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Article

# MiR-6747-5p suppresses angiogenesis in esophageal squamous cell carcinoma by targeting EGFL6

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**Abstract:** Epidermal growth factor-like domain 6 (EGFL6) plays a crucial role in angiogenesis in various malignant tumors. This study aimed to screen microRNAs (miRNAs) targeting EGFL6 and explore their mechanisms in regulating angiogenesis in esophageal squamous cell carcinoma (ESCC) cells. After analyzing the miRNA expression profiles of ESCC and using the target prediction algorithm, we screened three miRNAs that could potentially target EGFL6. By dual luciferase reporter gene assay and western blot, we found that miR-6747-5p could directly target EGFL6 and down-regulate EGFL6 expression in ESCC cells. The results of clone formation, CCK-8, Transwell, wound healing, and endothelial cell tube formation assay showed that miR-6747-5p exerted a significant inhibitory effect on the proliferation, migration, invasion, and angiogenesis of ESCC cells. At the same time, we observed that the phosphorylation levels of AKT and MAPK were decreased, the epithelial-mesenchymal transition (EMT) related E-cadherin expression was downregulated while N-cadherin was upregulated, and the protein expression of the pro-angiogenic factors, including platelet-derived growth factor subunit B (PDGFB), fibroblast growth factor 2 (FGF2), and angiogenin (ANG) were inhibited transfected with miR-6747-5p mimics. Further studies showed that the overexpression vectors of EGFL6 transfected into ESCC cells could reverse the inhibitory effects induced by miR-6747-5p. These findings reveal that miR-6747-5p could target EGFL6 and inhibit tumor angiogenesis and ESCC progression. miR-6747-5p may be a promising biomarker for the anti-angiogenic treatment of ESCC.

**Keywords:** miR-6747-5p; EGFL6; esophageal squamous cell carcinoma; angiogenesis

## 1. Introduction

Esophageal cancer (EC) ranks among the most aggressive malignancies and is the eighth most frequently diagnosed cancer worldwide [1]. It is noteworthy that the incidence of esophageal cancer remains consistently high in regions such as Asia and Africa, underscoring its regional public health burden [2]. ESCC predominates among all histological subtypes of esophageal cancer, exhibiting the highest frequency of occurrence [3,4]. As a result of its propensity for metastasis, ESCC frequently presents in advanced stages upon diagnosis [5]. Despite ongoing advancements in diagnostic and therapeutic modalities, the 5-year survival rate of ESCC continues to pose a significant challenge [6,7]. Thus, there is an immediate requirement for more precise and sensitive molecular indicators for the development of more precise and sensitive molecular markers. These markers would significantly

enhance both the diagnostic accuracy and the effectiveness of targeted treatments for patients with ESCC [8].

The process of tumor angiogenesis constitutes a pivotal stage in the progression and dissemination of malignant neoplasms [9]. Neovascularization can support the growth of tumor cells and serve as a channel for tumor cell migration [10]. Clinical practice has shown that the application of anti-angiogenic medications has demonstrated a certain degree of efficacy in enhancing the prognosis of ESCC. However, the comprehensive efficacy of such interventions was not satisfactory [11,12]. The currently available anti-angiogenic pharmaceutical agents, exemplified by bevacizumab, primarily focus on the inhibition of vascular endothelial growth factor (VEGF) [13]. However, these drugs often have side effects, including hypertension and impaired wound healing [14]. Therefore, identifying potential therapeutic targets remains a research priority.

EGFL6 is a secreted protein first discovered by Yeung in 1999 [15,16]. An intriguing observation is the elevated expression of EGFL6 within tumor tissues, contrasting with its negligible expression in normal adult tissue counterparts [17]. It can promote angiogenesis by regulating the migration of vascular endothelial cells [18]. The prior investigation demonstrated that the inhibition of EGFL6 expression effectively suppressed both the growth of ovarian cancer and its associated angiogenic processes [19]. EGFL6 has emerged as a prospective target for suppressing tumor angiogenesis owing to its distinctive involvement in this biological process. However, the precise molecular mechanism underlying EGFL6-mediated regulation of angiogenesis in ESCC remains unclear.

miRNAs are a class of small non-coding RNAs known for their regulatory roles in gene expression Their mechanism of action involves either suppressing translation or inducing degradation of target mRNAs via binding to the 3’UTR of target genes [20]. Some studies have demonstrated that miRNAs could serve as crucial regulators in inhibiting tumor progression [21,22]. In ESCC, miR-130b has been found to be overexpressed and enhanced the proliferation and invasion by downregulating SAM and SH3 domain containing 1 (SASH1) [23]. miR-338-5p has been identified as a promising factor in reversing the chemoresistance in ESCC through directly targeting inhibitor of DNA binding 1 (Id-1) [24]. Overexpression of miR-4707-3p could inhibit angiogenesis in ESCC by targeting fork head box protein C2 (FOXC2) [25]. Thus, inhibiting EGFL6 through miRNA appears to be a viable therapeutic strategy for ESCC.

In this study, miRNAs targeting EGFL6 were analyzed and mimics of initially identified candidate miRNAs were synthesized. Then the transfection cell experiment and double luciferase reporter gene experiment were carried out with mimics. The results further confirmed the targeting relationship between miR-6747-5p and EGFL6 among the candidate miRNAs. The functions of miR-6747-5p for ESCC cell proliferation, migration, invasion and angiogenesis were investigated. The assays of signal pathway and EGFL6 regulations were researched. The findings may offer potential target for anti-angiogenic treatment in ESCC.

## 2. Materials and methods

### 2.1. Cell culture

ESCC cell lines (KYSE70, KYSE150, KYSE410, EC18, EC9706, and TE12), HEK-293T and HUVEC cells were kept in our laboratory. ESCC cells were cultured in RPMI-1640 (Biological Industries) medium, while HEK-293T and HUVEC cells were cultured in DMEM medium. All cells were cultured in the medium supplemented with 100 U/mL pen/strep (Beyotime) and 10% fetal bovine serum (FBS) at 37 ℃ with 5% $CO_2$.

### 2.2. Western blot

The process of total protein extraction and immunoblotting was carried out following established protocols as previously described [26]. The antibodies of EGFL6 (ab140079), PDGFB (ab23914), FGF2 (ab92337) were bought form Abcam. The antibodies of t/p-AKT (4691S, 4060S), t/p-MAPK (4695S, 4370S), E-cadherin (3195S), N-cadherin (13116S), GAPDH (5174S) were bought from Cell Signaling Technology. The antibodies of Vimentin (WL01960) and ANG (WL04508) were bought form Wanleibio. The bands on the blot were visualized by chemiluminescent imaging system (Tanon).

### 2.3. Cell transfection

The cells were cultured in plates and grown to 80% confluence. Following this, ESCC cells were transfected with miRNA mimics and pcDNA3.1-EGFL6 plasmids by Lipofectamine 2000 (GLPBIO).

EGFL6 gene fragment was synthesized through polymerase chain reaction (PCR) utilizing specific primers (forward primer: 5′-ATGCCTCTGCCCTGGAGCCTTG-3′ and reverse primer: 5′-TCAGTCATCCACAGATAAAAGGCTATC-3’). The pcDNA3.1-EGFL6 vector was constructed by cloning the EGFL6 gene fragment into the pcDNA3.1 vector. mimics-NC (5′-UCACAACCUCCUAGAAAGAGUAGA-3′), miR-1224-5p mimics (5′-GUGAGGACUCGGGGAGGUGG-3′), miR-6747-5p mimics (5′-AGGGGGUGGUGGAAAGAGGCAGAACA-3′), miR-6759-5p mimics (5′-UUGGUGGGGUGGGCAGAAGUCUGU-3′) were synthesized by Tsingke.

### 2.4. Bioinformatics analysis

The dbDEMC 3.0 [27] (https://www.biosino.org/dbDEMC/index) database was used to analyze the miRNA microarray datasets of ESCC. The miRWalk 2.0 [28] (http://mirwalk.umm.uni-heidelberg.de/) and RNA22 v2 [29] (https://cm.jefferson.edu/rna22-full-sets-of-predictions/) databases were utilized to predict EGFL6 3’UTR in the binding site of miRNA.

### 2.5. Dual luciferase reporter gene assay

The pmirGLO-EGFL6-3’UTR vector was constructed. Incorporating either the wild-type (WT) or mutant (mut) 3’UTR of EGFL6 containing the miR-6747-5p binding site into the pmirGLO vector (Promega). The vectors and miR-6747-5p

mimics were co-transfected into HEK-293T cells. The luciferase activity was assayed via the Dual Luciferase Reporter Gene Assay System (Yeasen) and then normalized based on the Renilla luciferase activity.

