MicroRNA-206 exerts anti-oncogenic functions in esophageal squamous cell carcinoma by suppressing the c-Met/AKT/mTOR pathway

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Abstract. Increasing evidence suggests that the dysregulation of microRNAs (miRNAs) has an important role in the progression of human cancer, including ESCC. However, the exact functions and mechanisms of miRNAs in ESCC remain largely unclear. The aim of the present study was to investigate the expression and biological functions of miRNAs in ESCC and reveal the underlying molecular mechanisms. miRNA microarray and reverse transcription-quantitative polymerase chain reaction analyses were performed, which identified and confirmed that miR‑206 was significantly downregulated in ESCC tissues and cell lines. Its low expression was associated with lymph node metastasis, advanced TNM stage and N classification, as well as poorer overall survival in patients with ESCC. CCK‑8 and flow cytometry assays demonstrated that ectopic miR‑206 expression inhibited ESCC cell proliferation and induced cell apoptosis. In addition, MET proto-oncogene, receptor tyrosine kinase (c-Met), a well-known oncogene, was a direct target of miR‑206. An inverse correlation between the levels of miR‑206 and c‑Met mRNA in ESCC tissue samples was confirmed. Notably, c-Met overexpression inhibited the effects of miR-206 on the proliferation and apoptosis of ESCC cells. Additionally, it was confirmed that the tumor-suppressive functions of miR-206 may have contributed to the inactivation of the c-Met/protein kinase B (AKT)/mechanistic target of rapamycin (mTOR) signaling pathway. In conclusion, the findings of the present study suggested that miR-206 exerts its anti-cancer functions via the c-Met/AKT/mTOR signaling pathway, providing a novel candidate prognostic factor and a potential therapeutic target in ESCC.
Subsequently, the mechanisms underlying the effects of miR-206 on ESCC cell proliferation and apoptosis were investigated, and the collective signaling pathways enriched by the predicted targets of miR-206 in ESCC carcinogenesis were analyzed. The findings of the present study suggested that miR-206 may be a potential target for the treatment of ESCC.

Materials and methods

Clinical specimens. ESCC and paired normal esophageal tissues (>5 cm from the tumor margin) were obtained from 52 patients (median age 63.5 years, range 48-76 years, female: male=9:17) who underwent esophagus resection between January 2014 and May 2016 at the Department of Thoracic Surgery, the Second Affiliated Hospital of Zhengzhou University (Zhengzhou, China). Patient characteristics are presented in Table I. The ESCC patients were recruited according to the following inclusion and exclusion criteria: First, all ESCC patients were newly diagnosed and confirmed by histopathological examination in accordance with the 7th edition of the TNM-UICC/AJCC classification (17); second, all included patients underwent a total or subtotal esophagectomy with resection of at least 12 regional lymph nodes; third, all enrolled patients were diagnosed and had not received any other treatment before esophagectomy; fourth, follow-up data could be obtained from all eligible patients. Patients with acute or chronic infection, autoimmune, hematological or liver disease or other malignancies and those without clinical characteristics or survival data were excluded in the present study. Following excision, tissue specimens were immediately frozen in liquid nitrogen for subsequent analysis. Informed consent was obtained, and the present study was approved by the Ethics Committee of the Second Affiliated Hospital of Zhengzhou University (Zhengzhou, China; approval no. 2016-008).

Microarray analysis. Total RNA was extracted from ESCC tissues and adjacent normal tissues using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and Cy3- or Cy5-labeled cDNAs were hybridized on the miRCURY™ LNA Array kit (version 16.0; Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. Following washing with PBS, the slides were scanned using an Axon GenePix 4000 B microarray scanner (Molecular Devices, LLC, Sunnyvale, CA, USA). Scanned images were subsequently imported into the GenePix Pro 6.0 program (Molecular Devices, LLC) for grid alignment and data extraction. Replicated miRNAs were averaged, and miRNAs with intensities of ≥50 in all samples were used to calculate a normalization factor. Expressed data were normalized by median normalization. Following this, the miRNAs that were significantly differentially expressed were identified by Volcano Plot filtering with fold change values >2 and P<0.05 as the screening conditions using the R software package (version 3.1.3, 2015; R Core Team, Vienna, Austria). Finally, the expression data were subjected to hierarchical clustering and subsequently depicted in a heat map format using GeneSpring GX (version 7.3; Agilent Technologies, Inc., Santa Clara, CA, USA).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). miRNA was isolated from tissue samples and cell lines using the mirVANA RNA isolation kit (Ambion; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Total RNA was reverse transcribed into complementary DNA (cDNA) by using a miScript reverse transcription kit (Qiagen, Inc.) in a volume of 20 µl containing 10 µl RNA, 4 µl miScript RT Buffer, 1 µl miScript Reverse Transcriptase Mix and 6 µl RNase-free H₂O. The reaction was incubated for 1 h at 37 ºC. miRNA PCR was performed on an Applied Biosystems Prism 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). Relative expression was determined by normalization to U6 or GAPDH. The primers for RT-qPCR analysis were as follows: miR-206 forward, 5'-TGGAAATGTA AGGAAATG-3' and reverse, 5'-AGATCGGGAGTGCTGG AGT-3'; U6 forward, 5'-TGCGGGTGCCTGCTCGACG-3' and reverse, 5'-CCAGTGCCAGGTCGAGT-3'. The amplification protocol was set as follows: Initial denaturation at 95 ºC for 5 min, followed by 50 cycles of denaturation at 94ºC for 15 sec, annealing at 55ºC for 30 sec and extension at 70ºC for 30 sec.

