Madison, WI, USA), respectively. The vector (WT-circ_0001742, MUT-circ_0001742, ATF3 3'UTR-WT or ATF3 3'UTR-MUT) and miRNA mimic (miR-431-5p or miR-NC) were co-transfected into CAL27 and SCC4 cells. At 48 h after transfection, the activities of luciferases were measured by a Dual-Luciferase reporter system (Promega, Madison, WI, USA).

RNA Immunoprecipitation (RIP) Assays

An RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was used for RIP assay. Cells were lysed with RIP lysis buffer. Magnetic beads and human Anti-Ago2 (ab32381; Abcam, Cambridge, UK) or negative control Anti-IgG were added to incubate the cell lysates. The co-precipitated RNAs were isolated and purified for qRT-PCR.

RNA Pull-Down Assay

Biotinylated miRNAs (Bio-miR-NC or Bio-miR-431-5p) were obtained from Sangon (Shanghai, China). CAL27 and SCC4 cells transfected with Bio-miR-NC or Bio-miR-431-5p were lysed and incubated with streptavidin magnetic beads (Thermo Fisher Scientific, Waltham, MA, USA). After that, the level of circ_0001742 was detected by qRT-PCR.

Statistical Analysis

Data were presented as mean ± standard deviation. Statistical analysis was carried out by SPSS 22.0 (IBM, Armonk, NY, USA). Each experiment was repeated three times. The differences between two groups or among multiple groups were eval-

uated by Student's *t*-test or one-way analysis of variance (ANOVA) with the Tukey HSD post-hoc test. *p*-value <0.05 was considered as statistically significant.

Results

Circ_0001742 Was Upregulated in TSCC Tissues and Cells

To investigate whether circ_0001742 played a role in TSCC, we first determined the expression of circ_0001742 in TSCC tissues and cells. The qRT-PCR result revealed that the expression of circ_0001742 was significantly higher in TSCC tissues than that in normal tissues (Figure 1A). Besides, circ_0001742 was significantly upregulated in CAL27 and SCC4 cells when compared with HOK cells (Figure 1B). These results implied that circ_0001742 might play a crucial role in the progression of TSCC.

Knockdown of circ_0001742 Inhibited Proliferation, Migration, Invasion and EMT and Promoted Apoptosis in TSCC Cells

To further explore the role of circ_0001742 in TSCC, si-circ_0001742#1, si-circ_0001742#2 or si-circ_0001742#3 was transfected into CAL27 and SCC4 cells to silence circ_0001742. The expression of circ_0001742 was greatly reduced in CAL27 and SCC4 cells transfected with si-circ_0001742#1, si-circ_0001742#2 or si-circ_0001742#3 and lowest when transfected with si-circ_0001742#1 (Figure 2A). Hence, si-circ_0001742#1 was used for following experiments. MTT assay showed that knockdown of circ_0001742 repressed proliferation of both CAL27 and SCC4 cells (Figure 2B and C). Conversely, apoptosis was enhanced in CAL27 and SCC4 cells transfected with si-circ_0001742#1 (Figure 2D). Then, migration and invasion of CAL27 and SCC4 cells were detected by transwell assay. The results indicated that circ_0001742 knockdown suppressed migration and invasion of CAL27 and SCC4 cells (Figure 2E and F). In addition, the protein levels of EMT-related proteins (MMP9, vimentin, N-cadherin and E-cadherin) were measured by Western blot, and the results showed that MMP9, vimentin, and N-cadherin were decreased while E-cadherin was elevated in CAL27 and SCC4 cells transfected si-circ_0001742#1 (Figure 2G and H). These data suggested that circ_0001742 might play an oncogenic role in TSCC.

MiR-431-5p was a Direct Target of circ_0001742

Bioinformatics analysis identified miR-431-5p as a potential target of circ_0001742, and the putative binding sites between miR-431-5p and circ_0001742 were also predicated. To confirm the relationship between miR-431-5p and circ_0001742, WT-circ_0001742 and MUT-circ_0001742 luciferase reporter vectors were constructed (Figure 3A). The dual-luciferase assay indicated that the activities of luciferases were markedly reduced by miR-431-5p in CAL27 cells transfected with WT-circ_0001742 rather than MUT-circ_0001742 (Figure 3B).

