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MiR-671-5p sponging activity of circMMP1 promotes esophageal squamous cancer progression

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Abstract

Background: The aim of this study was to explore the function and mechanism of circular RNA (circRNA) matrix metallopeptidase 1 (circMMP1) in the progression of esophageal squamous cell carcinoma (ESCC).

Methods: CircMMP1 expression was detected by quantitative real-time PCR (qRT-PCR), and its relationship with the prognosis of ESCC patients was evaluated by Kaplan–Meier analysis. Cells were transfected using corresponding plasmids, and the cell proliferation activity, migration and invasion capabilities in vitro were assessed. The protein level in tissues and cells was analyzed using western blotting. RNA pull-down, dual-luciferase reporter assay and RNA immunoprecipitation assay were performed in ESCC cells to detect the interaction between circMMP1 and miR-671-5p, or the correlation between miR-671-5p and ANO1. Xenograft tumor experiment was carried out to uncover the function of circMMP1 in vivo.

Results: The high level of circMMP1 in tumor tissues was associated with poor prognoses of ESCC patients. Knockdown of circMMP1 suppressed ESCC cell proliferation, migration and invasion in vitro. MiR-671-5p was the target of circMMP1 and mediated the inhibition effect of circMMP1 on ESCC cells. CircMMP1 targeted miR-671-5p to regulate ANO1 expression, which was downstream of miR-671-5p. Overexpression of ANO1 weakened tumor-repressive function of circMMP1 knockdown in ESCC cells. Moreover, silencing of circMMP1 impeded ESCC tumor growth in vivo.

Conclusion: Our study provided novel evidence that circMMP1 accelerated ESCC progression by acting as a miR-671-5p sponge to enhance ANO1 expression.

KEYWORDS
ANO1, circMMP1, ESCC, miR-671-5p

INTRODUCTION

Esophageal squamous cell carcinoma (ESCC) is the principal histological type of esophageal cancer in China.[1] ESCC has a high malignancy and lack of typical early symptoms and precise biomarkers, which results in most patients not being diagnosed until middle or late stages.[2,3] It is estimated that less than 30% of ESCC patients survive 5 years despite multidisciplinary therapy.[4] With the advent of precision medicine, revealing the pathogenesis of cancer at the molecular level and targeting therapy for patients have become an important development direction of tumor therapy. Therefore, identification of the therapeutic targets in ESCC for improving the prognosis of patients is of great clinical significance.

Circular RNAs (circRNAs) are not affected by RNA exonuclease and present greater stability then linear noncoding RNA.[5] CircRNAs have previously been indicated to be expressed in a tissue and cell-specific manner in various physiological and pathological conditions, which is significantly related to the progression of numerous diseases, including malignancies.[6–8] MicroRNAs (miRNAs), which further influence the biological activity and function of their target mRNAs, is the most classical functional model of

circRNA.[9] For instance, in bladder cancer, circPPP1CB inhibited the malignant progression by regulating miR-1307-3p/SMG1 axis.[10] Circ_0043280 regulated RAQR3 expression by interacting with miR-203-3p and played an antitumor effect in cervical cancer.[11] CircLGMN accelerated glioma cell proliferation and invasion through relieving the degradation of LGMN by miR-127-3p.[12] Moreover, circ_0000277 and circ_0006948 have previously been reported to be highly expressed in ESCC and participate in tumorigenesis of ESCC via the circRNA-miRNA-mRNA network.[13,14] CircMMP1 (hsa_circ_0024108) located on chr11: 102660640-102 667 893 with a length of 1562 nt, has been identified to be upregulated in ESCC tissues by circRNA microarray analysis and qRT-PCR.[15] In addition, circMMP1 facilitated glioma progression via enhancing HMGB3 level by sponging miR-433.[16] However, the functions and mechanisms of circMMP1 in ESCC are still unclear.

Herein, we verified that circMMP1 was overexpressed in ESCC tissues and cells, and exerted cancer-promoting effect by regulating miR-671-5p/ANO1 axis, providing evidence for the involvement of circMMP1 in ESCC progression.

METHODS

Tissue sample collection

Human ESCC tissue samples, including 77 tumor tissues samples and 77 matched adjacent normal tissues, were collected from ESCC patients who had undergone surgery at The First Affiliated Hospital of Xi'an Jiaotong University from 2018 to 2020 with written informed consent. Patients had not received chemoradiotherapy or immunotherapy prior to radical operation. The present study received the approval of the Ethics Committee of The First Affiliated Hospital of Xi'an Jiaotong University.

Cell culture and transfection

The Het-1A cells, representative of normal human esophageal cells, and four ESCC cell lines (ECA-109, KYSE-180, KYSE-150, and KYSE-30) were acquired from Chuan Qiu Biotechnology (Shanghai, China). Cells were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) from Gibco. Short hairpin RNA (shRNA) targeting circMMP1 (sh-circMMP1) and nonsense shRNA (sh-NC) were acquired from Ribobio and transfected into lentivirus vectors. miRNA mimics (miR-NC), miR-671-5p mimics (miR-671-5p), miRNA inhibitors (in-miR-NC), or miR-671-5p inhibitors (in-miR-671-5p) were purchased from Sangon. The ANO1 overexpression plasmid (pcDNA-ANO1) and pcDNA empty plasmid (pcDNA-NC) were provided by GenePharma. Transfection was conducted using Lipofectamine 2000 (Invitrogen).

Quantitative real-time PCR (qRT-PCR)

The TRIzol method was used to extract RNA and Takara reverse transcription kit to reverse transcribe the RNA. Next, quantitative real-time polymerase chain reaction (qRT-PCR) was done on an ABI real-time PCR system utilizing the SYBR Green method. Finally, circMMP1, miR-671-5p and ANO1 mRNA levels were determined by the $2^{-\Delta\Delta ct}$ method. The sequences of primers used in this experiment are presented in Table 1.

Cell proliferation assays

For cell counting kit-8 (CCK-8) assay, cells (4×10^3/well) were added to a 96-well plate and treated with CCK-8 reagent for 2 h at designated time pints, respectively. Subsequently, absorbance at 450 nm was read. For the colony formation experiment, cells were collected and resuspended in DMEM media containing 10% FBS, and seeded (2×10^3 cells/well) in six-well plates. Then, 10–15 days after incubation, paraformaldehyde was applied to fix the colonies and crystal violet was exploited for staining. Finally, colonies with over 50 cells were counted. For 5-ethynyl-2′-deoxyuridine (EdU) staining assay, cells were exposed to an EdU solution for 2 h, followed by staining with 4′,6-diamidino-2-phenylindole (DAPI). The EdU positive rates represented the proliferative ability of cells.

