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RESEARCH ARTICLE

Nicotine upregulates microRNA-21 and promotes TGF-β-dependent epithelial-mesenchymal transition of esophageal cancer cells

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Abstract A consistent positive association between cigarette smoking and the human esophageal cancer has been confirmed all over the world. However, details in the association need to be more focused on and be identified. Recently, aberrantly expressed microRNAs (miRNAs) have been shown to be promising biomarkers for understanding the tumorigenesis of a wide array of human cancers, including the esophageal cancer, and the deregulation on the epithelial to mesenchymal transition (EMT) by miRNAs is involved in the tumorigenesis. In present study, we were going to identify the role of nicotine-induced miR-21 in the EMT of esophageal cells. We found that there was an overexpression of miR-21 in esophageal specimens, having an association with cigarette smoking, and the upregulation of miR-21 was also induced by nicotine in esophageal carcinoma cell line, EC9706. Moreover, the upregulated miR-21 by nicotine promoted EMT transforming growth factor beta (TGF-β) dependently. Thus, the present study reveals a novel oncogenic role of nicotine in human esophageal cancer.

Keywords Nicotine · Epithelial-mesenchymal transition · microRNA-21 · TGF-β

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Introduction

As the eighth most common cancer worldwide and the sixth most common death cause from cancer [1], esophageal cancer occurs geographically [2, 3] and mainly in developing countries. In China, esophageal cancer ranks as the fourth most common cause of cancer death [4], posing great threat to Chinese health. Cigarette smoking has been a well-established risk factor for cancers, i.e., liver cancer [5], breast cancer [6], and lung cancer [7]. A consistent positive association between cigarette smoking and esophageal cancer has also been confirmed all over the world [8–13]. However, the association of the severe cigarette smoking problem in China with the high prevalence of esophageal cancer has not been well confirmed, though the chronic smoking problem in China is particularly acute because China has the largest population of smokers in the world, about 350 million currently [14]. Therefore, the details in the association of smoking cigarette and esophageal cancer need to be more focused and identified.

Cigarette smoke contains multiple classes of established carcinogens including benzo(a)pyrenes, polycyclic aromatic hydrocarbons, and tobacco-specific nitrosamines, most of which exert their genotoxic effects by forming DNA adducts and generation of reactive oxygen species, causing mutations in vital genes such as K-Ras and p53 [15]. Furthermore, it has been demonstrated that nicotine, the major tobacco component, is the most active carcinogen in cigars and can induce cell-cycle progression, angiogenesis, and metastasis of multiple cancers [15–19]. However, there is little known about the molecular pathways activated by nicotine in cancers throughout the gastrointestinal tract, potentiating cancer growth and/or inducing the formation of cancer on their own [19]. Thus, it is important to unlock the carcinogenic mechanisms induced by nicotine in the gastrointestinal cancers, including esophageal cancer. The epithelial-mesenchymal transition (EMT) was reported to be induced by nicotine in human airway

epithelial cells via Wnt/β-catenin signaling [20]. And, the EMT was also induced by nicotine during its promotion to cell proliferation and invasion in a variety of human cancer cell lines [21]. It was shown that nicotine enhanced, via periostin, proliferation, invasion, and EMT of nicotine-promoted gastric cancer [22]. And, the activation of 5-lipoxygenase was required for nicotine-mediated EMT gastric tumor cell growth [23]. Thus, to clarify the oncogenic role of nicotine in esophageal cancer, it is a cut way to identify the EMT and the regulation or signaling details of EMT in the esophageal epithelial cells.

microRNAs (miRNAs) are 18–24 nt endogenous non-coding RNAs that regulate gene expression [24] in a wide variety of cell processes of various organisms, including humans [25–27]. Aberrantly expressed miRNAs have been shown to be promising biomarkers for understanding the tumorigenesis of a wide array of human cancers [28, 29]. Recent years, the deregulation of numerous oncogenic or tumor suppressive miRNAs has been reported to be associated with cancer tumorigenesis. And, the deregulation on the EMT by miRNA is proved to be involved in the tumorigenesis. It has demonstrated that induction of the EMT can generate cells with properties of stem cells or cancer stem cells (CSCs) [30], which are defined operationally as tumor-initiating cells [31]. Hence, the tumorigenesis cascade may involve a cell-type conversion process, which has been shown to be orchestrated by transcription factors and miRNAs. Recently, several miRNAs have been identified to inhibit EMT and tumor metastasis. Activated miRNA-200 transcription maintains epithelial characteristics and prevents EMT induction in epithelial cells [32]. It is reported that the repression of miR-203 or miR-34c expression promotes EMT and tumor metastasis in breast cancer [33, 34]. Interestingly, the promotion to EMT by miRNAs has rarely been reported.

In the present study, we determined that cigarette smoking is associated miR-21 overexpression in esophageal (cancer) specimens and in esophageal cancer cell line, EC9706. And, we confirmed the oncogenic role of nicotine, the major tobacco component, in EC9706 cells, via promoting EMT in a miR-21-dependent pathway and transforming growth factor beta (TGF-β)-dependent pathway. The present study reveals a novel oncogenic role of nicotine in esophageal cancer.

