CircHIPK3 Acts as an Oncogene by Sponging miR-124 and Regulating AKT3 Expression in Esophageal Squamous Cell Carcinoma

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Research

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Abstract

Purpose: Circular RNAs (circRNAs) are an important type of RNA regulatory factor. Recent studies have demonstrated that circHIPK3 is closely related to the malignant behavior of cancer cells. However, the function of circHIPK3 in esophageal squamous cell carcinoma (ESCC) remains unclear. The aim of the current study was to investigate the value of circHIPK3 for the prognosis of patients with ESCC.

Methods: The expression of circHIPK3 in 32 pairs of ESCC and normal tissues were detected by quantitative Real-time polymerase chain reaction (RT-qPCR); the correlation between circHIPK3 expression and the pathological features of patients was also analyzed. Cell biology experiments and bioinformatics were used to explore the function of circHIPK3 in the development of ESCC.

Results: The expression of circHIPK3 in tumor tissues of ESCC patients was significantly higher than that of adjacent tissues. Moreover, knockdown the expression of circHIPK3 retarded esophageal cancer cell proliferation *in vitro* and *in vivo*. Mechanistically, we found that circHIPK3 acted as a sponge to absorb miR-124 and promoted *AKT3* expression.

Conclusion: Our work revealed that circHIPK3, acting as an oncogene, promotes tumor progression in ESCC, and that the circHIPK3- *AKT3* axis is a potential therapeutic target for patients with ESCC.

Introduction

Esophageal cancer is one of the most common cancers in human beings with a high mortality[1]. China is a high incidence area of esophageal cancer, and about 150,000 patients die of the disease every year[2]. The histological types of esophageal cancer mainly include esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma. The latter is common in European and American countries, while the incidence of esophageal cancer in China is mainly ESCC, accounting for about 90% [3]. Esophageal cancer is characterized by high metastasis and invasiveness, and lacks markers for early diagnosis. When diagnosed, it is mostly in the middle or late stage. Therefore, the 5-year survival rate of esophageal cancer is only 15% – 25%. Therefore, if we can further clarify the molecular mechanism of the occurrence and development of esophageal cancer and improve the early diagnosis of esophageal cancer, it is expected to improve the cure rate and reduce the mortality rate of esophageal cancer.

Circular RNA (circRNA) is a class of non-coding single stranded RNA molecules. The median length of the circRNA was 530 nucleotides[4, 5]. circRNAs play an important role in biological and pathological processes, affect cell apoptosis and metabolism, and work as oncogenes or tumor suppressor genes[6]. The closed-loop structure of circRNA determines its highly stable and conservative biological genetic characteristics. Currently, research on the circRNA has shown that the realization of its biological function mainly takes place as the "molecular sponge" of miRNA. By competitive adsorption of miRNA, it leads to its inactivation, and further affects the biological function of the cell. Zheng, Q. et al. have shown that there are three different kinds of splicing bodies in the circRNAs of HIPK3 gene, among which circHIPK3 is the most abundant and has significant function in cells [7]. Many studies have found that the

expression of circHIPK3 in human tumor cells and normal cells is very different[8–10]. Therefore, researchers speculate that circHIPK3 may affect the progression of tumors. At the same time, other studies have shown that circHIPK3 plays an important role in the development of bladder cancer, liver cancer and lung cancer. However, the functions of circHIPK3 in ESCC remain unclear.

As far as we know, this work is the first to investigate the expression and function of circHIPK3 in ESCC. We found that circHIPK3 was significantly upregulated in 32 pairs of ESCC tissues. Mechanistically, we found that circHIPK3 acted as a sponge to absorb miR-124 and promoted *AKT3* expression. These observations indicate a possible novel therapeutic strategy involving circular RNAs in ESCC.

Materials And Methods

Patients and tissue specimens

The collection and manipulation of human patients' tissues were approved by the Human Research Ethical Committee of Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University. Tissues used for research were collected from 32 patients with ESCC who underwent resection at Shenzhen Second People's Hospital from 2018 to 2020. All the patients signed informed consent forms. The tissues were obtained and instantly transported in an ice box to the laboratory. Half part of the tissue was frozen in liquid nitrogen for RNA and protein extraction, and the other part was fixed in formaldehyde solution for RNAscope analysis.

Real time-quantitative PCR (RT-qPCR) analysis and In Situ RNA Detection

Total RNA was extracted by Trizol (DP424, Tiangen, China) from tissues and cells. The cDNA and RT-qPCR assays were performed using SuperReal PreMix Plus (FP205, Tiangen, China) in the LightCyclerR480II System (Roche) following the manufacturer's instructions. The primers used in this study were in the supplementary table1. RNAscope manual procedure was conducted at Shenzhen Hospital laboratories following standard protocol as previously described with minor modifications[11].

Nucleic Acid Electrophoresis and Treatment with RNase R.

