

Original Article

MiR-19b promotes growth and migration of human esophageal squamous cell carcinoma Eca109 cells by negatively regulating PTEN

Liang Shen^{1,2}, Hao Zhu², Shengguang Ding², Yiming Xu², Chongjun Zhong², Shiyong Zheng¹

¹Department of Thoracic Surgery, The First Affiliated Hospital of Soochow University, Suzhou 215006, P. R. China;

²Department of Thoracic and Cardiovascular Surgery, The Second Affiliated Hospital of Nantong University, Nantong 226001, P. R. China

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Abstract: Esophageal cancer is one of the most common malignant tumors, and it makes a leading cause of death worldwide. Esophageal squamous cell carcinoma (ESCC) is the main histological characteristic of esophageal cancer in China, and it has a poor prognosis. Therefore, novel therapeutic strategies for ESCC are highly needed. MiR-19b is a critical regulator for development of tumors. However, its functional roles in ESCC are rarely elucidated. In our study, we reported that miR-19b accelerates growth and migration of ESCC cells by negatively regulating PTEN. First, Eca109 cells were transfected with miR-19b mimics, inhibitors or their respective negative controls. Second, proliferative role of cells was evaluated by Cell Counting Kit-8 (CCK-8) and EdU incorporation assay, while cell migration was determined by the width of the initial scratched area. Third, PTEN was confirmed as a target of miR-19b by bioinformatics analysis. Finally, knockdown of PTEN was able to promote cell multiplication and migration of Eca109 cells, while the growth inhibiting effect of miR-19b inhibitor in both proliferative and migratory roles of Eca109 cells could be hindered by PTEN siRNAs. Taken together, our present work indicates that inhibition of miR-19b might be a new therapeutic strategy for esophageal squamous cell carcinoma (ESCC).

Keywords: miR-19b, proliferation and migration, ESCC, PTEN

Introduction

Esophageal carcinoma is one of the most commonest malignant tumors with high morbidity and mortality worldwide [1]. About 70% of the world's esophageal cancer occurs in China, and esophageal squamous cell carcinoma is the most common histopathological type of esophageal cancer [2]. Despite intensive research of the molecular and clinical features of ESCC as well as great progress in surgical techniques and perioperative management, however, the prognosis of esophageal cancer is still poor and the overall five-year survival rate is still very low. [3-8]. Therefore, new therapeutic strategies for esophageal cancer are urgently needed for improving the outcome of this fatal disease.

MicroRNAs are small noncoding RNAs, 21-25 nucleotides in length, which usually negatively regulate gene expression in the level of post transcription by degradation and/or transla-

tional inhibition of the target mRNAs [9, 10]. They are involved in various biological pathways and may promote or inhibit tumors [11, 12]. Numerous studies demonstrated that abnormal expression of miRNA contributes to multiple types of cancers, including esophageal cancer [13-16]. With regard to ESCC, several relevant studies have shown the aberrant expression of miRNAs in ESCC. MiR-133a, miR-29c, miR-30-2b, miR-520a, and miR-375 were decreased in human ESCC tissues [17-21], while miR-34b, miR-373, miR-16, miR-208 and miR-223 were increased [22-26]. In the current study, miR-19b expression level was positively associated with tumor size, lymph node metastasis, and clinicopathological stage [27]. However, the biological significance of miR-19b and its molecular mechanisms in ESCC remain to be elucidated.

Here, we performed a systematic study to identify functional role of miR-19b using ESCC cell

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line Eca109. Real-time quantitative PCRs (qRT-PCRs) were applied to confirm the effects of miRNA-19b mimics and inhibitors in Eca109 cells, while Cell Counting Kit 8 (CCK-8), as well as 5-Ethynyl-2'-deoxyuridine (EdU) assay was used to evaluate the proliferation properties. The width of the scratched area was used to confirm cell migration. We then used qRT-PCR and western blot to determine the candidate target genes in both mRNA and protein levels. Finally, the two PTEN siRNAs, PTEN si-01 and PTEN si-02, were used for further investigating if PTEN was critical for the functional roles of miR-19b in ESCC cell's growth and migration.

