


# Circ\_0005231 promotes the progression of esophageal squamous cell carcinoma via sponging miR-383-5p and regulating KIAA0101

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## Abstract

**Background:** Circular RNAs (circRNAs) can act as key regulators in human cancers, including esophageal squamous cell carcinoma (ESCC). However, the role and mechanism of circ\_0005231 in ESCC have not previously been reported.

**Methods:** RNA levels and protein levels were detected by real-time quantitative polymerase chain reaction (RT-qPCR) and Western blot assay, respectively. Cell proliferation was assessed by colony formation assay and 5-ethynyl-2'-deoxyuridine (EdU) assay. Wound healing and transwell assays were used to assess cell migration and invasion, respectively. The intermolecular interaction was predicted by bioinformatic analysis and verified by RNA immunoprecipitation (RIP), RNA pulldown and dual-luciferase reporter assays. Xenograft tumor model was used for exploring the biological function of circ\_0005231 in vivo.

**Results:** Circ\_0005231 was upregulated in ESCC plasma, tissues and cells. Cell proliferation, migration and invasion were significantly restrained by knockdown of circ\_0005231 in ESCC cells. Circ\_0005231 acted as a sponge of miR-383-5p, and circ\_0005231 regulated ESCC cellular behavior by sponging miR-383-5p. Moreover, miR-383-5p directly targeted KIAA0101, and circ\_0005231 positively regulated KIAA0101 expression by sponging miR-383-5p. Furthermore, circ\_0005231 knockdown suppressed the malignant behavior of ESCC cells by downregulating KIAA0101. Importantly, knockdown of circ\_0005231 blocked xenograft tumor growth in vivo.

**Conclusion:** Circ\_0005231 acted as a sponge of miR-383-5p to promote ESCC progression by upregulating KIAA0101, which provided a potential therapeutic strategy for ESCC treatment.

## KEYWORDS

esophageal squamous cell carcinoma, circ\_0005231, miR-383-5p, KIAA0101

## INTRODUCTION

Esophageal cancer (EC) is a common gastrointestinal malignancy, which ranks sixth in mortality and seventh in incidence.<sup>1,2</sup> Esophageal squamous cell carcinoma (ESCC), a main subtype of EC, is prevalent in China and accounts for about 90% of all cases.<sup>3</sup> Although a series of advanced techniques have been applied in clinical therapy, the 5-year survival rate for ESCC patients remains poor.<sup>4,5</sup> Hence, it is essential to identify novel biomarkers

and molecular targets for improving the treatment of ESCC.

Circular RNAs (circRNAs), a kind of non-coding RNAs (ncRNAs), are generated by pre-mRNA back splicing of precursor mRNA and can form covalently closed structures.<sup>6</sup> In recent years, circRNAs have been reported to be extensively present in the cytoplasm of eukaryotic cells and they have higher stability and can avoid degradation by RNases.<sup>7</sup> CircRNAs participate in the progression and development of human diseases, including cancers.<sup>8,9</sup> In addition, several

circRNAs have been found to be involved in the tumorigenesis of ESCC by acting as tumor promoters or suppressors.<sup>10,11</sup> Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE112496>; <https://www.ncbi.nlm.nih.gov/geo/geo2r/?acc=GSE112496>) showed that circ\_0005231 was remarkably upregulated in ESCC tissue and plasma samples. However, the role of circ\_0005231 in ESCC has not previously been illustrated.

As previously reported, circRNAs can act as sponges/decoys for microRNAs (miRNAs) to affect target gene expression by competitively binding to miRNA response elements, which is known as the competing endogenous RNAs (ceRNA) mechanism.<sup>12,13</sup> Accumulating evidence shows that miRNAs (small ncRNAs) is tightly related to the progression of cancer.<sup>14</sup> Recent research has suggested that miR-383-5p acts as a crucial regulator in some tumors.<sup>15</sup> However, its role in ESCC is still largely unknown. KIAA0101 has been identified as a tumor-promoting gene in multiple human malignancies, including ESCC.<sup>16,17</sup> Coincidentally, we observed that both circ\_0005231 and KIAA0101 had complementary binding sites for miR-383-5p using bioinformatic tools. According to these findings, we assumed that circ\_0005231 might regulate ESCC progression by interacting with miR-383-5p to affect KIAA0101 expression.

In our study, the levels and functions of circ\_0005231, miR-383-5p and KIAA0101 in ESCC tissues and cells were explored. The potential interactions among circ\_0005231, miR-383-5p and KIAA0101 were investigated using bioinformatic databases and a series of experiments. Our study aimed to provide promising therapeutic strategies for ESCC.

## METHODS

### Specimen collection

A total of 40 ESCC tissues and corresponding adjacent normal tissue samples were obtained from ESCC patients who underwent surgery at The Fourth Hospital of Hebei Medical University. Peripheral blood samples were obtained from 40 ESCC patients prior to the operation and plasma was then isolated. In addition, normal plasma was obtained from 22 healthy volunteers at The Fourth Hospital of Hebei Medical University. Written informed consent was obtained from each participant before recruitment. This study received approval from the Research Ethics Committee of The Fourth Hospital of Hebei Medical University.

### Cell culture and transfection

ESCC cells (TE-1 and Eca-109) and human normal esophageal cells (HET-1A) were provided by BeNa Culture Collection. RPMI-1640 medium (Invitrogen) including 10% fetal bovine serum (FBS; Invitrogen) was used to incubate the cells which were then placed into a humidified atmosphere with 5% CO<sub>2</sub> at 37°C to grow.

Short hairpin RNA targeting circ\_0005231 (sh-circ\_0005231), miR-383-5p mimic and inhibitor (miR-383-5p and anti-miR-383-5p), KIAA0101-overexpressing plasmid (KIAA0101), and their controls (sh-NC, miR-NC, anti-miR-NC, and pcDNA3.1) were constructed by RiboBio. Lipofectamine 3000 reagent (Invitrogen) was applied for transfection when the cell confluence reached 70%.

### Real-time quantitative polymerase chain reaction

TRIzol reagent (Invitrogen) was utilized to isolate total RNA from ESCC tissues and cells, and BIOLOG lysis Easy Kit (BIODAI) was used to extract plasma RNA. The cDNA was synthesized by a Primescript RT reagent (for circRNA and mRNA; TaKaRa) or miScript II RT kit (for miRNA; Invitrogen). Thereafter, real-time quantitative polymerase chain reaction (RT-qPCR) reaction was manipulated using a Bio-Rad CFX96 system (Bio-Rad) using SYBR Premix Ex Taq II (Takara). The RNA levels were analyzed via the  $2^{-\Delta\Delta Ct}$  method. U6 and  $\beta$ -actin were utilized as the reference controls for miRNA and circRNA/mRNA, respectively. The primer sequences are shown in Table 1.

### RNase R treatment

RNase R was utilized to validate the closed-loop structure of circ\_0005231. Total RNA (5  $\mu$ g) was incubated for 0.5 h at 37°C with/without RNase R (Seebio). After that, the enrichment of circ\_0005231 and EIF3H was detected by agarose gel electrophoresis.

### Subcellular localization

Cytoplasm and nucleus fractions were isolated using the PARIS kit (Invitrogen). The expression levels of circ\_0005231, 18S rRNA (a cytoplasm control) and U6 (a nucleus control) were determined by RT-qPCR.

### Cell proliferation assays

For the colony formation assay, ESCC cells were cultivated in a 6-well plate and culture medium was changed after 3 days. Then, 14 days later, cell colonies were washed twice with PBS (Beyotime) after removing the culture medium. These colonies were stained with 0.1% crystal violet (Beyotime) after fixing with 4% paraformaldehyde (Beyotime). Cell colonies (one colony with more than 50 cells) were counted.

