MicroRNA-145 Inhibits Cell Migration and Invasion and Regulates Epithelial-Mesenchymal Transition (EMT) by Targeting Connective Tissue Growth Factor (CTGF) in Esophageal Squamous Cell Carcinoma

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Background: This study investigated the mechanism of miR-145 in targeting connective tissue growth factor (CTGF), which affects the proliferation, migration, invasion, and epithelial-mesenchymal transition (EMT) of ESCC cells.

Material/Methods: A total of 50 ESCC tissues and their corresponding normal adjacent esophageal tissue samples were collected. Then, miR-145 expression in both ESCC clinical specimens and cell lines was detected using quantitative real-time PCR. CTGF protein was detected using immunohistochemistry. Dual luciferase reporter gene assay was employed to assess the effect of miR-145 on the 3’UTR luciferase activity of CTGF. Eca109 cells were transfected with miR-145 mimics and CTGF siRNA, respectively, and changes in cellular proliferation, migration, and invasion were detected via MTT assay, wound-healing assay, and Transwell assay, respectively. Western blotting assay was used to detect the expression of marker genes related to EMT.

Results: MiR-145 was significantly down-regulated in ESCC tissues and cell lines compared with normal tissues and cell lines (P<0.05). We found significantly more positively expressed CTGF protein in ESCC tissues was than in normal adjacent esophageal tissues (P<0.01). Dual luciferase reporter gene assay showed that miR-145 can specifically bind with the 3’UTR of CTGF and significantly inhibit the luciferase activity by 55% (P<0.01). Up-regulation of miR-145 or down-regulation of CTGF can suppress the proliferation, migration, invasion, and EMT process of ESCC cells.

Conclusions: MiR-145 was significantly down-regulated in ESCC tissues and cell lines, while the protein expression of CTGF exhibited the opposite trend. MiR-145 inhibited the proliferation, migration, invasiveness, and the EMT process of ESCC cells through targeted regulation of CTGF expression.

MeSH Keywords: Cell Proliferation • Enoxaparin • Esophageal Diseases

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**Background**

Esophageal cancer (EC) is a common cancer that is often diagnosed at an advanced stage. Patients with EC do not have specific symptoms until they reach a very late stage in which unfavorable prognostic outcomes usually occur [1]. Esophageal squamous cell carcinoma (ESCC) is defined as a major histological type of esophageal carcinoma and it has been ranked as the fourth most dangerous malignancy in China, with an unexpectedly high fatality rate [2]. Radical surgery alone or combined with radiotherapy or chemotherapy has substantially improved the prognosis of ESCC patients [3,4]. However, potential radiation hazards and limited effectiveness of surgery have motivated researchers to introduce alternative therapeutic approaches for managing patients with ESCC. Also, clarification of the molecular mechanisms in ESCC progression is believed to provide improvements in disease diagnosis.

As noncoding RNAs, microRNAs (miRNAs) contain 21–25 nucleotides and mature miRNAs play vital regulatory roles in cell proliferation, differentiation, and apoptosis [5]. Cell proliferation generally exerts bad influences in some diseases [6,7]. Regulating gene expression at the transcriptional level is the main function of miRNA; growing evidence indicates that miRNAs are abnormally expressed in various human cancers and they have significant effects on cancer progression and carcinogenesis [8–10]. As predicted by bioinformatic analyses, single miRNA has various targets; therefore, miRNAs are able to mediate numerous protein-coding genes and approximately one-third of human genes are regulated by miRNAs [11,12]. Among all miRNAs, down-regulated miR-145 has been observed in multiple types of cancers, including lung cancer [13], renal cell cancer [14], prostate cancer [15], bladder cancer [16], and colon cancer [17]. Moreover, miR-145 has been verified to inhibit tumor cell proliferation and invasion; therefore, it is considered as a presumptive tumor suppressor [18]. Studies also have confirmed that miR-145 plays an important tumor-suppressing role in ESCC [7,19–21]. The relationship between miR-145 and ESCC should be further studied.

