

Original Article

Expression of circRNA-0036474 in esophageal cancer and its clinical significance

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Abstract: Circular RNAs are considered to play important roles in the progression of different cancers such as esophageal squamous cell carcinoma. However, the functions of circular RNAs in esophageal squamous cell carcinoma are still not clear. This study aimed to investigate the role and mechanism of circRNA-0036474 in the progression of esophageal squamous cell carcinoma. The hsa_circ_0036474 expression levels were found to be elevated in both EC109 cells and esophageal squamous cell carcinoma tissue samples. Moreover, knockdown of circRNA-0036474 expression in the EC109 cells induced migration and invasion, characterized by the down-regulation of E-cadherin, and up-regulation of N-cadherin and vimentin. In addition, the over-expressed hsa_circ_0036474 significantly decreased the activity of EC109 cells, elevated E-cadherin expression but declined N-cadherin and vimentin expression. Moreover, over-expressed mir-223-3p levels and interfered RERG expression verified the role of hsa_circ_0036474 in inhibiting the invasion and migration of EC109 cells, reducing the expression of N-cadherin and vimentin, and promoting the expression of E-cadherin. In conclusion, circRNA-0036474 mitigated the progression of esophageal squamous cell carcinoma through regulating mir-223-3p/RERG axis, presenting a potential therapeutic target for the treatment.

Keywords: Circular RNAs, overexpression, cell biological behavior, molecular mechanism, esophageal squamous cell carcinoma

Introduction

Esophageal cancer is considered as one of the common malignant tumors in the digestive system, with an overall 5-year survival rate of 18% [1]. Esophageal squamous cell carcinoma, as the primary pathological type, accounted for about 90% of cases [2]. Despite significant advancements in the treatment of esophageal cancer in recent years, the mortality is still high due to unknown genetic mechanisms. Notably, the pathogenesis of esophageal cancer is quite complex. Previous studies suggested that the abnormal proliferation, differentiation, infiltration, and apoptosis of cells could result in the onset of esophageal cancer [3]. Studies have demonstrated a variety of abnormal expressions of miRNAs in esophageal cancer, which could be related to esophageal cancer genesis and progression [4]. Investigating the specific mechanisms underlying esophageal cancer

development could provide new strategies for its prevention and treatment.

Circular RNAs (CircRNAs) are a large group of endogenous RNAs, which are characterized by the formation of covalently closed continuous loops and are widely expressed in eukaryotes [5]. At present, considerable efforts have been made to understand the roles of circular RNAs in the development of tumors [6]. Various studies have indicated that most circRNAs modulate target genes expression downstream by competitively binding to miRNAs, thus involving in the post-transcriptional regulation of gene expression and affecting the biological functions of the body [7]. It has been demonstrated that circRNAs are associated with the progression of multiple diseases, including breast cancer, gastric cancer, malignant melanoma, and colorectal cancer [8]. CircRNA-0036474 dysregulation has been implicated in multiple dis-

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eases [9]. However, its role in esophageal cancer progression remains unknown. Moreover, many circRNAs play a role in the development of esophageal cancer by interacting with related miRNAs, but the effect of CircRNA-0036474 on esophageal squamous cell carcinoma cells has yet to be determined. Considering these aspects, this study aimed to elucidate the expression level of CircRNA-0036474 and its effects on the occurrence of esophageal cancer. Accordingly, the observed CircRNA-0036474 expression levels within esophageal cancer were correlated to the mechanisms of migration, invasion, and epithelial-mesenchymal transition (EMT) pathways, so that the findings of this study could propose a promising clinical marker and treatment target for esophageal squamous cell carcinoma.

Materials and methods

General information

Thirty-six paired samples of esophageal squamous cell carcinoma and adjacent noncancerous tissues (located at least 6 cm away from the cancer tissues) were obtained from patients during surgical operation at The First Affiliated Hospital, College of Clinical Medicine of Henan University of Science and Technology. The esophageal squamous cell carcinoma tissues were confirmed by the histological examination. These tissue samples were immediately snap-frozen in liquid nitrogen and then stored at -80°C . Before the study, all patients provided written informed consent, and this study was approved by the Ethics Committees of The First Affiliated Hospital, College of Clinical Medicine of Henan University of Science and Technology (Approval No. 2020-067).

Cell cultures

Human esophageal cancer cell lines (EC109) and human normal esophageal epithelial cell line (Het-1A) were obtained from the Type Culture Collection of the Chinese Academy of Sciences, Shanghai, China. All the cell lines were cultured in Dulbecco's Modified Eagle Medium (Gibco, USA), followed with incubation at 37°C and 5% CO_2 . The culture medium included 10% fetal bovine serum (FBS, Gibco, USA) and 1% Penicillin-Streptomycin reagents (Solarbio, China).

Cell transfection

Cells (1×10^5 /well) were seeded in 6-well culture plates and incubated overnight at 37°C until they reached about 60% density. Then, the medium was replaced with serum-free medium. The sequences were as follows: si-NC: antisense: 5'-ACGUGACACGUUCGGAGAATT-3', sense: 5'-UUCUCCGAACGUGUCACGUTT-3'. si-CircRNA-0036474#1: antisense: 5'-CACUAAUCUUCUCUCCCUGAAUGACG-3', sense: 5'-CACCAGCCAUAACAGAUGAAGAGGA-3'. si-CircRNA-0036474#2: antisense: 5'-AGAUACGACUGCUCUACACAA-3', sense: 5'-CCUCCAAACGCGUCACGUCA-3'. si-NC and either si-CircRNA-0036474#1 or si-CircRNA-0036474#2 were co-transfected into cells for 6 h using Lipofectamine (Invitrogen, USA), following specific instructions. Further, the cells were incubated in medium containing 10% FBS for 42 h before being harvested to evaluate the knockdown efficiency.

Dual-luciferase reporter gene assay

Potential transcription factors binding sites within the promoter region were analyzed and predicted by bioinformatics analysis. Initially, primers were designed to clone the target promoter fragment from genomic DNA by PCR, and the fragment was later inserted into a luciferase reporter gene plasmid. Then, we selected and sequenced those positive clones. Following sequencing, the chosen clone was amplified, and the plasmid was purified. In addition, the transcription factor plasmid was amplified and purified. Meanwhile, the corresponding empty plasmid control was prepared and purified. Furthermore, HEK293 target cells were seeded in 24-well cell culture plates and incubated for a period of 10-24 h until reaching 80% confluence. After that, the transcription factor plasmid and reporter gene plasmid were co-transfected into the cells. Proteins were then isolated to measure luciferase activities, which involved introducing a substrate. Finally, the relative fluorescence intensities were determined.