The cDNA and gDNA PCR products were detected by 2% agarose gel electrophoresis. The DNA was separated by electrophoresis at 180 V for 15 min. The results were then illustrated by UV irradiation. RNase R treatment was conducted for 15 min at 37°C using RNase R (NEB) 5 U/mg.

Cell culture and shRNA transfection

KYSE-150 and KYSE-410 cells were gifts from Professor Xinchen Sun (Department of Radiology, the First Affiliated Hospital of Nanjing Medical University). The ECA-109, KYSE180 cells and a normal human esophageal epithelial cell line (HEEC) were purchased from GeneChem (Shanghai, China). All cell lines

were cultured according to ATCC guidelines at 37°C in a 5% CO2 incubator. KYSE-150 and ECA-109 cells were transfected with circHIPK3 knockdown lentivirus and negative control vectors, following the standard manufacturer's instructions (GENECHEM, Shanghai, China). The stable knockdown cell lines were selected with 4μg/ml of puromycin treatment after 72h of transfection. The efficiency of knockdown was tested by RT-qPCR.

Cell proliferation and Colony formation assays

To conduct cell proliferation assays, CCK-8 kit (Dojindo, Kumamoto, Japan) and EdU Apollo® 567 In Vitro Imaging Kit (Ribobio, Guangzhou, China) were used according to the manufacturer's instruction. To conduct the colony formation assay, 2.5×10^3 cells were seeded into six-well plate. 10 days later, the colonies were fixed and stained according to the manufacturer's instruction (Beyotime, Beijing, China). Then, visible colonies were photographed (Nikon, Tokyo, Japan) and calculated. All experiments were repeated three times.

Transwell analysis

The KYSE-150 and ECA-109 cells were prepared into serum-free cell suspension. Each Transwell chamber was inoculated with 2×10^4 cells, and the lower Transwell chamber was added with 500 μ l serum containing DMEM. The cells were incubated in 5% CO2 incubator at 37 ℃ for 24 h, the cells were wiped with cotton swab, fixed with 4% paraformaldehyde for 30 min, and stained with 0.1% crystal violet for 20 min at room temperature. Five visual field cells were randomly selected under microscope, photographed and counted. The experiment was repeated three times.

Western Blotting

Western Blotting was performed as described previously[12, 13]. The antibodies used for western blot are as follows: Primary antibody: AKT3 (22028-1-AP, Proteintech, 1:2000), anti-GAPDH antibody (66009-1-Ig, Proteintech ,1:100000), E-cadherin (20874-1-AP, Proteintech, 1:2000), Vimentin (10366-1-AP, Proteintech, 1:2000). Secondary antibody: Goat Anti-Rabbit (SA00001-1, Proteintech, 1:5000).

Renilla luciferase

The fragment of *AKT3* 3' UTR containing the binding site of miR-124 was spliced to the 3'-end of the Renilla luciferase reporter gene. The wildtype or mutant circHIPK3 and miR-124 binding sites were sub-cloned into psiCHECK-2 system (Promega, Madison, WI, United States).

Animals

A total of 50 BALB/c nude mice were chosen and assigned to 2 groups: shCtrl group (injected with ECA-109 cells) and sh-circHIPK3 group (injected with ECA-109 cells with circHIPK3 knockdown). 200 ul of the above cell suspension containing $2 *10^5$ cells was injected into the left or right back of each mice. Tumor sizes and tumor volume were measured as described previously[14]. We euthanized mice with carbon dioxide. The mice were anesthetized with ether.

Statistical Analysis

Statistical analyses were performed using excel or Graphpad Prism software version 8.0 (USA). Experimental data are described as mean ± SEM. The significance of the observed differences was measured via the Student's t-test or chi-square test. $P < 0.05$ was served to be statistically significant.

Results

The expression patten of circHIPK3 in ESCC tumor tissues.

To elucidate the functional roles of circHIPK3 in ESCC, we investigated the expression of circHIPK3 in 32 patients' tumor tissues and adjacent tissues by using RT-qPCR and RNAscope. Two sets of primers for circHIPK3 were designed. The first set contained a divergent primer that amplifies only circHIPK3. The second set of primers contained an opposite-directed primer to detect the HIPK3 mRNA. Using cDNA and gDNA (genomic DNA) from ESCC tissues, circHIPK3 was only amplified by divergent primers in cDNA, and no amplification product was observed in gDNA (Fig. 1A). Sanger sequencing confirmed the cyclization site sequence (Fig. 1B). RNase R was used to digest the RNA in ESCC tissues. RT-qPCR results showed that the expression level of circHIPK3 did not change significantly before and after RNase R treatment, but the expression level of linear HIPK3 mRNA decreased significantly (Fig. 1C, $P < 0.01$). We then identified that the expression of circHIPK3 was markedly higher in ESCC tissues compared with the adjacent normal tissues. High expression of circHIPK3 was detected in 21/32 (66%) ESCC (Fig. 1D and Table 1). Higher expression of circHIPK3 was also proved by RNAscope analysis (Fig. 1E). In addition, we also found a significant correlation between circHIPK3 expression and clinical features. The results revealed that patients with higher expression of circHIPK3 exhibited lymph node metastasis, larger tumor size and poorly tumor differentiation (Table 1). In order to choose the suitable cell lines for *in vitro* experiments, the expression of circHIPK3 was detected by RT-qPCR. Consistent with results in tumor tissue, the expression of circHIPK3 was higher in ESCC cell lines than the normal human esophageal epithelial cell line (HEEC) (Fig. 1F). Then, we selected KYSE-150 and ECA-109 cell lines with the highest circHIPK3 expression to investigate the functions of circHIPK3 *in vitro*.

