**MicroRNA-590 inhibits migration, invasion and epithelial-to-mesenchymal transition of esophageal squamous cell carcinoma by targeting low-density lipoprotein receptor-related protein 6**

HONGYA GUAN¹, JIA LIU¹, PENGJU LV¹, LIJUAN ZHOU¹, JIANYING ZHANG² and WEI CAO¹

¹Department of Translational Medicine Center, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou, Henan 450007; ²Henan Academy of Medical and Pharmaceutical Sciences, Zhengzhou University, Zhengzhou, Henan 450001, P.R. China

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**Abstract.** MicroRNA-590 (miR-590) has been revealed as a tumor suppressor, while low-density lipoprotein receptor-related protein 6 (LRP6) is considered to act as a tumor promoter. However, their roles and underlying molecular regulatory mechanisms in esophageal squamous cell carcinoma (ESCC) have yet to be fully elucidated. Therefore, the present study aimed to investigate these mechanisms. The expression levels of miR-590 and LRP6 in human ESCC samples and cell lines were determined using reverse transcription-quantitative PCR. Bioinformatics analysis was used to predict the relationship between miR-590 and LRP6, and luciferase assay was performed to validate the relationship between these factors. Transwell assays were used to determine cell migration and invasion, while western blotting assays were used to detect the protein expression levels of LRP6, E-cadherin, N-cadherin and Vimentin. The present study demonstrated that miR-590 was downregulated and LRP6 was upregulated in ESCC tissues and cell lines. Furthermore, it was found that miR-590 overexpression and LRP6 knockdown inhibited cell migration, invasion and epithelial-to-mesenchymal transition (EMT) in ESCC cell lines. Additional mechanistic studies identified that LRP6 was a target of, and was inhibited by, miR-590. Collectively, the present findings suggested that miR-590 inhibited the invasion, migration and EMT of ESCC cells by mediating LRP6.

**Introduction**

Esophageal cancer (EC) is the 6th leading cause of cancer-related mortality worldwide and the incidence is 45,600 cases each year (1). The etiology of EC varies with histological type and population. Moreover, the histopathology of EC is composed of ESCC and esophageal adenocarcinoma (EA), and ESCC accounts for ~90% of the EC incident each year. ESCC is a complex disease, and metastasis and invasion significantly promote the development and progression of ESCC. Moreover, epithelial-to-mesenchymal transition (EMT) contributes to the metastasis and invasion of ESCC cells by disassembling the cell-cell junctions, increasing cell motility and conferring invasive properties (2). Therefore, identifying the key inducers and understanding the molecular mechanisms underlying EMT in ESCC is crucial.

MicroRNAs (miRNAs/miRs) are a class of endogenous non-coding RNAs, ~18-25 nucleotides in length, which are found in eukaryotes (3). miRNAs regulate gene expression by combining with the 3'-untranslated region (3'-UTR) of their target mRNAs to then induce translational repression and/or mRNAs degradation (4). Previous studies have shown that miRNAs are implicated in numerous important physiological and pathological processes, such as stem cell differentiation (5,6), cell proliferation and apoptosis (7,8), immune responses (9), viral infection (10) and tumorigenesis (11,12). miR-590, a newly identified miRNA, was reported to act as an anti-oncogene in osteosarcoma (13), non-small cell lung cancer (14,15) and hepatocellular carcinoma (16,17). However, the clinical significance and biological mechanism of action of miR-590, as well as its association with EMT in ESCC progress remain elusive.

The present study aimed to investigate the association between the expression levels of miR-590 and low-density lipoprotein receptor-related protein 6 (LRP6) in ESCC tissues and cell lines. The effects of miR-590 and LRP6 on the migration, invasion and EMT process in ESCC cells were also examined in order to identify a novel regulatory mechanism involving the miR-590/LRP6/EMT axis, which may be of therapeutic value in the treatment of ESCC.
Materials and methods

**Tissue specimens.** A total of 28 paired human ESCC and corresponding adjacent non-tumor tissues (age range, 43-77 years; mean age, 62.79±7.09 years; males, 20; females, 8) were obtained from the Zhengzhou Central Hospital between July 2017 and June 2018. The inclusion criteria included patients who diagnosed pathologically with ESCC who had not undergone radiotherapy or chemotherapy prior to surgery. The exclusion criteria included patients who had undergone radiotherapy and chemotherapy before operation or whose tissues did not meet the pathology requirements, which were independently determined by two pathologists. All patients signed an informed consent form for having their data collected and analyzed for research purposes. The clinical specimens were promptly snap-frozen in liquid nitrogen (-196°C) after resection and then maintained at -80°C for further use. The research protocol was approved by the Ethics Review Committee of Zhengzhou University.