Methods

Cell culture and cell transient transfection

The human ESCC cell line Eca109 was provided by Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, and was cultured in RPMI-1640 medium (Gibco company) supplemented with 10% FBS, 100 µg/mL of streptomycin, and 100 U/mL of penicillin. Cells were cultured in 5% CO₂ incubator at 37°C with medium changed every 48 h.

The miR-19b mimics, inhibitors and their negative controls (NC), as well as PTEN siRNA were purchased from RiboBio (China), and were transfected into Eca109 cells using the lipofectamine 2000 reagent (Invitrogen, USA), according to the manufacturer's instructions. The concentrations of miR-19b mimics for the transfection were (50 nM), and the concentrations of their inhibitors were (100 nM). A similar procedure was used to transfect PTEN siRNAs (100 nM).

Cell proliferation assay

We use Cell Counting Kit (CCK-8, Dojindo, Japan) and 5-ethynyl-20-deoxyuridine (EdU) Kit (RiboBio, Guangzhou, China) to analyze cell proliferation. Cells were seeded in 96-well plates and incubated for 48 h after transfection. After 1 hour, CCK-8 was added before the terminal time of the experiment and was cultured in the 37°C incubator. The results were determined by a Microplate Absorbance Reader (Biorad, Richmond, CA, USA) under the optical density (OD) of 450 nm. For EdU assays, Eca109 cells were incubated with 10 µM EdU for 48 hrs, and the proliferative rate was then tested.

Cell migration assay

We used a classical wound healing assay to assess migration of Eca109 cells. In brief, the migration ability of Eca109 cells was assessed using 6-well plates. After transfection, the Eca109 cells were permitted to grow to converge after forming the monolayer. The cell monolayer was lightly lacerated with a 200-µl pipette tip and cleaned using PBS before the culture medium was replaced. The bottoms of the plates were noticeable to show where the initial images of the wounded areas were occupied. The migration ability of Eca109 cells was analyzed by measuring the width of the initial scratched area at 0 hr and the width of the terminal scratched area at 48 hrs.

RNA extraction and quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

MiRNeasy Mini Kit (Qiagen, Germany) was used to obtain the Eca109 cells' total RNA after transfection following producers' manual. For mRNA evaluation, Bio-Rad iScript™ cDNA Synthesis Kit (Bio-Rad) was used to synthesize cDNA. For miRNA expression analysis, reverse transcription and qRT-PCR were performed using TaqMan miRNA assay kits (RiboBio, China) with miR-19b. 5S ribosomal RNA (internal control) was used to normalize target miRNA expression. Relative expression levels for each mRNA and miRNA were determined using the 2^{-ΔΔCt} method according to the manufacturer's instructions. The relative expression level of target genes was standardized with that of β-actin gene by the 2^{-ΔΔCt} way as previously described [28].

Western blot analysis

Cells were harvested and lysed using RIPA lysis buffer (Beyotime Institute of Biotechnology, China) with 1% phenylmethanesulfonyl fluoride (PMSF). Lysates equivalent of 30 µg of protein were subjected to electrophoreses on 10% SDS-PAGE gels, transferred onto PVDF membranes, and probed with primary antibodies as following: anti-PTEN (1:1000; Epitomics, ab15-4812), and β-actin (1:10000; Abclonal, AC004) used as a loading control. After incubation with the appropriate HRP-conjugated secondary antibodies for 2 h at room temperature, protein bands were visualized using enhanced chemiluminescence (ECL) system (Pierce Biotechno-

