

Article

MiR-128-3p Inhibits Esophageal Squamous Cell Carcinoma Progression by Targeting TTC9

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Abstract

Background: MicroRNAs (miRNAs) serve as targets for cancer diagnosis and therapy and have the potential to regulate the occurrence and progression of Esophageal squamous cell carcinoma (ESCC). Tetratricopeptide repeat domain 9 (TTC9) gene may involve in the invasion and metastasis of cancer cells. This study aimed to investigate the effects of miR-128-3p and TTC9 on ESCC progression and to elucidate the underlying molecular mechanisms.

Methodology: RT-qPCR was employed to measure miR-128-3p levels in ESCC tissues and adjacent non-cancerous tissues from 50 patients, as well as in ESCC cells and normal human esophageal squamous epithelial HET1A cells. Cell metabolic activity, migration, and invasion were assessed through MTT assays, scratch healing and transwell assays; and the protein expression of epithelial-mesenchymal transition (EMT)-related markers and TTC9 was measured by Western blot. The targeted regulatory relationship between miR-128-3p and TTC9 was verified by dual-luciferase reporter assay. The effect of miR-128-3p on xenograft tumor growth in nude mice was examined, and TTC9 expression in xenograft tumors was detected by immunohistochemistry.

Results: miR-128-3p expression was significantly lower in ESCC tissues and cells than in adjacent non-cancerous tissues and HET1A cells. Knocking miR-128-3p down markedly intensified the metabolic activity, invasion and moving of ESCC cells, whereas upregulating miR-128-3p excessively exhibited the opposite effect, notably suppressed tumor growth in nude mice. Through miR-128-3p's targeted regulation, TTC9 was suppressed. Conversely, TTC9 overexpression attenuated the effects of miR-128-3p and enhanced the proliferative and metastatic capacities of ESCC cells. Additionally, miR-128-3p overexpression suppressed ESCC growth in nude mice, whereas TTC9 overexpression partially reduced this suppressive effect.

Conclusion: miR-128-3p is significantly downregulated in ESCC. Overexpression of miR-128-3p suppresses the malignant biological behaviors of ESCC cells, and inhibits tumor growth in nude mice, at least in part by targeting TTC9.

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Highlights:

1. In ESCC cells, miR-128-3p was downregulated, whereas TTC9 was upregulated.
2. Overexpression of miR-128-3p inhibited the progression and metastasis of ESCC cells, while its downregulation promoted these processes.
3. Increased TTC9 expression attenuated the suppressive effects of miR-128-3p overexpression on the biological behaviors of ESCC cells.
4. In mouse xenograft models, miR-128-3p overexpression inhibited ESCC growth.

1. Introduction

Esophageal cancer, notably aggressive in the categories of digestive tract malignancies, holds the sixth position

in global cancer death rankings [1,2]. Within this category, 90% of the cases are esophageal squamous cell carcinoma (ESCC) [3]. Over fifty percent of ESCC instances are in China [4]. ESCC progresses rapidly and is less sensitive to chemotherapy [5]. This condition leads to poor prognosis and high mortality rates in clinical settings [6]. Hence, finding the gene related to ESCC growth and metastasis and probing into the related molecular mechanism are meaningful for preventing and treating ESCC.

MicroRNAs (miRNAs) can bind to the 3'UTRs of messenger RNAs to involve in gene modification post transcription [7,8]. Serving as novel cancer biomarkers, miRNAs can function as suppressors or oncogenes [9], taking part in various processes (e.g., the cycle, proliferation, apoptosis, invasion, and migration) of cells [10]. MiRNAs if dysfunctional may boost cancer onset and development [11,12]. As extensively reported,

miR-128-3p, acting mainly as a tumor suppressor, restrains the cells of breast cancer [13], ovarian cancer [14], colorectal cancer [15], prostate cancer [16], cervical cancer [17], and non-small cell lung cancer [18] from proliferating, invading and undergoing enhanced epithelial-mesenchymal transition (EMT). Nevertheless, miR-128-3p's involvement in ESCC is rarely investigated, thus is to be explained in detail.

Located on chromosome 14q24.2, the tetratricopeptide repeat domain 9 (TTC9) gene is encoded with a 5094 bp-long transcript that can be translated into a protein containing three repeated sequences of tetrapeptide [19,20]. Cao et al. [21] discovered that the expression of TTC9 in breast cancer cells was inhibited by hormones and this inhibition was reversed by the ERK inhibitor PD98059. Further studies reveal that one of the binding proteins of TTC9 is Tm5NM-1, a tropomyosin associated to mesenchymal cell motility [22]. Hence, the author deduced that TTC9 might take part in the invasion and metastasis of cancer cells. By investigation, Huang et al. [19] confirmed that decreasing TTC9 expression could inhibit lung adenocarcinoma cells from proliferating, migrating, and invading, while intensifying apoptosis. However, there haven't been literature reporting TTC9's involvement in controlling ESCC.

In this circumstance, the author firstly conducted RT-qPCR assays, discovering different expression levels of miR-128-3p in the tissues of ESCC and adjacent normal tissues, hence affirming the involvement of miR-128-3p in ESCC progress. Next, a further exploration was made on miR-128-3p's involvement in the processes like growth, moving, invasion, and EMT of *in vitro* ESCC cells and the growth of *in vivo* tumor. Over database prediction and luciferase assays, the TTC9 gene was verified as a target gene downstream of miR-128-3p; this gene was depressed by miR-128-3p and, if excessively expressed, was capable of attenuating miR-128-3p's effects. Therefore, targeting the miR-128-3p/TTC9 pathway might be a novel and achievable approach to better control the growth and metastasis of ESCC clinically.

2. Materials and Methodology

2.1 Collection of Clinical Specimens

For the objective of this survey, the author acquired the biopsy specimens of esophageal cancer from such 50 patients as pathologically confirmed as suffering from ESCC. These patients were admitted to The First Affiliated Hospital of Zhengzhou University and had been informed about the survey content and method and signed a written form of informed consent before the acquisition of the specimens. This study was performed in accordance with the principles of the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University (Approval No. 2025-KY-0540).

2.2 Cell Culture

Normal human esophageal squamous epithelial HET1A

cells and the ESCC cell lines EC9706, KYSE-30, TE-1, and TE-3 were obtained from the Cell Center of the Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. All cell lines were authenticated by STR profiling before use, and were tested for mycoplasma contamination and were negative. After resuscitation, the cells were seeded in DMEM medium (11965, Gibco, Big Cabin, OK, USA) supplemented with 10% fetal bovine serum (FBS, C0235, Beyotime, Shanghai, China) and 1% penicillin-streptomycin (15140122, Gibco) and cultured at 37°C under 5% CO₂. When cell confluence exceeded 80%, the cells were digested with 0.25% trypsin (T4799, Sigma-Aldrich, St. Louis, MO, USA) and passaged.

2.3 Cell Transfection

miR-128-3p mimics/inhibitors/agomir, OE-TTC9, and sh-TTC9, as well as the corresponding negative control (NC) were provided by Ribobio (Guangzhou, China). Lipofectamine 3000 (L3000001, Invitrogen, Austin, TX, USA) was used for transfection according to the manufacturer's instructions. The transfection steps are detailed below: 1) inoculate TE-1 and KYSE-30 cells into a plate comprising six wells; 2) upon the cell confluency fallen into the range of 70%-80%, transfect the cells using Lipofectamine 3000 and then test the transfection efficiency with the Reverse Transcription quantitative Polymerase Chain Reaction (RT-qPCR) method or Western Blot.