## 2.6. RT-qPCR

The total RNA extraction and RT-qPCR procedures carried out as previously outlined [30]. U6 was employed as a control, and the relative expression level of miR-6747-5p was determined using the $2^{-\Delta\Delta CT}$ method. The primers of U6 were 5′-CTCGCTTCGGCAGCACA-3′, and 5′-AACGCTTCACGAATTTGCGT-3′. The primers of miR-6747-5p were 5′-GAGGGGGTGTGGAAAGAGGC-3′, and 5′-AGTGCAGGGTCCGAGGTATT-3′.

## 2.7. CCK-8 and clone formation assays

For CCK-8 assay, $2 \times 10^3$ cells of EC9706 and KYSE70 were cultured in each well of a 96-well plate for 24 h, 48 h, 72 h, and 96 h. Then 10 μL of CCK-8 solution (Meilunbio) was added and the absorbance at 450 nm was measured using a microplate reader (BioTek Instruments). In the clone formation assay, $2 \times 10^3$ cells of EC9706 and KYSE70 were cultured in each well of a 6-well plate. After incubating for approximately seven days, the cells were fixed and stained. Photographs were taken to count the number of clones.

## 2.8. Transwell assay

The Transwell assay were conducted using a Transwell-Matrigel system (Costar) in 24-well plates. In the migration assay, $2 \times 10^4$ cells in 200 μL of basal medium were cultured in the upper chamber, while 500 μL of complete medium was supplemented to the lower chamber. Cells were fixed and stained after 24 h of incubation and imaged using the inverted microscope (Olympus). Invasion experiments were performed using a similar method, except that the chamber was supplemented with 50 μL of Matrigel. The cells of EC9706 and KYSE70 were counted in 3 randomized areas using Image J software. The migration and invasion rates were quantified.

## 2.9. Wound healing assay

The cells of EC9706, KYSE70, or HUVEC were cultured in 6-well plates. Cells were scraped with a pipette tip. Imaging was performed using Image J software at 0 h, 24 h, or 48 h, and the wound area were quantified.

## 2.10. Endothelial cell tube formation assay

The cells of HUVEC were cultured in Matrigel-coated 96-well plates. Conditioned medium from EC9706 or KYSE70 was used to incubate HUVEC cells at 37 ℃with 5% $CO_2$. Imaging was performed after 6 h of culture, and tube formation was analyzed using the Image J software.

### 2.11. Statistical analysis

The experiments were repeated three times independently, and quantitative data were presented as mean ± standard deviation (SD). To analyze statistical differences, one-way analysis of variance (ANOVA) was used. Statistical analysis was conducted utilizing GraphPad Prism 9.5 software, $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. The screening of miRNA targeting EGFL6

To screen miRNAs targeting EGFL6, we analyzed four datasets (GSE106817, GSE112264, GSE113740, and GSE122497) from the dbDEMC database. The results showed that 92 miRNAs were downregulated in expression in all four datasets (**Figure 1A**). In addition, the potential miRNAs that target the 3'UTR of EGFL6 were predicted via a combination of target prediction algorithms (miRWalk and RNA22). The results showed that 16 miRNAs could potentially target EGFL6 (**Figure 1B**). After comparing the two sets of data, three candidate miRNAs were identified: miR-1224-5p, miR-6747-5p, miR-6759-5p (**Figure 1C**). It was showed that EGFL6 is significantly upregulated in esophageal cancer by analyzing the TCGA dataset through the GEPIA database (**Figure 1D**). The western blot showed an elevated expression level of EGFL6 in EC9706 and KYSE70 cell compared to KYSE150, KYSE410, EC18, and TE12 cell (**Figure 1E**). Thus, the cells of EC9706 and KYSE70 were chosen for further experimental investigations.

To determine whether EGFL6 expression is regulated by these three miRNAs, the cells of EC9706 and KYSE70 were transfected with miR-1224-5p mimics, miR-6747-5p mimics, and miR-6759-5p mimics. The western blot results showed that only miR-6747-5p mimics could significantly reduce EGFL6 levels in EC9706 and KYSE70 cells (**Figure 1F**). To validate the interaction of miR-6747-5p with EGFL6, vectors containing either the WT or mut sequences of the miR-6747-5p binding site within the 3'UTR of EGFL6 were constructed for dual-luciferase reporter gene experiments. The results showed that co-transfection of WT vectors with miR-6747-5p mimics could led to a reduction in luciferase activity by 67%, when the binding site is mutated, miR-6747-5p mimics do not affect the luciferase activity (**Figure 1G**). Thus, the results demonstrate that miR-6747-5p could target EGFL6 and negatively regulate EGFL6 expression in ESCC cells.

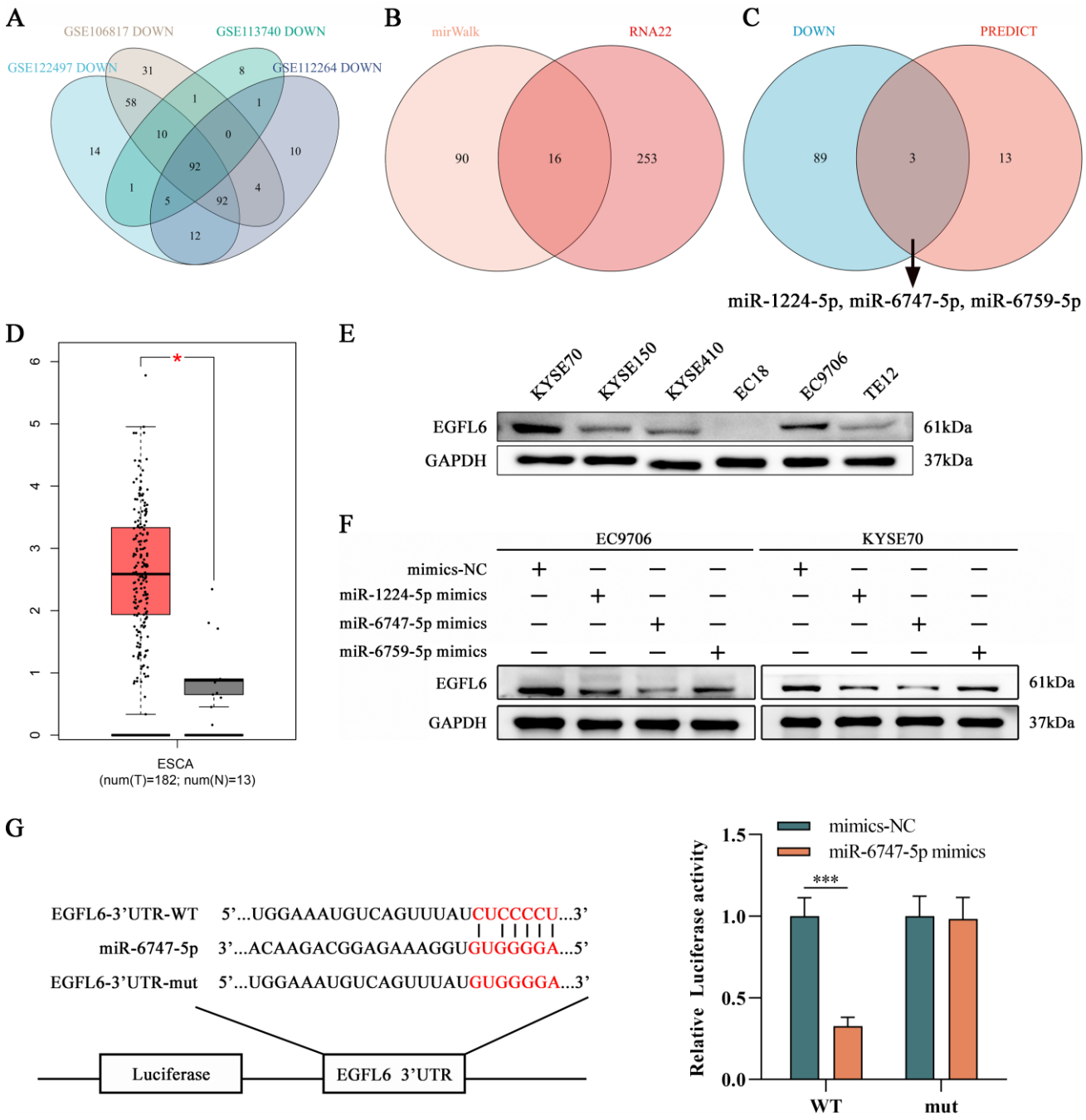


**Figure 1.** The screening of miRNA targeting EGFL6 **(A)** The overlap of downregulated miRNAs in the GSE106817, GSE112264, GSE113740, and GSE122497 datasets, employing stringent criteria of adj. $p < 0.05$, log FC < −2.0; **(B)** the Venn diagram showing the predicted results of miRNAs potentially regulating EGFL6 in the miRWalk and miRNA22 databases; **(C)** The intersection of miRNAs with down-regulated expression and predicted miRNAs; **(D)** The analysis of EGFL6 expression in ESCA was conducted through the GEPIA database; **(E)** The protein expression of EGFL6 were assessed across six ESCC cell lines; **(F)** The expression levels of EGFL6 were assessed in EC9706 and KYSE70 cells following transfection with miR-1224-5p mimics, miR-6747-5p mimics, or miR-6759-5p mimics by western blot; **(G)** The dual luciferase reporter gene assays for miR-6747-5p binding with EGFL6. *** $P < 0.001$.