TABLE 1 Primers sequences used for qRT-PCR.

Name		Primers for qRT-PCR (5′-3′)
circMMP1	Forward	CCTGGATAGGCAAGGGATAA
	Reverse	TTGGAAGGCTTTCTCAATGG
miR-556-5p	Forward	GTATGAGATGAGCTCATTGTAA
	Reverse	CTCAACTGGTGTCGTGGA
miR-217	Forward	GTATGATACTGCATCAGGAACTG
	Reverse	CTCAACTGGTGTCGTGGA
miR-654-3p	Forward	GTATGATATGTCTGCTGACCATG
	Reverse	CTCAACTGGTGTCGTGGA
miR-324-5p	Forward	GTATGACGCATCCCCTAGGGCATT
	Reverse	CTCAACTGGTGTCGTGGA
miR-671-5p	Forward	GTATGAAGGAAGCCCTGGAGGGGC
	Reverse	CTCAACTGGTGTCGTGGA
miR-769-5p	Forward	GTATGATGAGACCTCTGGGTTCT
	Reverse	CTCAACTGGTGTCGTGGA
ANO1	Forward	GCCAAGTTTGGCTACAGCAC
	Reverse	AGACTAGGGAGCGACGAAGT
GAPDH	Forward	GGAGCGAGATCCCTCCAAAAT
	Reverse	GGCTGTTGTCATACTTCTCATGG
U6	Forward	GCTTCGGCAGCACATATACTAA
	Reverse	AACGCTTCACGAATTTGCGT

Abbreviation: qRT-PCR, quantitative real-time polymerase chain reaction.

Transwell migration and invasion assay

Migration and invasion experiments were carried out with a 24-well transwell chamber (for migration, Corning) and transwell chamber precoated with Matrigel matrix (for invasion, Corning), respectively. Briefly, cells resuspended in 200 μL of DMEM with 1% FBS were seeded into the top chambers, while the bottom chambers were suffused with 600 μL of DMEM with 10% FBS. Following 24 h incubation, migratory and invading cells were cultured, fixed, stained and then photographed.

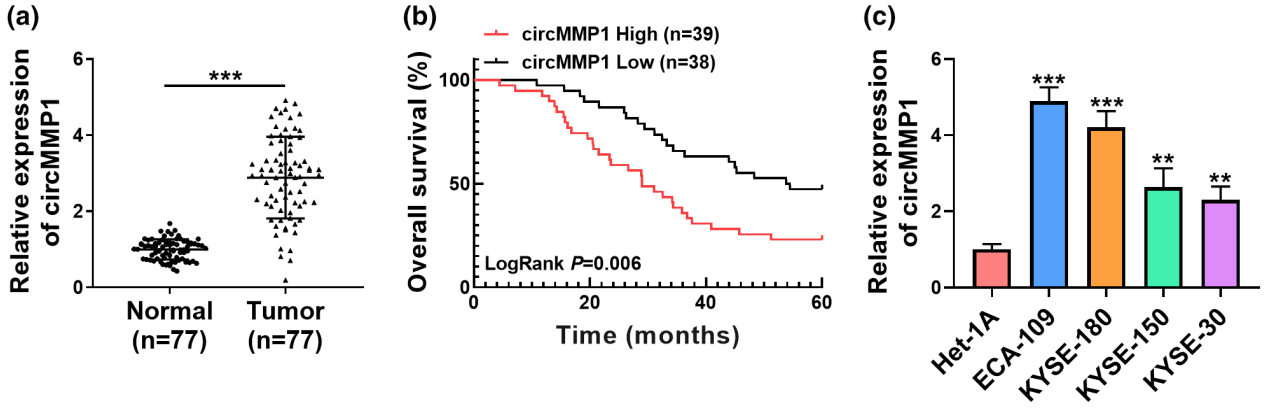

FIGURE 1 CircMMP1 was upregulated in esophageal squamous cell carcinoma (ESCC) tissues and cells. (a) Relative expression of circMMP1 in ESCC tissues and adjacent normal tissues was analyzed using quantitative real-time polymerase chain reaction (qRT-PCR). (b) Kaplan–Meier survival curve of overall survival in 77 ESCC patients with high or low circMMP1 expression. (c) Relative expression of circMMP1 in cells was detected by qRT-PCR. $^{**}p < 0.01$, $^{***}p < 0.001$.