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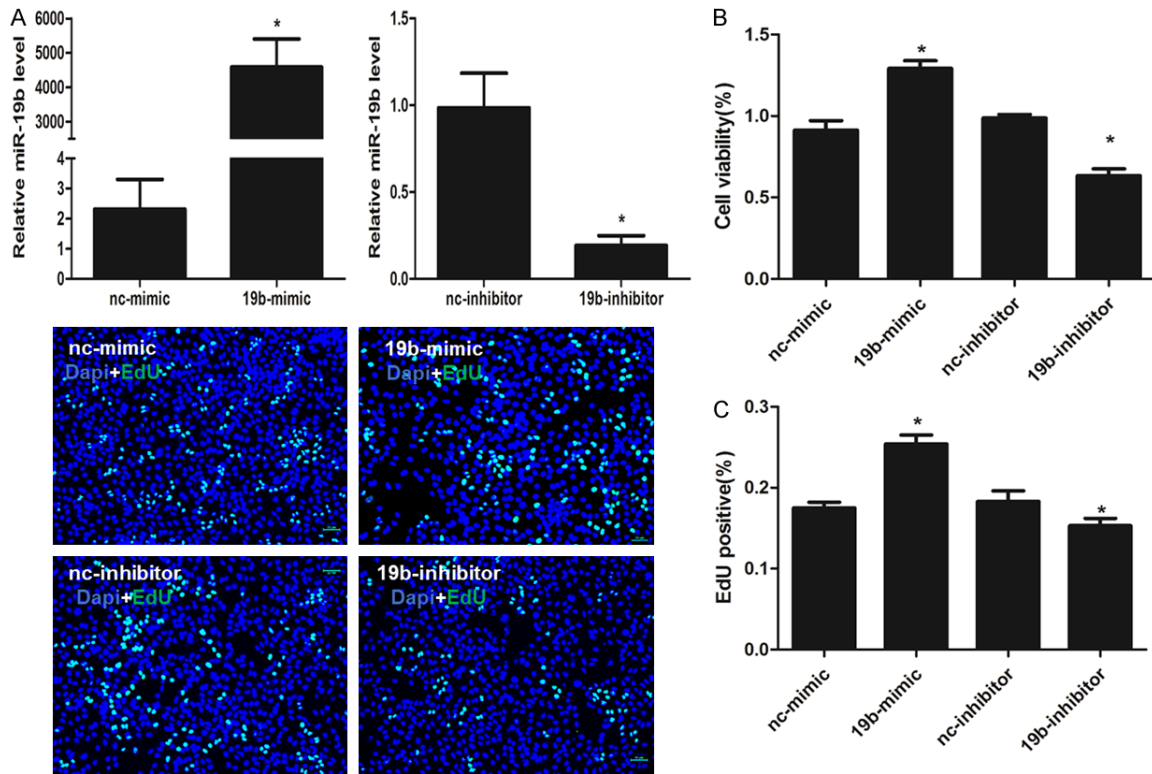


Figure 1. miR-19b increases proliferation of Eca109 cells. QRT-PCR illustrated that overexpression of miR-19b promoted miR-19b expression level, while depression of miR-19b reduced miR-19b expression in Eca109 cells (A). MiR-19b increased Eca109 cells' proliferation as indicated by CCK-8 (B) and EdU staining assays (C). * $P < 0.05$ versus control.

logy Inc., Rockford, IL, USA) with the ChemiDoc XRS Plus luminescent image analyzer (Bio-Rad). Densitometric analysis of protein bands was confirmed using Image Lab software (Bio-Rad).

Statistical analysis

All data were presented as mean \pm SEM using SPSS (version 20), and an independent t-test or one-way ANOVA followed by Bonferroni's tests were utilized. P -value, less than 0.05, was regarded as statistically significant.

Results

MiR-19b overexpression enhances Eca109 cells' viability and proliferation

To know the potential cellular functions of miR-19b on Eca109 cells, Eca109 cells were transfected with miR-19b mimics, inhibitors or their negative controls for 48 h. QRT-PCRs were applied to evaluate the roles of miR-19b mimics and inhibitors. As indicated in (Figure 1A), miR-

19b expression levels were remarkably upregulated by miR-19b mimics while downregulated by miR-19b inhibitors, indicating that miR-19b mimics and inhibitors significantly took effects in increasing or decreasing miR-19b expression levels. Based on that model, overexpression of miR-19b with miR-19b mimics enhanced cell vitality, and knockdown of miR-19b with miR-19b inhibitors decreased Eca109 cell vitality (Figure 1B). To confirm if the change of cell viability was due to higher proliferation rate, we investigated the effects of miR-19b using EdU incorporation. The EdU assay showed that Eca109 cells transfected with miR-19b mimics had a higher proliferative rate when compared with negative control Eca109 cells, and that inhibition of miR-19b reduced that effect (Figure 1C).