For 5-ethynyl-2'-deoxyuridine (EdU) assay, EdU cell proliferation kit (RiboBio) was used to measure DNA synthesis for reflecting proliferative capability. In short, cells were inoculated into a 24-well plate. After being treated with EdU, the cells were fixed and treated with Triton-X-100

**TABLE 1** Primer sequences used for polymerase chain reaction (PCR)

Name		Primers for PCR (5'-3')
circ_0005231	Forward	CCTTGCCAGCAGTCCAATATC
	Reverse	GTGAGAGAGATCCTTGGGCA
EIF3H	Forward	TCGGGAGATTTCAGCCGTGA
	Reverse	CTCCGCATCATTTCATCTGATA
miR-383-5p	Forward	CTCCGAGAGATCAGAAGGTGA
	Reverse	CTCAACTGGTGTCTGGGAG
UBE2T	Forward	ATCCCTCAACATCGCAACTGT
	Reverse	CAGCCTCTGGTAGATTATCAAGC
KIAA0101	Forward	ATGGTGCGGACTAAAGCAGAC
	Reverse	CCTCGATGAAACTGATGTCGAAT
U6	Forward	CTTCGGCAGCACATATACT
	Reverse	AAAATATGGAACGCTTCACG
β-actin	Forward	CTTCGCGGGCGACGAT
	Reverse	CCACATAGGAATCCTTCTGACC
18S rRNA	Forward	GGAGTATGGTTGCAAAGCTGA
	Reverse	ATCTGTCAATCCTGTCCGTGT

(0.5%), followed by staining with Apollo reaction for 0.5 h in a dark place. DAPI was then applied to stain the nuclei in the cells. Finally, EdU-positive (EdU<sup>+</sup>) cells were photographed using a fluorescence microscope (Leica).

### Migration and invasion assays

Migrative ability of ESCC cells was assessed by wound healing assay. In short, cells were inoculated into a 24-well plate. When the cell confluence reached about 80%, a sterile pipette tip (200 μl) was employed to scratch wounds. After being washed with PBS, cells were photographed with a microscope (Leica) to record the wound width at 0 and 24 h.

Transwell assay was used for the detection of cell invasion. In short, transwell inserts were coated with 10% Matrigel (BD Biosciences) for 0.5 h at 37°C. Thereafter, cell suspension (200 μl) without FBS was seeded into the top chamber. Meanwhile, complete medium (0.6 ml) was placed into the lower chamber. Then, 24 h later, cells in the upper chamber were carefully removed and invaded cells were fixed with paraformaldehyde (Beyotime). The cells were then photographed using an inverted microscope (Leica) at ×100 magnification after staining with crystal violet solution (Beyotime).

### Western blot assay

RIPA lysis buffer (Beyotime) was used for cell lysis and to extract proteins, and the proteins were denatured via heating for 3–5 min at 100°C. Protein samples (about 30 μg/lane) were loaded onto SDS-PAGE for separating the proteins after quantification of total proteins and then blotted onto

PVDF membranes (Millipore). After sealing with 5% nonfat milk (Beyotime), the blocked membrane was immunoblotted for 12–14 h at 4°C using primary antibodies, followed by incubation for 1–2 h with a goat anti-rabbit IgG secondary antibody (ab205718; 1:4000; Abcam). Lastly, the enhanced chemiluminescence solution (Vazyme) was applied to observe the combined signals. The antibodies including KIAA0101 (ab226255; 1:1000) and β-actin (ab8227; 1:2000) were bought from Abcam, while E-cadherin (20874-1-AP; 2000) and N-cadherin (22018-1-AP; 1:2000) were purchased from Proteintech.

### RNA immunoprecipitation

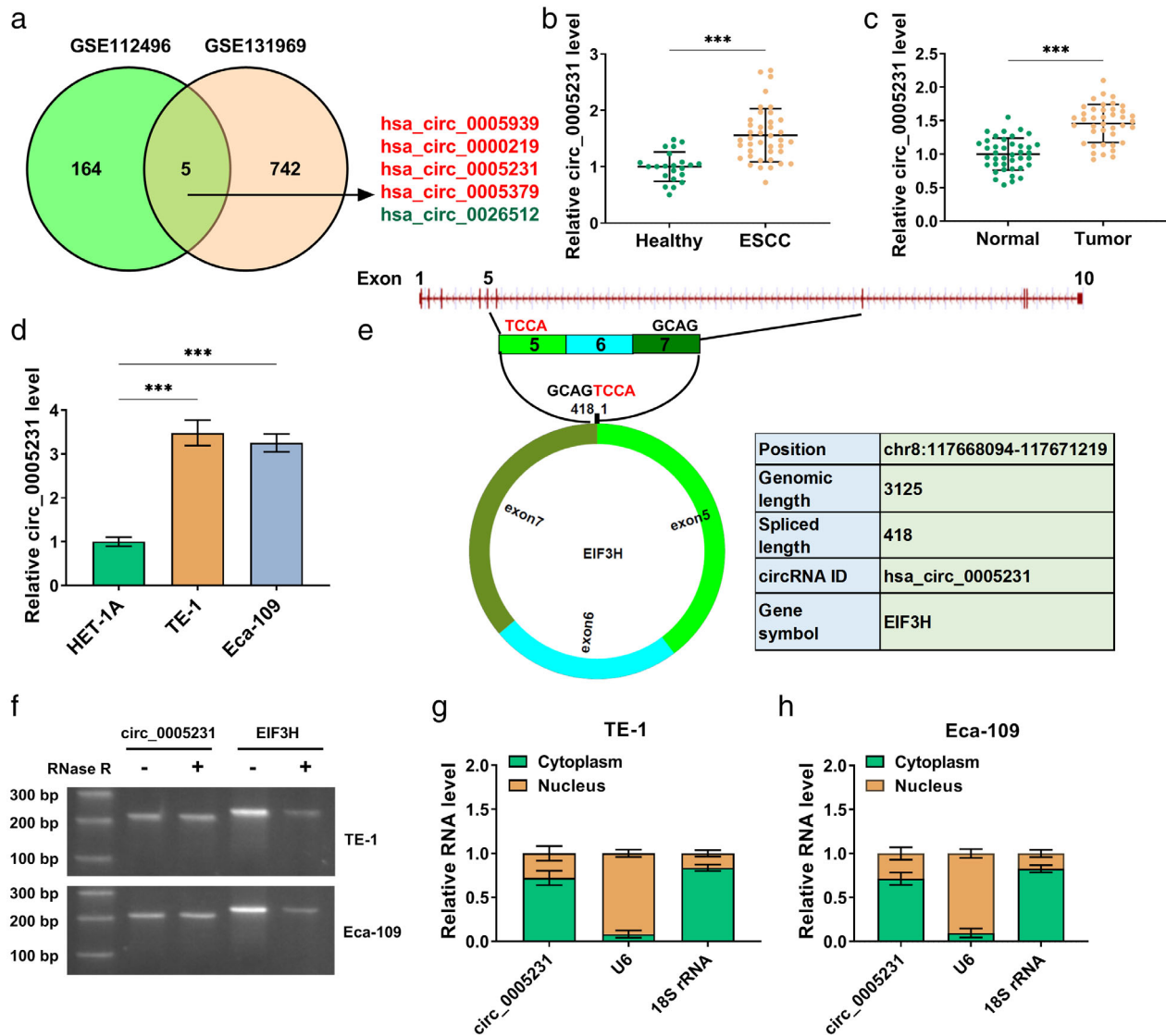
We used the EZMagna RIP kit (Millipore) for RNA immunoprecipitation according to the manufacturer's protocol. RNA immunoprecipitation (RIP) lysis buffer was employed to lyse TE-1 and Eca-109 cells, and the cell extracts were treated with magnetic beads conjugated with the antibody against immunoglobulin G (anti-IgG; as a control) or Argonaute2 (anti-AGO2) for 6 h at 4°C. After incubation with proteinase K for 0.5 h at 55°C, immunoprecipitated RNA was subjected to RT-qPCR analysis.

### RNA pulldown assay

Biotinylated negative control (bio-NC) and biotinylated miR-383-5p (bio-miR-383-5p) were purchased from RiboBio and transfected into ESCC cells for 24 h. After that, the cells were lysed with RIPA buffer (Beyotime), followed by incubation with M-280 streptavidin magnetic beads (Invitrogen). The RNAs attached to the beads were purified using TRIzol reagent (Invitrogen). Finally, RNA levels were tested via RT-qPCR after RNA isolation from magnetic beads.