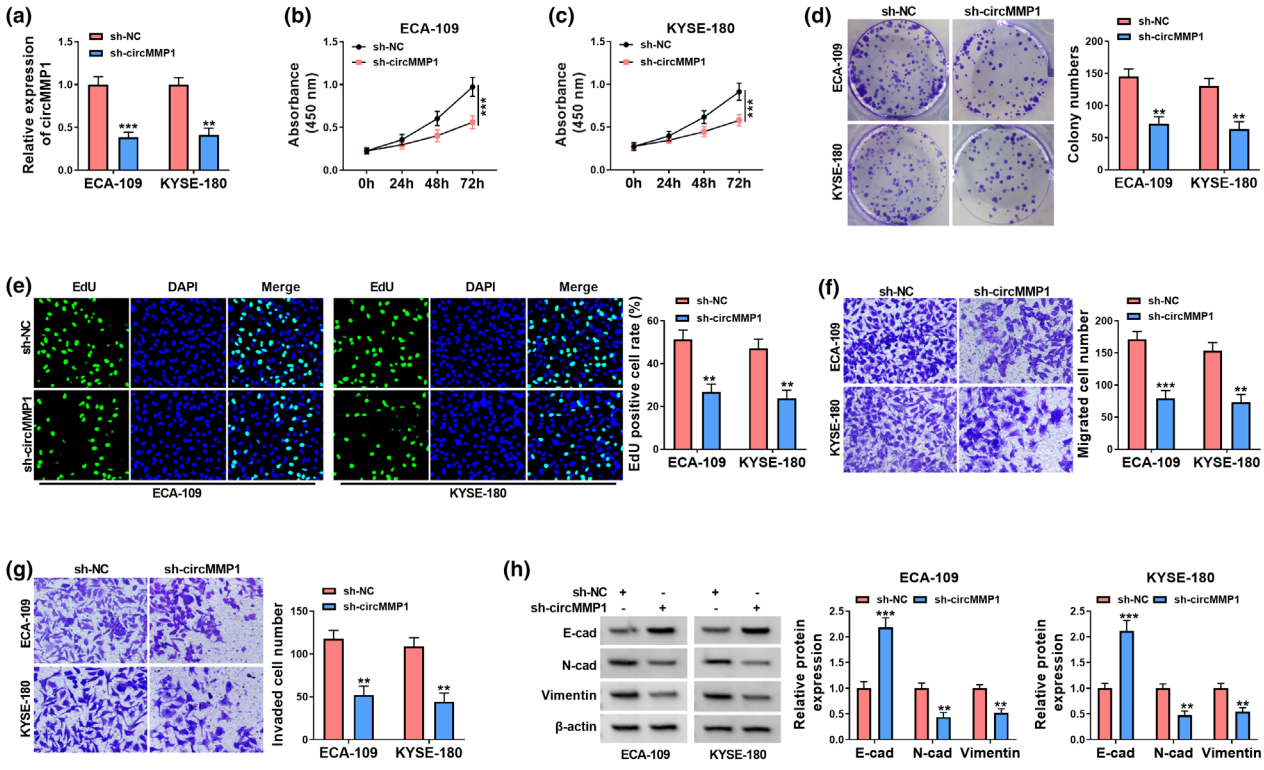

FIGURE 2 CircMMP1 silencing inhibited ESCC cell proliferation, migration and invasion in vitro. ECA-109 and KYSE-180 cells were transfected with sh-circMMP1 or sh-NC. (a) Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the transfection efficiency of sh-circMMP1 in ESCC cells. (b, c) The growth curves of cells were evaluated by cell counting kit-8 (CCK8). (d) Cell colony formation ability was assessed by colony formation assay. (e) Cell proliferation was determined by 5-ethynyl-2′-deoxyuridine (EdU) assay. (f, g) Cell migration and invasion were tested by transwell assay. (h) Western blot was executed to detect the protein expression of E-cad, N-cad and vimentin. $^{**}p < 0.01$, $^{***}p < 0.001$.

Western blotting assay

Total protein from tissues and cells extraction were performed by radioimmunoprecipitation assay (RIPA) lysates (Beyotime). Next, 10% SDS-PAGE electrophoresis was applied to detach proteins, and then immunoblotting was performed on polyvinylidenefluoride (PVDF) membranes after protein was transferred. Blocking membranes with 5% nonfat milk was followed by incubation with the primary antibodies: anti-E-cadherin (E-cad) (ab231303, 1:250, Abcam), anti-N-cadherin (N-cad) (ab245117, 1:1000, Abcam), anti-vimentin (ab16700, 1:100, Abcam), anti-ANO1 (ab84915, 1:1000, Abcam) or anti-β-actin (ab8227, 1:1000, Abcam), and then subjected to secondary antibodies incubation, and imaged with enhanced chemiluminescence (ECL) reagents (Bio-Rad).

RNA pulldown assay

To pulldown RNA, oligo probe and circMMP1 probe were obtained from Sangon. The lysate samples of ECA-109 and KYSE-180 cells were incubated with oligo probe and circMMP1 probe. Then, the RNA–RNA complex conjugated with streptavidin magnetic beads was purified with TRIzol, and the enrichments of miRNAs were analyzed by qRT-PCR.

Dual-luciferase reporter gene

CircMMP1 or ANO1 3′UTR wild-type fragments containing miR-671-5p complementary binding sites (circMMP1-WT or ANO1 3′UTR-WT) and the corresponding mutant