CTGF is an immediate-early gene product of the CCN family and has certain appropriate expression levels under normal physiological conditions [22,23]. Imbalanced CTGF expression may trigger several pathological states, such as arthritis, fibrosis, and cancers [24]. Previous studies reported that CTGF as a downstream effector of the TGF-β pathway was over-expressed and indirectly exacerbated the cellular invasiveness and proliferation in ESCC [25,26]. Furthermore, connective tissue growth factor (CTGF) has been identified as a unique target of miR-145 in glioma cells [27], but such a relationship has not been confirmed between miR-145 and CTGF in ESCC. Epstein-mesenchymal transition (EMT) is a signal pathway that contains a key step involved in the evolution of tumor cell metastasis, including successive cell detaching, migrating, invading, dispersing, and residing [28]. EMT has been classified as a hallmark of tumor metastasis and it is associated with various transcriptional factors [29–31]. Tumor biology studies indicated that signaling pathways, including TGF-β, Notch, and Wnt, as well as growth factors such as FGF and PDGF may induce EMT [32]. Mechanism analysis suggested that CTGF can regulate EMT in several types of cancer [33,34].

Therefore, in the current study, the expression level of miR-145 and CTGF in ESCC and corresponding normal adjacent esophageal tissues were detected. We found that miR-145 can suppress cell proliferation, migration, invasion, and EMT process by directly targeting CTGF. The aims of this study were to further systematically clarify the potential function of miR-145 targeting CTGF in ESCC and promote the development of an effective diagnosis or therapeutic strategy for ESCC.

**Material and Methods**

**Clinical specimens and cell lines**

Fifty pairs of primary ESCC tissues and corresponding normal adjacent esophageal tissues were taken from patients (38 males and 12 females) undergoing esophagectomy at the Fifth Affiliated Hospital of Sun Yat-Sen University between January 2013 and July 2015. Ages of involved patients ranged from 47 to 76 years with a median age of 63. No patients underwent radiotherapy, chemotherapy, or hormone therapy before surgery. Tissue samples were immediately frozen in liquid nitrogen and stored at –80°C until the commencement of RNA extraction. Another part of tumor tissues was fixed with 10% formalin, embedded in paraffin, and analyzed using immunohistochemistry. Histological diagnoses of ESCC were conducted by a pathologist. Informed consent was obtained from all patients and the Research Ethics Committee of the Fifth Affiliated Hospital of Sun Yat-Sen University approved this study.

Human esophageal endothelial cell line HEEC was purchased from ScienCell Research Laboratories (ScienCell, USA) and cultured in Epithelial Cell Medium-2 (ScienCell) containing epithelial cell growth supplement-2 (ScienCell). All human ESCC cell lines used in this study, including EC1, Eca109, KYSE150, and KYSE180, were acquired from the Cell Center of the Shanghai Institute of Life Science (Chinese Academy of Science, Shanghai, China) and were cultured in RPMI1640 medium ( Gibco, USA) containing 10% fetal bovine serum (FBS; Gibco), streptomycin (100 mg/ml; Gibco), and penicillin (100 units/ml; Gibco) at 37°C in an incubator with 5% CO₂.
Cell transfection

Eca109 cells were transfected into 5 different groups: blank group, scramble group, miR-145 mimics group, miR-145 inhibitor group, and CTGF siRNA group. Eca109 cells in the blank group were transfected without any sequence. Eca109 cells in the other 4 groups were separately transfected with scramble miRNA mimics as the negative control, miR-145 mimics, miR-145 inhibitors, and CTGF siRNA, which were all synthesized from GenePharma (Shanghai, China). Transfection was carried out using Lipofectamine 2000 (Invitrogen, USA) and cells were cultured at 37°C in an incubator with 5% CO₂. Complete medium was replaced after 6–8 h and then cell culture was continued. Cells were harvested for further experiments after 48-h transfection.