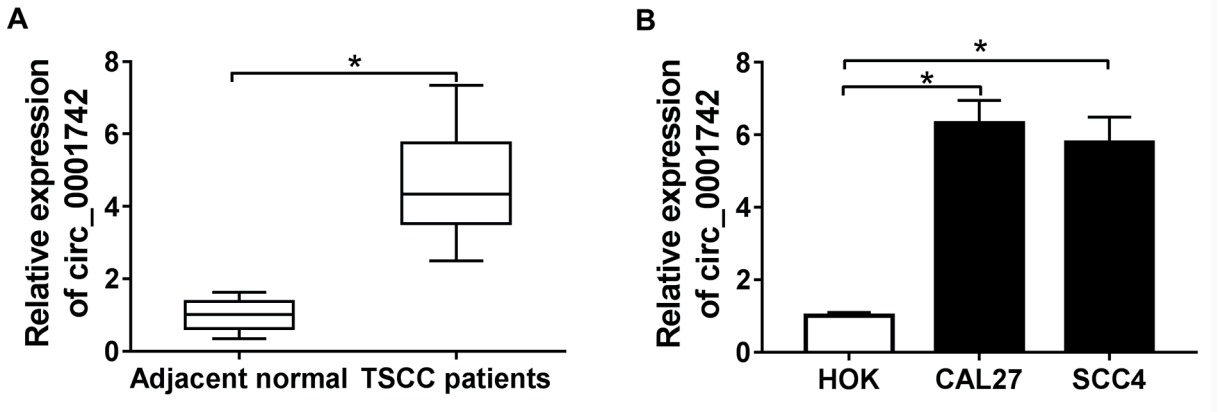

Figure 1. Circ_0001742 was upregulated in TSCC tissues cells. **A**, the expression of circ_0001742 in TSCC tissues and normal tissues was detected by qRT-PCR. **B**, the expression of circ_0001742 in human oral keratinocytes cell line (HOK) and TSCC cell lines (CAL27 and SCC4) was detected by qRT-PCR. *$p<0.05$.

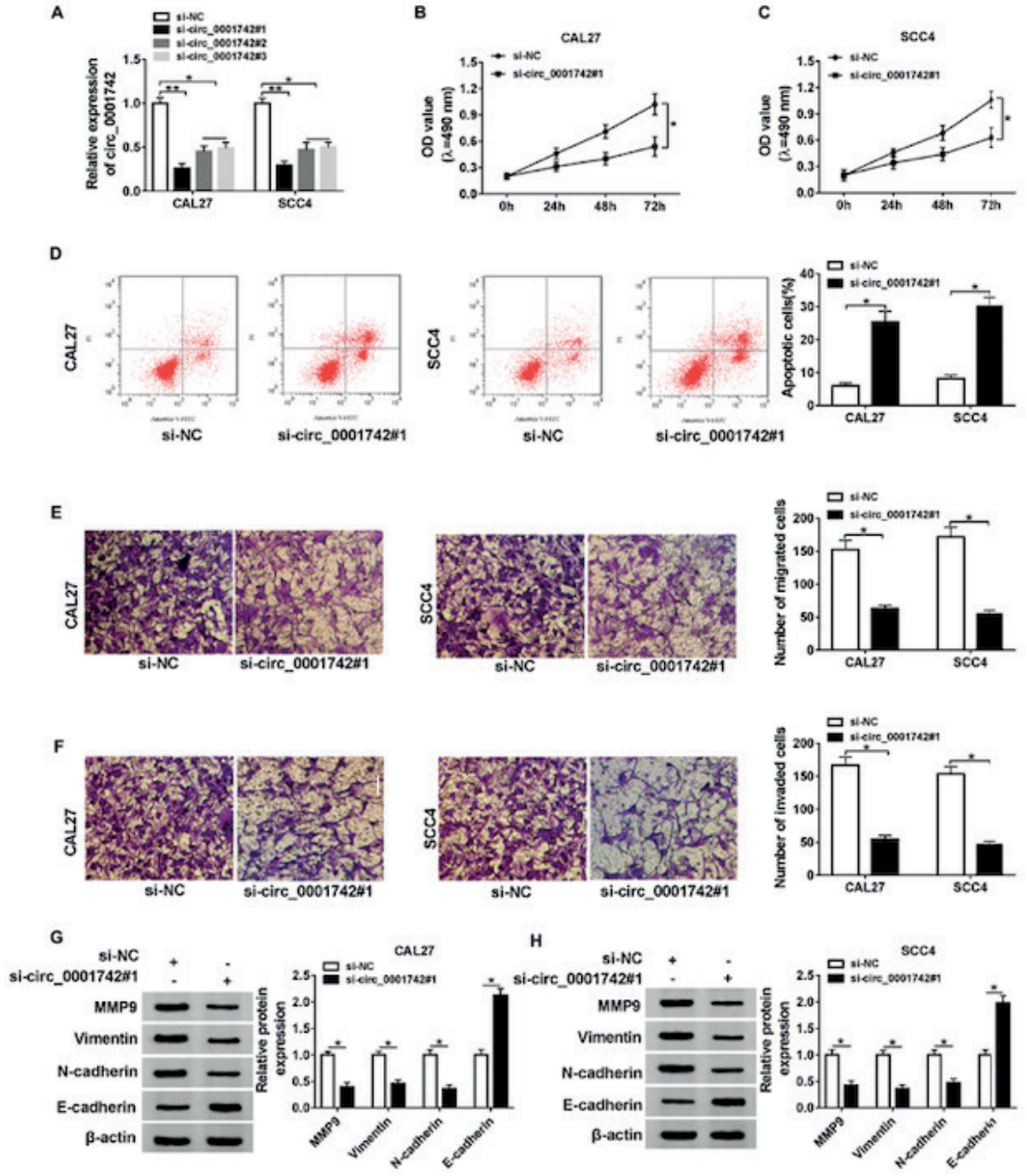

Figure 2. Knockdown of circ_0001742 suppressed proliferation, migration, invasion and EMT and induced apoptosis in TSCC cells. **A**, the expression of circ_0001742 was detected by qRT-PCR in CAL27 and SCC4 cells transfected with si-NC, si-circ_0001742#1, si-circ_0001742#2 or si-circ_0001742#3. **B** and **C**, proliferation of CAL27 and SCC4 cells transfected with si-NC or si-circ_0001742#1 was measured by MTT assay. **D**, apoptosis of CAL27 and SCC4 cells transfected with si-NC or si-circ_0001742#1 was detected by flow cytometry. **E** and **F**, transwell assay was used to assess migration and invasion in CAL27 and SCC4 cells transfected with si-NC or si-circ_0001742#1. **G** and **H**, the protein levels of MMP9, vimentin, N-cadherin and E-cadherin were detected by Western blot in CAL27 and SCC4 cells transfected with si-NC or si-circ_0001742#1. *p<0.05.

