MicroRNA-134 prevents the progression of esophageal squamous cell carcinoma via the PLXNA1-mediated MAPK signalling pathway

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A B S T R A C T

Background: MicroRNAs (miRNAs) are involved in oncogenesis of esophageal squamous cell carcinoma (ESCC). miR-134 is reported to have a tumour-suppressive role but its role in ESCC is not known. The present study was designed to examine whether miR-134 inhibits ESCC development and further explored relevant underlying mechanisms.

Methods: Differentially expressed genes related to ESCC were identified from microarray gene expression profiles. Immunohistochemical staining and RT-qPCR assays identified elevated PLXNA1 expression levels and low miR-134. The relationship between miR-134 and PLXNA1 was predicted and further verified by a dual-luciferase reporter assay. The expression levels of miR-134 and PLXNA1 in ESCC cells were modified by miR-134 mimic/inhibitor and siRNA against PLXNA1, respectively. Thereafter, the expression of MAPK signalling pathway-related proteins, as well as the viability, migration, invasion, cell cycle and cell apoptosis of ESCC cells was investigated.

Findings: The results showed that miR-134 could block the MAPK signalling pathway by downregulating PLXNA1. When miR-134 was overexpressed or PLXNA1 was silenced, cell apoptosis was enhanced, the cell cycle was retarded, and the cell proliferation, migration and invasion were suppressed. In vivo experiments confirmed that miR-134 overexpression or PLXNA1 silencing restrained tumour growth and lymph node metastasis.

Interpretation: These findings demonstrate that cancer cell proliferation, migration, invasion, and tumour metastasis of ESCC can be suppressed by overexpression of miR-134 through downregulating PLXNA1, which subsequently blocks the MAPK signalling pathway. These results provide new potential targets and strategies for the treatment of ESCC.

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1. Introduction

Esophageal squamous cell carcinoma (ESCC) is a highly prevalent cancer globally, with a poor prognosis, and is frequently reported in the Chinese population. The factors associated with the progression of ESCC include cigarette smoking, alcohol consumption, and low intake of fruits and vegetables [1]. Typically, ESCC is already at an advanced stage at initial diagnosis as its carcinogenesis is asymptomatic [2]. Consequently, the survival rate in ESCC has not remarkably improved despite the advent of multimodal treatments including surgery, radiation and chemotherapy [3]. Furthermore, lymph node metastasis (LNM) is of great prognostic significance in ESCC [4]. There exists a need for molecular investigations that can direct the development of biomarkers and therapeutic targets.

MicroRNAs (miRs) participate in a series of cellular processes and regulate gene expression via targeting multiple molecules [5]. Currently, accumulating evidence suggests that some miRs exert regulatory effects on cell growth, invasion, and LNM in ESCC [6]. For instance, miR-518b has been observed to function as an anti-oncogene in ESCC suggesting...
Research in context

Evidence before this study

Esophageal squamous cell carcinoma (ESCC) is one of the highly prevalent cancers around the world with a poor prognosis, and frequently occurs in Chinese population, and the factors leading to the progression of ESCC include cigarette smoking, alcohol consumption, and low intake of fruits and vegetables. Currently, there are accumulating evidences suggesting that some of miRs exert regulatory effects on cell growth, invasion and LNM in ESCC. For instance, miR-518b has been observed to function as an anti-oncogene in ESCC and therefore has important the clinical and prognostic values. Although the tumour suppressive role of miR-134 has been demonstrated in other cancers, such as breast cancer and hepatocellular carcinoma, the function of miR-134 in ESCC remains elusive. Besides, according to a biological prediction website MicroRNA.org, plexin A1 (PLXNA1) is identified as a target gene of miR-134 in our study. As a member of Plexin A family, PLXNA1 has been reported to be regulated in malignant cells and nerves of cancerous specimens. In addition, the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signalling pathway is implicated in the modulation of human cancers by miR-134. MAPKs, serine-threonine kinases, can regulate intracellular signalling involving in a diversity of cellular activities such as cell differentiation, proliferation and death, which have been found to affect the development of ESCC. Therefore, in the current study, we hypothesized miR-134 may be involved in the development of ESCC through its regulation on MAPK signalling pathway by regulating PLXNA1.

Added value of this study

In our study, the inhibitory effect of miR-134 was identified in the development and progression of ESCC. We found that miR-134 could restrain the cancer cell proliferation, migration, invasion, and tumour metastasis of ESCC through its regulation on PLXNA1-mediated MAPK signalling pathway. Specifically, miR-134 was downregulated in ESCC and PLXNA1 was upregulated, which was proved to be negatively regulated by miR-134. After establishment of an in vitro cell model of ESCC, miR-134 overexpression treatment or PLXNA1 silencing treatment was observed to inhibit ESCC cell proliferation, migration and invasion but promote apoptosis. Moreover, as further confirmed in vivo, the inhibitory effects on tumour growth and lymph node metastasis were observed in the nude mice xenografted with ESCC cells with an upregulated miR-134 or a downregulated PLXNA1 content. Additionally, further investigation based on MAPK inhibitor treatment suggested that miR-134 downregulated the expression of PLXNA1 to inactivate the MAPK signalling pathway.