2.4 RT-qPCR

Total RNA was extracted from tissues or cells using a TRIzol reagent kit (DP424, Tiangen Biotech Co., Ltd, Beijing, China), and RNA concentration and purity were determined with a UV spectrophotometer. The extracted total RNA was reversely transcribed into cDNAs by using the Prime Script RT reagent kit numbered RR047 A. This reagent kit was operated as per its user manual. An ABI PRISM 7300 RT-PCR system (7300, Thermo Fisher Scientific, MA, USA), in conjunction with SYBR Green PCR reagent kits (4309155, Thermo Fisher Scientific), was employed to examine mRNA expression, taking cDNA as a template. The detailed test operations adhered to the kits' instructions. The relative expression levels of the genes were figured out in the form of $2^{-\Delta\Delta C_t}$. U6 and GAPDH were used as the internal control genes of miR-128-3p and its targeting genes (LYPD3 and TTC9) respectively. The specific sequences of the primers for detecting the genes are presented below (5'-3'):

miR-128-3p: F: 5'-CCACAGGAACCGGCC-3'; R: 5'-AGTGCAGGGTCCGAGGTATT-3';

U6: F: 5'-GGAGACACGCAAACGGAAG-3'; R: 5'-AGTGCAGGGTCCGAGGTATT-3';

LYPD3: F: 5'-CCCCACCCAGTTCTCAACTC-3'; R: 5'-GCTTTAGCAAGGAGGGCTCA-3';

TTC9: F: 5'-AACCAACAGGTAAGGCGGAA-3'; R: 5'-GAAGAGAAGAGGGGCCTGTG-3';

GAPDH: F: 5'-AAAGGGCCCTGACAACCTCTT-3'; R: 5'-TGGTACATGACAAGGTGCGG-3'.

2.5 Western Blot

Cells were lysed with pre-chilled RIPA buffer (20101ES60, Yeasen Biotechnology, Shanghai, China) to extract total protein, and protein concentrations were determined using a BCA protein assay kit (P0012, Beyotime Biotechnology). Equal amounts of protein (20 µg per lane) were separated by 10% SDS-PAGE and transferred onto a PVDF membrane. After transfer, the membrane was blocked with 5% bovine serum albumin at room temperature for 1 h, washed with TBST, and incubated overnight at 4°C with rabbit primary antibodies against N-cadherin (ab76011, 1:5000), E-cadherin (ab40772, 1:2000), Vimentin (ab16700, 1:2000), Snail (ab216347, 1:1000), Slug (ab27568, 1:1000), ZEB1 (ab203829, 1:500), and TTC9 (ab235537, 1:1000), as well as GAPDH (ab9485, 1:2500) as an internal control, respectively. All primary antibodies were purchased from Abcam (Waltham, MA, USA). On the following day, the primary antibody solution was removed, and the membrane was washed with TBST and incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (Abcam) at a dilution of 1:2000 at room temperature for 30 min. The membrane was washed again with TBST solution and further dosed with a chemiluminescent solution ECL (P0018S, Beyotime biotechnology) for color exposure. The protein strip picture was taken using a chemiluminescence imaging system numbered 5200 and provided by Tanon (Shanghai, China) and the grayscale values of the protein strips were assessed using Image J 1.8.0 software designed by the National Institutes of Health, Bethesda (MD, USA).

2.6 Methylthiazolyldiphenyl-tetrazolium Bromide (MTT) Assay

MiR-128-3p's involvement in regulating the vitality of ESCC cells was tested through MTT assays. Each group was set with three replicates. The TE-1 and KYSE-30 cells transfected were put into a plate containing 96 wells. The plate was positioned in an incubator till the cells stuck onto the well walls. A MTT solution was prepared as per the instructions of the MTT reagent (ST316, Beyotime biotechnology) and then administered to the ESCC cells for incubation. The culture medium was abandoned and then each well was added with dimethyl sulfoxide (numbered ST1276 and provided by Beyotime Biotechnology). A microplate reader setting at the wavelength of 490 nm was utilized to examine the absorbance and assess ESCC cell viability.

2.7 Scratch Healing Assay

The transfected TE-1 and KYSE-30 cells were resuspended in a PBS solution, then seed the cells into 6-well plate at a density of 1×10^6 cells per well (1 mL cell suspension per well). After cell attachment, the cells were pretreated with mitomycin C (1 µg/mL, M5353, Sigma-Aldrich) for 1 h to inhibit proliferation. A 200 µL pipette tip was used to draw a straight, uniform vertical scratch across the bottom of each well. After the suspended cells were washed away, serum-free culture medium was added, and the plate was incubated at 37°C

with 5% CO₂ for 24 h. Scratch widths were observed and photographed under a microscope at 0 h and 24 h. The scratches were measured using the Image J 1.8.0 software, and the relative scratch width was calculated: $\text{relative scratch width} = (\text{scratch width at 0 h} - \text{scratch width at 24 h}) / \text{scratch width at 0 h}$.

2.8 Transwell Assay

Assay for testing the cells' ability to migrate: place Transwell chambers in a plate containing 24 wells and add 100 µL of the cell suspension (density: 1×10^5 cells/mL) to the upper chamber, with each well of the lower chamber dispensed with 500 µL of FBS (10%)-contained RPMI-1640 culture medium; position the plate into an incubator (parameters: 5% CO₂; 37 °C) for 24 hours of incubation; take out the plate, discard the mediums in the upper and lower chambers and use a cotton swab to wipe off the cells in the upper chamber; fix the cells by adding 4% paraformaldehyde (P6148, Sigma) to the cells, staying for 30 min; then stain the cells with 0.1% dose crystal violet (C0121, Beyotime Biotechnology) for 30 min; use a PBS solution to rinse the cells for two repetitions and dry up the cells. Finally, a high-power microscope was applied to take pictures in six random fields of each chamber and count and average the number of cells.

Assay for testing the cells' ability to invade: 50 µL of Matrigel matrix gel (354234, Corning Inc., Corning, NY, USA) was placed in the upper chambers in the 24 wells of the plate and maintained in an unregulated temperature condition for 4h for drying up. The cell suspension and culture medium were injected into the upper/lower chambers separately. The subsequent operation steps followed the steps conducted in the assay for testing the cells' ability to migrate.

2.9 Dual-luciferase Assay

In this assay, the first was to create wild-type (3'UTR-WT) and mutant-type (3'UTR-MUT) dual-luciferase reporter gene vectors for the TTC9 gene. The second was to have miR-128-3p mimics and the 3'UTR-WT or 3'UTR-MUT reporter vectors of the TTC9 gene transfected concurrently into ESCC cells by means of the mentioned Lipofectamine 3000. The mimics-NC group was regarded as the control group. Forty-eight hours post-transfection, the procedures outlined in the user manual for dual-luciferase reporter assay kits (Numbered RG027 and sourced from Beyotime Biotechnology) were implemented to acquire the relative activity data of luciferase in the groups of ESCC cells.

2.10 Analysis Based on Bioinformatic Data

The target gene downstream of the miR-128-3p was speculated based on such databases as miRWalk, miRPathDB, targetScan, and GEPIA, with corresponding websites as shown below:
<http://mirwalk.umm.uni-heidelberg.de/>;
<https://mpd.bioinf.uni-sb.de/>;
https://www.targetscan.org/vert_80/;
<http://gepia.cancer-pku.cn/>.