Also, the same result was found in SCC4 cells (Figure 3C). Subsequently, RIP assay was performed to confirm whether circ_0001742 could interact with miR-431-5p. The data displayed that the RNA enrichments of circ_0001742 and miR-431-5p were significantly increased when

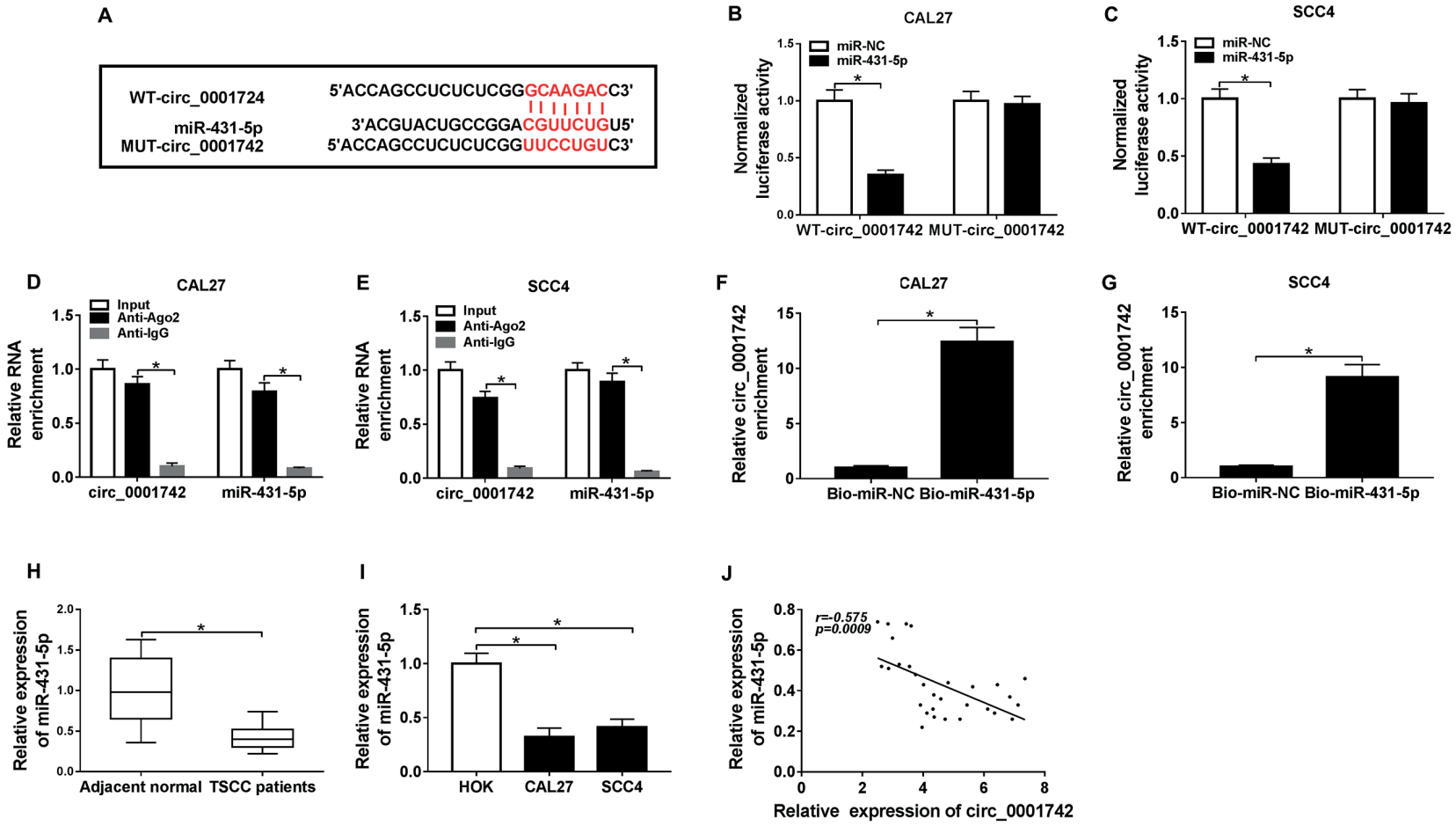

Figure 3. MiR-431-5p was a direct target of circ_0001742. **A**, The putative target sites between circ_0001742 and miR-431-5p were predicted by Circular RNA Interactome. **B** and **C**, The luciferase activities of WT-circ_0001742 and MUT-circ_0001742 were measured in CAL27 and SCC4 cells transfected with miR-NC or miR-431-5p. **D** and **E**, RIP assay was used to explore the interaction between circ_0001742 and miR-431-5p in CAL27 and SCC4 cells. **F** and **G**, RNA pull-down assay was used to confirm the targeted relationship between circ_0001742 and miR-431-5p in CAL27 and SCC4 cells. **H**, The expression of miR-431-5p in TSCC tissues and normal tissues was detected by qRT-PCR. **I**, The expression of miR-431-5p in HOK, CAL27 and SCC4 cells was detected by qRT-PCR. **J**, the correlation between the expression of circ_0001742 and miR-431-5p in TSCC tissues was analyzed. *$p<0.05$.