Transwell migration and invasion assays

Initially, the cells cultured in 200 μl of serum-free medium were introduced in each of the upper chamber at a density of 2×10^4 per well. The lower chamber was filled with 600 μl of

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Table 1. Primer sequences used in this study

Gene	Primers
miR-223-3p	F: 5'-TATCAGTATATCAGAGA-3' R: 5'-GAGACCTATATCCATATTGA-3'
U6	F: 5'-ACCCATACACCATATAT-3' R: 5'-TCAGGAGGAGACAGGAGTTTATAT-3'
β -actin	F: 5'-GATTCCTATGTGGGCGACGA-3' R: 5'-TGGGTCTAGTACAACTCTGGA-3'
CircRNA-0036474	F: 5'-AGACTGTTTGAATCTGTCTCACT-3' R: 5'-TACGATCAATCACCATGGCAAC-3'
GAPDH	F: 5'-CAGGAGGCATTGCTGATGAT-3' R: 5'-GAAGGCTGGGGCTCATT-3'

F: Forward primer; R: Reverse primer.

DMEM containing 20% FBS, serving as chemo-attractants. After incubation for 24 h, the invading cells were fixed and stained using 95% hematoxylin solution mixed with ethanol. Non-invasive cells were then removed using swabs. Finally, the number of stained cells was counted to evaluate the invasion index, and images were captured using a light microscope (Nikon, Tokyo, Japan). For the invasion assay, in addition to pre-coating the Transwell microfilters with 40 μ l of working Matrigel, the following procedures of the experiment were the same as those of the cell migration assay. After incubation, the cells reaching the lower chamber were measured by the same approaches as those employed in the cell migration assay.

RNA pull-down

The total RNA from the EC109 cell line overexpressing circRNA-0036474 was extracted by the Trizol method at room temperature. The probe solution was mixed with streptavidin magnetic beads. The total RNA was gently mixed with the above mixture and allowed to incubate for 30 min. Then, the bound RNA complex was collected by adding elution buffer. Finally, the circRNA-0036474 expression level was measured using the qRT-PCR method.

Western blotting analysis

The cells, which had been incubated and transfected in the 6-well (5×10^4 /well) plates for 24 h, were further subjected to RIPA Lysis-Buffer (Solarbio, China) containing Protease Inhibitor Cocktail (Roche, USA). The concentration of total protein was determined using the BCA

Protein Detection kits (Solarbio, China). The protein was isolated using 10% SDS-PAGE. Then, the gel was transferred onto Immobilon-NC membranes a thickness of 0.45- μ m (Solarbio, China), followed by incubation of the membranes with corresponding primary antibodies against E-cadherin, Vimentin, N-cadherin, RERG, and β -actin (Abcam) overnight. The relative protein expressions were detected using a secondary antibody (Boster, China) with ECL chemiluminescent solution.

RNA isolation, cDNA synthesis, and qRT-PCR

Cells (5×10^4 /well) were cultured and inoculated into the 6-well plates for 24 h. Following lysis, cell transfection was performed using iPrep™ Trizol™ Plus RNA Kits (Thermo Fisher Scientific, USA). Further, the total purified RNA samples were converted to cDNA (2 μ g) by utilizing High-Capacity cDNA Reverse Transcription Kit and the RNase Inhibitor Kits (Thermo Fisher Scientific, USA). The relative expression levels of circRNA-0036474, miR-223-3p, β -actin, GAPDH, and U6 were examined using FastFire qPCR PreMix (SYBR Green, Tiangen, China) in a ProFlex™ PCR system (Thermo Fisher) with the analysis of a standard curve. The PCR amplification was conducted using the primers listed in **Table 1**, and all PCR products were subjected to the following reaction conditions: denaturation at 95°C; followed by 40 cycles of 10 s at 95°C, 15 s at 60°C, and 8 s at 72°C. The expression levels of circRNAs and mRNAs were normalized to GAPDH, and the expression of miRNAs was normalized to U6. The relative expression levels of genes were calculated by the $2^{-\Delta\Delta Ct}$ method.

Statistical method

SPSS 22.0 software was employed to statistically analyze the data in this research. The data regarding Transwell, PCR, RNA pull-down and Western blot were present in the form of mean \pm SD. The comparisons between two groups were conducted by independent t-test. For the comparison of paired tissues, paired t test was used. In addition, Bonferroni post hoc test following One Way ANOVA was performed for comparing average values among 3 cohorts and

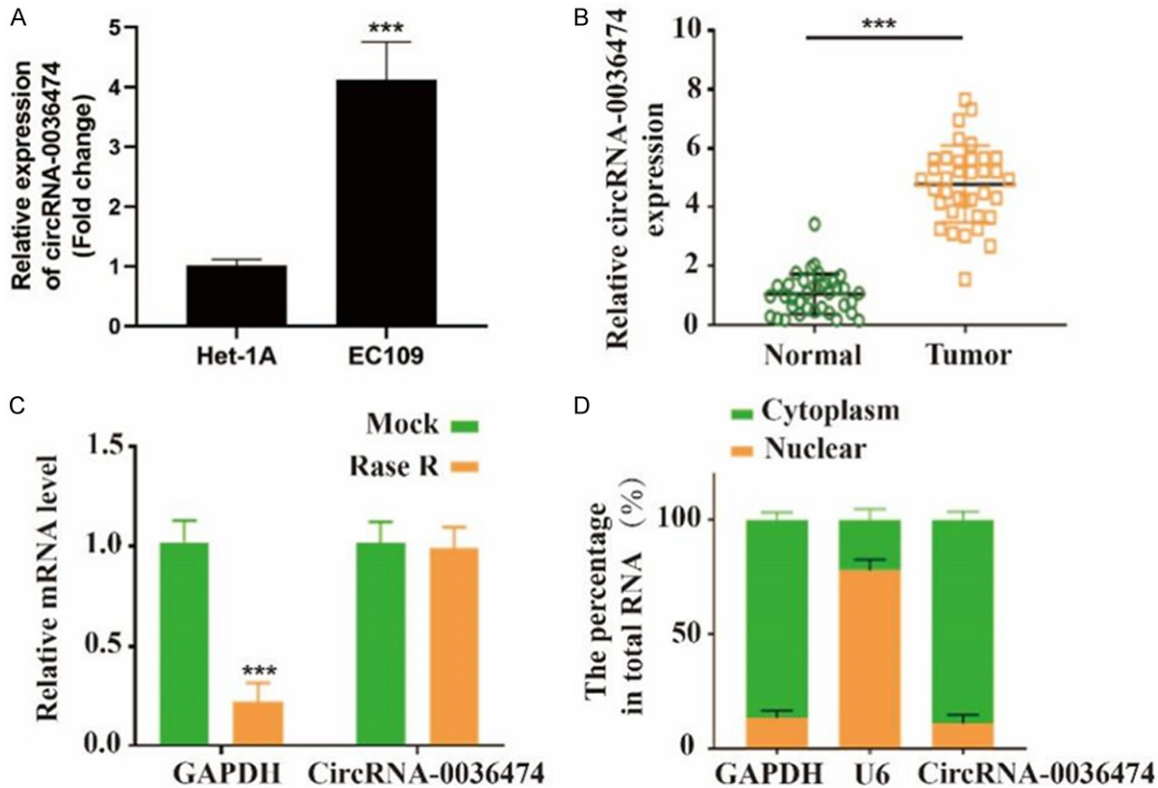


Figure 1. Elevated expression of circRNA-0036474 in esophageal squamous cell carcinoma cells lines and tissues. A. Expression of circRNA-0036474 in Het-1A and EC109 cells lines, ***P<0.001 vs. Het-1A. B. qRT-PCR revealed the markedly elevated circRNA-0036474 level within esophageal squamous cell carcinoma tissues, ***P<0.001 vs. normal tissues. C. qRT-PCR showed that the relative mRNA level of circRNA-0036474 in EC109 cells lines increased significantly, ***P<0.001 vs. the mock group. D. qRT-PCR showed that the expression of circRNA-0036474 in cytoplasm increased significantly.