Results

Overexpression of miR-21 in esophageal specimens is associated with cigarette smoking

Little reports revealed the promotion of nicotine to the miR-21 expression [35]. In order to uncover the details of nicotine-induced esophageal cancer, we measured the expression of miR-21, which is one of the well-identified oncogenic miRNAs in a variety of cancers, in esophageal specimens from esophageal cancer patients or normal subjects, both of which are cigarette smokers. The miR-21 expression was determined by TaqMan quantitative real-time polymerase chain reaction (qRT-PCR), with U6 as an internal reference. The results indicated that upregulated miR-21 occurred in normal esophageal tissues of cigarette smoking subjects ($n=21$), significantly higher than specimens from nonsmoking subjects ($n=10$) ($p=0.0104$) (Fig. 1a). To assess the association of miR-21 with cigarette smoking in esophageal cancer, 25 tissue samples from esophageal cancer subjects with cigarette smoking habit and 28 tissue samples from nonsmoking esophageal cancer patients were utilized to examine the miR-21 level. The miR-21 was also upregulated in the esophageal cancer tissues of cigarette smokers significantly ($p=0.0013$), compared with the nonsmoking samples. As shown in Fig. 1b, the average Ct (ΔCt) value of miR-21 expression in smoker's samples was 1.95, comparing 1.00 in nonsmoker's samples. The results indicated that the increased expression of miR-21 could be concerned with the cigarette smoking either in normal esophageal tissues or in esophageal cancer specimens.

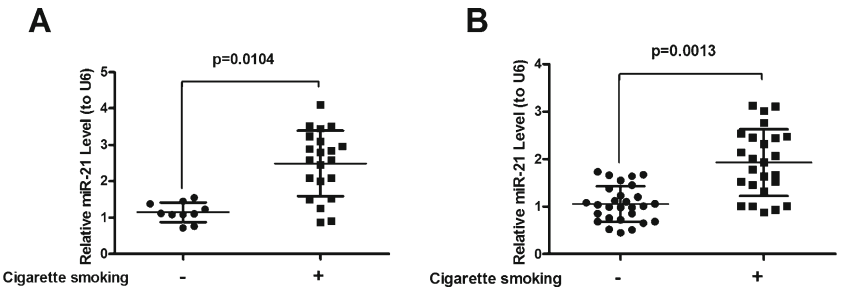


Fig. 1 miR-21 was upregulated in esophageal cancer specimens. **a** RT-qPCR assay for the relative miR-21 expression to U6 in normal esophageal epithelia from subjects with (21) or without (10) a consistent cigarette smoking habit, respectively. **b** Relative miR-21 expression to U6 in primary esophageal squamous cell carcinoma tissues from patients with (25) or without (28) a consistent cigarette smoking habit, respectively. RT-qPCR assay for each specimen was performed in duplicate, from which the average relative miR-21 result was calculated. And, statistical significance was considered with a p value <0.05

Nicotine induces miR-21 in esophageal carcinoma cell line, EC9706

Nicotine is the major toxic tobacco component and is the most active carcinogen in cigars [15–19]. To evaluate the promotion to miR-21 expression by nicotine, we used quantitative reverse transcription (RT)-PCR to examine the expression of miR-21 in EC9706 cells post nicotine treatment. Figure 2a showed that miR-21 was upregulated in the EC9706 cell line, 24 h post 5- or 10-nM nicotine treatment (p=0.022 and 0.010, respectively, vs control). Then, we reconfirmed the significant miR-21 upregulation in EC9706 cells, the miRNA samples were examined by northern blot, and it was shown in Fig. 2b that the nicotine group (either 5 or 10 nM) was still significantly higher than the normal group (p=0.042 and p=0.008, respectively). To further evaluate the upregulation of miR-21 by nicotine, two key miRNA processing enzymes, Drosha and Dicer, were quantitatively determined in both mRNA and protein levels; Fig. 2c–f showed that Drosha and Dicer were upregulated post the nicotine treated of 10 nM, in protein level (24- or 36-h post treatment, p=0.037 or 0.031 for Drosha and p=0.030 or 0.023 for Dicer, respectively) and mRNA level (12- or 24-h post treatment, p=0.007 or 0.004 for Drosha and p=0.026 or 0.015 for Dicer, respectively). Therefore, the nicotine treatment promotes miR-21 expression in esophageal carcinoma cell line, EC9706.