### Dual-luciferase reporter assay

Circinteractome (<https://circinteractome.nia.nih.gov/>), starBase (<http://starbase.sysu.edu.cn/>), circbank (<http://www.circbank.cn/>) were applied to predict the target miRNAs for circ\_0005231. Targetscan (<http://www.targetscan.org/>) was employed to predict the target mRNAs for miR-383-5p. To generate wild-type reporter vectors (wt-circ\_0005231 and wt-KIAA0101 3'UTR), the fragments of circ\_0005231 or KIAA0101 3'UTR including the miR-383-5p-binding sequence were amplified and cloned into psiCHECK2 dual-luciferase vector (Promega). Meanwhile, mutated reporter vectors (mut-circ\_0005231 and mut-KIAA0101 3'UTR) without miR-383-5p binding sites were generated as described above. Thereafter, the psiCHECK2 luciferase reporter vector (wt or mut) was cointroduced with miR-NC/miR-383-5p into TE-1 and Eca-109 cells for 48 h. A dual-luciferase reporter gene assay kit (LMAI Bio) was utilized to analyze the luciferase activities.



**FIGURE 1** Circ\_0005231 was overexpressed in esophageal squamous cell carcinoma (ESCC). (a) Venn diagram showing differentially expressed circRNAs in ESCC. (b) circ\_0005231 expression was detected by RT-qPCR in plasma samples of ESCC and healthy controls. (c) The level of circ\_0005231 was determined by RT-qPCR in ESCC tissues and normal tissues. (d) RT-qPCR was applied to measure circ\_0005231 expression in ESCC cells (TE-1 and Eca-109) and HET-1A cells. (e) circ\_0005231 information is presented. (f) Agarose gel electrophoresis was used to detect circ\_0005231 expression in RNAs treated with or without RNase R. (g and h) The localization of circ\_0005231 was determined by RT-qPCR in the nuclear and cytosolic fractions of TE-1 and Eca-109 cells. \*\*\* $p < 0.001$ .

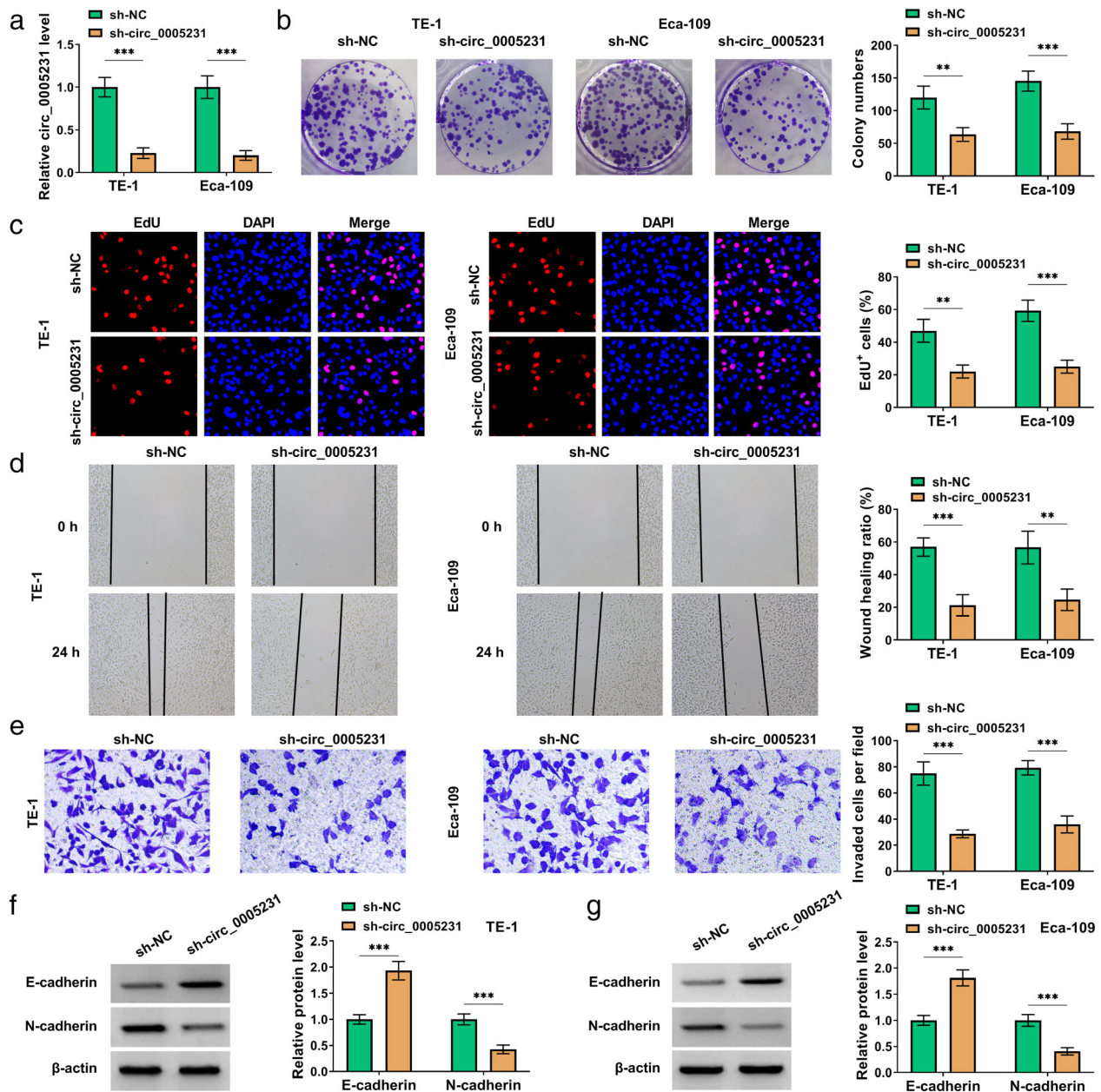
## Tumor formation assay in vivo

Animal experiments were conducted with the permission of the Animal Care and Use Committee of The Fourth Hospital of Hebei Medical University. BALB/c nude mice (female;  $n = 10$ ; 5 weeks) were commercially provided by Vital River. The mice were divided into two groups ( $n = 5$  per group). Briefly, mice were subcutaneously inoculated with Eca-109 cells ( $1 \times 10^7$ , circ\_0005231 silencing or control). At 3 days after injection, tumor volume was tested every 3 days by caliper calculated using the formula:  $1/2 \times \text{width}^2 \times \text{length}$ . Then, 27 later, the mice were killed, and all tumors were excised and harvested for further study.

## Immunohistochemistry (IHC) analysis

Tumor tissues were fixed in paraformaldehyde (4%) and embedded in paraffin. After cutting into 5- $\mu\text{m}$  thick sections, the sections were incubated with primary antibodies against KIAA0101 (ab226255; 1:500; Abcam), Ki-67 (1:200; ab15580; Abcam), E-cadherin (20874-1-AP; 1:1000; Rosemont) and N-cadherin (22018-1-AP; 1:1000; Rosemont). Thereafter, the sections were incubated with corresponding secondary antibodies (1:4000; ab205718; Abcam). After staining with diaminobenzidine (DAB; Beyotime) and counterstaining with hematoxylin (Beyotime), a microscope (Leica) was used to observe the immunohistochemistry images.





**FIGURE 2** Knockdown of circ\_0005231 inhibited esophageal squamous cell carcinoma (ESCC) cell proliferation and metastasis. TE-1 and Eca-109 cells were transfected with sh-NC or sh-circ\_0005231. (a) circ\_0005231 expression was measured by RT-qPCR. (b) Colony formation assay was performed to detect the number of colonies. (c) DNA synthesis was assessed using EdU assay. (d) Cell migration was assessed by wound healing assay. (e) Cell invasion was evaluated by transwell assay. (f and g) E-cadherin and N-cadherin protein levels were measured by Western blot analysis. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