CAL27 and SCC4 cells were incubated with Anti-Ago2 compared with Anti-lgG (Figure 3D and E). Moreover, RNA pull-down assay showed that the expression of circ_0001742 was significantly elevated when CAL27 and SCC4 cells were transfected with Bio-miR-431-5p (Figure 3F and G). These results indicated that circ_0001742 could bind to miR-431-5p. In addition, we detected the expression of miR-431-5p in TSCC tissues and cells. The results revealed that miR-431-5p was downregulated in TSCC tissues and cells (Figure 3H and I). Besides, the expression of miR-431-5p was negatively correlated with the expression of circ_0001742 in TSCC tissues (Figure 3J).

Silencing of miR-431-5p Reversed the Effects of circ_0001742 Knockdown on Proliferation, Apoptosis, Migration, Invasion and EMT in TSCC Cells

To further investigate the interaction between circ_0001742 and miR-431-5p in TSCC cells, CAL27 and SCC4 cells were transfected with si-NC, si-circ_0001742#1, si-circ_0001742#1 + inhibitor-NC or si-circ_0001742#1 + miR-431-5p inhibitor. The expression of miR-431-5p was enhanced in CAL27 and SCC4 cells transfected with si-circ_0001742#1 and reversed when co-transfected with miR-431-5p inhibitor (Figure 4A). Proliferation was suppressed by knockdown of circ_0001742 in both CAL27 and SCC4 cells but rescued by miR-431-5p inhibitor (Figure 4B and C). Moreover, apoptosis of CAL27 and SCC4 cells was induced by si-circ_0001742#1 and restored by si-circ_0001742#1 + miR-431-5p inhibitor (Figure 4D). Also, the inhibiting effects of circ_0001742 knockdown on migration and invasion of CAL27 and SCC4 cells were abolished by silencing miR-431-5p (Figure 4E and F). The protein levels of MMP9, vimentin and N-cadherin were downregulated in CAL27 and SCC4 cells transfected with si-circ_0001742#1 and recovered when transfected with si-circ_0001742#1 + miR-431-5p inhibitor; results showed that there was an inverse effect on the expression of E-cadherin (Figure 4G and H). These results suggested that the downregulation of miR-431-5p restored the effect of circ_0001742 knockdown on TSCC cells progression.

MiR-431-5p Targeted ATF3 and Regulated ATF3 Expression

MiRNAs have been proved to play functions via regulating target gene expression. StarBase 3.0 predicated ATF3 as a potential target of miR-431-5p. Then ATF3 3'UTR-WT and ATF3 3'UTR-MUT were cloned and inserted into pGL3 luciferase reporter vectors (Figure 5A). The luciferase activity of ATF3 3'UTR-WT was remarkably reduced in CAL27 and SCC4 cells transfected with miR-431-5p, while the luciferase activity of ATF3 3'UTR-MUT had no change (Figure 5B and C). The mRNA and protein levels of ATF3 in CAL27 and SCC4 cells were downregulated by miR-431-5p (Figure 5D and E). In addition, the expression of ATF3 in TSCC tissues and normal tissues was detected, and the result revealed that the mRNA and protein levels of ATF3 were remarkably increased in TSCC tissues (Figure 5F and G). Also, ATF3 was significantly upregulated in CAL27 and SCC4 cells when compared with HOK cells (Figure 5H and I). The mRNA level of ATF3 was negatively correlated with the expression of miR-431-5p and positively correlated with the expression of circ_0001742 (Figure 5J and K).

Overexpression of ATF3 Rescued the miR-431-5p-Mediated Effects on Proliferation, Apoptosis, Migration, Invasion and EMT in TSCC Cells

To further investigate the interaction between miR-431-5p and ATF3 in TSCC cells, CAL27 and SCC4 cells were transfected with miR-NC, miR-431-5p, miR-431-5p + pcDNA or miR-431-5p + pcDNA-ATF3. The mRNA and protein levels of ATF3 were decreased in CAL27 and SCC4 cells transfected with miR-431-5p and restored when transfected with miR-431-5p + pcDNA-ATF3 (Figure 6A and B). Then MTT assay indicated the proliferation was inhibited by miR-431-5p and rescued by ATF3 overexpression in CAL27 as well as SCC4 cells (Figure 6C and D). The enhanced apoptosis of CAL27 and SCC4 cells, which mediated by miR-431-5p, was reversed by overexpression of ATF3 (Figure 6E). Additionally, the migration and invasion were suppressed by miR-431-5p in CAL27 and SCC4 cells, and overexpression of ATF3 recovered this effect (Figure 5F and G). MMP9, vimentin and N-cadherin were downregulated while E-cadherin was upregulated in CAL27 cells transfected with miR-431-5p and reverted when co-transfected with pcDNA-ATF3 (Figure 6H and I); further, the same trend was found in SCC4 cells (Figure 6J and K). These results revealed that overexpression of ATF3 recovered the miR-431-5p-mediated effects in TSCC cells.