MiR-19b enhances Eca109 cells' migration

The regulating function of miR-19b on migration was evaluated based on the unhealing distance. The smaller unhealing distance indicat-

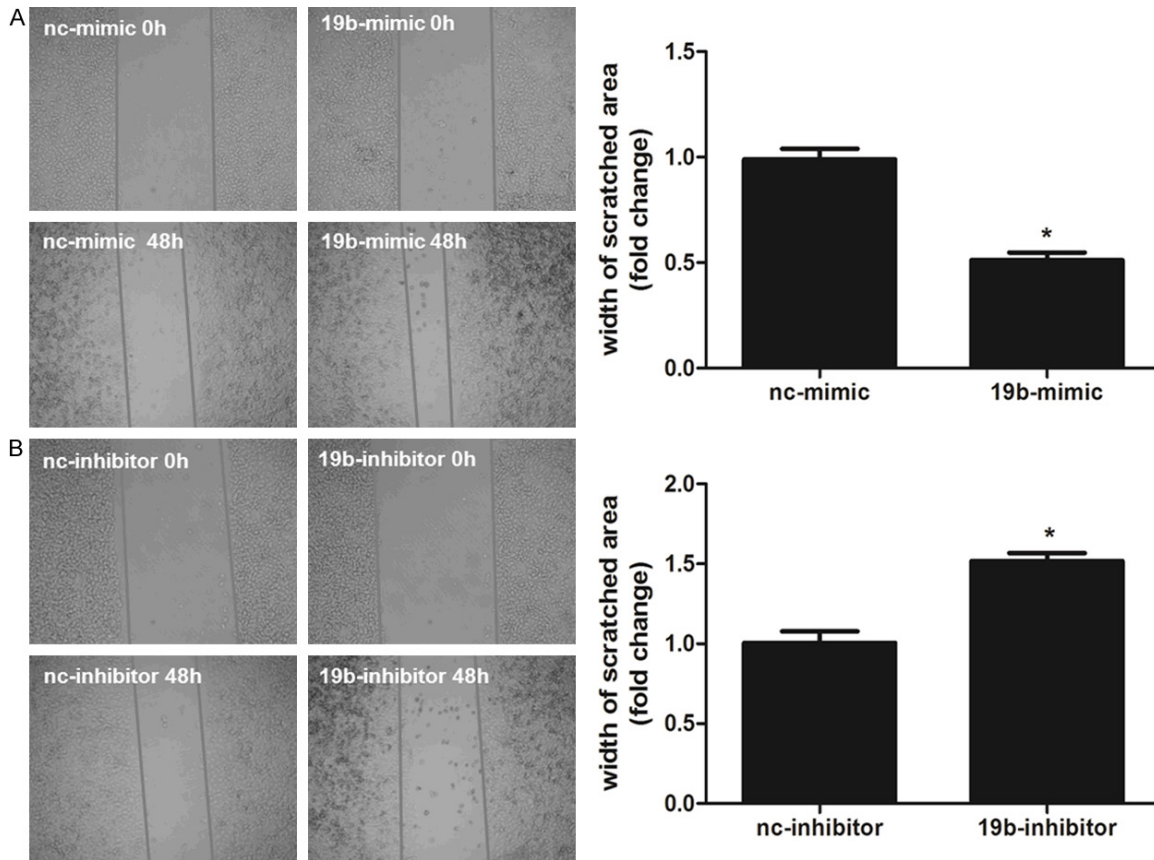


Figure 2. MiR-19b enhances Eca109 cells' migration. MiR-19b mimics promoted Eca109 cells' migration (A), while suppression of miR-19b decreased that effect (B). *P < 0.05 versus control.

ed the greater migration ability. We showed that overexpression of miR-19b significantly reduced the unhealing distance in Eca109 cells, while inhibition of miR-19b increased that (Figure 2), suggesting that miR-19b was responsible for regulating ESCC migration.

PTEN is critical for effects of miR-19b in Eca109 cells

To determine the interrelation between miR-19b and PTEN in Eca109 cells, we transfected Eca109 cells with miR-19b mimics and inhibitors, then estimated both mRNA and the protein levels of PTEN using real time quantitative PCR and western blotting assay. We found that miR-19b mimics reduced while miR-19b inhibitors raised PTEN at the protein level (Figure 3A, 3B), indicating that PTEN might be a potential target of miR-19b in Eca109 cells.