For mRNA reverse transcription, cDNA was synthesized using Primerscript RT Reagent (Takara Bio, Inc.). The RT-qPCR reaction system (30 µl) contained 5 µl cDNA, 15 µl 2X qPCR mix, 1 µl upstream primer, 1 µl downstream primer and 8 µl double distilled H₂O. PCR was performed with an Applied Biosystems Prism 7900HT Fast Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) and the protocol was: 95 ºC for 15 min, followed by 40 cycles of 94ºC for 15 sec, 55ºC for 30 sec and 70ºC for 30 sec with a final extension step of 72 ºC for 5 min using. Relative quantification was determined by normalization to U6 or GAPDH. The primer sequences were as follows: c-Met, forward 5'-CCC CACCCCTTGTTCAG-3' and reverse 5'-TCAGCCCTTTGCTC TTCTC-3'; GAPDH forward, 5'-GAAGATGATGTAGGG ATTTCC-3' and reverse, 5'-GAAGTGCTAAGGTCGAGT-3'; RT-qPCR assays were performed in triplicate and expression alterations were calculated using the 2^ΔΔCq method (18).

Cell lines and cultures. ESCC cell lines Ec9706, EcA109 and KYSE410, as well as the normal esophageal cell line Het-1A, were obtained from the American Type Culture Collection (Manassas, VA, USA). All the cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) at 37°C in a humidified atmosphere of 95% air and 5% CO₂.

Cell transfection. miR-206 mimics, miR-206 inhibitor, and their negative controls (NCs) were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). The coding domain sequences of c-Met mRNA were amplified by PCR, and inserted into a pcDNA 3.0 vector (Invitrogen; Thermo Fisher Scientific, Inc.) to enhance its expression, and was termed pcDNA-c-Met. EcA109 and KYSE410 cells (5.0x10⁴/well) were seeded and cultured to 60-80% confluence in six-well plates. According to the manufacturer's protocol, they were transiently transfected with the 20 nM interference oligonucleotide which included negative control (inhibitor NC: 5'-CAG UACUUCUUUUGUGAAGACCA-3'; mimics NC: 5'-UUUCUCC GAACUGUCAGCUTT-3'), miR-206 inhibitor (5'-CCACAC ACUUCUUCAUACUCA-3') or miR-206 mimics (5'-UGG AAUGUAAGGAGUGUGUGGG-3') using Lipofectamine®

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Following 6 h transfection at 37˚C, the medium was replaced with RPMI1640 media containing 10% FBS and the cells were harvested for further experiments after 48 h.

**Cell proliferation.** A Cell Counting Kit-8 (CCK-8) assay was performed to assess the proliferation of Eca109 and KYSE410 cells following transfection in 96-well plates at a density of 5x10^3 cells/well. After 24, 48 and 72 h of incubation, 10 µl CCK-8 was added to each well. After incubation for 4 h at 37˚C, the absorbance rates were measured at a wavelength of 450 nm using a microplate reader (Infinite M200; Tecan Group, Ltd., Mannedorf, Switzerland). All experiments were performed in triplicate.

**Cell apoptosis.** Cell apoptosis was determined using an Annexin V-FITC Apoptosis Detection kit (cat no. ab14085; Abcam, Cambridge, UK) according to the manufacturer’s protocol. Briefly, Eca109 and KYSE410 cells were seeded in 6-well plates at a density of 1.0x10^5 cells/well, and subjected to the various treatments as described above. At the end of the exposure, the cells were harvested and washed twice with PBS, and stained with 5 µl of FITC Annexin V (Abcam, Cambridge, UK), and 5 µl of propidium iodide (PI; Abcam, Cambridge, UK) in the dark for 15 min at room temperature. Following this, cell apoptosis was analyzed on Becton Dickinson FACScan flow cytometer (Beckman Coulter, Inc., Brea, CA, USA). Data was analyzed with the ModFit LT software package version 4.0 (Verity Software House, Inc., Topsham, ME, USA).

**Target genes prediction of miR-206.** The target genes of miR-206 were predicted by bioinformatics analysis. The analysis was performed by TargetScan 5.1 (targetscan.org) (19), miRanda (2010 release; microRNA.org) (20) and PicTar 5 (pictar.mdc-berlin.de) (21). Results of the forecast targets were intersected by miRWalk V2.0 (www.ma.uni-heidelberg.de/apps/zmf/mirwalk) website tools (22).