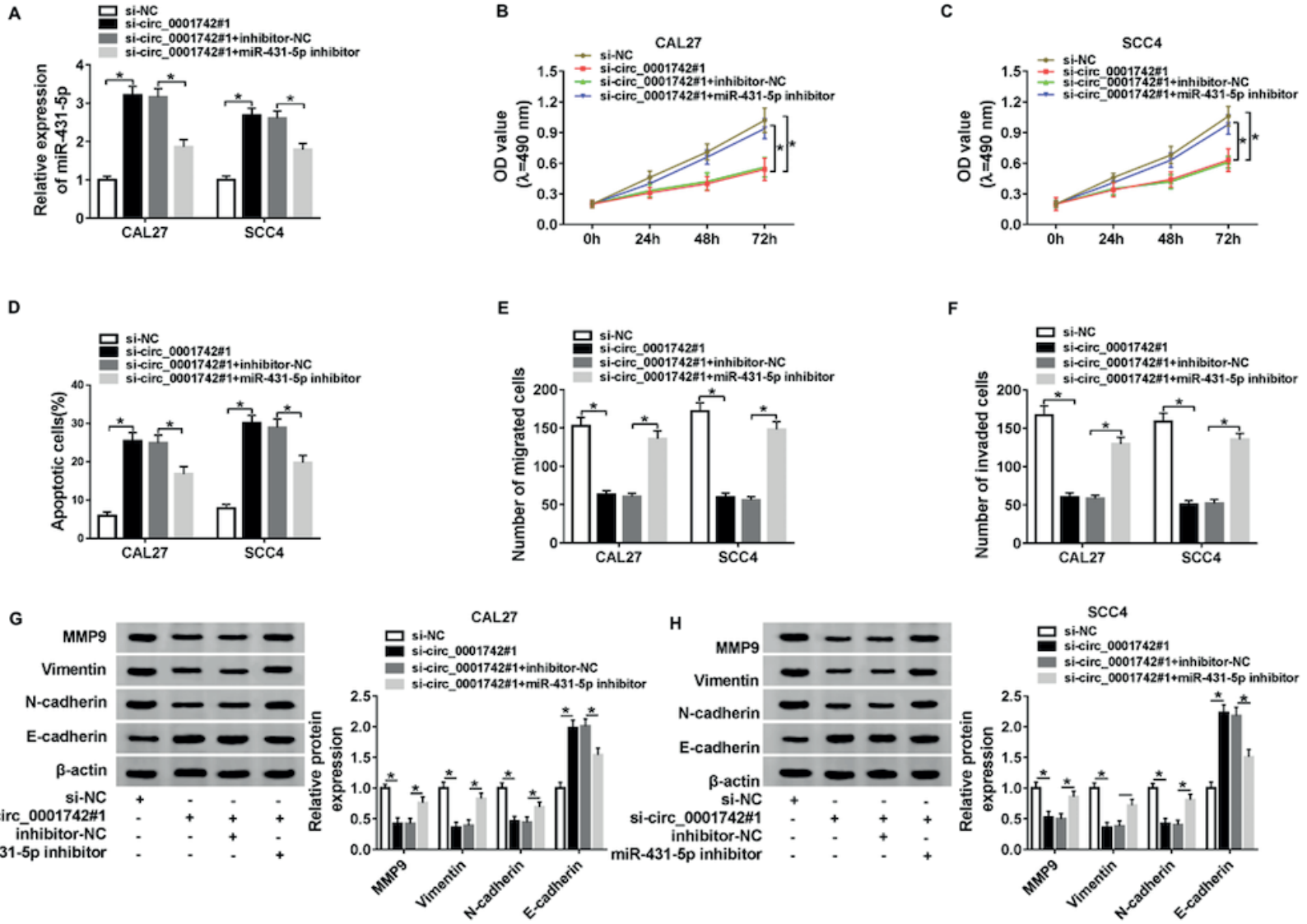

Figure 4. Silencing of miR-431-5p reversed the effects of circ_0001742 knockdown on proliferation, apoptosis, migration, invasion and EMT in TSCC cells. CAL27 and SCC4 cells were transfected with si-NC, si-circ_0001742#1, si-circ_0001742#1 + inhibitor-NC or si-circ_0001742#1 + miR-431-5p inhibitor. **A**, The expression of miR-431-5p was detected by qRT-PCR. **B** and **C**, Proliferation of CAL27 and SCC4 cells was measured by MTT assay. **D**, Apoptosis of CAL27 and SCC4 cells was detected by flow cytometry. **E** and **F**, Migration and invasion of CAL27 and SCC4 cells were assessed by transwell assay. **G** and **H**, The protein levels of MMP9, vimentin, N-cadherin and E-cadherin were detected by Western blot. *$p<0.05$.