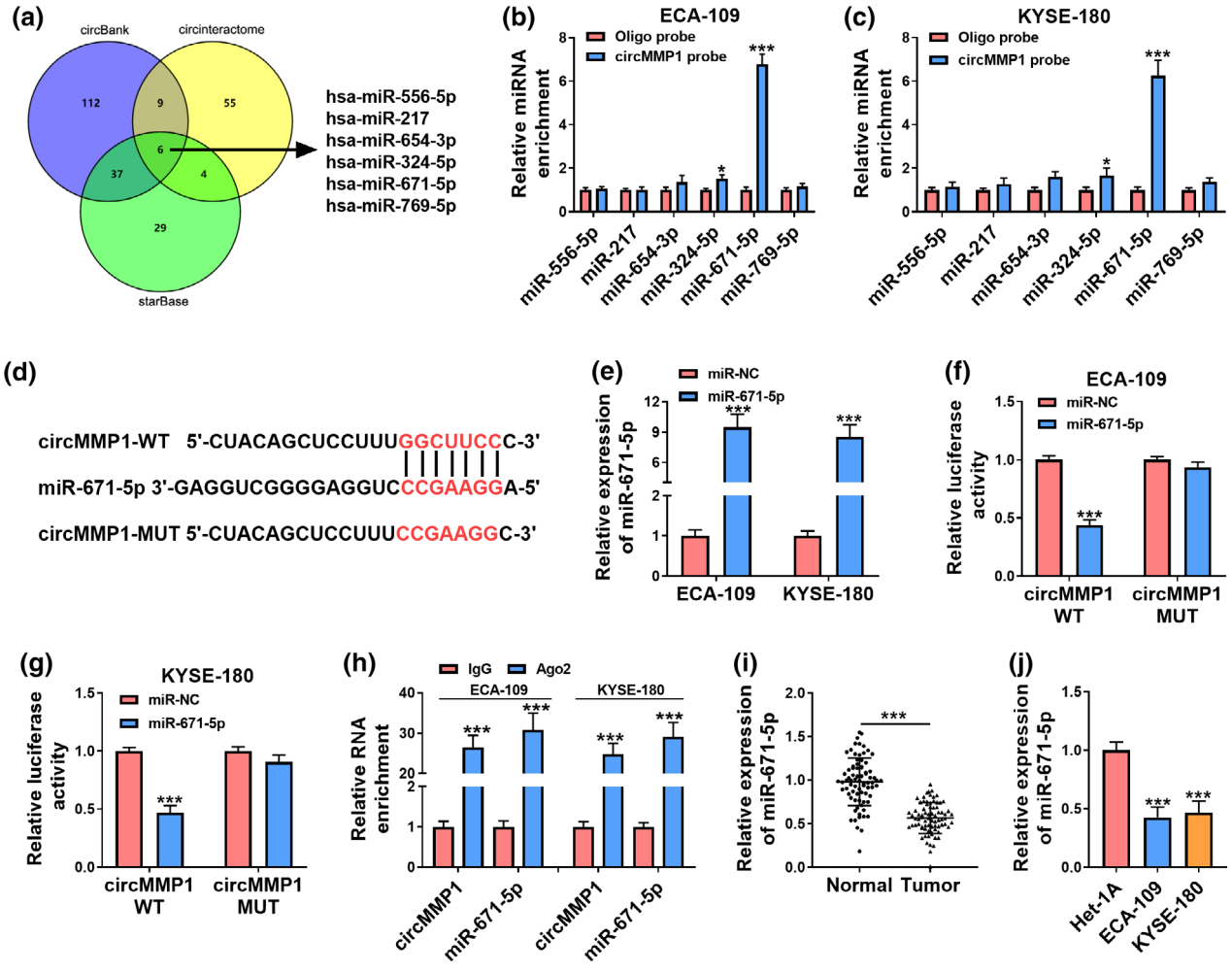

FIGURE 3 CircMMP1 targeted miR-671-5p. (a) Venn diagram for the potential target miRNAs of circMMP1. (b, c) The potential target miRNAs of circMPP1 were identified by RNA pulldown assay. (d) The miR-671-5p binding site on circMMP1 predicted by starBase. (e) Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the transfection efficiency of miR-671-5p mimics in esophageal squamous cell carcinoma (ESCC) cells. (f, g) The binding relationship between circMMP1 and miR-671-5p was verified by dual-luciferase reporter assay. (h) RNA immunoprecipitation (RIP) assay was carried out and the enrichments of circMMP1 and miR-671-5p were detected by qRT-PCR. (i) Relative expression level of miR-671-5p in ESCC tissues and adjacent normal tissues was analyzed by qRT-PCR. (j) Relative expression level of miR-671-5p in ESCC cells was detected by qRT-PCR. ***$p < 0.001$.

binding sequences (circMMP1-MUT or ANO1 3′UTR-MUT) were constructed luciferase vectors. Next, the above mentioned vectors and miRNA mimics or miR-671-5p mimics were cotransfected into ECA-109 and KYSE-180 cells. After 48 h, the luciferase assay system was applied to measure the relative luciferase activity.

RNA immunoprecipitation (RIP) assay

For RIP assay, ECA-109 and KYSE-180 cells were lysed and then hatched with Ago2 or IgG antibody-conjugated magnetic beads in RIP buffer. After treatment with proteinase K, the enrichments of circMMP1 and miR-671-5p in precipitated RNA were measured by qRT-PCR.

Animal experiments

A total of 10 BALB/c nude mice (6–8 weeks old) from Vital River Laboratory Animal Technology Co., Ltd., (Beijing, China) were subcutaneously injected with ECA-109 cells (5×10^6 cells) that were stably transfected with either sh-circMMP1 or sh-NC, with five mice per group. Tumor volumes were monitored every 5 days for a period of 25 days, after which all mice were sacrificed and the xenograft tumors were excised, weighed, and preserved for subsequent analysis.

For immunohistochemical (IHC) assay, the tissue slices were blocked with 5% goat serum for 30 min. Subsequently, the slices were incubated with ANO1 (ab84915, 1:200) or Ki67 (ab15580, 1:500, Abcam) antibody overnight at 4 °C, and then subjected to secondary antibody treatment for 30 min at room temperature.

Statistical analysis

Data analysis were performed using GraphPad Prism 8.0 software. Student's *t*-test one-way ANOVA was used to analyze the differences among groups. The overall survival of patients was analyzed by Kaplan–Meier curves. $p < 0.05$ was considered as a statistically significant difference between groups.

RESULTS

CircMMP1 is highly expressed in ESCC

We detected the expression of circMMP1 in 77 paired ESCC tissues and adjacent normal tissue samples. There was