Upregulated miR-21 by nicotine promotes EMT in EC9706 cells

Morphologically, the EC9706 cells post nicotine treatment lost their close contact to each other and were more similar to mesenchymal cells (data not shown). It implies a possible EMT in those cells. Then, to evaluate whether there was an associated EMT which was promoted by the nicotine-upregulated miR-21, we identified the EMT in nicotine treated and/or miR-21 mimics (miR-21 inhibitor) transfected EC9706 cells, via the Western blot assay of

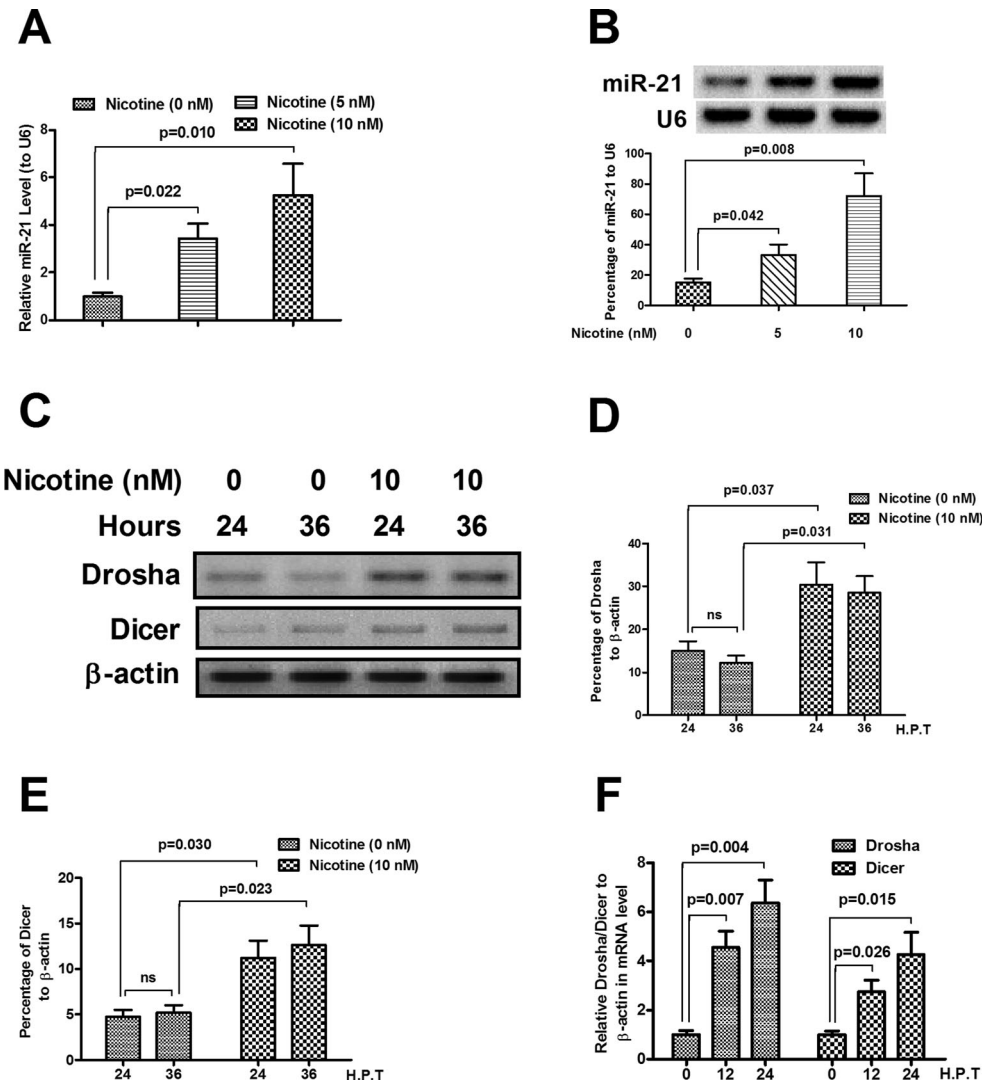


Fig. 2 miR-21 was upregulated by nicotine in esophageal cancer cell line, EC9706. **a** RT-qPCR assay for miR-21 expression to U6 in EC9706 cells post nicotine stimulation. **b** Northern blotting assay for miR-21 expression to U6 in EC9706 cells post nicotine stimulation. **c** Western blotting analysis of Drosha and Dicer in EC9706 cells treated with or without nicotine (24 h). **d**, **e** Percentage of Drosha or Dicer to β-actin. **f** Relative expression of Drosha or Dicer in mRNA level in the nicotine-treated EC9706 cells. All results were expressed as mean values ± SE for three independent experiments. Statistical significance was considered with p<0.05

E-cadherin, occludin, collagen I, and vimentin. First, we evaluated the promotion or reduction of miR-21 level by miR-21 mimics or miR-21 inhibitor in EC9706 cells. Figure 3a indicated that the miR-21 mimics transfection (25 or 50 nM) significantly promoted the miR-21 level (*t* test, $p<0.001$, respectively), while Fig. 3b showed that the miR-21 inhibitor (25 or 50 nM) blocked the miR-21 promotion by nicotine (*t* test, $p<0.05$ or $p<0.01$). The Western blot assay indicated that the nicotine treatment (10 nM) or miR-21 (50 nM) transfection significantly reduced the expression of E-cadherin and occludin, both of which were of epithelial cell characteristics, and significantly promoted the collagen I and vimentin, both of which were of mesenchymal cell characteristics (*t* test, $p<0.05$ or $p<0.01$), while the miR-21 inhibitor could reversed the promotion or reduction significantly (*t* test, $p<0.05$) (Fig. 3c, d). Taken together, the upregulated miR-21 by nicotine promotes EMT in EC9706 cells.