Table 1
Relationship between circHIPK3 expression in patients with ESCC and clinicopathologic characteristics.

Feature	No.	circHIPK3 expression		P value
		High	Low	
Age(years)				
≥ 60	23	15	8	0.612
< 60	9	5	4	
Tumor size(cm)				
≥ 4.0	20	16	4	0.008*
< 4.0	12	4	8	
Tumor differentiation				
Poorly	19	15	4	0.020*
High	13	5	8	
Lymph node metastasis				
N0-N1	14	6	8	0.043*
N2-N3	18	14	4	
TNM Stage				
I/II	10	5	5	0.325
III/IV	22	15	7	

CircHIPK3 promotes proliferation and migration of ESCC cell lines in vitro and in vivo

To further investigate if circHIPK3 was correlated with ESCC progression, shRNA specifically targeting circHIPK3 was transfected into KYSE-150 and ECA-109 cells by lentivirus infection, respectively. The results from RT-qPCR displayed that shRNA specifically downregulated the expression of circHIPK3 (Fig. 2A). To explore the role of circHIPK3 knockdown on cell proliferation, CCK-8 and EdU proliferation assays were conducted. CCK-8 and EdU analysis showed that cell proliferation in both KYSE-150 and ECA-109 cell lines were blocked after circHIPK3 knockdown (Fig. 2B-D). Additionally, knockdown circHIPK3 expression decreased growth ability as a result of fewer colonies formed after 9 days than the shCtrl group in both cell lines (Fig. 2E-F). We further tested whether the expression of circHIPK3 influenced cell migration or not. While knockdown the expression of circHIPK3 significantly reduced the migration of KYSE-150 and ECA-109 cells (Fig. 2G). In addition, knockdown the expression of circHIPK3

clearly reduced the expression of E-cadherin and Vimentin (Fig. 2H); To explore whether knockdown the expression of circHIPK3 could reduce tumor growth and migration *in vivo*, normal expression of circHIPK3 and knockdown expression of circHIPK3 ECA-109 cells were seeded into the nude mouse, respectively. The tumor growth and migration were monitored (Fig. 2I–J). The *in vivo* experiments showed that knockdown expression of circHIPK3 reduced tumor growth and migration.

CircHIPK3 interacts with miR-124 to meditate ESCC cell lines proliferation and migration

It has been found that circHIPK3 functions as “miRNA sponge” in various cancers. To address whether circHIPK3 could sponge miRNAs in ESCC cells, we selected candidate miRNAs from the experiment results of miRNA recognition elements in circHIPK3 datasets by Circ2Disease (http://bioinformatics.zju.edu.cn/Circ2Disease/index.html). The Circ2Disease database suggested that miR-124 was the target gene of circHIPK3 that had been verified most times. To investigate the relationship between miR-124 and circHIPK3, we performed luciferase reporter assays in KYSE-150 and ECA-109 cells. As shown in Fig. 3, we observed that co-transfection of miR-124 and circHIPK3 markedly suppressed the luciferase activity compared with that from the co-transfection of miR-124 and circHIPK3-MUT (Fig. 3A and B). Moreover, the expression of miR-124 was up-regulated after knockdown of circHIPK3 (Fig. 3C). To explore the role of miR-124 in ESCC, we performed CCK-8 assays to detect the relationship between the expression of miR-124 and cell proliferation. As illustrated in Fig. 3, miR-124 overexpression markedly reduced cell proliferation which could be rescued by co-transfection with circHIPK3 in ESCC cell lines (Fig. 3D). Transwell analysis proved that miR-124 markedly reduced the migration of KYSE-150 and ECA-109 cells, whereas it was reversed by circHIPK3 overexpression (Fig. 3E). Western blot analysis illustrated that miR-124 overexpression-reduced the expression of E-cadherin and Vimentin (Fig. 3F). A reverse correlation was discovered between miR-124 and circHIPK3 in the real-world tumor tissues (Fig. 3G).

