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MEG3 inhibits cell proliferation, invasion and epithelial-mesenchymal transition in laryngeal squamous cell carcinoma

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Abstract. – OBJECTIVE: Dysregulation of long non-coding RNAs (lncRNAs) is associated with human carcinogenesis. The aim of the study is to explore the biological functions of MEG3 expression in laryngeal squamous cell carcinoma (LSCC).

PATIENTS AND METHODS: QRT-PCR analysis was performed to assess the expression of MEG3 in 35 pairs of LSCC tissues and adjacent non-cancerous tissues. Cell proliferation, cell migration, and cell invasion capacities were determined by CCK8 assay and transwell assay in Hep-2 cell. QRT-PCR and Western blot analysis were applied to detect the relative expression of Twist1, E-cadherin and Vimentin in Hep-2 cells.

RESULTS: In the study, our results showed that MEG3 expression was significantly lower in tumor tissues compared with that in adjacent non-cancerous tissues. Lower MEG3 expression was significantly associated with lymph node metastasis and advanced TNM stage of patients. Knockdown of MEG3 significantly promotes cell proliferation, cell migration, cell invasion and Epithelial-Mesenchymal Transition (EMT) process by upregulating Twist1 and Vimentin expression and reducing E-cadherin expression in Hep-2 cell. Conversely, upregulation of MEG3 had the inhibiting effects in Hep-2 cell.

CONCLUSIONS: These results suggested that MEG3 may serve as a novel potentially therapeutic target for LSCC treatment.

Key Words:
Laryngeal squamous cell carcinoma, MEG3, Cell invasion, Epithelial-mesenchymal transition.

Introduction

Laryngeal squamous cell carcinoma (LSCC) is a common aggressive head and neck cancer with high mortality and incidence[1]. Some novel advances for LSCC treatment including radiation therapy, chemotherapy and surgery have improved patients' prognosis at early stage of disease. However, patients who were diagnosed at late stage remain a poor prognosis[2,3]. Thus, it is important to identify novel target of therapy for LSCC patients.

Long non-coding RNAs (lncRNAs) are a novel class of non-protein coding RNA molecules with more than 200 bases in length[4, 5]. Deregulation of lncRNAs expression has recently been found to be involved in the pathogenesis of some types of cancer including laryngeal squamous cell carcinoma. Such as, decreased expression levels of AC026166.2-001 and RP11-169D4.1-001 were associated with poorer prognosis[6]. HOTAIR levels were significantly higher in laryngeal squamous cell carcinoma (LSCC) and regulated PTEN methylation in LSCC cells[7]. Combined detection of serum exosomal miR-21 and HOTAIR served as diagnostic and prognostic biomarkers for laryngeal squamous cell carcinoma[8]. Long non-coding RNA NEAT1 promoted laryngeal squamous cell cancer through regulating miR-107/CDK6 pathway[9]. H19 expression promoted LSCC cell migration, invasion and proliferation via miR-148a-3p and targets DNMT1[10]. However, the biological role of MEG3 in LSCC is still unknown.

We demonstrated that MEG3 was downregulated in human LSCC tissues. Lower MEG3 expression was associated with lymph node metastasis and advanced clinical stage. Moreover, upregulation of MEG3 in Hep-2 cells significantly inhibited cell proliferation, migration, invasion, and EMT process. Thus, our results suggest that MEG3 may serve as a novel therapeutic target in LSCC.

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Patients and Methods

Patient Tissue Samples

35 cases of pairs tumor tissues and adjacent non-cancerous matched tissues were obtained from patients who underwent partial or total laryngectomy at Department of Otolaryngology, Daqing Oil Field General Hospital between January 2008 and March 2014. The sample consisted of 29 males and 6 females with an average age of 50.6±3.85 years. All of patients did not receive any tumor therapy before surgery. The tissues were preserved in liquid nitrogen and then were transported frozen to the laboratory and stored at -80°C for analysis. The study was approved by the Ethics Committee of Daqing Oil Field General Hospital and informed consent was obtained from all patients.