more. The significance level was set at $\alpha = 0.05$.

Results

CircRNA-0036474 expression

Initially, the expression of circRNA-0036474 in esophageal squamous cell carcinoma cell lines and tissues from patients was determined. The expression levels of circRNA-0036474 were higher in EC109 cells than in the Het-1A cell line (**Figure 1A**). Furthermore, qRT-PCR analysis of 36 paired samples of normal and esophageal cancerous tissues revealed a significant elevation in circRNA-0036474 levels within esophageal cancerous tissues were markedly elevated (**Figure 1B**). In addition, circRNA-0036474 and GAPDH levels within RNase R-treated EC109 cells were measured through qRT-PCR. Relatively, the GAPDH levels within RNase R-treated cells were lower com-

pared to the mock group, while the circRNA-0036474 expression showed no significant change between the groups (**Figure 1C**). Moreover, the subcellular localization analysis by qRT-PCR revealed nuclear and cytoplasmic localization of circRNA-0036474 within cells. The circRNA-0036474 showed cytoplasmic localization, while the U6 and GAPDH were internal references of the nucleus and cytoplasm, respectively (**Figure 1D**).

Effects of circRNA-0036474 knockdown

The effects of circRNA-0036474 knockdown on the EC109 cells were explored in terms of invasion, migration, and EMT pathways. Initially, the efficacy of downregulating expression was confirmed by qRT-PCR following silencing of circRNA-0036474 within the cells. Compared to si-NC, the relative expression of circRNA-0036474 was measured following transfection with si-circRNA-0036474#1 or #2 for 48 hours

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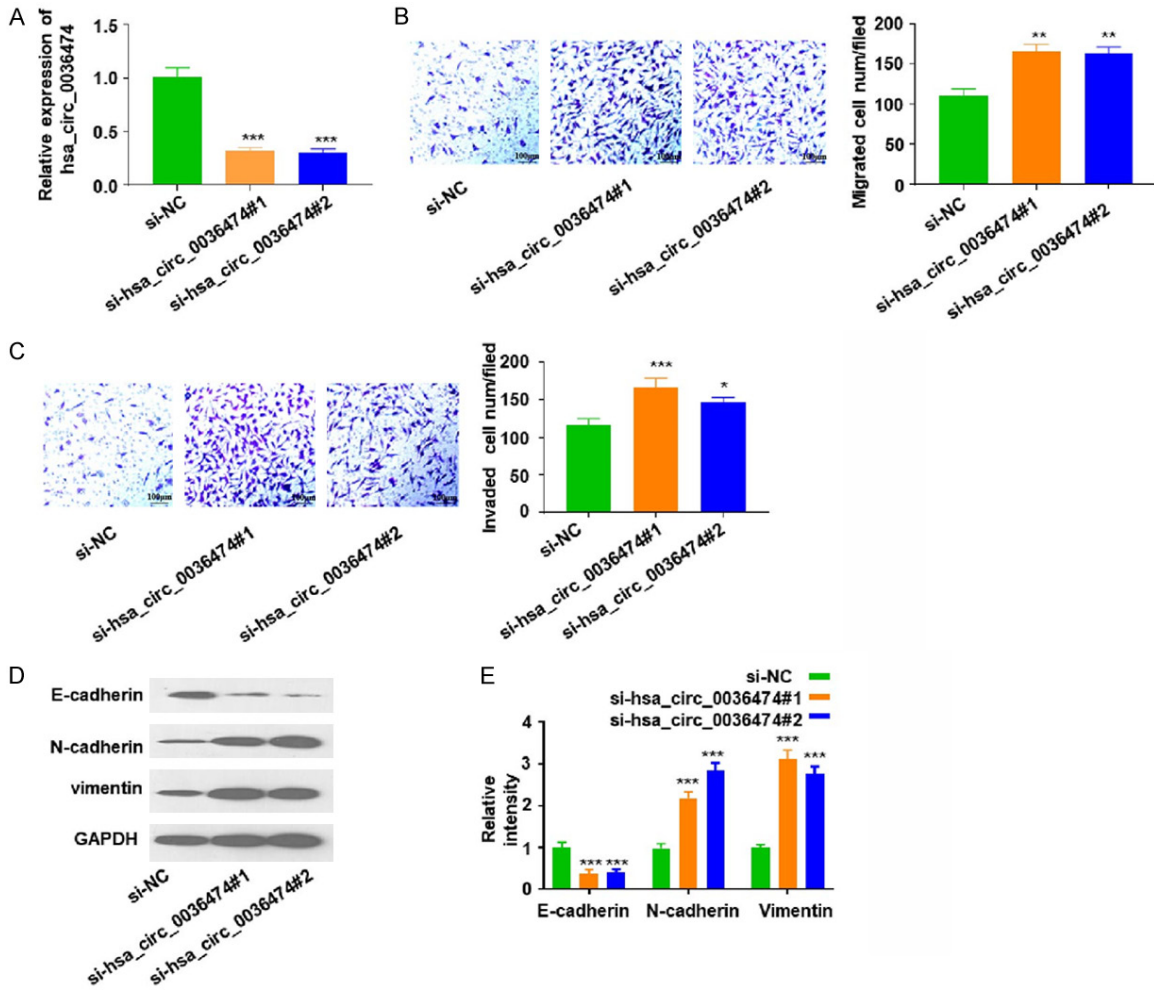


Figure 2. Knockdown of circRNA-0036474 augmented the invasion, migration and EMT pathways of EC109 cells. A. qRT-PCR showing circRNA-0036474 knockdown in EC109 cells, *** $P < 0.001$ vs. si-NC group. B, C. Transwell was used to detect the migration and invasion ability of circRNA-0036474-knockdown EC109 cells, * $P < 0.05$ vs. si-NC group, ** $P < 0.01$ vs. si-NC group, *** $P < 0.001$ vs. si-NC group. D, E. E-cadherin, N-cadherin, and vimentin levels in EC109 cells were determined by Western blot, *** $P < 0.001$ vs. si-NC group.