Nicotine-upregulated miR-21 promotes TGF-β in EC9706 cells

It is well known that the TGF-β plays an important role in EMT. TGF-β induces connective tissue growth factor (CTGF) expression, by activating the SMAD signals or by binding to the transcription enhancer factor (TEF) on the CTGF promoter element, and then promotes EMT [36, 37]. Moreover, the upregulation of miR-21 has been confirmed to promote TGF-β in myelodysplastic syndromes or in endothelial lineage differentiation [38, 39]. To identify the signaling pathways of and the possible role of TGF-β in nicotine-promoted EMT, we examined the regulation of TGF-β by nicotine or miR-21 in EC9706 esophageal cells. We first found that the cells significantly increased TGF-β expression in mRNA level (Fig. 4a) (*t* test, $p<0.05$ or $p<0.01$) as determined by RT-qPCR, 12-h post nicotine exposure or miR-21 transfection, or in protein level (Fig. 4b) (*t* test, $p<0.05$ or $p<0.01$), as

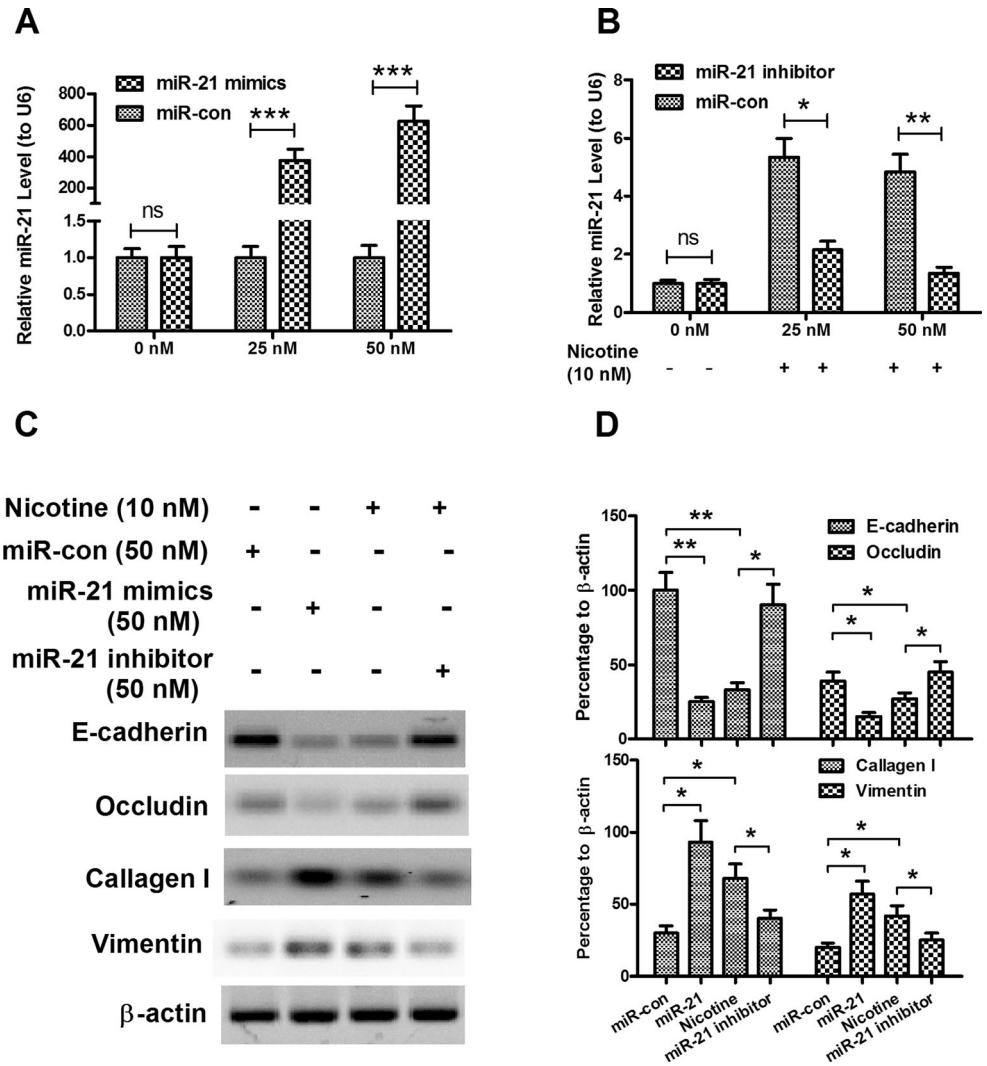


Fig. 3 Nicotine upregulated miR-21 promotes EMT in EC9706 cells. **a**, **b** Manipulation of miR-21 level in EC9706 cells by transfection with miR-21 mimics or miR-21 inhibitor. miR control was used as a control. **c**, **d** Expression of epithelial markers and mesenchymal markers in EC9706 cells post nicotine treatment, miR-21 mimics, and/or miR-21 inhibitor transfection, by Western blotting analysis. All results were expressed as mean values ± SE for three independent experiments. Statistical significance was considered with $p<0.05$. *ns* no significance; *$p<0.05$, **$p<0.01$, ***$p<0.001$