Implications of all the available evidence

Taken together, the available data indicate that overexpressed miR-134 targeting PLXNA1 may inhibit the development of ESCC through the down-regulation of MAPK signalling pathway, highlighting a new target and strategy for the treatment of ESCC.

its clinical and prognostic value [7]. Although a tumour suppressive role of miR-134 has been demonstrated in other cancers, such as breast cancer [8] and hepatocellular carcinoma [9], knowledge of the function of miR-134 in ESCC remains elusive. miR targets include Plexins, which are semaphorin receptors, and have important functions in the development of nervous system and vasculature [10]. According to a biological prediction website MicroRNA.org, plexin A1 (PLXNA1) is identified as a target gene of miR-134 in our study. PLXNA1 is a member of the Plexin A family, which is implicated in regulation of malignant cells and neural tissue in cancerous specimens [11]. In particular, PLXNA1 has been demonstrated to accelerate the progression of lung cancer [12].

miR-134 is reported to regulate the mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) signalling pathway which has been implicated in the modulation of human cancers [13]. MAPKs, serine-threonine kinases, can regulate intracellular signalling, involving in a diversity of cellular activities such as cell differentiation, proliferation and death [14]. For example, the disruption of MAPK signalling pathway can result in an induction of epithelial mesenchymal transition (EMT), thus affecting the development of ESCC [15]. In the current study, it was hypothesized miR-134 affected the proliferation, apoptosis, and LNM of ESCC through the MAPK signalling pathway by regulating PLXNA1. Therefore, the role of miR-134 in ESCC and its relevant mechanisms involving PLXNA1 and the MAPK signalling pathway were investigated both in vitro and in vivo.

2. Materials and methods

2.1. Ethical statement

All patients had signed written informed consents. The study was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University and carried out in accordance with the declaration of Helsinki. The animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

2.2. Bioinformatics prediction

Microarrays datasets related to gene expression in ESCC were downloaded from the Gene Expression Omnibus (GEO) database of National Centre for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/). Five datasets; GSE17351, GSE20347, GSE29001, GSE45168 and GSE45670, were obtained and differentially expressed genes (DEGs) in ESCC were determined. Detailed information regarding these microarray datasets is depicted in Table 1. Considering a p Value <.05 and Log Fold Change >2 as the thresholds, the “limma” package in the R software environment was applied to screen DEGs. A heat map of the obtained DEGs was plotted using the “pheatmap” package. Overlapping DEGs retrieved from the five microarrays were depicted using Venn diagrams plotted using the Venn online analysis tool (http://bioinformatics.psb.ugent.be/webtools/Venn/), and the potential key genes of ESCC were identified. The putative miRNAs targeting these DEGs were predicted using TargetScan (http://www.targetscan.org/vert_71/), miRDB (http://www.mirdb.org/), miRWalk (http://mirwalk.umm.uni-heidelberg.de/) and microRNA (http://34.236.212.39/microrna/getGeneForm.do).

Table 1

The gene expression dataset of ESCC.

| Accession | Platform | Organism  | Sample |
|-----------|----------|-----------|--------|
| GSE17351  | GPL570   | Homo sapiens | 5 primary ESCC tumour tissues and 5 adjacent normal esophageal mucosa tissues |
| GSE20347  | GPL571   | Homo sapiens | 17 micro-dissected ESCC tumour and matched normal tissue pairs |
| GSE29001  | GPL571   | Homo sapiens | 21 ESCC tumour and 24 normal esophageal epitheliums |
| GSE45168  | GPL13497 | Homo sapiens | 5 pairs of cancerous and normal tissues from ESCC patients |
| GSE45670  | GPL570   | Homo sapiens | pretreatment cancer biopsies from 28 ESCCs and 10 normal esophageal epithelia |

Note: ESCC, esophageal squamous cell carcinoma.
60.81 ± 7.87 years) pathologically diagnosed as having ESCC (including 57 males and 11 females) were enrolled in the study. ESCC was the primary tumour in all the 68 patients. All patients had not received any radiotherapy or chemotherapy before the operation and did not suffer from other malignant tumours. The patients underwent surgical resection at the First Affiliated Hospital of Zhengzhou University from June 2014 to December 2015. Among these patients, 10 patients were diagnosed with poorly differentiated ESCC and 58 patients were diagnosed with moderately/highly differentiated ESCC; 56 patients had no LNM and 12 patients had regional LNM. ESCC tissues and adjacent normal tissues (>5 cm away from the tumour) were collected in duplicate from each patient. One part was quickly stored in liquid nitrogen and the other part was soaked in 10% neutral formalin and embedded with paraffin. All patients were followed up for a detailed understanding of the patients’ post-treatment clinical outcomes and the improvement in clinical parameters. The follow-up period started after surgery up to December 2018 and ranged from 3 to 36 months.

### 2.3. Study subjects

A total of 68 patients aged between 50 and 75 years (median age: 60.81 ± 7.87 years) pathologically diagnosed as having ESCC (including

| Gene   | Sequence (5′–3′)          |
|--------|---------------------------|
| miR-134| Forward: TGTTTCCTCATGACTGCCCC | Reverse: CGATGCCGGTTGCCTGTTTA |
| PLXNA1 | Forward: ACCACACTGTGTCATGACA | Reverse: CGGTTAGCGGCATAGTCCA |
| ERK    | Forward: CGTACACCGAGTTGACATAC | Reverse: CATCCCAGCCTCCGTTATCC |
| JNK    | Forward: ACAGCTACGTCATCAGCTCC | Reverse: ACAGGTCAGTCTCCATCAGGTCC |
| p38    | Forward: CAATGATGTGTATCTGGTGAC | Reverse: CGGTTAGCGGCATAGTCCA |
| Bcl-2  | Forward: GAACTGAGGGAGGATTGTGG | Reverse: CGGTTAGCGGCATAGTCCA |
| BAX    | Forward: CCCAGAGCCTGTTTCTC | Reverse: CGGTTAGCGGCATAGTCCA |
| U6     | Forward: GCTGCAGGACCATACTAATAAT | Reverse: CGGTTAGCGGCATAGTCCA |
| GAPDH  | Forward: GTTACAAGGGAGAAGTTCACCAT | Reverse: CGGTTAGCGGCATAGTCCA |

Note: miR-134, microRNA-134; PLXNA1, plexin A1; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal protein kinase; Bcl-2, B-cell lymphoma-2; BAX, Bcl-2 associated X protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

### 2.4. Immunohistochemical staining

The formalin stored specimens were dehydrated, paraffin-embedded, and cut into 4 μm slices. The slices were dewaxed, hydrated, and incubated with citric acid buffer (pH = 6.0). Then the slices were treated with 30 mL/L H2O2 to inactivate endogenous peroxidase and blocked with goat serum. The slices were incubated with rabbit anti-human antibody to p38 (1: 200, ab217634, Abcam Inc., Cambridge, MA, USA) at 4 °C overnight, and then incubated with biotin-labelled