**Dual-luciferase reporter assays.** The predicted and mutated sequences targeting the 3’untranslated region (UTR) of c-Met were synthesized and ordered from Shanghai GenePharma Co., Ltd. 293T cells (1x10^4 cells/well) in 24-well plates were co-transfected with miR-206 mimics (50 nM), mimics NC (50 nM), miR-206 inhibitor (50 nM), inhibitor NC (50 nM) and luciferase reporter plasmids (50 ng) (pmiRGLO-c-Met-3’UTR Wt or pmiRGLO-c-Met-3’UTR Mut) using Lipofectamine® 2000, according to the manufacturer’s recommendations. The relative luciferase activities were determined using a Dual-Luciferase Reporter Assay system (Promega Corporation, Madison, WI, USA) at 48 h post-transfection. All

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Table I. Correlation between miR-206 expression and the clinicopathological features of patients with esophageal squamous cell carcinoma.

| Clinical variables                  | Total n=52 | High expression | Low expression | P-value |
|------------------------------------|------------|----------------|----------------|---------|
| Gender                             |            |                |                | 0.7165  |
| Male                               | 34         | 15             | 19             |         |
| Female                             | 18         | 7              | 11             |         |
| Age (years)                        |            |                |                | 0.8612  |
| ≥60                                | 30         | 13             | 17             |         |
| <60                                | 22         | 9              | 13             |         |
| Tumor location                     |            |                |                | 0.5386  |
| Upper or middle 1/3                | 40         | 16             | 24             |         |
| Lower 1/3                          | 12         | 6              | 6              |         |
| Lymph node metastasis              |            |                |                | 0.0015^b|
| Negative                           | 16         | 12             | 4              |         |
| Positive                           | 36         | 10             | 26             |         |
| TNM stage                          |            |                |                | 0.0209^a|
| I-II                               | 19         | 12             | 7              |         |
| III-IV                             | 33         | 10             | 23             |         |
| Differentiation                    |            |                |                | 0.4043  |
| Well and moderate                  | 37         | 17             | 20             |         |
| Poor                               | 15         | 5              | 10             |         |
| Distant metastasis                 |            |                |                | 0.3807  |
| M0                                 | 42         | 19             | 23             |         |
| M1                                 | 10         | 3              | 7              |         |

^aP<0.05, ^bP<0.01. miR-206, microRNA-206.
experiments were performed in triplicate. Renilla luciferase activity was normalized to firefly luciferase activity.

**Western blotting.** Following transfection, total protein was extracted from cells using radioimmunoprecipitation assay (RIPA) lysis buffer containing 1x protease inhibitory cocktail and phosphatase inhibitors (RIPA Lysis Buffer system, Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Protein concentration was determined using a bicinchoninic acid assay kit (Beyotime Institute of Biotechnology, Haimen, China). Proteins (25 µg/lane) were analyzed by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (GE Healthcare, Chicago, IL, USA) by electroblotting. The membranes were blocked with 5% bovine serum albumin (BSA, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 1 h at room temperature. Subsequently, the membranes were incubated with primary antibodies at 4°C overnight. Primary antibodies against c-Met (cat no. 8198; 1:1,000; Santa Cruz Biotechnology, Inc.), phosphorylated (p)-c-Met (cat no. ab5662; 1:1,000; Abcam; Cambridge, UK), p-mechanistic target of rapamycin (p-mTOR; cat no. 5536; 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), mTOR (cat no. 2983; 1:1,000; Cell Signaling Technology, Inc.), p-AKT (cat no. 4060; 1:1,000; Cell Signaling Technology, Inc.), AKT (cat no. 4691; 1:1,000; Cell Signaling Technology, Inc.) and β-actin (cat no. A1987; 1:2,000; Sigma-Aldrich; Merck KGaA). Thereafter, membranes were incubated with secondary antibodies (cat no. 8885; 1:10,000; Cell Signaling Technology, Inc.). Immunoreactivity was visualized using an Enhanced Chemiluminescence Western Blotting kit (Beyotime Institute of Biotechnology, Beijing, China) according to the manufacturer’s protocols. The intensity of the bands was analyzed by ImageJ software (version 1.46; National Institutes of Health, Bethesda, MD, USA).

**Statistical analysis.** Statistical analysis was performed with SPSS (version 18.0; SPSS, Inc., Chicago, IL, USA). Data were presented as the mean ± standard deviation. Student’s t-test or one-way analysis of variance followed by Tukey’s post-hoc test was used to analyze the differences among/between sample groups. Kaplan-Meier analysis was performed to calculate the overall survival (OS) rates, and a log-rank test was conducted to compare the survival distributions between two groups. Multivariate analysis for overall survival (OS) was performed using the Cox proportional hazard model, with factors identified to be statistically significant from univariate analysis. Correlations between miR-206 and c-Met expression levels in databases were assessed by Spearman’s correlation. P<0.05 was considered to indicate a statistically significant difference.

