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Mir-655 up-regulation suppresses cell invasion by targeting pituitary tumor-transforming gene-1 in esophageal squamous cell carcinoma

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Abstract

Background: MicroRNAs (miRNAs) can act as either oncogenes or tumor suppressor genes under different conditions and thus can play a significant role in cancer development. We investigated miR-655 expression in a cohort of esophageal squamous cell carcinoma (ESCC) to assess the impact of this miRNA on ESCC cell invasion and metastasis.

Methods: A qRT-PCR assay was used to quantify miR-655 expression levels in 34 paired ESCC samples and adjacent non-tumor tissues. Wound healing and transwell assays were used to evaluate the effects of miR-655 expression on the invasiveness of ESCC cells. Luciferase reporter and western blot assays were used to determine whether the mRNA encoding pituitary tumor-transforming gene-1 (PTTG1) is a major target of miR-655.

Results: The expression level of miR-655 in ESCC tissues was found to be lower than in adjacent non-tumor tissues ($P < 0.05$). This relatively low expression level was significantly associated with the occurrence of lymph node metastases ($P < 0.05$). Migration rates were significantly lower for two ESCC-derived cell lines (EC9706 and KYSE150) transfected with miR-429 mimics ($P < 0.05$). Subsequent western blot and luciferase reporter assays demonstrated that miR-655 could bind to putative binding sites within the PTTG1 mRNA 3'-untranslated region (3'-UTR) and thus reduce the expression.

Conclusions: miR-655 is expressed at low levels in primary ESCC tissues, and up-regulation of miR-655 inhibits ESCC cell invasiveness by targeting PTTG1. Our findings suggest that PTTG1 may act as a major target of miR-655. This study improves our understanding of the mechanisms underlying ESCC pathogenesis and may promote the development of novel targeted therapies.

Keywords: Esophageal squamous cell carcinoma, miR-655, Invasion, PTTG1

Background

MicroRNAs (miRNAs) are a recently discovered class of small (approximately 18–24 nucleotides in length), non-coding regulatory RNAs that negatively regulate gene expression at the post-transcriptional and/or translational level. miRNAs can trigger cleavage of target mRNAs or inhibit protein translation through sequence-specific interactions with the 3'-untranslated regions (3'-UTRs) of the target mRNAs [1-6]. Although the biological functions of

miRNAs have yet to be fully characterized, they are known to be intrinsic regulators of many cellular processes, including cell invasion, differentiation, proliferation and apoptosis [7-12]. Furthermore, aberrant expression of miRNAs has been linked to the development and progression of cancer and has prognostic significance for several tumor types, including lung and esophageal squamous cell cancer, neuroblastoma and lymphocytic leukemia [13-16].

Esophageal squamous cell carcinoma (ESCC) is a major cause of cancer-related death worldwide and is the fourth most lethal type of tumor in China. Altered expression of miRNAs has been observed in ESCC [17], suggesting that miRNA deregulation may play a role in

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ESCC carcinogenesis. We have previously performed a miRNA chip-based expression analysis of primary ESCC tissues and found that the expression of miR-655 in these tissues was lower than in adjacent, paired non-tumor tissues. Studies have also shown that miR-655 is an epithelial mesenchymal transition (EMT) suppressive microRNA [18]. However, the expression level of miR-655 and its role in ESCC have not yet been determined.

The pituitary tumor-transforming gene-1 (PTTG1) protein, also known as human securin, was originally isolated from rat pituitary tumor cells [19]. PTTG1 performs multiple physiologic functions critical to normal cellular mitosis and is active in the maintenance of sister chromatid separation [20]. PTTG1 levels correlate with tumor invasiveness [21], and PTTG1 has thus been identified as a key gene in tumor metastasis [22]. Previous research has shown that PTTG1 induces EMT through integrin $\alpha 4$ and $\beta 3$ focal adherin kinase signaling in lung cancer cells [23]. Also, PTTG1 promotes tumor malignancy via the EMT and expansion of the breast cancer stem cell population [24]. PTTG1 overexpression has been reported in a variety of cancers, including ESCC [25-27]. Nevertheless, whether miR-655 modulates PTTG1 in ESCC invasion and metastasis remains unknown.