AKT3 is a target of miR-124 and is meditated by circHIPK3

Notably, the RTK-MAPK-PI3K pathway is frequently dysregulated by multiple molecular mechanisms in ESCC, suggesting that the genes involved in this signaling pathway may be the target genes of miR-124. Based on bioinformatics prediction using TargetScan (http://www.targetscan.org/vert_72/), *Akt3* was screened out as a prime target, with a highly conserved complementary miR-124-binding site in its 3’ UTR across vertebrates from Lizard to Human (Fig. 4A). To confirm miR-124 function at the *AKT3* 3’ UTR, we performed luciferase reporter assays in KYSE-150 and ECA-109 cells. As shown in Fig. 4, we found that miR-124 up-regulation markedly suppressed the luciferase activity when co-transfected miR-124 with wild-type *AKT3* 3’ UTR vectors (Figs. 4B and C). Moreover, miR-124 overexpression repressed the expression of AKT3, whereas it was reversed by circHIPK3 overexpression (Figs. 4D). Knockdown the expression of AKT3 obviously suppressed the cell proliferation and migration (Figs. 4E and F). In order to further verify the synergy of circHIPK3/miR-124/*AKT3* in ESCC, we detected the correlation of

circHIPK3/miR-124/*AKT3* expression in ESCC tumor tissues. It is of note that the expression of *AKT3* was positively correlated with the high expression of circHIPK3 ($P < 0.01$, Figs. 4G and H), which suggested that circHIPK3 might regulate the expression of *AKT3* by sponging miR-124.

Discussion

ESCC is one of the most lethal malignant tumors in the world, and its treatment is limited [3]. In addition, the biological mechanism of the occurrence and development of ESCC is still largely unknown. Therefore, it is urgent to explore the biological mechanisms of ESCC to identify molecular biomarkers for early diagnosis and prognosis. CircRNA is a long noncoding RNA (ncRNA), whose transcript length is usually more than 200 and contains no coding region [15]. Emerging evidence has revealed that circRNA plays a critical role in different types of human cancers[16]. As a miRNA sponge, circRNA regulates many biological processes by regulating the function of miRNA, affecting RNA splicing, chromatin structure and mRNA stability [17, 18].

Current studies have found that circHIPK3 plays a dual role in a variety of human cancers. The overexpression of circHIPK3 can effectively reverse the miR-7-induced decrease in the progression of colorectal cancer cells by up-regulating the expression of several key miR-7 target genes (including EGFR, IGF1R, FAK and YY1) [8]. In gallbladder cancer cells, knockdown the expression of circHIPK3 could increase the expression of miR-124, thereby reducing tumor cell proliferation and survival [19]. Overexpression of circHIPK3 sponges miR-124, inhibits its activity and increases the expression of its target genes, such as IL6R and DLX2, thereby causing tumor cell growth. However, in some kinds of cancer, circHIPK3 also displays the function of inhibiting tumor growth. Overexpression of circHIPK3 significantly inhibited the growth of bladder cancer *in vivo* [20], while knockdown the expression of circHIPK3 promoted the proliferation of ovarian cancer cells (A2780 and SKOV3) and normal ovarian epithelial cells [21]. These studies proved that circHIPK3 participated in complex regulatory networks, and confers cell-type-specific regulation of cell function in different cancers.

In this study, we investigated the expression of circHIPK3 in ESCC tumor tissues and ESCC cell lines. We further identified that circHIPK3 negatively regulated the expression of miR-124 in ESCC

cell lines. Our *in vitro* experiments showed that miR-124 suppressed cell proliferation and migration in ESCC cells. Moreover, the expression of miR-124 was remarkably down-regulated in ESCC tumor tissues. Therefore, we suggested that miR-124 functioned as a tumor suppressor gene that was dependent on circHIPK3 in ESCC.

Moreover, our data for the first time proved that *AKT3* was suggested to be a target gene of miR-124. *AKT3* encodes a serine/threonine protein kinase that belongs to the AGC kinase family [22]. AKT-kinases play roles in signaling pathways involved in cell proliferation, oncogenic transformation, cell survival, cell migration, and intracellular protein trafficking [23, 24]. *AKT3* alterations have been related with tumor growth and migration. Previously, gain of function mutations in *AKT3* were found in several cancer types, such as breast cancer and endometrium cancer, which were relied on the mediation of PI3K signaling

pathways [25, 26]. In this study, using luciferase and western blot analysis, we found that miR-124 negatively modulated *AKT3* expression. In ESCC tumor tissues, AKT3 expression was highly increased, which was in line with the TCGA database. Intriguingly, promoting the expression of circHIPK3 enhanced *AKT3* expression in ESCC cells, while the opposite result was observed in circHIPK3-knockdown cells. Moreover, enhancing *AKT3* expression significantly enhanced ESCC cell proliferation and migration, which were in line with those promoting tumorigenesis in various cancers[25].

Conclusion

In summary, for the first time in the present study, our results indicated that circHIPK3 functioned as an oncogenic circRNA that enhanced ESCC tumorigenesis and progression through the miR-124/ *AKT3* pathway. The findings here indicate that a circHIPK3- miR-124/ *AKT3* axis may be a potential therapeutic target for ESCC.