(**Figure 2A**). Moreover, cell migration was enhanced in both si-circRNA-0036474#1 and si-circRNA-0036474#2 groups compared to the si-NC group (**Figure 2B**). The Transwell invasion assay was employed to measure EC109 cells invasion after circRNA-0036474 knockdown for 48 h. Compared to the si-NC group, cell invasion after circRNA-0036474 knockdown was enhanced in both treatment groups (**Figure 2C**). Comparatively, E-cadherin expression was down-regulated, whereas N-cadherin and vimentin expression were up-regulated relative to the si-NC group (**Figure 2D** and **2E**). Together, the experimental findings suggest that the knockdown of circRNA-0036474 can enhance the invasion, migration, and EMT pathways of EC109 cells.

Effects of overexpression of circRNA-0036474 on EC109 cells

The overexpression of circRNA-0036474 in EC109 cells was confirmed using qRT-PCR. Compared to the vector control, cells transfected with the pcDNA3.1-circRNA-0036474 plasmid exhibited significantly higher expression levels of circRNA-0036474 (**Figure 3A**). Then, a migration assay using the Transwell approach was performed to assess cell migration under conditions of circRNA-0036474 overexpression. After 48 h of incubation, the ability of cells to migrate following circRNA-0036474 overexpression was reduced compared to the vector group (**Figure 3B**). Moreover, the efficacy of cell invasion following overexpression of cir-

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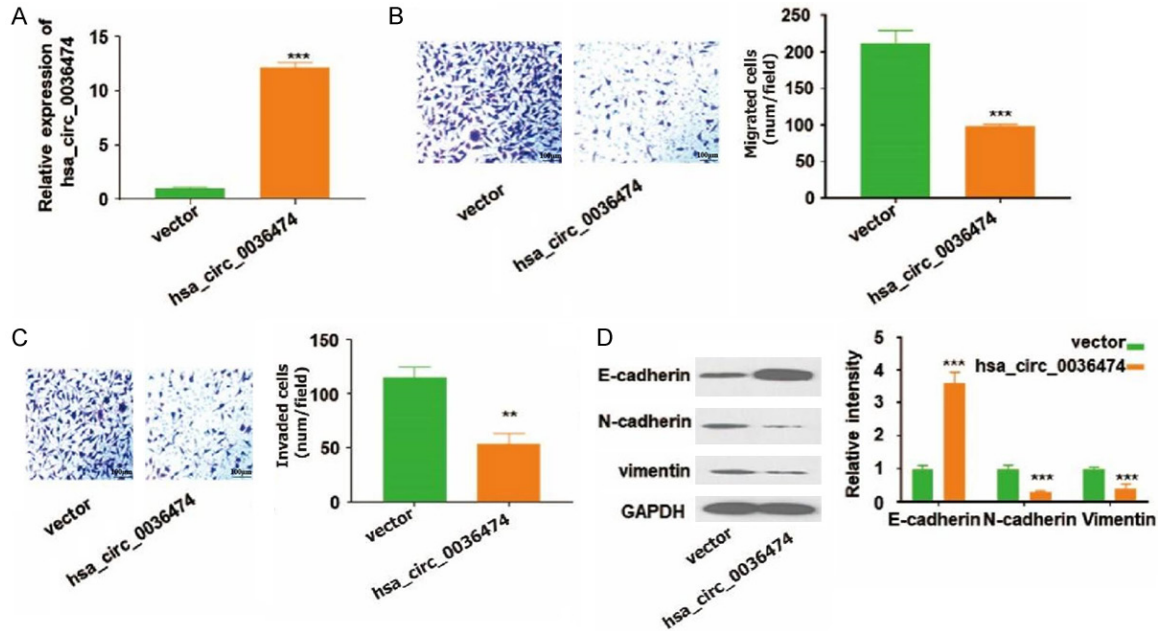


Figure 3. Overexpression of circRNA-0036474 reduced the invasion, migration, and EMT pathways of EC109 cells. A. qRT-PCR showing over-expressed circRNA-0036474 in EC109 cells, *** $P < 0.001$ vs. vector. B, C. EC109 cells invasion after overexpressing circRNA-0036474 measured by Transwell, ** $P < 0.01$ vs. vector, *** $P < 0.001$ vs. vector. D. E-cadherin, N-cadherin and vimentin levels in EC109 cells overexpressing circRNA-0036474 measured by Western blot assay, *** $P < 0.001$ vs. vector.

circRNA-0036474 was assessed using the invasion Transwell approach. The results showed that cell invasion following circRNA-0036474 overexpression was weaker than that of the vector group (Figure 3C). Further, changes in EMT-associated markers within cells overexpressing circRNA-0036474 were evaluated using Western blot analysis. Compared with the vector group, the E-cadherin expression level was elevated, while N-cadherin and vimentin expression levels were declined in the circRNA-0036474 overexpression group (Figure 3D). Together, the overexpression of circRNA-0036474 significantly inhibited the abilities of invasion and migration, and EMT in cells.

MiR-223-3p was the circRNA-0036474 target within EC109 cells

miR-223-3p was predicted as a target of circRNA-0036474 by Starbase software version 3.0, as shown in Figure 4A. Compared to the miR-NC treatment group, luciferase activity was further suppressed in 293T cells overexpressing miR-223-3p (Figure 4B). However, no suppression of luciferase activity was observed after mutating the predicted binding site of circRNA-0036474. Furthermore, the expression

levels of miR-223-3p within cells following circRNA-0036474 knockdown were analyzed using qRT-PCR. Compared to si-NC, circRNA-0036474 knockdown significantly upregulated the expression levels of miR-223-3p (Figure 4C). The levels of miR-223-3p within EC109 cells after the overexpression of circRNA-0036474 were measured using the qRT-PCR method. Compared with the vector, overexpression of circRNA-0036474 down-regulated the relative levels of miR-223-3p expression (Figure 4D). Relative to the NC probe, the miR-223-3p probe showed higher circRNA-0036474 levels in the EC109 cells (Figure 4E). Meanwhile, miR-223-3p levels within the 36 paired samples were measured through qRT-PCR. The results of qRT-PCR revealed that the miR-223-3p levels were declined within esophageal squamous cell carcinoma tissues, indicating that circRNA-0036474 expression was antagonistic to miR-223-3p within esophageal squamous cell carcinoma (Figure 4F, 4G).