RNA extraction and RT-PCR

Total RNA extraction from human ESCC tissues and cells was conducted using TRIzol reagent kit (Invitrogen) strictly following the manufacturer’s instructions. Complementary DNA (cDNA) was acquired using the Omniscript reverse transcription kit (Qiagen, Germany). Real-time quantitative RT-PCR assay was conducted by ABI7500 quantitative PCR instrument (Applied Biosystems) for detecting the relative expression levels of miR-145 and CTGF mRNA. The primers of miR-145 and CTGF (purchased from Invitrogen) were set as: miR-145 forward, 5'- TCGGTCCAGTTTTCCAG-3' and reverse, 5'-AGTGCGTGTCGTGGAGTC-3'; CTGF sense, 5'-CCCAAGGACCCAAACCGTG-3' and antisense, 5'-CTAATCATAGTTGGGTCTGGGC-3'. The relative expression level of miR-145 and CTGF mRNA were calculated using the 2ΔΔCT method and they were normalized to the expression quantity of U6 snRNA.

Immunohistochemistry

CTGF protein expression in ESCC tissues was detected using the immunohistochemistry universal PV-9000 two-step method. In brief, formalin-fixed and paraffin-embedded tissues from 50 ESCC patients were cut into 4-μm slices. Then, tissue slices underwent conventional dewaxing, graded ethanol dehydration, antigen retrieval, and addition of 3% hydrogen peroxide to block endogenous peroxidase. Primary antibodies (mouse anti-human CTGF monoclonal antibody, Bioss, China) were applied to tissues slices at 4°C overnight. Then, tissues were incubated for 20 min at room temperature after the addition of polymerase adjuvants. Secondary antibody labeled with HRP (Bioss) was also applied and tissues were incubated for another 30 min at room temperature. Staining was performed by diaminobenzidine (DAB) and slices were counterstained using hemalum. Phosphate-buffered saline (PBS) instead of a primary antibody was caused as the negative control and a known positive antibody was set as the positive control. The integral calculation of CTGF complied with both the product of staining intensity (3 – brown; 2 – yellow; 1 – light yellow; 0 – colorless) and positive cell percentage (4, >75%; 3, 51–75%; 2, 26–50%; 1, 6–25%; 0, ≤5%). Cells were randomly selected from 5 high-power fields (× 400) in each slice and 100 cells were counted in each field. As suggested by the double types of scores, the integral levels of CTGF were evaluated as: negative (−), 0–4 points; weakly positive (+), 5–8 points, and strongly positive (++), 9–12 points. Two independent pathologists were responsible for analyzing the slices.

Dual-luciferase reporter assay

MiRNA targets were predicted using the TargetScan system (https://www.targetscan.org). We constructed luciferase reporter vectors for the wild-type and mutant-type of CTGF 3’ untranslated regions (UTR). Either miR-145 mimics or control were co-transfected with the constructed wild-type or mutant-type luciferase reporter vector into Eco109 cells using Lipofectamine 2000 (Invitrogen). The pGL3 control vector (Promega, USA) was transfected and served as the control. The luciferase activity was assessed using the Dual-Luciferase Reporter Assay System (Promega) after 48-h cell transfection.

MTT assay

Cell proliferation was evaluated by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-tetrazolium bromide] assays. Briefly, transfected cells washed twice using PBS were cultured until a density of 80% was reached. Cells were digested with trypsin into cell suspensions and then the number of cells was counted using a cell counter. Eca109 cells were inoculated into 96-well plates with 3×10³ cells/well. When cells were cultured to the level of 90% confluence, monolayer cells were scratch-wounded using a sterilized 1000-µl pipette tip. Then cells were washed with PBS 3 times to remove the detached cells, then cells were
cultured for another 48 h. Migration distances of cells were imaged and monitored using an inverted microscope (Carl Zeiss AG, Germany) with 3 randomly selected fields in the wounded region at 0 h, 24 h, and 48 h. The percentage of wound width was calculated as: the wound width at 0, 24, or 48 h/the original wound width measured at 0 h.