### 3.2. miR-6747-5p inhibits the proliferation, migration and invasion of ESCC cells

To investigate the role of miR-6747-5p in ESCC, miR-6747-5p mimics were transfected into EC9706 and KYSE70 cells. Subsequent RT-qPCR analysis revealed an increase of miR-6747-5p expression, with a 43-fold increase in EC9706 cells and a 22-fold increase in KYSE70 cells following transfection (**Figure 2A**). Concurrently, western blot assays demonstrated a discernible suppression of EGFL6 protein expression in both EC9706 and KYSE70 cells subsequent to transfection with miR-6747-5p mimics (**Figure 2B**). The CCK-8 assay showed a notable decrease in the proliferation rates of EC9706 and KYSE70 cells subsequent to transfection with miR-6747-5p mimics, exhibiting reductions of 41% and 31%, respectively (**Figure 2C**). The results of CCK-8 showed that miR-6747-5p could inhibit the proliferation of EC9706 and KYSE70 cells. Similarly, the clone formation assay showed a diminished proliferative capacity in EC9706 and KYSE70 cells transfected with miR-6747-5p mimics, with reductions of 36% and 34%, respectively (**Figure 2D**). The results of clone formation experiment were consistent with those of CCK-8 experiment, which further indicated that miR-6747-5p could inhibit the proliferation of EC9706 and KYSE70 cells. In addition, AKT and MAPK signaling pathways contribute to cell proliferation by facilitating cell cycle operation. Therefore, we conducted a detection on them. Attenuated phosphorylation levels of AKT and MAPK were observed in EC9706 and KYSE70 cells transfected with miR-6747-5p mimics (**Figure 2E**). The results indicated that miR-6747-5p might inhibit the proliferation of EC9706 and KYSE70 cells by inhibiting AKT and MAPK signaling pathways.

(See **Figure 3A–G**) Following transfection with the miR-6747-5p mimic, the Transwell assay showed a reduction in both migration and invasion rates of EC9706 cells by 47% and 49%, respectively. KYSE70 cells exhibited a 44% and 36% reduction in migration and invasion rates, respectively (**Figure 3A,B,E**). In the wound healing assay, it was observed that the migration of EC9706 cells decreased by 51% and 44% at 24 h and 48 h, respectively. KYSE70 cells exhibited reductions in migration by 50% and 35% at 24 h and 48 h, respectively (**Figure 3C,D,F**). The results showed that miR-6747-5p could inhibit the migration and invasion ability of EC9706 and KYSE70. Additionally, western blot analysis showed decreased levels of N-cadherin and Vimentin, coupled with increased levels of E-cadherin in both EC9706 and KYSE70 cells following transfection with miR-6747-5p mimics (**Figure 3G**). The results indicated that miR-6747-5p might inhibit the metastasis of esophageal cancer cells by inhibiting EMT pathway. These findings collectively indicate the inhibitory role of miR-6747-5p in ESCC cell proliferation, migration, and invasion.

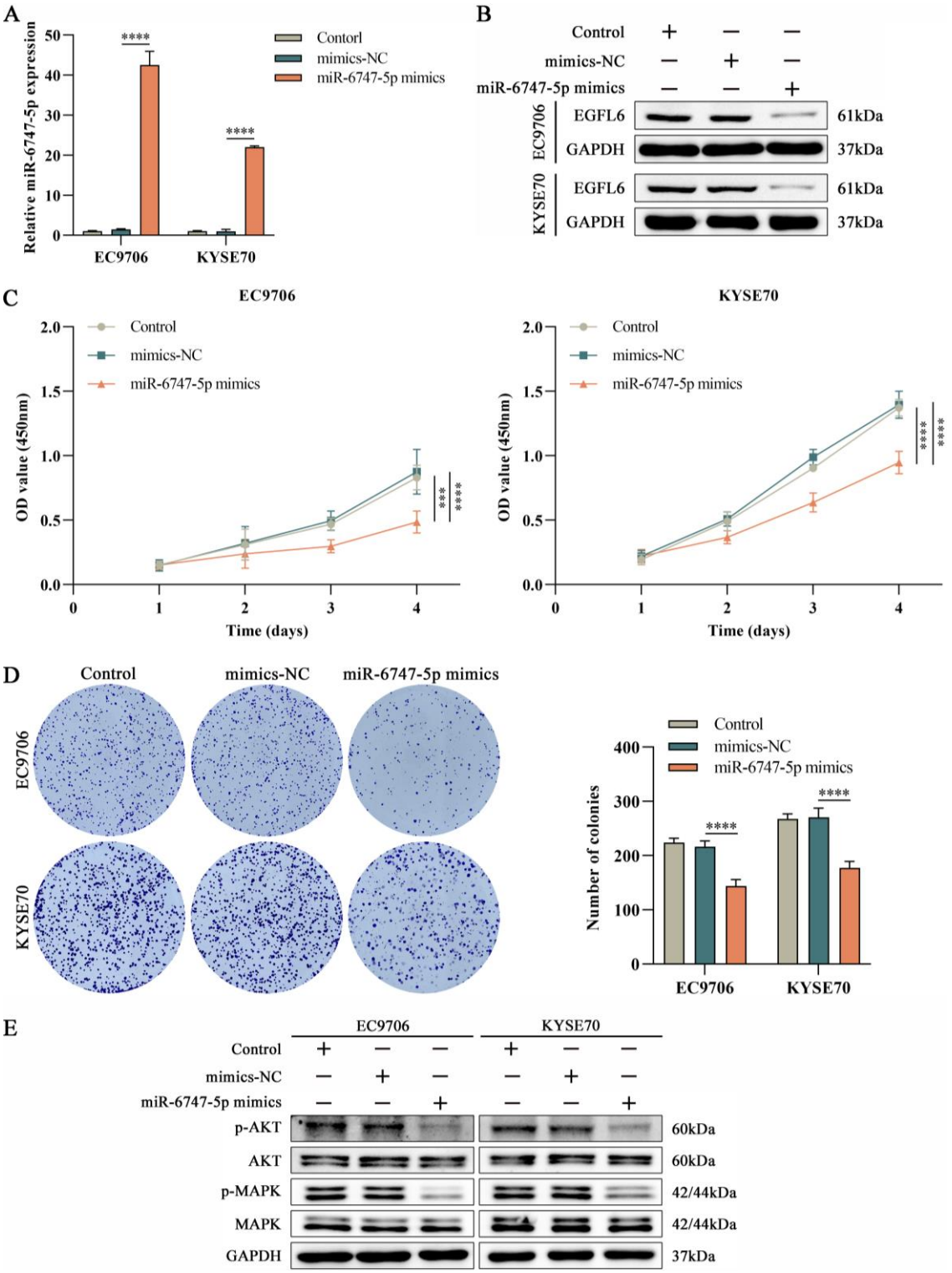


**Figure 2.** The inhibition of the proliferation of ESCC cells by miR-6747-5p; **(A)** The RT-qPCR results of the relative expression levels of miR-6747-5p in EC9706 and KYSE70 cells; **(B)** The western blot results of the expression levels of EGFL6 in EC9706 and KYSE70 cells; **(C)** The viability of EC9706 and KYSE70 cells was evaluated utilizing the CCK-8 assay; **(D)** Clone formation assay of EC9706 and KYSE70 cells; **(E)** The phosphorylation levels of AKT and MAPK in EC9706 and KYSE70 cells. *** $P < 0.001$, **** $P < 0.0001$.