**Results**

miR-206 expression is downregulated in ESCC tissues and cell lines, and correlated with clinicopathologic features. To determine the potential involvement of miRNAs in ESCC, miRNA microarray profiling was performed in ESCC tissues and matched normal tumor-adjacent tissues. The data revealed that compared with the normal group, 35 miRNAs were upregulated and 22 miRNAs were downregulated in the ESCC group (Fig. 1A). Among these differentially expressed miRNAs, miR-206 was one of the most downregulated miRNAs, and several studies have previously demonstrated that miR-206 suppresses cell growth and invasion in various types of cancers (23-25). However, the function and mechanism of miR-206 in ESCC has not been characterized. Thus, miR-206 was selected for further analysis.

Next, the expression levels of miR-206 in 52 paired ESCC tissues and matched tumor-adjacent tissues were validated by RT-qPCR. Consistent with the array data, miR-206 expression was significantly lower in ESCC tissues compared with that in the matched tumor-adjacent tissues (Fig. 1B). The expression of miR-206 was also examined in a series of ESCC cell lines (Eca9706, Eca109 and KYSE410). The results revealed that the expression of miR-206 was markedly downregulated in these cell lines when compared with the normal Het-1A cell line, particularly in Eca109 and KYSE410 cells (Fig. 1C). These data suggested that miR-206 may function as a tumor suppressor in the initiation and progression of ESCC.

In order to evaluate the clinical importance of miR-206 in ESCC, the association between miR-206 expression levels and the clinicopathological parameters of ESCC patients was investigated. A total of 52 ESCC patients were divided into two groups according to the median level of miR-206 (cut off value for miR-206 was 2.25). As presented in Table I, low expression of miR-206 was inversely associated with lymph node metastasis and TNM stage. However, there were no significant correlations between miR-206 expression levels with gender, age, tumor location, differentiation or distant metastasis in ESCC. In addition, it was demonstrated that patients with low miR-206 expression had shorter OS compared with patients with high miR-206 expression (Fig. 1D). These results indicated that miR-206 downregulation may be associated with the development of ESCC.

**Overexpression of miR-206 inhibits cell proliferation and induces cell apoptosis.** Considering that miR-206 was downregulated in ESCC tissues, it was hypothesized that miR-206 may function as a tumor suppressor in ESCC. To test this hypothesis, overexpression of miR-206 was established in Eca109 and KYSE410 cells, which had the lowest expression of miR-206 in the three ESCC cell lines. The efficiency of miR-206 mimic transfection in Eca109 and KYSE410 cells was confirmed through RT-qPCR (Fig. 2A). The results of the CCK-8 assay demonstrated that miR-206 overexpression significantly inhibited cell proliferation in Eca109 (Fig. 2B) and KYSE410 cells (Fig. 2C), compared with the mimic NC groups. To further understand the mechanisms by which cell proliferation was affected, flow cytometry was performed to analyze cell apoptosis. As presented in Fig. 2D, cell apoptosis was markedly promoted in the miR-206 mimics group compared with mimics NC group. These results implied that miR-206 may have inhibited ESCC cell proliferation by promoting apoptosis.

**c-Met is a direct target of miR-206.** To further elucidate the underlying molecular mechanisms involved in the anti-oncogenic role of miR-206 in ESCC cells, target genes of miR-206 were searched for using TargetScan 5.1, miRanda (19) and PicTar 5 (26). The predicted results indicated that miR-206 directly targeted the c-Met gene and that the target
sequences were highly conserved among different species (Fig. 3A and B). To confirm whether c-Met was a direct target of miR-206, a dual-luciferase reporter system containing the 3’-UTR of c-Met along with the putative miR-206 binding sites was constructed.

The results of the luciferase reporter gene assay demonstrated that the luciferase activity was remarkably decreased following co-transfection with the luciferase reporter plasmid harboring the c-Met 3’UTR wild-type (WT) and miR-206 mimics. By contrast, luciferase activity was notably increased...
Following co-transfection with c-Met 3'UTR wild-type (WT) plasmid and miR-206 inhibitor. However, these effects were abrogated when the c-Met sequence was mutated (Fig. 3C). To further identify the correlation between miR-206 expression and c-Met, c-Met protein expression was determined in Eca109 and KYSE410 cells by western blotting. The expression of c-Met at the protein level was significantly downregulated following overexpression of miR-206, whereas it was upregulated following knockdown of miR-206 in Eca109 and KYSE410 ESCC cells (Fig. 3D). In addition, the results revealed that c-Met expression at the mRNA level was significantly upregulated in ESCC tissues compared with that in matched normal adjacent tissues (Fig. 3E), as well as increased in the three ESCC cell lines, compared with the normal esophageal cell line (Fig. 3F). Furthermore, miR-206 expression was inversely correlated with c-Met expression in ESCC tissues (R^2=0.7922; P<0.001; Fig. 3G). These data suggested that miR-206 suppressed the expression of c-Met in ESCC.