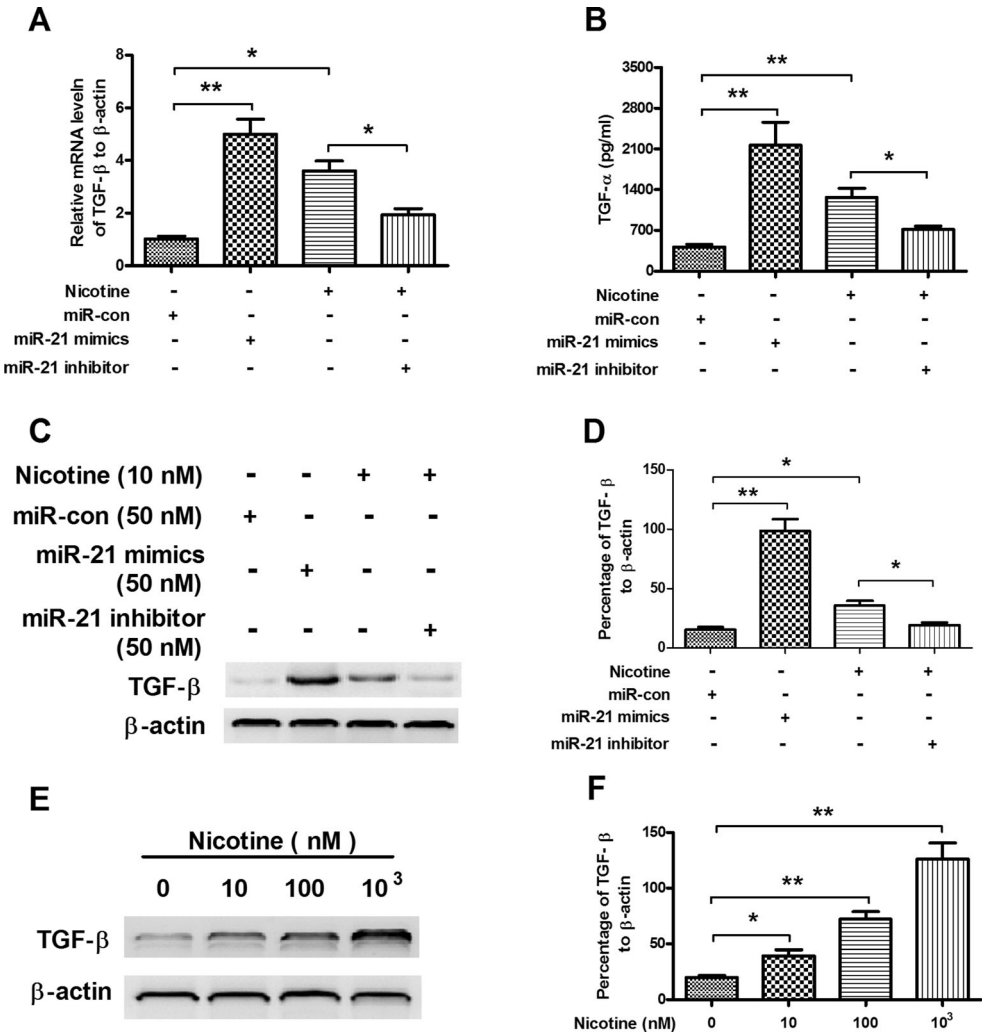


Fig. 4 Nicotine upregulated miR-21 promotes TGF-β expression in EC9706 cells. **a** miR-21 mimics upregulated, while miR-21 inhibitor downregulated the TGF-β expression in mRNA level in the nicotine-treated EC9706 cells (RT-qPCR assay). **b** TGF-β expression in the supernatant of EC9706 cells (ELISA), post nicotine treatment, miR-21 mimics, and/or miR-21 inhibitor transfection. **c**, **d** Western blotting revealed that miR-21 mimics upregulated, while miR-21 inhibitor downregulated the TGF-β expression in protein level in the nicotine-treated EC9706 cells. **e**, **f** Nicotine upregulated TGF-β expression dose dependently. All assays were repeated for triple times independently; results were expressed as mean values ± SE. *$p<0.05$, **$p<0.01$

determined by ELISA. Then, Western blotting analysis also confirmed the induction of TGF-β by nicotine or miR-21 (Fig. 4c, d) (*t* test, $p<0.05$ or $p<0.01$), and there was a dose dependence on the TGF-β induction by nicotine (Fig. 4e, f) (*t* test, $p<0.05$ or $p<0.01$). In addition, the mediation of miR-21 on the induction of TGF-β by nicotine was confirmed by the miR-21 inhibitor (Fig. 4a–d) (*t* test, $p<0.05$ or $p<0.01$). Therefore, TGF-β is dramatically induced in nicotine or miR-21-promoted EMT of EC9706 cells.