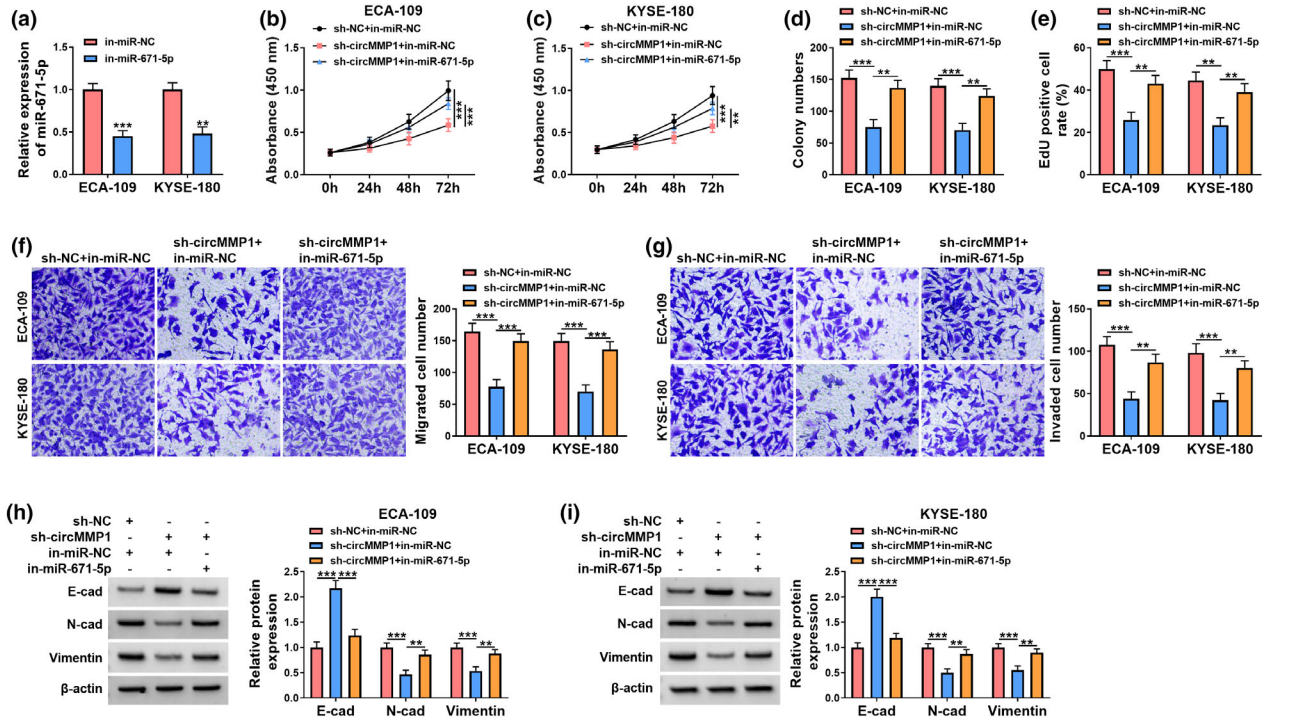

FIGURE 4 The inhibitory effects of circMMP1 knockdown on cell proliferation, migration and invasion of esophageal squamous cell carcinoma (ESCC) cells were partly reversed by miR-671-5p. (a) Quantitative real-time polymerase chain reaction (qRT-PCR) was used to detect the transfection efficiency of miR-671-5p inhibitors in ESCC cells. (b–i) ECA-109 and KYSE-180 cells were transfected with sh-NC + in-miR-NC, sh-circMMP1 + in-miR-NC or sh-circMMP1 + in-miR-671-5p. (b, c) The growth curves of cells were evaluated by cell counting kit-8 (CCK-8). (d) Cell clonogenic ability was assessed by colony formation assay. (e) Cell proliferation was determined by 5-ethynyl-2′-deoxyuridine (EdU) assay. (f, g) Cell migration and invasion were tested by transwell assay. (h) Western blot was executed to detect the protein levels of E-cad, N-cad and vimentin. $^{**}p < 0.01$, $^{***}p < 0.001$.

significantly increased expression of circMMP1 in ESCC tissues compared to adjacent normal tissues (Figure 1a). Kaplan–Meier survival analysis showed that the overall survival of ESCC patients with circMMP1 high expression were shorter in comparison with that in circMMP1 low expression group (Figure 1b). Consistently, circMMP1 expression was upregulated in ESCC cell lines (ECA-109, KYSE-180, KYSE-150 and KYSE-30) when compared with human normal esophageal epithelial cell line Het-1A (Figure 1c). These data confirmed that circMMP1 was highly expressed in ESCC and may exert an oncogenic role in ESCC.

Knockdown of circMMP1 inhibits cell proliferation, migration and invasion of ESCC in vitro

The biological function of circMMP1 in ESCC was studied by stably transfecting sh-circMMP1 or sh-NC into ECA-109 and KYSE-180 cells to silence circMMP1. The expression of circMMP1 was significantly decreased after transfection of sh-circMMP1 (Figure 2a). CCK-8 assay showed that circMMP1 knockdown suppressed cell viability (Figure 2b,c). In addition, cell clone formation was detected by clonogenic assay and the results showed that the cloning formation ability of cells was markedly inhibited (Figure 2d). Consistently, the EdU assay showed that circMMP1 knockdown depressed the proliferation of cells (Figure 2e). Moreover, the migrated and invaded cells were decreased in the sh-circMMP9 group compared with the sh-NC group (Figure 2f,g). Furthermore, EMT-associated protein (N-cad and vimentin) levels were decreased and E-cad was increased in the sh-circMMP1 group (Figure 2h). Collectively, these data suggested that circMMP1 knockdown inhibited cell proliferation, migration and invasion of ESCC. However, circMMP1 knockdown had no significant effect on Het-1A cell proliferation (Figure SS1).

CircMMP1 directly binds to miR-671-5p

It is well known that circRNA can act as a miRNA sponge to regulate the miRNA-targeted mRNA. In our study, the targets miRNA of circMMP1 were predicted by the circBank, Circinteractome and starBase online databases. The results