In the present study, we first investigated miR-655 expression levels in tumor and normal tissues from 34 ESCC patients and then observed concomitant alterations in ESCC cell invasion and metastasis.

Materials and methods

Patients and tissue specimens

Tumors and adjacent non-tumor tissues were obtained at the time of surgery from 34 patients who had undergone surgical treatment for ESCC in the First Affiliated Hospital of Zhengzhou University following pathologic identification in 2002 and 2005, and snap frozen in liquid nitrogen. All patients consented to the use of their tissue samples in this study. None had received chemotherapy or radiotherapy before surgery. This study was approved by the ethics committee of Zhengzhou University, and informed consent was obtained from each patient.

RNA extraction and quantitative real-time PCR

Total RNA was isolated from ESCC tissue samples and adjacent non-tumor tissue samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. To determine mature miRNA expression levels, quantitative real-time PCR (qRT-PCR) was performed using a high-specificity miR-655 qRT-PCR Detection Kit (Stratagene Corp, La Jolla, CA) in conjunction with an ABI 7500 fast thermal cycler and

the manufacturer's recommendations. We used U6 small nuclear RNA (U6 snRNA) as an endogenous control for normalization. The qRT-PCR results were expressed relative to the miR-655 expression level at the threshold cycle (Ct) and were converted to the fold change ($2^{-\Delta\Delta C_t}$).

Cell lines

Human ESCC cell lines (EC9706 and KYSE150), purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China), were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS; Gibco BRL, Gaithersburg, MD, USA) and incubated at 37°C and 5% CO₂.

miRNA transfection

The miR-655 mimics (GMR-miR™ microRNA-655 mimics) and scrambled miR-655 used in this study were synthesized by Shanghai GenePharma Co. Ltd. Prior to transfection in complete medium, 2×10^5 cells were plated in 6-well plates for 24 h. Transfection was performed with a BTX ECM 2001 square wave electroporator (Genetronics Inc., San Diego, CA, USA) with electroporation settings adjusted according to the BTX ECM2001 protocol. Transfection efficiencies were evaluated in every experiment by qRT-PCR 24 h post-transfection. Cells from each cell line were subdivided into three groups: the non-transfected blank group (Blank), the scrambled miR-655 transfected negative control group (NC) and the miR-655 mimic-transfection group (miR-655).

Wound healing assay

For the wound-healing assay, 2×10^5 cells/well were plated onto 6-well plates. Cell cultures at varying confluences were serum starved for 8 h. After scratching the monolayer, cells were washed with PBS, cultured in RPMI-1640 containing 10% fetal bovine serum, and photographed with a 10× objective lens after 24 h.

Transwell assay

The concentration of cells in each group was adjusted to 2×10^5 cells/mL at 48 h post-transfection. The upper chamber of a 24-well transwell permeable support (Costar, USA) with an 8 μ m pore size was loaded with 200 μ l of cell suspension, and the lower chamber was filled with 500 μ l of medium containing 10% FBS. Cells were then incubated at 37°C/5% CO₂ for 48 h. Five wells were used for each group. After incubation, the media was removed from the upper chamber, and cells were scraped out of the upper chamber with a cotton swab. Cells that had migrated to the other side of the membrane were fixed with methanol, stained with hematoxylin, mounted and dried at 80°C for 30 min. The number of cells invading the

matrigel was counted in three randomly selected fields using an inverted microscope (200× magnification).

Enzyme-linked immunosorbent assay (ELISA)

Cells (2×10^5 cells/well) were added to 6-well plates in RPMI-1640 containing 10% fetal bovine serum. The cells were grown for 48 h until they were approximately 60–70% confluent. The growth medium was then removed and replaced with fresh RPMI-1640 containing 1% fetal bovine serum. 10 ng/ml EGF was added to growth medium to stimulate the expression of MMP-2 and MMP-9. MMP-2 and MMP-9 levels in the growth medium were measured using a human MMP-2 and MMP-9 ELISA kit (R&D Systems, USA) according to the manufacturer's instructions. Each experiment was performed three times, and the mean concentrations of MMP-2 and MMP-9 are presented.