**RNA extraction and reverse transcription-quantitative PCR (RT-qPCR) analysis.** Total RNA was extracted from tissue samples and cultured cells using TRIzol® reagent (Thermo Fisher Scientific, Inc.). Subsequently, a Reverse Transcripase kit (Thermo Fisher Scientific, Inc.; cat. no. AB1454LDB) was used to reverse transcribe RNA into cDNA at 50°C for 15 min. qPCR was performed using Applied Biosystems™ SYBR™ Green (Thermo Fisher Scientific, Inc.) on the ABI Real-Time PCR System (Thermo Fisher Scientific, Inc.) following the manufacturer’s protocols. The relative expression was calculated using the 2-ΔΔct method (18). The expression of LRP6 was normalized to β-actin expression, and the expression of miR-590 was normalized to U6. The PCR cycle parameters for miR-590 and U6 were as follows: Initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 30 sec. The RT-qPCR parameters for LRP6 and β-actin were as follows: Initial denaturation at 95°C for 10 min, followed by 40 cycles at 95°C for 15 sec and 60°C for 1 min. The primer sequences used included: miR-590 forward, 5'-TCT AAG GCA ATA GCT CTG GGT-3'; and reverse, 5'-TGT CAG CGA AGA AGC CAT TAA A-3' and TGT CGT GGA GTC G-3'; U6 forward, 5' -TCC GAT CGT GAA and si-NC, respectively, were used as corresponding controls.

**qPCR.** The primary antibodies used (all from Abcam) were as follows:

- miR-590 mimics (or control miRNAs) and luciferase plasmids using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The sequences were as follows: miR-590 mimics forward, 5'-UAA UUU AUG UAU AAG CUAU-3' and reverse, 5'-UAGCUU AUACAUAA AUAUUU-3'; miR-590 mimics NC forward, 5'-UUUCGCAACGUGUCAGGUTT-3' and reverse, 5'-ACG UGACAGUUCGGAGAATT-3'; si-LRP6 forward, 5'-UUG CAUAAAGCAAAAGGGG-3' and reverse, 5'-CCUUUG UUGCUUUAUGCAAC-3'; and si-NC forward, 5'- UCCGAACUGUACGUGUTT-3' and reverse, 5'-ACGUGA CACGUUCGGAGAATT-3'.

**Bioinformatics predictions and luciferase assay.** Putative binding sites of miR-590 and LRP6 were predicted using Phusion® High-Fidelity DNA polymerase (New England Biolabs, Inc.) from human genomic DNA using PCR. The PCR thermocycling conditions were as follows: Initial denaturation for 3 min at 94°C, followed by 35 cycles at 94°C for 30 sec, 58°C for 30 sec and 72°C for 45 sec. The sequences of LRP6 for PCR were as follows: forward, 5'-CCGCTCGAGCTCTCTGTA CTGCCCTCAA-3' and reverse, 5'-GCTCTAGATATGCG ACAAGCAGA-3'. The LRP6 3'-UTR (including WT and mutant fragments) were inserted into pmirGLO reporter vectors (Promega Corporation). When EC-109 and EC-9706 cells had grown to 80% confluence, they were co-transfected into 40 nM miR-590 mimics (or control miRNAs) and luciferase plasmids using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After transfection for 36 h, firefly and Renilla luciferase activities were detected using the Dual-Luciferase Reporter Assay system (Promega Corporation).

**Transwell assay.** For invasion assays, the upper chamber of a 24-well Transwell insert (size, 8-µm pores; Merck KGaA) was pre-coated with 100 µl Matrigel (pre-coating at 4°C for overnight). 3x10^4 cells in serum-free medium (200 µl) were added to the upper chamber, while 500 µl culture medium was added to the lower chamber. The loaded device was then placed into an incubator for 24 h at 37°C. The invading cells were fixed with 4% paraformaldehyde for 20 min at room temperature, stained with 0.1% crystal violet for 30 min at room temperature and counted under inverted fluorescent microscope (Olympus Corporation) from five randomly selected fields (magnification, x200). For the migration assay, the experimental procedures were similar, but no Matrigel was added to the upper chamber.