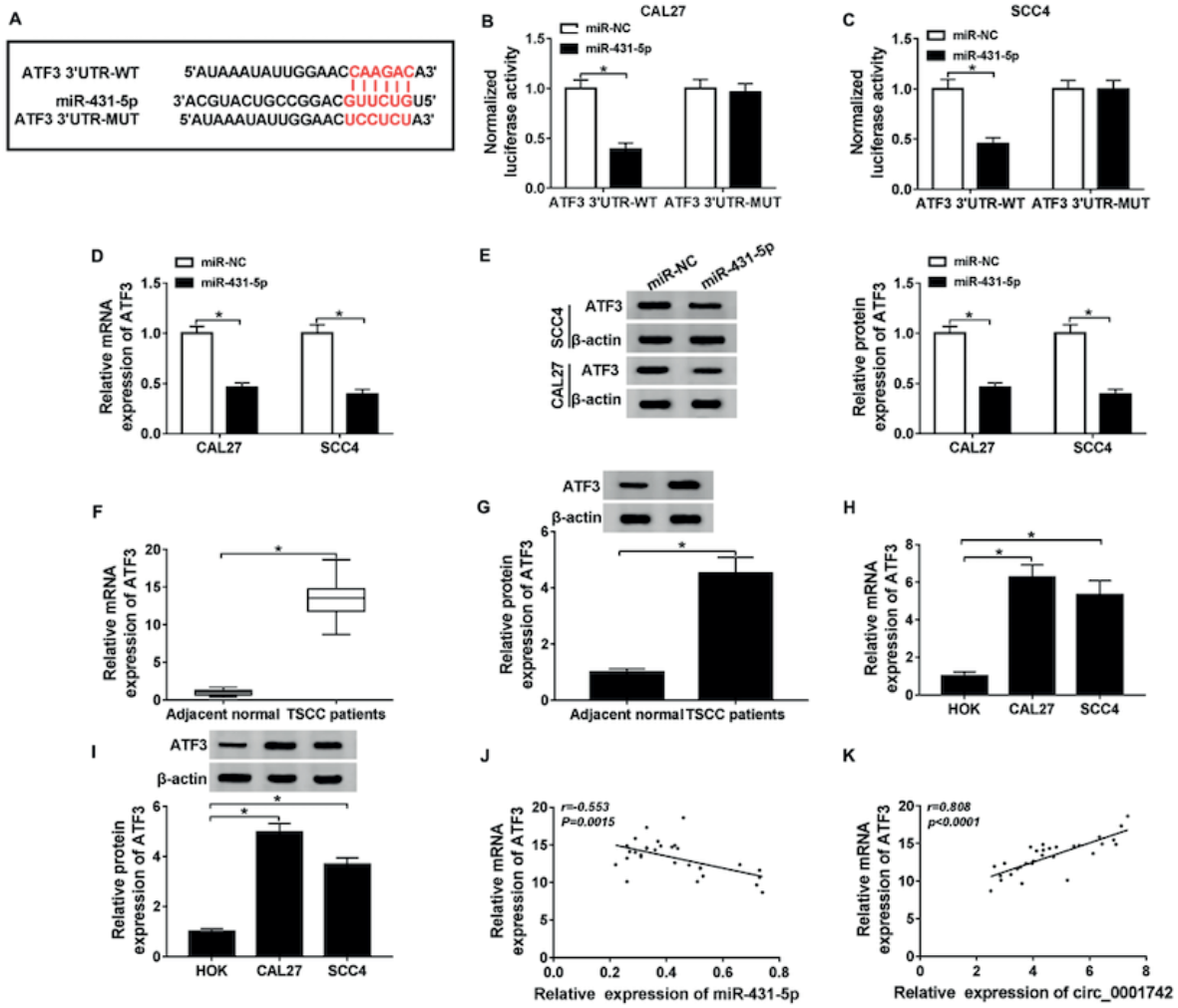

Figure 5. MiR-431-5p regulated ATF3 expression by target the 3' UTR of ATF3. **A**, The putative target sites between miR-431-5p and ATF3 were predicted by starBase 3.0. **B** and **C**, The luciferase activities of ATF3 3'UTR-WT and ATF3 3'UTR-MUT were measured in CAL27 and SCC4 cells transfected with miR-NC or miR-431-5p. **D**, The mRNA level of ATF3 was detected by qRT-PCR in CAL27 and SCC4 cells transfected with miR-NC or miR-431-5p. **E**, The protein level of ATF3 was detected by western blot in CAL27 and SCC4 cells transfected with miR-NC or miR-431-5p. **F** and **G**, The mRNA and protein levels of ATF3 were detected in TSCC tissues and normal tissues. **H** and **I**, The mRNA and protein levels of ATF3 were detected in HOK, CAL27 and SCC4 cells. **J**, The correlation between the levels of miR-431-5p and ATF3 mRNA in TSCC tissues was analyzed. **K**, The correlation between the levels of circ_0001742 and ATF3 mRNA in TSCC tissues was analyzed. *$p<0.05$.

Circ_0001742 Regulated ATF3 Expression Through Sponging miR-431-5p in TSCC Cells

To explore the regulating mechanism among circ_0001742, miR-431-5p and ATF3 in TCCC cells, the mRNA and protein levels of ATF3 were detected in CAL27 and SCC4 cells transfected with si-NC, si-circ_0001742#1, si-circ_0001742#1 + inhibitor-NC or si-circ_0001742#1 + miR-431-5p inhibitor. The qRT-PCR result showed that the mRNA level of ATF3 was reduced by knockdown of circ_0001742, and silencing miR-431-5p reversed the effect in CAL27 and SCC4 cells (Figure 7A). The western blot result was in keeping with the qRT-PCR result (Figure 7B). These results indicated that circ_0001742 regulated ATF3 expression via miR-431-5p in TSCC cells.