Western blotting

Total protein from cultured cells was extracted using RIPA buffer containing phenylmethanesulfonyl fluoride. A BCA protein assay kit (Beyotime, Haimen, China) was used to determine protein concentrations. Proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto poly-

vinylidene difluoride (PVDF) membranes. After blocking, the membranes were incubated overnight at 4°C with diluted (1:500) primary antibody (polyclonal rabbit anti-PTTG1; Santa Cruz). Following extensive washing, the membranes were incubated with diluted (1:3000) horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz). Signal was detected using a chemiluminescence detection kit (Amersham Pharmacia Biotech, Piscataway, NJ). An antibody against β -actin (Santa Cruz Biotechnology) served as an endogenous reference.

Plasmid construction and the Luciferase reporter assay

Wild type and mutant human PTTG1 3'UTR fragments (bases 655–713, NM_004219) were created by two single-strand annealing (Sangon Biotech Co., Ltd, Shanghai). These fragments were cloned into a pGL3 promoter vector (Promega) downstream of the luciferase gene to generate, respectively, the recombinant vectors pGL3-PTTG1-wt and pGL3-PTTG1-mut.

For the luciferase reporter assay, EC9706 and KYSE150 cells were transiently co-transfected with the pRL-TK *Renilla* plasmid (Promega, Madison, WI), matched reporter vectors (wild type reporter vectors or mutant reporter vectors), and miR-655 mimics or scrambled miR-655. Each plasmid set was transformed with a BTX

Table 1 Clinicopathological characteristics of ESCC patients

Variables	n	miRNA-655 expression (Median \pm SD)	P Value	Relative PTTG-1 protein expression (Median \pm SD)	P Value
Gender					
Male	22	0.3914 \pm 0.09025	0.522	0.7773 \pm 0.13088	0.992
Female	12	0.4117 \pm 0.08189		0.7768 \pm 0.15969	
Age					
\geq 60	22	0.3891 \pm 0.08788	0.398	0.7806 \pm 0.13393	0.846
<60	12	0.4158 \pm 0.08544		0.7707 \pm 0.15456	
Tumor location					
Middle	27	0.3856 \pm 0.08675	0.087	0.7837 \pm 0.14907	0.594
Lower	7	0.4486 \pm 0.07105		0.7516 \pm 0.09727	
Lymph node metastasis					
Negative	19	0.4574 \pm 0.05445	0.000*	0.6995 \pm 0.09244	0.000*
Positive	15	0.3240 \pm 0.05705		0.8753 \pm 0.12743	
Differentiation					
Well	9	0.4467 \pm 0.08396		0.7756 \pm 0.11179	
Moderate	17	0.4071 \pm 0.08091	0.010*	0.7184 \pm 0.11237	0.005*
Poor	8	0.3263 \pm 0.05731		0.9035 \pm 0.14727	
TNM stage					
I	8	0.4475 \pm 0.07996		0.7344 \pm 0.16531	
II	19	0.4137 \pm 0.07135	0.001*	0.7421 \pm 0.08004	0.006*
III	7	0.3014 \pm 0.06067		0.9209 \pm 0.15870	

*Indicated statistical significance ($P < 0.05$).