**Overexpression of c-Met inhibits the tumor suppressive role of miR-206 in ESCC cells.** Given that c-Met is a target of miR-206, and c-Met has been widely documented to regulate proliferation and migration during the development of human cancers (27), whether miR-206 mediated the inhibitory effects on ESCC via c-Met was examined. First, the effects
of c-Met overexpression in miR-206 mimic transfected cells were investigated. Western blotting results demonstrated that pcDNA-c-Met vector transfection significantly increased c-Met expression in miR-206 mimic transfected Eca109 and KYSE410 cells (Fig. 4A). Subsequently, cell proliferation was assessed with a CCK-8 assay. As presented in Fig. 4B and C, overexpression of c-Met in Eca109 and KYSE410 cells partly suppressed the inhibitory effects of miR-206 on cell proliferation. In addition, it was demonstrated that overexpression of c-Met abrogated the promotive effects of miR-206 on cell apoptosis in Eca109 and KYSE410 cells (Fig. 4D and E). These data suggested that miR-206 exerted its tumor suppressive effects by modulating c-Met expression.

**Overexpression of miR-206 blocked the activation of c-Met/AKT/mTOR pathway.** To investigate the molecular mechanism underlying the inhibition of cell proliferation by miR-206, The expression of c-Met, p-c-Met, AKT, p-AKT, mTOR and p-mTOR was determined by western blot analysis (Fig. 5A). The results revealed that overexpression of miR-206 inhibited the p-c-Met, p-AKT and p-mTOR expression, whereas the expressions of Met, AKT and mTOR were not noticeably altered in Eca109 or KYSE410 cells (Fig. 5B). These data suggested that miR-206 regulated the c-Met/AKT/mTOR pathway, which was at least partially responsible for the effects of miR-206 on cell proliferation and apoptosis in ESCC cells.

**Discussion**

In the present study, miR-206 was significantly downregulated in ESCC and its expression was correlated with poor prognosis. Overexpression of miR-206 inhibited ESCC cell proliferation and induced ESCC cell apoptosis. In addition, miR-206 upregulation in ESCC cells inactivated the AKT/mTOR signaling pathway by targeting c-Met. The data from the present study indicated that miR-206 may be a novel biomarker and therapeutic target in ESCC.

Increasing evidence has revealed that miRNAs have pivotal function in cancer development and progression, including ESCC (28-31). In the present study, differentially expressed miRNAs were profiled in ESCC tissues, and it was demonstrated that miR-206 was one of most downregulated miRNAs. miR-206 was selected for further analysis, as miR-206 has important involvement in various types of
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For example, the expression levels of miR-206 are markedly decreased in breast cancer tissues, and low miR-206 expression is associated with advanced disease progression and poor prognosis (32). Pan et al (24) demonstrated that miR-206 suppresses medulloblastoma cell viability and invasion, at least partially via the targeting of LIM and SH3 protein 1. Another study reported that the expression level of miR-206 is significantly lower in hepatocellular carcinoma (HCC) tissues, and miR-206 inhibits the growth of HCC cells through targeting cyclin-dependent kinase 9 (33). In the present study, it was observed that miR-206 is significantly downregulated in human ESCC tissues and cell lines, and the low expression level of miR-206 was closely correlated with key clinico-pathological properties, as well as overall survival in ESCC patients. In addition, overexpression of miR-206 reduced ESCC cell proliferation and induced cell apoptosis in vitro. These data were consistent with the results of a previous study, demonstrating that miR-206 functions as a tumor suppressor in clear-cell renal cell carcinoma (34).

Several recent reports have proven that c-Met is overexpressed in several cancer types, including gastric and breast cancer (35-37). c-Met, a well-known oncogene, has been reported to promote cell proliferation and invasion (27). Of note, many studies have reported that the c-Met is regulated by miRNAs, such as miR-146a, miR-181a-5p and miR-101 (38-40). Furthermore, c-Met has been identified to be a direct target of miR-206 in lung cancer cells (41,42). In the present study, it was demonstrated that miR-206 directly bound to the 3’UTR of c-Met and negatively regulated its expression in ESCC cells. The restoration of c-Met expression inhibited the anti-tumor activities of miR-206 in ESCC cells.