Declarations

Ethics approval and consent to participate

The researchers were granted approval to conduct the research by the Departmental Research Ethics Committee of Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University. The study protocol was approved by the institutional review board of Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University. All the procedures were performed in accordance with the Declaration of Helsinki and relevant policies in Shenzhen Second People's Hospital, The First Affiliated Hospital of Shenzhen University. The animal studies were approved by the Institutional Animal Care and Use Committee of Shenzhen Hospital, and were carried out according to institutional guidelines at animal experimental center of Shenzhen Hospital (Approval number: SZDL2020334452).

Consent for publication

Participants have given their consent that data from this study can be published in an anonymized form.

Data availability statement

All data generated or analyzed during the present study are included in this published article.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Author contributions

Designing research studies: WZ and YD. Conducting experiments: YD, LS and CS. Preparing the manuscript: WZ and YJ. Grammar Check: LS and CS. Supervision: WZ. Funding Acquisition: WZ; The authors read and approved the final manuscript.

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Figures

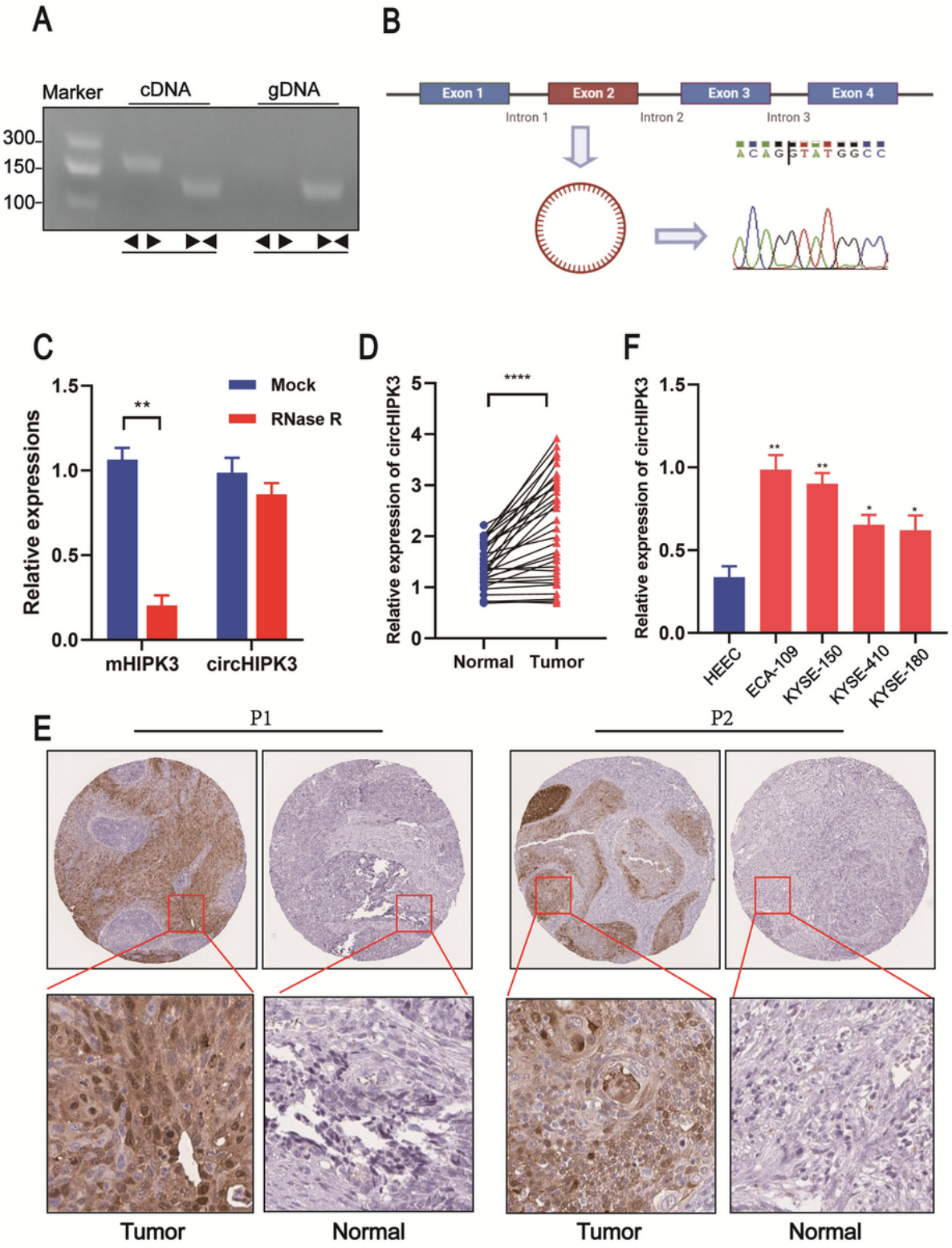

Figure 1

circHIPK3 validation and expression in ESCC tissues and cell lines. (A) The existence of circHIPK3 was validated in ECA-109 cell lines by RT–qPCR. Divergent primers amplified circHIPK3 in cDNA but not genomic DNA (gDNA). (B) Schematic illustration showing the circularization of HIPK3 exon 2 forming circHIPK3 (blue arrow). The presence of circHIPK3 was confirmed by Sanger sequencing. (C) The expression of circHIPK3 and HIPK3 mRNA in ECA-109 cells treated with or without RNase R was detected

by RT-qPCR. (D) The expression of circHIPK3 was detected by real-time PCR in 32 pairs of ESCC and normal tissues. (E) Representative RNAscope images of circHIPK3 expression in ESCC tissues and adjacent normal tissue of two patients (P1–P2). (F) The expression of circHIPK3 in ESCC cell lines was determined using RT-qPCR. (** P<0.01, ****P < 0.0001, Student's t-test.)