2.11 Xenograft Tumor Experiment *In Vivo*

For this experiment, twenty four BALB/c nude mice (five weeks old) were purchased from Shanghai Model Organisms Center (Shanghai, China). These mice were maintained under conventional breeding conditions. After 1 week of adaptation, the nude mice were randomly divided into 4 groups according to the random number table method: miR-128-3p agomir NC group, miR-128-3p agomir group, miR-128-3p agomir + OE-NC group, miR-128-3p agomir + OE-TTC9 group, with 6 mice in each group. Nude mice in miR-128-3p agomir NC group and miR-128-3p agomir group were subcutaneously inoculated with 200 μ L KYSE-30 cell suspension (1×10^7 cells/mL) in the axillae, whereas the miR-128-3p agomir + OE-NC and miR-128-3p agomir + OE-TTC9 groups received OE-NC- or OE-TTC9-transfected KYSE-30 cells, respectively. 24 h later, mice in the miR-128-3p agomir NC group were intravenously inoculated with miR-128-3p agomir NC and the other three groups were intravenously treated with miR-128-3p agomir once a day. The injection dose was 1.45 mg/kg, and treatment continued until day 20. Both miR-128-3p agomir NC and miR-128-3p agomir were obtained from Ribobio (Guangzhou, China). Tumor volume was measured every 5 days from the first day of agomir administration (day 0) until day 20. On day 20, mice were euthanized by cervical dislocation, and tumors were excised and photographed. Tumor volume (V) and weight were then measured, and V was calculated as: $V = 1/2 \times L \times W^2$ (L: length; W: width). Tumor volume measurement, weighing, and data analysis were performed by investigators blinded to group allocation. All animal experimental procedures were performed following the ARRIVE guidelines and approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University (Approval No. 2025-KY-0540).

2.12 Immunohistochemistry

Tumor tissues were fixed in formalin, embedded in paraffin, and sectioned at a thickness of 5 μ m. The sections were dewaxed, rehydrated, and subjected to antigen retrieval using citrate antigen retrieval solution (C1032, Solarbio, Beijing, China). After antigen retrieval, the sections were blocked with avidin/biotin blocking buffer (C-0005, HaoRan Biotech, Shanghai, China) at

room temperature and then further blocked with 3% BSA for 30 min. The sections were incubated overnight at 4°C with rabbit anti-TTC9 primary antibody (ab235537, 1:200, Abcam), followed by incubation at 37°C for 1 h with goat anti-rabbit IgG secondary antibody (ab150077, 1:500, Abcam) diluted. Subsequently, the sections were treated with horseradish peroxidase-conjugated streptavidin (SE068, Solarbio) at 37°C for 20 min. Color development was performed using a Pierce DAB Substrate Kit (24002, Thermo Fisher Scientific). The sections were then counterstained with hematoxylin (C0107, Beyotime Biotechnology) for 5 min, dehydrated with graded ethanol, cleared in xylene, and mounted with neutral resin (G8590, Solarbio). Finally, the sections were observed and imaged under a microscope.

2.13 Statistical Analysis

Statistical analyses were performed using SPSS 26.0 (SPSS Inc., Chicago, IL, USA), and graphs were generated using GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA). Sample sizes were determined based on previous studies and our preliminary experiments. For cell experiments, three biologically independent replicates were performed. For *in vivo* experiments, six mice were used per group. Data are presented as the mean $\pm$ standard deviation (SD). Normality and variance homogeneity were assessed using the Shapiro-Wilk and Levene's tests, respectively. When these assumptions were met, unpaired Student's t-test was used for two-group comparisons, and one-way ANOVA with Tukey's post hoc test were used for multiple-group comparisons. When normality or variance homogeneity assumptions were violated, non-parametric tests or Welch's correction were applied. Paired t-tests were used to compare adjacent normal tissues with tumor tissues from the same patients. Tumor volume was measured repeatedly over time and analyzed using repeated-measures ANOVA. Effect sizes (Cohen's d for t-tests and partial η^2 for ANOVA) and 95% confidence intervals were calculated where applicable. A P value < 0.05 was considered statistically significant.

3. Results

3.1 The Expression of MiR-128-3p in ESCC

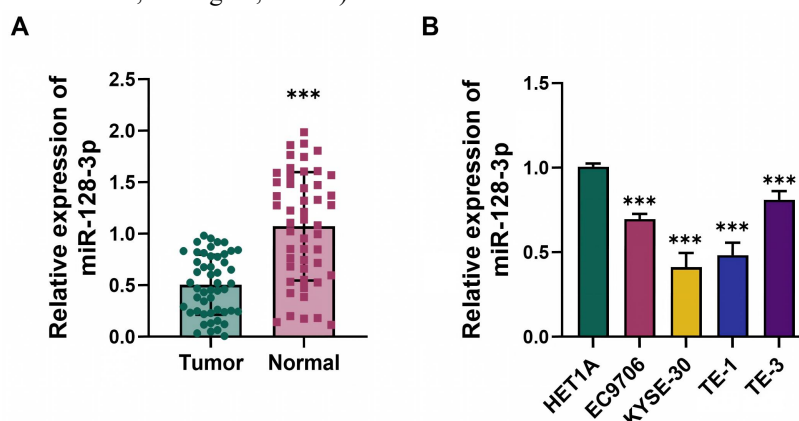


Figure 1. MiR-128-3p expression in ESCC. A: Relative expressions of miR-128-3p in cancer tissue and its adjacent tissues (n=50). B: Relative expressions of miR-128-3p in HET1A and ESCC cell lines (EC9706, KYSE-30, TE-1, and TE-3). n=3. ***P<0.001.

As found in the RT-qPCR assays on 50 specimens of ESCC tissues and adjacent tissues, miR-128-3p was dampened in the cancer tissues (Figure 1A). The subsequent assays on the ESCC cells exhibited similar trend: namely, compared with HET1A, miR-128-3p levels were seen sharply dropped in such ESCC cell lines as KYSE-30, EC9706, TE-3, and TE-1 (Figure 1B), particularly in such cells as TE-1 and KYSE-30. Hence, TE-1 and KYSE-30 were selected to do the subsequent cellular experiments *in vitro*.

3.2 Overexpression of miR-128-3p Suppresses the Biological Functions of ESCC Cells

KYSE-30 and TE-1 cells were transfected to establish ESCC cell lines stably overexpressing miR-128-3p, and transfection efficiency was verified by RT-qPCR. miR-128-3p expression was markedly elevated in transfected KYSE-30 and TE-1 cells compared with control cells (Figure 2A), confirming successful

establishment of miR-128-3p-overexpressing ESCC cell lines. MTT assay showed that miR-128-3p overexpression reduced the metabolic activity of TE-1 and KYSE-30 cells (Figure 2B). To analyze the involvement of raised miR-128-3p levels in the migration and invasion of ESCC cells, wound healing and Transwell assays were performed. Compared with the control group, miR-128-3p-overexpressing cells exhibited a decreased wound healing rate and fewer migrated and invaded cells (Figures 2C-2H), indicating that miR-128-3p overexpression attenuated the migratory and invasive capacities of ESCC cells. Furthermore, Western blot analysis revealed that miR-128-3p overexpression upregulated E-cadherin and downregulated N-cadherin, Vimentin, Snail, Slug, and ZEB1 (Figures 2I-2M), suggesting that miR-128-3p inhibited EMT. Collectively, these findings indicate that miR-128-3p overexpression reduces cell viability, and suppresses the migration, invasion, and EMT of ESCC cells.