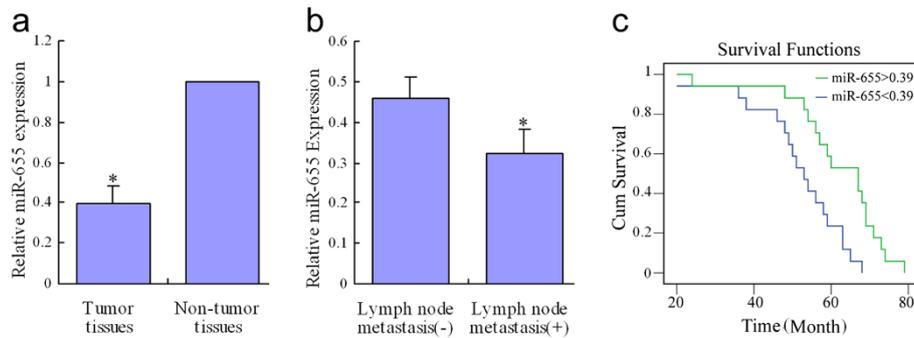


Figure 1 miR-655 expression in ESCC tissues and adjacent non-tumor tissues. (a) The relative expression levels of miR-655 in paired ESCC tissues and adjacent non-tumor tissues were determined by qRT-PCR. A statistically significant difference in expression levels ($P < 0.05$) was observed. (b) qRT-PCR results showing that miR-655 expression in the metastasis-positive group was notably lower than in the metastasis-free group ($P < 0.05$). (c) Kaplan–Meier curves of the clinical outcome for miR-655. Primary ESCCs with moderate to strong miR-655 expression (>0.39) had significantly better survival than did those with weak miR-655 expression (<0.39) ($P < 0.05$, log-rank test). T: tumor tissue (n = 34); N: adjacent non-tumor tissue (n = 34). * $P < 0.05$ compared to the control group.

ECM 2001 square-wave electroporator (Genetronics Inc., San Diego, CA, USA), and electroporation settings were adjusted according to the BTX ECM2001 protocol. Luciferase activity was measured at 24 h post-transfection using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer’s instructions.

Statistical analysis

Statistical analysis was performed using SPSS17.0 software. Data were expressed as the mean \pm the standard deviation (SD). The student’s t-test and a one-way analysis of variance (ANOVA) were used in the comparison of means from different samples. The follow-up data were analyzed using the Kaplan–Meier method and log-rank test. P values of less than 0.05 were considered statistically significant.

Results

Down-regulation of miR-655 in ESCC

Using adjacent non-tumor tissues as a reference, miR-655 expression in ESCC tissues was found to be significantly reduced ($P < 0.05$; Table 1; Figure 1a). We found that miR-655 expression levels in ESCC tissues were associated with the occurrence of lymph node metastases, differentiation status and TNM stage ($P < 0.05$; Table 1; Figure 1b). No significant differences were observed between miR-655 expression and gender, age or tumor location ($P > 0.05$; Table 1).

Kaplan–Meier curves for patients with esophageal squamous cell cancer categorized according to miR-655 expression are shown in Figure 1c. The patients were divided into two groups using a cutoff of 0.39 for miR-655 expression. The log-rank test indicated that there were significant differences between the survival curves of the