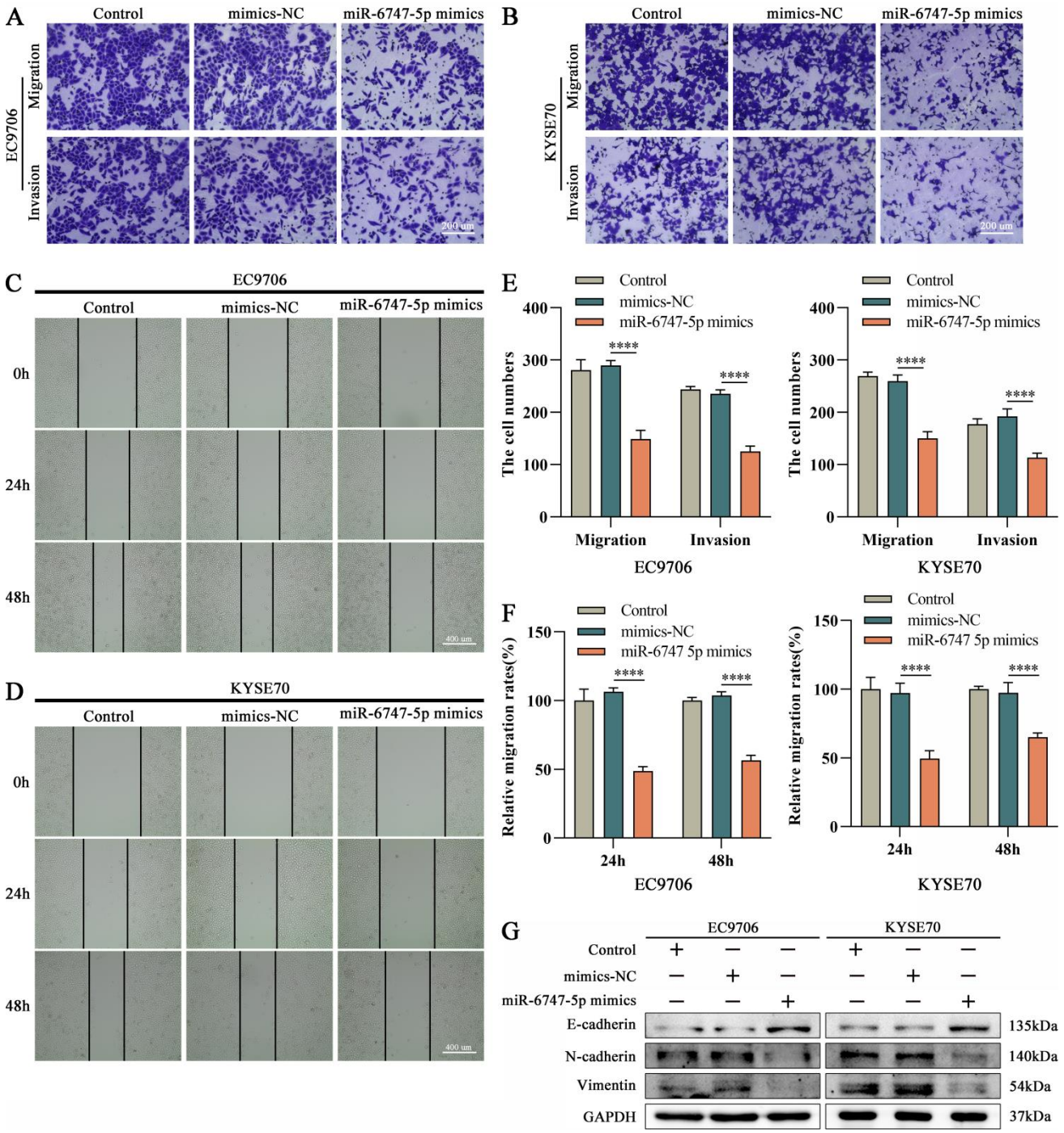


**Figure 3.** The inhibition of both migration and invasion of ESCC cells by miR-6747-5p **(A,B)** transwell assays for evaluating the migratory and invasive capacities of EC9706 and KYSE70 cells; **(C,D)** Wound healing assay showed the effect of miR-6747-5p mimics on the migration of EC9706 and KYSE70 cells; (**E,F**) The statistical graph of A-D; **(G)** Alterations in the protein levels of E-cadherin, N-cadherin, and Vimentin in EC9706 and KYSE70 cells subsequent to transfection with miR-6747-5p mimics. **** $P < 0.0001$.

### 3.3. miR-6747-5p inhibits the tube formation and migration of HUVEC cells

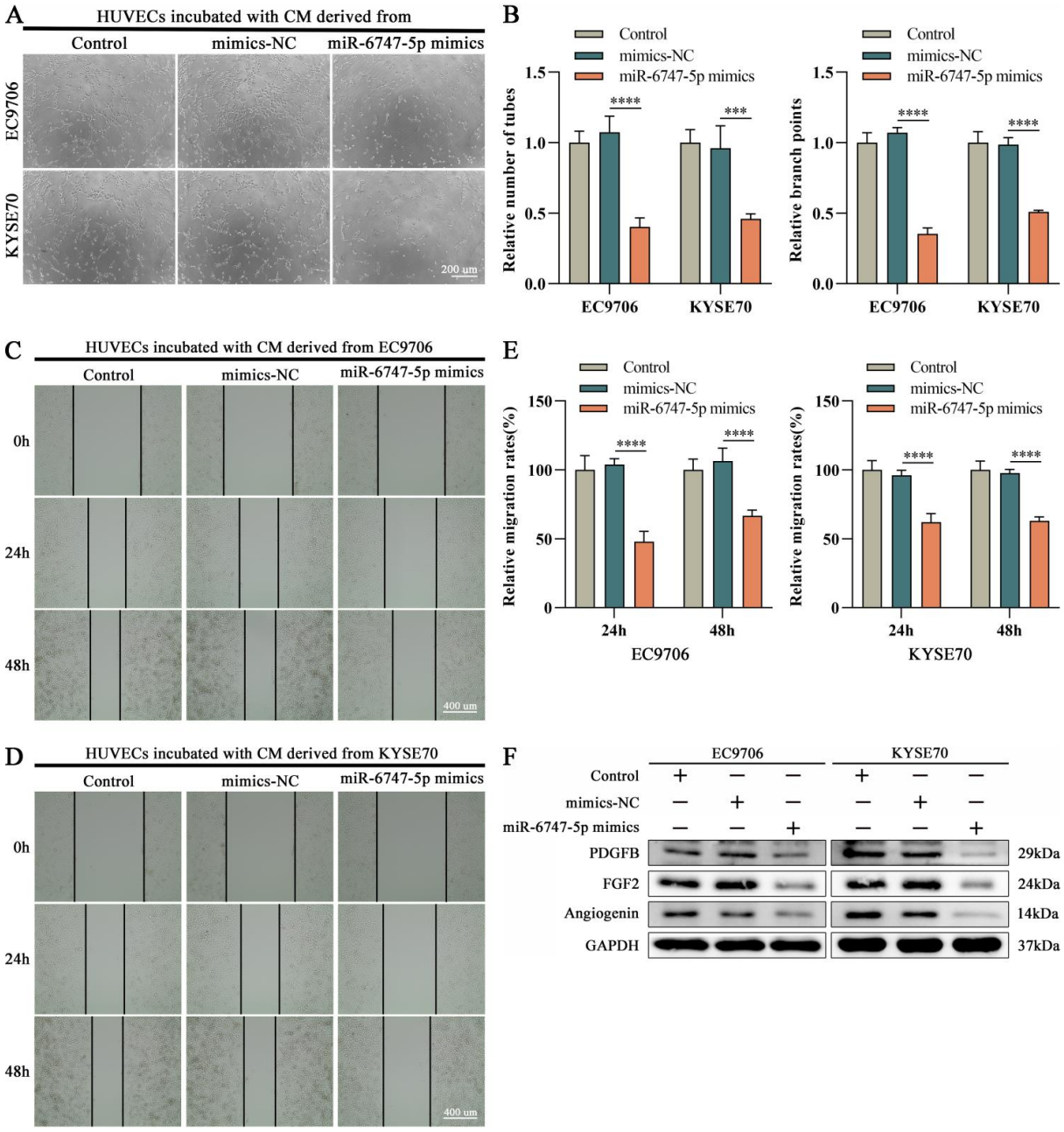


**Figure 4.** The inhibition of both tube formation and migration of HUVEC cells by miR-6747-5p **(A)** The tube formation assay of HUVEC cells cultured with conditioned medium derived from EC9706 and KYSE70 cells; **(B)** The statistical graph of endothelial cell tube formation assay of HUVEC cells; **(C,D)** The wound healing assay of HUVEC cells cultured with conditioned medium derived from EC9706 and KYSE70 cells; **(E)** The statistical graph of wound healing assay of HUVEC cells; **(F)** The protein expression of PDGFB, FGF2 and ANG in EC9706 and KYSE70 cells. *** $P < 0.001$, **** $P < 0.0001$.