Nicotine-promoted EMT is TGF-β-dependent

To test whether TGF-β is involved in the nicotine-mediated EMT, the loss-of-function approach, by RNAi technology was employed to knockdown the TGF-β expression. Transfection of TGF-β small interfering RNA (siRNA) into the cells significantly decreased the TGF-β expression in mRNA level in both nicotine-treated and untreated cells. Compared with the negative control siRNA, which had no inhibitory effect on enhanced TGF-β expression after nicotine treatment, TGF-β siRNA transfection suppressed this increase in TGF-β expression; however, TGF-β expression was still higher in the nicotine-treated cells with TGF-β knockdown than that in the untreated control (*t* test, $p<0.05$ or $p<0.01$) (Fig. 5a). The TGF-β knockdown was also confirmed in protein level with the ELISA assay or Western blotting assay. Similar kind of suppression of TGF-β expression by TGF-β siRNA transfection was observed in nicotine-treated and untreated cells, compared to the cells with control siRNA transfection (Fig. 5b–d). We next examined whether transfection of TGF-β siRNA would block nicotine-induced EMT. As expected, cell transfection with our TGF-β-specific siRNA inhibited nicotine-induced EMT, whereas no inhibitory effect was observed in cells transfected with a control siRNA. Cells transfected with a TGF-β-specific siRNA showed enhanced E-cadherin and occludin expression and decreased collagen I and vimentin expression than the control siRNA-transfected cells (*t* test, $p<0.05$ or $p<0.01$) (Fig. 5e–g). Thus, we confirmed that TGF-β knockdown attenuated the nicotine-promoted upregulation of mesenchymal markers and downregulation of epithelial markers; in the other words, the nicotine-mediated EMT is TGF-β dependent.

Discussion

The biological processes involved in the esophageal tumorigenesis are still largely unknown; EMT has been reported to play a crucial role in this process and further in the tumor invasion and metastasis [40, 41]. And, multiple oncogenic factors promote the EMT in esophageal cancers. It was reported that the increased expression of EIF5A2, via hypoxia or gene amplification, contributes to metastasis and angiogenesis of esophageal squamous cell carcinoma [42]; the glioma-associated oncogene homolog 1 promotes EMT in human esophageal squamous cell cancer by inhibiting E-cadherin via Snail [43], and IGFBP3 promotes esophageal cancer growth by regulating EMT [44]. Recent studies have revealed the high importance of miR-21 in the EMT of breast cancer and other cancers [45–47]. miR-21 is one of the best established oncomir that is overexpressed in most types of cancer analyzed [48] and targets a number of tumor suppressors, including PDCD4, PTEN, TPM1, and RHOB [49–52]. And, miR-21 has been confirmed to promote TGF-β [38, 39], which is well known to play an important role in EMT [36, 37]. Given the high importance of miR-21 in the tumorigenesis and tumor progression, we focused on the miR-21 regulation by nicotine in esophageal cancer (cells) in the present study.

Our study reveals that there is an increased expression of miR-21 which is concerned with the cigarette smoking either in normal esophageal tissues or in esophageal cancer specimens. And, the in vitro results demonstrate that the nicotine, the major tobacco component, also upregulates miR-21 level in esophageal carcinoma cell line, EC9706 cells. Further experiments underline the high importance of miR-21 in the EMT promotion, in a TGF-β-dependent pathway, in the esophageal cells post exposure to nicotine. First, an upregulated miR-21 occurred in normal esophageal tissues of cigarette smoking subjects and in tissue samples from esophageal cancer subjects with cigarette smoking habit, implying that an increased expression of miR-21 could be concerned with the cigarette smoking. And, the miR-21 upregulation was also identified, by quantitative PCR or northern blotting assay, in the EC9706 cell line, post exposure to nicotine, and also, there was an upregulation of two key miRNA processing enzymes, Drosha and Dicer, in both mRNA and protein levels. Second, we found that the nicotine-upregulated miR-21 promoted EMT in the EC9706 cells, by Western blot assay of E-cadherin and occludin, both of which were of epithelial cell characteristics, and of collagen I and vimentin, both of which were of mesenchymal cell characteristics. Taken together, the upregulated miR-21 by nicotine promotes EMT in EC9706 cells.