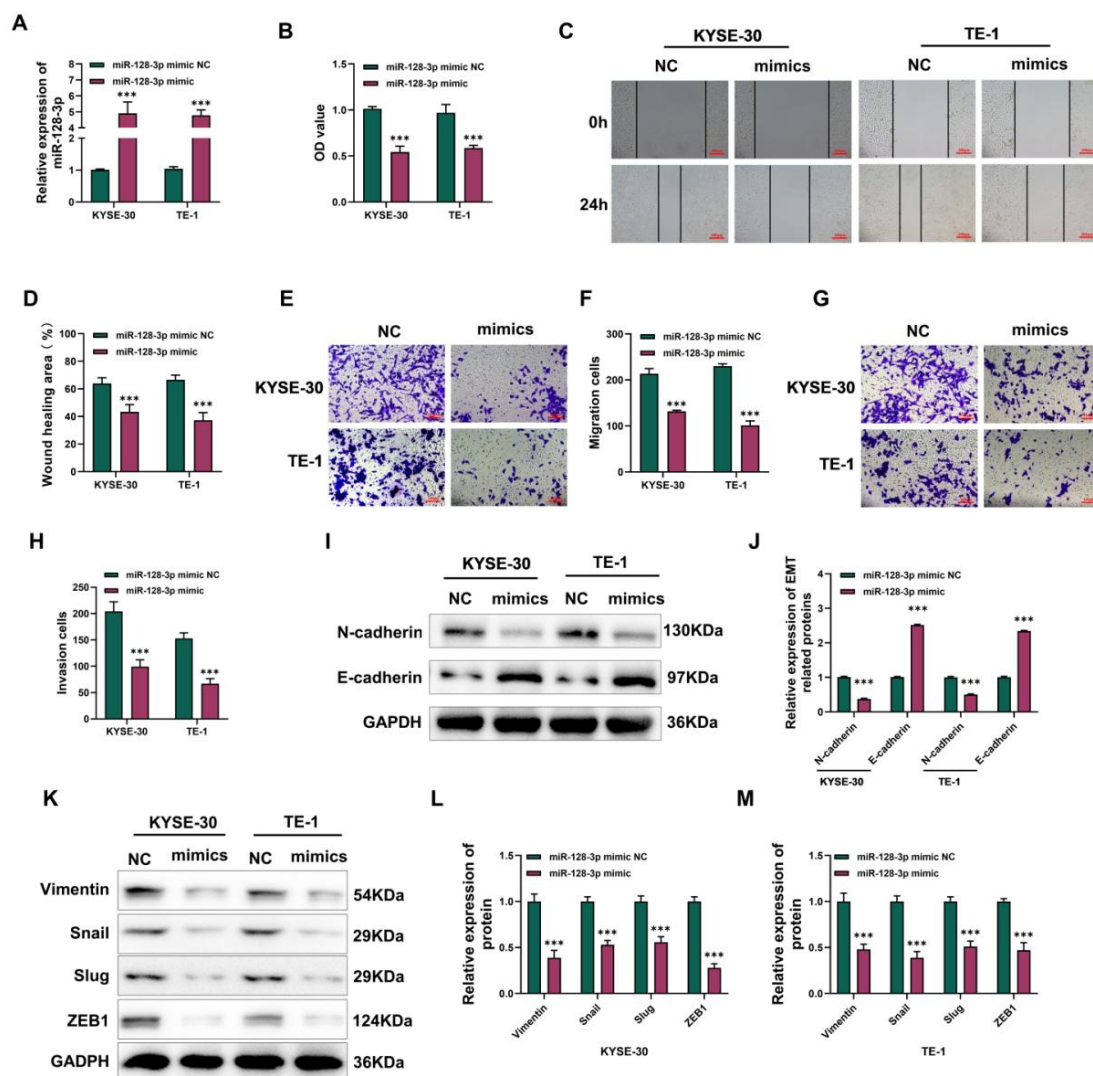


Figure 2. miR-128-3p overexpression inhibits the malignant biological behaviors of ESCC cells. A: MiR-128-3p expression in TE-1 and KYSE-30 cells after transfection with miR-128-3p mimic NC or miR-128-3p mimic. B: OD values examined through MTT assays. C-D: Wound healing assay was used to evaluate cell migration at 0 and 24 h after scratch creation (10 $\times$, 200 μ m). E-F: Transwell assays were used to determine the number of migrated ESCC cells following miR-128-3p overexpression (20 $\times$, 100 μ m). G-H: Transwell assays were used to determine the number of invaded ESCC cells following miR-128-3p overexpression (20 $\times$, 100 μ m). I-J: Western blot analysis of E-cadherin and N-cadherin protein expression levels. K-M: Western blot analysis of Vimentin, Snail, Slug, and ZEB1 protein expression levels. n=3. ***P<0.001.

3.3 Silencing of miR-128-3p Promotes the Biological Functions of ESCC Cells

Similarly, ESCC cell lines with miR-128-3p knockdown were successfully established (Figure 3A) to evaluate the effects of miR-128-3p silencing on the biological functions of ESCC cells. MTT, wound healing, Transwell, and Western blot assays were performed to assess metabolic activity, migratory and invasive

capacities, and EMT status in TE-1 and KYSE-30 cells. Knockdown of miR-128-3p markedly enhanced metabolic activity and promoted the migration and invasion of ESCC cells, and also induced EMT (Figures 3B-3M). These findings indicate that the biological functions of ESCC cells are regulated by miR-128-3p expression levels, suggesting that miR-128-3p may function as a tumor suppressor in ESCC.