To investigate the role of miR-6747-5p in ESCC angiogenesis, HUVEC cells were cultured with conditioned medium obtained from EC9706 and KYSE70 cells following transfection with miR-6747-5p mimics. The endothelial cell tube formation assay showed a reduction in the number of tubes by 60%–65% in the

conditioned medium derived from EC9706 cells, and a reduction by 49%–54% in the conditioned medium derived from KYSE70 cells (**Figure 4A,B**). The results showed that miR-6747-5p could inhibit the ability of EC9706 and KYSE70 cells to induce the tube formation of HUVEC cells. In the wound healing assay, treatment with conditioned medium derived from EC9706 cells resulted in a reduction of HUVEC cell migration by 48% and 67% at 24 h and 48 h, respectively. Similarly, treatment with conditioned medium derived from KYSE70 cells decreased the migration of HUVEC cells by 38% and 37% at 24 h and 48 h, respectively (**Figure 4C–E**). The results showed that miR-6747-5p could inhibit the ability of EC9706 and KYSE70 cells to induce HUVEC cell migration. The western blot results demonstrated that the protein expression of pro-angiogenic factors including PDGFB, FGF2, and ANG were down-regulated in both EC9706 and KYSE70 cells (**Figure 4F**). These experimental findings suggest that miR-6747-5p could inhibit angiogenesis in ESCC cells.

### 3.4. Overexpression of EGFL6 reverses the effects of miR-6747-5p on proliferation, migration and invasion of ESCC cells

To determine whether EGFL6 overexpression could reverse the impact of miR-6747-5p on ESCC cells, miR-6747-5p mimics and EGFL6 overexpression vectors were transfected individually or co-transfected into EC9706 and KYSE70 cells. The western blot results confirmed the efficacy of the EGFL6 overexpression vectors (**Figure 5A**). The CCK-8 assay showed that overexpressing EGFL6 led to a 39% and 41% increase in the viability of EC9706 and KYSE70 cells, respectively. The results showed that EGFL6 overexpression could promote the proliferation of EC9706 and KYSE70 cells, and reverse the inhibitory effect of miR-6747-5p on the proliferation of EC9706 and KYSE70 cells. The clone formation assay demonstrated a 30% and 38% increase in the number of clones in EC9706 and KYSE70 cells, respectively, upon EGFL6 overexpression. Remarkably, EGFL6 overexpression counteracted the inhibition of EC9706 and KYSE70 cell proliferation induced by miR-6747-5p mimics (**Figure 5B,C**). Western blot indicated that the levels of phosphorylated AKT and MAPK were elevated in EC9706 and KYSE70 cells transfected solely with EGFL6 overexpression vectors. When miR-6747-5p mimics and EGFL6 overexpression vector were co-transfected into EC9706 and KYSE70 cells, the levels of phosphorylated AKT and MAPK were restored (**Figure 5D**).

(See **Figure 6A–G**) In addition, in the Transwell assay, EGFL6 overexpression led to a 39% increase in EC9706 cell migration, a 38% increase in EC9706 cell invasion, a 35% increase in KYSE70 cell migration, and a 40% increase in KYSE70 cell invasion (**Figure 6A,B,E**). In the wound healing assay, EGFL6 overexpression resulted in a 50% and 35% increase in EC9706 cell migration at 24 h and 48 h, respectively, and a 57% and 32% increase in KYSE70 cell migration at 24 h and 48 h, respectively (**Figure 6C,D,F**). EGFL6 overexpression exerted a reversal effect on the inhibitory influence of miR-6747-5p mimics on EC9706 and KYSE70 cell migration and invasion (**Figure 6A–F**). The Western blot results indicated that EGFL6 overexpression led to an upregulation of N-cadherin and Vimentin protein expression and a downregulation of E-cadherin protein expression in both EC9706

and KYSE70 cells. When miR-6747-5p mimics and EGFL6 overexpression vectors were co-transfected into EC9706 and KYSE70 cells, the expression of these proteins was restored (**Figure 6G**). The results suggest that EGFL6 overexpression could promote the proliferation, migration, and invasion capabilities of ESCC cells and counteract the inhibitory effects of miR-6747-5p.

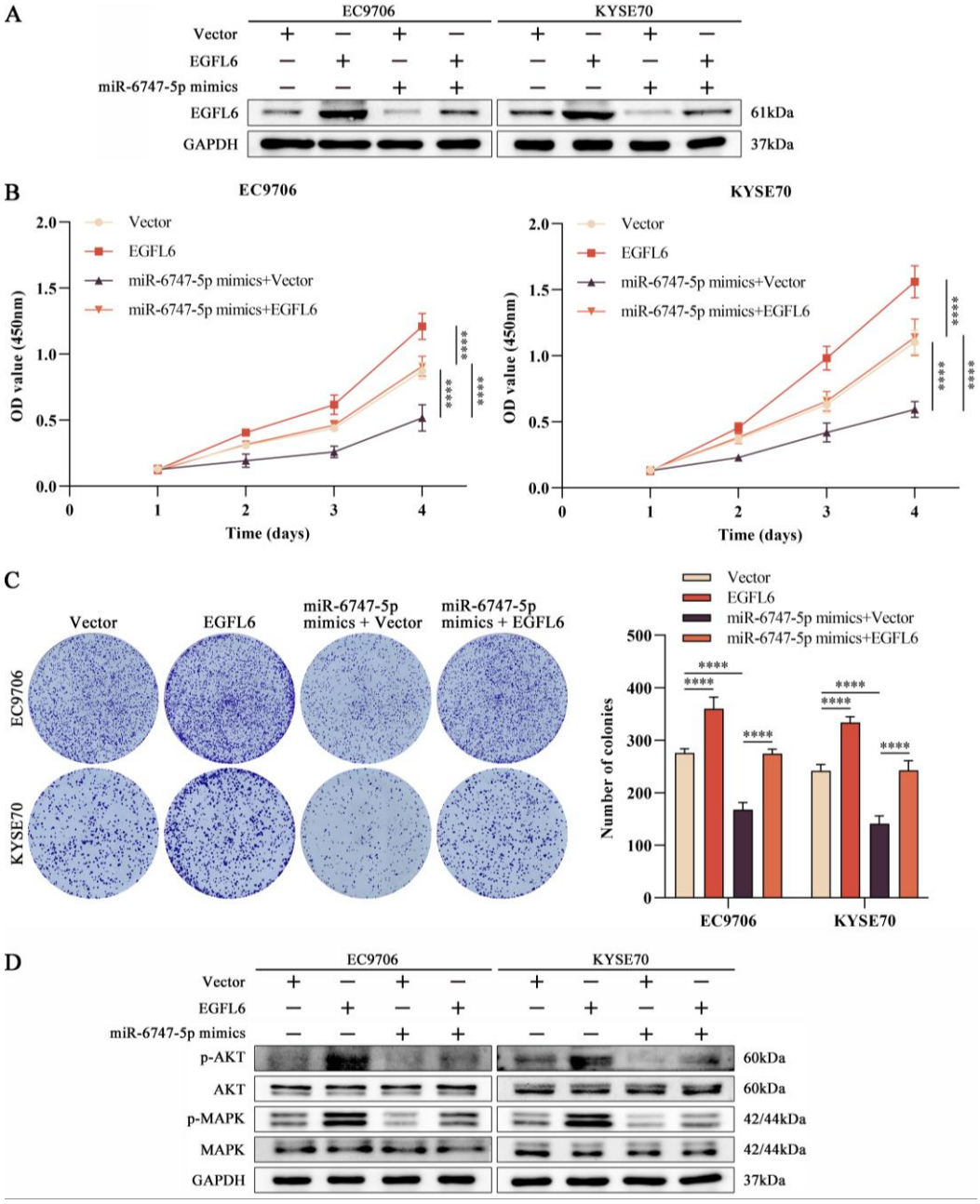


**Figure 5.** The reversal of miR-6747-5p effect on ESCC cell proliferation by EGFL6 overexpression; **(A)** The expression of EGFL6 were evaluated in EC9706 and KYSE70 cells subsequent to transfection with miR-6747-5p mimics or EGFL6 overexpression vectors; **(B)** The CCK-8 assay for detecting the viability of EC9706 and KYSE70 cells; **(C)** Clone formation assay for the proliferation of EC9706 and KYSE70 cells; **(D)** The levels of phosphorylated AKT and MAPK in EC9706 and KYSE70 cells. **** $P < 0.0001$.