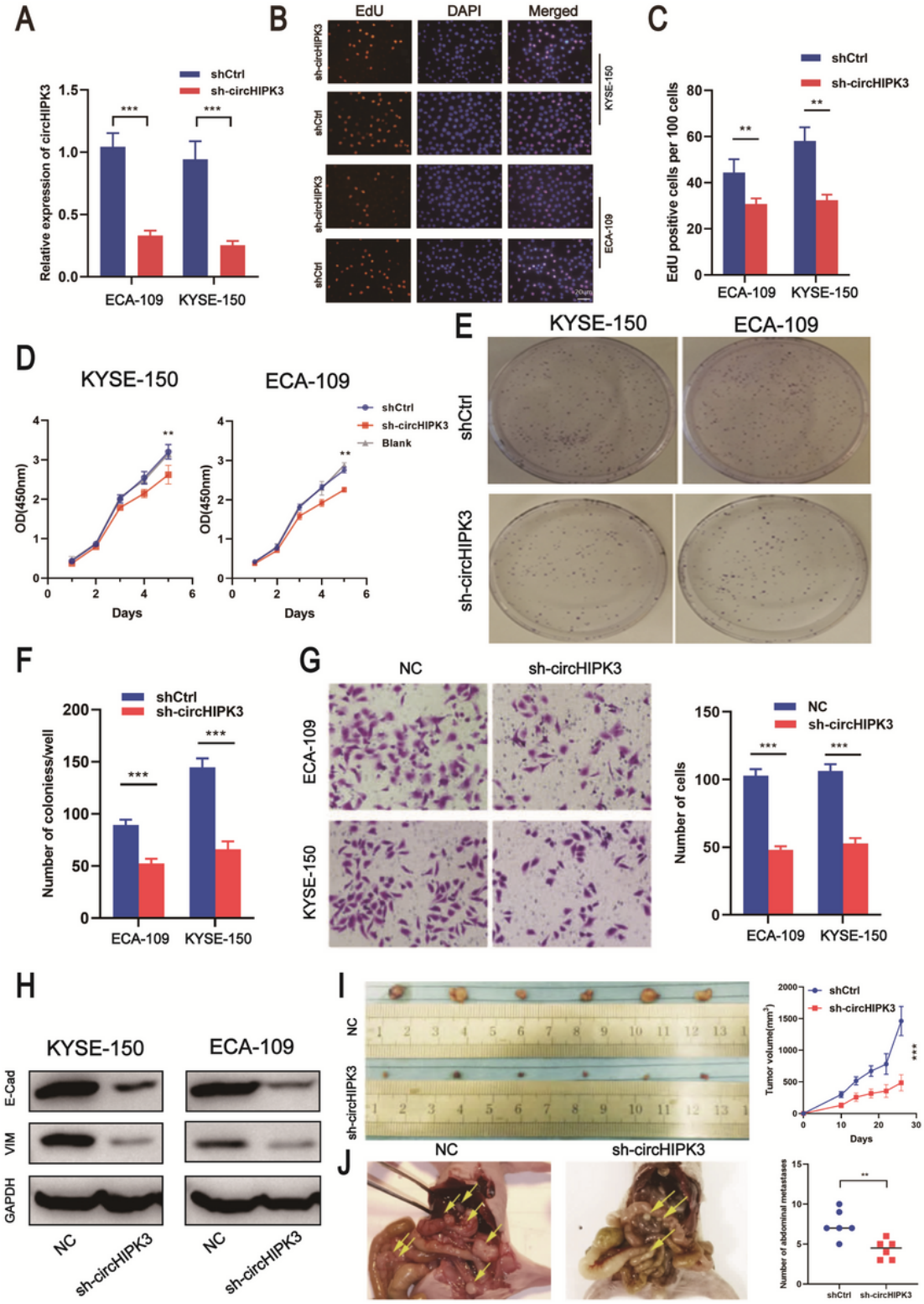

Figure 2

circHIPK3 Promotes ESCC Cells Tumorigenicity in vitro and in vivo. (A) Expression level of circHIPK3 knockdown efficiency in ECA-109 and KYSE-150 cell lines was detected by RT-qPCR. (B-D) The influences of circHIPK3 knockdown on cell proliferation were confirmed using the CCK-8 assay and EdU assay. (E-F) The representative picture of colony formation assay, and the quantification of colonies per well. (G) Transwell analysis was used to determine the effects of circHIPK3 on migration in ECA-109 and KYSE-150 cell lines. (H) WB exhibited the protein levels of E-cadherin and Vimentin in ESCC cells transfected with shCtrl or sh-circHIPK3. (I-J) Knockdown of circHIPK3 effectively inhibited ECA-109 cells subcutaneous tumor growth and migration in nude mice. (** $P<0.01$, *** $P<0.001$, Student's t-test.)