Quantitative Real-time PCR (QRT-PCR) Aanalysis

RNA was extracted from LSCC tissues and Hep-2 using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The RNA was quantified by using the NanoDrop 1000 (NanoDrop Technologies, Rockland, DE, USA). The cDNA was synthesized from RNA using the PrimeScript RT reagent kit (TaKaRa, Dalian, China). SYBR Green RT-PCR Reagents was applied to perform the quantitative PCR (TaKaRa, Dalian, China). The mRNA expression levels were measured using Applied Biosystems 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Relative mRNA was normalized against GAPDH and mRNA was calculated using the $2^{-\Delta\Delta Ct}$ method. The primers sequences used in the study are MEG3-forward: 5'-ATCATCCGTCCACCTCCTTGTCTTC-3', MEG3-reverse: 5'-GTATGAGCATAGCAAAGGTCAGGGC-3'. GAPDH-forward: 5'-AATGCCTCCTGCACCACCAAC-3', GAPDH-reverse: 5'-AAGGCCATGCCAGTGAGCTTC-3'.

Cell Line Culture

Hep-2 cell was obtained from the Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). Cells are all cultured in Roswell Park Memorial Institute (RPMI)-1640 medium supplemented with 10% fetal bovine serum (FBS, Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin, and 100 mg/ml streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA) at 37°C in humidified air containing 5% of CO_2.

Cell Transfection

The si-negative control (NC), si-MEG3-1, si-MEG3-2 oligos and the MEG3 overexpression plasmid (pcDNA3.1-MEG3) or control vector (pcDNA3.1-vector) were constructed and purchased from Genechem (Shanghai, China). Cell transfection was performed using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's instructions. After 48 h, the cells were collected for following experiments.

Cell Proliferation Assay

The transfected Hep-2 cells were detected using cell count kit 8 (CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) according to the manufacturer's instructions. Cells (2×10^3 cells/well) were seeded into 96-well plates. Cell were cultured and measured at indicated time (1, 2, 3 and 4 days). The 10 µl CCK-8 solution were added to each well and incubated for 2 h at 37°C in humidified air containing 5% of CO_2. The absorbance (A) at 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA, USA).

Cell Migration and Invasion Assay

Cell migration or invasion was performed using 24-well transwell (8 mm pore size; Corning, NY, USA) coated without or with 1 mg/mL Matrigel (BD Biosciences, Franklin Lakes, NJ, USA). The transfected cells (1×10^5) in the upper chamber were added 200 µL free fetal bovine serum (FBS) medium, and the 500 µL of 10% FBS medium was added in the lower chambers. Following incubation for 48 h, the cells were removed using cotton wool in the upper membrane. Next, cells on the lower membrane were stained with 100% methanol and 0.1% crystal violet. Then, the invasive cells were assessed in five random microscopic fields.

Western Blot Analysis

The transfected cells were washed twice with phosphate-buffered saline (PBS) and lysed on ice using radioimmunoprecipitation assay (RIPA) buffer for for 30 min (Sigma-Aldrich; St. Louis, MO, USA) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Protein were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) and incubated with specif-

ic primary antihuman antibodies with E-cadherin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Vimentin (1:500, Santa Cruz Biotechnology, Santa Cruz, CA, USA), Twist1 (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and GAPDH (1:1000, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA). An enhanced chemiluminescence kit (Millipore, Billerica, MA, USA) chromogenic substrate was performed to analyze the bands. GAPDH was used as the internal control.

Statistical Analysis

All of the experiments were independently repeated at least three times and presented as the mean ± S.D. The two-tailed Student's *t*-test or a one-way analysis of variance (ANOVA) was used to evaluate between different groups by the SPSS 18.0 software program (SPSS Inc., Chicago, IL, USA). A $p < 0.05$ was considered as statistically significant.

Results

The Expression of MEG3 is Downregulated in LSCC Tissues

The expression levels of MEG3 in 35 paired of LSCC samples and adjacent normal tissues were detected by qRT-PCR assay. As shown in Figure 1A, the MEG3 expression levels were significantly downregulated in LSCC tissues compared with adjacent normal tissues ($p < 0.05$). We then investigated the relationship between MEG3 expression and the clinical features of LSCC patients. The patients were divided into two groups based on the median expression levels of MEG3 in tumor tissues. The results revealed that lower MEG3 expression levels were significantly associated with lymph node metastasis and advanced clinical stage ($p < 0.05$, Table I).