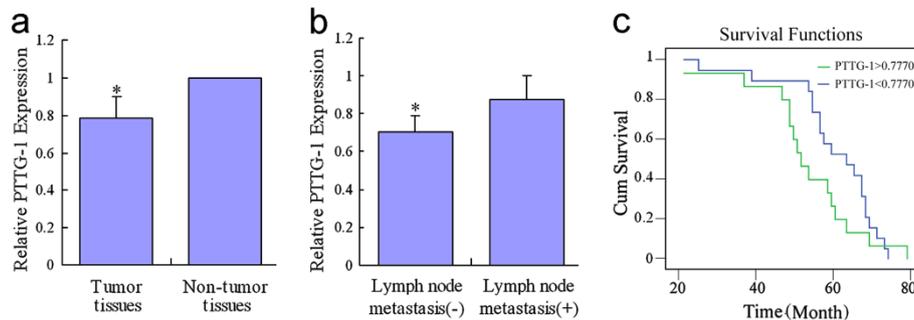
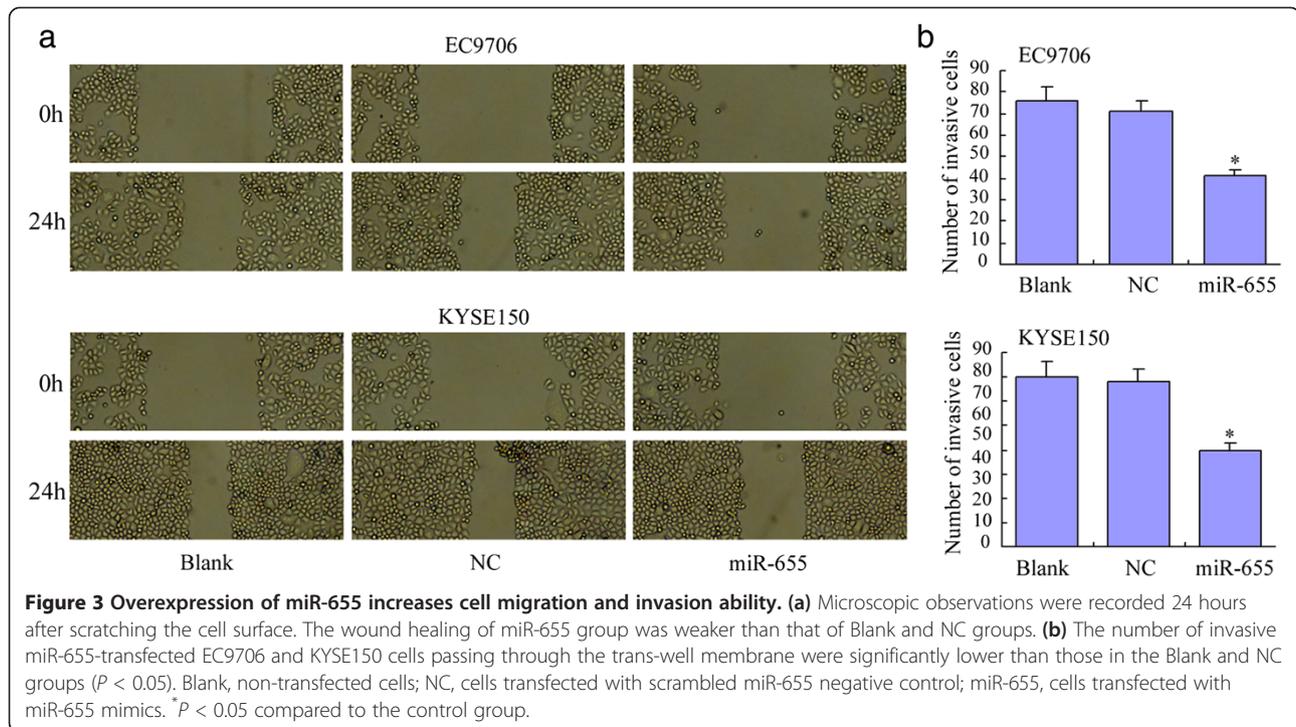


Figure 2 Expression levels of PTTG1 in ESCC tissues and adjacent non-tumor tissues. (a) Compared with adjacent non-tumor tissues, PTTG1 expression levels in ESCC tissues were significantly higher ($P < 0.05$). (b) Among ESCC tissues, expression levels of PTTG1 in cases with lymph nodes positive for metastases were higher than those in cases with lymph nodes negative for metastases ($P < 0.05$). (c) Kaplan–Meier curves of the clinical outcome for PTTG1. Primary ESCCs with moderate to strong PTTG1 expression (>0.7770) had significantly worse survival than did those with weak PTTG1 expression (<0.7770) ($P < 0.05$, log-rank test). T: tumor tissue (n = 34); N: adjacent non-tumor tissue (n = 34). * $P < 0.05$ compared to the control group.



two groups ($P < 0.05$; Figure 1c). Primary ESCCs with moderate to strong miR-655 expression (>0.39) were associated with significantly better survival than those with weak miR-655 expression (<0.39).