To further study whether PTEN is critical for the effect of miR-19b in Eca109 cell proliferation

and migration, two siRNAs, siRNA PTEN 01 and siRNA PTEN 02, were used in the present study to remove the off-target effects. Both PTEN siRNAs could synchronously suppress PTEN, at least, at mRNA level (Figure 3C). Therefore, in the following studies, as confirmed by CCK-8 and wound healing assays, knockout of PTEN could increase Eca109 cells' proliferation and migration, while the inhibiting effect of miR-19b inhibitor in both Eca109 cells' proliferation and migration could be reversed by the two PTEN siRNAs (Figures 3D and 4). These data suggest that miR-19b functions as a tumor enhancer and can promote ESCC proliferation and migration via regulating PTEN.

Discussion

Esophageal cancer is one of the common malignant tumors of digestive tract and patients with advanced esophageal cancer generally display poor survival rates [29]. ESCC constitutes the main histopathological subtype of

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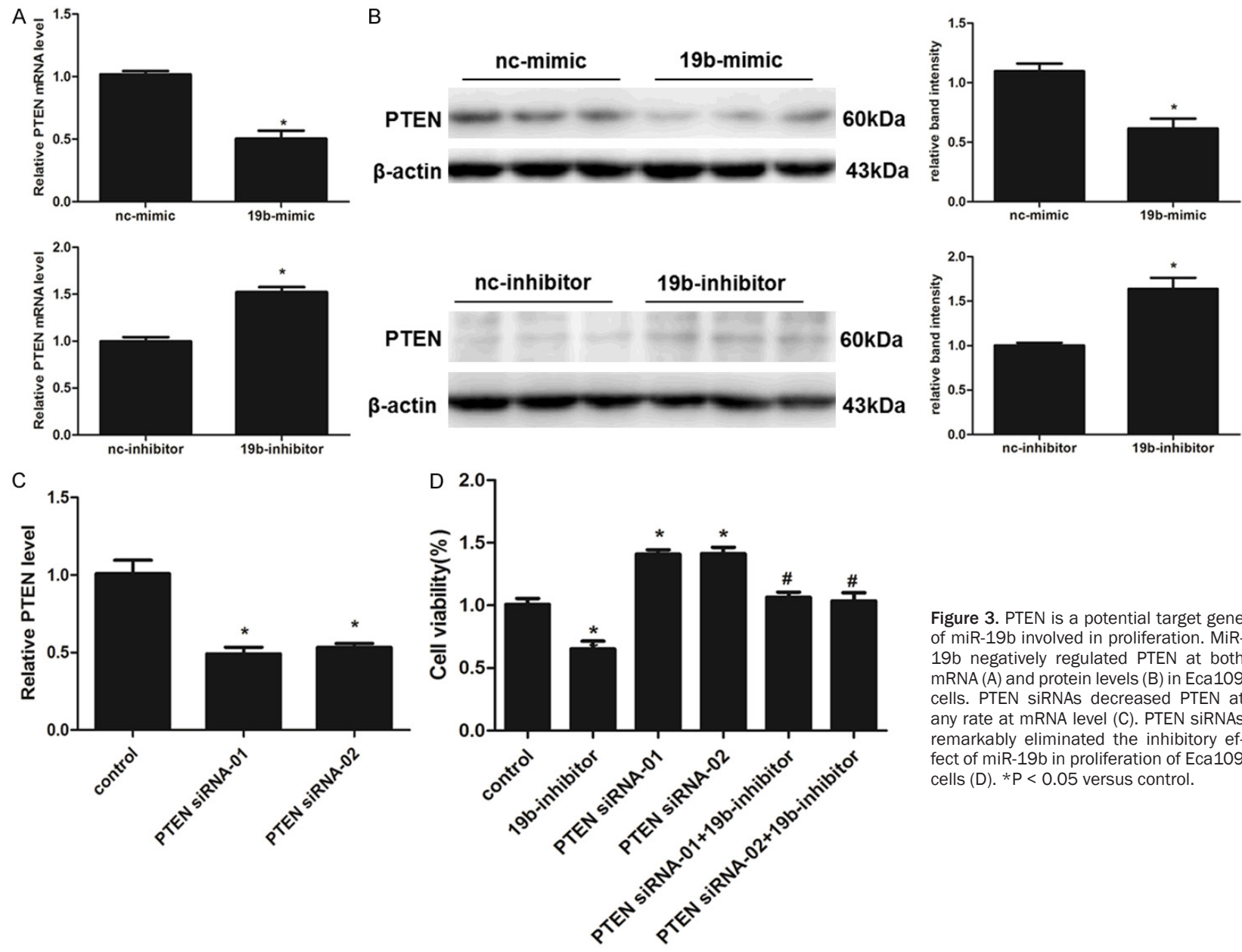