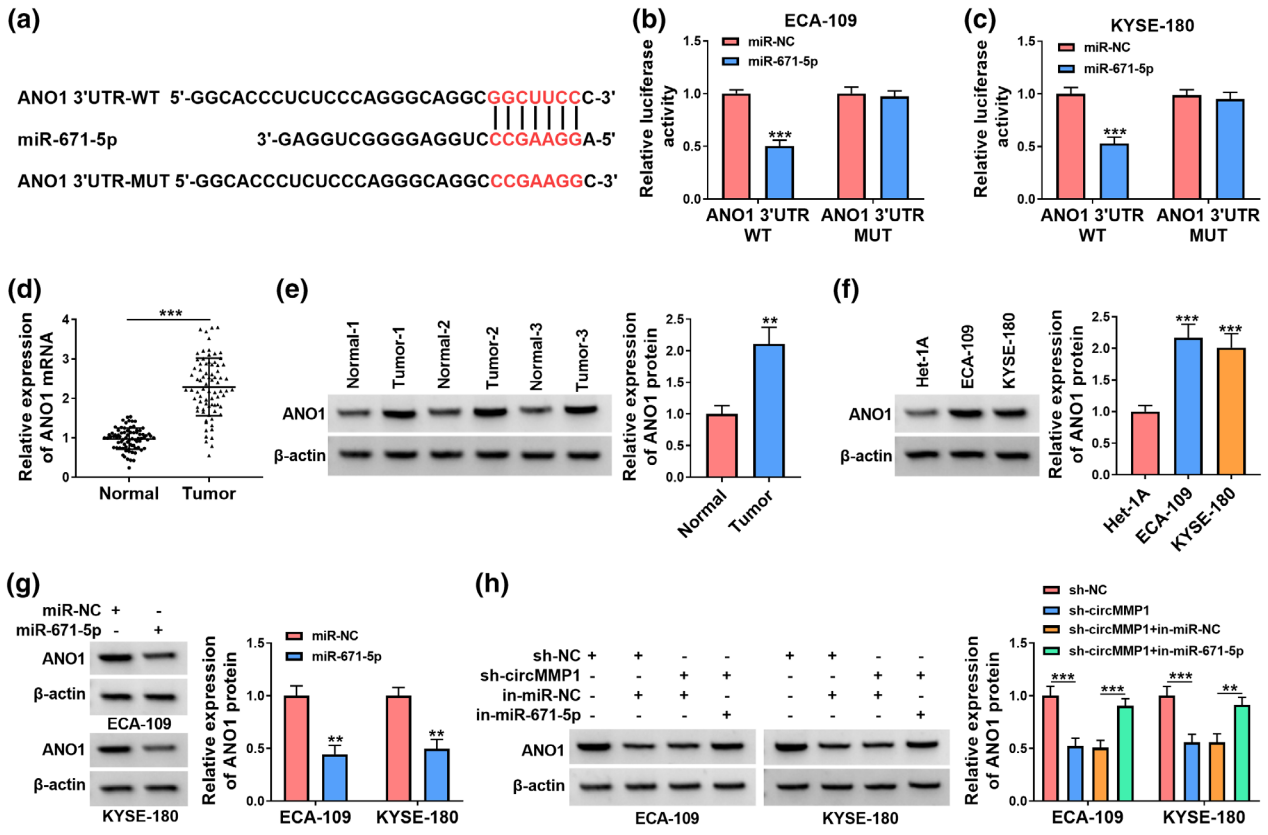

FIGURE 5 ANO1 was targeted by miR-671-5p and regulated by circMMP1. (a) The binding sites between miR-671-5p and ANO1 3′UTR were predicted by starBase. (b, c) The binding relationship between circMMP1 and miR-671-5p was verified by dual-luciferase reporter assay. (d) Relative expression level of ANO1 mRNA in esophageal squamous cell carcinoma (ESCC) tissues and adjacent normal tissues was analyzed by quantitative real-time polymerase chain reaction (qRT-PCR). (e) Western blot was used to analyze the protein of ANO1 in ESCC tissues and adjacent normal tissues. (f) Western blot was used to analyze the protein of ANO1 in cells. (g) The level of ANO1 in ESCC cells was determined by western blot after transfection of miR-671-5p mimics or miR-NC. (h) The level of ANO1 in ESCC cells was determined by western blot after transfection with sh-NC, sh-circMMP1, sh-circMMP1 + in-miR-NC or sh-circMMP1 + in-miR-671-5p. $^{**}p < 0.01$, $^{***}p < 0.001$.

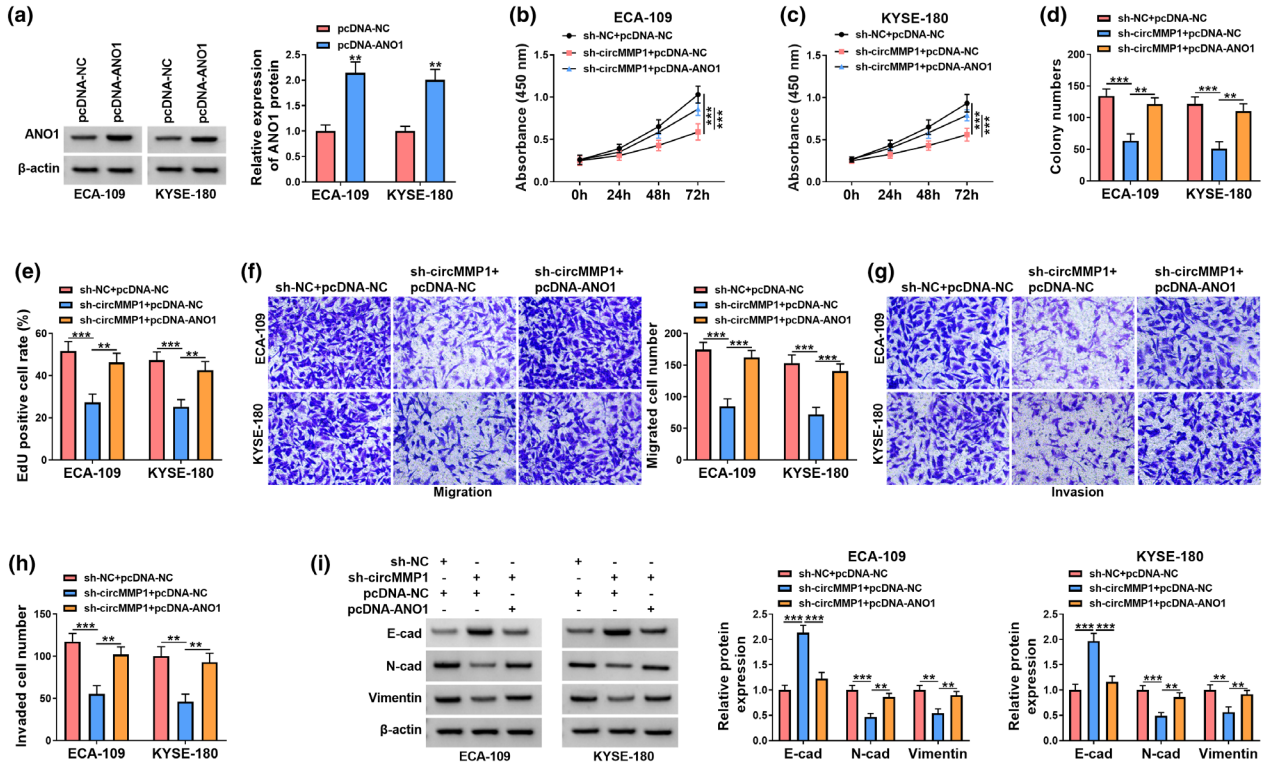

FIGURE 6 Overexpression of ANO1 reinstated the inhibitory effect of circMMP1 knockdown on cell proliferation, migration and invasion in esophageal squamous cell carcinoma (ESCC) cells. (a) The transfection efficiency of pcDNA-ANO1 in ESCC cells was detected by western blot. (b–i) ECA-109 and KYSE-180 cells were transfected with sh-NC + pcDNA-NC, sh-circMMP1+ pcDNA-NC or sh-circMMP1+ pcDNA-ANO1. (b, c) The growth curves of cells were evaluated by cell counting kit-8 (CCK-8). (d) Cell clonogenic ability was assessed by colony formation assay. (e) Cell proliferation was determined by 5-ethynyl-2′-deoxyuridine (EdU) assay. (f–h) Cell migration and invasion were tested by transwell assay. (i) Western blot was executed to detect the protein levels of E-cad, N-cad and vimentin. $^{**}p < 0.01$, $^{***}p < 0.001$.

showed that there were binding sites between circMMP1 and miR-556-5p, miR-217, miR-654-3p, miR-324-5p, miR-671-5p or miR-769-5p (Figure 3a). Next, RNA pulldown assay confirmed that miR-671-5p directly targeted circMMP1 in both ECA-109 and KYSE-180 cells (Figure 3b,c). Then, we performed dual-luciferase reporter and RIP assays to further verify the targeted binding of circMMP1 and miR-671-5p. The putative binding sequence between circMMP1 and miR-671-5p is shown in Figure 3d. Transfection of miR-671-5p mimics successfully increased miR-671-5p expression in both ECA-109 and KYSE-180 cells (Figure 3e). Subsequently, the correlation between circMMP1 and miR-671-5p was verified by dual-luciferase reporter assay (Figure 3f,g) and RIP assay (Figure 3h). Furthermore, qRT-PCR analysis revealed that miR-671-5p was significantly downregulated in ESCC tissues and cells (Figure 3i,j). These data indicated that circMMP1 could function as a sponge of miR-671-5p in ESCC cells.