It is well established that c-Met is an important regulator of the Akt/mTOR pathway (43-45), and activation of the phosphoinositide 3-kinase (PI3K)/mTOR signaling pathway has been reported to have a pivotal role in the progression of ESCC (46-48). For example, Liu et al (25) demonstrated that miR-206 inhibits head and neck squamous cell carcinoma progression via the Akt/mTOR pathway. Another study from Liu et al (49) reported that using LY294002, a PI3K specific inhibitor, significantly inhibits ESCC cell proliferation and migration. As previously mentioned, the present study demonstrated that c-Met may be a direct target of miR-206 in ESCC cells. Therefore, it was speculated that miR-206 affected the proliferation and apoptosis of ESCC by mediating the c-Met/AKT/mTOR signaling pathway. The expression levels of a number of c-Met/AKT/mTOR signaling pathway-associated proteins were examined, including p-c-Met, c-Met, p-AKT, p-AKT/mTOR pathway. Eca109 and KYSE410 cells were transfected with miR-206 mimics or mimics NC for 48 h and subjected to western blot analysis. (A) c-Met, p-c-Met, AKT, p-AKT, p-mTOR and mTOR were detected by western blotting and (B) bands were quantitatively analyzed using ImageJ software, normalized to β-actin density. Data represent the mean ± standard deviation of three independent experiments. **P<0.01 vs. mimics NC. miR, microRNA; AKT, protein kinase B; mTOR, mechanistic target of rapamycin; -Met, MET proto-oncogene, receptor tyrosine kinase; NC, negative control; p, phosphorylated.

Figure 5. Overexpression of miR-206 inhibits the activation of the AKT/mTOR pathway. Eca109 and KYSE410 cells were transfected with miR-206 mimics or mimics NC for 48 h and subjected to western blot analysis. (A) c-Met, p-c-Met, AKT, p-AKT, p-mTOR and mTOR were detected by western blotting and (B) bands were quantitatively analyzed using ImageJ software, normalized to β-actin density. Data represent the mean ± standard deviation of three independent experiments. **P<0.01 vs. mimics NC. miR, microRNA; AKT, protein kinase B; mTOR, mechanistic target of rapamycin; -Met, MET proto-oncogene, receptor tyrosine kinase; NC, negative control; p, phosphorylated.
AKT, p-mTOR and mTOR. The results demonstrated that the expression of these proteins was significantly inhibited, suggesting that miR-206 exerted its anti-tumor effects via the c-Met/Akt/mTOR pathway.

Although the present study supported the conclusion that miR-206 functioned as a suppressor of cell growth and as a prognostic marker for longer survival in ESCC, other target genes of miR-206 or other differentially expressed miRNAs identified in the present work should also be carefully tested for their significance in ESCC. In addition, the cohort for the survival analysis was small, and larger cohorts are required in order to verify the results of the present study.

In conclusion, the present study demonstrated that miR-206 was downregulated in ESCC and overexpression of miR-206 suppressed ESCC cell proliferation and induced cell apoptosis, potentially via the c-Met/Akt/mTOR signaling pathway. Overall, these findings suggested that miR-206 may be a novel target for ESCC treatment.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
XF designed the experiments which were performed by JZ and QZ. JZ and XF analyzed data, and XF contributed reagents and other essential material. JZ wrote the paper and the manuscript was reviewed by JZ, XF and QZ.

Ethics approval and consent to participate
Informed consent was obtained, and the present study was approved by the Ethics Committee of the Second Affiliated Hospital of Zhengzhou University (Zhengzhou, China; approval no. 2016-008).