Discussion

Emerging evidence indicated that circRNAs are key regulators in the progression and development of oral cancer. For instance, the expression of circRNA_100290 was elevated in oral squamous cell carcinomas, and silencing of

circRNA_100290 impeded oral squamous cell carcinomas cells proliferation[27]. Low expression of circ_0007059 was associated with poor prognosis, and overexpression of circ_0007059 inhibited cell growth in oral squamous cell carcinoma [28]. However, there are little studies about the

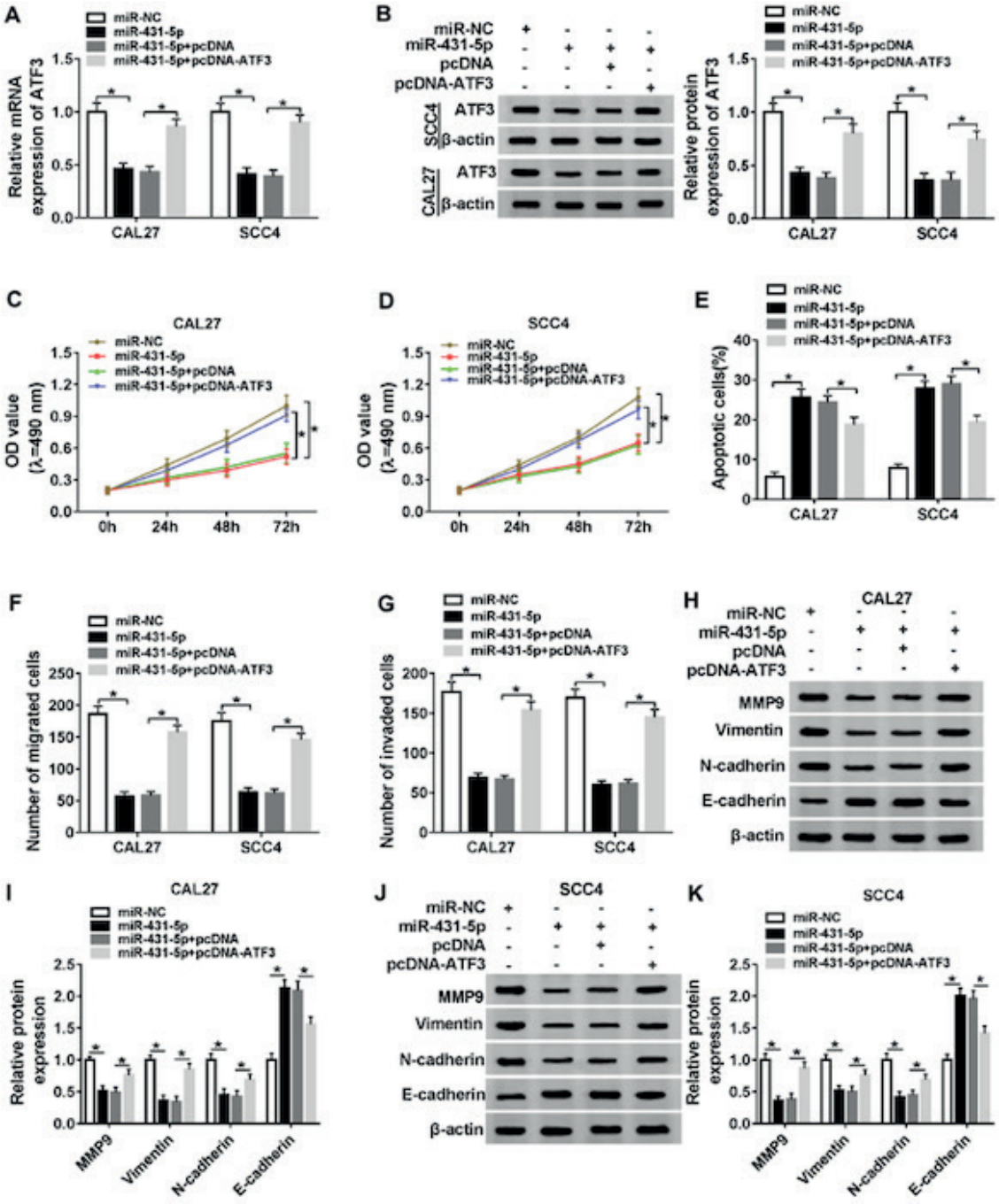

Figure 6. Overexpression of ATF3 rescued the effects of miR-431-5p on proliferation, apoptosis, migration invasion and EMT in TSCC cells. CAL27 and SCC4 cells were transfected with miR-NC, miR-431-5p, miR-431-5p + pcDNA or miR-431-5p + pcDNA-ATF3. **A**, The mRNA level of ATF3 was detected by qRT-PCR. **B**, The protein level of ATF3 was detected by western blot. **C** and **D**, Proliferation of CAL27 and SCC4 cells was measured by MTT assay. **E**, Apoptosis of CAL27 and SCC4 cells was detected by flow cytometry. **F** and **G**, Migration and invasion of CAL27 and SCC4 cells were assessed by transwell assay. **H** and **I**, The protein levels of MMP9, vimentin, N-cadherin and E-cadherin were detected by Western blot in CAL27 cells. **J** and **K**, The protein levels of MMP9, vimentin, N-cadherin and E-cadherin were detected by western blot in SCC4 cells. *p<0.05.