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**Fig. 1.** miR-134 is predicted as a candidate miRNA that affects the progression of ESCC through regulation of PLXNA1. (a) the top 500 DEGs determined from GSE17351, GSE20347, GSE29001, GSE45168 and GSE45670 datasets; (b) and (c) a heat map depicting the top 50 DEGs from GSE45168 and GSE29001 datasets (the x-axis represents the sample number and the y-axis represents the DEGs; the upper right histogram represents the colour gradation; each rectangle in the graph corresponds to one sample’s gene expression; red represents high expression and green represents low expression); (d) the expression of PLXNA1 in ESCC in the GSE45670 dataset; (e) the expression of PLXNA1 in ESCC in the GSE17351 dataset; (f) the expression of PLXNA1 in ESCC in the GSE20347 dataset; (g) the top 200 miRNAs predicted from four web-based resources, TargetScan, miRDB, miRWalk and microRNA. miR-134, microRNA-134; ESCC, esophageal squamous cell carcinoma; PLXNA1, plexin A1; MAPK, mitogen-activated protein kinase.
goat anti-rabbit antibody at 37 °C for 30 min. After that, the slices were treated with horseradish peroxidase-streptomyein, developed using diaminobenzidine (DAB) and counterstained using hematoxylin. Positively stained cells were counted using the image analysis software (Nikon Eclipse 80i, Nikon, Tokyo, Japan) with five visual fields (200×) randomly selected from each slice. The ratio of positively stained cells in the measured area was then calculated.

2.5. Dual-luciferase reporter assay

The relationship between PLXNA1 and miR-134 was predicted using MicroRNA.org, a biological prediction website. The 3' untranslated region (UTR) sequence of PLXNA1 was synthesized and then inserted into pMIR-reporter (Promega, Madison, WI, USA). Mutant (Mut) fragment with mutated binding sites of miR-134 in the PLXNA1 3' UTR was also cloned into pMIR-reporter. The pMIR-Wt-PLXNA1 and pMIR-Mut-PLXNA1 were respectively co-transfected with miR-134 into HEK-293 T cells (Shanghai Beinuo Biotechnology Co., Ltd., Shanghai, China). At the 48th h after transfection, the cells were collected and lysed. Then luciferase activity was assessed using the luciferase activity detection kit (Promega).

2.6. Cell line screening

Human normal esophageal epithelial cells (HEEC) and ESCC cell lines (EC9706, Eca109, NEC, TE-1, and KYSE30) were purchased from BeiNa Culture Collection (Beijing, China) and cultured. The culture medium was renewed every 24 h. The cell line NEC was found to have the highest expression of miR-134 as determined by reverse transcription quantitative polymerase chain reaction (RT-qPCR) and was used in subsequent experiments.

2.7. Cell transfection

siRNA for PLXNA1 (GCTTCCCTCTGTGAAATAA) was designed by Thermo Fisher Scientific’s siRNA design software (Thermo Fisher Scientific, Waltham, MA, USA). The selected human ESCC cell line (NEC) cells were seeded into 6-well plates 24 h before transfection. When the cell density reached 70% - 80%, cells were treated with miR-134 mimic, miR-134 inhibitor, siRNA targeting PLXNA1 (si-PLXNA1) alone, or SB203580 (a p38MAPK inhibitor), LY3214996 (a ERK inhibitor), SP600125 (a JNK inhibitor) in the presence of si-PLXNA1, with dimethylsulfoxide (DMSO) in the presence of si-PLXNA1 as control (the final concentration of inhibitors was 10 nM, respectively) according to the instructions of lipofectamine 2000 (11668–019, Invitrogen, Carlsbad, CA, USA) [16]. The total RNA and protein were extracted after 48 h for subsequent experiments.

2.8. RT-qPCR

Total RNA was extracted from tissues and cells by Trizol reagent (Invitrogen, Carlsbad, MA, USA). miRNA was isolated using the PureLink FFPE Total RNA Isolation Kit (Haoran Biotechnology Co., Ltd., Shanghai, China). RNA was reverse transcribed into cDNA using the ALL-in-One miRNA reverse transcription kit (GeneCopoeia Inc., Rockville, MD, USA). Thereafter, real time quantitative PCR was performed based on the specifications of the SYBR®Premix Ex Taq™ II kit (TaKaRa Biotechnology Co., Dalian, Liaoning, China) using an ABI 7500 PCR instrument (Applied Biosystems, Inc., Foster City, CA, USA). U6 was taken as the internal reference for miR-134 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was taken as the internal reference for PLXNA1, ERK, JNK, p38, Bcl-2, and BAX. All the primer sequences were designed and synthesized by Bio Just Biomart Company (Wuhan, Hubei, China).

Fig. 2. miR-134 has a low expression level in ESCC tissues while PLXNA1 is highly expressed. (a) miR-134 expression and PLXNA1 mRNA expression levels in ESCC tissues and in adjacent normal tissues as determined by RT-qPCR; (b and c) the protein expression of PLXNA1 in ESCC tissues and in adjacent normal tissues as determined by Western blot assay; (d) immunohistochemical staining of PLXNA1 in ESCC tissues and in adjacent normal tissues (200×; scale bar = 50 um); (e) the positive expression rate of PLXNA1 in ESCC tissues and in adjacent normal tissues. *p < .05 vs. the adjacent normal tissues. Measurement data were expressed as mean ± standard deviation and analysed by paired t-test, n = 68. ESCC, esophageal squamous cell carcinoma; RT-qPCR, reverse transcription quantitative polymerase chain reaction; miR-134, microRNA-134; PLXNA1, plexin A1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
miR-134 exhibits an association with degree of differentiation, the presence of LNM and TNM stage in ESCC.