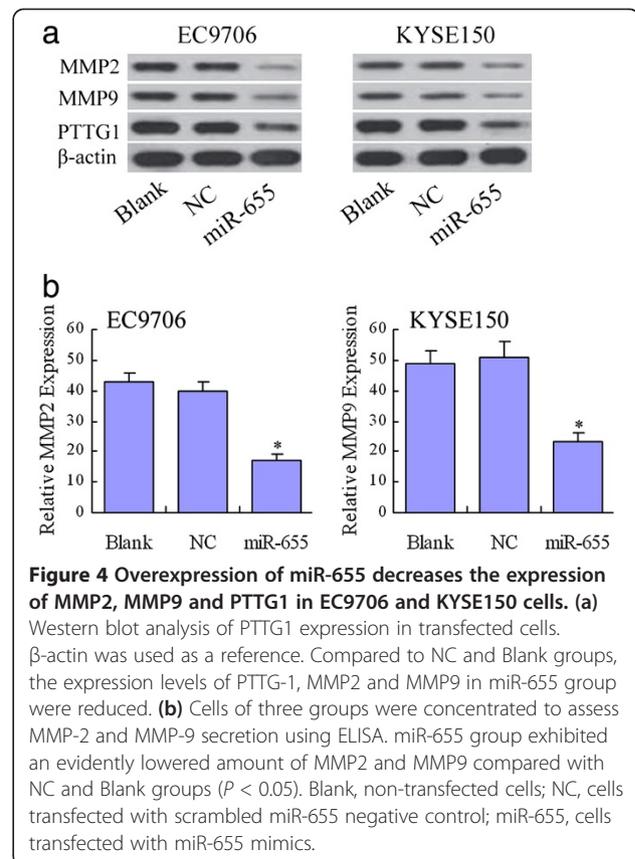
Up-regulation of PTTG1 in ESCC

In western blot analysis, the expression of PTTG1 in ESCC tissue was higher than in adjacent non-tumor tissues ($P < 0.05$; Figure 2a; Table 1). The PTTG1 expression level in ESCC tissues was associated with the occurrence of lymph node metastases, differentiation status and the TNM stage ($P < 0.05$; Figure 2b; Table 1) and again was not associated with gender, age or tumor location ($P > 0.05$; Table 1).

Kaplan–Meier curves for patients with esophageal squamous cell cancer categorized according to PTTG1 expression levels are shown in Figure 2c. Patients were divided into two groups by a cutoff of 0.7770 for PTTG1 expression. A log-rank test indicated that primary ESCCs with moderate to strong PTTG1 expression (>0.7770) were associated with significantly worse survival than ESCCs with weak PTTG1 expression (<0.7770) ($P < 0.05$).

Up-regulation of miR-655 restricts cell migration and invasion in EC9706 and KYSE150 cells

To test whether miR-655-overexpressing cells possessed a reduced propensity for migration and invasion, we performed wound-healing and transwell assays. For the wound-healing assay, microscopic observations of the three groups were recorded 24 h after scratching the cell



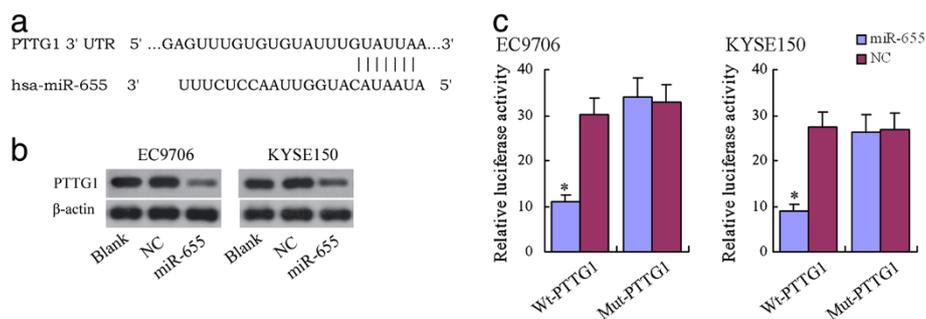


Figure 5 PTTG1 was identified as a target of miR-655 in EC9706 and KYSE150 cells. **(a)** The putative miR-655 binding sequences for the PTTG1 3' UTR. **(b)** Western blot analysis of PTTG1 expression in transfected cells. β-actin was used as a reference. Blank, non-transfected cells; NC, cells transfected with scrambled miR-655 negative control; miR-655, cells transfected with miR-655 mimics. **(c)** Luciferase activity determined 48 h after transfection. NC, scrambled-miRNA; miR-655, miR-655 mimics; Wt-PTTG1, pGL3-PTTG1-wt; Mut-PTTG1, pGL3-PTTG1-mut. **P* < 0.05 compared to the control group.

surface. The capacity for wound healing was lower for the miR-655 group than for the blank and NC groups (*P* > 0.05; Figure 3a).