Figure 3. PTEN is a potential target gene of miR-19b involved in proliferation. MiR-19b negatively regulated PTEN at both mRNA (A) and protein levels (B) in Eca109 cells. PTEN siRNAs decreased PTEN at any rate at mRNA level (C). PTEN siRNAs remarkably eliminated the inhibitory effect of miR-19b in proliferation of Eca109 cells (D). *P < 0.05 versus control.

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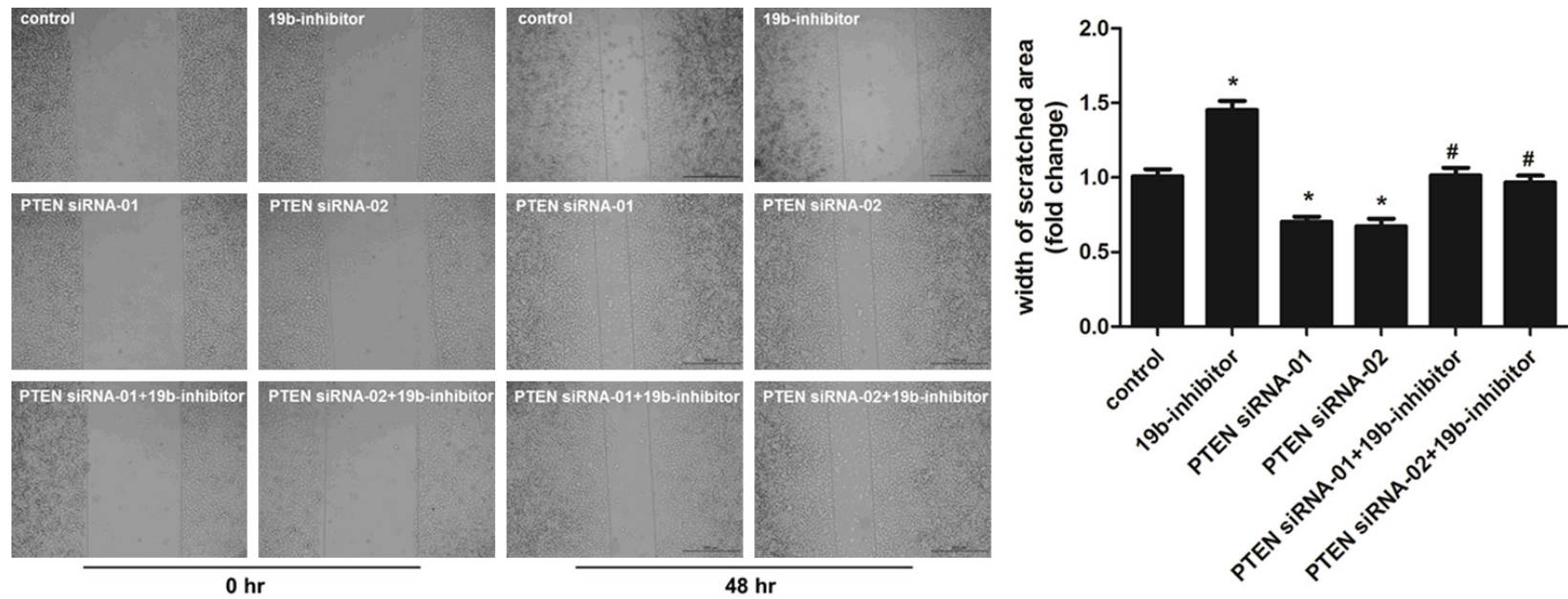