**Western blotting.** After transfected cells were lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology) and determined using a bicinchoninic acid protein assay kit (Beyotime Institute of Biotechnology), an equal amount of protein (40 µg per lane) in each group was separated via SDS-PAGE (10%) and transferred onto PVDF membranes. After blocking at room temperature for 2 h with 10% skimmed milk, primary antibodies were incubated with the PVDF membranes overnight at 4°C. The primary antibodies used (all from Abcam) were as follows:
Anti-N-cadherin (1:500; Mouse; cat. no. ab98952), anti-vimentin (1:800; Mouse; cat. no. ab20346), anti-E-cadherin (1:500; Mouse; cat. no. ab219332) and anti-β-actin (1:30,000; Mouse; cat. no. ab49900). The primary antibodies were discarded, and the membranes were washed with PBS/0.1% (v/v) Tween-20 (three times; 10 min/time). Subsequently, the membranes were incubated with a horseradish peroxidase-conjugated secondary goat anti-mouse IgG antibody (1:4,000; cat. no. ab205719; Abcam) for 2 h at room temperature. Then, the membranes were washed with PBS/0.1% (v/v) Tween-20 (three times; 10 min/time) and incubated with chemiluminescence (ECL) solution (Beyotime Institute of Biotechnology.) for 1 min at room temperature on a Bio-Rad ChemiDoc MP system (Bio-Rad Laboratories, Inc.) and exposed in a dark room. ImageJ software version 1.46 (National Institutes of Health) was used to measure the density of the protein bands.

Statistical analysis. SPSS 21.0 software (IBM Corp.) was used to analyze data. Data are presented as the mean ± standard deviation. Each experiment was repeated ≥3 times. Student's t-test was used to analyze the differences between two groups, and one-way ANOVA followed by Tukey's post hoc test was applied to analyze the differences among ≥3 groups. A paired t-test was used to analyze the statistical differences between tumors and adjacent non-tumor samples from the same individual. Fisher's exact test was used to assess the association between the expression of LRP6 or miR-590 and clinicopathological features. The correlation of LRP6 and miR-590 in tissue samples was evaluated using Pearson's correlation coefficient. P<0.05 was considered to indicate a statistically significant difference.

Results

Expression of miR-590 is low, while that of LRP6 is high in human ESCC tissues and cell lines. The RT-qPCR results demonstrated that miR-590 was significantly downregulated and LRP6 was upregulated in tumor tissues compared with healthy tissues (Fig. 1A and B). It was then examined whether the expression pattern of miR-590 and LRP6 was the same in ESCC cell lines; miR-590 was also significantly downregulated and LRP6 was upregulated in ESCC cell lines, compared with HET-1A cells (Fig. 1C and D). Pearson's correlation analysis identified that there was a negative correlation between miR-590 and LRP6 expression in ESCC tissues (Fig. 1E).

Overexpression of miR-590 inhibits migration and invasion in EC-109 and EC-9706 cells. Following transfection of miR-590 mimics, its expression was significantly increased in EC-109 and EC-9706 cells (Fig. 2A). Moreover, the Transwell assay results demonstrated that cell migratory and invasive activities were significantly reduced in EC-109 and EC-9706
Overexpression of miR-590 inhibits EMT of EC-109 and EC-9706 cells. Subsequently, the expression levels of EMT-related molecular markers were assessed. It was found that miR-590 overexpression led to downregulation of N-cadherin and vimentin, and upregulation of E-cadherin expression in EC-109 and EC-9706 cells compared with the NC group (Fig. 3A-D), indicating that miR-590 inhibited the EMT process in ESCC. Together with the aforementioned results, it may be inferred that miR-590 inhibits the invasive phenotype of ESCC cells.

Knockdown of LRP6 inhibits migration and invasion of EC-109 and EC-9706 cells. To evaluate the effects of LRP6 on ESCC cells, siRNAs targeted LRP6 and NC were designed and transfected into EC-109 and EC-9706 cells. After transfection of si-LRP6, LRP6 expression was significantly decreased in EC-109 and EC-9706 cells (Fig. 4A). In addition, Transwell assay results identified that cell migratory and invasive abilities were significantly reduced in EC-109 and EC-9706 cells following transfection with si-LRP6 (Fig. 4B-E), which indicated that downregulation of LRP6 exerted the same effect as overexpression of miR-590.