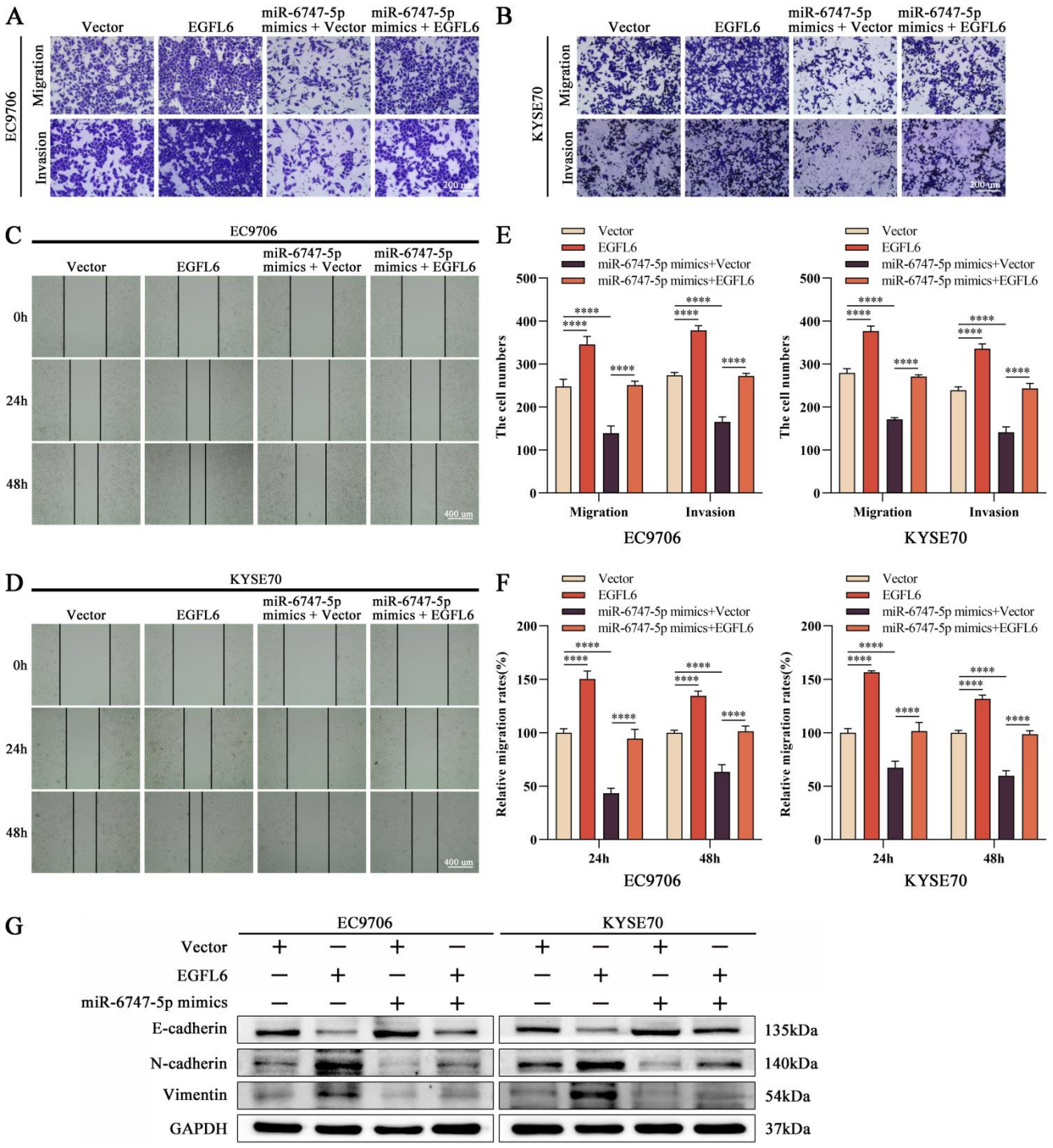


**Figure 6.** The reversal of miR-6747-5p effect on ESCC cell migration and invasion by EGFL6 overexpression **(A, B)** The Transwell assay for EC9706 and KYSE70 cell migration and invasion; **(C, D)** The wound healing assay for EC9706 and KYSE70 cell migration; **(E, F)** The statistical graph of A–D; **(G)** Protein levels of E-cadherin, N-cadherin and Vimentin in EC9706 and KYSE70 cells. **** $P < 0.0001$.

### 3.5. Overexpression of EGFL6 reverses the effects of miR-6747-5p on tube formation and migration in HUVEC cells

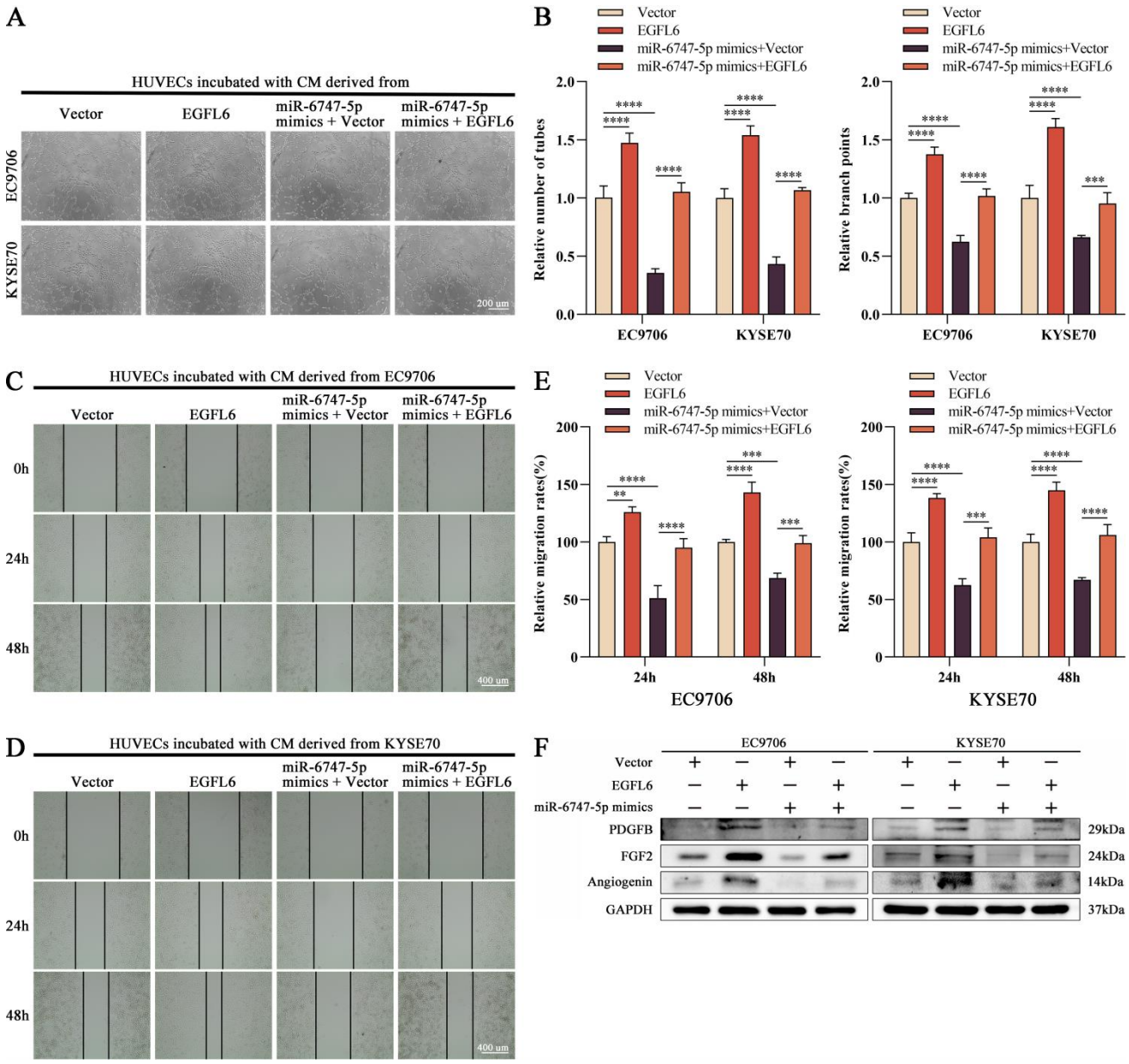


**Figure 7.** The reversal of miR-6747-5p effect on the tube formation and migration of HUVEC cells by EGFL6 overexpression **(A)** The endothelial cell tube formation assay of HUVEC cells cultured with conditioned medium from EC9706 and KYSE70 cells; **(B)** The statistical graph of endothelial cell tube formation assay; **(C,D)** The wound healing assay of HUVEC cells cultured in conditioned medium from EC9706 and KYSE70 cells; **(E)** The statistical graph of wound healing assay of HUVEC cells; **(F)** Protein levels of PDGFB, FGF2, and ANG in EC9706 and KYSE70 cells. ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