MiR-671-5p mediates the inhibition of circMMP1 knockdown on cell proliferation, migration and invasion of ESCC

To explore whether circMMP1 exerted its biological function by sponging miR-671-5p, rescue experiments were designed using miR-671-5p inhibitors. The expression of miR-671-5p in both ECA-109 and KYSE-180 cells were reduced after transfection with miR-671-5p inhibitors (Figure 4a). Downregulation of miR-671-5p could rescue the suppressive effects of circMMP1 knockdown on cell proliferation (Figure 4b–e), migration (Figure 4f) and invasion (Figure 4g) in ECA-109 and KYSE-180 cells. Furthermore, western blot assay demonstrated that the increased levels of E-cad protein and the decreased levels of N-cad and vimentin caused by silencing of circMMP1 were reversed by miR-671-5p inhibitors (Figure 4h,i). In summary, these data suggested that circMMP1 might function as a sponge of miR-671-5p and participate in the development of ESCC.

ANO1 is a target of miR-671-5p and is regulated by circMMP1 through sponging miR-671-5p

Subsequently, we utilized starBase to predict the underlying targets genes of miR-671-5p. As shown in Figure 5a, the binding sites of miR-671-5p in the 3′UTR of ANO1 were predicted. According to dual-luciferase reporter experiment, the luciferase activity was noticeably decreased by cotransfection of miR-671-5p mimics and ANO1 3′UTR-WT luciferase reporter

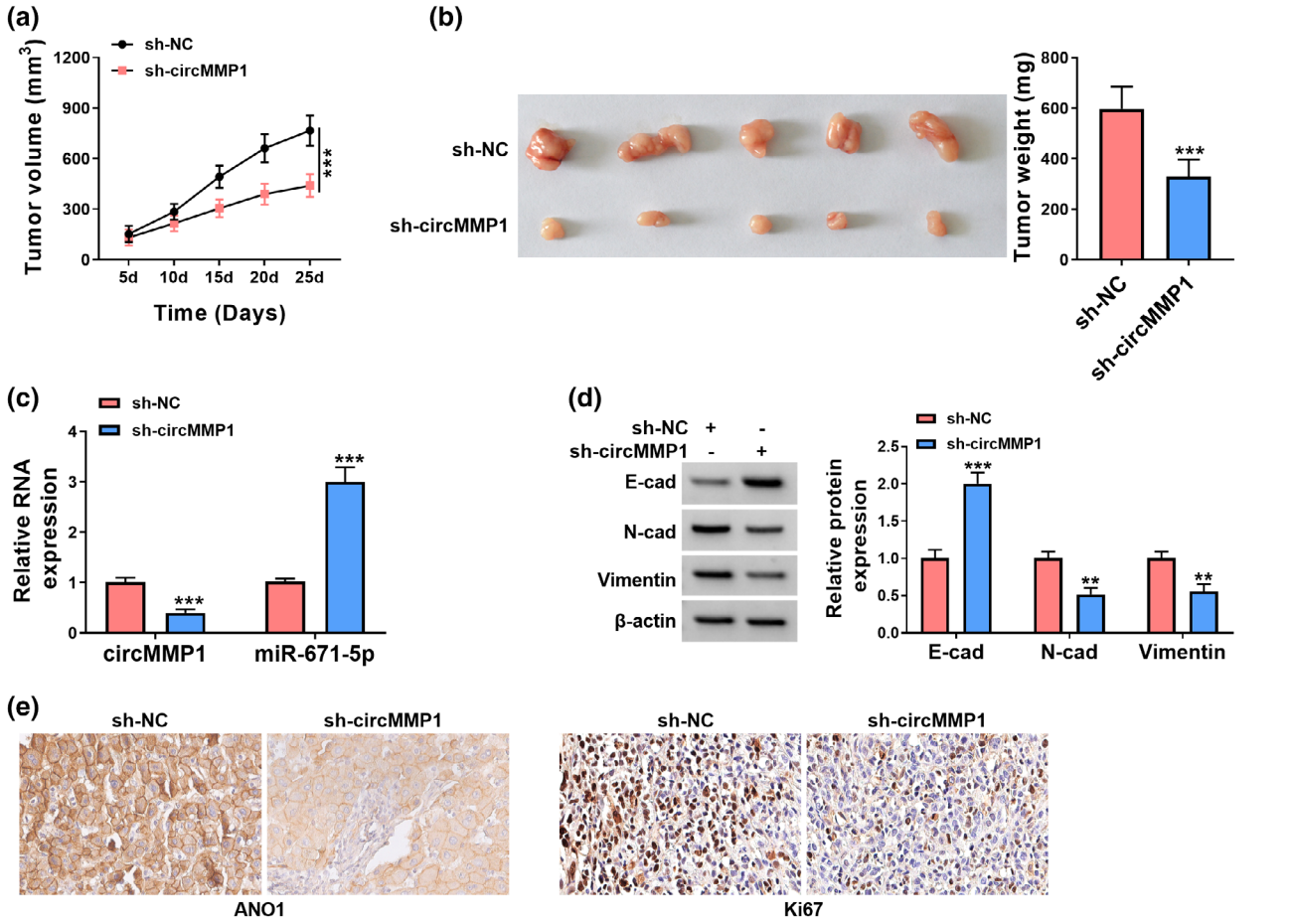

FIGURE 7 CircMMP1 knockdown suppressed esophageal squamous cell carcinoma (ESCC) tumor growth in vivo. ECA-109 cells stable expressing sh-NC or sh-circMMP1 were subcutaneously implanted into nude mice ($n = 5$/group). (a) The tumor growth volume in nude mice was assessed. (b) Representative tumor image and tumor weight in each group. (c) CircMMP1 and miR-671-5p expression levels in xenograft tumors were examined using qRT-PCR. (d) E-cad, N-cad and vimentin protein levels in xenograft tumors were detected using western blot. (e) Immunohistochemistry (IHC) was performed to analyze the expression of ANO1 and Ki67 in xenograft tumors. $^{**}p < 0.01$, $^{***}p < 0.001$.