Knockdown of LRP6 inhibits EMT of EC-109 and EC-9706 cells. Since LRP6 silencing inhibited cell invasion and migration, it was then investigated whether these effects were mediated via inhibition of the EMT process. The protein expression levels of N-cadherin and vimentin were significantly downregulated, while the expression of E-cadherin was upregulated after LRP6 silencing (Fig. 5A-D). These results suggested that downregulation of LRP6 suppresses EMT in ESCC.

LRP6 is a potential target of miR-590. miRNAs can regulate their targets to exert their biological effects (19,20). For example, in glioma, miR-320a inhibits cell invasion and migration by targeting aquaporin 4 (21), and in liver cancer, miR-498 inhibits cell proliferation and metastasis by targeting zinc finger E-box binding homeobox 2 (22). Therefore, the association between miR-590 and LRP6 was examined, and it was identified that LRP6 served as a potential target of miR-590 (Fig. 6A), which was further assessed with a luciferase reporter assay. The luciferase results demonstrated the interactions of miR-590 with LRP6 (Fig. 6B). In addition, western blotting was used to demonstrate that miR-590 suppressed the protein expression of LRP6 in EC-109 and EC-9706 cells (Fig. 6C and D). Collectively, the results suggested that LRP6 may serve as a potential target of miR-590.

Discussion

Over the past few years, previous studies have reported that non-coding RNAs, including miRNAs and long non-coding RNAs, serve important roles in cancer (23-25). For example, miR-543 was observed to promote ESCC cell migration, invasion and EMT via targeting phospholipase A2 Group IVA (26). It has also been shown that miR-145 can promote the proliferation and metastasis of ESCC cells by targeting SMAD5 (27). Moreover, miR-590 was revealed to inhibit the invasion and metastasis of triple-negative breast cancer (28). Similar
anti-tumor effects have been reported in osteosarcoma (13) and tongue SCC (29). The present study investigated miR-590 expression, which was found to be significantly decreased in ESCC tissues and cell lines.

LRP6, a member of the low-density lipoprotein receptor family, has been reported to be closely associated with the invasion, metastasis and EMT of cancer cells (30,31). Furthermore, LRP6 is a key receptor protein in the Wnt signaling pathway, and activates Wnt signaling by binding to the Wnt ligand protein (32). In the present study, LRP6 expression was found to be upregulated, which was consistent with other studies reporting that LRP6 expression was also increased in different tumor types, such as human triple negative breast cancer (31) and prostate cancer (33). It is well known that miRNAs may exert their biological roles via the regulation of their target mRNAs (19-22). Therefore, the current study investigated the association between miR-590 and LRP6 by analyzing their expression in 28 pairs of ESCC tissues, and subsequently identified a weak negative correlation between the two factors.

The functional roles of miR-590 and LRP6 in ESCC cells were also examined in the present study. Following transfection of miR-590 mimics into EC-109 and EC-9706 cells, Transwell assays were performed, the results of which demonstrated that miR-590 suppressed cell migration and invasion. EMT is a key mechanism involved in cancer metastasis, which includes the process of polarized epithelial cells transforming into motile stromal cells (34,35). EMT is also considered to be one of the most important mechanisms for promoting tumor migration and invasion. For instance, it has been shown that a number of key EMT-related factors, such as E-cadherin, N-cadherin and vimentin, serve important roles in tumor

Figure 2. miR-590 overexpression suppresses cell migration and invasion in ESCC cells. (A) Relative expression of miR-590 in ESCC cell lines transfected with miR-590 mimics or NC was measured using reverse transcription-quantitative PCR. (B) Transwell assays demonstrated that overexpression of miR-590 (C) significantly decreased EC-109 and EC-9706 cell migration (magnification, x200). (D) Transwell assay results also indicated that miR-590 overexpression (E) reduced EC-109 and EC-9706 cell invasiveness (magnification, x200). **P<0.01 vs. NC. NC, negative control; miR, microRNA; LRP6, low-density lipoprotein receptor-related protein 6; ESCC, esophageal squamous cell carcinoma.