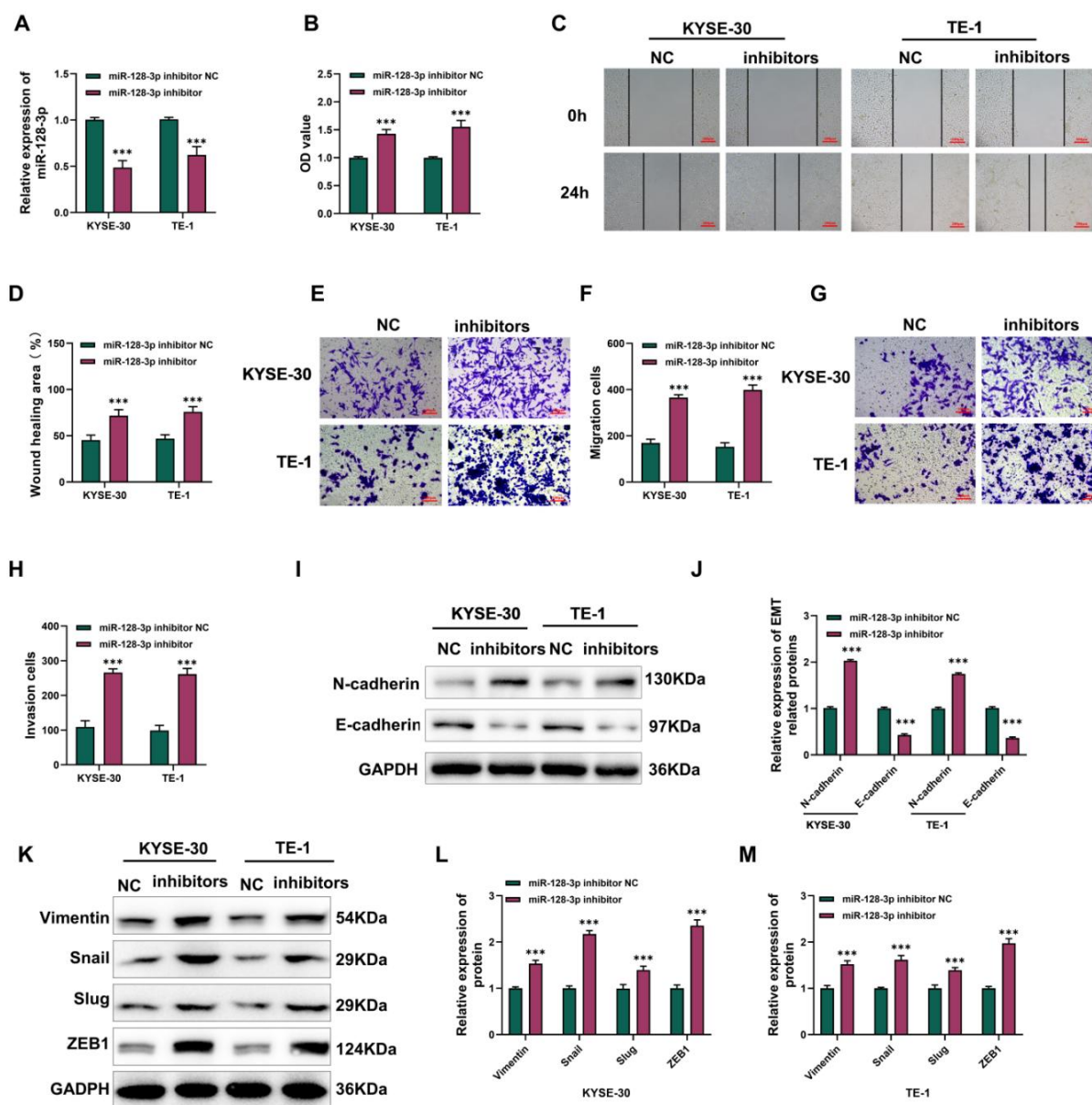


Figure 3. miR-128-3p knockdown enhances the malignant biological behaviors of ESCC cells. A: RT-qPCR analysis of miR-128-3p expression in TE-1 and KYSE-30 cells after transfection with miR-128-3p inhibitor NC or miR-128-3p inhibitor. B: OD values examined through MTT assays. C-D: Wound healing assay was used to evaluate cell migration at 0 and 24 h after scratch creation (10×, 200 μm). E-F: Transwell assays were used to determine the number of migrated ESCC cells following miR-128-3p knockdown (20×, 100 μm). G-H: Transwell assays were used to determine the number of invaded ESCC cells following miR-128-3p knockdown (20×, 100 μm). I-J: The effect of miR-128-3p knockdown on EMT in ESCC cells was assessed by measuring E-cadherin and N-cadherin protein expression via Western blot. K-M: Western blot detected Vimentin, Snail, Slug, and ZEB1 protein levels in TE-1 and KYSE-30 cells. n=3. ****P*<0.001.

3.4 Targeted Regulation of TTC9 by miR-128-3p

To elucidate the specific regulatory role of miR-128-3p in ESCC cells, its downstream target genes, LYPD3 and

TTC9, were first identified from four databases by Venn diagram analysis (Figure 4A). By comparing the mRNA expression in the two types of genes, the TTC9 gene showed a more significant difference in its expression

between the miR-128-3p mimics group and the control group (Figure 4B). Hence, TTC9 was chosen as miR-128-3p's downstream target gene; the binding site between TTC9 and miR-128-3p is illustrated in Figure 4C. As found from the luciferase assay results, ESCC cells concurrently-transfected with miR-128-3p mimics and TTC9 gene 3'UTR-WT showed a sharp decline in the luciferase activity, compared to cells in the control group; however, the ESCC cells concurrently transfected by miR-128-3p mimics and TTC9 gene 3'UTR-MUT displayed no large difference from the control group regarding this activity (Figures 4D-4E). This phenomenon proves the targeting regulatory relationship

between miR-128-3p and the TTC9 gene. As discovered from the TCGA database, TTC9 is intensified in esophageal cancer tissues (Figure 4F). The protein and mRNA levels of TTC9 in ESCC tissue specimens were elevated highly than those levels in normal tissue specimens (Figures 4G-4H); the expressed levels of TTC9 in ESCC cell lines (EC9706, KYSE-30, TE-1, and TE-3) were markedly elevated compared to those in HET1A cells (Figure 4I). Collectively, these findings indicate that TTC9 is upregulated in ESCC and is directly targeted by miR-128-3p, suggesting that TTC9 may serve as a downstream mediator of miR-128-3p in ESCC.