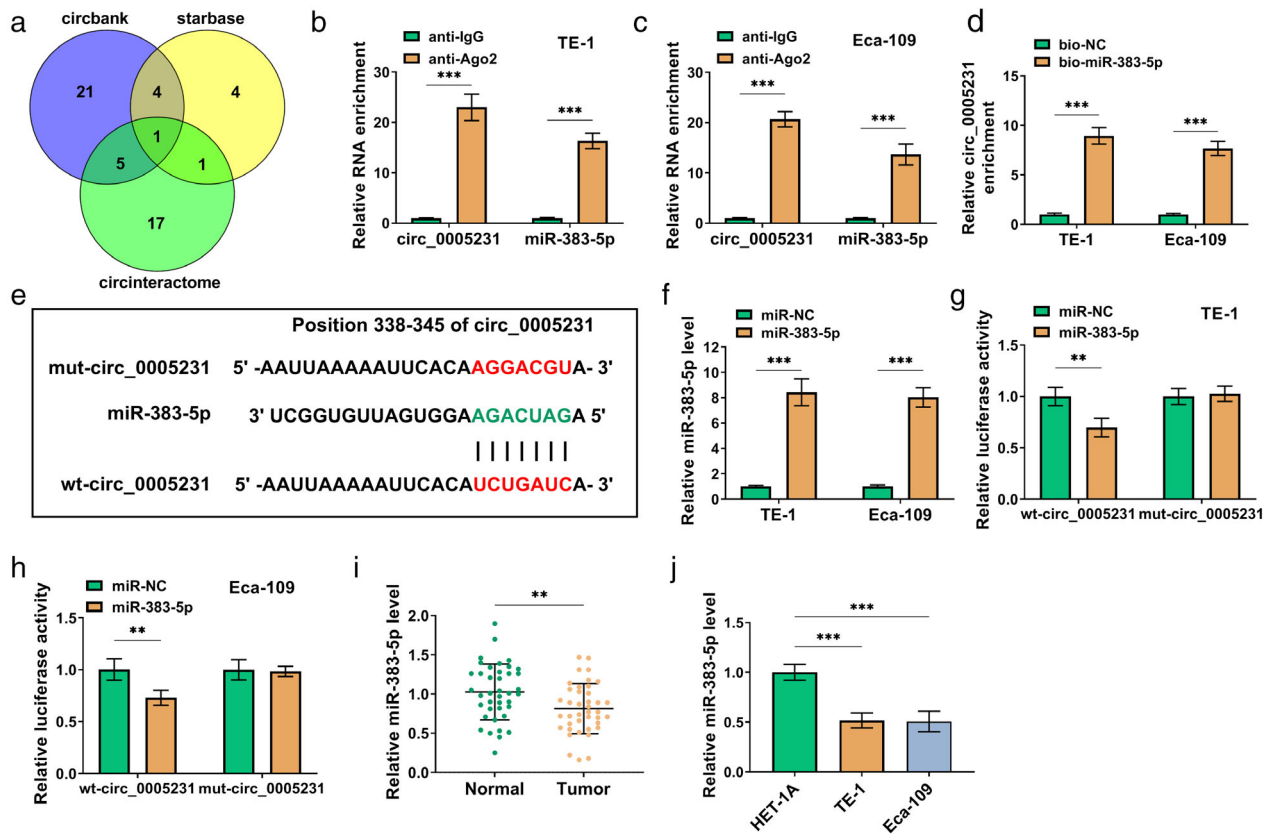
## Statistical analysis

All data are displayed as mean  $\pm$  standard deviation and were obtained from three repeated experiments. Statistical analysis was carried out using GraphPad Prism 7.0 software. Comparison of two or more groups (more than two groups) was determined using a Student's *t*-test or one-way analysis of variance (ANOVA). A statistically significant difference was represented when  $p < 0.05$ .

## RESULTS

### Circ\_0005231 was upregulated in ESCC patients and cells

In an attempt to identify novel circRNAs involved in ESCC, we searched the circRNAs microarray datasets. The data from GEO database (accession numbers GSE112496 and GSE112496) showed that five circRNAs were found in the intersection part (Figure 1a). Among



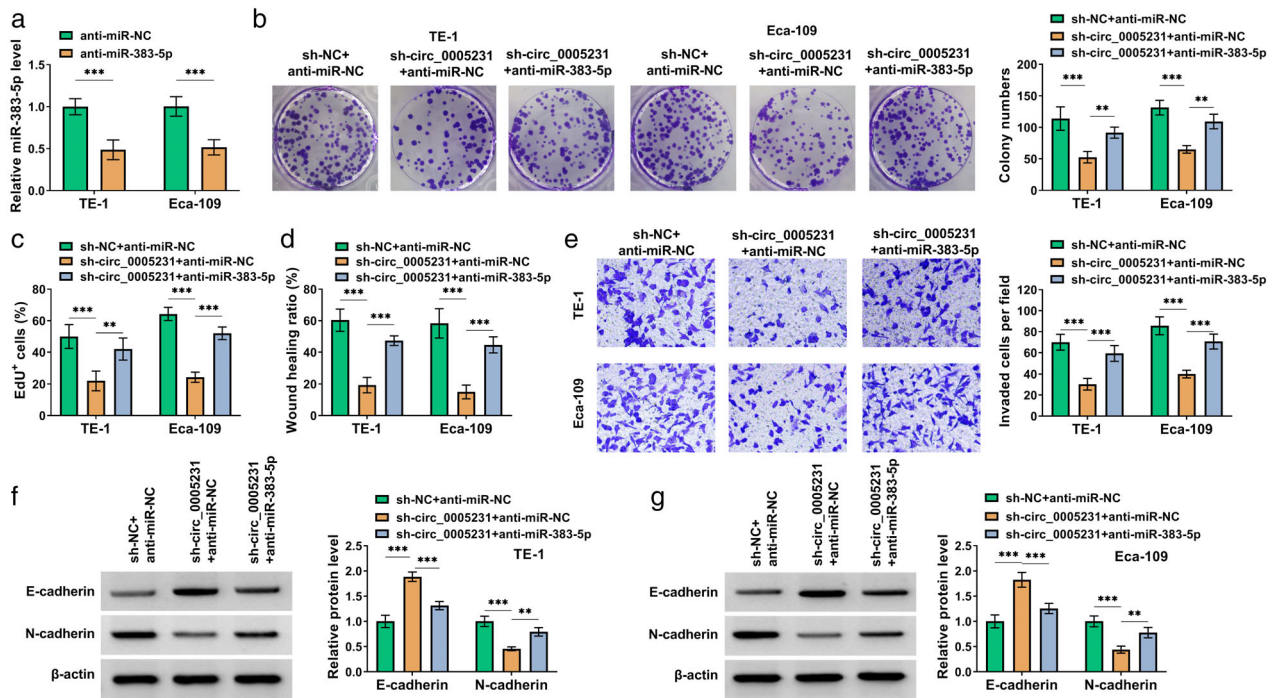
**FIGURE 3** Circ\_0005231 interacted with miR-383-5p. (a) Venn diagram shows the potential target genes of circ\_0005231. (b–d) The interaction between circ\_0005231 and miR-383-5p was confirmed by RIP and RNA pull-down assay in TE-1 and Eca-109 cells. (e) The complementary binding sequence of circ\_0005231 and miR-383-5p is shown. (f) miR-383-5p expression was examined by RT-qPCR in TE-1 and Eca-109 cells transfected with miR-NC or miR-383-5p. (g and h) The interaction between circ\_0005231 and miR-383-5p was also verified by dual-luciferase reporter assay. (i and j) RT-qPCR was performed to measure miR-383-5p expression in normal tissues, esophageal squamous cell carcinoma (ESCC) tissues, HET-1A cells, and ESCC cells (TE-1 and Eca-109).  $^{**}p < 0.01$ ,  $^{***}p < 0.001$ .

these, hsa\_circ\_0005939, hsa\_circ\_0000219, hsa\_circ\_0005231, and hsa\_circ\_0005379 are upregulated circRNAs, while hsa\_circ\_0026512 is a downregulated circRNA in ESCC tissues and plasma. Next, we analyzed the expression of the five circRNAs in ESCC tissues and the adjacent normal tissues. As shown in Figure S1, circ\_0026512 expression was most significantly dysregulated in the tissues compared with controls. Thus, hsa\_circ\_0005231 was chosen for further research. To further confirm the expression of circ\_0005231 in ESCC, RT-qPCR was conducted. As displayed in Figure 1b,c ( $^{***}p < 0.001$ ), circ\_0005231 expression was increased in ESCC plasma and tissues. Likewise, circ\_0005231 level was enhanced in ESCC compared to normal cells (Figure 1d,  $^{***}p < 0.001$ ). Circ\_0005231 is generated from 5–7 exons of eukaryotic translation initiation factor 3 subunit H (EIF3H) gene and is located on chr8:117668094–117671219, whose spliced mature sequence length is 418 nt (Figure 1e). Agarose gel electrophoresis showed that circ\_0005231 bands were apparent after RNase R treatment, whereas the bands corresponding to EIF3H were weakened (Figure 1f), indicating that circ\_0005231 forms a circular RNA resistant to RNase R treatment. Moreover, we found that circ\_0005231 was mainly located in the cytoplasm

(Figure 1g,h). These results suggested that circ\_0005231 might be associated with ESCC progression.