MEG3 Suppresses Cell Proliferation, Migration and Cell Invasion in Hep-2 Cell

To investigate the functional role of MEG3 in LSCC progression, Hep-2 cells were transfected with two si-MEG3 oligos for MEG3 knockdown and pcDNA3.1-MEG3 for MEG3 overexpres-

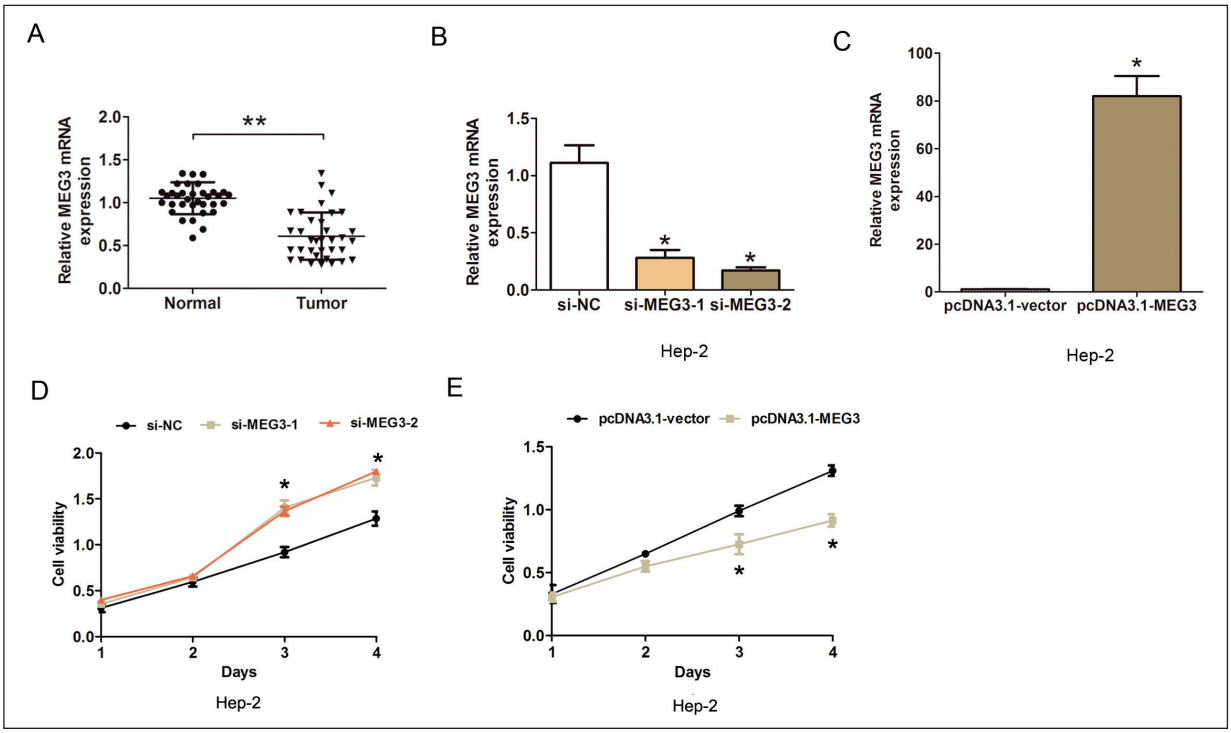

Figure 1. MEG3 was lower in LSCC tissues. ***(A)*** The relative expression of MEG3 in 35 cases LSCC tissues and adjacent non-cancerous matched tissues. ***(B)*** The relative expression of MEG3 in Hep-2 cells transfected with si-NC, si-MEG3-1, si-MEG3-2. ***(C)*** The relative expression of MEG3 in Hep-2 cells transfected with pcDNA3.1-vector or pcDNA3.1-MEG3. ***(D)*** Cell proliferation was evaluated in Hep-2 cells transfected with si-NC, si-MEG3-1, si-MEG3-2. ***(E)*** Cell proliferation was evaluated in Hep-2 cells transfected with pcDNA3.1-vector or pcDNA3.1-MEG3. $^{**}p < 0.01$, $^{*}p < 0.05$.

Table I. The relationship between expression of MEG3 and clinical factors was analyzed.