Using a transwell assay, we found that the mean number of cells penetrating the membrane was not significantly different for the blank and NC groups (*P* > 0.05; Figure 3b). However, the mean number of cells penetrating the transwell membrane was significantly lower in the miR-655 group (*P* < 0.05; Figure 3b) than in the blank and NC groups. Based on these results, we conclude that exogenous over-expression of miR-655 decreases the invasive ability of both EC9706 and KYSE150 cells.

Up-regulation of miR-655 decreased the expression of PTTG1, MMP2 and MMP9

In western blotting, the expression levels of PTTG1, MMP2 and MMP9 were reduced in the miR-655 group relative to the NC and blank groups (*P* < 0.05; Figure 4a). There was no significant difference between the NC and blank groups, however (*P* < 0.05; Figure 4a). As is shown in Figure 4b, the ELISA showed that the miR-655 group exhibited a reduced level of MMP2 and MMP9 relative to the NC and blank groups. Previous studies have demonstrated that high expression of PTTG1, MMP-2 and MMP-9 can promote ESCC invasion. Our results strongly suggest that the up-regulation of miR-655 is connected to the reduced expression of PTTG1, MMP2 and MMP9 and thus restricts cell migration and invasion in ESCC cell lines.

PTTG1 is a direct target of miR-655

Bioinformatic analyses using TargetScan and miRanda predicted that the 3'UTR of PTTG1 would contain binding sites for miR-655 (Figure 5a). Subsequent western blot analysis did in fact show that PTTG1 expression was down-regulated in the EC9706 and KYSE150 cell lines following transfection with miR-655 mimics (Figure 5b). To verify whether PTTG1 is a direct target of miR-655,

we used a Dual-Luciferase reporter system containing either the wildtype or mutant 3' UTR of PTTG1. Co-transfection with miR-655 significantly suppressed the luciferase activity of the reporter containing the wildtype 3' UTR (Figure 5c). These results indicated that miR-655 negatively regulates PTTG1 expression by directly binding to putative binding sites in the 3' UTR.

Expression of PTTG1 restores the anti-migration function of miR-655

Western blotting showed that co-transfection of miR-655 mimics and PTTG1 lacking the 3' UTR sequence (pcDNA3.1- PTTG1) led to an increase in PTTG1 expression, and abrogated the effects of miR-655 mimics (Figure 6a). In the transwell assays, we found that co-

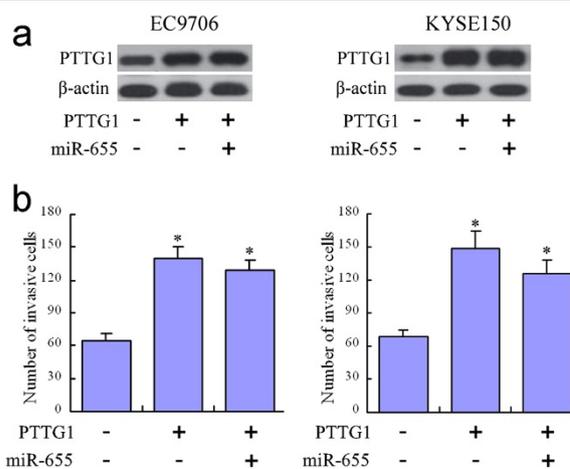


Figure 6 Expression of PTTG1 abrogates the anti-migratory functions of miR-655. **(a)** Transfection with pcDNA3.1-PTTG1 and the miR-655 mimics increased PTTG1 expression. **(b)** Transfection with pcDNA3.1-PTTG1 and the miR-655 mimics increased the average number of migrating cells, abrogating the effects of the miR-655 mimics on decreasing cell numbers. Blank, non-transfected cells; NC, cells transfected with scrambled miR-655 negative control; miR-655, cells transfected with miR-655 mimics. **P* < 0.05 compared to control.

transfection of pcDNA3.1-PTTG1 and miR-655 mimics increased the average number of migrating cells ($P < 0.05$; Figure 6b). From these results we conclude that PTTG1 expression restores the anti-migration function of miR-655.