### Circ\_0005231 knockdown inhibited the proliferation, migration and invasion of ESCC cells

RT-qPCR showed that transfection of sh-circ\_0005231 significantly reduced circ\_0005231 expression in TE-1 and Eca-109 cells indicating a high silencing efficiency of sh-circ\_0005231 (Figure 2a,  $^{***}p < 0.001$ ). Colony formation assay showed that the colony-forming ability of circ\_0005231-downregulating cells was decreased in contrast to control cells (Figure 2b,  $^{**}p < 0.01$  and  $^{***}p < 0.001$ ). Similarly, the EdU assay revealed a decrease in the number of EdU<sup>+</sup> cells after knockdown of circ\_0005231, indicating circ\_0005231 downregulation inhibited DNA synthesis (Figure 2c,  $^{**}p < 0.01$  and  $^{***}p < 0.001$ ). We next investigated whether circ\_0005231 affected ESCC cell migration and invasion using wound healing and transwell assays, respectively. The migration and invasion abilities of TE-1



**FIGURE 4** Circ\_0005231 silencing inhibited the malignant behavior of esophageal squamous cell carcinoma (ESCC) cells by targeting miR-383-5p. (a) miR-383-5p expression was tested by RT-q PCR in TE-1 and Eca-109 cells transfected with anti-miR-NC or anti-miR-383-5p. (b–g) TE-1 and Eca-109 cells were transfected with sh-NC + anti-miR-NC, sh-circ\_0005231 + anti-miR-NC, or sh-circ\_0005231 + anti-miR-383-5p. (b and c) Cell proliferation was assessed by colony formation assay and EdU assays. (d and e) Wound healing and transwell assays were performed to assess cell migration and invasion, respectively. (f and g) Western blot assay was performed to detect E-cadherin and N-cadherin protein levels. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

and Eca-109 cells were found to be obviously reduced after circ\_0005231 silencing (Figure 2d,e, \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ). Epithelial-mesenchymal transition (EMT) is a key step toward cancer metastasis<sup>18</sup>. Hence, EMT-related proteins (E-cadherin, an epithelial marker; N-cadherin, a mesenchymal marker) were measured using Western blot analysis. The data showed that circ\_0005231 interference increased E-cadherin protein level and reduced N-cadherin protein expression (Figure 2f,g, \*\*\* $p < 0.001$ ). Taken together, these results suggested that circ\_0005231 acted as a tumor promoter.

### Circ\_0005231 was a sponge of miR-383-5p

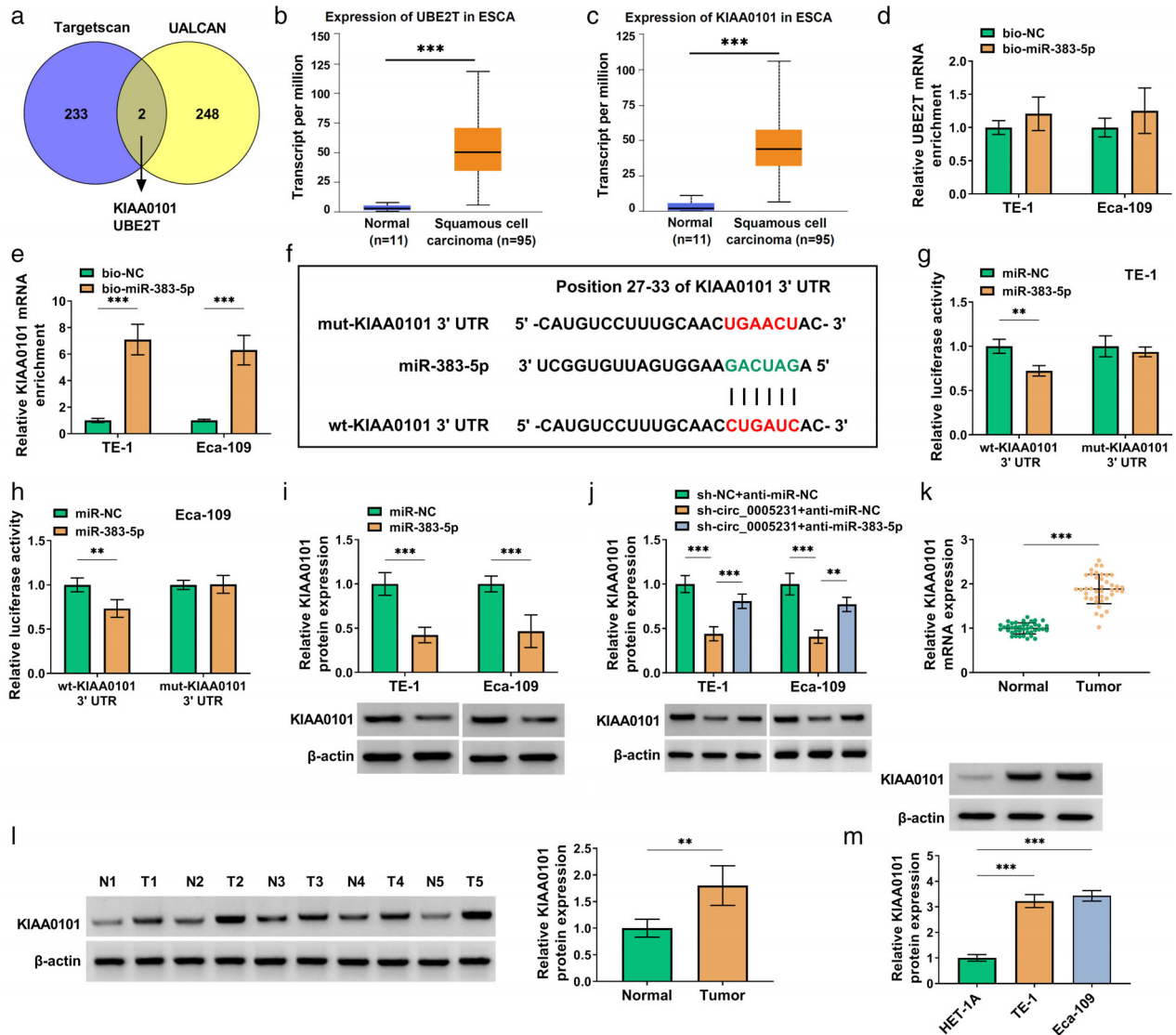
Circbank, Circinteractome and starBase were utilized to search for possible miRNAs binding with circ\_0005231. MiR-383-5p was identified since it was predicted by the three bioinformatic tools (Figure 3a). To confirm the interaction between circ\_0005231 and miR-383-5p, RIP and RNA pulldown assays were performed. RIP assay results manifested that both circ\_0005231 and miR-383-5p were abundant in the anti-Ago2 complex instead of anti-IgG complex (Figure 3b,c, \*\*\* $p < 0.001$ ). Furthermore, the enrichment of circ\_0005231 was markedly increased in the bio-miR-383-5p probe (Figure 3d, \*\*\* $p < 0.001$ ). The binding sequence of circ\_0005231 and miR-383-5p is shown in Figure 3e. The overexpression efficiency of miR-383-5p is

shown in Figure 3f (\*\* $p < 0.01$ ). Consistent with the RIP and RNA pulldown assays, dual-luciferase reporter assay proved that the luciferase activity of wt-circ\_0005231 rather than that of mut-circ\_0005231 vector could be reduced after overexpression of miR-383-5p (Figure 3g,h, \*\* $p < 0.01$ ). Moreover, an apparent decrease of miR-383-5p expression was observed in ESCC tissues and cells (Figure 3i,j, \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ). These results suggested that miR-383-5p could be sponged by circ\_0005231.