Characteristics	Patients (n=35)	MEG3 expression levels		p-value
		High (n = 19)	Low (n = 16)	
Sex				0.582
Male	29	16	13	
Female	6	3	3	
Age (years)				0.505
≤ 55	23	12	11	
> 55	12	7	5	
Lymph node metastasis				0.036[a]
Negative	22	15	7	
Positive	13	4	9	
Smoking				0.194
No	17	11	6	
Yes	18	8	10	
Classification				0.942
Supraglottic LSCC	14	8	6	
Glottic LSCC	11	6	5	
Subglottic LSCC	10	5	5	
TNM stage				0.015[a]
I and II	21	15	6	
III and IV	14	4	10	

[a]p-value < 0.05.

sion, respectively (Figure 1B-C). Cell proliferation, migration and invasion were determined by CCK8 and transwell assays. The results indicated that cell proliferation capacity in si-MEG3-1 and si-MEG3-2 groups were significantly promoted compared with si-NC group in Hep-2 cells (Figure 1D). Conversely, cell proliferation capacity in pcDNA3.1-MEG3 group was significantly inhibited compared with the pcDNA3.1-vector group in Hep-2 cells (Figure 1E). Furthermore, cell migration and cell invasion assay showed that cell migration and invasive number was increased in si-MEG3-1 or si-MEG3-2 group compared with the si-NC group in Hep-2 cell (Figure 2A-2B). However, cell migration and invasive number were decreased in pcDNA3.1-MEG3 group compared with the pcDNA3.1-vector group in Hep-2 cells (Figure 2C-2D). These results showed that MEG3 suppressed cell proliferation, migration and invasion in Hep-2 cell.

MEG3 Suppresses Epithelial-Mesenchymal Transition Process in Hep-2 Cell

To investigate the role of MEG3 in LSCC cell EMT process, the Western blot assay was performed in Hep-2 cells. We selected si-MEG3-2 for the knockdown of MEG3 due to its higher silencing efficiency for MEG3. The results confirmed that the EMT relative transcription factor Twist1 and mesenchymal maker Vimentin expression levels were increased, while E-cadherin expression was reduced in si-MEG3 group compared with the si-NC group in Hep-2 cell (Figure 3A). Conversely, EMT relative transcription factor Twist1 and mesenchymal maker Vimentin expression levels were decreased, while E-cadherin expression was increased in pcDNA3.1-MEG3 group compared with the pcDNA3.1-vector group in Hep-2 cell (Figure 3B). Thus, we confirmed that MEG3 suppressed epithelial-mesenchymal transition process in Hep-2 cell.

Discussion

Laryngeal squamous cell carcinoma (LSCC) is an aggressive malignant cancer. The regulatory roles of LSCC development and progression by long non-coding RNAs (lncRNAs) were not well understood. Recently, studies had reported that MEG3 exerted as tumor suppressors in some tumors[11], such as, decreased expression of long noncoding RNA MEG3 suppresses cell proliferation and predicts a poor prognosis for patients with colorectal cancer[12]. LncRNA-MEG3 inhibits cell proliferation of endometrial carcinoma by inhibiting Notch signaling pathway[13]. Downregulation of long non-coding