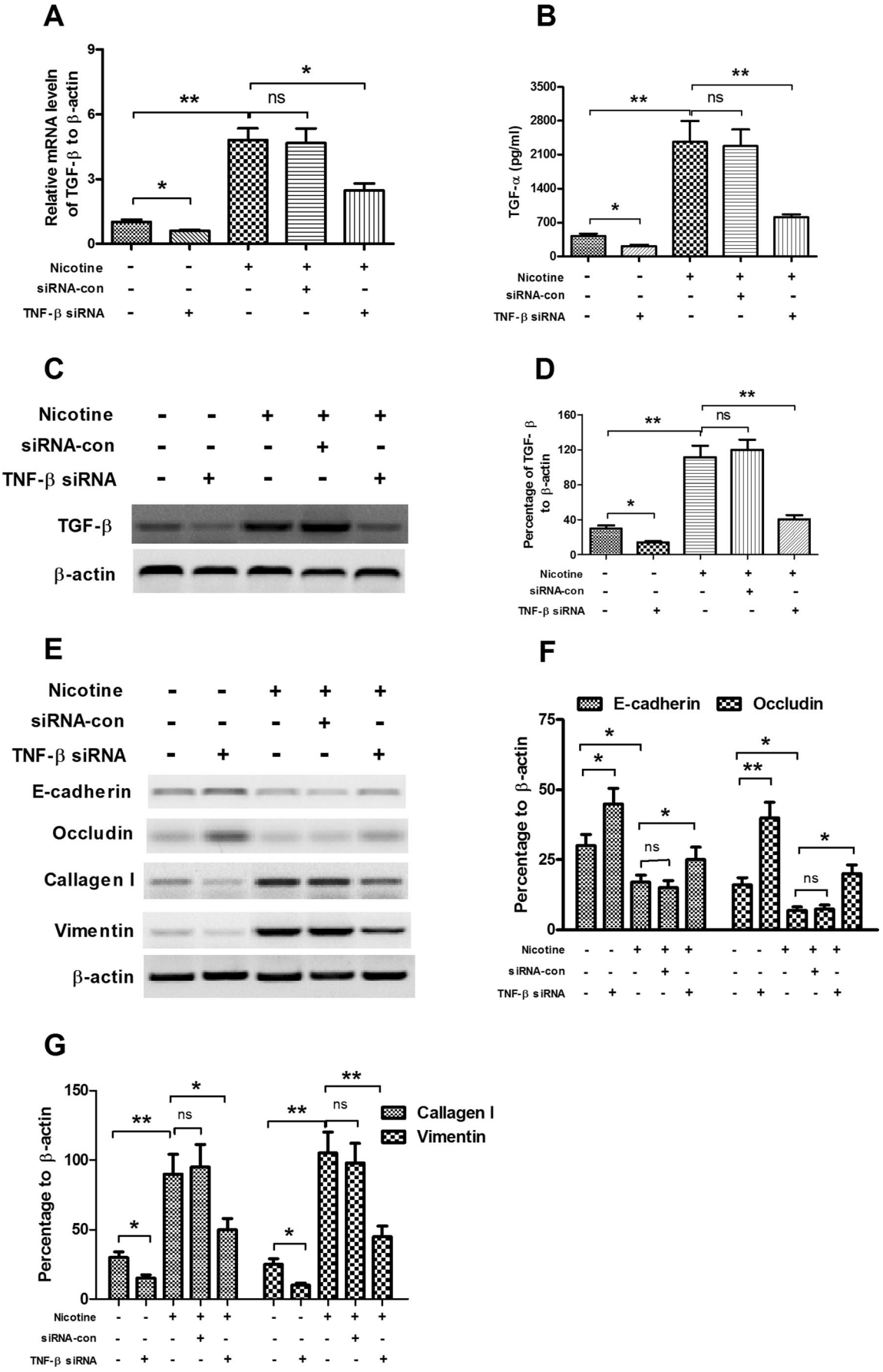
It is well known that the TGF-β plays an important role in EMT [36, 37]. And, miR-21 has been confirmed to promote TGF-β [38, 39]. In the present study, we identified the key role of TGF-β in nicotine-promoted EMT. First, TGF-β was positively regulated by nicotine or miR-21 in EC9706 esophageal cells, and the EC9706 cells significantly increased TGF-β expression in both mRNA and protein levels. Second, the loss-of-function approach, by RNAi technology to knockdown the TGF-β expression, confirmed that the nicotine or nicotine-upregulated miR-21-promoted EMT was TGF-β dependent; knockdown of TGF-β blocked the EMT induced by nicotine or miR-21 in EC9706 esophageal cells.

In summary, we found that an overexpression of miR-21 in esophageal specimens is associated with cigarette smoking, and upregulation of miR-21 is also induced by nicotine in esophageal carcinoma cell line, EC9706. And, additionally, we confirmed that the upregulated miR-21 by nicotine promotes EMT and upregulated TGF-β in EC9706 cells, and the nicotine-promoted EMT is TGF-β dependent.