| Clinical characteristics | Case (n) | miR-134 expression | p     |
|--------------------------|---------|--------------------|-------|
| Age (years)              |         |                    | .47   |
| < 60                     | 32      | 0.471 ± 0.117      |       |
| ≥ 60                     | 36      | 0.451 ± 0.110      |       |
| Gender                   |         |                    | .63   |
| Male                     | 57      | 0.458 ± 0.116      |       |
| Female                   | 11      | 0.440 ± 0.095      |       |
| Tumour diameter (cm)     |         |                    | .865  |
| < 4                      | 46      | 0.458 ± 0.114      |       |
| ≥ 4                      | 22      | 0.463 ± 0.112      |       |
| Degree of differentiation|         |                    | <.010 |
| Poor/undifferentiation   | 10      | 0.358 ± 0.064      |       |
| High/moderate undifferen-| 58      | 0.478 ± 0.110      | <.010 |
| Lymph node metastasis    |         |                    | <.050 |
| Yes                      | 12      | 0.367 ± 0.063      |       |
| No                       | 56      | 0.480 ± 0.111      |       |
| TNM stage                |         |                    |       |
| I-II                     | 54      | 0.476 ± 0.119      |       |
| III                      | 14      | 0.399 ± 0.055      |       |

Note: miR-134, microRNA-134; ESCC, esophageal squamous cell carcinoma; TNM, tumour, node and metastasis.

and are listed in Table 2. Relative gene expression levels were calculated using the 2^−ΔΔCt method as described by Zhang et al. [17]. All experiments were conducted independently in triplicate.

2.9. Western blot assay

Western blot assay was performed to determine the protein expression levels of ERK, JNK, p38, BAX and Bcl-2 as previously described [18]. The tissues and cells were lysed with lysis buffer and centrifuged at 12000 r/min for 30 min at 4 °C to collect total protein. The protein (50 μg) was separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride (PVDF) membrane, which was then blocked using 5% skimmed milk for 1 h at room temperature. Afterwards, the membrane was incubated with the following primary antibodies at 4° overnight: ERK (1: 1000, ab196883), JNK (1: 1000, ab4821), p38 (1: 1000, ab170099), BAX (1: 2000, ab32503), Bcl-2 (1: 1000, ab32124), p-ERK (1: 200, ab214362), p-JNK (1: 1000, ab124956) and p-p38 (1: 1000, ab4822). All antibodies were procured from Abcam Inc. (Cambridge, MA, USA). Next, the membrane was incubated with horseradish peroxidase (HRP)-labelled secondary antibody for 1 h. Subsequently, the membrane was developed with enhanced chemiluminescence (ECL) solution (ECL808-25, Biomiga, San Diego, CA, USA) for 1 min at room temperature. Resulting protein bands were observed using an X ray machine (36209E01, Shanghai Qcbio Science & Technologies Co., Ltd., Shanghai, China).

2.10. Cell count kit-8 (CCK-8)

The transfected cells were seeded into 96-well plates at a density of 2 × 10^3 cells/mL 12 h after transfection, and cultured at 37 °C. At the 24th, 48th and 72nd h of culture, each well was added with 10 μl CCK-8 reagent (C0037, Beyotime Biotechnology Co., Ltd., Shanghai, China) for 2 h at 37 °C, and the optical density (OD) value was recorded by a Multiskan FC microplate reader (Thermo Fisher Scientific Inc., Walltham, MA, USA) at a wavelength of 450 nm. Each group was plated in three parallel wells, and the experiment was repeated three times. The cell viability curve was plotted with time point on the x-axis and OD value on the y-axis.

2.11. Flow cytometry

Annexin-V-FITC, propidium iodide (PI) and N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES) were mixed at a ratio of 1: 2: 50 to prepare the Annexin-V-FITC/PI staining solution, according to the instructions of Annexin-V-fluorescein-isothiocyanate (Annexin-V-FITC) cell apoptosis detection kit (C1065, Beyotime Biotechnology Co., Ltd., Shanghai, China). At the 48th h after transfection, 1 × 10^5 cells were resuspended with 100 μl Annexin-V-FITC/PI staining solution. After an incubation at room temperature for 15 min, the cells were washed with 1 ml HEPES buffer. Fluorescence from FITC and PI was measured at 488 nm excitation, 525 nm and 620 nm bandpass filters, using the BD-Aria flow cytometer (FACS Calibur). For each group, three parallel wells were used, and the experiment was repeated three times independently.

2.12. Scratch test

After 24 h of transfection, the cells in each group were inoculated in a 6-well plate at a density of 5 × 10^5 cells each well. When the cell...
Confluence reached about 90%, a sterile pipette was used to gently cross the central axis of the well. Any cells on the surface were removed by washing with PBS and the cells were cultured in serum-free medium for 0.5–1 h for recovery. The cells were photographed at 0 and 24 h after recovery. The cell-migration distance over 24 h was determined using the Image-Pro Plus Analysis software (Media Cybernetics, Bethesda, MD, USA), and an average value was obtained.

### 2.13. Transwell assay

Matrigel (356234, BD Biosciences, San Jose, CA, USA) was dissolved overnight at 4 °C, diluted with serum-free medium at a ratio of 1:3, added to the apical chamber of a Transwell chamber at 50 μL per well, and kept in an incubator for 30 min. The cell suspension (1 × 10⁵ cells/mL) was inoculated in the apical chamber, and the culture medium

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**Fig. 4.** PLXNA1 is a target gene of miR-134. (a) the sequence of miR-134 binding to PLXNA1 predicted using the biological prediction online resource microRNA.org; (b) the luciferase activities of PLXNA1-Wt and PLXNA1-Mut in response to miR-134 upregulation; *p < .05 vs. the NC group (cells transfected with NC plasmid). Measurement data were expressed as mean ± standard error of mean, and analysed by unpaired t-test; the experiment was repeated three times. miR-134, microRNA-134; PLXNA1, plexin A1; NC, negative control; Wt, wild-type; Mut, mutant.