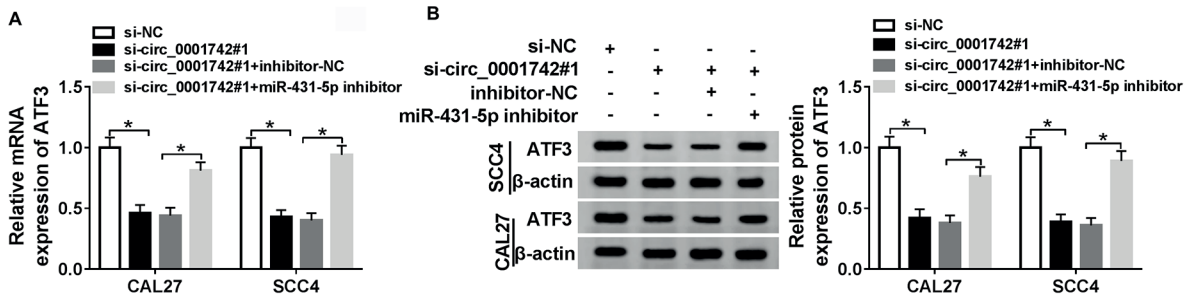

Figure 7. Circ_0001742 regulated ATF3 expression through sponging miR-431-5p in TSCC cells. CAL27 and SCC4 cells were transfected with si-NC, si-circ_0001742#1, si-circ_0001742#1 + inhibitor-NC or si-circ_0001742#1 + miR-431-5p inhibitor. **A**, The mRNA level of ATF3 was detected by qRT-PCR. **B**, The protein level of ATF3 was detected by Western blot. *p<0.05.

functions of circRNAs on TSCC. Pin et al[29] identified 322 differentially expressed circRNAs in TSCC tissues compared with the normal tissues; among them, circ_0001742 was significantly increased in TSCC tissues. Shao and He[30] reported that circ_0001742 facilitated the progression of TSCC. In agreement with these studies, our data exhibited that circ_0001742 expression was elevated in TSCC cells, and knockdown of circ_0001742 suppressed proliferation, migration and invasion and facilitated apoptosis in TSCC cells. In addition, the level of metastasis-related proteins was determined. MMP9 has been shown to promote tumor metastasis and induce EMT[31]. Vimentin, a canonical marker of EMT, has been reported to contribute to the cancer progression[32]. N-cadherin and E-cadherin play key roles in cell-cell adhesion. Overexpressed N-cadherin downregulated E-cadherin and enhanced cell migration[33]. In our study, knockdown of circ_0001742 decreased the levels of MMP9, vimentin and N-cadherin and upregulated E-cadherin, suggesting that circ_0001742 played an oncogenic function in TSCC.

CircRNAs have been elucidated to regulate miRNAs expression by acting as competing endogenous RNA (ceRNAs)[34]. CircRNAs, long non-coding RNAs (lncRNAs) and mRNA can competitively bind to the same miRNA, thereby forming a ceRNA network to regulate the progression of cancers. Here, we identified miR-431-5p as the downstream target of circ_0001742. MiR-431-5p was downregulated and exerted a tumor inhibition effect in lung cancer[35]. Overexpression of miR-431-5p repressed EMT in hepatoma cells[36]. Besides, the expression of miR-431 was reduced in hepatocellular carcinoma, papillary thyroid carcinoma and pancreatic cancer[37-39]. Nevertheless, the effect of miR-431-5p in TSCC has not been reported. We found that the expression of miR-431-5p was decreased in TSCC tissues and cells. Silencing of miR-431-5p rescued the inhibiting effects of circ_0001742 on TSCC cells, implying that miR-431 served as an anti-oncogene in TSCC.

Usually, ATF3 has a low expression under normal conditions, while it can be immediately induced by various stress signals[40]. Many reports have demonstrated that ATF3 is associated with tumor development, but the role of ATF3 is controversial. It seems to play different functions in different cell types and contexts[41]. ATF3 was reported to suppress cell proliferation in hepatocellular carcinoma[25]. Conversely, ATF3 can be an oncogene in breast cancer[21]. As for the role of ATF3 in oral cancers, Wang et al[42] revealed that overexpression of ATF3 in mice promoted tumorigenesis of oral squamous cell carcinoma. In TSCC, ATF3 was found to be upregulated, and ATF3 knockdown impeded EMT and invasion[23]. Our data also showed an increased level of ATF3 in TSCC tissues and cells. And overexpressed ATF3 reversed the inhibiting effect mediated by miR-431-5p in TSCC cells, indicating the tumor-promoting role of ATF3, which was consistent with Shi et al[23].

Conclusions

An augmented circ_0001742 expression was showed in TSCC tissues, and then the effects of circ_0001742 in TSCC cells were investigated by knockdown of circ_0001742. The results showed that knockdown of circ_0001742 inhibited proliferation, migration, invasion and EMT and enhanced apoptosis in TSCC cells. Additionally, miR-431-5p was identified as a target of

circ_0001742, and ATF3 was indicateded to be a downstream target of miR-431-5p. Restoration experiments demonstrated that circ_0001742 restrained TSCC cell progression by regulating ATF3 expression through miR-431-5p. Our results may provide a new light for the improvement of diagnosis and treatment strategies of TSCC.

Conflict of Interests

The Authors declare that they have no conflict of interests.

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