Fig. 5 TGF-β knockdown attenuated the nicotine-promoted EMT in EC9706 cells. **a**, **b** TGF-β siRNA blocked the promotion by nicotine of TGF-β expression in mRNA level in EC9706 cells (RT-qPCR assay) and in protein level in the cell supernatant (ELISA). **c**, **d** TGF-β siRNA blocked the promotion by nicotine of TGF-β expression in protein level in EC9706 cells (Western blotting assay). **e**–**g** TGF-β siRNA blocked the nicotine-promoted the upregulation of mesenchymal markers and the downregulation of epithelial markers in protein level (Western blotting assay) in EC9706 cells. All assays were conducted for triple times independently; results were expressed as mean values ± SE. *ns* no significance;* $p<0.05$, ** $p<0.01$

Materials and methods

Tissue samples and reagents Twenty-five and 28 primary esophageal squamous cell carcinoma tissues were obtained from patients with or without a consistent cigarette smoking habit, respectively. And, 21 and ten matched normal esophageal epitheliums were obtained from patients in the The Third Affiliated Hospital of Jianghan University, from 2009 to 2011, with informed consent and agreement. The cigarette smoking habit was uniformized with 20 to 40 cigarettes each day and a continuous smoking duration of 15 to 20 years, while the totally blank smoking history quantified the participants without smoking history. All tissue samples were from untreated patients undergoing surgery and were snap frozen in liquid nitrogen and stored at −80 °C until the RNA extraction. For all the samples, clinicopathologic information (age, gender, pathology, differentiation, tumor-node metastasis classification) was available. The study was approved by the medical ethics committee of Jianghan University. miR-21 mimics, miR-21 inhibitor oligonucleotide, and control oligonucleotide were purchased from Ambion. siTGF-β and siRNA control oligomers were synthesized by GenePharma Technology (Shanghai, China). Mouse antibodies to β-actin, E-cadherin, occludin, vimentin, or collagen I were purchased from Santa


A
Relative mRNA leveln of TGF-β to β-actin
Nicotine
siRNA-con
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B
TGF-α (pg/ml)
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Cruz Biotechnology (Santa Cruz, CA). Nicotine was purchased from Calbiochem (San Diego, CA). miR-21 mimics or miR-con (QIAGEN) were utilized to elevate the miR-21 level.

Cell culture and treatment Human esophageal squamous cell carcinoma cell line EC9706 was purchased from American Type Cell Collection and was grown in RPMI 1640 (Invitrogen) supplemented with 10 % fetal bovine serum (Invitrogen), 2 mmol/L glutamine, 100 units of penicillin/mL, and 100 μg of streptomycin/mL (Sigma-Aldrich) and incubated at 37 °C in a humidified chamber supplemented with 5 % CO_2. Prior to the experiments, the cells were serum starved for 24 h and then stimulated with 10, 100, or 10^3 nM of nicotine in the growth medium for 24 h. The cells were then harvested for further analysis. Cells were plated in growth medium without antibiotics so that they were approximately 80 % confluent at the time of transfection. The cells were transfected with 25- or 50-nM miR-21 mimics, miR-con, or miR-21 inhibitor and were replaced with Opti-MEM with or without nicotine (10 nM). The TGF-β knockdown was conducted by transfection of 5-nM siRNA duplexes (si-control or siTGF-β) using transfection reagent and transfection medium according to the manufacturer's recommendations.

Real-time quantitative PCR and northern blotting RNeasy plus mini kit (Qiagen) was used to extract total RNA from cells according to the manufacturer's instructions, and RNA was reverse transcribed into cDNA (TaKaRa). The cDNA was then amplified by real-time quantitative TaqMan PCR in a reaction containing 1 TaqMan Universal PCR Master Mix, 1-m primers, and 0.3-m probe and analyzed by the use of a Lightcycler 480 II (Roche, Mannheim, Germany). The housekeeping gene β-actin was used as an internal control. The data were normalized to β-actin and expressed as the fold change over control and calculated with the ΔΔCt method [53]. A nonradioactive northern blot method, LED, for small RNA (about 15–40 bases) detection using digoxigenin (DIG)-labeled oligonucleotide probes containing locked nucleic acids (LNA) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide was utilized to confirm the miR-21 and U6 expressions, according to the protocol [54].

Protein extraction and Western blotting The cells were lysed with cell lysis reagent (Promega) and supplemented with cOmplete Mini protease inhibitor cocktail (Roche). After protein concentration, determination using Bradford reagent (Bio-Rad) was loaded on and separated by SDS-PAGE. The proteins were transferred to PVDF membranes, which were then blocked in 5 % skimmed milk for 1 h at room temperature and probed with an antibody to E-cadherin, occludin, collagen I, vimentin, or β-actin. Antibody binding was detected using chemiluminescence according to the manufacturer's instructions with a peroxidase-conjugated anti-mouse antibody. The housekeeping gene β-actin was used as an internal control. The data were expressed as percentage to β-actin.

ELISA The cell supernatants were collected, and TGF-β was measured with human TGF-β ELISA kit (ExCell Bio, Shanghai) according to the manufacturer's instructions. Samples with a concentration exceeding the standard curve limits were diluted until an accurate reading could be obtained. Four replicate wells were used to obtain all the data points, and all of the samples were processed in duplicate and averaged.

Statistical analysis The data for analysis were obtained from at least three independent sets of experiments and expressed as means SD. The data were analyzed with the SPSS 17.0 statistical package. Statistical evaluation of the continuous data was performed by ANOVA or the independent sample *t* test for between-group comparisons. The level of significance was considered to be $p<0.05$.

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