Figure 4. PTEN is an underlying target gene of miR-19b involved in tumor migration. Two PTEN siRNAs successfully reversed the inhibiting function of miR-19b in Eca109 cells' migration. *P < 0.05 versus control.

esophageal cancer [30]. To improve the survival rate of patients with advanced esophageal cancer, a better understanding of the genetic and/or epigenetic alterations in potential tumorigenesis and metastasis of ESCC is urgently required. In this paper, we first transfected miR-19b mimics, inhibitor and their negative controls into Eca109 cells, a human EC cell line. We confirmed that miR-19b enhances proliferation and migration of Eca109 cells. Then we detected the potential target gene of miR-19b at both mRNA and protein levels. We demonstrated that overexpression of miR-19b significantly suppressed the expression of PTEN, while suppression of miR-19b increased that. Finally, the siRNAs of PTEN were applied to further evaluate if PTEN was pivotal for the role of miR-19b in ESCC proliferation and migration. We showed that both Eca109 cells' proliferation and migration could be reversed by two PTEN siRNAs, indicating that the targeting miR-19b/PTEN axis might be a promising therapeutic strategy for ESCC.

At present, certain studies have confirmed that miRNAs could contribute to the formation and development of various cancers and they played a functional role in the diagnosis and treatment for tumors [31]. Among which, miR-19b is particularly an important one. MiR-19b belongs to the miR-17-92 cluster which is located at 13q31.3, encoding six microRNAs including miR-17, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a, and is implicated in the carcinogenesis of multiple cancers [32]. It has been found that miR-19b is highly expressed in bladder carcinoma, vesicular rhabdomyosarcoma, colon cancer, and its high expression is closely related to tumor angiogenesis, disease prognosis and survival rate [33-35]. Gu and colleagues confirmed that miR-19a and miR-19b co-regulate tumor suppressor MTUS1 to promote cell proliferation and migration in lung cancer [36]. MiR-19b-3p promotes colon cancer proliferation and oxaliplatin-based chemoresistance by targeting SMAD4 as validated by bioinformatics and experimental analyses [37]. Osip'yants showed that changes in the level of circulating hsa-miR-297 and hsa-miR-19b-3p miRNA are associated with generalization of prostate cancer [38]. MiR-19b promotes breast cancer metastasis by targeting MYLIP and its related cell adhesion molecules [39]. We previously reported that miR-19b controls cardiac

fibroblast proliferation and migration [28]. A recent study showed that overexpression of miR-19b was negatively correlated with tumor size, lymph node metastasis, and clinical stage [27]. However, the biological significance of miR-19b and its potential role in ESCC remains to be poorly elucidated. It is urgently needed to detect the effects of miR-19b in ESCC.

PTEN, a classic tumor suppressor gene, is an underlying target of miR-19b, which controls cancer cell proliferation [40-43]. Based on that, we examined whether PTEN could be a downstream regulator of miR-19b mediating its effect in Eca109 cells. As expected, PTEN was reversely modulated by miR-19b at both mRNA and protein levels in Eca109 cells. Importantly, silencing PTEN could eliminate the aggravated proliferative effect of miR-19b inhibitor. Our present data found that PTEN is a downstream effector of miR-19b in regulating Eca109 cells' proliferation and migration.

In conclusion, our data provide a regulatory link between miR-19b and PTEN expression in esophageal cancer cell line, suggesting that PTEN is suppressed by miR-19b. Inhibiting miR-19b could mean an alternative way to increase therapeutically PTEN expression, thereby attenuating aggressive tumor properties. In summary, our present work not only helps us further understand the potential molecular mechanism of esophageal cancer, but also gives us a strong rationale to further investigate miR-19b as a new promising biomarker and therapeutic target for esophageal carcinoma.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Shiyong Zheng, Department of Thoracic Surgery, The First Affiliated

Hospital of Soochow University, Shizi Street No 188, Suzhou 215006, Jiangsu, P. R. China. E-mail: syzheng88@sina.com

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