| 3’ ggGGAGACCAGUUGGUUCAGu  | 5’ hsa-miR-134 |
|-----------------------------|----------------|
| 1587:5’ guUUUUAUUAUUAUCAGUCu | PLXNA1         |

**PhastCons score:** 0.5972

**miR-SVR score:** -0.7742

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**Fig. 5.** Overexpressed miR-134 elevates BAX expression while reducing PLXNA1, ERK, JNK, p38 and Bcl-2 expression along with ERK and JNK phosphorylation in ESCC cells. ESCC cells were treated with miR-134 expression and/or si-PLXNA1. (a) the miR-134 expression and mRNA expression of PLXNA1, ERK, JNK, p38, BAX and Bcl-2 relative to GAPDH in ESCC cells as determined by RT-qPCR; (b) the protein bands and the expression of PLXNA1, ERK, JNK, p38, BAX and Bcl-2 relative to GAPDH in ESCC cells as measured by Western blot assay. *p < .05 vs. the blank and NC groups (cells without transfection or transfected with empty plasmid); #p < .05 vs. the miR-134 and si-PLXNA1 groups (cells transfected with miR-134 or si-PLXNA1). Measurement data were expressed as mean ± standard error of mean and analysed by one-way ANOVA; the experiment was repeated three times. miR-134, microRNA-134; ANOVA, analysis of variance; PLXNA1, plexin A1; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal kinase; p-ERK, phosphorylated-ERK; p-JNK, phosphorylated-JNK; Bcl-2, B-cell lymphoma-2; BAX, Bcl-2 associated X protein; RT-qPCR, reverse transcription quantitative polymerase chain reaction.
Fig. 6. MiR-134 overexpression suppresses the proliferation and promotes the apoptosis of ESCC cells. ESCC cells were treated with miR-134 expression and/or si-PLXNA1. (a) the proliferation of ESCC cells assessed by CCK-8 assay and analysed by repeated measures ANOVA; (b) a flow chart depicting cell cycle distribution in ESCC cells as assessed by flow cytometry; (c), the cell proportion/ratio in each cell-cycle phase; (d) the flow cytometry scatter plot of cell apoptosis in ESCC cells; (e) the ESCC cell apoptosis rate determined by Annexin V/PI double staining. *p < .05 vs. the blank and NC groups (cells without transfection or transfected with empty plasmid); #p < .05 vs. the miR-134 and si-PLXNA1 groups (cells transfected with miR-134 or si-PLXNA1). Measurement data were expressed as mean ± standard error of mean and analysed by one-way ANOVA, followed by Tukey’s post hoc test; the experiment was repeated three times. ESCC, esophageal squamous cell carcinoma; miR-134, microRNA-134; ANOVA, analysis of variance; PLXNA1, plexin A1; NC, negative control; OD, optical density.
containing 10% fetal bovine serum was added to the basolateral chamber. The number of cells passing through the Matrigel in each group was used as an index to evaluate the invasion ability of the cells.

2.14. Nude mouse tumorigenecity assay

Seventy-two BALB/c nude mice (4–6 weeks old; Institute of Laboratory Animal Research, Chinese Academy of Medical Sciences, Beijing, China) were raised under conventional conditions. Each nude mouse was subcutaneously injected with 200 μL (containing 1 × 10^7 cells) transfected cells on the left side of the ribs. Four days after inoculation, tumours were visible with naked vision. The tumour size was measured with a vernier calliper on the 5th day after inoculation and the tumour volume was computed using the formula as: 0.52 × length × width. Four weeks later, the mice were euthanized, the tumours were weighed and LNM was observed. Lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) was detected as a lymphatic marker using rabbit polyclonal antibody to LYVE-1 (Upstate Biotechnology Inc., Lake Placid, NY, USA). Microlymphatic density (MLD) was determined by the streptavidin-peroxidase (SP) method.

2.15. Statistical analysis

All data were processed using SPSS 21.0 software (IBM Corp. Armonk, NY, USA). Normality and homogeneity of variance tests were performed. The data conforming to normal distribution and homogeneity of variance were expressed as mean ± standard deviation, and analysed with paired t-test for intra-group comparison and non-paired t-test for inter-group comparison. One-way analysis of variance (ANOVA) and repeated-measures ANOVA were conducted for multiple group comparison, and post-hoc tests were employed for pairwise comparison. If the data did not conform to normal distribution or homogeneity of variance, a rank sum test was applied. The relationship between miR-134 expression and total survival (OS) and disease-free survival (DFS) was analysed by Kaplan-Meier method. p < .05 was accepted as indicative of statistical significance.

3. Results

3.1. The significances of PLXNA1 and miR-134 are implicated in ESCC progression

Initially, 619, 1010, 1964, 3068, and 2726 DEGs were determined in GSE17351, GSE20347, GSE29001, GSE45168 and GSE45670, respectively, at a p Value < .05 and Log Fold Change > 2. The top 500 DEGs in each dataset were then determined and three overlapping genes among these were identified, which were ABCA8, MYBL2 and PLXNA1 (Fig. 1a). Heatmaps were plotted with the top 50 DEGs of GSE45168 (Fig. 1b) and GSE29001 (Fig. 1c). These analyses showed that PLXNA1 was upregulated in ESCC. PLXNA1 was detected as highly expressed in ESCC from the data in GSE45670 (Fig. 1d), GSE17351 (Fig. 1e) and GSE20347 datasets (Fig. 1f). The high expression of PLXNA1 in multiple datasets suggested that PLXNA1 might affect the progression of ESCC. Putative miRNAs targeting PLXNA1 gene were predicted using TargetScan, miRDB, miRWalk, and microRNA. By analysing the top 200 miRNAs from each prediction website, it was found that there were two common predictive miRNAs, hsa-miR134-5p and hsa-miR-29a-3p (Fig. 1g), indicating that these two miRNAs might importantly target PLXNA1. A previous study has shown that miR-29a is differentially expressed in ESCC tissues and cell lines [19], but the role of miR-134 in ESCC remains uninvestigated.