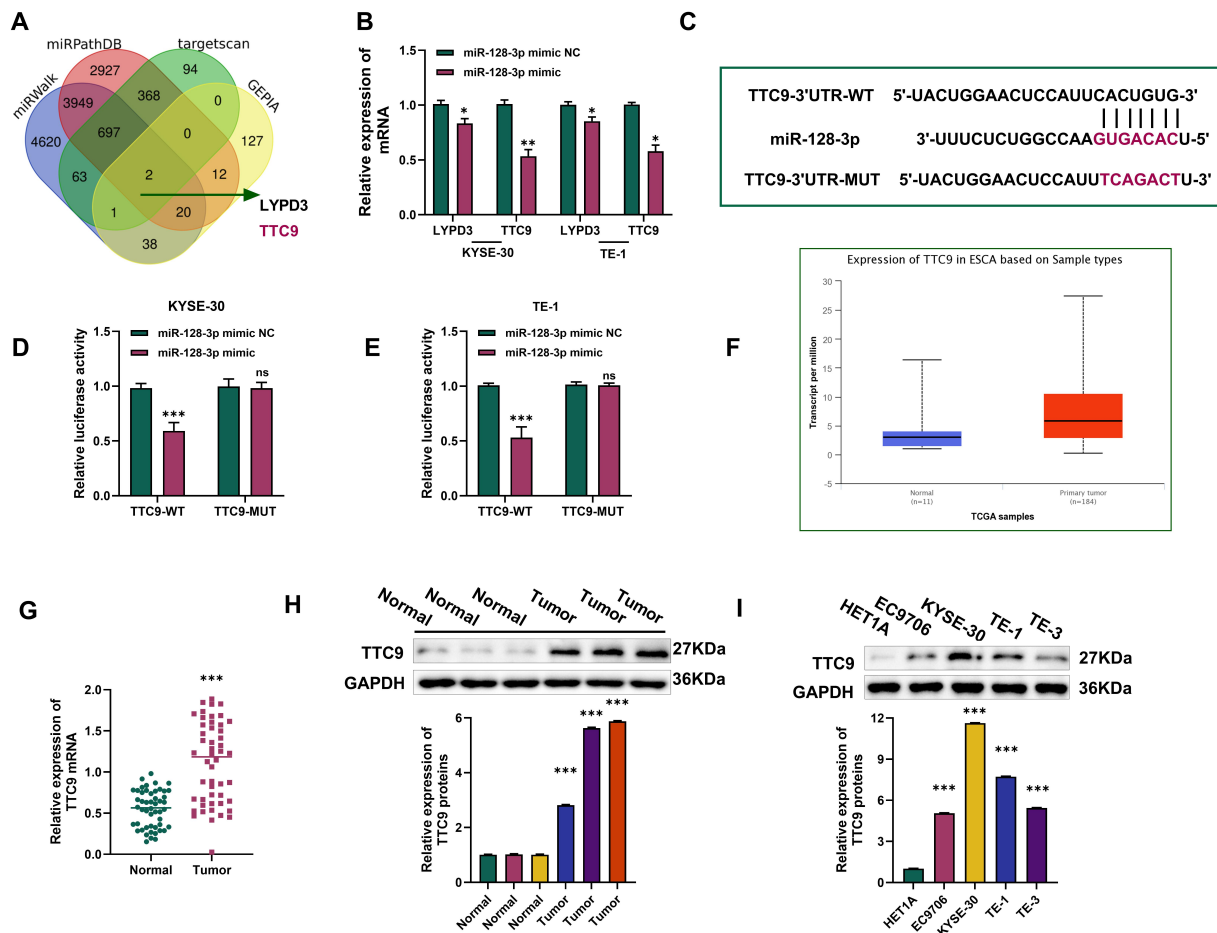


Figure 4. Screening and testing of the target genes. A: The target genes (LYPD3 and TTC9) screened based on Venn diagram and four databases. B: LYPD3 and TTC9 mRNA expression levels in TE-1 and KYSE-30 cells after transfection with miR-128-3p mimic NC or miR-128-3p mimic. C: Binding site between TTC9 and miR-128-3p. D-E: Dual-luciferase reporter assays in TE-1 and KYSE-30 cells. F: TTC9 expression in normal tissues and esophagus cancer tissue as recorded in the TCGA database. G: Relative mRNA expression of TTC9 in ESCC tissues and normal tissues (n=50). H: Western blot analysis of TTC9 protein expression in ESCC tissues and normal tissues. I: Western blot analysis and quantification of TTC9 protein expression in HET1A cells and ESCC cell lines (EC9706, KYSE-30, TE-1, and TE-3). n=3. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

3.5 TTC9 Overexpression Attenuates miR-128-3p-Mediated Suppression of ESCC Cell Biological Functions

The expressed protein levels of TTC9 in ESCC cells transfected with OE-NC or OE-TTC9 were examined through western blot. The findings demonstrated that the OE-TTC9 markedly increased TTC9 protein levels in KYSE-30 and TE-1 cells (Figure 5A), indicating

successful overexpression. Whether TTC9 overexpression could reverse the inhibitory effects of miR-128-3p mimics on ESCC cells was assessed by co-transfecting KYSE-30 and TE-1 cells with miR-128-3p mimics plus OE-NC or OE-TTC9. Compared with the mimics + OE-NC group, transfection with mimics + OE-TTC9 significantly increased metabolic activity (Figure 5B), wound healing rate (Figure 5C), and the numbers of migrated and invaded

cells (Figures 5D-5E), and enhanced EMT, as shown by the downregulation of E-cadherin and the upregulation of N-cadherin, Vimentin, Snail, Slug, and ZEB1 (Figures 5F-5G). Collectively, these findings indicate that TTC9

overexpression attenuates the suppressive effects of miR-128-3p on the malignant biological behaviors and EMT of ESCC cells.

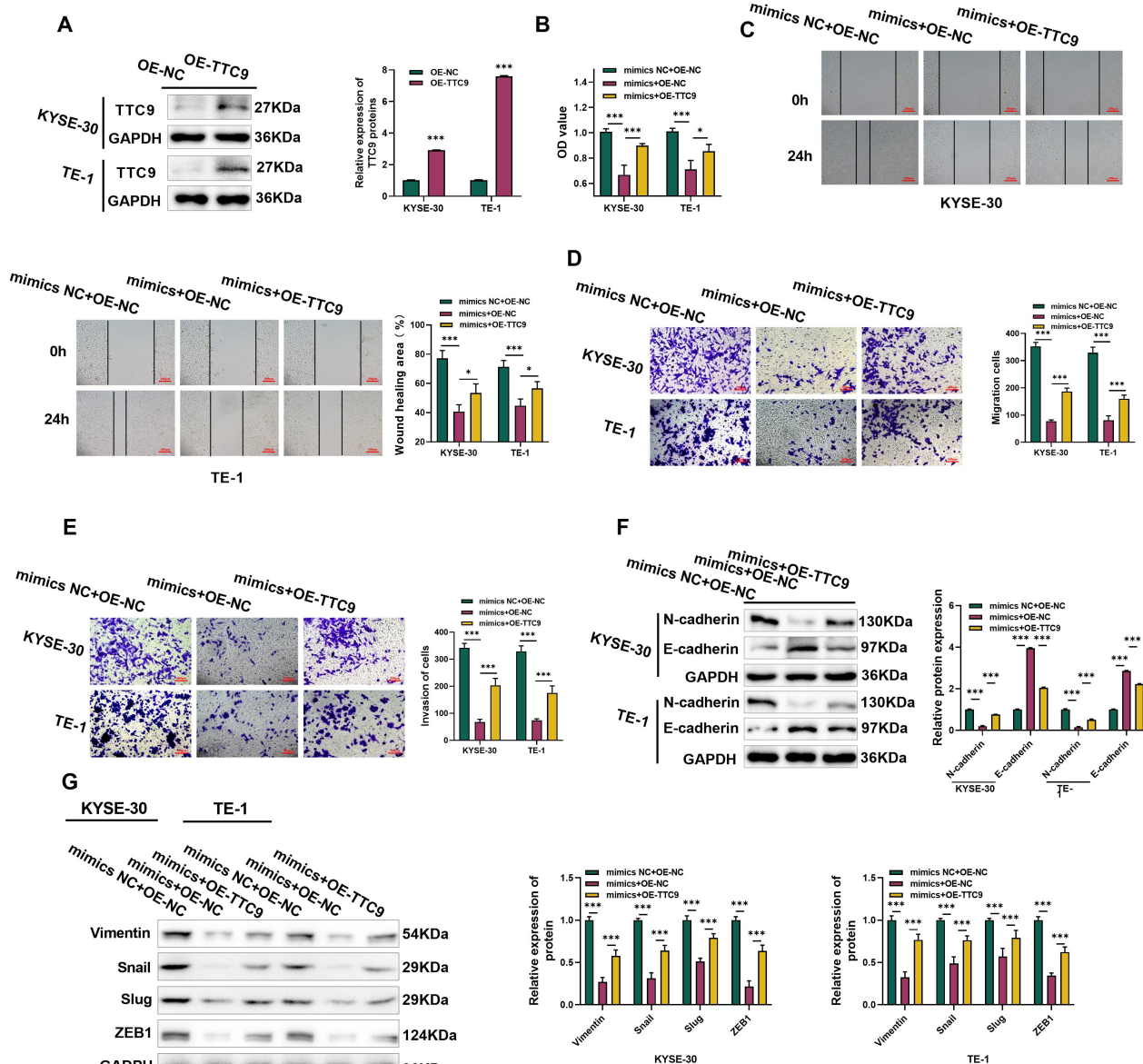


Figure 5. TTC9 overexpression attenuates the inhibitory effect of miR-128-3p on ESCC cell malignancy. A: Western blot analysis of TTC9 protein expression in TE-1 and KYSE-30 cells transfected with OE-NC or OE-TTC9. B: MTT assay was employed to assess the metabolic activity of TE-1 and KYSE-30 cells. C: Wound healing assay and quantitative analysis of TE-1 and KYSE-30 cells at 0 and 24 h after scratch creation (10 $\times$, 200 μ m). D: Transwell migration assay and quantitative analysis of TE-1 and KYSE-30 cells (20 $\times$, 100 μ m). E: Transwell invasion assay and quantitative analysis of TE-1 and KYSE-30 cells (20 $\times$, 100 μ m). F: Western blot analysis of E-cadherin and N-cadherin expression in TE-1 and KYSE-30 cells. G: Western blot detected Vimentin, Snail, Slug, and ZEB1 protein levels in TE-1 and KYSE-30 cells. n=3. * P <0.05, *** P <0.001.