### Knockdown of circ\_0005231 suppressed the malignant behavior of ESCC cells

The transfection efficiency of anti-miR-383-5p was confirmed by detecting its low expression after transfection (Figure 4a, \*\*\* $p < 0.001$ ). To study whether circ\_0005231 exerted its role via sponging miR-383-5p, a series of rescue experiments were conducted in TE-1 and Eca-109 cells. MiR-383-5p deficiency abolished the suppression of colony formation ability and DNA synthesis caused by knockdown of circ\_0005231 (Figure 4b,c and Figure S2a, \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ). Moreover, the inhibitory effects of circ\_0005231 silencing on migration and invasion were counteracted by miR-383-5p downregulation (Figure 4d,e and Figure S2b, \*\*\* $p < 0.001$ ). In addition, sh-circ\_0005231-induced promotion of E-cadherin protein expression and reduction of N-cadherin protein level were also reversed by inhibition of miR-383-5p (Figure 4f,g,





**FIGURE 5** KIAA0101 was directly targeted by miR-383-5p. (a) Venn diagram showing the potential target genes of miR-383-5p. (b and c) KIAA0101 and UBE2T expression in ESCA were shown from the UALCAN website. (d and e) RNA pull-down assay was used to explore the interaction between miR-383-5p and KIAA0101 or UBE2T in TE-1 and Eca-109 cells. (f) The complementary binding sites between miR-383-5p and KIAA0101 3'UTR were presented. (g and h) The interaction between miR-383-5p and KIAA0101 was also confirmed using dual-luciferase reporter assay. (i) The protein expression of KIAA0101 was measured by Western blot assay in TE-1 and Eca-109 cells transfected with miR-NC and miR-383-5p. (j) KIAA0101 protein expression was examined by Western blot assay in TE-1 and Eca-109 cells transfected with sh-NC + anti-miR-NC, sh-circ\_0005231 + anti-miR-NC, or sh-circ\_0005231 + anti-miR-383-5p. (k) KIAA0101 mRNA expression was determined by RT-qPCR in normal and tumor tissues. (l and m) KIAA0101 protein expression was determined by Western blot assay in normal tissues, ESCC tissues, HET-1A cells, and ESCC cells (TE-1 and Eca-109). \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

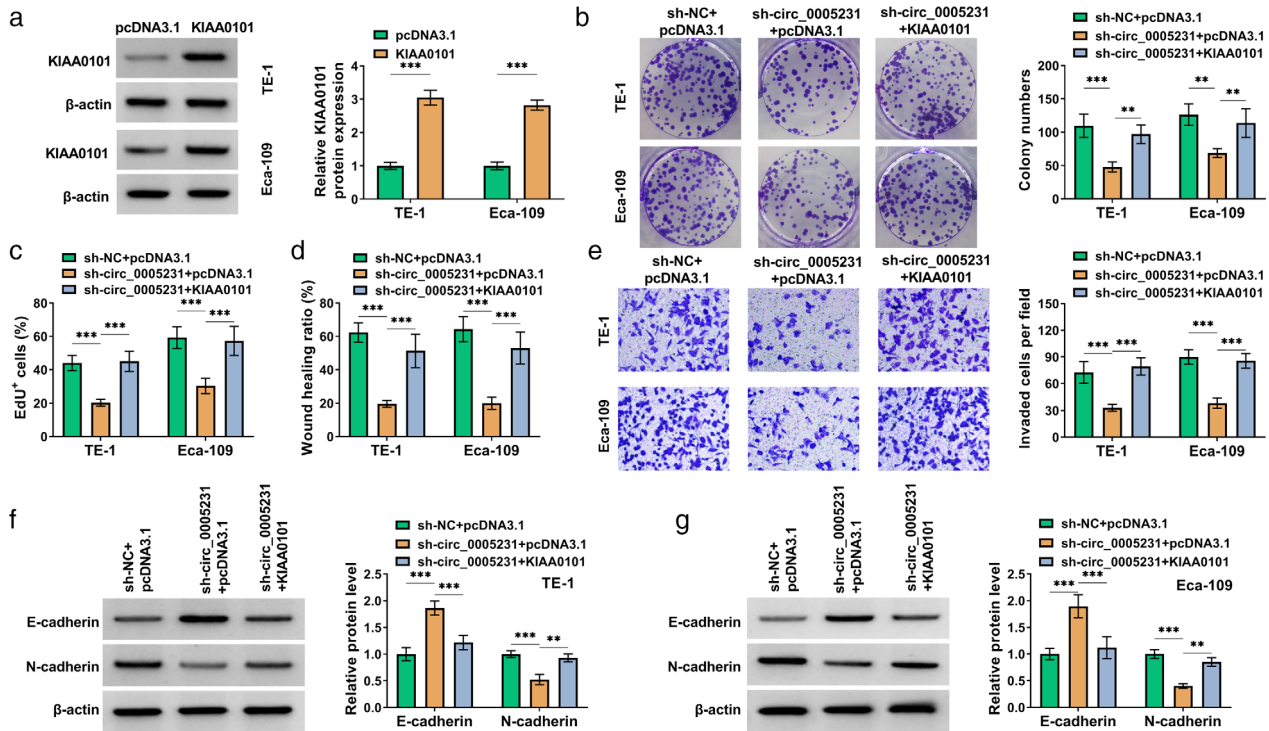
\*\* $p < 0.01$  and \*\*\* $p < 0.001$ ). Overall, miR-383-5p down-regulation rescued the inhibitory effect of circ\_0005231 knock-down on malignant phenotypes of ESCC cells.

### KIAA0101 was a direct target of miR-383-5p

To elucidate which genes are regulated by the circ\_0005231/miR-383-5p axis in ESCC, Targetscan was used to predict the targets of miR-383-5p. As shown in Figure 5a, KIAA0101 and UBE2T overlapped in these candidate genes

and UALCAN (<http://ualcan.path.uab.edu/analysis.html>). As shown by UALCAN, KIAA0101 and UBE2T expression were significantly upregulated in esophageal carcinoma (ESCA) (Figure 5b,c, \*\*\* $p < 0.001$ ). RNA pull-down assay showed that KIAA0101 mRNA enrichment was significantly increased in TE-1 and Eca-109 cells with transfection of bio-miR-383-5p, whereas UBE2T mRNA enrichment did not increase significantly (Figure 5d,e, \*\*\* $p < 0.001$ ), indicating that KIAA0101 could interact with miR-383-5p. The complementary binding sites of KIAA0101 and miR-383-5p are shown in Figure 5f. Dual-luciferase reporter assay was





**FIGURE 6** Knockdown of circ\_0005231 suppressed ESCC cell proliferation and metastasis via reducing KIAA0101 expression. (a) The protein expression of KIAA0101 was determined using Western blot assay in TE-1 and Eca-109 cells transfected with pcDNA3.1 or KIAA0101. (b–g) TE-1 and Eca-109 cells were transfected with sh-NC + pcDNA3.1, sh-circ\_0005231 + pcDNA3.1, or sh-circ\_0005231 + KIAA0101. (b and c) Colony formation and EdU assays were used to assess cell proliferation. (d and e) Cell migration and invasion were evaluated by wound healing and transwell assays, respectively. (f and g) Western blot assay was carried out to analyze the protein levels of E-cadherin and N-cadherin. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

performed to further confirm the interaction between KIAA0101 and miR-383-5p. The results of the dual-luciferase reporter assay showed that overexpression of miR-383-5p repressed the luciferase activity of wt-KIAA0101 3'UTR, while changes in miR-383-5p expression could not affect the luciferase activity mut-KIAA0101 3'UTR in TE-1 and Eca-109 cells (Figure 5g,h, \*\* $p < 0.01$ ). Moreover, miR-383-5p overexpression reduced KIAA0101 protein expression in TE-1 and Eca-109 cells (Figure 5i, \*\*\* $p < 0.001$ ). In addition, circ\_0005231 knockdown inhibited KIAA0101 protein expression, while miR-383-5p downregulation abated this inhibition (Figure 5j, \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ), indicating that circ\_0005231 regulated KIAA0101 expression by interacting with miR-383-5p. We observed an overt upregulation of KIAA0101 mRNA in ESCC tissues (Figure 5k, \*\*\* $p < 0.001$ ). Similarly, KIAA0101 protein level was also increased in ESCC tissues and cells (Figure 5l,m, \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ). Collectively, these data demonstrated that miR-383-5p directly targeted KIAA0101 in ESCC cells.