Figure 3. miR-590 overexpression suppresses the EMT process in esophageal squamous cell carcinoma. (A) Western blotting results of the relative expression levels of EMT-related molecular markers in (B) EC-109 and (C) those in (D) EC-9706 cells. **P<0.01 vs. NC. NC, negative control; miR, microRNA; EMT, epithelial-mesenchymal transition.
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Figure 4. Knockdown of LRP6 suppresses cell migration and invasion in ESCC cells. (A) Relative expression of LRP6 in ESCC cell lines transfected with si-LRP6 or NC was tested using reverse transcription-quantitative PCR. (B) Transwell assays suggested that LRP6 knockdown (C) significantly decreased EC-109 and EC-9706 cell migration (magnification, x200). (D) Transwell assays also demonstrated that LRP6 knockdown (E) reduced EC-109 and EC-9706 cell invasiveness (magnification, x200). *P<0.05 and **P<0.01 vs. NC. NC, negative control; si, small interfering; LRP6, lipoprotein receptor-related protein 6; ESCC, esophageal squamous cell carcinoma.

Figure 5. Knockdown of LRP6 suppresses the EMT process in esophageal squamous cell carcinoma cells. (A) Western blotting results of the relative expression levels of EMT-related molecular markers in (B) EC-109 and (C) those in (D) EC-9706 cells. *P<0.05 and **P<0.01 vs. NC. NC, negative control; si, small interfering; LRP6, lipoprotein receptor-related protein 6.

invasion and metastasis (36). Moreover, the findings of the present study suggested that miR-590 reduced the expression levels of N-cadherin and vimentin, while enhancing E-cadherin expression. This indicated that miR-590 not only inhibited cell migration and invasion, but also suppressed the EMT process in ESCC cells. However, the mechanism underlying the role of miR-590 in EMT remains unknown. Therefore, the function of LRP6 in ESCC cells was assessed, which identified that LRP6 knockdown exerted the same effects as miR-590 overexpression. The Wnt signaling pathway is often abnormally activated and is the key signal transduction pathway that induces EMT (37,38). In addition, the Wnt signaling pathway can coordinate with or antagonize other signaling pathways to regulate the proliferation, migration, invasion and EMT of tumor cells (39,40). LRP6 is a key receptor protein in the Wnt signaling pathway (41), which may explain why LRP6 knockdown inhibited the migration, invasion and EMT of ESCC cells. In order to evaluate whether the changes caused by miR-590 were mediated by LRP6 in ESCC cells, biological analysis and luciferase experiments were performed, and LRP6 was identified as one of potential targets of miR-590. Western blotting results demonstrated that the expression of LRP6 was reduced following overexpression miR-590. Therefore, these findings may explain how the overexpression miR-590 can inhibit the migration, invasion and EMT in ESCC cells. It was suggested that miR-590 targeting of LRP6 leads to inactivation of the Wnt signaling pathway, thus suppressing EMT and invasion of ESCC cells (Fig. 7). Therefore, miR-590 appears to act as a tumor inhibitor by targeting LRP6 and restricting cell migration, invasion and EMT in ESCC. However, there
were certain limitations to the present study, such as the limited number of tissue samples. The focus of future studies will be to further elucidate the roles of miR-590 and LRP6 in ESCC tissues, and identify additional potential targets and signaling pathways of miR-590, to determine whether these may interact with LRP6 to affect cell migration, invasion and EMT in ESCC.

In conclusion, the findings of the present study demonstrated that miR-590 acted as an anti-oncogene in ESCC, and inhibited ESCC cell migration, invasion and EMT by targeting LRP6. These findings suggested that the miR-590/LRP6/EMT axis may be a novel potential therapeutic target for ESCC.

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**Availability of data and materials**

The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

**Authors’ contributions**

HG performed all of the experiments and draft the manuscript. JL revised the manuscript. PL and LZ performed the statistical analysis. JZ and WC designed the study. All authors read and approved the final manuscript.

**Ethics approval and consent to participate**

The present study was approved by the Ethics Committee of the Zhengzhou University and all the participants signed...
informed consent. All patients provided informed consent to undergo the procedures and for having their data collected and analyzed for research purposes.

Patient consent for publication
Not applicable.

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

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