Circ_0036474 enhanced RERG expression through sponging miR-223-3p

The miR-223-3p binding sites and RERG were evaluated by the Starbase version 3.0 software

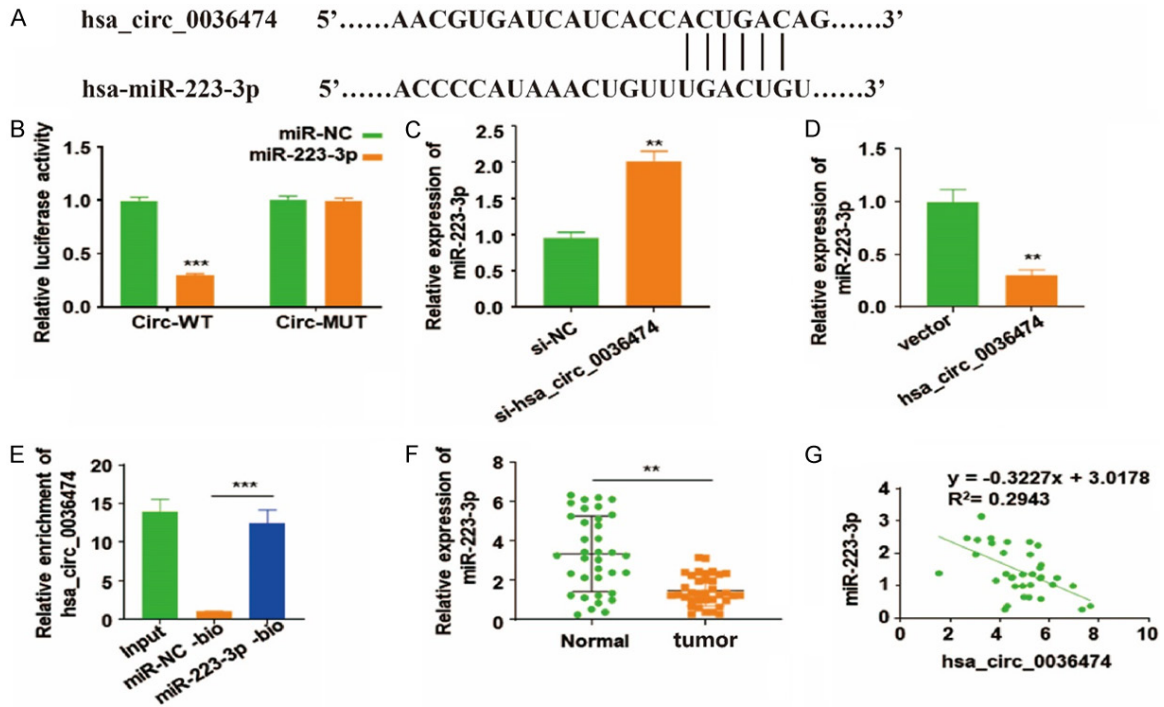


Figure 4. The association between hsa_circ_0036474 and miR-223-3p. A. miR-223-3p was predicted as the hsa_circ_0036474 target by Starbase software version 3.0. B. Luciferase activity was determined in 293T cells co-transfected with circRAN-0036474-WT or circRAN-0036474-Mut and miR-223-3p mimics or miR-NC, ***P<0.001 vs. miR-NC. C. The comparison of miR-223-3p expression between si-NC and si-hsa_circ_0036474 groups, **P<0.01 vs. si-NC group. D. The comparison of miR-223-3p expression between vector group and has_circ_0036474 group, **P<0.01 vs. vector group. E. Elevated circRNA-0036474 levels in the EC109 cells with miR-223-3p probe in contrast to miR-NC probe, ***P<0.001 vs. miR-NC Probe. F. The comparison of miR-223-3p expression between normal tissue and tumor tissue, **P<0.01 vs. normal tissues. G. The relationship of hsa_circ_0036474 expression with miR-223-3p expression.

(**Figure 5A**). Compared to miR-NC, overexpression of miR-223-3p suppressed luciferase activity in 293T cells, which, however, was reversed after mutating the predicted RERG binding site (**Figure 5B**). qRT-PCR and Western blot assays were employed to examine RERG protein and mRNA levels within EC109 cells after miR-223-3p knockdown or overexpression (**Figure 5C**). The experimental results revealed that miR-223-3p knockdown increased RERG mRNA and protein expression levels, while miR-223-3p overexpression decreased RERG mRNA and protein expression levels. Moreover, the hsa_circ_0036474 levels within cells were analyzed through qRT-PCR and Western blot. The experimental results exhibited increased protein and mRNA levels of RERG (**Figure 5D**). Notably, the RERG levels were partially declined following miR-223-3p mimics co-transfection. RERG mRNA levels within the 36 paired samples also were analyzed through qRT-PCR, showing increased RERG levels in the

esophageal squamous cell carcinoma tissue samples. The RERG level within esophageal squamous carcinoma was directly proportional to hsa_circ_0036474 expression and inversely proportional to miR-223-3p expression (**Figure 5E, 5F**).

Effects of circRNA-0036474 on migration, invasion, and EMT through targeted regulation of miR-223-3p/REG in EC109 cells

EC109 cells migration of different groups (control, hsa_circ_0036474, hsa_circ_0036474 + miR-223-3p, hsa_circ_0036474 + si-REG) was assessed. Compared to the control treatment group, the overexpression of hsa_circ_0036474 significantly attenuated the migration efficacy of cells (**Figure 6A**). Furthermore, compared to the hsa_circ_0036474 group, the migration ability in groups treated with hsa_circ_0036474 + miR-223 and hsa_circ_0036474 + si-REG mimics co-transfec-