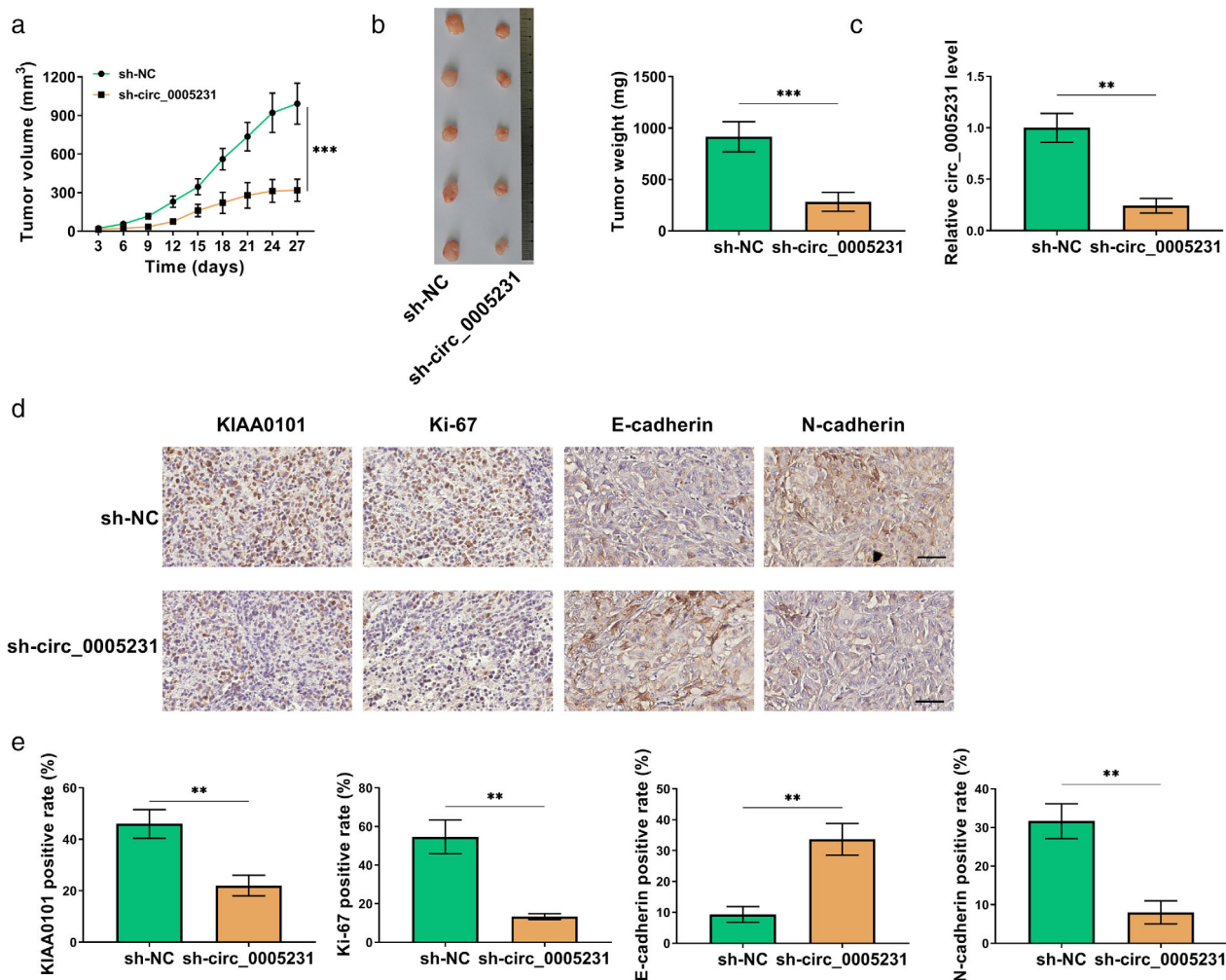
### Circ\_0005231 silencing repressed malignancy of ESCC cells by targeting KIAA0101

Transfection of KIAA0101 increased KIAA0101 protein expression in TE-1 and Eca-109 cells (Figure 6a,

\*\*\* $p < 0.001$ ), suggesting a high transfection efficiency. To determine whether circ\_0005231 regulated the malignancy of ESCC cells by targeting KIAA0101, we performed rescue experiments in TE-1 and Eca-109 cells. The results confirmed that the inhibitory effects of circ\_0005231 silencing on colony formation ability, DNA synthesis, migration and invasion were overturned by the addition of KIAA0101 (Figure 6b,e and Figure S3, \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ). Moreover, knockdown of circ\_0005231 increased E-cadherin protein expression and decreased N-cadherin protein expression, which could be rescued by elevating KIAA0101 (Figure 6f,g, \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ). Overall, these results suggested that knockdown of circ\_0005231 inhibited ESCC cell proliferation and metastasis by decreasing KIAA0101 expression.

### Circ\_0005231 downregulation inhibited ESCC tumorigenicity in vivo

We next explored the effect of circ\_0005231 silencing on ESCC tumorigenicity in vivo. We found that circ\_0005231 knockdown obviously reduced tumor volume and tumor weight (Figure 7a,b, \*\*\* $p < 0.001$ ). Moreover, the results of RT-qPCR showed that circ\_0005231 knockdown reduced the expression of circ\_0005231 in tumor tissues (Figure 7c, \*\* $p < 0.01$ ). IHC analysis showed that circ\_0005231



**FIGURE 7** Downregulation of circ\_0005231 blocked tumor growth in vivo. Eca-109 cells transfected with sh-NC or sh-circ\_0005231 were subcutaneously injected into nude mice. (a) Tumor volume was monitored every 3 days. (b) Tumor weight was measured after injection for 27 days. (c) The level of circ\_0005231 was detected by RT-qPCR in tumor tissues. (d and e) Immunohistochemistry (IHC) analysis was used to detect KIAA0101, Ki-67, E-cadherin, and N-cadherin in tumor tissues. \*\* $p < 0.01$ , \*\*\* $p < 0.001$ .

interference suppressed KIAA0101, Ki-67 (a proliferation marker) and N-cadherin expression as well as increased E-cadherin expression in tumor tissues (Figure 7d,e). Collectively, these in vivo data indicated that circ\_0005231 knockdown could inhibit the tumorigenicity of ESCC.

## DISCUSSION

ESCC is a frequent malignant tumor in the digestive system with short survival time due to the limitations of conventional treatment strategies and lack of early diagnosis.<sup>19</sup> Hence, it is critical to find effective ESCC biomarkers to strengthen its diagnosis and treatment. Herein, we found that circ\_0005231 knockdown restrained the proliferation and metastasis of ESCC cells and also inhibited tumor growth and metastasis in vivo via regulating the miR-383-3p/KIAA0101 axis, offering a possible treatment strategy for ESCC patients.

With the development of high-throughput sequencing, circRNAs have gradually received greater attention in tumor research.<sup>20</sup> circRNAs are considered promising biomarkers for the prognosis and diagnosis of diverse cancers due to their abundance and stability in plasma and tissues.<sup>21</sup> As for ESCC, more and more circRNAs have been reported to regulate ESCC cell behavior. For example, CiRS-7 could promote the growth and metastasis of ESCC cells.<sup>22</sup> Moreover, the proliferative and migratory capacities of ESCC cells were decreased and apoptosis was increased by hsa\_circ\_0000700 knockdown.<sup>23</sup> In this study, a novel upregulated circRNA named circ\_0005231 was identified based on the GEO database, and we further confirmed the level of circ\_0005231 was remarkably upregulated in ESCC plasma, tissues and cells. Next, we knocked down circ\_0005231 expression and evaluated the effects on ESCC cell behavior. The data showed that circ\_0005231 silencing limited ESCC cell growth, migration and invasion, implying that circ\_0005231 could function as an oncogene in ESCC.

CircRNAs can regulate various biological processes by serving as miRNA sponges, thereby decreasing the miRNA levels and releasing the targeted genes of miRNA.<sup>24</sup> We analyzed circ\_0005231-miRNA interactions using bioinformatic tools. After performing a series of experiments, miR-383-5p was demonstrated to be sponged by circ\_0005231 and inversely regulated by circ\_0005231. MiR-383-5p is able to exert an important role in the progression of various human tumors. For instance, miR-383-5p serves as tumor-suppressing miRNA in ovarian cancer,<sup>25</sup> nasopharyngeal carcinoma,<sup>26</sup> breast cancer,<sup>27</sup> gastric carcinoma,<sup>28</sup> and lung adenocarcinoma.<sup>29</sup> On the contrary, miR-383-5p acts as tumor-promoting miRNA in cholangiocarcinoma.<sup>30</sup> In ESCC, miR-383 was downregulated and inhibited ESCC cell growth.<sup>31</sup> In our study, a low level of miR-383-5p was detected in ESCC tissues and cells. Additionally, miR-383-5p inhibition abated the repressive influence of circ\_0005231 depletion on ESCC cell growth and metastasis. According to the above results, knockdown of circ\_0005231 increased miR-383-5p expression to restrain ESCC malignant behavior.