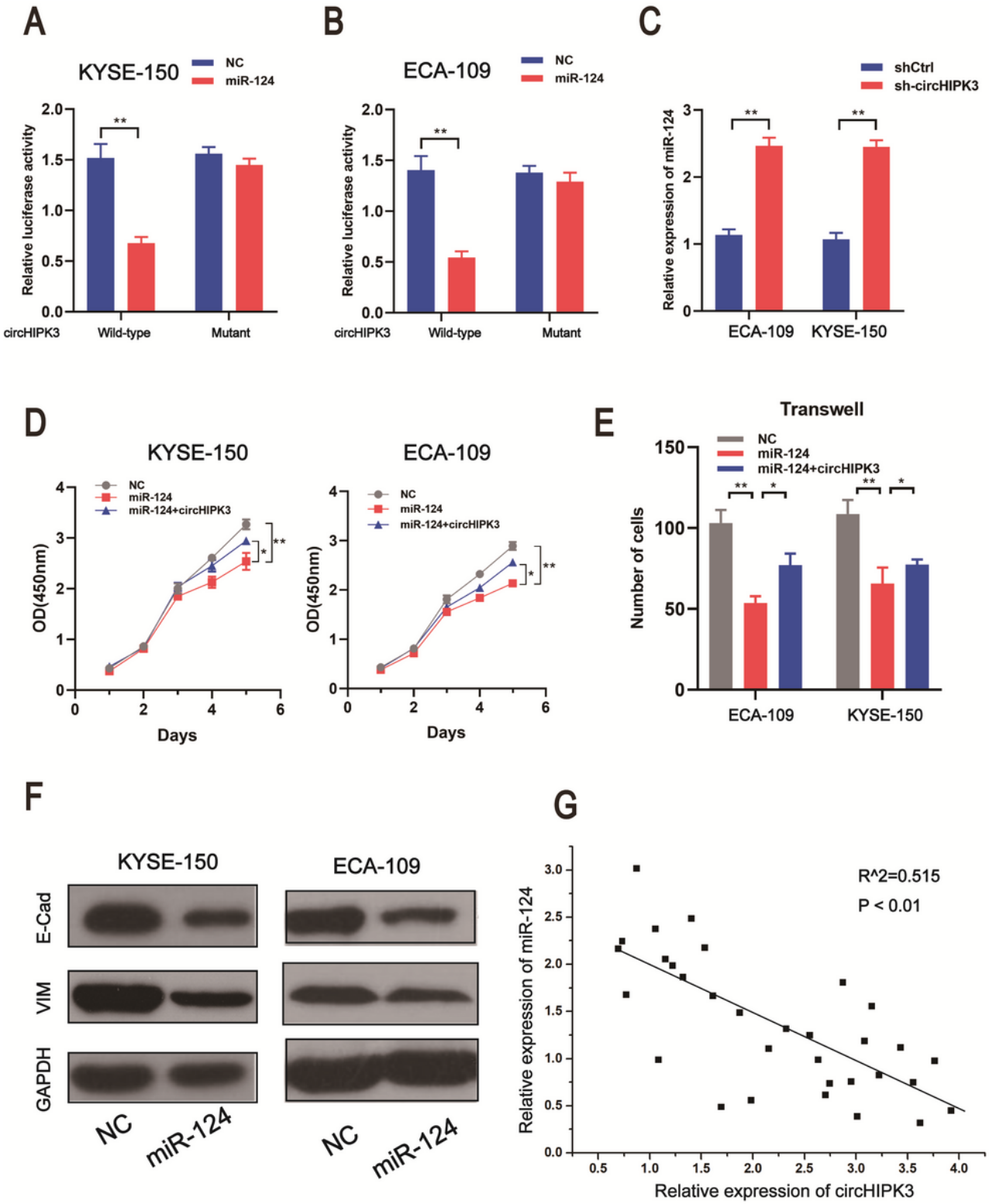

Figure 3

circHIPK3 interacts with miR-124 to meditate ESCC cells proliferation and migration. (A-B) Luciferase report analysis of ESCC cells co-transfected with miR-124 or control and circHIPK3 WT or circHIPK3 MUT. (C) RT-qPCR was used to measure miR-124 expression in ESCC cells with circHIPK3 or circHIPK3 knockdown. (D) CCK-8 analysis of ESCC cell proliferation after co-transfection with NC-mimics, miR-124, or circHIPK3 as indicated. (E) Transwell analysis of ESCC cell migration after co-transfection with NC-

mimics, miR-124, or circHIPK3 as indicated. (F) WB exhibited the protein levels of E-cadherin and Vimentin in ESCC cells transfected with NC-mimics or miR-124. (G) A negative correlation between circHIPK3 and miR-124 in ESCC tissues. (** P<0.01, Student's t-test.)