3.2. MiR-134 is downregulated and PLXNA1 is upregulated in ESCC tissues

The expression pattern of miR-134 and PLXNA1 in ESCC was characterized by Western blot assay and RT-qPCR. As shown in Fig. 2a–c, when compared with the adjacent normal tissues, the expression of miR-134 in ESCC tissues was decreased significantly (p < .05), whereas the mRNA and protein expression of PLXNA1 was significantly increased (p < .05). Immunohistochemical staining was carried out to detect the positive expression rate of PLXNA1 in ESCC. As shown in Fig. 2d, brown yellow-stained PLXNA1 was mainly located in the cell membrane. The positive expression rate of PLXNA1 in the ESCC tissues (75.99 ±
3.4. miR-134 could target and downregulate PLXNA1 expression

The online biological prediction website microRNA.org illustrated that miR-134 could bind to PLXNA1 (Fig. 4a). The results of a dual luciferase reporter gene assay showed that miR-134 mimic had no significant effect on luciferase activity of PLXNA1-Mut plasmid, but the luciferase activity of PLXNA1-Wt plasmid was downregulated (Fig. 4b). These results suggested that miR-134 could specifically bind to the PLXNA1 gene, and verified a targeting relationship between miR-134 and PLXNA1.

3.5. Elevation of miR-134 downregulates PLXNA1 to block the MAPK signalling pathway

In order to examine the regulatory network of miR-134 in ESCC, gain-of-function and loss-of-function experiments were conducted with ESCC cells treated with miR-134 mimic, miR-134 inhibitor, or si-PLXNA1. The cells without transfection and those transfected with empty plasmid were set as the blank and NC groups, respectively. Subsequently, the miRNA and protein expression levels of PLXNA1, MAPK signalling pathway-related factors (ERK, p38) and cell apoptosis-related factors (BAX, Bcl-2) were determined (Fig. 5a–c). The expression of the aforementioned factors had no significant notable differences between the blank and NC groups (all \( p > 0.05 \)). The expression of miR-134 was significantly increased by transfection with miR-134 mimic or co-transfection with miR-134 mimic plus si-PLXNA1, but

Fig. 8. Downregulated PLXNA1 modulated the ESCC cell biological properties via the involvement of the MAPK signalling pathway. ESCC cells were treated with DMSO, SB203580, LY3214996 and SP600125 in the presence of si-PLXNA1. (a) the proliferation of ESCC cells assessed by CCK-8 assay and analysed by repeated measures ANOVA; (b) the flow chart depicting cell cycle distribution in ESCC cells as assessed by flow cytometry; (c) the cell proportion/ratio in each cell-cycle phase; (d) the flow cytometry scatter plot of cell apoptosis in ESCC cells; (e) the ESCC cell apoptosis rate determined by Annexin V/PI double staining; (f) the protein expression of Bcl-2 and BAX in the ESCC cells as determined by western blot analysis; (g) the grey value of Bcl-2 and BAX protein bands in the ESCC cells as determined by western blot analysis. \( p < 0.05 \) vs. the si-PLXNA1 + DMSO group (cells treated with DMSO in the presence of si-PLXNA1). Measurement data were expressed as mean ± standard error of mean and analysed by one-way ANOVA, followed by Tukey’s post hoc test; the experiment was repeated three times. ESCC, esophageal squamous cell carcinoma; miR-134, microRNA-134; ANOVA, analysis of variance; PLXNA1, plexin A1; NC, negative control.
significantly decreased by transfection of miR-134 inhibitor ($P < .05$). The silencing of PLXNA1 did not affect the expression of miR-134 ($P > .05$). The expression of BAX was elevated when the cells were transfected with miR-134 mimic, si-PLXNA1, or co-transfected with miR-134 mimic plus si-PLXNA1, while that of Bcl-2 was reduced ($P < .05$). However, when miR-134 in the ESCC cells was inhibited, the expression of BAX was decreased and that of Bcl-2 was increased ($P < .05$). The mRNA and protein expression levels of PLXNA1 as well as the ratios of p-ERK/ERK, p-JNK/JNK and p-p38/p38 were significantly increased in the cells transfected with miR-134 inhibitor, but decreased in the cells transfected with miR-134 mimic, si-PLXNA1, or co-transfected with miR-134 mimic plus si-PLXNA1 ($all P < .05$). Collectively, these results indicated that miR-134 elevation might suppress the activation of the PLXNA1-regulated MAPK signalling pathway in ESCC cells.

3.6. Elevation of miR-134 inhibits ESCC cell proliferation and induces ESCC cell apoptosis by targeting PLXNA1

The regulatory effects of miR-134 on ESCC cell proliferation, cell cycle, and apoptosis in ESCC cells expressing miR-134 and in those with the absence of miR-134 or PLXNA1 were each determined by CCK-8 assay, PI staining, and Annexin V/PI double-staining assay, respectively. As depicted in Fig. 6a, no significant difference was detected regarding the cell proliferation between cells without transfection and cells transfected with empty plasmid 24 h after transfection ($P > .05$). At the 48th and 72nd h after transfection, the viability of ESCC cells transfected with miR-134 mimic or si-PLXNA1 was decreased significantly ($P < .05$), but increased remarkably in the ESCC cells transfected with miR-134 inhibitor ($P < .05$). In addition, co-transfection with miR-134 mimic and si-PLXNA1 further reduced the viability of cells when compared to the cells transfected with miR-134 mimic or si-PLXNA1 alone ($P < .05$). Taken together, these data demonstrated that miR-134 overexpression and PLXNA1 silencing inhibited the proliferation of ESCC cells.

The results depicted in Fig. 6b, c indicated that as compared with the cells without transfection and cells transfected with empty plasmid, the proportion of cells in G0/G1 phase increased and that in S phase decreased when cells were transfected with miR-134 mimic or si-PLXNA1. The cells transfected with miR-134 inhibitor exhibited an increased cell proportion in S phase and a decreased cell proportion in G0/G1 phase ($P < .05$). Moreover, the combined transfection of miR-134 mimic and si-PLXNA1 resulted in an enhanced cell cycle arrest than either alone ($P < .05$). These data revealed that the elevation of miR-134 or silencing of PLXNA1 arrested more cells in the G0/G1 phase but fewer cells in the S phase.