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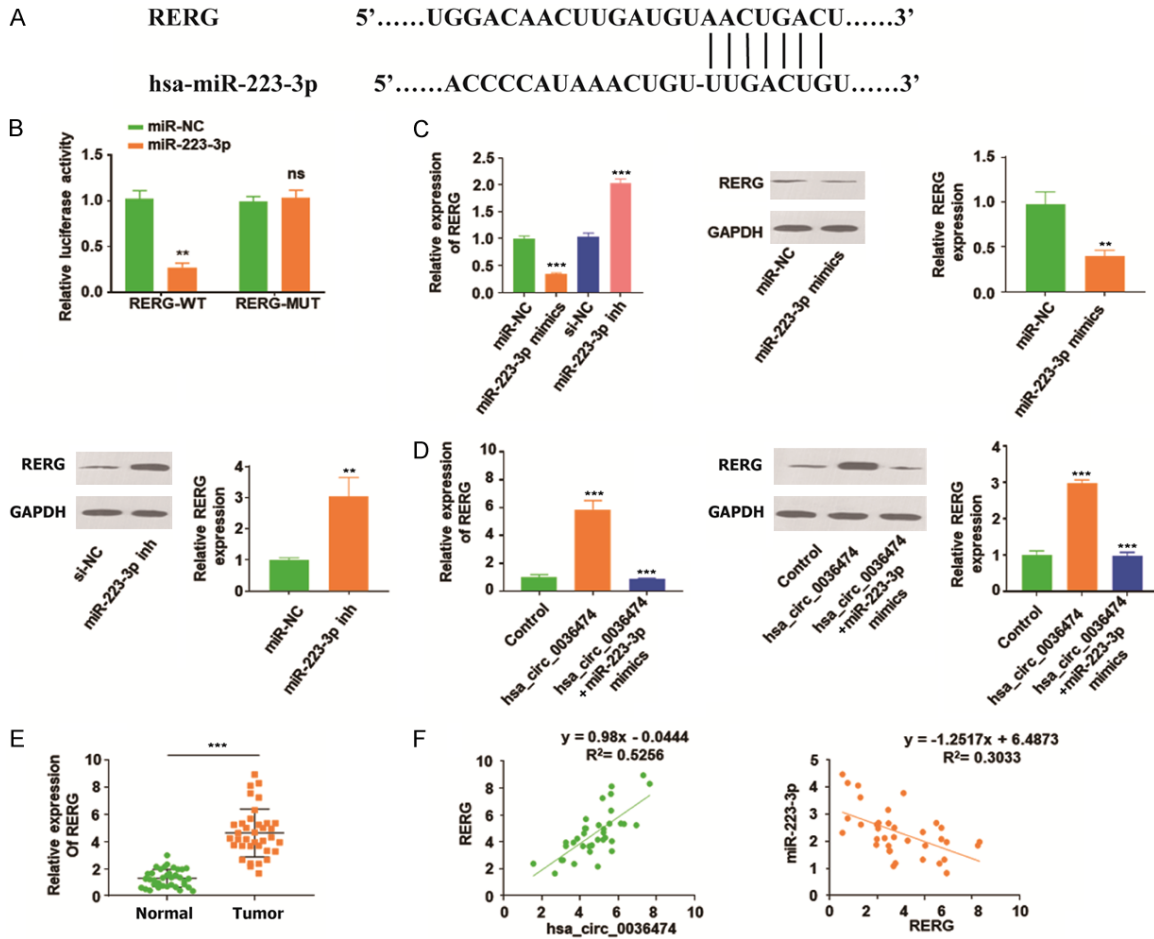


Figure 5. CircRNA-0036474 promoted RERG expression via sponging miR-223-3p. A. Starbase version 3.0 software predicted miR-223-3p and RERG as binding sites. B-F. 293T cells were adopted for luciferase reporter gene assay. The RERG mRNA and protein expression in EC109 cells after miR-223-3p overexpression or knockdown were measured through qRT-PCR and Western blot. **P<0.01 vs. miR-NC group, ***P<0.001 vs. miR-NC group, si-NC group, control group or normal tissues.

tion was partially enhanced. Moreover, the Transwell approach was employed to detect EC109 cells invasion. Similarly, the overexpression of circRNA-0036474 attenuated cells invasion over the control treatment group. Compared to the hsa_circ_0036474 group, after miR-223-3p and hsa_circ_0036474 mimics co-transfection, the cell invasion was partially enhanced (**Figure 6B**). In addition, hsa_circ_0036474 and si-RERG co-transfection enhanced the cell invasion compared to that in the hsa_circ_0036474 group (**Figure 6B**). Finally, the changes in the EMT-related markers in cells from different groups were measured via Western blot assay. Compared with the control group, E-cadherin levels were elevated, while N-cadherin and vimentin expressions were declined within the hsa_circ_0036474

overexpression group (**Figure 6C**). Compared with the hsa_circ_0036474 group, E-cadherin levels within miR-223-3p and hsa_circ_0036474 mimics cells were decreased, whereas N-cadherin and vimentin levels were partially elevated. Compared with the hsa_circ_0036474 group, the hsa_circ_0036474 and si-RERG co-transfected treatment group presented declined E-cadherin expression, and elevated N-cadherin and vimentin expressions.

Discussion

Advancements in molecular biology have led to significant breakthroughs in targeted treatments for esophageal squamous cell carcinoma research. Metastasis and progression are the key determinants of poor prognosis for