### Transwell migration and invasion assays

Transwell invasion assay was performed using 24-well transwell plates (8-µm pores) and a coating of Matrigel (BD Biosciences, US) was introduced to measure the invasion status of Eca109 cells. Transwell migration assay was performed without the Matrigel coating. Briefly, cells were trypsinized and resuspended in serum-free medium after 48-h transfection and 2×10^4 cells were placed in the upper transwell chamber. Then, 0.5 ml complete RPMI 1640 medium was added in the lower chamber as the chemoattractant. After cells were incubated for 24 h at 37°C, non-migratory or non-invasive cells in the top chamber were removed with cotton swabs. Migrated or invaded cells at the bottom of the membrane were fixed with methanol and then stained with crystal violet. The number of migrated or invaded cells was counted to assess the migration and invasion status of cells.

### Western blotting assay

The expression levels of CTGF, E-cadherin, N-cadherin, fibronectin, and vimentin were examined by Western blotting assay. Cellular proteins were extracted after 48-h transfection. The BCA approach was used to detect the protein density. An equal amount of proteins for each group was loaded, separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), transferred onto polyvinylidene fluoride membranes, and blocked with 5% skim milk. Membranes were incubated with primary antibodies (CTGF, E-cadherin, N-cadherin, fibronectin, and vimentin) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (CST, American), respectively, at 4°C overnight. Membranes were washed with TBST 3 times (10 min each) and incubated with horseradish-peroxidase-linked secondary antibodies at room temperature for 1 h. After that, membranes were washed again with TBST another 3 times (10 min each) and signal detection was carried out using Super ECL Plus Detection Reagent (Applygen Technologies Inc., China).

### Statistical analysis

Statistical analyses were performed using SPSS 19.0 software. Significant differences in continuous data (mean ±SD) were estimated using the analysis of variance (ANOVA). Differences between 2 groups were analyzed by unpaired t tests. Results from the immunohistochemistry of CTGF protein were analyzed by the rank sum test. Two-sided P value <0.05 was defined as the cut-off value for evaluating statistical significance.

### Results

**MiR-145 was down-regulated in human ESCC cell lines and clinical specimens**

Quantitative real-time PCR was used to evaluate the expression level of miR-145 in 50 ESCC tissues and matched normal adjacent esophageal tissues (Figure 1A) as well as in ESCC cell lines (Figure 1B). MiR-145 expression in ESCC tissues was significantly down-regulated compared to that in normal adjacent esophageal tissues (P<0.05). MiR-145 expression was also significantly down-regulated in ESCC cell lines in comparison to normal esophageal endothelial cell line HEEC (P<0.05),
while the lowest level of miR-145 expression was observed in Eca109 cell lines. Therefore, Eca109 cell lines were used to perform subsequent experiments.

CTGF expression in ESCC clinical specimens

The protein expression of CTGF in ESCC tissues and their corresponding normal adjacent esophageal tissues was detected by immunohistochemistry. Positively expressed CTGF was mainly located in cell cytoplasm with yellow granules (Figure 2). Positively expressed CTGF proteins in ESCC tissues significantly exceeded those in normal adjacent esophageal tissues (P<0.01) (Table 1).

Targeting CTGF by miR-145

A putative conserved binding site of miR-145 at nucleotide position 31–37 of human CTGF 3’UTR was predicted by the TargetScan database. Perfect base pairing was observed between the seed sequence of mature miR-145 and the 3’UTR of CTGF mRNA (Figure 3A). Dual luciferase reporter gene assays revealed that miR-145 weakened the luciferase activity of CTGF wild-type by 55% (P<0.01). However, miR-145 did not significantly affect the luciferase activity of CTGF with mutant-type 3’UTR (Figure 3B). As suggested by RT-PCR and Western blot assays, CTGF mRNA and protein expressions were significantly inhibited in the miR-145 mimics group and stimulated in the miR-145 inhibitors group compared to the reference scramble group (P<0.05) (Figure 3C, 3D). Collectively, the above findings indicated that CTGF was a direct target of miR-145.

MiR-145 targeted CTGF for suppressing ESCC cell proliferation

As suggested by MTT assays, significantly constrained proliferation of Eca109 cells was observed when cells were transfected with miR-145 mimics and CTGF siRNA for 48 h compared with the scramble group and blank group (all P<0.05). Furthermore, this trend appeared to become more significant over time. In contrast, no significant difference in the proliferation status of Eca109 cells was observed between the miR-145 mimics and CTGF siRNA group (P>0.05). No notable difference in cell survival ability was detected between the blank and scramble group at each time point (all P>0.05) (Figure 4).