As displayed in Fig. 6d, e, no significant change with regard to the cell apoptosis rate was found between the cells without transfection and cells transfected with empty plasmid ($P > .05$). The apoptosis rate was however, markedly and significantly reduced in the cells transfected with miR-134 inhibitor ($P < .05$), whereas it was significantly elevated in the cells transfected with miR-134 mimic or si-PLXNA1 or miR-134

![Graphs showing tumor volume, tumor weight, MLD, and LNM over time](image.png)

Fig. 9. Upregulation of miR-134 prevents tumour growth, MLD, and LNM of ESCC. Nude mice were injected with ESCC cells transfected with miR-134 expression and/or si-PLXNA1. (a) the tumour volume in mice; (b) the tumour weight in mice; (c) MLD in mice; (d) LM in mice. *$P < .05$ vs. the blank and NC groups (nude mice injected with cells without transfection and transfected with empty plasmid, respectively). Measurement data were expressed as mean value ± standard deviation. The differences in tumour volume were analysed by repeated measures ANOVA; the differences in tumour weight and LNM rate were analysed by one-way ANOVA, $n = 12$. ESCC, esophageal squamous cell carcinoma; miR-134, microRNA-134; ANOVA, analysis of variance; PLXNA1, plexin A1; NC, negative control; MLD, microlymphatic density; LNM, lymph node metastasis.
mimic plus si-PLXNA1 (p < .05). The above findings suggested that miR-134 overexpression or PLXNA1 silencing promoted the apoptosis of ESCC cells.

3.7. Elevation of miR-134 represses ESCC cell migration and invasion by targeting PLXNA1

We next investigated whether miR-134 restoration and PLXNA1 silencing might mediate the migration and invasion of ESCC cells by scratch test and Transwell assay (Fig. 7). There was no significant difference in cell migration and invasion between cells without transfection and cells transfected with empty plasmid (p > .05), but cell migration and invasion following miR-134 inhibitor treatment were significantly enhanced, whereas miR-134 mimic and/or si-PLXNA1 treatment exerted the opposite effects on cell migration and invasion in relative to cells without transfection or transfection with empty plasmid (p < .05). Furthermore, decline in migration rate was more pronounced in cells co-treated with miR-134 mimic and si-PLXNA1 (p < .05). Collectively, these results suggest that miR-134 overexpression and PLXNA1 silencing contributed to suppression of ESCC cell migration and invasion.

3.8. PLXNA1 silencing blocks the activation of the MAPK signalling pathway, thereby disrupting the progression of ESCC

In order to further explore whether PLXNA1 has an effect on the MAPK signalling pathway, thus affecting the biological properties of ESCC cells, we treated the ESCC cells with related inhibitors. The results showed that (Fig. 8a-d) as compared with DMSO treatment in the presence of si-PLXNA1, SB203580, LY3214996, and SP600125 in the presence of si-PLXNA1 treatments exerted an inhibitory effect on cell proliferation, migration and invasion, but a promotive effect on cell apoptosis (p < .05). In addition, the results of western blot analysis (Fig. 8f, g) showed that Bcl-2 expression decreased and BAX expression was elevated after treatment with SB203580, LY3214996, and SP600125 in the presence of si-PLXNA1 versus that after co-treatment of si-PLXNA1 and DMSO (p < .05). Collectively, these results suggest that the PLXNA1-dependent MAPK signalling pathway blockage is required for impairing ESCC progression.

3.9. Elevation of miR-134 represses tumour growth and metastasis of ESCC

Finally, a xenograft tumour experiment in nude mice was conducted to verify the potential anti-tumour effect of miR-134. There was no death of nude mice in all groups. The growth of the tumour displayed significant differences between the different groups 12 days after inoculation (Fig. 9). There was no notable difference in tumour weight and tumour volume between the mice injected with non-transfected cells or with cells transfected with empty plasmid (p > .05). The tumour weight, tumour volume, and MLD were significantly decreased in the mice injected with cells transfected with miR-134 mimic or si-PLXNA1 or both, since the 12th day (all p < .05). The tumour growth rate and volume were the lowest in the mice injected with cells co-transfected with miR-134 mimic and si-PLXNA1 (p < .01). The size and volume of tumour were increased significantly after injection of cells transfected with miR-134 inhibitor (p < .05) and enlarged lymph nodes were observed in the groin and armpit, which were confirmed as tumour metastasis by hematoxylin-eosin (HE) staining. The LNM rate in the mice injected with cells transfected with miR-134 mimic, si-PLXNA1, or both was decreased significantly but found to be significantly increased in the mice injected with cells transfected with miR-134 inhibitor (p < .05). In the mice injected with non-transfected cells or cells transfected with empty plasmid, lymphatic vessels were dilated, and the MLD of lymphatic endothelial cells in these groups was (12.61 ± 2.35) and (13.28 ± 1.97), respectively. Only a few lymphatic vessels were found in strip shape in the mice injected with cells transfected with miR-134 mimic or si-PLXNA1 or both, and the MLD of these lymphatic vessels in these groups (8.68 ± 2.13, 9.75 ± 1.40, and 6.13 ± 1.18 respectively) was significantly lower than that in the mice injected with non-transfected cells or cells transfected with empty plasmid (p < .05). However, the injection of miR-134 inhibitor-transfected cells resulted in more dilated lymphatic vessels in tumour tissues, some cancer cells in lymphatic vessels, and a higher MLD (16.79 ± 2.97) than those in the mice injected with non-transfected cells or cells transfected with empty plasmid (p < .05). All the above results demonstrated that both the upregulation of miR-134 and downregulation of PLXNA1 repressed the tumour growth and metastasis of ESCC.