MiRNAs can bind with target mRNAs to regulate many biological and pathological processes.<sup>32</sup> Next, we analyzed the downstream target for miR-383-5p. KIAA0101 was demonstrated as a target for miR-383-5p. KIAA0101, a well-known oncogene, is a PCNA-associated protein and is involved in the modulation of many biological processes, such as cell cycle progression, DNA repair, and cell proliferation.<sup>33,34</sup> Previous research indicated that the abundance of KIAA0101 protein was upregulated in EC tissue samples and is related to poor survival of the patients, EC progression, and cisplatin resistance.<sup>17</sup> Another study showed that KIAA0101 promoted the migration and invasion of ESCC cells.<sup>35</sup> Consistent with the previous results, KIAA0101 expression was high in ESCC tissues and cells. Rescue assays showed that KIAA0101 overexpression could reverse the anticancer function of sh-circ\_0005231 in ESCC cells, indicating the tumor-promoting role of KIAA0101. In line with in vitro data, circ\_0005231 knockdown limited tumor growth and metastasis by downregulating KIAA0101.

In conclusion, circ\_0005231 was highly-expressed in ESCC. Circ\_0005231 silencing suppressed ESCC cell malignant behavior through regulation of miR-383-5p/KIAA0101 axis. These results may contribute to the understanding of ESCC pathogenesis and circ\_0005231 is able to serve as a potential target for ESCC.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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## REFERENCES

- Bray F, Ferlay J, Soerjomataram I, et al. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin*. 2018;68:394–424.
- Kato K, Cho BC, Takahashi M, et al. Nivolumab versus chemotherapy in patients with advanced esophageal squamous cell carcinoma refractory or intolerant to previous chemotherapy (ATTRACTION-3): a multicentre, randomised, open-label, phase 3 trial. *Lancet Oncol*. 2019;20:1506–17.
- Arnold M, Soerjomataram I, Ferlay J, et al. Global incidence of esophageal cancer by histological subtype in 2012. *Gut*. 2015;64:381–7.
- Huang F, Yu S. Esophageal cancer: Risk factors, genetic association, and treatment. *Asian J Surg*. 2016;41:210–5.
- Abnet CC, Arnold M, Wei W. Epidemiology of Esophageal Squamous Cell Carcinoma. *Gastroenterology*. 2017;154:360–73.
- Liu J, Liu T, Wang X, et al. Circles reshaping the RNA world: from waste to treasure. *Mol Cancer*. 2017;16:58.
- Chen LL. The biogenesis and emerging roles of circular RNAs. *Nat Rev Mol Cell Biol*. 2016;17:205–11.
- Arnaiz E, Sole C, Manterola L, et al. CircRNAs and cancer: Biomarkers and master regulators. *Semin Cancer Biol*. 2019;58:90–9.
- Vo JN, Cieslik M, Zhang Y, et al. The Landscape of Circular RNA in Cancer. *Cell*. 2019;176:869–81.e13.
- Chen X, Jiang J, Zhao Y, et al. Circular RNA circNTRK2 facilitates the progression of esophageal squamous cell carcinoma through up-regulating NRIP1 expression via miR-140-3p. *J Exp Clin Cancer Res*. 2020;39:133.
- Zhang Y, Wang Q, Zhu D, et al. Up-regulation of circ-SMAD7 inhibits tumor proliferation and migration in esophageal squamous cell carcinoma. *Biomed Pharmacother*. 2019;111:596–601.
- Bach DH, Lee SK, Sood AK. Circular RNAs in Cancer. *Mol Ther Nucleic Acids*. 2019;16:118–29.
- Panda AC. Circular RNAs Act as miRNA Sponges. *Adv Exp Med Biol*. 2018;1087:67–79.
- Kwan JY, Psarianos P, Bruce JP, et al. The complexity of microRNAs in human cancer. *J Radiat Res*. 2016;57(Suppl 1):i106–i111.
- Zhang J, Kong X, Shi Q, et al. MicroRNA-383-5p acts as a potential prognostic biomarker and an inhibitor of tumor cell proliferation, migration, and invasion in breast cancer. *Cancer Biomark*. 2020;27:423–32.
- Zhu K, Diao D, Dang C, et al. Elevated KIAA0101 expression is a marker of recurrence in human gastric cancer. *Cancer Sci*. 2013;104:353–9.
- Cheng Y, Li K, Diao D, et al. Expression of KIAA0101 protein is associated with poor survival of esophageal cancer patients and resistance to cisplatin treatment in vitro. *Lab Invest*. 2013;93:1276–87.
- Kudo-Saito C, Shirako H, Takeuchi T, et al. Cancer metastasis is accelerated through immunosuppression during Snail-induced EMT of cancer cells. *Cancer Cell*. 2009;15:195–206.
- Alsop BR, Sharma P. Esophageal Cancer. *Gastroenterol Clin North Am*. 2016;45:399–412.
- Patop IL, Kadener S. circRNAs in Cancer. *Curr Opin Genet Dev*. 2018;48:121–7.
- Li Y, Zheng Q, Bao C, et al. Circular RNA is enriched and stable in exosomes: a promising biomarker for cancer diagnosis. *Cell Res*. 2015;25:981–4.
- Li RC, Ke S, Meng FK, et al. CiRS-7 promotes growth and metastasis of esophageal squamous cell carcinoma via regulation of miR-7/HOXB13. *Cell Death Dis*. 2018;9:838.
- Fang J, Ji WH, Wang FZ, et al. Circular RNA hsa\_circ\_0000700 promotes cell proliferation and migration in Esophageal Squamous Cell Carcinoma by sponging miR-1229. *J Cancer*. 2021;12:2610–23.
- Militello G, Weirick T, John D, et al. Screening and validation of lncRNAs and circRNAs as miRNA sponges. *Brief Bioinform*. 2017;18:780–8.
- Jiang J, Xie C, Liu Y, et al. Up-regulation of miR-383-5p suppresses proliferation and enhances chemosensitivity in ovarian cancer cells by targeting TRIM27. *Biomed Pharmacother*. 2019;109:595–601.
- Ma R, Gao P, Yang H, et al. Inhibition of cell proliferation and radio-resistance by miR-383-5p through targeting RNA binding protein motif (RBM3) in nasopharyngeal carcinoma. *Ann Transl Med*. 2021;9:123.

27. Azarbarzin S, Hosseinpour-Feizi MA, Banan Khojasteh SM, et al. MicroRNA -383-5p restrains the proliferation and migration of breast cancer cells and promotes apoptosis via inhibition of PD-L1. *Life Sci.* 2021;267:118939.
28. Xu G, Li N, Zhang Y, et al. MicroRNA-383-5p inhibits the progression of gastric carcinoma via targeting HDAC9 expression. *Braz J Med Biol Res.* 2019;52:e8341.
29. Mu X, Wu H, Liu J, et al. Long noncoding RNA TMPO-AS1 promotes lung adenocarcinoma progression and is negatively regulated by miR-383-5p. *Biomed Pharmacother.* 2020;125:109989.
30. Wan P, Chi X, Du Q, et al. miR-383 promotes cholangiocarcinoma cell proliferation, migration, and invasion through targeting IRF1. *J Cell Biochem.* 2018;119:9720–9.
31. Wang X, Ren Y, Wang Z, et al. Down-regulation of 5S rRNA by miR-150 and miR-383 enhances c-Myc-rpL11 interaction and inhibits proliferation of esophageal squamous carcinoma cells. *FEBS Lett.* 2015; 589:3989–97.
32. Felekis K, Touvana E, Stefanou C, et al. microRNAs: a newly described class of encoded molecules that play a role in health and disease. *Hippokratia.* 2010;14:236.
33. Turchi L, Fareh M, Aberdam E, et al. ATF3 and p15PAF are novel gatekeepers of genomic integrity upon UV stress. *Cell Death Differ.* 2009;16:728–37.
34. Hosokawa M, Takehara A, Matsuda K, et al. Oncogenic role of KIAA0101 interacting with proliferating cell nuclear antigen in pancreatic cancer. *Cancer Res.* 2007;67:2568–76.
35. Sun T, An Q, Yan R, et al. MicroRNA-216a-5p suppresses esophageal squamous cell carcinoma progression by targeting KIAA0101. *Oncol Rep.* 2020;44:1971–84.

## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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