MiR-145 inhibited ESCC cell migration

As suggested by the transwell migration assay, there were significantly fewer migrated cells in the miR-145 mimics (65.5±5.8) and CTGF siRNA (69.3±4.6) group than in the blank (117.2±7.5) and scramble groups (112.6±6.2) (all P<0.05), while the difference between the miR-145 mimics group and CTGF siRNA group was insignificant (P>0.05) (Figure 5A). The wound healing assay also found that the percentage of wound width in the blank and scramble group were significantly less than those in the miR-145 mimics and CTGF siRNA group, both at 24 h and 48 h (all P<0.05) (Figure 5B). Therefore, up-regulation of miR-145 or down-regulation of CTGF can restrict the migration ability of Eca109 cells.
MiR-145 inhibited ESCC cell invasion

As shown in Figure 6, transwell matrigel invasion assay indicated that the number of invaded cells in the blank and scramble groups were 78.5±4.9 and 75.6±5.2, respectively, with no significant difference (P>0.05). Invaded Eca109 cells transfected with miR-145 mimics (42.8±5.5) and CTGF siRNA (45.2±4.2) were notable fewer than those in the blank and scramble group (all P<0.05), while the difference between the miR-145 mimics and CTGF siRNA groups was insignificant (P>0.05). These data indicate that up-regulation of miR-145 or down-regulation of CTGF can suppress the invasive ability of Eca109 cells.

MiR-145 repressed EMT in ESCC cells

In order to understand how miR-145 and CTGF siRNA inhibited the migration and invasion of Eca109 cells, the protein expressions of EMT markers were examined via Western blot analysis. The results indicated that N-cadherin, which is a mesenchymal marker and supposed to be up-regulated during EMT, was decreased in Eca109 cells transfected with miR-145 mimics or CTGF siRNA when compared with the blank and scramble groups. Moreover, fibronectin and vimentin, another mesenchymal marker, was also down-regulated in Eca109 cells when transfected with miR-145 mimics or CTGF siRNA. However, E-cadherin, which is an epithelial marker and should be down-regulated during EMT, was promoted in Eca109 cells transfecteded with miR-145 mimics or CTGF siRNA when compared to the blank and scramble groups (Figure 7). These findings indicate that miR-145 repressed the migration and invasion of Eca109 cells through inhibiting the EMT process.

Discussion

Many studies have demonstrated that miRNAs regulate the progression of various tumors [35], and miR-145 plays a particularly crucial role in many cancers, including glioma [27], breast cancer [36], renal cell carcinoma [37], and prostate cancer [38].
Our results show that miR-145 is dramatically down-regulated in ESCC cell lines and ESCC tissues and these conclusions are similar to those from previous studies [7,39]. CTGF is a member of the CCN family and has been reported to participate in cell proliferation, adhesion, and tumor angiogenesis [22]. Several studies have reported that CTGF was not only up-regulated in ESCC, but also stimulated the development and progression of ESCC [25,26]. Results from immunohistochemistry confirmed that CTGF protein levels in ESCC tissues were significantly elevated compared to those in normal adjacent esophageal tissues (P<0.01).

Generally, miRNAs mediate a series of biological processes via different target sites and they also regulate the expression of their downstream targeting mRNAs [40,41]. Many studies demonstrated that miR-145 influence tumor development by mediating different downstream targeting mRNAs, including c-Myc in breast cancer [42] and human lung cancer [43], FSCN1 in prostate cancer [44], ANGPT2 and NEDD9 in renal cell carcinoma [37], and Sox2 in human choriocarcinoma cells [45]. In this study, we predicted that CTGF was a target gene of miR-145 using the TargetScan database. Furthermore, dual luciferase reporter gene assay enabled us to prove that miR-145 is able to directly bind with the 3’UTR of CTGF mRNA. Moreover, our study demonstrated that miR-145 mimics inhibited the mRNA and protein expression level of CTGF in Eca109 cells, and miR-145 inhibitors can significantly elevate CTGF expression in Eca109 cells. Thus, our results provide evidence that gene and transcription levels of CTGF are modulated by miR-145.