4. Discussion

A panel of seven serum miRNAs (miR-10a, miR-22, miR-100, miR-148b, miR-223, miR-133a, and miR-127-3p) has been identified and proposed as a noninvasive, highly accurate biomarker of ESCC diagnosis [20]. The current study characterized the expression of miR-134 in ESCC, and proved that upregulation of miR-134 could reduce the expression of PLXNA1, thus repressing the MAPK signalling pathway, by which the proliferation, tumour growth, and metastasis of ESCC cells was inhibited, and cell apoptosis was enhanced, therefore restraining the progression of ESCC (Fig. 10).

Primarily, the current study elucidated that miR-134 had low expression levels in ESCC tissues and its low expression was correlated with the poor differentiation, LNM, and advanced TNM stage of ESCC. Similarly, miR-134 has been demonstrated to be downregulated in colorectal cancer (CRC) tissues and cell lines; and decreased miR-134 expression may serve as an independent predictor of poor survival due to its negative correlation with LNM, tumour size, and clinical stage [21]. Moreover, low expression of miR-134 has been significantly associated with LNM, TNM stage, and diminished cell differentiation in breast cancer [8]. It has also been found that miR-134 can impede gastric cancer cell proliferation by downregulating GOLPH3 [22]. PLXNA1 was
predicted in-silico as a target gene of miR-134 and high PLXNA1 expression was determined in ESCC tissues. In our precious investigation, the integrative analysis of mRNA and lncRNA profiles related to ESCC had revealed PLXNA1 is expressed at a high level in ESCC [23]. In a similar finding, it has been demonstrated that PLXNA1 functions as a tumour promoter in prostate cancer [24]. PLXNA1 knockdown is shown to reduce the proliferation-marker genes at the mRNA level and downregulate cell proliferation in Lewis lung carcinoma (LLC) cells [12]. Besides, it has been validated that a stress signal like isoprorenaline promotes tumour angiogenesis through the activation of the PLXNA1/VEGFR-2 signalling pathway in gastric cancer [25]. Therefore, we speculate that miR-134 may exert a suppressive effect on the development of ESCC via downregulating PLXNA1.

In addition, our study revealed that upregulating miR-134 or downregulating PLXNA1 induced ESCC cell apoptosis and inhibited cell proliferation, migration, and invasion in vitro, and suppressed tumour growth and LNM in vivo. Several mechanisms have been reported in context of miR-134 mediated tumour regulation. In osteosarcoma, the upregulation of miR-134 reportedly inhibits cell invasion and reduces the invasive potential of NSCLC cells [27] and inhibit the growth of NSCLC by targeting the epidermal growth factor receptor [28]. Bcl-2 encodes for an apoptotic protein that resists cancer cell apoptosis [29]. It has been found that the Bcl-2 level in ESCC tissues is significantly higher than that in normal esophageal epithelial tissues and dysplasia tissues [30]. Besides, BAX is downregulated in ESCC cells and is associated with a lower apoptosis [31]. In this study, the level of BAX was found increased, while the level of Bcl-2 was noted as decreased after miR-134 overexpression, suggesting that miR-134 potentially induces the apoptosis of ESCC cells. Hence, it is reasonable to conclude that upregulation of miR-134 suppresses tumour metastasis and induces cell apoptosis, thus preventing the progression of ESCC by downregulation of PLXNA1.

In this study, miR-134 was further demonstrated to inhibit the expression of MAPK signalling pathway-related proteins via downregulating PLXNA1. A former study has demonstrated that MAPK/ERK signalling pathway activation induced by Collagen Triple Helix Repeat Containing 1 (CHTRC1) facilitated the progression of ESCC [32]. Consistent with our study, MAPK signalling pathway has been implicated in previous findings regarding miR mediated tumour regulation mechanisms. For instance, upregulated miR-143 was found to suppress osteosarcoma invasion via inhibition of EGRF signalling through its downstream ERK/MAPK signalling cascades [33] and increased miR-126 expression decelerated angiogenesis of gastric cancer through negative regulation of vascular endothelial growth factor A (VEGF-A) via inactivation of the MAPK/ERK signalling [34]. In agreement with our findings, miR-134 suppressed proliferation and EMT of renal cell carcinoma cells by downregulating the MAPK/ERK signalling pathway [35]. Thus, our results can be considered confirmatory that miR-134 can mediate the activation of MAPK signalling pathway [36], which has been implicated in ESCC [37,38]. Thus, it seems rational to summarize that miR-134 targets PLXNA1 to block the MAPK signalling pathway, which prevents the progression of ESCC.

Collectively, the current study provides evidence that the disruption of the MAPK signalling pathway induced by PLXNA1 downregulation underlies the tumour repressive role of miR-134 in the development and progression of ESCC. These findings suggest a translational value of miR-134 elevation towards formulating novel therapeutic strategies for ESCC. However, further research is warranted to explore the potential of miR-134 in ESCC therapeutics and elucidate the biologic interaction between PLXNA1 and the MAPK signalling pathway.

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Ethical statement

All patients have signed written informed consents. The study was approved by the Ethics Committee of The First Affiliated Hospital of Zhengzhou University and carried out in accordance with the declaration of Helsinki. The animal experiments were performed in accordance with the ‘Guide for the Care and Use of Laboratory Animals’ of the National Institutes of Health.

Author contributions

Wei-Wei Wang designed the study. Wei-Wei Wang and Zhi-Hua Zhao collated the data, carried out data analyses and produced the initial draft of the manuscript. Li Wang, Pan Li, Kui-Sheng Chen and Jian-Ying Zhang collated the data, carried out data analyses. Wen-Cai Li, Guo-Zhong Jiang and Xiang-Nan Li designed the study and contributed to drafting the manuscript. All authors have read and approved the final submitted manuscript.

Consent for publication

Written consent for publication was obtained from all the participants.

Availability of data and material

The datasets generated/analysed during the current study are available.

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Conflicts of interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the review of the manuscript.

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