Discussion

MicroRNAs have been estimated to regulate up to 30% of all human gene, and control a variety of cellular processes [17,28]. Recent studies have also shown that miRNAs are deregulated in various cancers and their expression is relevant to the diagnosis and prognosis of a diverse array of tumors [29,30]. Various publications have associated miRNAs with cancer [31], however, the relation between miR-655 and ESCC remains unknown. In the current study, we first demonstrated that miR-655 is significantly down-regulated in human ESCC tissues. We also found that altered miR-655 expression levels are associated with the occurrence of lymph node metastases and the ESCC differentiation status and TNM stage. Using wound healing and transwell assays, we found that up-regulation of miR-655 in EC9706 and KYE150 cells could suppress the migration and invasion capability of these ESCC strains. The observed aberrant expression of miR-655 suggests that miR-655 functions as a tumor suppressor in ESCC.

The protein product of the PTTG was first isolated from GH4 rat pituitary tumor cell [19,32] and then shown to be transforming in vitro and tumorigenic in vivo [33]. The PTTG family includes PTTG1, PTTG2, and PTTG3. PTTG1 is the most abundant and widely studied form of this protein and is referred to as PTTG [34]. PTTG1 overexpression has been reported in a variety of cancers including ESCC [25-27]. PTTG1 levels correlate with tumor invasiveness, and PTTG1 has been identified as a key signature gene in tumor metastasis [21,22]. These findings agree with our results that indicate that PTTG1 is significantly up-regulated in human ESCC tissues. In addition, we also found that altered PTTG1 expression levels are associated with the occurrence of lymph node metastases and ESCC differentiation status and TNM stage.

MMPs are a highly regulated superfamily of zinc-dependent endopeptidases that are causally associated with the development and progression of tumors [35]. The proposed role of MMPs in tumor invasion is primarily based on their observed overexpression in invasive malignant tumors [36,37]. Esophageal squamous cell carcinoma is primarily derived from squamous cells in the Japanese population, and it has been demonstrated that MMP-2 and MMP-3 expression is positively correlated with the depth of invasion, lymph node metastasis, and vessel permeation [38]. MMP-7, MMP-9, and MT1-MMP have also been shown to be closely associated with

invasion depth and venous invasion in esophageal squamous cell carcinomas [39]. In western blotting and ELISA, we found that up-regulation of miR-655 in EC9706 and KYE150 cells could reduce the expression of PTTG1, MMP2 and MMP9. These findings suggest that miR-655 negatively regulates the expression of PTTG1, MMP2 and MMP9 in these ESCC cell lines.

Previous studies have demonstrated that PTTG1 down-regulates MMP2 and MMP9 [40]. Therefore, we confirmed that PTTG1 acts as a direct functional target of miR-655 using western blotting and luciferase reporter and rescue assays. We also verified that miR-655 influences the invasive and metastatic ability of ESCC. Taken together, our findings indicate that miR-655 can act as a metastasis suppressor by targeting PTTG1.

In conclusion, we have shown that miR-655 is down-regulated in ESCC. We have also shown that the up-regulation of miR-655 inhibits invasion and metastasis in ESCC-derived cell lines. Based on these findings, we propose that miR-655 could potentially serve as a therapeutic agent for ESCC.

Abbreviations

miRNAs: MicroRNAs; ESCC: Esophageal squamous cell carcinoma; PTTG1: Pituitary tumor-transforming gene-1; 3'-UTRs: 3'-untranslated regions; EMT: Epithelial mesenchymal transition.

Competing interest

The authors have declared that no competing interest exists.

Authors' contributions

YYW, WQZ, YWD, XDC performed and participated in analysis of laboratory experiments data. GQZ, YYW and ZMD participated in the design of experiments. YYM, PL and TW acquired, preserved clinical samples. GQZ provided administrative support and funded experiments. YYW, WQZ and GQZ drafted the manuscript. All authors have contributed and approved the final manuscript.

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