The findings from the endothelial cell tube formation assay demonstrated that upregulation of EGFL6 resulted in a significant increase in tube formation, with a respective increase of 37%–47% and 54%–61% observed in conditioned medium derived from EC9706 and KYSE70 cells. Overexpression of EGFL6 was observed to counteract the suppressive influence exerted by miR-6747-5p mimics on tube

formation in conditioned medium derived from both EC9706 and KYSE70 cells (**Figure 7A,B**). In the wound healing assay, EGFL6 overexpression in EC9706 cells resulted in a 26% and 43% increase in the migration of HUVEC cells at 24 h and 48 h, respectively. EGFL6 overexpression in KYSE70 cells resulted in a 38% and 45% increase in the migration of HUVEC cells at 24 h and 48 h, respectively. EGFL6 overexpression effectively counteracted the inhibitory impact of miR-6747-5p mimics on HUVEC cell migration (**Figure 7C–E**). Additionally, the western blot assay demonstrated that EGFL6 overexpression in EC9706 and KYSE70 cells resulted in increased expression of pro-angiogenic factors PDGFB, FGF2 and ANG. EGFL6 overexpression effectively reversed the inhibitory effect exerted by miR-6747-5p mimics on the expression of these pro-angiogenic factors (**Figure 7F**). The results indicate that the EGFL6 overexpression could promote angiogenesis and reverse the inhibitory impact of miR-6747-5p in ESCC cells.

## 4. Discussion

Tumor angiogenesis is a crucial factor in sustaining malignant growth and is associated with hypoxia within the tumor [31]. In solid tumors larger than 2 mm, the hypoxic environment promotes the formation of the tumor vascular system, a process governed by the regulation of various angiogenic factors [32,33]. Emerging evidence suggests that miRNAs exhibit varying expression profiles within tumor tissues and play a role in modulating tumor angiogenesis [34–36]. Shi et al. screened miR-1224-5p as an ESCC-related miRNA by analyzing the distinct expression profiles of miRNAs within ESCC tissues, as well as GSE55856 and GSE43732 data sets. Functioning as a tumor suppressor miRNA, miR-1224-5p could impede the proliferation and metastasis of ESCC cells by targeting TNS4 [37]. Additionally, Wang et al. [38] characterized miR-181b-5p as an oncogenic miRNA by analyzing miRNA expression profiles in ESCC tissues. The elevated expression levels of miR-181b-5p could counteract the inhibition on ESCC angiogenesis mediated by PTEN and PHLPP2.

In this study, we analyzed the GSE106817, GSE112264, GSE113740, and GSE122497 data sets and found that 92 miRNAs were downregulated in ESCC. miR-1224-5p, miR-6747-5p and miR-6759-5p were identified as potentially targeting EGFL6 through a target prediction algorithm. The interaction of miR-6747-5p with EGFL6 was verified through western blot and a dual-luciferase assay. The results of in vitro functional experiments showed that miR-6747-5p could downregulate AKT and MAPK signaling pathways to inhibit ESCC cell proliferation, metastasis, and angiogenesis.

EGFL6 is a significant contributor to tumorigenesis and progression [39]. The involvement of EGFL6 in ESCC angiogenesis remains to be fully understood, but previous studies have shown that EGFL6 promotes angiogenesis in various malignancies, such as nasopharyngeal carcinoma, colorectal and ovarian cancer [40–42]. EGFL6 regulates the asymmetric cell division by activating ERK signaling in ovarian cancer, which promotes tumor metastasis [43]. In breast cancer cells, the upregulation of EGFL6 expression promotes metastasis and inhibits apoptosis [44]. Conversely, reduced level of EGFL6 inhibits ovarian cancer cell migration and

angiogenesis [45]. Our results showed that EGFL6 overexpression could promote ESCC cell proliferation, migration, invasion, and angiogenesis. The suppressive impact exerted exerted by miR-6747-5p on ESCC cell was restored upon EGFL6 overexpression. Thus, the miR-6747-5p/EGFL6 axis could be worth further exploration.

At present, anti-angiogenesis drugs for targeted therapy of esophageal cancer are mainly developed for VEGF targets. Bevacizumab has been applied to the clinical treatment of esophageal cancer, and the results show that it can partially relieve the symptoms of patients. Remolumab, a monoclonal antibody that targets VEGFR2, also improves the survival time of patients with esophageal cancer to some extent, but may cause side effects such as hypertension and impaired wound healing [46,47]. Although some breakthroughs have been made in anti-angiogenesis therapy for esophageal cancer, the safety of anti-angiogenesis-targeted drugs needs to be further confirmed due to their late start [48]. Therefore, further research on the mechanism of anti-angiogenesis therapy, and exploration of more effective and specific targets for application are feasible strategies for future anti-angiogenesis therapy of esophageal cancer. The potential clinical value of EGFL6 in inhibiting angiogenesis needs to be explored. Follow-up in vivo experiments are needed to explore the synergistic effect of EGFL6 and bevacizumab or other anti-angiogenic drugs, so as to further promote the study of ESCC treatment strategies.

In summary, we analyzed miRNA expression profiles in ESCC and identified miR-6747-5p specifically targeting EGFL6. miR-6747-5p could downregulate AKT and MAPK signaling pathways and inhibit ESCC cell proliferation, migration, invasion and angiogenesis by reducing the EGFL6 expression level. The findings suggest that EGFL6 is a potential target for antiangiogenic therapy in ESCC. Downregulation of EGFL6 by miR-6747-5p is a feasible strategy for ESCC treatment. Additionally, further research is necessary to elucidate whether miR-6747-5p could regulate ESCC progression in vivo. The study of ESCC treatment strategy is promoted through the study of combination therapy with other anti-angiogenesis drugs.