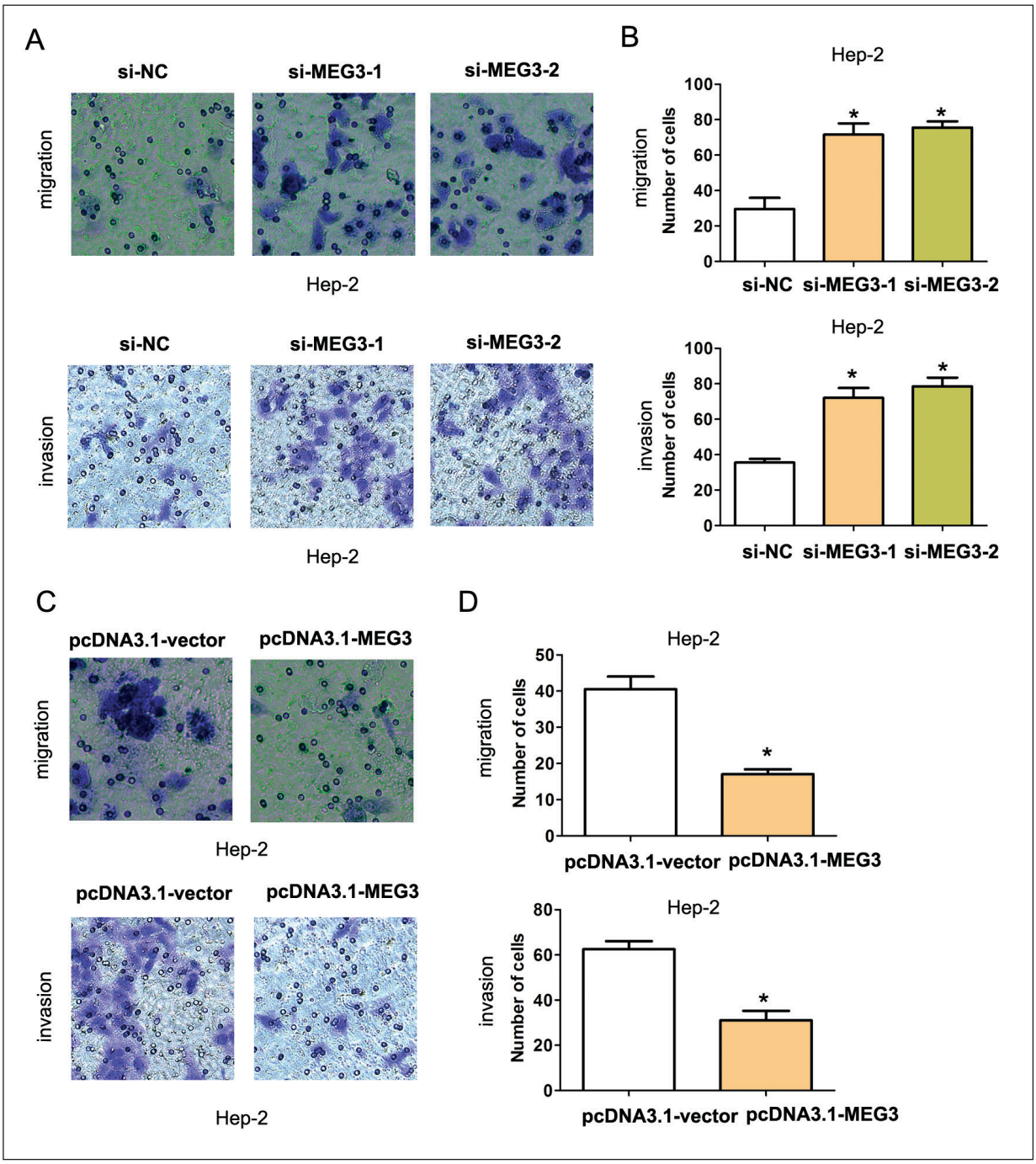

Figure 2. MEG3 suppressed cell proliferation, migration, and invasion in Hep-2 cell. ***(A-B)*** Cell migration and invasion number was evaluated in Hep-2 cells transfected with si-NC, si-MEG3-1, si-MEG3-2. ***(C-D)*** Cell migration and invasion number was evaluated in Hep-2 cells transfected with pcDNA3.1-vector or pcDNA3.1-MEG3. *$p < 0.05$.

RNA MEG3 affects proliferation, migration and invasion by depending on p53's transcriptional activity in breast cancer[14]. Reduced expression of long non-coding RNA MEG3 serves as a potential predictor biomarker in progression and poor prognosis of osteosarcoma[15]. Long noncoding RNA MEG3 is lower in cervical cancer and affects cell proliferation and cell apoptosis by regulating miR-21 expression[16]. These previous studies indicated that MEG3 act as regulators of tumor progression. In the study, we demonstrated that the MEG3 expression levels were