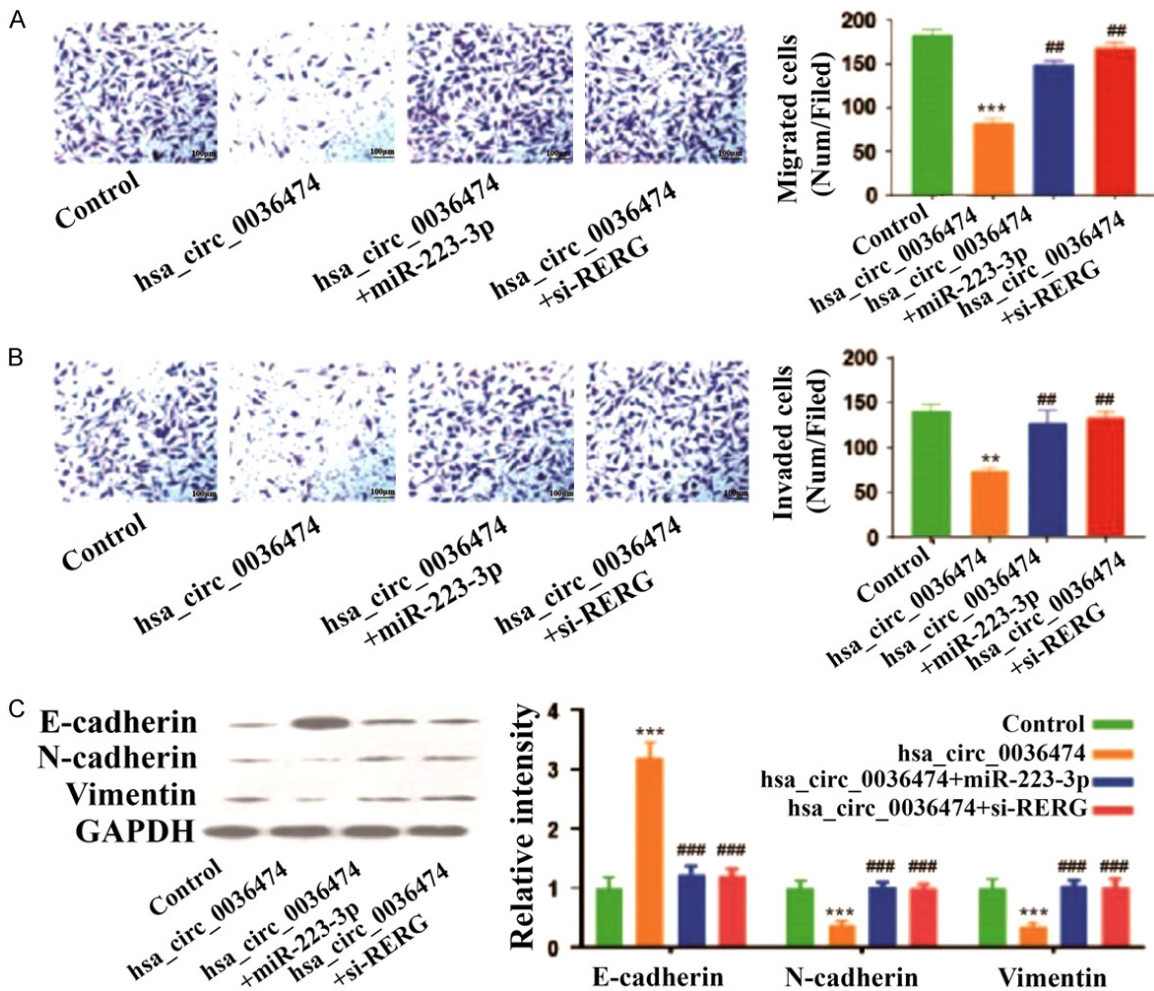


Figure 6. CircRNA-0036474 inhibited the migration, invasion, and EMT of EC109 cells. A, B. EC109 cells migration in different groups was detected by Transwell. ** $P < 0.01$, *** $P < 0.001$ vs. control. ## $P < 0.01$ vs. hsa_circ_0036474. C. E-cadherin, N-cadherin, and Vimentin levels in EC109 cells of different groups measured by Western blot. *** $P < 0.001$ vs. control. ### $P < 0.001$ vs. hsa_circ_0036474.

patients with cancers. It was reported that approximately 90% of tumor deaths are attributed to metastasis [10]. Throughout the intricate process of tumor metastasis, cancer cells encounter various obstacles, requiring distinct cellular characteristics to successfully colonize distant organs and tissues. As important initiating factors for the metastasis process, cell biological behaviors are controlled by epigenetic and transcriptional mechanisms [11].

With the rapid development of biological information analysis and RNA-seq technology, many circRNAs have been found to exhibit abnormal expression patterns in various tumors and participate in the progression of tumor via transcriptional and post-transcriptional regulation of gene expression [12]. CircRNAs are firmly

associated with occurrence and progression of complications in patients with esophageal squamous cell carcinoma. Several reports indicated that many circRNAs have been found to be dysregulated in esophageal squamous cell carcinoma [13, 14]. In this study, hsa_circ_0036474 levels were markedly increased within the EC109 cells. After circRNA-0036474 down-regulation, EC109 cell migration and invasion were increased, demonstrating that the circRNA-0036474 was closely related to the invasion of esophageal squamous cell carcinoma. Moreover, it was observed that the circRNA-0036474 expression in esophageal squamous cell carcinoma tissue samples was significantly up-regulated. Further, the inhibition of circRNA-0036474 could promote the migration and invasion of cells, and EMT pro-

cess, suggesting that regulating the expression of circRNA-0036474 might serve as a possible way to improve the prognosis of patients with esophageal squamous cell carcinoma. Therefore, circRNA-0036474 expression plays a role in regulating cell biological behaviors. It was found that hsa_circ_0036474 can sequester miR-223-3p, leading to inhibition of miR-223-3p expression. This indicates an opposing effect between hsa_circ_0036474 and miR-223-3p in EC109 cells, and circRNA-0036474 could have significant effects by modulating miR-223-3p expression. These obtained results were in agreement with the other studies [15, 16], indicating that some key circRNAs could play a role through related miRNAs. Notably, the hsa_circ_0036474 levels were markedly increased within the EC109 cells. Moreover, hsa_circ_0036474 overexpression within EC109 cells led to the sequestration of endogenous miR-223-3p and subsequent inhibition of its activity. This resulted in increased expression of the downstream target protein RERG, thereby resisting EC109 cells invasion and migration and protecting against esophageal squamous cell carcinoma. The over-expression of circRNA-0036474 showed a negative effect on regulating miR-223-3p, thus inhibiting the growth and migration of cells, and improving prognosis of esophageal squamous cell carcinoma through miR-223-3p/RERG pathway. Several reports indicated that hsa_circ_0036474 could regulate the expression of downstream target genes by sequestering miRNAs and play a key role in other cancers [17]. In this regard, circRNA-0036474 expression could counteract the deleterious effects miR-223-3p in esophageal squamous cell carcinoma. Additionally, it is worth noting that elevated levels of circRNA-0036474 have also been observed in other types of tumors [18]. Moreover, the miR-223-3p suppressed RERG levels but promoted the EMT process. Inhibiting hsa_circ_0036474 expression could reduce the EMT process in other cancers [19]. Silencing hsa_circ_0036474 substantially up-regulated the EMT related proteins. Given that hsa_circ_0036474 is the target of miR-223-3p, its overexpression in vitro effectively reversed miR-223-3p-induced metastasis in esophageal squamous cell carcinoma, along with alterations in miR-223-3p and its targeted protein RERG. Therefore, hsa_circ_0036474 silencing and miR-223-3p overexpression together could

have a more substantial inhibitory effect on esophageal squamous cell carcinoma cells than either hsa_circ_0036474 silencing or miR-223-3p overexpression. Together, these experimental results demonstrated the critical role of circRNAs in regulating esophageal squamous cell carcinoma metastasis and their potential as important targets for the treatment.

This mechanistic study showed that hsa_circ_0036474 could competitively sequester miR-223-3p and decrease its expression, thus enhancing the expression of target protein RERG. Therefore, hsa_circ_0036474/miR-223-3p/RERG is an essential pathway in metastasis of esophageal squamous cell carcinoma. miR-223-3p is a miRNA specifically expressed in the esophageal squamous cell carcinoma, promoting the progress of cells infiltration. Previous reports suggested that miR-223-3p was linked to cardiovascular diseases [20]. For example, miR-223-3p could cause myocardial injury of rats with acute myocardial infarction by increasing the expression of endothelin [21]. Moreover, inhibiting miR-223-3p expression could reduce the activity of cells in other cancers [22]. In this study, the database predictions and experimental verifications indicated that RERG serves as a target gene of miR-223-3p, whose overexpression could reduce the expression of EMT proteins, indicating that RERG could also inhibit the development of EMT. In addition, the expression levels of miR-223-3p were increased, but RERG levels were decreased in EC109 cells, which are consistent with the results of a previous study [23]. RERG is often expressed in the heart, lung, liver, and other tissues, participating in various signaling pathways and regulating cell differentiation, proliferation, and apoptosis [24, 25]. The observed decrease in E-cadherin levels and concurrent increase in N-cadherin and vimentin levels suggest an activation of the EMT process, which could promote the development and progression of esophageal squamous cell carcinoma. RERG, as a negative regulator, play a vital role in the mechanism of EMT, indirectly inhibiting the migration and invasion of cancer cells.

There are some limitations in this study. Firstly, due to constraints in time and resources, we focused solely on cell experiments, without including animal models. Secondly, the sample

size was relatively small in this study. Future studies should aim to address this limitation by conducting multi-centers and recruiting more patients. These efforts would contribute to a robust investigation of hsa_circ_0036474 as a therapeutic target for esophageal squamous cell carcinoma.