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## References


1. Thrift A P. Global burden and epidemiology of Barrett oesophagus and oesophageal cancer[J]. Nature Reviews Gastroenterology & Hepatology, 2021.
2. Morgan E, Soerjomataram I, Rumgay H, et al. The Global Landscape of Esophageal Squamous Cell Carcinoma and Esophageal Adenocarcinoma Incidence and Mortality in 2020 and Projections to 2040: New Estimates from GLOBOCAN 2020[J]. Gastroenterology, 2022;163(3):649-658.e2.
3. Grover F L.Squamous cell carcinoma of the esophagus[J].Tex Med, 1984, 80(5):38-40.
4. Shah M A, Altorki N, Patel A A J A.Improving outcomes in patients with oesophageal cancer[J].Nature reviews. Clinical oncology, 2023, 20(6):390-407.
5. Li H, Yang X, Zhang A,et al.Age-period-cohort analysis of incidence, mortality and disability-adjusted life years of esophageal cancer in global, regional and national regions from 1990 to 2019[J].BMC Public Health, 2024, 24(1).
6. He S, Xu J, Liu X,et al.Advances and challenges in the treatment of esophageal cancer[J].Acta Pharmaceutica Sinica B, 2021;11(11):3379-3392.
7. Ooki A, Osumi H, Chin K,et al.Potent molecular-targeted therapies for advanced esophageal squamous cell carcinoma[J].Therapeutic Advances in Medical Oncology, 2023.
8. Hassanabad A F, Chehade R, Breadner D,et al.Esophageal carcinoma: Towards targeted therapies.[J].Cell Oncol (Dordr), 2020(2).
9. Lugano R, Ramachandran M, Dimberg A. Tumor angiogenesis: causes, consequences, challenges and opportunities[J]. Cell Mol Life Sci, 2020;77(9):1745-1770.
10. Wang K, Chen Q, Liu N,et al.Recent advances in, and challenges of, anti-angiogenesis agents for tumor chemotherapy based on vascular normalization.[J].Drug discovery today, 2021, 26(11):2743-2753.
11. Zhang B, Qi L, Wang X, et al. Phase II clinical trial using camrelizumab combined with apatinib and chemotherapy as the first-line treatment of advanced esophageal squamous cell carcinoma[J]. Cancer Commun (Lond), 2020;40(12):711-720.
12. Zhao J, Lei J, Yu J,et al.Clinical efficacy and safety of apatinib combined with S-1 in advanced esophageal squamous cell carcinoma[J].Investigational New Drugs, 2020, 38(2):500-506.
13. Li X, Zhou J, Wang X, et al. New advances in the research of clinical treatment and novel anticancer agents in tumor angiogenesis[J]. Biomed Pharmacother, 2023;163:114806.
14. Lopes-Coelho F, Martins F, Pereira S A,et al.Anti-Angiogenic Therapy: Current Challenges and Future Perspectives[J].International Journal of Molecular Sciences, 2021, 22(7):3765.
15. Kang J, Wang J, Tian J,et al.The emerging role of EGFL6 in angiogenesis and tumor progression[J].International Journal of Medical Sciences, 2020, 17(10):1320-1326.
16. Yeung G, Mulero J J, Berntsen R P,et al.Cloning of a novel epidermal growth factor repeat containing gene EGFL6: expressed in tumor and fetal tissues.[J].Genomics, 1999, 62(2):304-307.
17. Song X, Cheng X, Jin X,et al.EGFL6 promotes bone metastasis of lung adenocarcinoma by increasing cancer cell malignancy and bone resorption[J].Clinical & Experimental Metastasis, 2023, 40:357 - 371.
18. Sung TY, Huang HL, Cheng CC, et al. EGFL6 promotes colorectal cancer cell growth and mobility and the anti-cancer property of anti-EGFL6 antibody[J]. Cell Biosci, 2021;11(1):53.
19. Noh K, Mangala L S, Han H D,et al.Differential Effects of EGFL6 on Tumor versus Wound Angiogenesis[J].Cell Reports, 2017, 21(10):2785-2795.
20. A M B, A A S P, A D M,et al.Micro-RNA: The darkhorse of cancer[J].Cellular Signalling, 2021.83:109995.
21. Shou Y, Wang X, Chen C, et al. Exosomal miR-301a-3p from esophageal squamous cell carcinoma cells promotes angiogenesis by inducing M2 polarization of macrophages via the PTEN/PI3K/AKT signaling pathway[J]. Cancer Cell Int, 2022;22(1):153.
22. Zhang C, Luo Y, Cao J, Wang X, Miao Z, Shao G. Exosomal lncRNA FAM225A accelerates esophageal squamous cell carcinoma progression and angiogenesis via sponging miR-206 to upregulate NETO2 and FOXP1 expression[J]. Cancer Med, 2020;9(22):8600-8611.
23. Zhu Y, Ma Y, Peng H, Gong L, Xiao M, et al. MiR-130b promotes the progression of oesophageal squamous cell carcinoma by targeting SASH1[J]. J Cell Mol Med, 2019;23(1):93-103.

24. Han L, Cui D, Li B, Xu WW, Lam AKY, et al. MicroRNA-338-5p reverses chemoresistance and inhibits invasion of esophageal squamous cell carcinoma cells by targeting Id-1[J]. Cancer Sci, 2019;110(12):3677-3688.
25. Bi Y, Guo S, Xu X,et al.Decreased ZNF750 promotes angiogenesis in a paracrine manner via activating DANCR/miR-4707-3p/FOXC2 axis in esophageal squamous cell carcinoma[J].Cell Death & Disease, 2020, 11(4).
26. Xu M, Zhang J, Lu X, Liu F, Shi S, et al. MiR-199a-5p-Regulated SMARCA4 Promotes Oral Squamous Cell Carcinoma Tumorigenesis[J]. Int J Mol Sci, 2023;24(5):4756.
27. Xu F, Wang Y, Ling Y, et al. dbDEMC 3.0: Functional Exploration of Differentially Expressed miRNAs in Cancers of Human and Model Organisms [J]. Genomics Proteomics Bioinformatics, 2022;20(3):446-454.
28. Dweep H, Gretz N, Sticht C. miRWalk database for miRNA-target interactions[J]. Methods Mol Biol, 2014;1182:289-305.
29. Loher P, Rigoutsos I. Interactive exploration of RNA22 microRNA target predictions[J]. Bioinformatics, (2012) 28:3322–3.
30. Sun H, Song X, Li C,et al.Humanized disulfide-stabilized diabody against fibroblast growth factor-2 inhibits PD-L1 expression and epithelial-mesenchymal transition in hepatoma cells through STAT3[J].IUBMB Life, 2023, 75(11):957-968.
31. Liu Z L, Chen H H, Zheng L L,et al.Angiogenic signaling pathways and anti-angiogenic therapy for cancer[J].Signal Transduction & Targeted Therapy, 2023, 8(1).198
32. Vimalraj S. A concise review of VEGF, PDGF, FGF, Notch, angiopoietin, and HGF signalling in tumor angiogenesis with a focus on alternative approaches and future directions[J]. Int J Biol Macromol, 2022;221:1428-1438.
33. Jiang X, Wang J, Deng X, et al. The role of microenvironment in tumor angiogenesis[J]. J Exp Clin Cancer Res, 2020;39(1):204.
34. Su Z, Li W, Lei Z, Hu L, Wang S, Guo L. Regulation of Angiogenesis by Non-Coding RNAs in Cancer[J]. Biomolecules, 2024;14(1):60.
35. Ding M H, Lozoya E G, Rico R N,et al.The Role of Angiogenesis-Inducing microRNAs in Vascular Tissue Engineering[J].Tissue engineering, Part A, 2020;26(23-24).
36. Nazari-Khanamiri, Fereshteh, Abdyazdani N, Abbasi R,et al.Tumor cells-derived exosomal noncoding RNAs in cancer angiogenesis: Molecular mechanisms and prospective[J].Cell Biochemistry & Function, 2023;41(8):1008-1015.
37. Shi Z Z, Wang W J, Chen Y X,et al. The miR-1224-5p/TNS4/EGFR axis inhibits tumour progression in oesophageal squamous cell carcinoma[J]. Cell Death & Disease, 2020;11(7):597.
38. Wang Y, Lu J, Chen L, et al. Tumor-Derived EV-Encapsulated miR-181b-5p Induces Angiogenesis to Foster Tumorigenesis and Metastasis of ESCC[J].. Mol Ther Nucleic Acids, 2020;20:421-437.
39. Shi S, Ma T, Xi Y.A Pan-Cancer Study of Epidermal Growth Factor-Like Domains 6/7/8 as Therapeutic Targets in Cancer[J].Frontiers in Genetics, 2020, 11:598743.
40. Zhu Z, Ni H, You B,et al.Elevated EGFL6 modulates cell metastasis and growth via AKT pathway in nasopharyngeal carcinoma[J].Cancer Medicine, 2018, 7(12):6281-6289.
41. Zhu W, Liu C, Lu T,et al.Knockout of EGFL6 by CRISPR/Cas9 mediated inhibition of tumour angiogenesis in ovarian cancer[J].Frontiers in Oncology, 2020;10:1451.
42. He G, Li W, Zhao W,et al.Formin-like 2 promotes angiogenesis and metastasis of colorectal cancer by regulating the EGFL6/CKAP4/ERK axis[J].Cancer Science, 2023, 114(5):2014-2028.
43. Bai S, Ingram P, Chen YC, et al. EGFL6 Regulates the Asymmetric Division, Maintenance, and Metastasis of ALDH+ Ovarian Cancer Cells[J]. Cancer Res, 2016;76(21):6396-6409.
44. An J, Du Y, Fan X,et al.EGFL6 promotes breast cancer by simultaneously enhancing cancer cell metastasis and stimulating tumor angiogenesis[J].Oncogene, 2019;38(12):2123-2134.
45. Wu B, Zhang L, Yu Y,et al.miR-6086 inhibits ovarian cancer angiogenesis by downregulating the OC2/VEGFA/EGFL6 axis[J].Springer Science and Business Media LLC, 2020;11(5):345.
46. Fukuda T, Baba H, Okumura T, et al. miR-877-3p as a Potential Tumour Suppressor of Oesophageal Squamous Cell Carcinoma[J]. Anticancer Res, 2023;43(1):35-43.
47. Wei Q Y, Jin F, Wang Z Y,et al.MicroRNAs:A novel signature in the metastasis of esophageal squamous cell carcinoma[J].World Journal of Gastroenterology, 2024, 30(11):1497-1523.
48. Xiao Z, Zhao J, Ji G,et al.miR-493-5p Silenced by DNA Methylation Promotes Angiogenesis via Exosomes and VEGF-A-Mediated Intracellular Cross-Talk Between ESCC Cells and HUVECs[J].International Journal of Nanomedicine, 2024, 19:7165-7183.