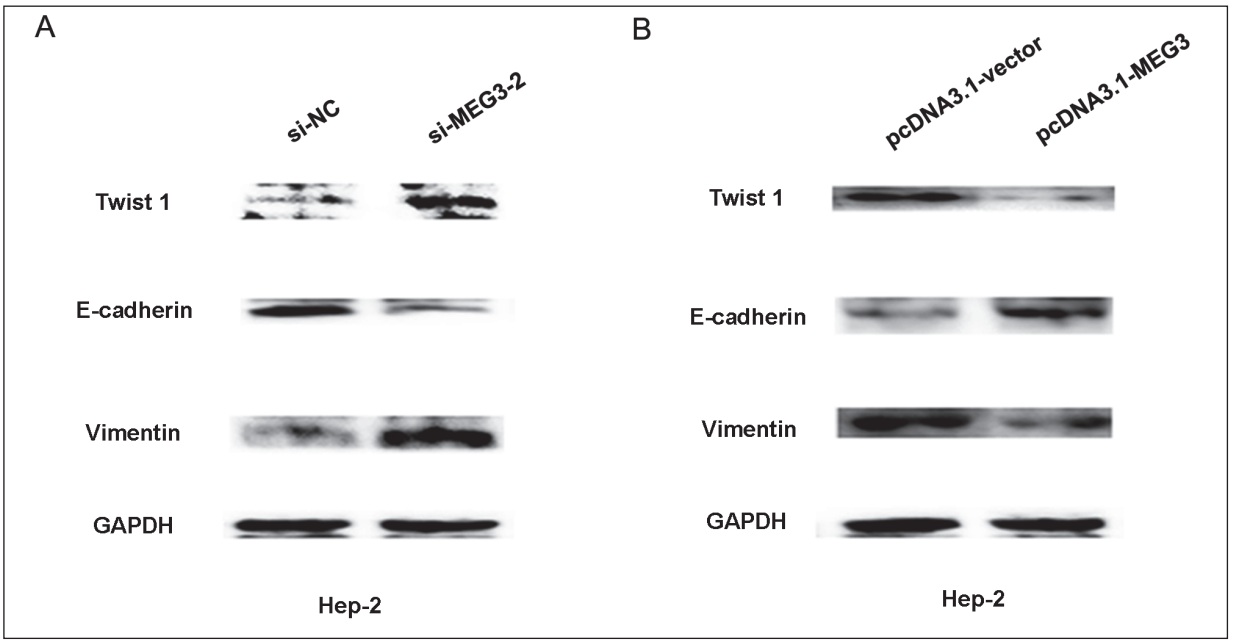

Figure 3. MEG3 suppressed cell EMT process in Hep-2 cell. ***(A)*** The relative protein expression was showed in Hep-2 cells transfected with si-NC or si-MEG3-2. ***(B)*** The relative protein expression was showed in Hep-2 cells transfected with pcDNA3.1-vector or pcDNA3.1-MEG3. $*p < 0.05$.

significantly downregulated in LSCC tissues, compared with adjacent normal tissues. Moreover, we analyzed the association of MEG3 expression with clinical factors of LSCC patients. Our results showed lower MEG3 expression levels were associated with lymph node metastasis and advanced clinical stage in LSCC patients. Moreover, we evaluated cell proliferation, migration and invasion ability by MEG3 knockdown or MEG3 overexpression in LSCC cells. The results indicated that upregulation of MEG3 inhibited cell proliferation, migration, and invasion, which suggested that MEG3 may function as a tumor suppressor in LSCC. Epithelial-mesenchymal transition (EMT) process is one of the mechanisms of metastasis, which is controlled by specific transcription factors including Snail1/2, Slug and Twist1[12,17]. MEG3 had been found to promoted cell migration, invasion and EMT process in previous study. Long non-coding RNA MEG3 suppresses migration and invasion of thyroid carcinoma by targeting of Rac1[18]. Knockdown of MEG3 inhibited TGF-β-mediated changes in cell morphology and cell motility characteristic of EMT in lung cancer cell Lines[19]. However, the association of MEG3 expression with EMT process in LSCC cells remains unknown. In the study, our findings showed that upregulation of MEG3 expression significantly inhibited cell invasion and Epithelial-Mesenchymal Transition (EMT) process by suppressing Twist1 and Vimentin expression and up-regulated E-cadherin expression in Hep-2 cells. However, downregulating the expression of MEG3 had opposite effects in Hep-2 cell. Thus, we demonstrated that MEG3 significantly affected LSCC cell EMT process.

Conclusions

We found that MEG3 was lower in LSCC tissues. Furthermore, we showed that MEG3 inhibited cell proliferation, migration, invasion and EMT process. These results provided that MEG3 may be a potential target of LSCC treatment.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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