Accordingly, Lee et al. reported that miR-145 regulates cell migration in glioma by targeting CTGF [27].

MiRNAs are known for their multifunctional roles in cell differentiation, apoptosis, proliferation, and metabolism [46,47]. To determine the regulatory roles of miR-145 that target CTGF in ESCC, Eca109 cells were separately transfected with miR-145

![Figure 5. The migration of Eca109 cells was suppressed by miR-145. (A) Transwell migration assay. (B) Wound healing assay. Photomicrographs were taken at 0, 24, and 48 h after cell wounding. The results (mean ±SD) were from 6 independent experiments. * P<0.05 vs. the blank and scramble group.](image-url)
mimics and CTGF siRNA and changes in cellular proliferation, migration, and invasion were assessed. Our findings reveal that up-regulated miR-145 significantly inhibits the proliferation, migration, and invasion of Eca-109 cells. Consistent with our findings, it was reported that over-expression of miR-145 inhibits the proliferation and invasion of human choriocarcinoma [45] and prostate cancer [44]. In addition, we discovered that the proliferation, migration, and invasion of Eca-109 cells were inhibited by miR-145. The invasion ability of cells at 48 h after transfection was detected by a transwell invasion assay. The results (mean ±SD) were from 6 independent experiments. * P<0.05 vs. the blank and scramble group.

Figure 6. The invasion of Eca109 cells were inhibited by miR-145. The invasion ability of cells at 48 h after transfection was detected by a transwell invasion assay. The results (mean ±SD) were from 6 independent experiments. * P<0.05 vs. the blank and scramble group.

Figure 7. The effect of miR-145 on EMT by targeting CTGF in Eca109 cells. Results from Western blot analysis showed that over-expression of miR-145 and down-regulation of CTGF were associated with increased E-cadherin expression and suppressed N-cadherin, fibronectin, and vimentin expression.

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were restricted by the knockdown of CTCF. Deng et al. reported that knockdown of CTGF in ESCC cells significantly inhibit cell growth, colony formation, and tumorigenicity in vivo [26]. These results suggest that miR-145 and CTGF may have significant effects on ESCC pathogenesis.

EMT not only triggers the invasion and metastasis of tumors, but also endows cancer cells with immortalized proliferation [48]. Importantly, many studies have validated that miRNAs play a key role in tumor metastasis by regulating EMT. For instance, previous studies demonstrated that over-expression of miR-145 can repress EMT in both prostate cancer [38,49] and breast cancer [36]. Our experiments indicated that miR-145 mimics inhibit the expression of several mesenchymal markers, including N-cadherin, fibronectin, and vimentin, and stimulate the expression of E-cadherin, which is a typical epithelial cell maker. Additionally, siRNA-mediated knockdown of CTGF specifically suppressed N-cadherin, fibronectin, and vimentin expression, and this is associated with increased E-cadherin expression. Results from our study are consistent with previous reports concluding that CTGF contributes to changes in epithelial cells both in vivo and in vitro, and CTGF promotes EMT in neuroblastoma cells [50,51]. Hence, all of these results suggest that miR-145, which targets CTGF, inhibits ESCC cell migration and invasion through the suppression of EMT.

The present study has several limitations. Although we demonstrated the relationship between miR-145 and CTGF in ESCC, our study had a relatively small sample size due to limited resources. In addition, only the Eca109 cell line was used to perform experiments of cell transfection. The molecular mechanism of miR-145 and CTGF in ESCC progression needs further study.

Conclusions

In conclusion, miR-145 expression exhibited down-regulation in ESCC cell lines and ESCC tissues. Our experiments further demonstrated that both over-expression of miR-145 and knockdown of CTGF inhibited the proliferation, migration, and invasion of ESCC cells through their influence on EMT. Therefore, miR-145 and CTGF can potentially be considered as therapeutic targets or diagnostic biomarkers of ESCC.

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