(Figure 5b,c). In addition, the results of qRT-PCR and western blot showed that the mRNA and protein levels of ANO1 in ESCC tissues were upregulated (Figure 5d,e). The protein levels of ANO1 were also decreased in ESCC cells (Figure 5f). Strikingly, as determined by western blot analysis, the protein level of ANO1 was substantially decreased after overexpression of miR-671-5p (Figure 5g). Then, we found that silencing of circMMP1 observably decreased the protein level of ANO1, while miR-671-5p inhibitors could reverse this effect (Figure 5h). These results indicated that circMMP1 could regulate the expression of ANO1 through sponging miR-671-5p in ESCC cells.

Inhibitory effect of circMMP1 knockdown on ESCC cells was partly overturned by ANO1

Based on the above findings, we explored whether circMMP1 performed its role through the regulation of ANO1. ANO1 was overexpressed in both ECA-109 and KYSE-180 cells after pcDNA-ANO1 transfection (Figure 6a). Functionally, overexpression of ANO1 reinstated cell proliferation that was inhibited by circMMP1 knockdown (Figure 6b–e). Consistently, the inhibition of circMMP1 knockdown on cell migration and invasion were abolished by ANO1 overexpression (Figure 6f–h). In addition, overexpression of ANO1 could reverse the increased E-cad protein level and reduced N-cad and vimentin protein levels induced by circMMP1 silencing (Figure 6i). Taken together, these results disclosed that circMMP1 regulated the development of ESCC through upregulating ANO1.

CircMMP1 knockdown suppressed ESCC tumor growth in vivo

Finally, we validated the inhibitory effect of silencing circMMP1 on tumor growth by subcutaneously injecting ECA-109 cells stable expressing sh-circMMP1 into nude mice to establish a xenograft tumor model. Slower growth (Figure 7a) and decreased tumor weight (Figure 7b) were found in the circMMP1 knockdown group as compared with the control group. The qRT-PCR results showed that

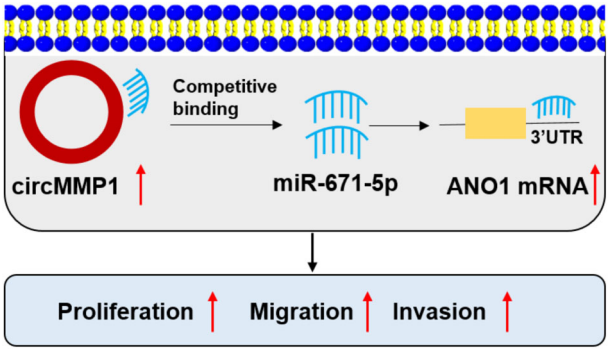

FIGURE 8 Schematic diagram of circMMP1 in esophageal squamous cell carcinoma (ESCC) via the miR-671-5p/ANO1 axis. Upregulated circMMP1 promoted ANO1 gene expression by sponging miR-671-5p, thereby facilitating cell proliferation, migration, and invasion of ESCC.

expression of circMMP1 was reduced while miR-671-5p expression was upregulated in the circMMP1 knockdown group (Figure 7c). We then examined the EMT markers by western blot and the results showed that the level of E-cad was enhanced and N-cad, vimentin levels were suppressed in the circMMP1 knockdown group, which was consistent with our in vitro analysis (Figure 7d). Moreover, circMMP1 knockdown inhibited the expression of ANO1 and proliferation marker Ki67 in xenograft tumor tissues (Figure 7e). These data demonstrated that knockdown of circMMP1 might impede ESCC tumor growth in vivo through targeting miR-671-5p/ANO1 axis (Figure 8).

DISCUSSION

Recently, the abnormal expression of circRNA in ESCC and relationship between circRNA and ESCC progress have been studied. Zhou et al. found that circNRIP1 was obviously upregulated in ESCC and its knockdown inhibited cell growth and invasion.[17] Pan et al. found that overexpression of circ_0006948 in ESCC cell could promote cell proliferation and induce EMT.[14] In this study, our results proved that circMMP1 functions as a miR-671-5p sponge to inhibit its suppressive effect on ANO1, thus accelerating ESCC cell growth, migration and invasion.

In EMT, adherent epithelial cells become invasive mesenchymal cells that are mainly characterized by reduced expression of cell adhesion protein marker E-cad, then mesenchymal protein markers N-cad and vimentin levels increased.[18–20] Tumor metastasis depends heavily on EMT.[20] Herein, we found that knockdown of circMMP1 in ESCC cells distinctly increased E-cad level and decreased the levels of N-cad and vimentin, suggesting that circMMP1 participated in the migration and invasion process of ESCC cells by regulating the EMT process. Furthermore, a number of circRNAs have been shown to promote cancer metastasis through influencing EMT, such as circMAT2B (non-small cell lung cancer),[21] circ_0092314 (pancreatic cancer)[22] and circPTPN22 (gastric cancer).[23]

At present, circMMP1 has been confirmed to exert its biological function through sponging miRNA.[15] Based on current research, we affirmed that miR-671-5p was a target of circMMP1. MiR-671-5p has previously been shown to suppress a number of cancers.[24–26] Herein, miR-671-5p was proven to be downregulated, and functionally, miR-671-5p deletion attenuated the inhibition effect of circMMP1 knockdown on ESCC cells. These results prompted that circMMP1 might act as a miR-671-5p sponge to promote ESCC progression.

Anoctamin 1 (ANO1) is one of the calcium-activated Cl-channel proteins.[27] So far, numerous cancers, such as colorectal cancer,[28] head and neck squamous cell carcinoma[29] and gastrointestinal stromal tumor,[30] have been reported to express high levels of ANO1. Herein, miR-671-5p was found to directly target ANO1. In concordance with Yu et al.,[31] we verified that ANO1 was overexpressed in ESCC tissues and cells. Furthermore, ANO1 was regulated by the circMMP1/miR-671-5p axis. Experimental rescue studies demonstrated that ANO1 overexpression abrogated circMMP1 knockdown-induced inhibitory malignant behaviors in ESCC cells. These data elucidated that circMMP1 regulated the malignant progression of ESCC through the miR-671-5p/ANO1 axis.

In conclusion, we determined that the high expression of circMMP1 in ESCC could promote tumor growth, migration and invasion partially through sponging miR-671-5p and enhancing ANO1 expression. Our results suggest that circMMP1 functions as a miRNA sponge in ESCC, and that circMMP1 could therefore be a new target for ESCC treatment.

AUTHOR CONTRIBUTIONS

Xiulin Wen conceived, designed and revised the current study. Rong Li analyzed the data and wrote the manuscript. Linyan Chai, Lei Lei, Rong Guo analyzed the data. All authors read and approved the final manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no financial conflicts of interest.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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