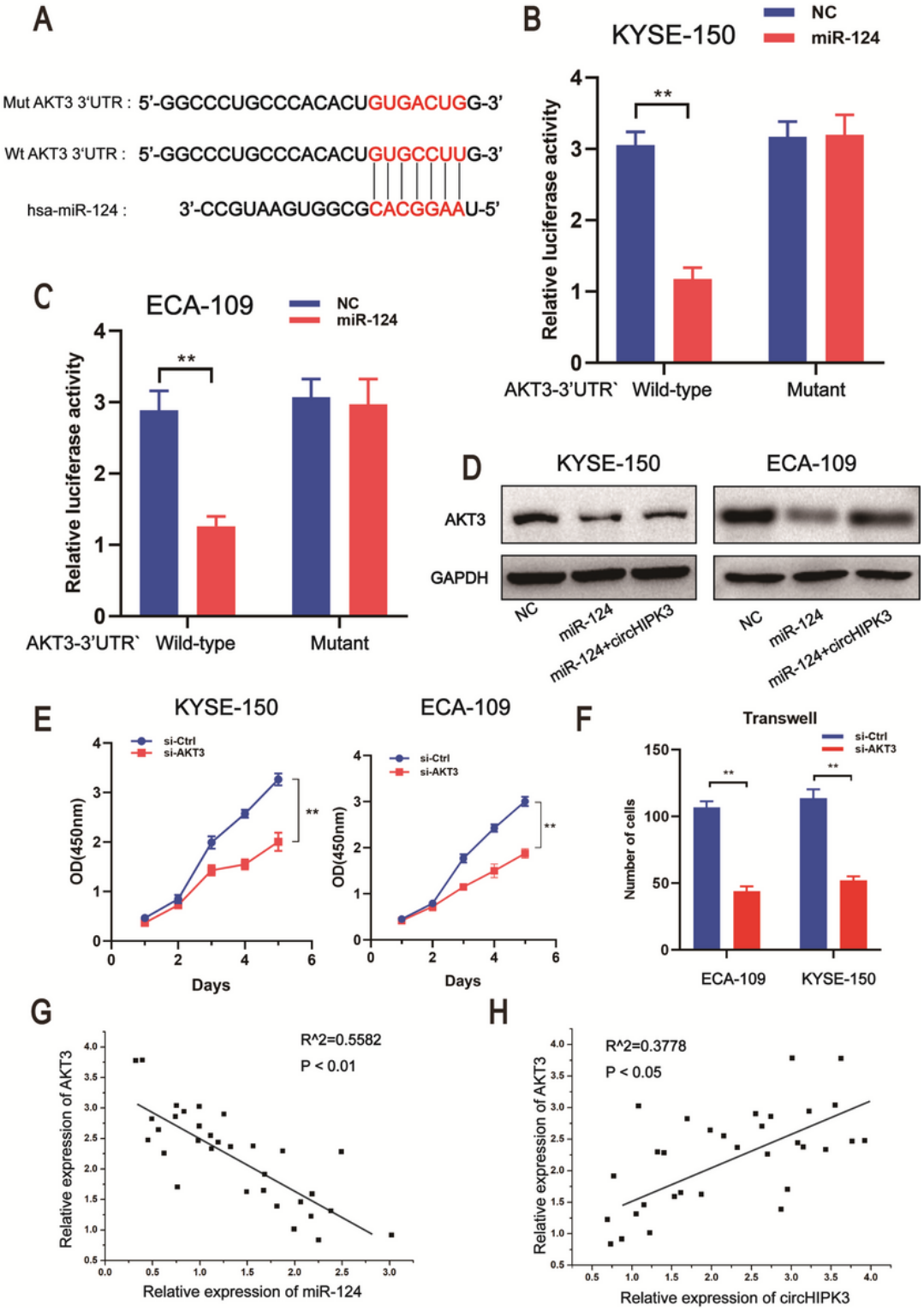

Figure 4

AKT3 is the target of miR-124 in ESCC cells. (A) Putative and mutant binding sites of miR-124 on AKT3. (B-C) Luciferase report analysis of ESCC cells co-transfected with miR-124 or control and AKT-3'UTR

WT or $AKT-3'UTR$ MUT. (D) WB was used to measure AKT3 expression in ESCC cells with miR-124 or circHIPK3. (E) CCK-8 analysis of ESCC cell proliferation after co-transfection with si-Control or si-AKT3 as indicated. (F) Transwell analysis of ESCC cell migration after co-transfection with si-Control or si-AKT3 as indicated. (G-H) A correlation between circHIPK3, miR-124 and AKT3 in ESCC tissues. (** P<0.01, Student's t-test.)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementaryTable1.xls