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References
1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J and Jemal A: Global cancer statistics, 2012. CA Cancer J Clin 65: 87-108, 2015.
2. Ferlay J, Soerjomataram I, Dikshit R, Eser S, Mathers C, Rebelo M, Parkin DM, Forman D and Bray F: Cancer incidence and mortality worldwide: Sources, methods and major patterns in GLOBOCAN 2012. Int J Cancer 136: E359-E386, 2015.
3. Abnet CC, Arnold M and Wei Q: Epidemiology of esophageal squamous cell carcinoma. Gastroenterology 154: 360-373, 2018.
4. Siegel R, Naishadham D and Jemal A: Cancer statistics, 2013. CA Cancer J Clin 63: 11-30, 2013.
5. Kim T, Grobmyer SR, Smith R, Ben-David K, Ang D, Vogel SB, and Hochwald SN: Esophageal cancer-The five year survivors. J Surg Oncol 103: 179-183, 2011.
6. Barthel DP: MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116: 281-297, 2004.
7. Kloosterman WP and Plasterk RH: The diverse functions of microRNAs in animal development and disease. Dev cell 11: 441-450, 2006.
8. Schmittgen TD: Regulation of microRNA processing in development, differentiation and cancer. J Cell Mol Med 12: 1811-1819, 2008.
9. Hiyoshi Y, Kamohara H, Karashima R, Sato N, Imamura Y, Nagai Y, Yoshida N, Toyama E, Hayashi N, Watanabe M and Baba H: MicroRNA-21 regulates the proliferation and invasion in esophageal squamous cell carcinoma. Clin Cancer Res 15: 1915-1922, 2009.
10. Kurashige J, Watanabe M, Iwatsuki M, Kinoshita K, Saito S, Hiyoshi Y, Kamohara H, Baba Y, Mimori K and Baba H: Overexpression of microRNA-223 regulates the ubiquitin ligase FBXW7 in oesophageal squamous cell carcinoma. Br J Cancer 106: 182-188, 2012.
11. Kimura S, Nagamura S, Susuki D, Hirono Y, Yamaguchi A, Fujieda S, Sano K and Itoh H: Expression of microRNAs in squamous cell carcinoma of human head and neck and the esophagus: miR-205 and miR-21 are specific markers for HNSCC and ESCC. Oncol Rep 23: 1625-1633, 2010.
12. Gopalan V, Islam F, Pillai S, Tang JC, Tong DK, Law S, Chan KW and Lam AK: Overexpression of microRNA-1288 in oesophageal squamous cell carcinoma. Exp Cell Res 348: 146-154, 2016.
13. Sun J, Song K, Feng X and Gao S: MicroRNA-367 is a potential diagnostic biomarker for patients with esophageal squamous cell carcinoma. Biochem Biophys Res Commun 473: 363-369, 2016.
14. Wang M, Wang L, Zhang M, Li X, Zhu Z and Wang H: MiR-214 inhibits the proliferation and invasion of esophageal squamous cell carcinoma cells by targeting CDC25B. Biomed Pharmacother 95: 1678-1683, 2017.
15. Dai C, Xie Y, Zhuang X and Yuan Z: MiR-206 inhibits epithelial ovarian cancer cells growth and invasion via blocking c-Met/Akt/mTOR signaling pathway. Biomed Pharmacother 104: 763-770, 2018.
16. Zhang WL, Lv W, Sun SZ and Zhang JH: miR-206 inhibits metastasis-relevant traits by degrading MRTF-A in anaplastic thyroid cancer. Int J Oncol 47: 133-142, 2015.
17. Gao QF, Qiu JC, Huang XH, Xu YM, Li SQ, Sun F, Zhang J, Yang WM, Min QH, Jiang YH, et al: The predictive and prognostic role of a novel ADS score in esophageal squamous cell carcinoma patients undergoing esophagectomy. Cancer Cell Int 18: 153, 2018.
18. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2-DDelta CT method. Methods 25: 402-408, 2001.
19. Friedman RC, Farh KB, Burge CB and Bartel DP: Most mammalian mRNAs are conserved targets of microRNAs. Genome Res 19: 92-105, 2009.
20. Betel D, Wilson M, Gabow A, Marks DS and Sander C: The microRNA.org resource: Targets and expression. Nucleic Acids Res 36: D149-D153, 2008.
21. Saito T and Settrom P: MicroRNAs-Targeting and target prediction. N Biotechnol 27: 243-249, 2010.
22. Osman A and Fäkler K: Charakterization of human platelet microRNA by quantitative PCR coupled with an annotation network for predicted target genes. Platelets 22: 433-441, 2011.
23. Ding W, Ren J, Ren H and Wang D: Long noncoding RNA BONAR modulates microRNA-206-mediated Bel-2 signaling to facilitate cell proliferation in breast cancer. Sci Rep 7: 17261, 2017.
24. Pan X, Wang Z, Wan B and Zheng Z: MicroRNA-206 inhibits the viability and migration of medulloblastoma cells by targeting LIM and SH3 protein 1. Exp Ther Med 14: 3894-3900, 2017.
25. Liu F, Zhao X, Qian Y, Zhang J and Yin R: MicroRNA-206 inhibits head and neck squamous cell carcinoma cell progression by targeting HDAC6 via PTEN/AKT/mTOR pathway. Biomed Pharmacother 96: 229-237, 2017.
26. Lewis BP, Burge CB and Bartel DP: Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15-20, 2005.
27. Organ SL and Tao MS: An overview of the c-MET signaling pathway. Ther Adv Med Oncol 3: S7-S19, 2011.