3.6 TTC9 Knockdown Inhibited the Malignant Biological Behaviors of ESCC Cells

KYSE-30 and TE-1 cells were transfected with sh-NC or sh-TTC9. Western blot analysis showed that sh-TTC9 markedly decreased TTC9 protein expression compared with sh-NC, confirming successful knockdown (Figures 6A-6B). MTT assay revealed that TTC9 knockdown reduced metabolic activity, as indicated by decreased OD values (Figure 6C). The scratch healing rate of cells in

the sh-TTC9 group was decreased (Figures 6D-6E), and the number of migrating and invading cells was decreased (Figures 6F-6I), indicating that knockdown of TTC9 resulted in reduced cell migration and invasion ability. Furthermore, TTC9 knockdown suppressed EMT, as evidenced by the up-regulation of E-cadherin, and the downregulation of N-cadherin, Vimentin, Snail, Slug, and ZEB1 (Figures 6J-6N). Collectively, these results indicate that TTC9 knockdown inhibits malignant biological behaviors of ESCC cells.

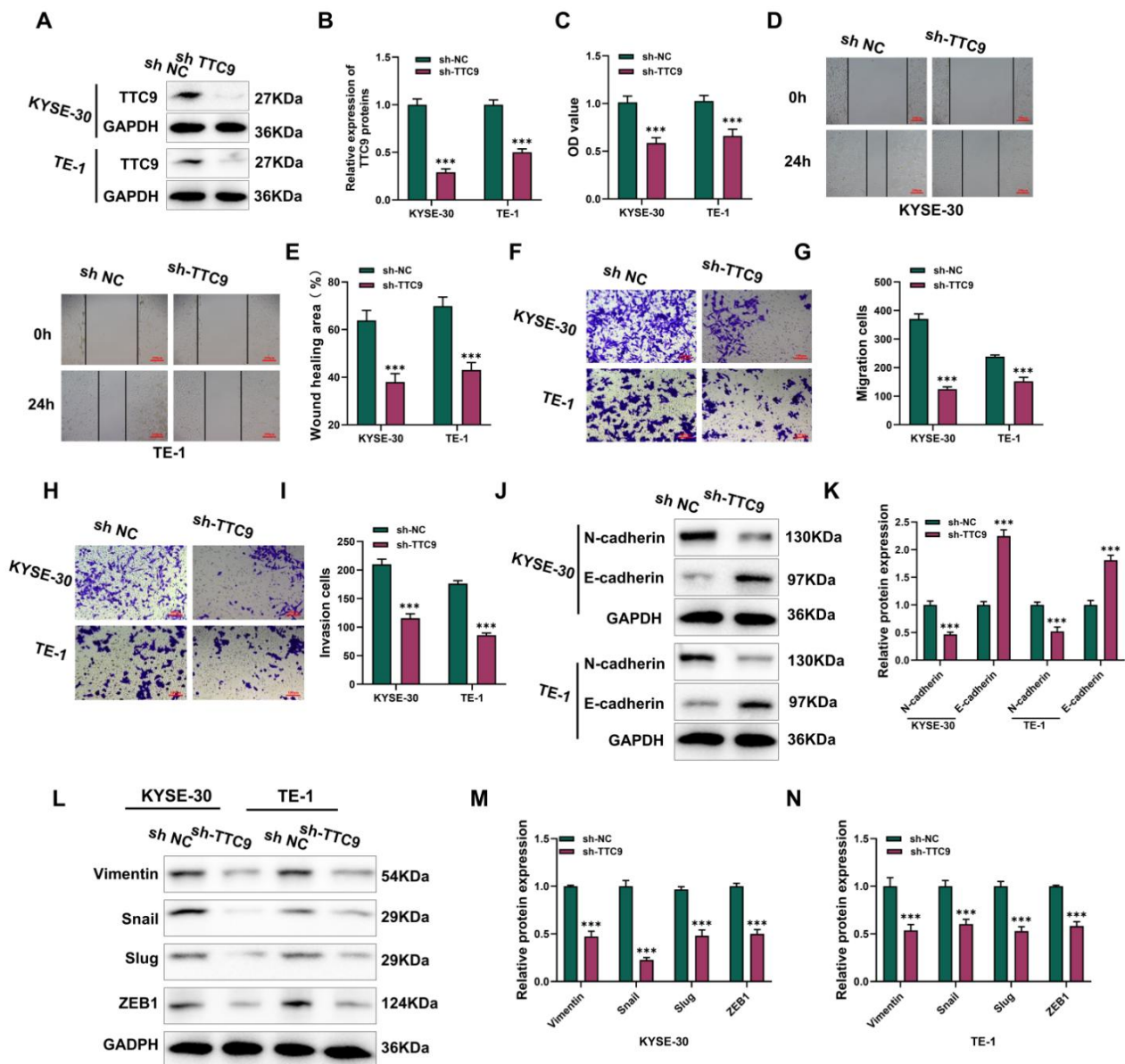


Figure 6. TTC9 knockdown inhibits the malignant biological behaviors of ESCC cells. A-B: Western blot analysis of TTC9 protein expression in TE-1 and KYSE-30 cells after transfection with sh-NC or sh-TTC9. C: OD values of KYSE-30 and TE-1 cells detected by MTT assay. D-E: Wound healing assay and quantitative analysis of KYSE-30 and TE-1 cells (10 $\times$, 200 μ m). F-G: Transwell migration assay and quantitative analysis of KYSE-30 and TE-1 cells (20 $\times$, 100 μ m). H-I: Transwell invasion assay and quantitative analysis of KYSE-30 and TE-1 cells (20 $\times$, 100 μ m). J-K: Western blot analysis of E-cadherin and N-cadherin protein levels in KYSE-30 and TE-1 cells. L-N: Western blot analysis of Vimentin, Snail, Slug, and ZEB1 protein levels in KYSE-30 and TE-1 cells. n=3. ***P<0.001.

3.7 MiR-128-3p Overexpression Inhibits ESCC Tumor Growth *In Vivo*

Figures 7A-7C show representative images, volumes, and weights of xenograft tumors in nude mice within 20 days after implantation of KYSE-30 cells. Compared with the miR-128-3p agomir NC group, the miR-128-3p agomir group exhibited slower tumor growth and significantly reduced tumor volume and weight. Moreover, compared with the miR-128-3p agomir + OE-NC group, the miR-128-3p agomir + OE-TTC9 group showed accelerated tumor growth and increased tumor volume and weight, indicating that TTC9

overexpression attenuated the tumor-suppressive effect of miR-128-3p overexpression. Immunohistochemical analysis after 20 days of breeding revealed that the proportion of TTC9-positive cells was markedly lower in the miR-128-3p agomir group than in the miR-128-3p agomir NC group (Figures 7D-7E), confirming that miR-128-3p negatively regulates TTC9 expression. In addition, the number of TTC9 positive cells in the miR-128-3p agomir + OE-TTC9 group was significantly increased. Collectively, these results demonstrate that miR-128-3p overexpression suppresses ESCC growth, whereas TTC9 overexpression partially reduced this suppressive effect.