In summary, our findings suggest that hsa_circ_0036474 expression is significantly upregulated in esophageal cancer patients. Moreover, hsa_circ_0036474 appears to play a role in inhibiting the invasion and migration of EC109 cells, as well as regulating EMT-related proteins through targeted regulation of the miR-223-3p/RERG pathway. Considering the above aspects, circRNA-0036474 emerges as a promising candidate for non-invasive biomarker development in detecting esophageal squamous cell carcinoma. Furthermore, it holds potential as a novel therapeutic target for the treatment of this malignancy.

Disclosure of conflict of interest

None.

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References

- [1] Uhlenhopp DJ, Then EO, Sunkara T and Gaduputi V. Epidemiology of esophageal cancer: update in global trends, etiology and risk factors. *Clin J Gastroenterol* 2020; 13: 1010-1021.
- [2] Waters JK and Reznik SI. Update on management of squamous cell esophageal cancer. *Curr Oncol Rep* 2022; 24: 375-385.
- [3] Rogers JE, Sewastjanow-Silva M, Waters RE and Ajani JA. Esophageal cancer: emerging therapeutics. *Expert Opin Ther Targets* 2022; 26: 107-117.
- [4] Mohammadi E, Aliarab A, Babaei G, Habibi NK, Jafari SM, Mir SM and Memar MY. MicroRNAs in esophageal squamous cell carcinoma: application in prognosis, diagnosis, and drug delivery. *Pathol Res Pract* 2022; 240: 154196.
- [5] Guo C, Mi J, Li H, Su P and Nie H. Dysregulated circular RNAs as novel biomarkers in esophageal squamous cell carcinoma: a meta-analysis. *Cancer Med* 2021; 10: 7895-7908.
- [6] Ju C, He J, Wang C, Sheng J, Jia J, Du D, Li H, Zhou M and He F. Current advances and future perspectives on the functional roles and clinical implications of circular RNAs in esophageal squamous cell carcinoma: more influential than expected. *Biomark Res* 2022; 10: 41.
- [7] Tang R, Zhou Q, Xu Q, Lu L and Zhou Y. Circular RNA circ_0006948 promotes esophageal squamous cell carcinoma progression by regulating microRNA-3612/LASP1 axis. *Dig Dis Sci* 2022; 67: 2158-2172.
- [8] Lei M, Zheng G, Ning Q, Zheng J and Dong D. Translation and functional roles of circular RNAs in human cancer. *Mol Cancer* 2020; 19: 30.
- [9] Hu X, Ao J, Li X, Zhang H, Wu J and Cheng W. Competing endogenous RNA expression profiling in pre-eclampsia identifies hsa_circ_0036877 as a potential novel blood biomarker for early pre-eclampsia. *Clin Epigenetics* 2018; 10: 48.
- [10] Lambert AW, Pattabiraman DR and Weinberg RA. Emerging biological principles of metastasis. *Cell* 2017; 168: 670-691.
- [11] Chatterjee A, Rodger EJ and Eccles MR. Epigenetic drivers of tumourigenesis and cancer metastasis. *Semin Cancer Biol* 2018; 51: 149-159.
- [12] Chen LL. The expanding regulatory mechanisms and cellular functions of circular RNAs. *Nat Rev Mol Cell Biol* 2020; 21: 475-490.
- [13] Wang Q, Liu H, Liu Z, Yang L, Zhou J, Cao X and Sun H. Circ-SLC7A5, a potential prognostic circulating biomarker for detection of ESCC. *Cancer Genet* 2020; 240: 33-39.
- [14] Zheng Y, Chen Y, Jiang H, Zhang H, Wang H, Xu J and Yu Z. Circ_0058063 upregulates GLUT1 expression and promotes glucose-uptake in esophageal squamous-cell carcinomas. *J Thorac Dis* 2020; 12: 925-931.
- [15] Zhang M, Bai X, Zeng X, Liu J, Liu F and Zhang Z. circRNA-miRNA-mRNA in breast cancer. *Clin Chim Acta* 2021; 523: 120-130.
- [16] Zhang ZH, Wang Y, Zhang Y, Zheng SF, Feng T, Tian X, Abudurexiti M, Wang ZD, Zhu WK, Su JQ, Zhang HL, Shi GH, Wang ZL, Cao DL and Ye DW. The function and mechanisms of action of circular RNAs in urologic cancer. *Mol Cancer* 2023; 22: 61.
- [17] Kristensen LS, Jakobsen T, Hager H and Kjems J. The emerging roles of circRNAs in cancer and oncology. *Nat Rev Clin Oncol* 2022; 19: 188-206.
- [18] Chen L and Shan G. CircRNA in cancer: fundamental mechanism and clinical potential. *Cancer Lett* 2021; 505: 49-57.

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- [19] Sabouni E, Nejad MM, Mojtavavi S, Khoshdruz S, Mojtavavi M, Nadafzadeh N, Nikpanjeh N, Mirzaei S, Hashemi M, Aref AR, Khorrami R, Nabavi N, Ertas YN, Salimimoghdam S, Zandieh MA, Rahmanian P, Taheriazam A and Hushmandi K. Unraveling the function of epithelial-mesenchymal transition (EMT) in colorectal cancer: metastasis, therapy response, and revisiting molecular pathways. *Biomed Pharmacother* 2023; 160: 114395.
- [20] Zhang MW, Shen YJ, Shi J and Yu JG. MiR-223-3p in cardiovascular diseases: a biomarker and potential therapeutic target. *Front Cardiovasc Med* 2020; 7: 610561.
- [21] Hromadka M, Motovska Z, Hlinomaz O, Kala P, Tousek F, Jarkovsky J, Beranova M, Jansky P, Svoboda M, Krepelkova I, Rokyta R, Widimsky P and Karpisek M. MiR-126-3p and MiR-223-3p as biomarkers for prediction of thrombotic risk in patients with acute myocardial infarction and primary angioplasty. *J Pers Med* 2021; 11: 508.
- [22] Wei Y, Peng J, He S, Huang H, Lin L, Zhu Q, Ye L, Li T, Zhang X, Gao Y and Zheng X. miR-223-5p targeting ERG inhibits prostate cancer cell proliferation and migration. *J Cancer* 2020; 11: 4453-4463.
- [23] Zhu S, Kong X, Song M, Chi M, Liu Y, Zhang P, Zhang Q, Shang P and Feng F. MiR-223-3p attenuates the migration and invasion of NSCLC cells by regulating NLRP3. *Front Oncol* 2022; 12: 985962.
- [24] Huang L, Tang X, Shi X and Su L. miR-532-5p promotes breast cancer proliferation and migration by targeting RERG. *Exp Ther Med* 2020; 19: 400-408.
- [25] Xiong Y, Huang H, Chen S, Dai H and Zhang L. ERK5-regulated RERG expression promotes cancer progression in prostatic carcinoma. *Oncol Rep* 2019; 41: 1160-1168.