28. Pandima Devi K, Rajavel T, Daglia M, Nabavi SF, Bishayee A and Nabavi SM: Targeting miRNAs by polyphenols: Novel therapeutic strategy for cancer. Semin Cancer Biol 46: 146-157, 2017.
29. Deng X, Zheng H, Li D, Xue Y, Wang Q, Yan S, Zhu Y and Deng M: MicroRNA-34a regulates proliferation and apoptosis of gastric cancer cells by targeting silent information regulator 1. Exp Ther Med 15: 3705-3714, 2018.
30. Liu W, Li M, Chen X, Zhu S, Shi H, Zhang D, Cheng C and Li B: MicroRNA-1 suppresses proliferation, migration and invasion by targeting Notch2 in esophageal squamous cell carcinoma. Sci Rep 8: 5183, 2018.
31. Li F, Lv JH, Liang L, Wang JC, Li CR, Sun L and Li T: Downregulation of microRNA-21 inhibited radiation-resistance of esophageal squamous cell carcinoma. Cancer Cell Int 18: 39, 2018.
32. Li Y, Hong F and Yu Z: Decreased expression of microRNA-206 in breast cancer and its association with disease characteristics and patient survival. J Int Med Res 41: 596-602, 2013.
33. Pang C, Huang G, Luo K, Dong Y, He F, Du G, Xiao M and Cai W: miR-206 inhibits the growth of hepatocellular carcinoma cells via targeting CDK9. Cancer Med 6: 2398-2409, 2017.
34. Xiao H, Xiao W, Cao J, Li H, Guan W, Guo X, Chen K, Zheng T, Ye Z, Wang J and Xu H: miR-206 functions as a novel cell cycle regulator and tumor suppressor in clear-cell renal cell carcinoma. Cancer Lett 374: 107-116, 2016.
35. Zhang J and Babic A: Regulation of the MET oncogene: Molecular mechanisms. Carcinogenesis 37: 345-355, 2016.
36. Marano L, Chiari R, Fabozzi A, De Vita F, Boccardi V, Roviello G, Petrioli R, Marrelli D, Roviello F and Patriti A: c-Met targeting in advanced gastric cancer: An open challenge. Cancer Lett 365: 30-36, 2015.
37. Ho-Yen CM, Jones JL and Kermorgant S: The clinical and functional significance of c-Met in breast cancer: A review. Breast Cancer Res 17: 52, 2015.
38. Bleau AM, Redrado M, Nistal-Villan E, Villalba M, Exposito F, Redin E, de Aberasturi AL, Larrazabal L, Freire J, Gomez-Roman J and Calvo A: miR-146a targets c-met and abolishes colorectal cancer liver metastasis. Cancer Lett 414: 257-267, 2017.
39. Korhan P, Erdal E and Atabey N: MiR-181a-5p is downregulated in hepatocellular carcinoma and suppresses motility, invasion and branching-morphogenesis by directly targeting c-Met. Biochem Biophys Res Commun 450: 1304-1312, 2014.
40. Hu Z, Lin Y, Chen H, Mao Y, Wu J, Zhu Y, Xu X, Xu X, Li S, Zheng X and Xie L: MicroRNA-101 suppresses motility of bladder cancer cells by targeting c-Met. Biochem Biophys Res Commun 435: 82-87, 2013.
41. Chen QY, Jiao DM, Wu YQ, Chen J, Wang J, Tang XL, Mou H, Hu HZ, Song J, Yan J, et al: MiR-206 inhibits HGF-induced epithelial-mesenchymal transition and angiogenesis in non-small cell lung cancer via c-Met/Pi3K/Akt/MAPK pathway. Oncotarget 7: 18247-18261, 2016.
42. Chen QY, Jiao DM, Wang J, Hu H, Tang X, Chen J, Mou H and Lu W: miR-206 regulates cisplatin resistance and EMT in human lung adenocarcinoma cells partly by targeting MET. Oncotarget 7: 24510-24526, 2016.
43. Yao Y, Dou C, Lu Z, Zheng X and Liu Q: MACC1 suppresses cell apoptosis in hepatocellular carcinoma by targeting the HGF/c-MET/akt pathway. Cell Physiol Biochem 35: 983-996, 2015.
44. Trovato M, Torre ML, Ragonese M, Simone A, Scarfi R, Barresi V, Giuffrè G, Benveniga S, Angileri FF, Tuccari G, et al: HGF/c-Met system targeting PI3K/AKT and STAT3/phosphorylated-STAT3 pathways in pituitary adenomas: An immunohistochemical characterization in view of targeted therapies. Endocrine 44: 735-743, 2013.
45. Jiang J, Feng X, Zhou W, Wu Y and Yang Y: MiR-128 reverses the gefitinib resistance of the lung cancer stem cells by inhibiting the c-Met/Pi3K/Akt pathway. Oncotarget 7: 73188-73199, 2016.
46. Li B, Tsao SW, Li YY, Wang X, Ling MT, Wong YC, He QY and Cheung AL: Id-1 promotes tumorigenicity and metastasis of human esophageal cancer cells through activation of PI3K/AKT signaling pathway. Int J Cancer 125: 2576-2585, 2009.
47. Yoshio A, Miyata H, Doki Y, Yasuda T, Yamasaki M, Motoori M, Okada K, Matsuyama J, Makari Y, Sohma I, et al: The activation of Akt during preoperative chemotherapy for esophageal cancer correlates with poor prognosis. Oncol Rep 19: 1099-1107, 2008.
48. Zhao H, Yang J, Fan T, Li S and Ren X: RhoE functions as a tumor suppressor in esophageal squamous cell carcinoma and modulates the PTEN/PI3K/Akt signaling pathway. Tumour Biol 33: 1363-1374, 2012.
49. Liu M, Hu Y, Zhang MF, Luo KJ, Xie XY, Wen J, Fu JH and Yang H: MMP1 promotes tumor growth and metastasis in esophageal squamous cell carcinoma. Cancer Lett 377: 97-104, 2016.

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