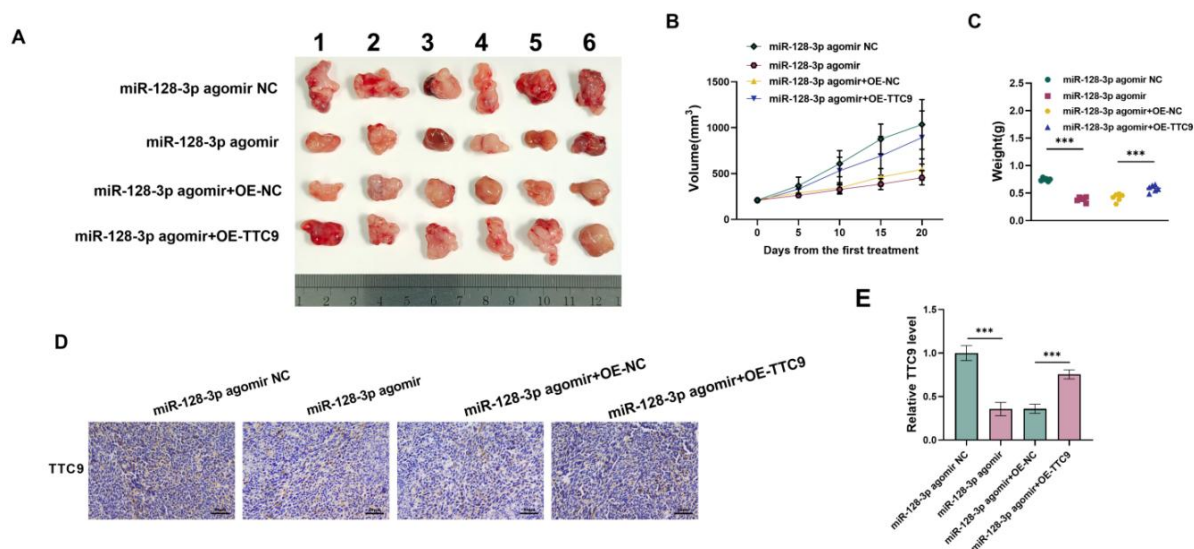


Figure 7. Subcutaneous tumor-bearing experiment in mice. A: Representative images of xenograft tumors excised on day 20. B: Tumor volume measured on days 0, 5, 10, 15, and 20. C: On day 20 of treatment, the weights of tumors were measured. D-E: TTC9 expression in tumor tissues was assessed by immunohistochemistry, and TTC9-positive levels were quantified (40 $\times$, 50 μ m). n=6. *** P <0.001.

4. Discussion

Due to the high proliferative and invasive capacity of ESCC, most patients present with tumor metastasis or advanced-stage disease at initial diagnosis, leading to a poor prognosis [23]. To improve the clinical prognosis of such patients, identifying novel biomarkers for early prognostic prediction in clinical settings remains necessary.

In cells of many cancers, including ESCC cells, miR-128-3p is expressed at a low level [24-26]. However, its precise regulatory role in the growth and metastasis of ESCC remains poorly understood and warrants further investigation. EMT is a process in which epithelial tumor cells lose polarity and intercellular adhesion and acquire a mesenchymal phenotype. This process is associated with stem cell-like properties in various malignant tumors [27] and is accompanied by decreased expression of the epithelial marker E-cadherin and increased expression of the mesenchymal marker N-cadherin [28,29], thereby contributing to cancer cell metastasis. Notably, Vimentin, a canonical EMT marker, was reported to correlate inversely with CD8⁺ T-cell infiltration and to promote nuclear translocation of PD-L1 in ESCC, suggesting that EMT also closely linked to immune evasion in ESCC [30]. As demonstrated in a published article [27], human ESCC cells in a mixed EMT state exhibit enhanced stem cell-like properties and spontaneous metastasis, which provides the condition for the onset and progression of ESCC; the metastasis of ESCC is accompanied by a decline in E-cadherin and a rise in N-cadherin [31,32]. Consistent with these findings, our results showed that miR-128-3p levels in ESCC patient tissues and ESCC cells were lower than those in adjacent non-tumor tissues and HET1A cells. After miR-128-3p overexpression, ESCC cells showed reduced cell viability, migration, and invasion, with increased E-cadherin and decreased

N-cadherin, Vimentin, Snail, Slug, and ZEB1; miR-128-3p downregulation produced the opposite effects. These results suggest that miR-128-3p may function as a tumor suppressor that inhibits ESCC growth and metastasis.

Following database prediction and luciferase reporter assays, TTC9 was identified as a downstream target of miR-128-3p. Previous studies have shown that TTC9 contributes to cancer cell migration and invasion [33], which is consistent with our finding that TTC9 was markedly upregulated in ESCC tissues and cells. TTC9 protein expression was significantly lower in ESCC cells in the miR-128-3p mimic group, and TTC9 overexpression partially reversed the inhibitory effects of miR-128-3p mimics on ESCC cells. These results indicate a targeted negative regulatory relationship between miR-128-3p and TTC9. This interaction may represent one of the mechanisms by which miR-128-3p suppresses ESCC growth and metastasis. In mouse xenograft experiments, miR-128-3p overexpression slowed ESCC progression and reduced TTC9 expression, while TTC9 overexpression weakened the effect of the overexpression of miR-128-3p. Thus, these findings support that miR-128-3p may inhibit ESCC growth and metastasis, at least in part, by targeting and suppressing TTC9.

Recent studies have highlighted the important role of the tumor immune microenvironment in ESCC progression. For instance, NME4 was shown to restrict CD8⁺ T-cell infiltration by suppressing the NF κ B2/CCL5 axis in ESCC [34], while SLC1A5 was associated with CD8⁺ T-cell exclusion in the tumor microenvironment of squamous cell carcinoma, including ESCC [35]. These findings indicate that ESCC progression is shaped not only by intrinsic oncogenic signaling but also by tumor-immune interactions. Therefore, the effect of miR-128-3p on the immune microenvironment of ESCC can be explored in the future.

This study has certain limitations. This study focused only on TTC9 as a downstream target of miR-128-3p. Given that a single miRNA can regulate multiple target genes, TTC9 is likely to be only one of the downstream mediators through which miR-128-3p exerts its biological functions in ESCC. Therefore, the complete molecular network by which miR-128-3p regulates ESCC progression remains to be fully elucidated. Future studies are warranted to systematically identify and validate additional downstream targets of miR-128-3p in ESCC. In addition, although functional experiments at cell and animal levels suggested that miR-128-3p targeted TTC9 to regulate ESCC malignant phenotypes, the endogenous inverse correlation between miR-128-3p and TTC9 was not verified in clinical patient samples in this study. Future studies will include larger clinical cohorts to validate the endogenous relationship between miR-128-3p and TTC9.

5. Conclusion

To sum up, overexpression of miR-128-3p suppresses the malignant biological behavior of ESCC cells and inhibits the growth of ESCC in nude mice, while overexpression of TTC9 partially reversed the tumor suppressive effect of miR-128-3p. Therefore, the miR-128-3p/TTC9 axis may provide a candidate direction for further investigation, but its clinical or therapeutic potential requires validation in future studies.

Author Contribution

Ruitai Fan: Developed and planned the study, performed experiments, and interpreted results. Edited and refined the manuscript with a focus on critical intellectual contributions.

Yiliang Wang, Xiaoxia Han, Yuhao Ping, Wenfu Tang: Participated in collecting, assessing, and interpreting the data. Made significant contributions to data interpretation and manuscript preparation.

Ruitai Fan, Yiliang Wang, Xiaoxia Han, Yuhao Ping, Wenfu Tang: Provided substantial intellectual input during the drafting and revision of the manuscript.

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Conflicts of Interest

The authors affirm that they do not have any financial conflicts of interest.

Data Availability Statement

The data supporting the findings of this study can be obtained from the corresponding author, Ruitai Fan,

upon request.

Ethical Approval

This research was approved by Ethics Committee of The First Affiliated Hospital of Zhengzhou University (Approval No. 2025-KY-0540)

Consent to Participate

We secured a signed informed consent form from every participant.

AI Statement

During the preparation of this manuscript, only Grammarly was used for English language polishing. All scientific content, data analysis and figure preparation were completed by the authors.

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